Ramon Guevara-Gonzalez
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Biosystems Engineering: Biofactories for Food Production in the Century XXI

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Foreword

Today's world is subject to important changes from the environmental point of view. Based on this, it is valid to assume that the way we produce foods must necessarily evolve to the generation of systems that consider the aspect of "sustainability." Thus, for future production systems, food production with these features (Biosystems) should be the most suitable option to support the worldwide growth of the population in an environmentally friendly way. Biosystems, in order to be a real alternative in food production, should consider including basic studies of various disciplines with the production of plants, animals, microorganisms, among others. These efforts must be related to aspects of Production Control Engineering as well as recycling systems and waste inputs.

The study of *Biosystems Engineering* is emerging worldwide focusing on issues such as agricultural production, livestock and aquaculture, amalgamating aspects of basic biology, chemistry, mathematics, physics, and engineering in searching for strategies for sustainable food production in protected environments. Some important research fields regarding Biosystems Engineering for food production are new production strategies of plants with high content of beneficial compounds for human health, development of electronic instrumentation for monitoring production at different levels, recycling agroindustry residues, environmentally friendly approaches for food production, among others.

As an example of the aforementioned, it is clearly a global trend (especially in Europe) toward "healthy eating," rich in compounds with activity called "nutraceutical," due to the presence of specific secondary metabolites. Agricultural production systems that have documented significant nutraceutical compounds are called "organic." While these systems produce functional (nutraceutical) food of high quality and reduce pollution effects in the process, it cannot generate enough yields to support the global growth and thus, are of high economic cost and hardly accessible for most of the population. On the other hand, conventional extensive agriculture has produced the food needed to sustain the population growth worldwide. However, this achievement is reached using components given by the Green Revolution and incorporates some elements of biotechnology. This type of agriculture has the peculiarity that the food produced is not as high in nutraceuticals as organic products. Additionally, conventional systems are accused of having associated environmental problems.

In this context, it is proposed to generate a *Third Pathway for Agriculture*, not only organic and not only conventional. It will be necessary to pursue efforts to develop new strategies for food production using the necessary knowledge accumulated from different disciplines. For instance, applications of metabolites-inducer factors (MIFs) to increase nutraceuticals, biological control strategies, greenhouses with appropriate climate control, recycling food production residues, etc. Taking into account all the aforementioned aspects this book, *Biosystems Engineering: Biofactories for Food Production in the Century XXI*, is an attempt to depict researches from several experts in the proposed fields, in order to get a vision related to this new proposal for food production.

Ramon Guevara-Gonzalez
Irineo Torres-Pacheco

Contents

Chapter 1
Strategies for Sustainable Plant Food Production: Facing the Current Agricultural Challenges—Agriculture for Today and Tomorrow

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Rosalía V. Ocampo-Velazquez, Enrique Rico-García,
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Abstract Plants are fundamental elements of the human diet, either as direct sources of nutrients or indirectly as feed for animals. During the past years, the main goal of agriculture has been to increase yield in order to provide the food that is needed by a growing world population. However, as important as yield, but commonly forgotten in conventional agriculture, is to retain and, if possible, to increase the phytochemical content due to their health implications. By 2025, the global population will exceed 7 billion. In the short term, per capita availability of arable land and irrigation water will decrease from year to year while biotic and abiotic stresses expand. Food security, defined as economic, physical, and social access to a balanced diet and harmless drinking water will be a compromise, with a holistic approach to nutritional and non-nutritional factors needed to achieve success in the eradication of hunger. Science and technology will play a very important role in stimulating and sustaining agriculture leading to long-term increases in productivity without linked ecological harm.

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Contents

1.1 Introduction

In recent times, concerns about the impact of the food that people consume on their own health, as well as the social and environmental consequences that it entails, have led to major changes in all steps of the food chain including all the agents from the producer to the retailer. These phenomena comprise complex technologic, social, economic, and environmental linkages that require integrated research approaches, i.e., the changes in consumer's demand and their consequences need to be considered from all these different points of view. While food is a basic requirement for human survival and well-being, the ability to ensure food security, that is, access to sufficient nutritious food is determined by multiple social and political factors. At the same time, food production as the foundation of food security also provides the potential for building a new politics to work toward sustainability.

Currently, the agricultural challenge is to get crop yield and quality at the same time. Since ancient times, agricultural practices seek to confer comfort to plants through stress reduction. For this reason, the use of greenhouse has emerged in the perspective of reducing the stress produced by inadequate weather parameters such as temperature, radiation, and relative humidity parameters as well as pest diseases. Consequently, they have allowed growing crops in an efficient way in relative safety from the outside world. However, this practice has brought a decrease in the production of phytochemicals with health implications, because these compounds are produced by the plant in response to stress conditions. Besides, because plant are sessile organisms, synthesis of phytochemicals represents a major strategy for counteracting unfavorable conditions.

The cultivation of crops inside greenhouses is the most intensive form of vegetal production. Consequently, the challenge of feeding a global population could be faced through protected agriculture. Nevertheless, the commitment in today's agriculture is not only to provide abundant food but also to contribute to increase the health and wellness of a growing global population that cares about living with quality and environment issues. Then the question that arises is related to how to obtain both crop yield and secondary metabolites with health implications, since it is stated that the production of these metabolites jeopardized plant fitness.

In this context, the use of metabolite inducing factors represents a viable strategy to achieve both crop yield and bioactives, that is, the challenges assumed by conventional and organic agriculture, respectively. Indeed, questions around the use of metabolite inducing factors remain: what is the best inductor to use, in which doses, at what growing state, just to name a few. The advances around this topic along with the improvement in greenhouse building and climate control will lead to conquer the goal of the twenty-first century: produce more and better food for an increasingly demanding population in a sustainable manner. For this reason, this chapter addresses the following topics: first, transitions in food production systems; second, to what extent are emerging technologies and sustainable agriculture compatible; third, prospects to integrate technology and sustainability with functional food.

1.2 Different Agricultural Visions: Conventional Versus Organic

This section discusses the principles of conventional farming compared with organic farming to achieve sustainable agriculture allowing in future the global food supply.

1.3 Conventional

According to Van der Walt (2000) and Kitch et al. (2002) agriculture has gone through three great periods. The first comprises the traditional technology, which began about 10,000 BC ago, in which civilizations used the natural biological diversity and domesticated plants and animals, began selecting plant material for propagation and animals for breeding. The second period comprises the conventional technology, which began in the late nineteenth century, when Gregor Mendel in 1865 identifies the principles of heredity, laying the groundwork for classical breeding methods. Thereafter, were obtained commercial hybrid crops (1930). Mutagenesis, tissue culture, and plant regeneration were applied in the mid-twentieth century. Finally, the third period focuses from the 1970s to date, in which gene transfer was initiated by recombinant DNA techniques. It uses the tissue culture for large-scale propagation of plants and embryo transfer in animal production. Vaccines and hormones are genetically engineered and cloned animals. And to begin the twenty-first century appears bioinformatics, genomics, proteomics, and metabolomics tools of great contribution in agricultural biotechnology.

1.3.1 Green Revolution

Green Revolution, the term used for rapid increases in wheat and rice yields in developing countries brought about by improved varieties combined with the expanded use of fertilizers and other chemical inputs, has had a dramatic impact on incomes and food supplies in many developing countries (Pinstrup-Andersen and Hazell 1985). The Green Revolution was a major achievement for many developing countries and gave them an unprecedented level of national food security. It represented the successful adaptation and transfer of the same scientific revolution in agriculture that the industrial countries had already appropriated for themselves (Hazell 2002).

Briefly, the history of the Green Revolution had its beginning in the early twentieth century when the Japanese crossed a semi-dwarf wheat variety with American high-yielding varieties to produce Norin 10. Although it was of little importance in Japan, Norin 10 was used in breeding programs in the USA after 1945 to produce a number of high-yielding semi-dwarf cultivars. The cross Norin 10-Brevor was sent to Norman Borlaug at the Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT) in Mexico and was bred with varieties adapted to grow in tropical and subtropical climates. The progeny were then distributed to Latin America and South and Southeast Asia, where they were rapidly adopted, allowing amazing increases in wheat yields in these countries. Although the American Cultivars were winter wheat that required exposure to low temperatures to flower, the new CIMMYT lines were spring wheat that could be grown in warm climates in any season, allowing two crops a year. Norin 10-Brevor 14 was also the origin of dwarfing genes in many wheat cultivars suitable for temperate countries. The Norin 10 dwarfing genes are now present in >70 % of current commercial wheat cultivars worldwide (Evans 1998; Hedden 2003).

1.3.1.1 High-Yield Varieties

Although the term Green Revolution originally described developments for rice and wheat, high-yielding varieties (HYVs) have since been developed for other major food crops important in developing countries, including sorghum, millet, maize, cassava, and beans.

HYVs were characterized by (a) high-yield, improved varieties of crops that give us more yields per hectare, (b) dwarfness, due to short height, and plants are stronger and hence can withstand strong winds. The problem of lodging is less in these improved varieties. (c) Better response to fertilizers, this means that when fertilizers are supplied to HYV and their yield increases substantially but is not so in case of traditional varieties, and (d) early maturation, it takes less time for maturing; as a result, some additional crop can be raised in the spare time thus saved. However, there are also disadvantages of using HYV. For example, the dwarfing of HYVs produce yields less fodder than the tall, traditional varieties of crops that require higher inputs such as more water and more fertilizers as compared to the traditional varieties of crops; weeding is more frequent, needs a continuous use of pesticides, and is generally more susceptible to diseases than the traditional varieties (Dalrymple 1974, 1986).

Even with its drawbacks, the HYVs were adopted quickly by the characteristics they possessed. By 1970, about 20 % of the wheat area and 30 % of the rice area in developing countries were planted to HYVs, and by 1990, the share had increased to about 70 % for both crops. Yields of rice and wheat virtually doubled. It is estimated that between one-third and one-half of the rice areas in the developing world was grown with high-yielding varieties, in the decade of the 1980s varying from 9 % in Thailand to 78 % in the Philippines, the yield increases due to these varieties is about 1 ton/ha in irrigated areas and 0.75 tons/ha on favored upland rice areas (Pinstrup-Andersen and Hazell 1985). The impact was estimated to be about 10 million tons in the Far East and less than one-half of a million tons in Latin America for the year 1976/1977 (Pinstrup-Andersen and Hazell 1985). In the same sense, 27 million tons from production of rice in eight Asian countries produced 85 % of Asia's rice in 1980. Another 29 million tons was added by fertilizers, and irrigation contributed 34 million tons for modern rice varieties. Higher yields and profitability were characteristics that led farmers to increase the area of rice and wheat they grew at the expense of other crops. And with faster growing varieties and irrigation, they grew more crops on their land each year (Hazell 2002) HYVs play an important role in increasing crop yield due to their resistance against pests. Ahmad et al. (2007) showed that the main constraints faced by farmers in adopting HYVs were unavailability of seed and lack of

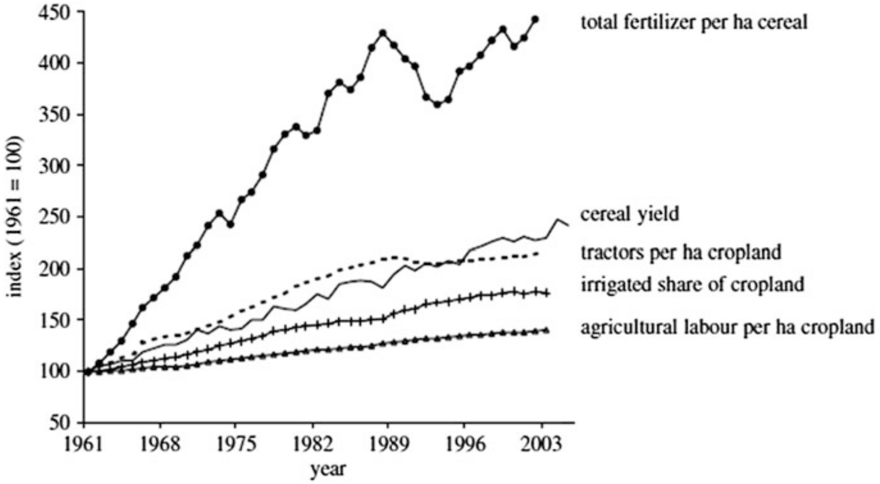

Fig. 1.1 Global trends in the intensification of crop production (index 1961–2002/2005). Reproduced from Hazell and Wood (2008). Updated from FAOSTAT (2006; tractor and fertilizer data to 2002, land use to 2003, production to 2005)

latest knowledge, in addition to access to water supply, lack of capital, shortage of cultivated land, shortage of labor, lack of technical know-how, and lack of inputs.

1.3.1.2 Agrochemicals and Machinery

At the global level, agricultural production has grown much faster than the population in recent decades, leading to a steady increase in per capita agricultural output (including food) and a steady decline in world prices for most agricultural commodities, particularly since the late 1970s. This has been achieved primarily with a technological revolution that has increased yields through increases in modern inputs irrigation, improved seeds, fertilizer, tractors, and pesticides as shown in Fig. 1.1 (Hazell and Wood 2008). As a result of this unprecedented growth in agricultural productivity, the world now produces more than enough food to feed the entire population to the minimum UN standards if it were distributed more equitably. Even more remarkably, this surplus has been achieved despite the diversion of considerable land, labor, and other rural resources to the production of higher value foods (meat, milk, fruits, vegetables, etc.) to meet the changing food demands of growing more urbanized and more affluent populations. This includes the additional cereals needed as feed grains in intensive livestock systems and oil crops for inland aquaculture (Hazell and Wood 2008).

1.3.1.3 Limitations of the Green Revolution

According to Pingali (2012) the Green Revolution contributed to widespread poverty reduction, averted hunger for millions of people, and avoided the conversion of thousands of hectares of land into agricultural cultivation. At the same time, the Green Revolution also spurred its share of unintended negative consequences, often not because of the technology itself but rather because of the policies that were used to promote rapid intensification of agricultural systems and increase food supplies. Some areas were left behind, and even where it successfully increased agricultural productivity, the Green Revolution was not always the panacea for solving the myriad of poverty, food security, and nutrition problems facing poor societies. The slowdown in yield growth that has been observed since the mid-1980s can be attributed, in part, to the above degradation of the agricultural resource base. These environmental costs are widely recognized as a potential threat to the long-term sustainability and replication of the Green Revolution success.

1.3.2 Biotechnology

Biotechnology is a complement, not a substitute, in many areas of conventional agricultural research. It offers a variety of tools to improve our understanding and management of genetic resources for food and agriculture. These instruments are already contributing to the improvement and conservation programs and facilitating the diagnosis, treatment, and prevention of diseases of plants and animals (FAO 2002).

1.3.2.1 Strategies to Increase Food Production

The agricultural solutions based in biotechnology may consist of new seeds or new breeds of cattle, which continue the tradition of selecting and improving crops and livestock produced over several centuries. However, the difference is that the new genetic technology can identify the desirable characteristics more quickly and accurately than conventional breeding practices of plants and animals.

According to Persley and Doyle (1999) modern biotechnology, composed of genomic, bioinformatic, molecular genetic improvement molecular diagnostic, and vaccination technology, will not solve all problems of food insecurity and poverty, unless it is governed by a set of policies directed to: (1) greater public investment in research and development, (2) standardize the regulation that informs the public and protects from any risk arising from the release of genetically modified organisms, (3) management of intellectual property to encourage greater investment in the private sector, and (4) the regulation of private sector seed production and agricultural research to protect the interests of small farmers and poor

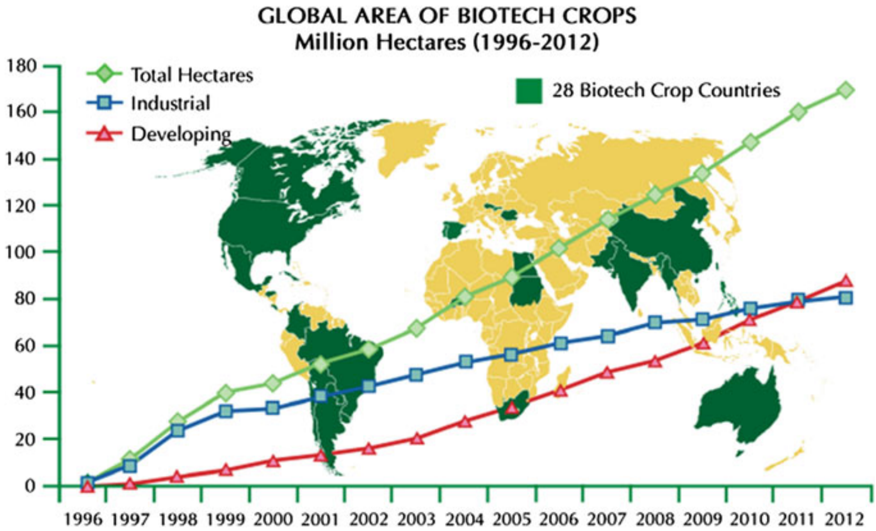

Fig. 1.2 Global area of biotech crops, 1996–2012: industrial and developing countries. Reproduced from ISAAA (2012)

consumers in developing countries; these could provide the key to a solution and discern whether biotechnology can benefit the poor in developing countries.

The use of biotechnology in agriculture and medicine has produced an increasing number of genetically modified organisms (GMO) and products derived from them. The rapid spread of genetically modified (GM) crops shows the biotechnology pace transforming the commercial landscape (Persley 1999). According to ISAAA (2012) biotech crop hectares increased by an unprecedented 100-fold, from 1.7 million ha in 1996, to 170 million ha in 2012 (Fig. 1.2).

Of the 28 countries which planted biotech crops in 2012 (Table 1.1), 20 were developing and 8 were industrial countries; and it is noted that more than half the world's population, 60 % or ∼4 billion people, live in the 28 countries planting biotech crops (ISAAA 2012).

1.3.2.2 Environment Benefits Offered by Biotech Crops

Impact on insecticide/herbicide use and impact on carbon emissions are two key aspects of environmental impact. From 1996 to 2011, biotech crops have contributed to food security, sustainability, and the environment/climate change by increasing crop production valued at US$98.2 billion; providing a better environment by saving 473 million kg of pesticides; in 2011 alone reducing CO_2 emissions by 23.1 billion kg, equivalent to taking 10.2 million cars off the road for 1 year; conserving biodiversity by saving 108.7 million ha of land; and helped alleviate poverty for >15.0 million small farmers and their families totaling >50 million

Table 1.1 Global area of biotech crops by country

Rank	Country	Area (million ha)	Biotech crops
1	USA[a]	69.5	Maize, soybean, cotton, canola, sugarbeet, alafalfa, papaya, squash
2	Brazil[a]	36.6	Soybean, maize, cotton
3	Argentina[a]	23.9	Soybean, maize, cotton
4	Canada[a]	11.6	Canola, maize, soybean, sugarbeet
5	India[a]	10.8	Cotton
6	China[a]	4	Cotton, papaya, poplar, tomato, sweet pepper
7	Paraguay[a]	3.4	Soybean, maize, cotton
8	South Africa[a]	2.9	Maize, soybean, cotton
9	Pakistan[a]	2.8	Cotton
10	Uruguay[a]	1.4	Soybean, maize
11	Bolivia[a]	1	Soybean
12	Philippines[a]	0.8	Maize
13	Australia	0.7	Cotton, canola
14	Burkina Faso[a]	0.3	Cotton
15	Myammar[a]	0.3	Cotton
16	Mexico[a]	0.2	Cotton, soybean
17	Spain[a]	0.1	Maize
18	Chile[a]	<0.1	Maize, soybean, canola
19	Colombia	<0.1	Cotton
20	Honduras	<0.1	Maize
21	Sudan	<0.1	Cotton
22	Portugal	<0.1	Maize
23	Czech Republic	<0.1	Maize
24	Cuba	<0.1	Maize
25	Egypt	<0.1	Maize
26	Costa Rica	<0.1	Cotton, soybean
27	Romania	<0.1	Maize
28	Slovakia	<0.1	Maize

[a] 18 biotech mega-countries growing 50,000 ha, or more, of biotech crops (ISAAA 2012)

people, who are some of the poorest in the world. Biotech crops are essential but not a panacea and adherence to good farming practices, such as rotations and resistance management, are a must for biotech crops as they are for conventional crops (ISAAA 2012).

1.3.2.3 Economic Benefits of Principal Biotech Crops Currently Grown

Principal biotech crops currently grown are maize, soybean, cotton, canola, and alfalfa. According to Kitch et al. (2002), of the 100 million ha of biotech soybean planted globally 81 % or (80.7 million ha) were biotech. During the 16-year period 1996–2011 the increase in income benefits for farmers growing biotech soybean was US$32.2 billion and for 2011 alone, it was US$3.9 billion (Brookes and Barfoot 2013). Maize is the second most dominant biotech crop after soybean in 2012, with a surface plantation of 39.9 million ha, up by 2.6 million ha from 2011, which occupied 23 % of the global biotech area. The stacked maize category includes three combinations of traits: a double stack with insect resistance (Bt) and herbicide tolerance (HT), Bt/HT; a double stack with two traits for insect resistance, Bt/Bt; and a triple stack with two types of insect resistance, plus herbicide tolerance, Bt/BtHT (ISAAA 2013). In the same sense, Bt cotton is the third most dominant crop globally, which occupied 18.8 million ha, equivalent to 11 % of the global biotech area, up by 0.9 million ha, or 5 % since 2011. The eighth most dominant trait was herbicide-tolerant cotton occupying 1.8 million ha, the same as last year, occupying 1 % of all biotech crops globally. HT cotton was planted in eight countries—USA, Brazil, Argentina, Paraguay, Australia, Mexico, Colombia, and South Africa. The increase in income benefits for farmers growing biotech cotton during the 16-year period 1996–2011 was US$32.5 billion and US$6.7 billion for 2011 alone (Brookes and Barfoot 2013). Another Bt crop is the alfalfa, which was first approved for commercialization in the USA in 2005. The 60,000 ha of RR®alfalfa represented approximately 5 % of the 1.3 million ha alfalfa seeded in 2006 (ISAAA 2013). Finally, the Bt canola increased globally by 1 million ha from 8.2 million ha in 2011 to 9.2 million in 2012, equivalent to 12 %. The increase in income benefits for farmers growing biotech canola during the 16-year period 1996–2011 was US$3.1 billion and US$0.42 billion for 2011 alone (Brookes and Barfoot 2013).

1.4 Organic

Organic agriculture refers to a farming system that has regulations to ban the use of agrochemicals such as synthetic fertilizers and pesticides and the use of GMO, as well as many synthetic compounds used as food additives (e.g., preservatives, coloring) (IFOAM 2013; Yussefi et al. 2008). Organic agriculture, along with other low input agriculture practices, results in less energy demand compared to intensive agriculture and could represent a means to improve energy savings and CO_2 abatement if adopted on a large scale. At the same time it can provide a number of important environmental and social services, such as preserving and improving soil quality, increasing carbon sink, minimizing water use, preserving biodiversity, halting the use of harmful chemicals, and thereby guaranteeing healthy food to consumers. Organic farming offers the most consistent approach to agroecological progress. Because of the ban or restricted use of many direct control techniques such as pesticides, herbicides, synthetic soluble fertilizers, and veterinary medicines, organic farmers rely heavily on preventive and system-oriented practices (Forster et al. 2012).

1.4.1 Food Quality

Whether organic food is better or equal in terms of quality (e.g., higher content of minerals, vitamins) compared to conventionally produced foods, is also an issue (Adam 2001; Brandt and Mølgaard 2001; Gomiero et al. 2008). Some experts have reported that organic food is not better than conventional, stating that data do not provide significant evidence of differences between the two (Brandt and Mølgaard 2001; Brandt et al. 2006; Trewavas 2001). Others, however, claim that differences do exist concerning the content of nutritional elements (such as vitamins and other beneficial micronutrients) (Heaton 2001; Winter and Davis 2006; Mitchell et al. 2007). Also, the avoidance of ingestion of food with harmful pesticides residues is a benefit (Curl et al. 2003; Lu et al. 2006; Winter and Davis 2006; Halweil 2007). Some authors have suggested that there is a potential for increased microbiological hazards from organic products (including animals) due to the prohibition of antimicrobial use as there is evidence that the use of pesticides, like herbicides, can decrease the number of toxic chemicals produced by plants. However, until now, this hypotheses has not been proven (Brandt and Mølgaard 2001; Brandt et al. 2006; Winter and Davis 2006; Halweil 2007).

1.4.2 Ecological Benefits

In ecological terms, organic agriculture offers energy efficiency and energy savings, because it performs much better than conventional in regard to energy efficiency (output/input). Generally, however, conventional crop production has the highest total net energy production per unit of cropped land. CO_2 and greenhouse gases (GHGs) abatement, as organic farming represents an important option to supply a carbon sink and GHGs abatement. Soil, however, has a limit to carbon sink. Long-term solutions concerning CO_2 emissions for global society should be searched for in new energy conservation techniques and strategies. Properly managed, organic agriculture represents an interesting option to reduce energy consumption, CO_2 and other GHG emissions, as well as to preserve soil health and biodiversity (Gomiero et al. 2008).

Although organic agriculture is one among the broad spectrum of methodologies which are based on the specific and precise standards with different names such as organic, biological, organic-biological, biodynamic, natural, and ecological agriculture, there are some commonly followed principles in organic agriculture. These principles are summarized as follows (Ma and Joachim 2006):

- Maintain long-term soil fertility through biological mechanism.
- Recycle wastes of plant and animal origin in order to return nutrients to the land, thus minimizing the use of external inputs outside systems, and keep the nutrients cycle within the system.

- Prohibit the use of synthetic materials such as pesticides, mineral fertilizers, chemical ingredients, and additives.
- Use natural mechanism and rely on renewable resources to protect the natural resources.
- Raise animals restricted to areas and guarantee the welfare of the animals.
- Adapt local environment and diversified organization.
- Produce high-quality products.

Since the beginning of the twenty-first century, the development of organic farming worldwide has shown a strong growth. Almost 23 million ha were managed organically worldwide in 2002. Compared to 2000 this is an increase of 3–1.4 %. The total world retail sales of organic products reached US$25 billion in 2002, which increased by 42.9 % compared to 2000 (Yussefi et al. 2003). Organic agriculture is practiced in almost all countries of the world, and its share of agricultural land and farms is growing (Scialabba and Hattaam 2002). The market for organic products is growing, not only in the major markets like Europe, North America, and Japan, but also in many other countries, including developing countries (Foster and Lampkin 2000; UNCTAD/WTO 2001; Lin 2003).

1.5 What Is the Necessity of Current and Future Agriculture

Agriculture currently appropriates a substantial portion of the Earth's natural resources. Land used for crop production, pasture, and livestock grazing systems amounts to 38 % of total land area (Wood et al. 2000; Spiertz 2010). Population growth and expanding demand for agricultural commodities constantly increase the pressure on scarce land and natural resources (Schade and Pimentel 2010). The drop in cropland in relation to population is very evident. Until the middle of the twentieth century, available cropland was of the order of 0.45 ha per person; by 1997 it had reduced by almost a factor of 2 resulting in 0.25 and the projection for 2050 is 0.15 ha per person (Lumpkin 2010). The question is whether we can produce enough food, feed, fiber, and fuel to meet the needs of a 50 % larger global population in 2050 in a sustainable manner. Cassman et al. (2003) concluded that "avoiding expansion of cultivation into natural ecosystems, increased N-use efficiency, and improved soil quality are pivotal components of a sustainable agriculture that meets human needs and protects natural resources." More resources are required for meeting the demands of the growing human population. Recent studies by Tilman et al. (2002) indicated that significant systems improvements and efficiency gains in agriculture are needed worldwide in the next decades, to be able to feed the increasing global population and at the same time to circumvent large-scale degradation of natural ecosystems and deterioration of ecosystem services through agricultural activities. The sustainability framework, comprising the balance between short-term and long-term objectives with respect to profitability, ecological health, and social-ethical acceptance gives guidance to research directions and policy measures. The conceptual framework of a sustainable gap was presented by Fischer et al. (2007b). They suggest a hierarchy of considerations with the biophysical limits of the Earth setting ultimate boundaries. The question is, whether this concept with the "economies" embedded in "human societies" does fit for major food production systems with free trade as drivers at a global scale. Transitions in agriculture are a response to external and/or internal "events" that provide the incentive for structural change (Godfray et al. 2010a). Possible events or "driving forces" for transitions in agriculture include gradual and sudden processes, like population pressure, changes in natural conditions (climate, diseases, and flooding), changes in markets and market prices, innovations, and applications of new technologies. Transitions in agriculture involve large-scale structural changes, which have a distinct impact on society. The difficulties in understanding the causes and effects of changes in agriculture arise from the diversity and complexity of agriculture, and the multitude of factors that affect agriculture (Godfray et al. 2010a; Spiertz 2010). Demands by society, economy, and environment determine the direction of change in agriculture. Decision making requires intensive mutual interaction and discussion to identify the challenges, trade-offs, discrepancies, and possibilities for synergy. A more effective strategy for the transition toward sustainable agriculture is setting suitable goals with clear targets and indicators to measure progress, when the gap between socioeconomic and ecological targets is too big. To meet the challenges of a global food security in a sustainable way requires the intensification of knowledge-intensive approaches and the use of modern agrotechnologies and biotechnologies.

1.6 Maximize Yield

In the near future, food availability will be threatened if the right agriculture measures are not adopted. Currently, the reduction of pesticides to control pests and pathogens, and the presence of health compounds in food is as important as food production. There is considerable agreement about the idea that increasing yields on existing agricultural land is a key component for minimizing further expansion (Wirsenius et al. 2010).

The world food situation is rapidly redefined by income growth, climate change, high energy prices, globalization, urbanization, and increased weeds, pests, and diseases. These issues are transforming food consumption, production, and ultimately, markets. By 2050, the world population will reach 9.1 billion and almost all of this population increase will occur in developing countries (Floros et al. 2010); it means a growth by 34 % from 6.8 billion in 2009. Also, life expectancy is increasing (Vaupel and Kistowski 2005) and people demand not only food, but quality food.

Whereas the achievements of global agriculture since the 1960s are among the greatest success stories, even greater challenges await in feeding more than

9 billion by 2050 (Lal 2013a). There are almost 1 billion hungry people in the world (Food and Nations 2009) and about 10.9 million children under five die in developing countries due to hunger related causes (UNICEF 2009). As an example, a third of the African population faces widespread hunger at present, especially those in rural communities and are dependent on traditional agriculture. Changes in weather patterns and extreme events may aggravate the situation (Haile 2005). This topic is further discussed in Sect. 4.4.

1.7 Production Protecting the Environment

Global warming represents a major threat for food security, especially in tropical countries. It is expected that global warming will worsen the drought and the irregularity of rainfall in many countries. Meanwhile, intensive dependence of agriculture on agrochemicals and nonrenewable fossil fuels has accounted for over 20 % of global anthropogenic greenhouse gas emissions (Scialabba 2003). Therefore, organic farming has been mentioned as a major thrust area for achievement of Millennium Development Goals (MDGs) and to secure sustainable development. Conversion to organic farming contributes to mitigate the contribution of agriculture to global warming. It therefore contributes to the stability of the food supply which is threatened by the climate change (Aubert 2007).

Organic farming was developed in the 1940s in Switzerland and the United Kingdom. Their developments were based in part on the concepts of biodynamic farming initiated in the 1920s by Rudolf Steiner in Germany. However, it was only in the 1980s when the negative impact of intensive conventional agricultural methods became apparent, that interest in organic farming reached a broader public. In the European Union (EU) organic farming has received policy support since the early 1990s for its potential to contribute to environmental protection, rural development, and animal welfare (Commission 2004; Häring et al. 2004; Nieberg et al. 2007). Organic farming is also linked to the production of quality food, and policies have been implemented to ensure transparency and fair competition on the market.

In 2005, around 4 % of the Utilized Agricultural Area of the 25 Member States of the EU was certified organic, representing over 6 million ha and nearly 158,000 organic producers (Llorens Abando and Rohner-Thielen 2007). In the last decade the market for organic products has grown steadily, both in Europe and elsewhere, and is expected to keep increasing (Michelsen et al. 1999; Yussefi et al. 2008). Research on agronomic aspects of organic farming has shown that crop yields tend to be lower in organic farming (Kaut et al. 2008), albeit less variable and less susceptible to drought. Organic farming methods tend to increase soil organic matter and thus enhance soil fertility (Darnhofer et al. 2011), making it less-dependent on external inputs. Organic farming may also provide ecosystem services, increase biodiversity, and have a positive impact on the landscape (Letourneau and Bothwell 2008; Norton et al. 2009). However, studies have pointed out that the potential benefits of organic farming are not always realized in all places and under all management systems (Darnhofer et al. 2011).

The growing concern about environmental degradation, dwindling natural resources, and urgency to meet the food needs of the increasing population are compelling farm scientists and policy makers to seriously examine alternatives to chemical agriculture. Nemecek et al. (2005) found greenhouse warming potential in organic systems to be 29–32 % lower on per ha basis than in a mineral fertilizer system and 35–37 % lower than in the conventional manure-based system. If one gets a premium price for the organically grown rice, the economic returns from the ecological farming system are really appreciable. The declines in productivity or no effect on yields is overturned by better prices farmers get for their organic produce. Various studies have confirmed that organic agriculture is productive and sustainable (Kaswan et al. 2012). Indeed, several studies have demonstrated that fruits and vegetables from organic farming generally are endowed with enhanced nutritional properties. A recent comparative study shows that organic tomato juice has a higher phenolic content and hydrophilic antioxidant activity when compared to conventional tomato juice. Organic tomatoes from Felicia, Izabella, and Paola varieties had higher vitamin C and carotenoid contents which were more pronounced when expressed on fresh matter than on dry matter. Organic strawberries present higher antioxidant concentrations and have been shown to inhibit the proliferation of human colon (HT29) and breast (MCF-7) cancer cells more effectively than conventional ones (Oliveira et al. 2013).

Organic agriculture avoids nutrient exploitation and increases soil organic matter content. In consequence, soils under organic agriculture capture and store more water than soils under conventional cultivation (Niggli 2007). Production in organic agriculture systems is thus less prone to extreme weather conditions such as drought, flooding, and water logging. Organic agriculture accordingly addresses key consequences of climate change, namely increased occurrence of extreme weather events, increased water stress and drought, and problems related to soil quality (Liverman 2008). Furthermore, organic agriculture reduces the vulnerability of the farmers to climate change and variability. First, it comprises highly diverse farming systems and thus increases the diversity of income sources and the flexibility to cope with adverse effects of climate change and variability, such as changed rainfall patterns. This leads to higher economic and ecological stability through optimized ecological balance and risk spreading. Second, organic agriculture is a low-risk farming strategy with reduced input costs and, therefore, lower risks with partial or total crop failure due to extreme weather events or changed conditions in the wake of climate change and variability (Eyhorn 2007). As such, it is a viable alternative for poor farmers. In addition, higher prices can be realized for the products via organic certification. Higher farm incomes are thus possible due to low input costs and high sale prices. The coping capacity of the farms is increased and the risk of indebtedness is lowered. Risk management, risk-reduction strategies, and economic diversification to build resilience are also prominent aspects of adaptation.

Crops and crop varieties used in organic agriculture are usually well adapted to the local environment. Local effects of climate variability cannot be foreseen in detail because at the local level climate change models are not very accurate or even available. Adaptation thus may utilize measures that build on self-adaptive capacity, such as local crop-breeding. The systemic character (on farm breeding,) of such sustainable agricultural system is especially adequate to provide such. Notwithstanding this potential, more research is needed on how organic farming systems perform under increased disease and pest pressures, which are important effects of climate change on agriculture (Liverman 2008) and on how local crop varieties adapt to climate change and variability. Organic agriculture also seems to perform better than conventional agriculture under water constraints (Darnhofer et al. 2011). By its nature, organic agriculture is an adaptation strategy that can be targeted at improving the livelihoods of rural populations and those part of societies that are especially vulnerable to the adverse effects of climate change and variability—for example, the rural population in sub-Saharan Africa; and improvements via reduced financial risk, reduced indebtedness, and increased diversity (Eyhorn 2007). By its systemic character, organic agriculture is an integrative approach to adaptation. Organic agriculture addresses many of the key challenges identified for adaptation to climate change and variability and it fulfills many of the criteria that are seen as important general prerequisites for such strategies as described in (Kaswan et al. 2012). Organic agriculture as a mitigation strategy addresses both emissions avoidance and carbon sequestration.

1.8 Functional Foods

Many biotechnological strategies have been hypothesized and used to enhance the production of secondary metabolites in plants such as: high-yielding cell line screening, optimization of cultivation media, biosynthesis pathways engineering, usage of elicitors, large-scale cultivation in bioreactor system, root culture, plant cell immobilization, and biotransformation (Peterhansel et al. 2008). Several strategies have been followed to improve yields of secondary metabolites in plant cell cultures. In the past years new approaches have been developed such as the culturing of differentiated cells, i.e., shoots, roots, and hairy roots, and induction by elicitors and metabolic engineering (Parsaeimehr et al. 2011). Cyanobacteria are an ancient group of photosynthetic prokaryotic organisms. The secondary metabolites produced in cyanobacteria play an important role in toxicity as iron chelators, protease inhibitors, growth inhibitors, as well as growth promoting properties and have been well documented (Prasanna et al. 2009; Yadav et al. 2011). Some metabolites such as microcystins, saxitoxins, or anatoxins are of global significance because of their toxicity while others display significant pharmaceutical potential (Sainis et al. 2010). It has been found that the cyanotoxins, anatoxin-a, microcystins, and cylindrospermopsin obtained from *Anabaena*, *Microcystis* and *Cylindrospermopsis* (respectively) show larvicidal activity with >50 % mortality. The aeruginosins particularly inhibit serine proteases (trypsin, chymotrypsin, thrombin, or elastase) and have been regarded as a promising drug candidate (Yadav et al. 2011).

Functional food products are nonhomogeneous food category; they are expanded to all segments of the food and drink market (Siro et al. 2008). Functional foods are predominantly found in the dairy, confectionery, soft drinks, bakery, and baby food market (Menrad 2003). The use of natural compounds as inhibitory agents for virulence factor production is a new approach to overcome increased antimicrobial resistance in pathogenic bacteria (Niraula et al. 2010). Medicinal plants are the most exclusive source of lifesaving drugs for a majority of the world's population. The utilization of plant cells for the production of natural or recombinant compounds of commercial interest has gained increasing attention over the past decades. The secondary metabolites are known to play a major role in the adaptation of plants to their environment and also represent an important source of pharmaceuticals (Sun et al. 2007; Jimenez-Garcia et al. 2013). Increasing epidemiological evidence associates diets rich in fruits and vegetables with reduced risk of heart disease, cancer, and other chronic diseases. A major benefit from such a diet may be increased consumption of antioxidants, including carotenoids, ascorbate, tocopherols, and phenolics. One phenolic fraction, flavonoids, are potent antioxidants and include compounds such as flavones, isoflavones, flavonones, catechins, and the red, blue, and purple pigments known as anthocyanins (Côté et al. 2010).

Dietary flavonoids (quercetin, kaempferol, and isorhamnetin) possess antiviral, anti-inflammatory, antihistamine, and antioxidant properties. They have been reported to inhibit lipid peroxidation, to scavenge free radicals, to chelate iron and copper ions (which can catalyze production of free radicals), and to modulate cell signaling pathways (Côté et al. 2010; Jimenez-Garcia et al. 2013). Production of peroxides and free radicals, which damage lipids, proteins, and DNA, has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation, and neurodegenerative diseases (Parkinson and Alzheimer). Flavonoids protect low-density lipoprotein cholesterol from being oxidized, preventing the formation of atherosclerotic plaques in the arterial wall. They stimulate enzymes involved in detoxification of cancerogenic substances and inhibit inflammation associated with local production of free radicals (Hounsome et al. 2008; Aires et al. 2009).

There are also reports about the usage of alkaloids in pharmaceuticals well as *Rauwolfia canescens* is a central nervous stimulator (Parsaeimehr et al. 2011). Dietary saponins cause a reduction of blood cholesterol, inhibit growth of cancer cells, and stimulate the immune system. Some saponins, such as sapotoxin, can be toxic for humans causing irritation of membranes of the respiratory and digestive tract, and increase the membrane permeability of red blood cells and urticaria (skin rash) (Satwadhar et al. 2011).

Plant glucoalkaloids, solanine, tomatine, and chaconine are called saponins; these are found in peas, beans, tomatoes, spinach, asparagus, onions, garlic, and potatoes. However, certain glucosinolates (glucoraphanin, glucobrassin, glucotropaeolin) and their breakdown products have been linked to a reduction in the prevalence of certain types of cancer (Hounsome et al. 2008; Aires et al. 2009; Jimenez-Garcia et al. 2013). A considerable pool of data shows a relationship between the risk of cancer and dietary carotenoid intake. A case–control study (2,706 cases of cancer of the oral cavity, pharynx, esophagus, stomach, colon and rectum vs. 2,879 controls) indicated that a high intake of tomatoes and tomato-based food, both of which are rich sources of lycopene, was strongly associated with reduced risk of digestive tract cancers, especially stomach, colon, and rectum (Rea et al. 2010). Also, resveratrol protects the cardiovascular system by mechanisms that include defense against ischemic-reperfusion injury, promotion of vasorelaxation, protection and maintenance of intact endothelium, anti-atherosclerotic properties, inhibition of low-density lipoprotein oxidation, suppression of platelet aggregation, and estrogen-like actions (Krzyzanowska et al. 2010).

1.9 Cheap Foods

Feeding the world requires local, national, and global solutions in view of the expected world population of 10 billion by 2050. Sustainability must characterize the socioeconomic infrastructures as well as the scientific and technical components. Today almost half the world population lives on $2 a day or less and although poor, most are not hungry (Hodges 2005). World Trade Organization (WTO) objectives for world trade include agriculture and food with the hope of supplying cheap food based on and generated by a capital intensive system. However, the current paradigm of producing ever cheaper food is found to be increasingly unsustainable and, if introduced globally, will create a variety of instabilities. A policy of feeding the growing cities of the developing world with imported food would remove domestic markets from poor farmers and would also risk large-scale famine when global food trade breaks down (Pretty et al. 2003).

The cheap food concept is a dinosaur left over from a previous age. It is big, powerful, anachronistic and consumes vast resources, harms the natural environment, emits negative influences far from its presence, is difficult to control and resists a quiet end. Disposing of the cheap food culture will require a colossal rethink by leaders and opinion makers and will not be easy because of the huge vested business interests in selling ever more food (Godfray et al. 2010a, b).

In most countries around the world, national food production capacity has been systematically dismantled and replaced by a growing capacity to produce agro exports, stimulated by enormous government subsidies to agribusiness, using taxpayer money (Rosset 2008). It is peasants and family farmers who feed the people of the world, by and large. Large agribusiness producers in most countries have an export "vocation." The result is that we now face one of the tightest margins in recent history between food reserves and demand, which generates both rising prices and greater market volatility. In other words, many countries no longer have either sufficient food reserves or sufficient productive capacity

(Godfray et al. 2010b). Another important short-term factor is the agro fuel boom. Agro fuel crops compete for planting area with food crops and cattle pasture. The major global price increase in the costs of chemical inputs for conventional farming, as a direct result of the high price of petroleum, is also a major short-term causal factor. Other factors of recent impact include droughts and other climates (Rosset 2008).

In Latin America and elsewhere, cheap food energy combined with low diversity and nutritional quality produces a pattern of obesity, particularly of women, in combination with household undernutrition (Doak et al. 2000). Early childhood malnutrition (fetal programming) probably increases susceptibility to diabetes and other conditions in later life (Popkin et al. 2001). Epidemics of chronic noncommunicable diseases can be expected to further accelerate in countries with aging populations.

1.10 Challenges to Reach the Proposed Vision for Food Production

1.10.1 Climate Change

Climate refers to an ensemble of both average and extreme conditions of its principal determinants including temperature, precipitation, humidity, wind velocity, radiation, and cloud cover observed over an extended period of time. Planet Earth has experienced numerous climate changes throughout its history. The last drastic climate change occurred between 10,000 and 12,000 BC, which turned out to be the defining moment in human history because it permitted the settling of agriculture. In this change, global temperature increased by ≈5 °C along with an increase in atmospheric concentration of CO_2 from 180 to 280 ppm. This increase in CO_2 concentration enhanced drastically the biomass production of C3 plants (e.g., wheat, barley, potato, sugarbeet) and moderately that of C4 plants (e.g., corn) (Lal 2013b).

Climate change has already caused significant impacts on water resources, food security, hydropower, and human health, especially for African countries, as well as to the whole world (Kang et al. 2009). The Intergovernmental Panel on Climate Change (IPCC) was set up jointly by the World Meteorological Organization and the United Nations Environment Programme (UNEP) to provide an authoritative international statement for scientific understanding of climate change. The IPCC has attributed the observed warming over the last century to anthropogenic emissions of greenhouse gases, especially carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) (Parry 2007). Understanding the potential impacts of climate change is essential if planned responses to avoid or minimize the negative impacts and taking advantage of positive impacts are to be successful (Taylor et al. 2013).

Climate change impacts on agriculture have been identified as potentially the most serious in terms of numbers of people affected and the severity of impacts on those least able to cope. Moreover, agriculture is identified as particularly vulnerable and particularly critical in terms of global impacts. Given that impacts are already occurring, and that expected future impacts have economic costs in the present day, adaptation is clearly necessary and inevitable. New challenges are emerging in terms of how to interpret the impacts of warming, how farming systems adapt or are adapted to these changes, and how near-term emissions mitigation requirements can take place in ways that are consistent with longer term adaptation plants (Wreford et al. 2010).

Climate factors constitute some of the main constraints on crop and livestock production and till recently have been assumed as exogenous and unchanging. While farming has a history of responding to changing conditions, whether they are economic, social, political, or climate-related, the potential increase in frequency and intensity of extreme climatic events, and other challenges posed by climate change, now gives rise to a need to reappraise the adaptive capacity of agricultural systems (Wreford et al. 2010).

Climate change may affect agriculture and food security by altering the spatial and temporal distribution of rainfall, and the availability of water, land, capital, biodiversity, and terrestrial resources (Hanjra and Qureshi 2010). The major climate factors contributing to these responses include increasing atmospheric carbon dioxide, rising temperature, and increasing extreme events, especially droughts and floods (Rosenzweig 2007). Climate change may influence the future occurrence of drought. Drought can have far-reaching consequences for agriculture, ecosystems, water availability, and society. Drought impacts can include water scarcity, crop failure, wildfires, and famines (Taylor et al. 2013). Moreover, observations have shown an increase in the severity and duration of droughts over larger areas since the 1970s (Parry 2007). Modeling by IIASA Fischer et al. (2007a) shows that future socioeconomic development and climate change may impact on regional and global irrigation requirements and thus on agricultural water withdrawals. Net irrigation requirements may increase by 45 % by 2080. Even with improvements in irrigation efficiency, gross water withdrawals may increase by 20 %. Global irrigation requirements with climate change will increase by 20 % above the reference base case scenario. The simulation shows that the global impacts of climate change on irrigation water requirements could be as large as the projected increase in irrigation due to socioeconomic development (Fischer et al. 2007a; Hanjra and Qureshi 2010).

Climate change could impact on rainfall and runoff and the availability of water for irrigation in many regions and countries in the world. A decline in rainfall along with an increase in temperature will increase crop water requirement due to high evapotranspiration while less rainfall will increase crop net irrigation water requirements. As a result, the already existing water scarcity problem will exacerbate in many regions and countries, and affect food production (Hanjra and Qureshi 2010).

Intergovernmental Panel on Climate Change Fourth Assessment Report (IPCC AR4) projected that mean annual precipitation will increase in the tropical regions and at high northern latitudes, and decrease in the subtropics. Meanwhile, precipitation may increase in one season, while it may decrease in another one. Over most parts of the globe, the mean annual runoff will increase; however, there are still some significant areas where runoff will decrease such as middle-east Europe, northern Africa, Central America, Southern Africa, major parts of southern and western Australia, and various areas of South America (Parry 2007).

Climate variability is one of the most significant factors influencing year-to-year crop production, even in high-yield and high-technology agricultural areas (Kang et al. 2009). Investments aimed at improving agricultural adaptation to climate change inevitably favor some crops and regions over others. Crop yields affected by climate change are projected to be different in various areas. Studies done to date show that negative and positive effects will occur both within countries and across the world and agricultural regions will be affected quite differently. In some areas crop yields will increase, and in other areas it will decrease depending on the latitude of the area and irrigation application (Kang et al. 2009). For example, some regions will experience increases in production and some will experience declines (Reilly et al. 2003). Also, developing countries are more vulnerable than developed countries. This is because most developing countries are located in lower latitude regions (some of which are indeed semi-arid), while most developed countries are located in the more humid mid to high latitudes. Thus, higher temperatures will intensify the evaporative demand of the atmosphere, leading to greater water stress, especially in semi-arid regions and as a result many crops will be pushed beyond their limits of optimal growth and yield. This suggests a divergence in vulnerability between these groups of nations (Rosenzweig 2007).

Farmers have dealt with climatic fluctuations since the advent of agriculture, and improving strategies for dealing with present climate extremes such as droughts, floods, and heat waves is an important way to prepare for climate change (Rosenzweig et al. 2001). The crop yield can be increased with irrigation application and precipitation increase during the crop growth; meanwhile, crop yield is more sensitive to the precipitation than temperature. If water availability is reduced in the future, soil of high water holding capacity will be better to reduce the frequency of drought and improve the crop yield (Kang et al. 2009). With climate change, the growing period will reduce, and the planting date also needs to change for higher crop production. Climate change can decrease the crop rotation period, so farmers need to consider crop varieties, sowing dates, crop densities, and fertilization levels when planting crops (Cuculeanu et al. 2002). So adaptation is a key factor that will shape the future severity of climate change impacts on food production. Although relatively inexpensive changes, such as shifting planting dates or switching to an existing crop variety, may moderate negative impacts, the biggest benefits will likely result from more costly measures including the development of new crop varieties and expansion of irrigation (Lobell et al. 2008).

Adverse effects of climate change on soil quality may be due to depletion of soil organic carbon (SOC) pool, decline in plant available water capacity (AWC), reduction in soil fertility and use efficiency of nutrients, decline in structure with attendant adverse impacts on vulnerability to degradation including crusting, compaction, accelerated erosion, and salinization. Climate change may also adversely affect water resources through alterations in the hydrologic balance, and high soil erosion may exacerbate nonpoint source pollution (Lal 2013a).

On the other hand, global warming has been speculated to increase yields due to the "fertilizer effect" of rising atmospheric carbon, but the impacts are likely to be net negative for poor countries. For example, global warming will reduce food production in countries closer to the equator (Droogers and Aerts 2005).

It is believed that climate change will increase water scarcity in the coming decades. With temperature increasing and precipitation fluctuating, water availability and crop production will decrease in the future. If the irrigated areas are expanded, the total crop yield will increase; however, food and environmental quality may degrade. And even if new supplies are added to existing ones, water might not be sufficient for increased food demand (Kang et al. 2009; Hanjra and Qureshi 2010).

1.10.2 Diminish in Water Availability

All organisms, including humans, require water for their survival. Although there is a lot of water on Earth, only a small amount of water is easily accessible (Oki and Kanae 2006). The Water Atlas reports a volume of 1,386 million Gm^3 of water on Earth, which consists of 97.5 % saline water and 2.5 % freshwater, most of which is stored as glaciers or deep groundwater. Out of 35 million Gm^3 of freshwater on Earth, approximately 30.5 % is available for human use. This amounts to 10.5 million Gm^3 water as groundwater and 0.13 million Gm^3 in lakes, soil, wetlands, etc. (Clarke and King 2004). The availability of freshwater for human use was mapped by the International Water Management Institute (IWMI). Irregularities in precipitation spatial distribution divide the globe into water-scarce and water abundant regions. The abundant water locations around the globe with less than 25 % water withdrawals were grouped by IWMI as regions of little or no water scarcity. If more than two-thirds of the available water was withdrawn in a location, this was accounted as physical water scarcity (IWMI 2008). The locations with more than 60 % withdrawals were labeled as approaching physical water scarcity.

Global demand for water has tripled since the 1950s, but the supply of freshwater has been declining. Half a billion people live in water-stressed or water-scarce countries, and by 2025 that number will grow to 3 billion due to an increase in population (Hanjra and Qureshi 2010).

Water availability will be one of the limiting constraints for crop production and food security. It is known that water resources play a vital role in human prosperity and crop productivity, being irrigated agriculture the dominant user of water, accounting for about 85 % of global water use and projected to double by 2050 (Molden 2007; Pfister et al. 2011). Irrigated area is expected to rise by a factor of 1.9 by 2050, while climate change is amplifying water stress by changing patterns of water availability in many parts of the world (Lobell et al. 2008). Because of that, the importance of cost analysis of water in the agricultural sector would increase.

The world's agriculture, hydroelectric power, and water supplies depend on different components of the hydrological cycle, including the natural replenishment of surface and groundwater resources (Kang et al. 2009). Fujihara et al. (2008) pointed out that water scarcity will not occur if water demand does not increase; however, if the irrigated area is expanded under present irrigation efficiency rates, water scarcity will occur. Moreover, water availability is under threat from changing climate because of possible precipitation decrease in some regions of the world. In light of the uncertainties of climate variability, water demand, and socioeconomic environmental effects, it is urgent to take some measures to use the limited water efficiently and develop some new water resources (Kang et al. 2009).

The challenges of water scarcity are heightened by the increasing costs of developing new water sources, land degradation in irrigated areas, groundwater depletion, water pollution, and ecosystem degradation (Hanjra and Qureshi 2010).

Rosegrant and Cai (2002) estimated that under their baseline scenario, total global water withdrawals for agricultural, domestic, and industrial use will increase by 23 % from 1995 to 2025. The availability of sufficient water resources is one of the major crises with overarching implications for many other world problems especially poverty, hunger, ecosystem degradation, desertification, climate change, and even world peace and security (Khan and Hanjra 2009).

In China, where more than 300 cities already are short of water, these shortages are intensifying (Khan et al. 2009). Water resources, critical for irrigation, are under great stress as populous cities, states, and countries require and withdraw more water from rivers, lakes, and aquifers every year (Hanjra and Qureshi 2010).

Recent flooding and heavy precipitation events worldwide have caused great damage to crop production (Rosenzweig 2007). If the frequency of these weather extremes were to increase in the near future, the cost of crop losses in the coming decades could rise dramatically. On the other hand, The Fourth assessment report (AR4) from the IPCC projected that drought-affected areas are likely to increase in extent in the future, with Europe, the Mediterranean, and southern areas of Australia at particularly high risk in the summer months (Gosling et al. 2011). Moreover, significant increases in drought have also been projected for West Africa, Central Asia, Central America, western Australia, the Middle-East, Indochina, and mid-latitude North American regions (Hirabayashi et al. 2008; Sheffield and Wood 2008; Sillmann and Roeckner 2008).

In the twenty-first century, global agriculture has met the new challenge, namely to increase food production for the growing population under increasing scarce water resources, which can be achieved by improving crop water productivity (Kijne et al. 2003).

Tebaldi and Lobell (2008) applied probabilistic methods to demonstrate that projected changes in temperature and precipitation negatively affect global crop yields by causing a decrease in yield of about 9 % (with 95 % probability intervals of 1.7–17 %) for barley, of 13 % (5–25 %) for maize and of 5 % (1–10 %) for wheat. So water for agriculture is critical for future global food security. However, continued increase in demand for water by nonagricultural uses, such as urban and industrial uses have put irrigation water demand under greater scrutiny and threatened food security (Hanjra and Qureshi 2010). Continued increase in demand for irrigation water over many years has led to changed water flows, land clearing, and therefore deteriorated stream water quality. Addressing these environmental concerns and fulfilling urban and industrial water demand will require diverting water away from irrigation. This will reduce irrigated area and its production and impact on future food security (Hanjra and Qureshi 2010).

Water productivity is a concept to express the value or benefit derived from the use of water and includes essential aspects of water management such as production for arid and semi-arid regions (Kijne et al. 2003). Increasing water productivity means either to produce the same yield with less water resources or to obtain higher crop yields with the same water resources (Zwart and Bastiaanssen 2004).

1.10.3 Few Fields for Agriculture

Land-use intensity is one of the most significant forms of land cover modification, and can have a major detrimental impact on terrestrial and aquatic ecosystems. Agricultural land transformation and occupation have direct ecological impacts on sites as well as on the surrounding landscape. Generally, crop production deprives the land of most ecological value.

Many developed countries are experiencing environmental pollution due to intensive agricultural activities, including intensive crop and livestock production. Intensive agricultural activities have been identified as the major sources of nonpoint source pollutants and are known to alter and impact the quality of the receiving water bodies (Zhang et al. 2012).

There are already 3.5 Gha (Gigahectare = 109 Ha) or 23.5 % of the total land which has been degraded to some degree (Bai et al. 2008) by a range of processes such as accelerated soil erosion (by water and wind), salinization, nutrient/elemental imbalance, depletion of soil organic C (SOC), and other processes which undermine the physical, chemical, and biological quality of soil (Lal 2013b). Urbanization, surface sealing, and brick making are other factors already affecting 300 Mha worldwide (Lal and Augustin 2011).

Over the 50-year period between 1961 and 2011, land area under agriculture increased modestly at the rate of 8.2 million ha for agricultural land, 6.3 for grazing land, 1.9 for cropland, and 3.0 for irrigated land. But in the recent years, cropland has been reduced by 13 % and pasture by 4 %. According to the Food and Agriculture Organization (FAO), world agricultural production growth is expected to fall by 1.5 % per year to 2030 and then a further reduction by 0.9 % to 2050, compared with 2.3 % growth per year since 1961 (Bruinsma 2003).

Food demands of the 7.2 billion population of 2013 are presently met through utilization of 4.9 gigahectare of land under agricultural production or 33.1 % of the total land area of 14.8 Gha. However, only 10.0 Gha is suitable for production of crops and/or livestock. The remaining 4.8 Gha (32.4 % of the total land area) is too dry, too hot, too cold, too shallow, or too steep. Moreover, land area available for agriculture is dwindling, and being degraded, eroded, salinized, contaminated, polluted, sealed up, and converted to nonagricultural uses (e.g., urban, recreational, industrial, biofuel) (Lal 2013b).

1.10.4 Necessary Increasing in Food Production

Food security is defined by the FAO (2002) as a "situation that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life." This definition involves four aspects of food security, namely food availability, food stability, food access, and food utilization (Schmidhuber and Tubiello 2007). Food security is increasingly important for humans all over the world. Food availability and food quality still are the big challenges for scientists due to changing climate. Further research on food security needs to integrate population, crop production, climate change, and water availability (Kang et al. 2009).

Coping with population growth as well as additional per capita food demand represents a major challenge in feeding humanity in the future. The world average caloric intake of about 2,800 kcal per person per day in the year 2000 is judged adequate for average activities. However, there are still about 570 million people living in countries with an average of less than 2,200 kcal per person per day, which is considered the minimal amount to meet the basic nutritional needs.

The high and widening inequality and income gap between the rich and the poor is a serious concern; though it is amazing that while 1 billion people are hungry in the developing world, a significant proportion of the population in the developed countries is obese (Barrett 2010).

Achieving food security under climate change is a complex public policy. Global food security threatened by climate change is one of the most important challenges in the twenty-first century to supply sufficient food for the increasing population while sustaining the already stressed environment (Kang et al. 2009). Alcamo et al. (2007) evaluated present and future climate scenario impacts on food security and water availability in 2020 and 2070 and provided some measures to enlarge potential crop production such as diversifying crops and expanding the rainfed and irrigated agriculture areas.

Current global food production comes from 1.5 billion ha of cultivated land, representing 12 % of the total land area. About 1.1 billion ha is rainfed with no irrigation systems. Thus rainfed agriculture is practiced on about 80 % of world's physical agricultural area and generates about 60 % of the world's staple food. The other 40 % was supplied by agricultural land cultivated through irrigation which only represents 19 % (Evenson and Gollin 2003; FAO 2010; Molden et al. 2010).

Pfister et al. (2011) illustrated the potential consequences of increasing agricultural production. Their study reveals possibilities to decrease the specific environmental impacts with good agricultural practice and well-distributed expansion and/or intensification on existing cropland. Also, they found that in order to feed the future generations, it seems necessary to develop agriculture in developing countries (particularly in Africa and parts of South America). But this needs to be done wisely because land and water resources will become increasingly stressed. Khan and Hanjra (2009) reviewed water management and crop production for food security in China, and pointed out that it is necessary to integrate climate, energy, food, environment, and population together to discuss future food security in China, and in the world as well.

The severity of the water crisis has prompted the United Nations (Watkins 2009) to conclude that it is water scarcity, and not a lack of arable land that will be the major constraint to increased food production over the next few decades. Australia is one of the major food-producing and land abundant countries but recent drought reduced its agricultural and food production substantially (Goesch et al. 2007). Drought in other food-producing countries such as parts of the United States of America and Europe is regarded as one of the major factors that contributed to the global food price crisis of 2008 (Piesse and Thirtle 2009).

Nevertheless, water scarcity remains the primary constraint to global food production. Reduction in irrigation water will cause decline in agricultural and food production. Major food-producing areas such as the Punjab of India and Pakistan, and the central and northern areas of China suffer from the depletion of aquifers and the transfer of water from irrigation to growing cities, with implications for food security. While irrigation almost always doubles productivity (Hanjra et al. 2009; Namara et al. 2010), higher energy and fertilizer prices present complex issues to these smallholder's irrigated systems. Loss of productive land to urbanization, and water logging and salinity are critical constraints. For example, in Indonesia in the last 5 years, about 1 million ha of farmland has been lost to urbanization due to industrial and infrastructure development (Halim et al. 2007).

A key challenge facing agriculture in the twenty-first century is how to feed a world with a continuously growing and increasingly affluent population which is projected to increase to about 9 billion by 2050. This will increase the demand for irrigation water to meet food production requirements and household and industrial demand. In response to population growth and rising incomes, worldwide cereals and meat demand has been projected to increase by 65 and 56 %, respectively (Hanjra and Qureshi 2010). The world demand for cereals was 1.2 billion tons in 1974, 1.84 in 1997, and is projected to be 2.50 billion tons in 2020. The global demand for meat was 109 million tons in 1974, 208 in 1997, and is projected to be 327 in 2020 (Rosegrant and Cai 2002). The rate of increase in food demand is expected to be greater in developing than developed countries. Due to strong economic growth, millions of people will buy diets far richer in protein, in the cases of China and India, three to five times richer.

World agriculture is at a crossroads and has limited resources. It must produce more from less per capita land and water resources and under changing and harsh climate (Vermeulen et al. 2012). FAO mentioned that biotechnology can be an approach to improve food security and reduce the environmental pressure (Bruinsma 2003). Meanwhile, modified crop varieties resisting drought, water logging, salinity, and extreme climate, can expand the crop planting area such as in the degraded soils, consequently, to increase food availability in the future. Feeding a growing, urbanized, and affluent population in a rapidly globalized world will be a global challenge. Thus unprecedented global cooperation will be inevitable in sustaining food production and improving global food security (Hanjra and Qureshi 2010). Additionally, future food supply will be determined by prudent management of the global agricultural resources and smart investments in technologies along with reforms in institutions and policies to achieve sizeable increase in food production (Herrero et al. 2010).

1.11 Current Options to Overcome the Challenges

1.11.1 Genetic Potential in Plants

Developing crops that are better adapted to abiotic stresses is important for food production in many parts of the world today. Anticipated changes in climate and its variability, particularly extreme temperatures and changes in rainfall, are expected to make crop improvement even more crucial for food production (Varshney et al. 2011).

The objective of plant breeding for stress environments is to accumulate favorable alleles that contribute to stress tolerance in a plant genome. Genes that confer stress resistance can be sourced from germplasm collections, including wild relatives of crops that are held in gene banks or organisms that currently live in habitats of water deficit or excess, extreme temperature, and salinity that have evolved to cope with those conditions (Nevo and Chen 2010; Varshney et al. 2011).

Superior genes or alleles where they have been identified in the same species can be transferred to elite genotypes through molecular breeding (MB). Molecular plant breeding has the potential to deliver improvements, once the component traits and the genes underlying these traits have been identified. These can be incorporated into new cultivars using conventional or biotechnological tools (Parry and Hawkesford 2012). Moreover, by using an approach such as genetic engineering (GE), there is no barrier to transferring useful genes or alleles across different species from the animal or plant kingdoms. Several key approaches for improved crop productivity in an environment with high temperature, high CO_2, and high ozone have been used.

These included manipulating leaf photosynthesis, photosynthate partitioning, total biomass production, and nitrogen use efficiency (NUE). NUE may be defined as the yield as a function of available nitrogen, and therefore improvements in yield will implicitly deliver enhanced NUE (Parry and Hawkesford 2012). Improved NUE in crops should lead to reduced fertilizer application and thereby lower emissions of greenhouse gases into the atmosphere (Varshney et al. 2011).

Significant advances have been made in the area of genomics over the past 10 years. Genome sequences are available now for many crop species. Furthermore, the advent of so-called “next-generation sequencing” (NGS) technologies has made it possible to sequence the transcriptomes or genomes of any species relatively quickly and cheaply (Varshney et al. 2009). These genome or transcriptome sequences coupled with genetic approaches can be used for identifying suitable genes conferring stress tolerance that can be deployed in crop improvement.

The molecular breeding approach involves first identifying quantitative trait loci (QTLs) for traits of interest, such as tolerance to abiotic stresses. Once the markers associated with QTLs were identified, the candidate QTLs or genes can be introgressed in elite lines through marker-assisted backcrossing (MABC), marker-assisted recurrent selection (MARS), or genome-wide selection (GWS) (Varshney et al. 2011).

The concern for global food security results from an impending imbalance between the supply and demand of the major food crops (wheat, rice, and maize). The predicted increase in temperatures, as well as decreased and more erratic rainfall as a result of global climate change, are projected to decrease global yields of crops. As a consequence, a need for a substantial acceleration in crop improvements is required (Parry and Hawkesford 2012). To do this, it is needed to increased productivity through plant breeding and improvements in agronomy to increase both the yield potential and the actual yields achieved by farmers. Genetic gains will be required in addition to agronomic improvements (Leegood et al. 2010).

Molecular breeding should best focus on constitutive traits that give good performance under drought stress. Traits related to root architecture and function are often difficult to assess under field conditions but are obvious targets for both selection and manipulation. Canopy traits that relate to sustaining high photosynthetic rate at low stomatal conductance are also important (Parry and Hawkesford 2012).

Knowledge gained from wide germplasm screening will inform on requirements for novel genes/alleles to be introduced through nonclassical means, such as gene transformation. Mutagenesis has been widely used to generate new variations in genomes. Mutations may be generated by irradiation or chemical treatment; the changes can result in large-scale deletions of DNA, or may only involve point mutations. While most often the performance of mutants is generally inferior to the wild type, occasionally lines with improved performance with respect to any trait can be selected. Moreover, mutagenesis also has the advantage over RNAi and similar methods of being able to target individual members of gene families and therefore potentially target gene function in specific tissues and at specific times (Parry et al. 2009).

1.11.2 Trying to Control Climate in Plant Production

Agriculture, broadly speaking, is the activity in which the farmer attempts to integrate agroecological factors and production inputs for optimum crop production. Greenhouses are one of the examples of modern agriculture that has emerged with the perspective of growing any plant in any place at any time by providing suitable environmental conditions inside them. This technology allows to optimize crop production in areas or periods of the year not appropriate for open field cultivation (Scarascia Mugnozza 1995). The greenhouse surface area nearly doubled from 1980 (150,000 ha) to 1995 (280,000 ha) (Zhang 2003). It is stated that the production per cultivate unit area of a greenhouse is higher than that in the field. For example, the production per cultivated unit area of a greenhouse tomato crop (>50 kg/m^2) is 10 times superior to that of a field crop.

Greenhouses are spaces covered with transparent materials, large enough to let plants grow under a partial of fully controlled environment. Through the manipulation of actions like heating, ventilation, and CO_2 enrichment the environment inside the greenhouse is different from the outside. Their presence will be considered a very common element of agricultural activities in the near future because they enable to grow crops by overcoming adverse weather conditions. Due to the adequate environmental conditions for plant growth and development, greenhouse protected agriculture could face the challenge to increase food production considering the growth of global population. Greenhouses are presented as a good alternative to tap water use and to protect crops from sudden temperature changes and adverse conditions such as wind, rain, pests, animals, and pollution. Inside them are created artificial microclimate conditions that allow cultivation in any season and create optimal levels of solar radiation, temperature, humidity of air and soil (Pawlowski et al. 2009), thereby producing higher yielding crops in open field cultivation (Mózner et al. 2012).

Despite their geographic location, greenhouse climate control systems consist of fundamental control components that can provide a greater or lesser amount of climate control, and subsequent plant growth and productivity depending on their design and complexity. As mentioned by Kittas et al. (2012), an accurate greenhouse climate control becomes more important at the end of the twentieth century, when the interest in topics on food, safety, and environmental pollution encouraged extensive research in the development of protected agriculture.

Temperature is the most significant variable of the greenhouse climate that needs to be controlled and it is usually controlled. A vast range of plants grown in greenhouses are warm-season species (Moretti et al. 2010). They are commonly adapted to average temperatures in the range 17–27 °C, with lower and upper temperature limits of 10 and 35 °C, for this reason in order to maintain adequate temperature inside the greenhouse, both heating or cooling systems are required. The second important variable to be controlled in the greenhouse climate is humidity. It is commonly expressed in terms of relative humidity. Relative humidity within the range 60–90 % is suitable for plant growth. Values below

60 % can cause water stress. A low-relative humidity may happen during ventilation in arid climates, or when plants are young with small leaves. On the other hand, serious problems can occur if the relative humidity exceeds 95 % for long periods because this favors the rapid development of fungus diseases.

Cooling and heating methods/systems to control greenhouses environment may include natural ventilation, shading screens, whitewashing, force ventilation, evaporative cooling (fogging, sprinklers, wet pads), piping hot, and hot air generation (Baille 2004). The option to be used for control of climate inside a greenhouse depends on the investment grade, the margin between the increase in crop value, and the cost.

Greenhouses provide many advantages as well as several disadvantages. The former are related to the harvest of high-quality agricultural products, increases in volume production, obtaining of off-seasonal products and pest and disease attacks are reduced to a minimum. The latter include high initial cost, high cost maintenance, and specialized operation to effectively control climate inside the greenhouse.

Much research is currently performed in order that in future greenhouses, control can be made by plant monitoring methods through image analysis which simulates the action of the greenhouse grower in evaluating the physiological processes and the plant growth stages. Also, relevant studies are carried out related to establishing setting points for the greenhouse microclimate control taking into account plant processes such as transpiration and photosynthesis that vary with the day and vegetative state of the plant. Advantages and disadvantages of control theories applied in greenhouse climate control systems are summarized by Galvan et al. (2012).

Horticulture in a protected greenhouse environment is increasing rapidly and it is consequently increasing in its economic and social importance. For this reason, the development of better greenhouse cover material, crop substrates, accurate ways to achieve appropriate climate conditions to ensure high-yield, and quality crops reducing energy consumption will continue to be topics of research, since they are closely related to the future of agriculture. They are related to the manner in which food products are going to be produced in order to satisfy the food requirements related to quantity and quality of an increasing population.

1.11.3 Strategies for Increasing Yield in Fresh Weight and Phytochemicals

At this point, it is necessary to define some important concepts that many times are misunderstood. To have this common frame of concepts will facilitate the understanding of the subsequent paragraphs.

Phytochemicals a variety of compounds made by plants. Chemical compounds from naturally occurring in plants. They are responsible for the odor, taste, and color of plants and act as a natural defense system against disease and infection. Phytochemicals include a wide range of chemical structures with diverse physiological functions such as carotenoids, phenolic compounds, glucosinolates, saponins, sulfides, phytosterols, phytoestrogens, monoterpenes, and protease inhibitors.

Bioactive a substance or a compound that has a biological activity on a living organism. The concept is usually only associated with positive effects on an organism. They are extranutritional constituents that usually occur in small quantities in foods and could display antimicrobial, antioxidant, anti-carcinogenic properties, etc.

Secondary metabolite chemicals produced by plants for which no role has yet been found in growth, photosynthesis, reproduction, or other “primary” function. It can serve as a signal molecule to attract pollinators and seed-dispersers, or mediate pathogenic, parasitic, or symbiotic interactions.

Nutraceuticals commodities derived from foods, but are used in the medicinal form of pills, capsules, potions and liquids, and again render demonstrated physiological benefits.

Functional food products that resemble traditional foods but possess demonstrated physiological benefits.

Conventional agricultural practices farming system that alter or change the natural environment through tilling of the soil and installation of irrigation systems. It uses inorganic fertilizers, pesticides, one crop. It has two objectives: maximize crop yield and earnings.

Organic agricultural practices system of farming that enhances biodiversity and soil biological activity. The use of off-farm inputs and on management practices is minimum. It is only allow use of organic amendments or organic fertilizers.

Integrated agricultural practices in this practice the use of pesticides and chemicals is limited. There is a sober-headed application of biotechnological, biological, chemical treatments, culture practices, and plant selection.

Elicitors chemicals or biofactors that can induce physiological changes in a target living organism. They can trigger physiological and morphological responses, as well as phytoalexin accumulation (Zhao et al. 2005).

It is expressed by the Royal Society that “food security is one of this century’s key global challenges” (Davies et al. 2009). The world population is estimated to increase up to 9 billion by 2050. Consequently demand for food will rise. As plants feed humans, either directly or indirectly, it remains a clear challenge to define how agricultural production could encourage health for all people. During the past years, the primary objective of agriculture has been to increase yield in order to feed a growing population; notwithstanding, the challenge is not just a matter of increasing crop yield, but also the amount of bioactive compounds in agricultural products (McGloughlin 2010).

Agricultural practices have usually focused on providing to plants all the necessary conditions (temperature, radiation, humidity, fertilizers, etc.) to increase yield (Bennett et al. 2012); however, these measures have depressed the synthesis of secondary metabolites since these products are produced during conditions of stress (Frost et al. 2008). Plants are not only a source of carbohydrates, proteins, and fats for food but also they are a valuable source of a wide range of secondary metabolites (Gry et al. 2007). These metabolites are needed by plants to defend themselves in a hostile environment (protection against predation, protection against fungal and bacterial diseases, or against adverse climatic conditions), but also they are useful to herbivores like humans, because they have beneficial effects on health (Rea et al. 2010). For this reason, phytochemicals with bioactive properties are attracting increased interest from consumers. Hence, improvements in agricultural practices, as mentioned before, should focus not only on yield, but also on the maintenance and/or augmentation of bioactive phytochemicals present in plants.

Epidemiological studies have demonstrated an inverse association between fruit and vegetable intake and chronic diseases (Alwan 2011). Some secondary metabolites have been indicated to be responsible for this observed protective effect. Nevertheless, population studies have demonstrated an inadequate consumption of fruit and vegetable. To increase consumption of fruit and vegetables has been a public health effort for years, but with minimal success. As the amount of fruits and vegetables does not seem to increase, it results in an approach that the development of fresh product containing a greater concentration of phytochemicals with biological activity. Controlled greenhouse environments provides an opportunity to modify not only the concentration of phytochemicals in fruit and vegetables but also the yield (Kubota et al. 2006). However, further knowledge to improve this practice is still needed.

Although the presence of bioactive compounds in vegetables and fruits depend both quantitatively and qualitatively on their genetic bases (Hervert-Hernández et al. 2010), they could be increased through modifications of environmental conditions and crop management strategies (fertilization, efficient water management, and techniques such as grafting). It is known that even the maturity state of a fruit or vegetable, harvest time, postharvest storage, and processing could affect the amount of secondary metabolites present in agricultural products (Ghasemnezhad et al. 2011).

Fertilization and agronomic condition such as organic and inorganic soil composition are relevant factors that affect not only crop yield, but also quality (Martínez-Ballesta et al. 2008). For example, sulfur and nitrogen fertilization has an impact in the plant glucosinolate content. An increased sulfur supply has been shown to result in higher levels of total glucosinolates. High N supply delayed the accumulation of phenolic compounds. Marín et al. (2008) report that sweet peppers grown in a greenhouse in a soil-less system showed similar or even higher concentrations of antioxidant compounds (vitamin C, provitamin A, total carotenoid, hydroxycinnamic acids, and flavonoids) than organic peppers. Chassy et al. (2006) indicate that no cropping systems differences exist between organic and conventional managed bell pepper, nonetheless it is also mentioned that these differences are difficult to establish due to uncontrollable conditions such as region, climate, soil quality, occurrence, and prevalence of pests and diseases. Conversely, Kim et al. (2010) reported in both green and red fruits, the ascorbic acid content in the organically grown hot pepper (OG) was significantly higher than the conventionally grown hot pepper (CG). Also, the content flavonoids (apigenin, luteolin, quercetin) and total phenolics in OG was typically higher than in CG. Organic practices also increased the ABTS radical-scavenging of red fruits. Mitchell et al. (2007) established similar findings in a 10-year comparison of the OG and CG on the flavonoid content in tomatoes.

Organic manure and mineral fertilizers have a positive effect on the antioxidant, polyphenols, and total carotenoid content, nevertheless, with the association of the two fertilizers major results are obtained. Combinations using doses of nitrogen, phosphorus, and potassium of 15, 30, and 45 kg/ha, respectively, associated with an annual or biennial fertilizer of 20 ton/ha gave the highest values of total antioxidant, polyphenolic, and carotenoid concentrations in Orange-fleshed sweetpotato tubers (Koala et al. 2013). It is postulated that fertilization with growth-limiting nutrients will lead to decreased concentrations of carbon-based secondary metabolites. This statement is known as the carbon/nitrogen balance (CNB) hypothesis (Bryant et al. 1983). Some studies have followed the predictions of this hypothesis while others have failed (Millard and Way 2011).

Roussos et al. (2011) stated that apricot cultivar subjected to fruit thinning during pit hardening, enhanced the morphological traits of apricot fruits as well as their phytochemical content. Biotic and abiotic stress could increase the presence of secondary metabolites in plants. Navarro et al. (2006) indicate that peppers collected in the red state presented an increase in antioxidant activity, total phenolics, ascorbic acid, lycopene, and β-carotene when moderate-saline (15 mM) irrigation water is used. It has been found that the content of β-carotene in tomato increased as the quality of irrigation water decreased. Decreased irrigation caused the glucosinolate content to double in broccoli. Deficit irrigation strategies are tools for managing plant growth and improving fruit quality and water use efficiency, while maintaining yields. Stefanelli et al. (2010) investigate the effect of minimal water use in a wide range of crops concluding that by carefully manipulating nutrient elements in horticulture it is possible to obtain both fruit quality and yield.

Many studies indicate that plants exposed to high or low (29–33 or 11–16 °C), rather than optimal intermediate growth temperatures (21 °C), produce the highest levels of glucosinolates (Björkman et al. 2011). Other factors that impact the plant's secondary metabolite content are the light intensity (irradiation), photoperiod, and light quality (wavelength). Plants have developed protective mechanisms against damaging elevated doses of UV radiation (280–315 nm) and extreme light intensities. UV-B radiation impacts on the levels of secondary metabolites like phenolic compounds, terpenoids, and alkaloids. Long periods seem to have a positive effect on glucosinolate content. Broccoli sprouting in light had higher total glucosinolate levels in roots and shoots when compared to the ones in darkness (Pérez-Balibrea et al. 2008).

Additionally to environmental factors, plants are subjected to biotic stress such as herbivore and pathogenic attacks. Perception, transduction, and propagation of

Table 1.2 Influence of some environmental factors on the production of secondary metabolite

Stress factor	Plant species	Secondary metabolite	References
Salinity	Pepper	Lycopene Phenols	Navarro et al. (2006)
Radiation	Pepper	Flavonoids	Sarghein et al. (2008)
Electrical conductivity	Tomatoes	Lycopene	Wu and Kubota (2008)
Heat shock, chilling, radiation	Lettuce	Phenolic compounds	Oh et al. (2009)

stress signals lead to the production of a great amount of secondary metabolites due to the activation of different defense pathways (Ncube et al. 2012).

Application of pre-postharvest elicitors can trigger distinct changes in the secondary metabolism of plants. The effect of elicitors in plant's metabolism is reviewed in the next section.

In summary, since plants are sessile organisms, they cannot escape from extreme environmental conditions, thus they have evolved complex mechanisms in order to adapt to the conditions to which they are exposed (Ramakrishna and Ravishankar 2011). Secondary metabolites are an integral component of the plant adaptive mechanisms. The relation between environment and agronomic practices on plant secondary metabolites may be direct or indirect, cultivar and crop specific, and short-lived or sustained. However, further knowledge of plant metabolism is still needed, because currently, although possible, it is a difficult issue to achieve both yield and phytochemical production (Martínez-Ballesta et al. 2008) (Table 1.2).

1.11.3.1 Elicitors Application in a Space–Time Manner in Plants

The first idea for the use of elicitors in agriculture was related to the increasing of plant resistance to pathogens (Boughton et al. 2006), however, it was found that they also increase the plant secondary metabolites because they mimic various stresses encountered by a plant. Consequently, they are seen as an alternative for obtaining plants with improved health properties (Ruiz-García and Gómez-Plaza 2013) and as a way to develop sustainable agriculture production; taking into account not only a minimized use of pesticides but also their use in crops could improve consumer's health (Capanoglu 2010).

Plant defense mechanisms are based on prevention, tolerance, or resistance (Thakur 2007). These mechanisms are activated in different ways, depending on the type of pathogens attacking the plant (or biotrophs appearing necrotrophic) (Jalali et al. 2006). Such mechanisms may be constitutive or induced. The first provides protection of initial invasion of a pathogenic agent by physical barriers or preformed biochemical. Whereas induced resistance is only activated as a response to pathogen attack (Cruz-Borruel et al. 2006). Activation of an effective defense by plant is based on speed with which it can recognize the pathogen (Fornoni et al. 2004). This is achieved through the activation signals induced by nonspecific elicitors and specific compounds that are first recognized by the plant to activate signaling cascade and therefore defense response (Ebel and Cosio 1994).

Chemical elicitors are compounds, which activate defense in plants through various biosynthetic pathways depending on the compound used; nevertheless it is possible to elicit the plant due to physical conditions such as UV and gamma radiation and high and low temperature, among others. These topics were discussed in Sect. 5.3.

Depending on the type of attack, the plant activates different signaling pathways to synthesize a specific set of defensive compounds such as terpenes, phenolics, and nitrogen and sulfur containing compounds (Mazid 2011). Some physiological responses to elicitors are not directly defensive, but can also serve to defend plants indirectly (Rohwer and Erwin 2008). It is commonly known that the salicylic (SA) pathway is initiated by biotrophic pathogens, while jasmonic acid (JA) and ethylene (ET) response is initiated by necrotrophic pathogens, although the final defense response may involve crosstalk among these biosynthetic pathways. Commonly, chemical elicitors include salicylic acid, methyl jasmonate, ethylene, and chitosan. Natural and synthetic compounds (carbohydrate polymers, lipids, glycopeptides, and glycoproteins) that can be used as elicitors have emerged due to the better understanding of plant signaling (Montesano et al. 2003), for example oxalic acid, calcium chloride, benzo(1,2,3)thiadiazole-7-Carbothioic Acid S-methyl Ester (BTH), potassium silicate, carrageenan, harpin, 2,6-Dichloroisonicotinic (INA), β-Aminobutyric acid (BABA) (Conrath 2009).

Elicitors could be delivered to the plant in different manners: fumigation, spraying, pellets, paste, and irrigation. They can be applied to the whole plant or to a selected part of it (pre or postharvest). To obtain fruit and vegetables with high concentration of phytochemicals, postharvest elicitor treatments might be used either singularly or in combination. Also, the concentration of the elicitor may differ as well as the growth stage of the plants at the time of elicitation and the contact time of elicitation (Rijhwani and Shanks 1998).

There is no consensus on these topics, since each plant family, genus, and species produces a characteristic mix of secondary metabolites, however, they have in common that elicitors induce plant signaling that serves as a guide to a series of intracellular events (e.g., production of ROS) (Shilpa et al. 2010), that finally lead to the expression of defense genes that are activated when the elicitor is recognized on plant by a receptor (protein), causing the release of defense strategies such as the production of secondary metabolites and plant volatiles. Jeong and Park (2005) state that further knowledge is needed about the mode of action of elicitors; consequently the effect of elicitation on a plant cannot be easily predicted. For this reason, it is common that elicitation methods are performed by trial and error.

It is indicated that the use of elicitors might result in a more resistant plant through the production of secondary metabolites, but their production reduces plant fitness (plant growth and reproduction). Nevertheless, other studies stated that it is possible to reconcile crop yield with the production of secondary metabolites with bioactive properties. García-Mier et al. (2013) discussed this option in a review.

Apart from inducing secondary metabolites when applied to plants, other advantages derived from the use of elicitors are the reduced environmental hazard due to their low toxicity compared with pesticides (Tripathi and Dubey 2004) and

Table 1.3 Examples of studies where crop yield and/or secondary metabolite production increased using elicitors

Elicitor	Plant species	Crop yield	Secondary metabolite	References
Carrageenan	Chickpea	Increased	Increased (total phenols)	Bi et al. (2011)
MJ	Lettuce, green beans, plums, and carrots.		Increased	Heredia and Cisneros-Zevallos (2009)
Prohexadione-calcium, benzoic acid, salicylic acid	Jalapeño, pepper	Increased	Increased	Ramírez et al. (2009)
Salicylic acid	Wheat	Increased		Tejeda et al. (1998)
MJ	Tomato		Increased	Chen et al. (2006)
Benzoic acid (AB) complex of poly (acrylic acid)-chitosan	Tomato	Increased		Benavides-Mendoza et al. (2007)
Hydrogen peroxide	Pepper		Increased	Bayoumi (2008)
Chitosan	Sweet pepper		Increased	Xing et al. (2011)
Ethyl-acetate MJ	Soybean		Increased	Eswaranandam et al. (2012)
Salicylic acid	Coriander		Increased	Puthusseri et al. (2012)
Laminarin	Pepper		Increased (capsaicinoids)	Gururaj et al. (2012)
Urea	Sweet pepper		Increased (anthocyanin/ flavonoids)	del Amor et al. (2009)
Nitrophenolates, gibberellic acid	Strawberry	Increased		Roussos et al. (2009)

that they represent an alternative to genetically modified (GM) plants. Some disadvantages may include a decrease in photosynthesis, reduced plant growth, and the cost of the products (Holopainen et al. 2009). Nevertheless, it is considered more knowledge of the mode of action of this compound could minimize these disadvantages.

To be sustainable, an activity must be viable from both economic and environmental points of view. Thus, the use of elicitors could become a viable strategy for the development of sustainable agriculture because when they are applied to crops, a reduction in the use of agrochemicals can be achieved (Mejía-Teniente et al. 2010). In addition, when they are applied at the right concentrations, the yields can be maintained or even improved. Here, it is not intended to go deep into the mechanisms of action of elicitors, but to present them as an alternative for sustainable agriculture. Information related to elicitor signal transduction is given by Mejía-Teniente et al. (2010), Zhao et al. (2005) and Bent and Mackey (2007) (Table 1.3).

1.12 Perspectives

1.12.1 Metabolites-Inducer Factors

Metabolism is the sum of all the chemical reactions that occur in an organism. Primary metabolites such as carbohydrates, proteins, lipids nucleic acids among others, are the end up molecules of carbon, nitrogen, and energy. These metabolites are required for the proper functions of cells and organisms. In contrast, plants produce molecules known as secondary metabolites, which once were thought to be the result of aberrant metabolism and possess no activity (Lewinsohn and Gijzen 2009). Nowadays, secondary metabolites are not only of commercial and industrial importance used as colors and scents, but also they are key ingredients in medicinals and nutraceuticals. It is recognized that they protect plants against being eaten by herbivores and against infection with microbial pathogens; they serve as attractants for pollinators and seed dispersing animals. Moreover, they function as agents of plant–plant competition and plant–microbe symbioses. Therefore, secondary metabolites are relevant in agriculture for plant defense purposes but their presence is also desirable in foods.

The difference between primary and secondary metabolites is not always easily to be made. Primary and secondary metabolites share many of the same intermediates and are derived from the same metabolic pathways. Primary metabolites are essential in order to fulfill daily nutrient requirements related to satisfy energy, regulation, and structural activities. Secondary metabolites usually are present in the plant in relatively low quantities and their production, as it was said before, may be restricted to particular families, genera, or even species.

Metabolites-inducer factors (MIFs) are conditions or compounds that enhance the production of primary and/or secondary metabolites in plants. Serrano et al. (2010) showed that applications of nitrophenolate increased the levels of glucose, fructose, ascorbic acid, citric acid, as well as total phenolics in sweet pepper. In a study by Nzanza et al. (2011) where *T. harzianum* and arbuscular mycorrhizal fungi (AMF) inoculation were used, it was concluded that these fungi inoculants have the potential to influence yield and nutrient content of greenhouse tomatoes. Folate content increased in coriander throughout elicitation (Puthusseri et al. 2012). Wang and Frei (2011) reviewed the impact of abiotic environmental stresses on crop quality. Here, they report that as a general trend stimulation of protein and antioxidant concentration or lower starch and lipid concentration occur, but these outcomes depend on numerous confounding physiological, environmental, and experimental factors.

The presence of both kinds of metabolites is of core relevance since they are required for the plant itself but also for their consumers. Consequently, further knowledge is needed that allows to carefully manipulate plant metabolism. Major knowledge of the direct and indirect causes that lead to the activation of metabolic pathways is still needed. It remains to be conclusive about what compounds can be used as MIFs, MIF’s time of application (at what stage to be used), site of applications, biochemical pathways activated. Also, it remains to elucidate as expressed by Lewinsohn and Gijzen (2009) the plant silent metabolism that is not silence, but is a pool of pathways that awake to be activated.

1.12.2 Use of Genotypes

Plant science is faced with a strong challenge: ensure yield security while shifting breeding goals in order to produce more with less. There is a predicted need to increase food production in a changing environment which affects agricultural yields. Therefore, it is urgent to improve the efficiency of crop production. Molecular plant breeding has the potential to deliver substantial improvements once the component traits and the genes underlying these traits have been identified. These traits are made available through genetic engineering or the mining of existing genetic variability. Moreover, yield enhancing genes have been the focus of intensive research during the last three decades or so and over the past 25 years, one new gene with an established phenotypic effect on crops has been identified every year on average using forward genetic approaches. Identified traits will be incorporated into new cultivars using conventional or biotechnological tools.

The first two classes of products of crop biotechnology, broad-spectrum herbicide-tolerant, and Bt-mediated insect resistance crops in corn, cotton, canola, and soybeans, have been widely adopted in the U.S., Canada, South America, India, and China because such traits have not been generated through classical breeding. By 2012, more than 10 % of the world croplands were growing transgenic crops, with an annual growth rate of 6 %. While 28 countries planted commercialized biotech crops in 2012, an additional 31 countries totaling 59 have granted regulatory approvals for biotech crops for import, food and feed use, and for release into the environment since 1996 (ISAAA 2012).

Several new developing countries are expected to plant biotech crops before 2015 led by Asia, and there is cautious optimism that Africa will be well-represented: the first biotech-based drought tolerant maize planned for release in North America in 2013 and in Africa by 2017; the first stacked soybean tolerant to herbicide and insect resistant will be planted in Brazil in 2013; subject to regulatory approval, Golden Rice could be released in the Philippines in 2013/2014; drought tolerant sugarcane is a possible candidate in Indonesia, and biotech maize in China with a potential of $\sim$30 million ha and for the future biotech rice which has an enormous potential to benefit up to 1 billion poor people in rice households in Asia alone (ISAAA 2012). Thus, genetic crop improvement is a reality and is necessary in order to increase production potential under water-scarce and climate change conditions. This will be an important avenue to improved food security over the next four decades and feed a growing human population.

1.12.3 Tools for Plant Study

Genomics deals with the discovery of all the sequences in the entire genome of a particular organism and since the success of the human genome project effort, several "omic" disciplines have emerged, with the goal of analyzing the components of a living organism in its entirety. Proteomics (related to the study of protein structure and function and what every protein in the cell is doing), epigenomics (the complete set of methylation alterations in the genome), transcriptomics (the study of the complete set of RNAs encoded by the genome of a specific cell or organism at a specific time or under a specific set of conditions), metabolomics (related to the study of the complete set of low molecular weight compounds in a sample) (Dixon et al. 2006). Lipidomics and glycomics are also included.

"Omics" sciences offer the opportunity to increase the knowledge on the fundamental biochemical basis of the things we eat. The research fields of molecular biology, biochemistry, plant physiology, and ecology have overlapped each other's boundaries as a result of the recent expansion in the interest on secondary metabolites. It has been possible to establish the variations in molecular mechanisms and metabolism underlying the diversity and production of these compounds in plants because they derive their synthesis from primary metabolism. The growing field of metabolomics complements information derived from genomics, transcriptomics, and proteomics in order to provide a systematic approach to the study of biological systems. The origin of the enormous chemical diversity of plants (200,000–1 million metabolites estimated can be unveiled by these studies Hall et al. 2008). Furthermore, knowledge derived from these studies would be the basis for future application of plants' function to agriculture and other industries.

The metabolome represents the final omic level in a biological system. It reflects changes in phenotype and functions. For this reason its study is as important as gene expression throughout genomics, transcriptomics, and proteomics (Putri et al. 2013). The information obtained from these sciences has the prospective to produce a more complete picture of food composition and feed products, in order to optimize crop trait to enhance diet and health.

The most widespread strategies for addressing the study of omic sciences involve the use of gas chromatography combined with mass spectrometry (GC–MS), liquid chromatography combined with MS (LC–MS), and nuclear magnetic resonance (NMR)—and capillary electrophoresis (CE)–MS which can profile the impact of time, stress, nutritional status, and environmental perturbation on a great number of metabolites simultaneously resulting in massive, complex datasets (Álvarez-Sánchez et al. 2010). The importance of a correct addressing of this data is fundamental since metabolites affect food quality, food functional properties, and attributes such as taste and aroma, which will influence consumer acceptability.

In the recent years terms such as phytochemical genomics and nutrigenomics (Hall et al. 2008; Saito 2013) have emerged. The former investigates the genomic basis of the synthesis and function of plant metabolites. Meanwhile, nutrigenomics tries to link nutrition and genome. It plays an important role in correlating bioactive food components and disease prevention. Both sciences have in common metabolomics as an essential component. However, one of the major bottlenecks of current metabolomics is the sample preparation and identification of metabolite peaks inspite of advances in computational methods. Another challenge is related to the possibility for interpretation and application to an understanding of the biological system of the complex information generated (Hendriks et al. 2011). For this reason, procedure and technical improvements are expected not only in this area but in all the above-mentioned.

1.12.4 Plant Nutrition for Increasing Phytochemicals

Currently, agricultural practices are focused on the optimization of nutrient management through a better control of plant water and nutrient requirements to improve plant health and crop yield. Nutrient solution is a key factor of yield and quality of plants grown in soil but mainly in soil-less culture where the availability of nutrient is inherently restricted (Sevil Kilinc et al. 2007). Soil-less culture is commonly used in greenhouse cultivation. Therefore, it is essential to know some important aspect when nutrient solutions are prepared such as pH, electrical conductivity (which is determined by the total ion concentration), the balance between anions and cations, temperature, and dissolved oxygen (Herrera 1999).

The first nutrient solutions emerged in the nineteenth century. Some nutrient solutions widely used are Douglas, Hoagland, and Steiner solutions (Colinas-León et al. 2006). Although these solutions are used for a vast array of cultures it is important to determine suitable nutrient solutions for each plant species as well as to adapt the nutrient solution when soil culture is issued since this substrate can have its own nutrient input.

Plants are formed by many chemical elements. Traditionally, some of them have been considered essential for their growth and development (nitrogen, phosphorus, calcium, magnesium, sulfur, and iron). However, at the beginning of the twentieth century it was realized that some other elements are needed for the plants. These elements are called microelements because they are required in less quantities, in minimal concentration than the ones mentioned before.

Several studies in plant nutrition have been carried out using nutrient formulations. Commonly, the main nutrient concentration (anion or cation) is modified, whereas the other nutrients remain at a constant level. Nevertheless, it is necessary to go beyond: what other elements need to be included in nutrient solution formulations in order to improve plant growth. These elements could be required in very minimum quantities that at the moment are not considered in the formulations. Moreover, soil gives to plant not only inorganic nutrient but also it can be found a biotic community that enhances plant growth (e.g., plant growth promoting rizhobacteria) (Andrews et al. 2012). For this reason, the development of new total nutrient cocktail solutions should take into account these elements, the proportions to be used, interaction with the whole component of the nutrient solution, and characteristics of the substrate in what are going to be used, temperature, just to mention a few.

To unravel the mechanisms behind the production of plants and plant compounds under specific agricultural and climatic conditions, without compromising on crop yield, it is of great scientific, economic, political, and social interest since population will continue to grow in the coming years, and this population will increasingly want not only to add years to life, but also add life to years. The response required to the current agricultural challenge is not a lone ranger view but a holistic approach.

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Chapter 2
Mathematical Modeling of Biosystems

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Abstract Mathematics is an important tool for system modeling allows us to describe the behavior of a phenomenon or system in the real world, in particular biological systems. This chapter gives an overview of mathematical models, their construction, types of models, and examples of possible applications in biosystems models. Essential for building a model is determining its scope. In addition, the mechanistic and phenomenological mathematical models are described. Applications on fish biomass estimation, quality of fruits and crops are presented.

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2.1 Introduction

When we meet with the problem of describing the behavior of a real-world phenomenon or system, there is a need to resort to models. These models allow us to answer the question about important features and the behavior of the system studied under different conditions. There are different types of models such as verbal, mental, physical, and mathematical (Ljung and Gland 1994). In mathematical modeling, a number of assumptions translate into the language of mathematics. This has many advantages

1. Mathematics is a very precise language. This helps us to formulate ideas.
2. Mathematics is concise language, with well-defined rules for manipulations.
3. The mathematical results that have been proven over years are available to us.
4. Computers can be used to perform numerical calculations.

There are different mathematical models related to biochemical, physiological, and physical variables related to biosystems. Biosystems consist in any system of living creatures, however this term has been widely used for either plant or animal groups. Systems as constructed wetlands (CWs) have been recognized as effective means of “green technology” for wastewater treatment because their use of biological processes (assimilation by the plant tissue and microbial transformations); thus they may be also considered as biosystems (Kumar and Zhao 2011). Biosystem’s technology uses mathematical models applications in biomass and productivity forecasting. This can be accomplished by using the instrumentation systems for measuring variables related to plant production (Espinosa-Calderon et al. 2011; Gómez et al. 2005; Guzmán-Cruz et al. 2009; Guzmán-Cruz 2010; Millan-Almaraz et al. 2009).

Mathematical models can be based on several methods and principles such as steady-state mechanistic, temperature functions, Gaussian integration, stomatal conductance, nitrogen analysis, dynamic biological systems models, PAR, 3-D gradients of climatologic parameters, linear regression, nonlinear regression, and diffusion-reaction (Bernacchi et al. 2009; Boonen et al. 2002; Farquhar et al. 2001; Gómez et al. 2005; Hoffman et al. 2004; Lombardozzi and Sparks 2012; Müller et al. 2009; Murray 2002).

2.2 Building a Mathematical Model

Essential for building a model is determining its scope. The process by which a mathematical model can be use to solve real world situations is usually called the mathematical modeling process (Miwa 1986a). Typically the process consists of the following four steps: (1) mathematical formulation, (2) programming, (3) parameter estimation, and (4) model evaluation (Soltani and Sinclair 2012). Sometimes, the developers of crop models do not always explicitly proceed through each step; it is valuable to be clear about these steps. If each step is not fully developed, the resultant model may be seriously compromised if its use is attempted outside the context in which the developer visualized it. It is likely a more robust model will result from the modeling process that recognizes each of these stages (Haefner 2005; Sinclair and Seligman 2000). The most important stage in this process is the formulation stage, assumptions based on deliberate suppression or neglect of irrelevant details are set up. If these assumptions are not appropriately setup, the nature of the situation is distorted, and the problem cannot be solved correctly. The setting up of adequate assumptions can be considered as the most important decision in performing mathematical modeling. Therefore, it is necessary to consider the role of assumptions in mathematical modeling process (Miwa 1986b).

2.2.1 Definition of Objectives

Before starting a modeling project, it is important to be clear about the model objectives. This step determines the future direction of the project in two ways. At the beginning of the modeling process, the objectives of the modeling effort should be explicitly and fully defined. A clear statement of specific objectives is essential to define needs and nature of a crop model (Sinclair and Seligman 1996).

It is more likely that success will be achieved when the objectives are clear, modest, and tractable (Sinclair and Seligman 1996). Criteria for judging the acceptability of a model should be defined in relation to the model's objectives. It is possible to quantitatively define stopping rules, in terms of statistical criteria concerning model predictions relative to a sample of observations.

Based on the objective, a list of specific hypotheses to be included in the model is prepared. Initially, it may be useful to list the hypotheses in the form of words and sentences. Some of the following points need to be considered in identifying hypotheses to be used to construct the model: (1) Models need to have generality, (2) Hypotheses will require input data either as parameters or driving variables,

(3) The most useful models will be those that include parameters and state variables that can be readily determined by simple measurements or observations, (4) Existing hypotheses/models are a useful resource, but they need to be evaluated in view of objectives of the current model construction (Sinclair and Seligman 1996).

2.2.2 Quantitative Description of Hypotheses

Qualitative hypotheses need to be expressed as mathematical functions. Equations need to be developed to express how each hypothesis can be expressed in the model system. The interaction of hypotheses also needs to be taken into account, which often introduces a whole new layer of assumptions. Commonly, it is assumed that there is no interaction among hypotheses other than what has been explicitly defined.

The quantification of the model can be the most challenging because it requires a thorough knowledge of the system being modeled, and an understanding of the most relevant relationships. Additional functions can always be added to the model, but do these functions enhance the performance of the model for the stated objective? Often, the most critical phase of the model construction will be selection of the quantitative functions that sufficiently and efficiently describe the model component being modeled. Assembling equations without understanding and evaluating their relevance to the objectives overlooks a critical aspect of the modeling (Soltani and Sinclair 2012).

2.2.3 Programming

Once the hypotheses have been defined and quantified, the model is finally constructed into computer code. That is, the knowledge and insight about the system should have been captured and it should be a straightforward, even a trivial task, to translate the hypotheses into computer code. However, considerable care is required to accurately express the model in computer code. It is necessary to verify the computer algorithms and the codes are correct from mathematical relationships defined. Preparing computer programs usually requires debugging to eliminate the errors that arise during programming.

The program code can usually be organized in individual sections as represented in a flow diagram. In more expensive models, it is often useful to structure the code so that each section is placed into its own submodel. Placing parameter values for the various functions in their own separate, initialization section can facilitate the use of the model in simulations using different parameters. That is, code the functions in the model using parameter names, and then define all parameters at the beginning of the program. In this way, simulations of other conditions such as a different cultivar are facilitated by adjusting the relevant parameters in the initialization (Haefner 2005).

2.2.4 Estimating Parameters

There are two acceptable general methods for parameter estimation:

1. *Literature* If the value of a parameter has been defined in research previously published, then these data are a strong starting point. This source of parameters is desirable because the research has been subjected to the rigors of peer review and the results are available to everyone.
2. *Measurement* Experiments or observations need to be done by the person constructing the model. Special research protocols need to be established and executed to obtain information about the desired parameters. Careful statistical analysis of the results is needed to obtain parameters. This method is time-consuming, laborious, and expensive. Soltani et al. (2004, 2006) presented examples of parameter estimation procedures related to different aspects of crop growth and development.

Another method that is too frequently used for parameter estimation is “calibration” of parameters so that the final output of the overall models matches the expected results. That is, the model is tested using different values for a specific parameter, then values are chosen that provide the closest match to the observations of the major outputs. Adjustments in parameters to achieve closer matches with observations by the complete model are difficult because it is not clear what parameters need adjusting. A major problem is that parameter adjustments can compensate each other and the parameters diverge from how the plant system operates.

The major limitation of the “calibration” approach is that reduces the model to an exercise in empirically fitting the model to the observations under a particular set of circumstances. Parameters are adjusted to achieve end results rather than relying on understanding about how the plants are performing and defining specific functions for the individual processes.

Sometimes “calibration” is done be reserving a separate data set for the calibration procedure. This approach does not overcome the basic empirical problem in this approach. Success of “calibrated” parameters in matching a second set of data only indicates success in splitting the two datasets, so that they represent the same population of data. That is, success with a calibrated model only assures that the calibration dataset were adequate to empirically math the second set of data (Soltani and Sinclair 2012).

2.2.5 Model Evaluation

Every model should be evaluated with respect to transparency and robustness. Transparency refers to how easy it is to understand a model and robustness refers to how closely the predictions of the model match with observed outputs from the system. However, after the above-mentioned steps in constructing a model, the model can be run and its output can be compared with measurements from the system. A test of the model output against observations is especially necessary when a model is to be used in an application mode. The users of the model need to be given some notion of situations in which the model has proven useful, with a disclaimer for reliability in any other situations (Sinclair and Seligman 1996). Model evaluation should be done based on predefined criteria established in the objective-definition stage. If the predictions are reasonably matched with the measurements from the system, based on predefined criteria, the modeling process is complete and the model is ready to use. If the results of the model evaluation stage are not satisfactory, it is necessary to reconsider the hypotheses, equations, and the quantitative methods use dot construct the model. If alternative methods and equations are identified, then the model evaluation should be repeated. If predictions and the output are not still satisfactory, it is likely that there is some basic problem with the hypotheses. More experimental investigation is required and the modeling process should be set aside until observations allow improved hypotheses (Soltani and Sinclair 2012).

There are two critical criteria in evaluating the suitability of a model: transparency and robustness. Transparency means that the model parameters, flow diagrams, and code can be readily understood by those that were not involved in its development. As much as possible, the functions are stand-alone descriptions of processes in the plant and crop. Transparency is facilitated by a minimum number of empirical coefficients, and these coefficients can be independently observed and measured. Robustness means that the model produces simulation results that compare favorably with observation. The judgment of “favorable” will depend directly on the original objectives for the model (Teh 2006).

2.3 Types of Models

Mathematical modeling can be divided in two principal groups: mechanistic (white box) and phenomenological (black box) models. The white box models are deterministic and use physical modeling, thus they are explicative about the modeled system. Black box models, also called identification models, are direct descriptions of the data. Black box models have the disadvantage of not giving an explanation of the subjacent mechanisms. A combination of these two models results in gray box models.

2.3.1 Mechanistic Models

The development of a mechanistic mathematical model requires sufficient understanding of the physical, chemical, and biological processes that occur in a system and its use demands a proper validation. These kind of models are explanatory models, and they can be static or dynamic (Kita 2011). In addition, the study and description of a system involves two processes construction of mathematical models and numerical solution of the set of equations that describe the behavior of the system, through the use of a digital computer.

Mechanistic models are based on the assumption that the state of a system can be quantified and that changes in the state can be described by mathematical equations, equations of rate of change or differential equations. These models include several components: state variables, differential equations, parameters, and inputs. Normally, a state variable is a variable that appears in the accumulation term of adynamic balance of mass or energy. A state variable is a variable that can be quantified (at least conceptually) and it allows knowing the behavior of the system at all future instant in time (Kita 2011).

2.3.1.1 Modeling Phases

There are three phases in the process of the mathematical modeling (Ljung and Glad 1994):

1. The problem is structured.
2. The basic equations are formulated.
3. The state-space model is formed.

If the model is not too complex in terms of state variables, it can be used to design control systems for example optimal control strategies (Seginer and Ioslovich 1998; Van Henten 1994; Tap 2000) for the best growth, production, and crop quality.

The problem is structured

It is important to understand the general structure of the system (Ljung and Glad 1994). Thus, we need to answer the following questions:

- What signals are outputs and inputs?
- What happen in the system?
- What quantities are constants?
- What signals are internal variables?

When we have decided what variables in the systems are of interest and how they interact, then we attempt to divide the system into subsystems. This phase puts the great demands on the modeler because it requires the understanding of the intuition for the physical system. In addition, in this phase, the level of complexity and degree of approximation are determined (Ljung and Glad 1994).

The basic equations are formulated
We have to formulate quantitative relationships between the inputs and outputs of the system (Ljung and Glad 1994). We must use knowledge about the mechanics, physics, chemists, and biological processes occurred in the system.

The relationships between the system variables can be of different kinds: conservation laws and constitutive relationships. Conservation laws are well-established laws like physics, chemises, or mechanics laws can be used. On the other hand, constitutive relationships might be given by experimental data or can be simple formulas that describe the general character of the relationship (Ljung and Glad 1994).

A good way of formulating the basic equation of a subsystem is the following:

1. Write down the conservation laws that are relevant for the subsystem.
2. Use suitable constitutive relationship to express the conservation laws in the model variables. Calculate the dimensions of the different quantities as a check (Ljung and Glad 1994).

The state-space model is formed
In this phase, we need to choose which internal variables will be considered state variables. Express the derivative of each state variable as function of state variables $x(t)$ and inputs $u(t)$. Express the outputs $y(t)$ as functions of the state and input variables (Ljung and Glad 1994). This is,

$$\begin{aligned} \frac{\mathrm{d}}{\mathrm{d}t}x(t) &= f(x(t), u(t)) \\ y(t) &= h(x(t), u(t)) \end{aligned} \tag{2.1}$$

2.3.2 *Phenomenological Models*

2.3.2.1 Linear Regression

Measurement accuracy depends on the model used and on the knowledge of the studied process. The specificity of the studied system to particular applications simplifies the equations of the model (André 2013).

One example of a linear black box model is the measurement of photorespiration at the biochemical whole plant and atmosphere levels. It solves the fitting of oxygen (O_2) and carbon dioxide (CO_2) gas exchanges, avoiding complex equations and revealing the main factors of regulation of photosynthesis with its inseparable association with photorespiration (André 2013). Models as this one might be simple, but they represent a useful tool with a good approximation to understand the global relationships between plants and biosphere.

By observing the fitting of different variable curves related to the process, it can be deduced if such variable plays, or not, a crucial role in the regulation of the studied process.

2.3.2.2 Multiple Linear Regression

In many cases, y will depend on several independent variables such as $x_1, x_2, x_3, \ldots, x_n$. This case can be treated by the multiple linear regression (MLR) method.

For example, Sousa et al. (2010) used MLR to model the pollen and fungal spores, considering nonbiological pollutant concentrations, the daily mean of ozone (O_3) and PM_{10} concentrations and meteorological parameters temperature (T), relative humidity (RH), precipitation (PP), and wind velocity (WV) as predictors. As results the models for each pollen and fungal spore were different depending on the analyzed period, which means that the correlations identified as statistically significant cannot be, even so, consistent enough.

2.3.2.3 Nonlinear Regression

In many applications, the regression functions will depend in a nonlinear way on the regression coefficients.

Nonlinear regression models are important tools as many crop and soil processes are better represented by nonlinear than by linear models, depending on the objective and the application domain, different priorities are set when fitting nonlinear models and these include obtaining acceptable parameter estimates, and a good model fit while meeting standard assumptions of statistical models.

A nonlinear regression model was used for Aufhammeret et al. (2006), in order to model the role of brown clouds using a panel of yields and weather outcomes in India.

2.3.2.4 Logistic Type

There are indeed many phenomena in nature exhibiting a fast (virtually exponential) initial growth that then slow down after a certain point (where the curve reaches an inflection point) until a point of equilibrium or saturation of the system (carrying capacity). The logistic curve was introduced by Verhulst in the nineteenth century. The objective of Verhulst was to study population growth. In the 1920s, the interest in such method revived because of its excellence for modeling the development and evolution of many other growth phenomena (Román-Román and Torres Ruiz 2012). This type of models has been used in Ecology, Demography, and in Biology and Medicine, for the analysis of the growth of bacteria, tumors, and several species of animals and plants.

The deterministic logistic model is defined in terms of a differential equation where the linear constant defines the growth rate, whereas the quadratic term serves to inhibit or retard this rate. In this sense, the quadratic term is usually smaller than the linear one. When the population is small, the quadratic term, or inhibiting term, has little effect on the rising, so the population starts off with almost exponential growth. However, as the population increases, the inhibiting term eventually slows the rate of growth dramatically. Environmental fluctuations and lack of precision measurements represent ubiquitous noises that contribute with randomness to the logistic model (Li et al. 2011).

For processes whose mean function will be a logistic curve, it is possible to explicitly determine the transition density function, which allows to answer questions like the estimation of parameters through discrete sampling of paths after finding the likelihood from the transition density functions and the initial distribution. Such improvement enables the researcher to calculate the first passage time density, which requires an explicit form of such densities (Román-Román and Torres Ruiz 2012).

Logistic models offer the possibility of finding explicitly transition densities. This characteristic allows analyzing inference from the discrete sampling of trajectories. They also permit the estimation of functions, which allow predictive uses of the model. Finally, by studying first passage times, these models help in the determination of time variables used to locate when a preset condition is verified.

2.3.2.5 Statistical Approaches

The statistical approaching methods for modeling systems and processes consist, mainly, in the evaluation through, and application of, statistical techniques to datasets. Their only limitation is large number of datasets with different experimental conditions required for the application of any statistical packages. This limitation maybe seems to be small, but it usually is a challenging task.

Some examples of statistical techniques are: Levenberge-Marquardt (LeM) method, Nonlinear mixed effects (NLME), and first-order kinetics (Stein et al. 2007).

Since these models use a large number of datasets, it is commonly found that the magnitude of the generated coefficients varies strongly by species.

2.3.2.6 Time-Dependent Retardation Model

Time-dependent retardation models assume that there is a continuous variation in the studied process, which is common for every biosystem process, where time is a common degradation variable. However, it is important to note that the performance of this kind of models is often limited by a residual outlet concentration of certain variables in the studied system.

This kind of modeling has been considered to be one of the most efficient methods for designing CWs, because it allows a steady-state decrease in chemical

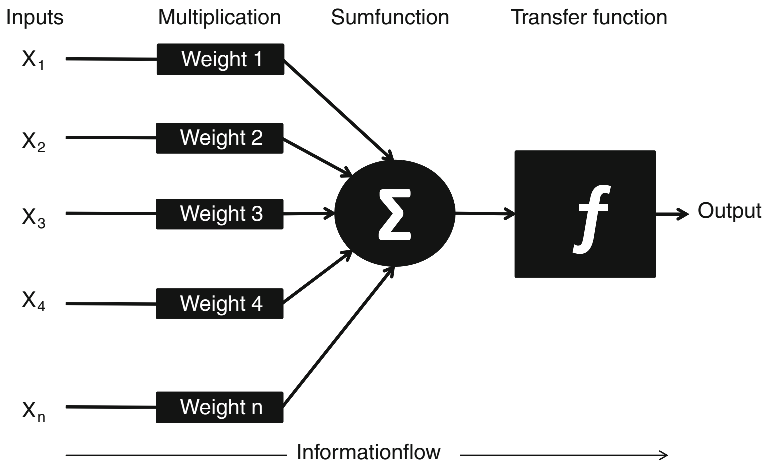

Fig. 2.1 Basic architecture of an artificial neuron

oxygen demand, or any other component. In such applications, it has been assumed that removal rates decrease during the course of time, because easily biodegradable substances are removed first and fast, thus leaving a solution with less biodegradable constituents and hence with slower removal kinetics. This continuous change in solution composition can be represented by a continuously varying volumetric first-order rate constant (Kumar and Zhao 2011).

2.3.2.7 Neuronal Networks

Artificial neural networks (ANNs) are parallel computational models, comprised of densely interconnected adaptive processing units. These networks are fine-grained parallel implementations of nonlinear static or dynamic systems. A very important feature of these networks is their adaptive nature where “learning by example” replaces “programming” in solving problems. This feature makes such computational models very appealing in application domains where one has little or incomplete understanding of the problem to be solved, but where training data is available (Hassoun 1995).

An ANN is a mathematical model that tries to simulate the structure and functionalities of biological neural networks. Basic building block of every ANN is artificial neuron, that is, a simple mathematical model (function). Such a model has three simple sets of rules: multiplication, summation, and activation. At the entrance of artificial neuron the inputs are weighted what means that every input value is multiplied with individual weight. In the middle section of artificial neuron is sum function that sums all weighted inputs and bias. At the exit of artificial neuron is sum of previously weighted inputs and bias is passing through activation function that is also called transfer function (Krenker et al. 2011) (Fig. 2.1).

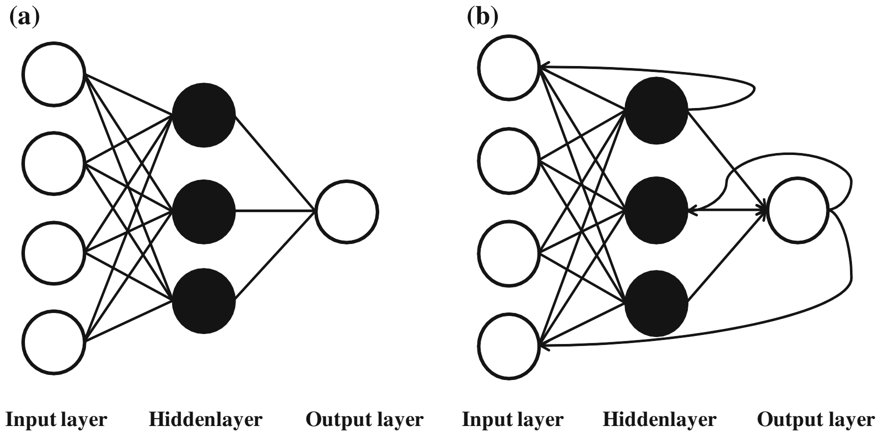

Fig. 2.2 Architecture of simple artificial neural networks: **a** Feed-forward (FNN) and **b** recurrent (RNN) topology of an artificial neural network

ANNs consist of two elements (i) formal neurons and (ii) connections between the neurons. The way that individual artificial neurons are interconnected is called topology, architecture or graph of an ANN. The full potential and calculation capabilities become clear when we start to interconnect neurons into ANNs. Neurons are arranged in layers, where at least two layers of neurons (an input layer and an output layer) are required for construction of a neural network. In any architecture every neuron of a layer is connected to every neuron of the following layer, and no intra-layer connections exist. This property allows referred to them as "multilayer perceptrons" although the classical perception contains only a single neuron (Minsky and Papert 1969). The fact that interconnection can be done in numerous ways results in different possible topologies that are divided into two basic types (Fig. 2.2). In the feed-forward topology (a) the information flows from inputs to outputs in only one direction and in the recurrent topology (b) some information flows not in only one direction from input to output but also in opposite direction. Fully connected feed-forward networks are by far the most frequently used neural networks for nonlinear modeling, pattern recognition, classification, signal filtering, and forecasting (Sumpter et al. 1994).

Formal neurons transform a numerical input to an output value, and the neuron connections represent numerical weight values. The weights and neurons' internal variables (termed bias or threshold values) are free variables of the system which must be determined in the so-called "training stage" of the network development. This training set the proper response of an ANN according to the characteristics of the problem to be solved.

2.4 Validation

Model verification and validation are the primary processes for quantifying and building credibility in numerical models. Verification is the process of determining that a model implementation accurately represents the developer's conceptual description of the model and its solution. Validation is the process of determining the degree to which a model is an accurate representation of the real world from the perspective of the intended uses of the model. Both verification and validation are processes that accumulative evidence of a model's correctness or accuracy for a real situation; thus verification and validation cannot prove that a model is correct and accurate for all possible scenarios, but, rather, it can provide evidence that the model is sufficiently accurate for its intended use. It is important to perform a proper validation and verification to the model to extrapolate the results to other similar situations and predict new events.

The data used for model validation have not been used to build the model. There is a strong reason for not using data as we used in parameter estimation, it will make us think that the model gives better predictions than it is really capable. In linear regression, we correct for the effect of estimating two parameters by dividing the residual sum of squares by ($n-2$) instead of (n). Since data often contain errors, it sometimes sufficient to prove that there is a not statistically significant difference between experimental data and model predictions (Bender 1978).

The differences between data and model predictions can be measured by graphical comparison, confidence intervals, and statistical tests. Hypothesis tests are particularly useful in comparing distributions, variances, or time series of model outputs to determine whether the model predictions are within an acceptable range of precision.

2.4.1 Validation Criteria

Some consist of comparisons with results available, others require the generation of new results for comparison, while others are based on responses from experts. Some of these criteria are used for verification to model validation. According to Godoy and Dardati (2001), the validation types can be classified as follows:

Comparison with other numerical solutions: the results of the model are associated with another solution identifying the proximity between them, that is, approximate results validate other approximate also obtained by other authors or other methods.

This technique does not involve a check of the representation of reality model. This type of comparison is also used to verify the model that can be verified when it is otherwise (Freedman and Ibaraki 2003).

Comparison with other analytical solutions: In this case, it is assumed that the researcher agrees with the theory behind the analytical formulation and with the same mathematical approach, as it also can be used for model verification. If this numeric code fails at playing an analytical solution, there must be a problem with the model formulation (Freedman and Ibaraki 2003).

Comparison with experimental results: This type of validation is the best, because it shows the consistency of the model with reality. However, obtained results of experiments are usually very complex and can be errors if not taken into account a correct consideration of environmental conditions. There is a risk that the model only works for a particular case and is not able to predict for another similar situation, or that the results do not accurately represent reality.

2.4.2 Statistical Tools

There are many statistical tools for model validation, but the primary tool for most process modeling applications is graphical residual analysis. These statistics can be classified in two categories:

(a) Differences between the predictions and the measurements.
(b) Correlation between the model outputs and the measurements from the system.

Deviation-based statistics has often used with correlation-based statistics. Although these different statistics may represent different aspects of the model measurement discrepancy, the deviation-based statistics (e.g., root mean square of deviation) and the correlation-based statistics (e.g., correlation coefficient) are not really with each other in their assumptions.

The residuals from a fitted model are the differences between the responses observed at each combination values of the explanatory variables and the corresponding predictions of the response computed using the model. Respectively, these values can be standardized by subtracting the mean and dividing by the standard deviation. Mathematically, the definition of the residual for the *i*th observation in the dataset is written.

$$e_i = y_i - x_i \tag{2.2}$$

The residuals plot can also be used to test the homogeneity of variance assumption. A residual score serves for determining the accuracy of your model (how much variability is explained by the model) and being used to test the assumptions inherent in the regression analysis (Shier and Wallenius 1999).

Mechanistic models provide a degree of understanding or explanation of the phenomena being modeled. To achieve this, the model must be constructed on (at least) two levels of description. A mechanistic model is based on our ideas of how the system works, what the important elements are and how they relate to each other (Thornley and France 2007).

A regression line between simulated and measured output is commonly included in the graph of simulated versus measured variable. This regression model will help to identify any bias in model prediction and determine correlation between model predictions and systems measurements.

Perhaps the most important statistic is the root mean square of deviation (RMSD), which is calculated from the following equation:

$$\mathrm{RMSD} = \left(\frac{\sum x_i - y_i}{n-1}\right)^{0.5} \tag{2.3}$$

With y_i the predicted variable with respect to the variable x_i and n the number of pairs of measured and simulated values. RMSD is usually reported and discussed as a percentage of average measured performance of the system.

In the second approach, the correlation coefficient between measured and simulated values are calculated. Correlation coefficient (r) can be obtained as follows:

$$r = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \bar{y})^2}} \tag{2.4}$$

where x_i and y_i are the measured data and estimated given respectively in time i, and $\bar{x}$ and $\bar{y}$ are the means of the measured and estimated data, respectively, verifying that $-1 \leq r \leq 1$.

With a high r one might conclude that the model is robust with a finite number of variable records y_i to predict with respect to the variable x_i, then the following criteria are used to determine the relationship between the model and the quantized data.

$$\text{Bias}\,(B) = \frac{1}{n}\sum_{i=1}^{n}(y_i - x_i) \tag{2.5}$$

$$\text{Standard deviation}\,(\mathrm{SD}) = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_i - x_i - B)^2} \tag{2.6}$$

$$\text{Prediction mean square}\,(\mathrm{MSE}) = \frac{1}{n}\sum_{i=1}^{n}(y_i - x_i)^2 = \mathrm{SD}^2 + B^2 \tag{2.7}$$

Each of these could be plotted against a chosen variable to test for homogeneity in performance. It is often a good idea to scale these summary statistics by the mean observation (Bender 1978).

For dynamics models that predict how quantities vary with time, so a dynamical model is generally presented as a set of ordinary differential equations with time to carry out the analyses of the obtained results some techniques of general statistics are used (Benes and Feiresl 2008).

Some measures of variance is the standard error of prediction percentage (%SEP); which establishes the degree of dispersion between the observed variable and the estimated variable. The coefficient of efficiency (E) and the average relative variance (ARV) are used to determine how the model can explain the total variation of the data (Rios Moreno et al. 2006). The percentage standard error of prediction is defined as:

$$\%\mathrm{SEP} = \frac{100}{\bar{x}}\sqrt{\frac{\sum_{i=1}^{n}(x_i - y_i)^2}{n}} \tag{2.8}$$

Efficiency (E) is a sum of squares that provide a relative index of model behavior. Values can be obtained less or equal to 1, values of 1 indicates a perfect fit. A value of zero indicates that the model predictions are no better than taking the average values of the observed data and a negative value can be interpreted as a bad approach is that the results are worse than using the mean values of the observed data. The coefficient of efficiency (E) and the ARV are defined as $E = \frac{S_{\text{obs}} - S}{S_{\text{obs}}}$ and $\text{ARV} = \frac{S}{S_{\text{obs}}}$ where $S_{\text{obs}} = \sum_{i=1}^{n}(x_i - \bar{x})^2$ and $S = \sum_{i=1}^{n}(x_i - y_i)^2$ to have a perfect match, r and E should be close to 1 and the values %SEP and ARV close to 0.

If the results of the model validation stage are not satisfactory, it is necessary to reconsider the hypotheses, equations, and the quantitative methods and the data used to construct the model. If alternative methods and equations are identified, the model validation should be repeated. If the predictions and the output are not still satisfactory, it is likely that there is some basic problem with the hypotheses (Soltani and Sinclair 2012). More experimental investigation is required and the modeling process should be set aside until observations allow improved hypotheses.

2.5 Applications

2.5.1 *Estimating the Biomass of Fish*

In crops of different aquacultural species it is important to determine the growth to know the development with respect to environmental conditions. In the literature, there are several studies which characterized the growth of various species of fish, but the authors recommend using the equations and statistical models obtained cautiously since the coefficients and constants involved in these equations depend mainly on the data analyzed.

The following is a study to determine the biomass of tilapia fish grown in ponds in greenhouses during the months of July and December of 2008, Fig. 2.4. Data were obtained from a random sample of fish to measure manually. Three measures and the weight were considered for the study of which are shown in Fig. 2.3.

One way to estimate the biomass of fish is using the lengths, for example, Hockaday et al. (2000) built two types of mathematical models for estimating fish

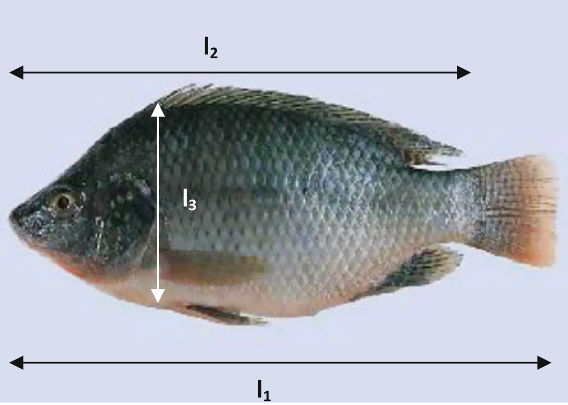

Fig. 2.3 Lengths measured during the growth cycle of the fish

biomass tilapia, first using least squares regression and through ANNs, these models are based on data on different lengths of fish. Another example is the research of Lines et al. (2001), which a system based on image analysis to estimate the mass of the salmon is proposed. The system was developed and tested under certain conditions. As well, Martínez et al. (2002) proposed a relation length–weight. In this case, a multiple regression was applied to relate the measured lengths with the weight of the fish. Thus, the mathematical model can be represented as follows:

$$p(l_1, l_2, l_3) = 0.155 l_1 l_2 l_3 - 0.7209 l_2 l_3 + 5.0869 l_1 - 7.6005 l_2 + 3.7588 l_3 \quad (2.9)$$

Figure 2.5 shows a comparison between the measurement data and estimated results by means of the Eq. (2.9). Some general statistics are used to analyze the obtained, such as: the correlation coefficient $r = 0.99$, bias $B = -0.05$, standard deviation $\mathrm{SD} = 2.28$. According to these results, a good fit of the estimated data to the measured data was obtained.

2.5.2 *Fruit Quality Changes*

What constitutes quality largely depends on the consumer and the final destination of the product (Sloof et al. 1996). Quality can be seen as the concerted action of several quality attributes each based on their own physiological or physical product property. To predict keeping quality of a product, monitoring of a single attribute suffices but not necessarily gives a complete picture of the quality. For this, a compound quality index is required (Hertog et al. 2004). Quality is not a static parameter in general it decreases with time. Depending on the position of the product in the postharvest chain, this might be interpreted as a gain or loss (Hertog and Tijskens 1998).

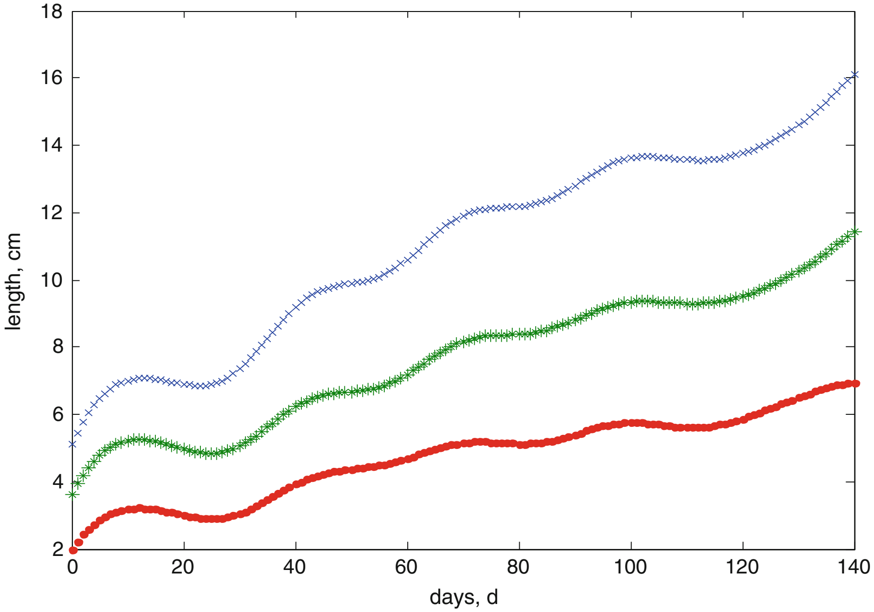

Fig. 2.4 Measurements obtained during the growth cycle of the fish: l_1 ($^{\mathbf{xxx}}$), l_2 (***) and l_3 ($\cdots$)

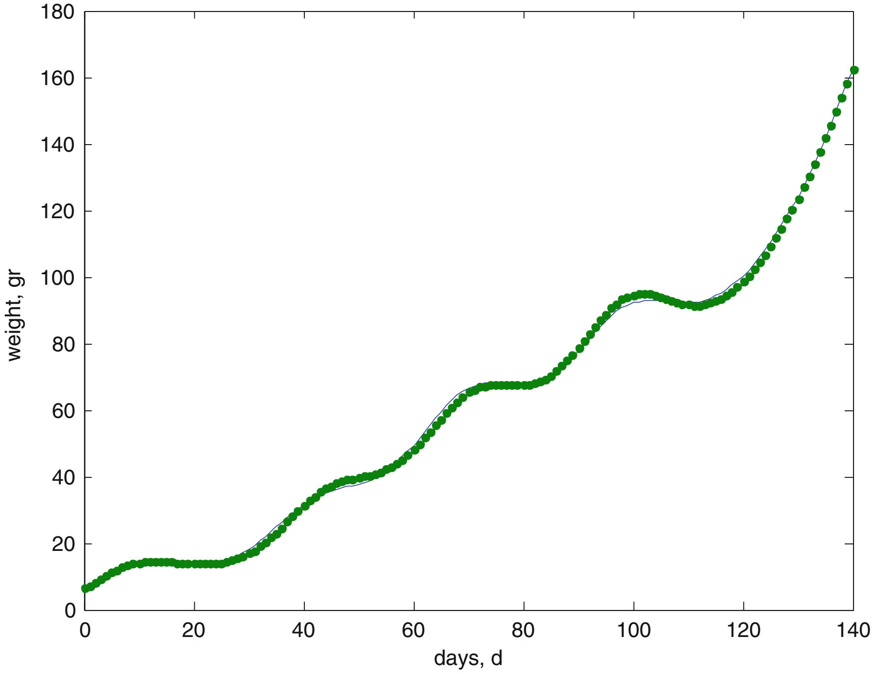

Fig. 2.5 Measurement data (**- - -**) and estimate data ($\cdots$)

2.5.3 Modeling of Fruit Quality

A wide range of mathematical models has found their application in the wider food area (Tijskens et al. 2001). Important quality traits are manifested at the fruit scale. This is especially true for fruit size, dry matter content, and percentage of edible tissues. Fruit size and dry matter content result from the exchange of resources with the plant and the atmosphere. The carbohydrate supply has often been modeled according to the source/sink concepts (Léchaudel et al. 2005). An important variable of these models is the fruit demand for carbohydrates which is positively correlated to the seed number (Lescourret et al. 1998). In some models, a more mechanistic approach has considered that processes involved in sugar unloading from the phloem to the fruit tissues. For example, Fishman and Génard (1998) and Bruchou and Génard (1999) modeled sugar phloem unloading through mass flow, diffusion, and active transport.

In a postharvest context, the gas (O_2, CO_2, ethylene, and water vapor) fluxes through the skin have been related to the concentrations gradients, according to physical laws of gas diffusion. A model of fruit surface conductance to water vapor has been proposed recently (Gibert et al. 2005), which could improve the prediction of fruit transpiration as a function of fruit growth. More generally, fruit surface conductance to gas needs to be modeled in the future because it has a strong implication for fruit quality through its effect on fruit physiology, ripening, and quality (Paul and Srivastava 2006). Biomass allocation to the fruit tissues (which determines the percentage of edible tissues) also needs to be considered at the fruit scale. However, to our knowledge, there is no mechanistic model of biomass allocation into fruit tissues. Establishing empirical laws relating the size of any given tissue to that of another one or to that of the whole fruit may be a solution. Such laws are common in biology, mainly in the framework of allometric growth (West et al. 1997). For example, the dry masses of each fruit tissue and the dry mass of the fruit have been linked by allometric relationships (Lescourret and Génard 2005).

In order to assess the key processes underlying sugar concentration, an approach combining ecophysiological modeling and quality trait loci (QTL) analysis was proposed by Prudent et al. (2010). For this purpose, a first model predicting tomato fruit sugar concentration was adapted from a previous model built on peach fruit (Quilot et al. 2004), allowing the dissection of three interrelated elementary processes: the assimilate supply provided to the fruit, the metabolic transformation of sugars into other compounds, and the dilution of sugars by water uptake. In this work, two sources of variation were used to modulate the sugar concentration: a genetic method and a physiological method. This approach allowed observation of the inter-genotypic relationships first among elementary processes and then between elementary processes and sugar concentration; estimation of whether two different sources of variation for sugar concentration lead to similar changes in the underlying processes; and identification at each QTL for sugar concentration those processes which were supposedly involved.

In recent years, fruit quality has become an increasingly important aspect of fruit production. For example, in Europe, the new market organization enjoins farmers to form producer organizations whose goal is to improve fruit quality. Research efforts directed toward understanding the effects of climate and management techniques on fruit quality are needed, and mathematical models are useful frameworks for these research efforts (Lescourret and Génard 2005). Fruit quality, even when reduced to organoleptic qualities (such as sweetness or acidity) that meet consumer's demand, is a multicriterion concept. Each quality trait is the result of a complex chain of biological processes that depend on environmental conditions. These processes are interrelated (sugar metabolism depends on the carbon fluxes) and their effects on quality traits may be opposite (enhancing water fluxes into fruit increases sugar concentration; Génard and Lescourret 2004). Clearly, a useful fruit quality model must take into account several quality traits, the underlying processes, and their interactions.

However, following the researches of C.T. de Wit (van Ittersum and Donatelli 2003), most process-based fruit models have focused on carbon relationships leading to predictions of fruit growth in dry mass. Such photosynthesis-driven models have been developed for apples (Baumgaertner et al. 1984), grapes (Gutierrez et al. 1985), kiwifruit (Buwalda 1991), olives (Abdel-Razik 1989), peaches (Grossman and DeJong 1994), and tomatoes (Heuvelink and Bertin 1994). Some models have dealt with nitrogen content, representing nitrogen and carbon dynamics on a similar conceptual basis. Researchers have modeled water accumulation in fruit, considering water uptake and transpiration per unit fruit area as constant or variable (Génard and Huguet 1996). In a more mechanistic work applied to tomatoes, the difference between water potentials in the stem and the fruit was assumed to be the driving force of water import rate (Bussières 1994). Another tomato water model focused on the role of pedicel resistance and calyx transpiration (Bussières 2002). A few models of fruit metabolism describing synthesis and degradation processes have been designed for sugar (Génard and Souty 1996) and citric acid accumulation (Lobit et al. 2003). However, few models consider several processes together. Nevertheless, the virtual peach fruit model proposed by Lescourret and Génard (2005) is the first model to integrate in systemic framework knowledge of many interrelated processes, resulting in a complex quality profile and emergent properties that are typical of complex systems. The model offers various possibilities as a research tool, both for performing theoretical experiments and for helping to understand experimental results when studying the effects of technical scenarios for which there is no literature. Lescourret and Génard (2005) examined relationships between quality traits, physiological variables, and between both and found patterns that did not derive just from the aggregation of modeled basic functions. Moreover, the virtual peach fruit model may help in assessing the relative importance of processes for a given complex function or trait.

Virtual plants are being viewed as a novel means to simulate the genetic variability of plant responses to environmental conditions (Tardieu 2003). Combining either gene regulatory networks or quantitative trait loci (QTL) and models (Reymond et al. 2003) are two possible avenues, assuming that a genotype is presented by ne parameter set (Tardieu 2003). Analyzing genotypic variation by means of models is a first step, and has been done with the carbon submodel on a contrasting population of peach genotypes (Quilot et al. 2004). In addition, incorporating the virtual peach fruit model in a crop model will be of value in simulating the combined effects of changes in climate, pest events changing leaf area or photosynthesis, genotype, and technical operations on profiles of quality traits, and will thereby help to improve breeding and crop management processes (Lescourret and Génard 2005).

2.5.3.1 Modeling Crop Quality: A Case of Study in Tomato

Fruit quality at harvest is a complex trait, including size, overall flavor (taste and texture), and visual attractiveness (color, shape), which depend on both genotype and environment. The improvement of fresh product quality is slowed down by its complexity. It is expected that the development of process-based models and their integrations in ecophysiological models should facilitate quality management, provided that integration properly accounts for interactions among biological processes (Bertin et al. 2006).

2.5.4 Applications of Neural Networks in Agriculture and Biosystems

Agricultural systems, such as environment–plant system, are quite complex and uncertain and they can be considered as ill-defined systems. They are characterized by nonlinearities, time-varying properties, climatic interactions, and many other factors. It is, therefore, difficult to quantify complex relationships between the input and the output of a system based on analytical methods. Recently, intelligent system control based on artificial intelligence has been one of the most prosperous technologies in the complex system science (Hashimoto 1997). ANN is also a promising tool for predicting crop yield and offers insight into the casual relationships through the use of sensitivity analyses, but the complex parameterization and optimum model structure require special attention (Park et al. 2005). The machine learning tools used to model the effects of environmental conditions on apparent photosynthesis provided a very powerful modeling alternative to ordinary curve fitting methods. Their major advantages are the flexibility to select between accuracy and generality and their robustness against outliers and mixtures of differentia responses (Dalaka et al. 2000). Resources capture and plant responses to energetic stimuli (solar radiation, temperature, CO_2 concentration, and humidity) present complex ecophysiological mechanisms, which conduct to dynamic modifications of the canopy development and architecture (Vazquez-Cruz et al. 2010).

In agronomic research, ANNs have been presented as alternative methodology to modeling and simulating crop biophysical properties. ANN models are specially designed for dynamic nonlinear systems (Rahimikhoob 2010). ANNs are now used in many computer-based applications where there is a need to identify patterns or "learn" relationships between a set of input variables and a set of output variables (Danson and Rowland 2003). During the last decade, there has been a significant increase in agronomic ANN applications (Huang et al. 2010) including crop development modeling, crop yield prediction, evapotranspiration estimations, soil water, and salt content assessments (Dai et al. 2011; Fortin et al. 2010; Liu et al. 2009). Another important variable, which is commonly used in modeling crop, is leaf area. There have been a few attempts to produce leaf area estimation models predicting leaf area by means of using simple linear measurements like length and width (Beyhan et al. 2008). Vazquez-Cruz et al. (2012) developed an ANN model to determine the response of tomato leaf area to different climate conditions such as CO_2 concentration, PAR, and temperature along with different salicylic acid treatments. The results showed that ANN model was a useful tool in research and understanding the complex relationships between greenhouse conditions and leaf area development.

More research is needed in order to increase performance of training algorithms to improve the ability of neural systems to learn from climatic and physiological data patterns for crop growth prediction, and the forecasting performance to provide a useful guidance or reference for yield estimation.

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Chapter 3
Water Recycling in Biosystems for Food Production

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Contents

3.1 Introduction

Nowadays, experts are discussing the issue of water taking into account the Declaration of Dublin, in which the topic of water is treated like a sustainable issue. This declaration was presented in 1992. It is a fact that the scarcity and

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inadequate use of freshwater are leading us to a nonrational and sustainable development from an ecological point of view (N.R.W.C. Committee 1993; Chapman 2006; Walsh et al. 2006). Health and well-being, food security, and industrialization are other threatened areas, which are affecting ecosystems on which all human beings depend. That is the reason why to secure a future, it is necessary to have an effective management of water and soil resources.

Within the acceptable minimum ethics, it should be noted that the general environment, including water resources in particular, is a heritage of mankind that we have inherited, and that we must protect for future generations. Proper management must certainly rely on the three commonly accepted principles of solidarity, subsidiary, and participation (F.I. Report 2009).

Solidarity recognizes that all men have equal rights to participate in environmental and water resources; subsidiary states that can make a lower social group, it is better than form a larger social group. Participation leads to the integration of the different users in the management of the environment and its resources (Sekoulov 2002).

In the agro-food industry, there is considerable water consumption due to the different kinds of processes and unit operation in which water is involved (e.g., cleaning, cooling, pasteurizing, etc.). In addition to this, good water quality and quantity are essential for food processing (Bazilian et al. 2011; Casani and Knøchel 2002; Palumbo et al. 1997). On the other hand, large volumes of wastewater are generated as a result of the need to use water in these processes. For instance, in Germany, 30 m^3 of wastewater is generated per ton of frozen carrot produced, 1.2 m^3 for apple juice, and 4.15 m^3 in beer production (Yang et al. 2007). Therefore, wastewater treatment and water management are fundamental concerns to ensure water supply over the years.

Wastewater effluents are continuous when water input and output exists in the different processes for food production. They can be derived from cooling and heating systems (condensers, cooling towers, and boilers) or industry cleaning processes (cleaning-in-place systems for the internal surface of pipes, vessels, process equipment, filters, and other accessories). Discontinuous wastewater effluents can also be generated from intermediate operations such as soaking, raw material cleaning, scalding, etc., and are prone to be the most polluted. Wastewater from the agro-food industry is characterized by high organic content, cleaning agents, salts, and suspended solids. High levels of Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) as well as considerable seasonal changes in flow and composition make their treatment difficult (Todd and Josephson 1996). Costs associated with water are the result of an elevated consumption of high-quality water plus wastewater treatments and the cost of wastewater disposal. Therefore, searching for profitable alternatives becomes necessary in order to minimize consumption as well as searching for environmentally friendly wastewater treatment strategies, which will be determined by the taxes applied to each effluent (Gray 2005). In the food and beverages industry, effluent charges were the principal factor in inducing water polluters to invest in biological wastewater technologies in the Netherlands (Casani and Knøchel 2002).

This is an essential and determinant factor for the development of new wastewater treatments and establishing their potential markets. An increasing need exists for creating an appropriate tax designed to encourage water treatment and savings.

A reconsideration of traditional irrigation practices is starting and a new appreciation of the advantages of traditional know-how is beginning to appear. However, pressures on irrigating farmers are continuing to require them to increase irrigation efficiency, achieve higher water productivity, and use less water. Yet there is often a lack of assistance for them to develop and adopt improved approaches and techniques appropriate to these changing farming objectives, however keeping farmers' objectives of financial and social nature. In addition, perceptions of an urban society that highly prioritizes environmental preservation for future water allocation are also challenging farmers' attitudes and requiring new approaches.

A new communication model has to be developed that could lead to a better understanding of water use in agriculture and demonstrate why performance improvement must occur within the context of the needs of the society and the objectives of farming (Elizaquível et al. 2011, 2012; Sánchez et al. 2012). New approaches are required to properly define and account for each item of water use and productivity with water conservation and saving being the primary drivers to achieve higher performance (García-Pérez et al. 2013). From this perspective, the performance concepts need to be differently defined, understood, and applied. In other words, we need a new model in terms of conceptualization of water use performance that can be understood by users, managers, and decision makers alike. This improved conceptualization can then provide a common framework around which actual water use (hopefully monitored water use—debate over words is not very useful if the actual volumes involved are only approximate) can become sustainable for large and small farmers or other users, in all climates and in societies with different degrees of development, utilizing a wide range of technologies (Chan et al. 2009; Kalbar et al. 2012; Muga and Mihelcic 2008; Tomar and Suthar 2011; Travis et al. 2012).

The terms water conservation and water saving are generally associated with the management of water resources under scarcity. However, these terms are often used with different meanings within specific scientific and technical disciplines or in the water user sector considered. Often, both terms are used synonymously. The term water conservation is used here to refer to every policy, managerial measure, or user practice that aims to conserve or preserve the water resource, as well as to combat the degradation of the water resource, including its quality. Differently, the term water saving describes the avoidance of loss of water by limiting or controlling water demand and use for any specific purpose (Muga and Mihelcic 2008; Jin et al. 2013), including the avoidance of wastes and the misuse of water.

In practice, these terms or perspectives are complementary and interrelated. Water conservation plays a major role in rain-fed agriculture and when irrigation is supplemental of rainfall (Chan et al. 2009; Muga and Mihelcic 2008; Wang et al. 2012), but it is essential in all water use systems, often as a means to achieve water saving. Water conservation can play a major role in agricultural and landscape irrigation considering that predictions for climate change indicate a concentration of rainfall and an increase of its intensity. A coupling of soil and water conservation is then essential to increase water infiltration and storage in the soil profile as well as to control soil evaporation.

Water conservation increases the amount of consumptive use by crops and natural vegetation, sometimes called the green water fraction, and assists in preserving the quality of flows that are often called the blue water, the general good quality environmental water (Rodriguez-Garcia et al. 2011). Water savings usually refer to the blue water fraction. Despite it often not being easy to distinguish between "conservation" and "saving," these terms should not be used synonymously. For example, questions related to preservation and upgrading of water quality are essential in water conservation but are rarely relevant to the usual ideas of water saving. It is arguably a modern tragedy that considerable volumes of the scarce resource can and are being lost or wasted due to lack of clarity of terms used and miscommunication between those involved. This is analyzed in most papers in this issue, with the various authors adopting a variety of approaches. Yet, communication must also apply to specific fields or scales: our main focus in this chapter is water use at the farm scale, or a group of users served by the same system, not basin planning, or water allocation. Therefore, the aim of this chapter is two-fold: (1) to demonstrate the confusion of the terms used both between and within disciplines and groups of users, and the resulting potential for poor use of water and (2) to suggest alternative terms that could gain wide acceptance and common usage. Some case study applications are used to illustrate the use of these terms and ideas.

Two important aspects must be analyzed in the field of water; (i) The physicochemical characteristics of water according to the nature and source of the water and (ii) Water as a fact of life. In this regard, considerations of water as life support, clean water and its uses, wastewater and the effects of pollution, and the health considerations of drinking water cannot be ignored (Muga and Mihelcic 2008).

The general principle in wastewater treatment is to remove pollutants from the water by getting them to either settle or float, and then removing this material. Some pollutants are easily removable. Others must be converted to a settleable form before they can be removed. Treatment facilities are designed in stages. Each stage either removes particles from the wastewater or changes dissolved and suspended material to a form that can be removed. A modern wastewater treatment plant (WWTP) may include the following stages: (i) influent, (ii) primary treatment, (iii) secondary treatment, and (iv) tertiary treatment and effluent discharge (Walsh et al. 2006).

In the final stage, the disinfection process has the objective to eliminate all the pathogen agents to prevent plant and human diseases. While most of these microorganisms are not pathogens, pathogens must be assumed to be potentially present. Thus, whenever wastewater effluents are discharged into receiving waters which may be used for water supply, swimming, shell fishing, or if its quality is really good, it can be used in food production, and the reduction of bacterial numbers to minimize health hazards is a very desirable goal (Abdel-Raouf et al. 2012).

A variety of physical or chemical methods are capable of destroying microorganisms under certain conditions. Physical methods might include, for example, heating to boiling or incineration or irradiation with X-rays or ultraviolet rays. Chemical methods might theoretically include the use of strong acids, alcohols, or a variety of oxidizing chemicals or surface active agents (such as special detergents). However, the treatment of wastewaters for the destruction of pathogens demands the use of practical measures that can be used economically and efficiently at all times on large quantities of wastewaters which have been treated at various degrees (Pablos et al. 2013).

The prevalent use of chlorine has come about because chlorine is an excellent disinfecting chemical and, until recently, has been available at a reasonable cost. However, the rising cost of chlorine coupled with the fact that chlorine even at low concentrations is toxic to fish and other biota, as well as the possibility that potentially harmful chlorinated hydrocarbons may be formed, has made chlorination less favored as the disinfectant of choice in wastewater treatment. As a result, newer technologies are showing promising results, such as the use of nanocatalysts like titanium dioxide (TiO_2) as disinfection agent by the photocatalysis process (Bodaghi et al. 2013; Fang et al. 2013; Hossain et al. 2014; Lin et al. 2013; Liu et al. 2013; Wang and Lim 2013).

Another important process related to wastewater treatment is that of metal removal to obtain the best water quality for reuse in food production (Bonnett et al. 2006; Lalov et al. 2000).

Heavy metal ions and waste dye are two major industrial pollutants, and many methods have been developed to remove them from wastewater (Ahluwalia and Goyal 2005; Nguyen et al. 2006; Monteagudo et al. 2006; Asma et al. 2006). In recent years, various methods including foam flotation, filtration, microbial reduction, chemical oxidation, reverse osmosis, coagulation and flocculation, biological treatments, photo degradation, and adsorption have been developed for treating dye contaminated wastewater. Among these methods, adsorption is known to be a promising technique due to the ease of operation and comparable low cost of application. These processes may be ineffective or extremely expensive, especially when the metals in solution are in the range of 1–100 mg/L (Liu and Lu 2011; Kasmuri et al. 2011).

Heavy metal pollution represents an important environmental problem; environmental contamination by heavy metals is of growing concern because of health risks on live organisms. At least 20 metals are classified as toxic and half of these are emitted into the environment in quantities that pose risks to human health. Accumulation of metals like Cd, Cr, Cu, Hg, and Zn, in humans has several consequences such as growth and developmental abnormalities, mental retardation, and a wide range of other illnesses (Liu and Lu 2011; Kortenkamp et al. 1996; Wang et al. 2008; Thiele 1995).

The above-mentioned are traditional methods of effluent treatment, nevertheless these methods have to be supplemented with modern and more effective means of treatment and this is where bioremediation becomes relevant providing several techniques for cleaning up pollutants by means of biological tools (Shetty 2006). Biological tools include living and nonliving microorganisms for removal or recovery of toxic substances from wastewater. The natural affinity of biological compounds for metallic elements could contribute to the purification of wastewater polluted with metals (Şahin and Öztürk 2005). The biosorption process is a feasible option as it is efficient and cheap. Compared with conventional methods for removing toxic metals from effluents, the biosorption process has the advantages of low-operating cost, minimization of volume of chemicals and biological sludge to be disposed of, and high efficiency in detoxifying very dilute effluents (Shetty 2006).

There are many different types of biosorbents like (i) Active biomass belonging to algae, bacteria, or fungi; (ii) Nonactive kind of biosorbent which is essentially a waste product or a by-product of a fermentation process; and (iii) Abundant natural materials or polymers.

There is a small degree of selectivity, in solution, from numerous biosorbents in the collection of heavy metals. The mechanism of metal sequestration can occur by processes such as complexation, chelation, ion-exchange or coordination; or physical mechanisms like adsorption or precipitation. However, since biomaterials used for sorption are complex, a number of these mechanisms could be occurring simultaneously. Biomaterials have several chemical groups: acetamide groups in chitin, amino and phosphate groups in nucleic acids, amino, amide, sulfhydryl and carboxyl groups in proteins, and hydroxyls in polysaccharides, which could potentially attract and sequester metal ions. The efficiency of a biosorption process depends not only on the binding properties of the biosorbents, but also on the composition of the wastewater that will be used for treating (Şahin and Öztürk 2005; Volesky and Holan 1995; Krishnani and Ayyappan 2006; Barros et al. 2006).

Polymers are used like biosorbents because they have different properties that should be considered, such as degradation, hydrolysis and mechanical shearing, and chemical and mechanical stability. Some polymers possess a flexible structure and at certain hydrodynamic conditions the shape of such polymers can change so that they will pass through the membrane even though their mass is more than the molecular weight cut-off (MWCO) of the membrane (Shetty 2006; Geckeler and Volchek 1996).

Among the many other low-cost absorbents identified, chitosan has the highest sorption capacity for several metal ions (Chauhan et al. 2012; Juang and Shiau 2000; Laus et al. 2007; Liu et al. 2011; Sankararamakrishnan et al. 2007; Sobahi et al. 2010). Chitosan ([β-(1→4)-2-amine-2-desoxy-D-glucose]) is a natural polysaccharide that is formed by altering the N-deacetylation of its precursor, Chitin. Chitin is the second most abundant natural polymer, and is widely distributed in nature, especially in the exoskeletons of marine invertebrates such as prawn, crab, and lobster.

The difference between chitin and chitosan is essentially related to the possibility to solubilize the polymer in dilute acidic media. Therefore the degree of acetylation (DA), which is related to the population balance of acetylated and deacetylated (100-DA) groups, is essential to define these two terms. When chitin is deacetylated in heterogeneous conditions, the solubility in aqueous acidic media is achieved for DA generally below 30 %. Nevertheless, on reacetylating chitosan it is possible to observe a solubilization up to DA close to 60 %. As a consequence, the frontier between chitin/chitosan can be located at a DA of 60 %. An effect of the DA can be appreciated in the chitosan, which is a polymer semicrystalline and the degree of crystallinity is a function of the degree of deacetylation. In addition, the amino groups in chitosan structure are responsible for complex formation between metal ions and the polymer chain (Bhatnagar and Sillanpää 2009; Bolto et al. 2004; Szyguła et al. 2009; Domard and Domard 2002).

Chitosan chelates five to six times greater amounts of metal than chitin, this effect is attributed to the free amino groups present in the chemical structure of chitosan caused by deacetylation process; this fact is used in several important applications, and specifically for wastewater treatment because it can absorb dye molecules, remove proteinaceous matter, and metallic ions, including Cu(II), Co(II), Ni(II), Hg(II), and Zn(II), either physically or chemically (Kasmuri et al. 2011; Alves and Mano 2008; Babel and Kurniawan 2003; Hu et al. 2013; Kamari and Ngah 2009; Milosavljević et al. 2011; Xi and Wu 2004).

Finally, after the above information was described, the main route for sustainability and water management is to produce recycled water or treated at high quality, which is sure to meet the demand in a sustainable way and which in turn benefits to the environment.

To obtain these results, we start with wastewater from the laboratory and sanitary services from the Airport Campus of the Universidad Autónoma de Querétaro (UAQ), where five WWTPs are located to treat the entire campus sewage. After this water is disinfected and made metal free, it can be potentially used in the food production field.

3.2 Experimental Conditions

3.2.1 Wastewater Treatment Plant

Treatment plants were designed primarily to address 0.5 Ls^{-1} flow. The plant consists of two round containers, which are contained one within the other, that is, are coupled concentrically. The first container is in the aerobic reactor which holds the water treatment process with a diameter of 3.68 m, while the second container is in the accumulated treated water, and both containers are connected by a pipe 10 cm in diameter. The second container has a double function: the first function is it serves as a settling process, while the second serves as a clarification process. The container has a diameter of 2.12 m and the plant receives wastewater continuously through a pipe 7.62 cm in diameter. The wastewater is pumped from a lift reception to the first container and has an air supply system and two pipes in which are located the second container in one of the pipes. The fats pump back to the main container and the other pipe is intended to return to the first deposit the settled sludge accumulated; finally the treated water is conducted from the second tank through a pipe 10 cm in diameter to the said pipe.

The physicochemical and biological characteristics of the wastewater were monitored monthly, and these measurements were pH, temperature, sludge volume index, and total and fecal coliform organisms.

3.2.2 Solar Photocatalysis Disinfection

3.2.2.1 Synthesis and Characterization of the Ag:TiO_2 Catalyst

The synthesis of the TiO_2 substrate was obtained by slowly dissolving the titanium precursor (titanium isopropoxide, 97 % Aldrich, TTIP) in an organic solvent (isopropanol, 99.9 %, J.T. Baker). The titanium solution was continuously magnetically stirred for 20 min under nitrogen atmosphere. The hydrolysis process was then performed by adding water into the flask containing precursor/solvent mixture and magnetically stirred for 1 h (Esquivel et al. 2013). The molar ratio of the precursors and solvents are, 0.03:4.2:1 for TTIP:H_2O:isopropanol, respectively. For the Ag-modified TiO_2 samples, the precursor was silver nitrate 99.9 % (Sigma-Aldrich). This precursor was added separately by dissolving it into the water used for the hydrolysis reaction at different weight percentages (0.005, 0.01, and 0.1 wt%). The obtained sol was transferred into Teflon-lined vessels and placed on a turn table for uniform heating using a microwave reaction system (Synthos 3000, Anton Paar). The change in temperature in the reaction vessel was monitored automatically by the dual-IR temperature probe and the solutions were continuously stirred at medium speed (300 rpm). The heating procedures were conducted for 30 min at 215 °C for 60 min using microwave radiation of 600 W, and finally the system was cooled down to room temperature. The obtained powder was filtered and washed with deionized water several times and room temperature dried for 18 h, and subsequently dried at 80 °C for 20 h in a conventional furnace. A calcination process was carried out at 550 °C for 3 h to improve the sample crystallinity. The catalyst characterization of the surface morphology analysis of the materials was carried out by Scanning Electron Microscopy (SEM) with a microscope JEOL JSM-6060 LV. The accelerating voltage employed was 15 kV. Elemental analysis was performed by Energy Dispersive X-ray Spectroscopy (EDS) (EDS Oxford Inca X-Sight coupled to a MT 1000, Hitachi). Also, the morphology analysis was carried out by high-resolution transmission electronic microscopy (HRTEM) using a JEOL JEM 2000FX microscope. The catalysts were crushed and dispersed ultrasonically in acetone at room temperature and then spread onto a perforated carbon–copper microgrid. The operational accelerating voltage was 200 kV using a filament of LaB_6. Bandgap energy (E_{bg}) values were determined from diffuse reflectance measurements (Cary 5000 UV–Vis-NIR Varian spectrophotometer) by applying the Kubelka–Munk function. X-ray diffraction analyses (XRD) were obtained using a Bruker D8 advanced diffractometer equipped with a Cu seal tube to generate Cu K_α radiation ($\lambda = 1.5406$ A) within the Bragg angles $10 < 2\theta < 80°$ in steps of 0.01°. Raman spectroscopy measurements were acquired with LabRAM HR, Horiba Scientific equipment with an Nd:YAG laser ($\lambda = 532$ nm, output power of 80 mW). The samples were analyzed with a power of 6 mW over an area with a diameter of 1.5 μm using a microscope on 10X objective.

3.2.2.2 Photocatalysis Tests

The photocatalytic activity of the synthesized samples was evaluated in disinfection process in a real residual-treated water at pH = 7, by quantification of the more likely number of pathogen agents to form colonies and by differentiation of those microorganisms by biochemical reactions in diverse mediums. In each test, 50 mg of the photocatalyst was dispersed in 100 mL of the residual-treated water magnetically stirred for 30 min in the darkness before irradiation. The system was illuminated using the solar radiation of a spring day in the city of Santiago de Querétaro, Qro. This represents between 800 and 1,000 Wm^{-2} around 12:00 am and 03:00 pm. At given irradiation time intervals and after 3 h of total reaction time, the samples were taken out and the pathogen microorganism removal was evaluated.

3.2.2.3 Microbiological Identification and Differentiation Test

Using a bacteriological water test based on the microorganism growth in two presumptive mediums (Lactose and Sodium Lauryl Sulfate, BD Bioxon) at concentrations of 10^{-1}, 10^{-2}, and 10^{-3}, the disinfection effectiveness process was tested. In the presumptive test, the mediums once seeded were incubated at 35 °C for 24 h. If the samples show the presence of gas, it can be noticed as a positive sample. After the presumptive test, the positive samples were seeded inoculant 1 ml in the confirmative mediums, EC, MIO, and Green Bright Billis (BD Bioxon). The tubes were incubated for 24 h at 35 °C, and the presence of gas means a positive sample. Finally for the differential tests, the positive samples from the confirmative tests, were seeded in a petri dish containing shigella and salmonella medium (BD Bioxon) and Macconkey agar (BD Bioxon) for 24 h at 35 °C (Ervin et al. 2010; Gilbride 2014; Scaglia et al. 2014).

3.2.3 Metal Removal

Chitosan synthesis was made in two ways: in the traditional method with several modifications, which include four-steps depigmentation, decalcification, deproteinization, and deacetylation and is reported elsewhere (Otakara and Yabuki 1991; Jing et al. 2012; Kumar et al. 2012; Reicha et al. 2012; Xu et al. 2011). The modification consists in reducing the concentrations of sodium hydroxide (NaOH) and hydrochloric acid (HCl), and substituting the alkali reagent for potassium hydroxide (KOH) (Elizalde-Peña et al. 2007). Additionally, decalcification, deproteinization, and deacetylation were carried out in a microwave reaction system (Synthos 3000, Anton Paar) with the following conditions: Decalcification: HCl 6 M for 30 min at 50 °C; Deproteinization: alkali solution 1 M for 30 min at 180 °C; and Deacetylation: alkali solution 30 % (v/v) for 15 min at 110 °C.

After deacetylation, the samples were washed thrice with hot deionized water and filtered, with a final oven dried process at 60 °C, for 2 h. The samples were characterized by infrared spectroscopy in a Perkin Elmer infrared spectrometer to obtain the acetylation degree by means of the integration of characteristic bands at 1320 and 1420 cm^{-1} according to Eq. (3.1), exposed by Brugnerotto et al. (2001).

$$N-\text{acetylation degree}\,(\text{AD}) = 31.92\left(\frac{A_{1320}}{A_{1420}}\right) - 12.20 \tag{3.1}$$

And therefore, deacetylation degree (DD):

$$\text{DD} = 100 - \text{AD}. \tag{3.2}$$

3.2.3.1 Flame Atomic Absorption Spectrophotometry

In order to observe chelating effect by chitosan, all products obtained with major DD were placed in contact with a stock solution of copper. Stock solution was diluted to concentrations of 1, 2, 3, 4, and 5 mg/L in 1 % nitric acid for calibration curve. The measurements were carried out in a Perkin Elmer absorption atomic spectrophotometer. In a vessel was placed 12.5 mL of a copper stock solution, with concentration of 5 mg/L, 12.5 mg of chitosan obtained, as blank Chitosan (Sigma Aldrich, without additional purification), and 1 mL of 5 % HCl with constant stirring, and placed in centrifuge to 3,400 rpm for 40 min. After this the samples were filtered to measure.

3.3 Results

3.3.1 Physicochemical Characteristics of the Wastewater

In Table 3.1 is presented the characteristics of the wastewater produced within the UAQ. Prior to the activated sludge treatment, it can be noticed that the pH is slightly basic and the pathogen agent's presence is above the regular value of highly polluted water. These characteristics are important to see if the biological

Table 3.1 Average values of wastewater produced within UAQ

Parameters	mlL^{-1}
Total solids (TS)	500
Total suspended solids (SS)	300
Total settleable solids (TSS)	180
Total dissolved solids (TDS)	200
Dissolved oxygen	0.1
Fats and oils	30
pH	7.5
Temperature	25 °C
Total fecal coliform organisms	>2,400/100 ml

process is working to achieve the elimination of the pathogen agents and to try to reach a neutral pH.

The increment of the sludge volume index shows how the microorganisms grow and how it helps in the organic matter removal. The amount of microorganisms let us know the behavior of the WWTP and how fast the organic matter can be eliminated from the wastewater to obtain treated water. The microorganisms' growth is considered to be an exponential growth and it can be confirmed with the data shown in Fig. 3.1.

Also from Fig. 3.2, it is possible to observe how the pH value from the wastewater changes by an average value of 7.5, showing how much the water is polluted with several substances such as chemical compounds with low toxicity from material wash from the lecturing laboratories.

It took almost 6 months to achieve the optimal and sustainable amount of microorganisms responsible in the organic matter removal. The flow of wastewater increased gradually as demand grew in the UAQ, which allows the proper microorganism growth.

After the wastewater is treated by the activated sludge process, the characteristics of the treated water are summarized in Table 3.2.

Once the majority of the organic matter has been removed, proper disinfection process is needed to avoid the propagation of diseases to animals, plants, and human beings.

3.3.2 Disinfection

Prior to the disinfection process, the titanium dioxide catalyst was synthesized by the sol–gel method. The morphological characterization is not shown in these results because they were already published (Esquivel et al. 2013). For the crystal analysis, X-ray diffraction test were carried out. In Fig. 3.3 is presented the results of the 0.1, 1, and 10 %w Ag-doped TiO_2, showing that with thermal treatment at 550 °C which was presented in the experimental conditions, it is possible to see

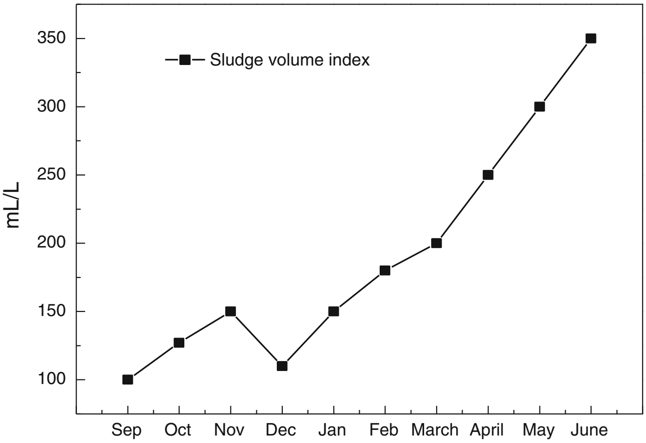

Fig. 3.1 Average sludge volume index from the WWTP at the UAQ, year 2012/2013

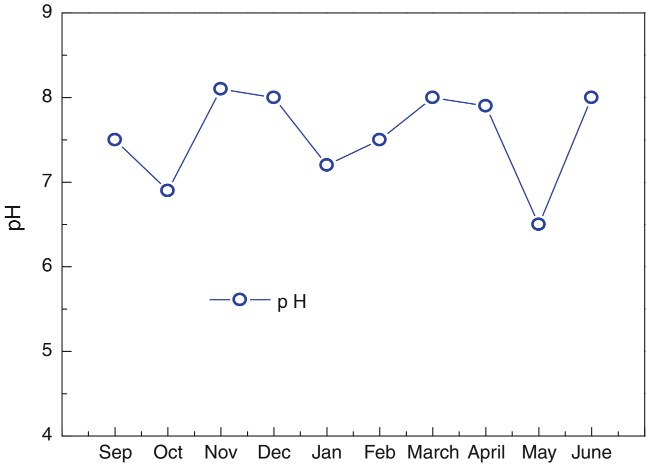

Fig. 3.2 Average potential hydrogen (pH) from the wastewater of the WWTP, year 2012/2013

Table 3.2 Average values of treated water produced within UAQ

Parameters	ml/L
Total solids (TS)	<25
Total suspended solids (SS)	<10
Dissolved oxygen	6
Fats and oils	5
pH	7.0
Temperature	25 °C
Total fecal coliform organisms	28/100 mL

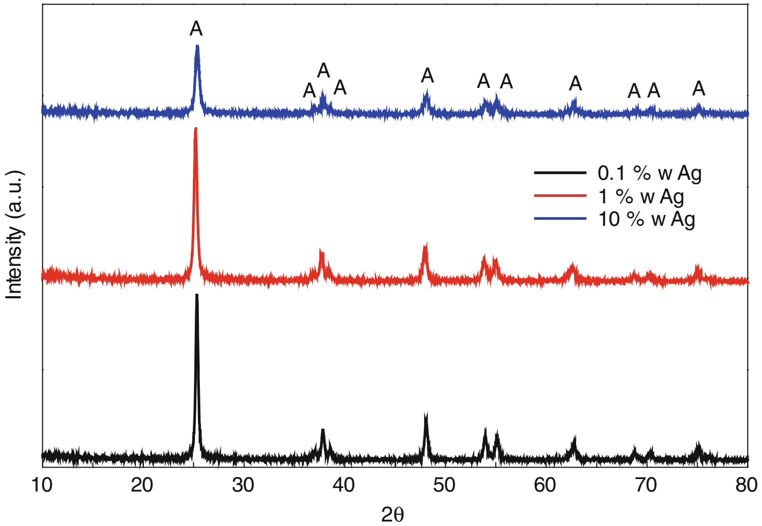

Fig. 3.3 X-ray diffraction patterns of Ag-doped TiO_2 at 550 °C calcination temperature

how the sample is getting ordered. The diffraction peaks detected in 2θ (25.4, 37.8, 48.1, 53.9, 55.2, 62.8, 68.6, 70.3, 75.1°) indicates the presence of the crystalline anatase phase (JCPDS no.: 00-004-0477).

From the diffraction pattern peak intensity, a preferential orientation of the plane (101) can be proposed. No diffraction signal appears showing the rutile phase. Also, no signal can be attributed to other crystalline phases of silver oxide compounds. As mentioned, no presence of rutile phase was observed in any of the tested materials, that is why the fraction of anatase XA is considered to be 1 (Petrovic et al. 2011). Even when no silver oxide or rutile phases are present in the samples, a decrease in the crystallinity is noticed and this can be attributed to the presence of silver in the TiO_2 crystal (Fujishima et al. 2008).

The grain crystal size calculated by the Debye–Scherrer equation for the unmodified TiO_2 shows an average crystal size of 29 nm. As regards the Ag-doped TiO_2, the crystal size is slightly smaller than the TiO_2 samples and the values are between 13 and 9 nm.

The crystal phase of the TiO_2 and Ag-TiO_2 photocatalyst was also confirmed with micro-Raman spectroscopy (Fig. 3.4a, b). Using this technique it was possible to observe that TiO_2 sample shows only four typical lines corresponding to anatase phase, 147 (Eg), 401(B1 g), 516(A1 g), and 640(Eg) cm^{-1}, having good agreement with those reported for the anatase phase (Horikoshi et al. 2011). In case of the Ag-doped samples at the different amounts of dopant used, the same signals are presented (Fig. 3.4a). No presence of the rutile phase is detected as was observed in the XRD results, too. Finally, as in the XRD analysis, the increment in the %w of the dopant, the intensity of the signals decreases, and in the 10 %w Ag-doped sample, three new signals are presented as can be noticed from Fig. 3.4b.

The new signals can be attributed to the presence of silver in the TiO_2 lattice (Hamal and Klabunde 2007) as oxide, which cannot be seen in the XRD analysis.

Another characterization test carried out was the bandgap value measurement by diffuse reflectance analysis and with the Kubelka–Munk function (Christy et al. 1995). According to the %w of silver aggregated to the synthesis the bandgap value does not change between them, it only changes in comparison with the pure TiO_2, from 3.2 to 3.15 eV (Table 3.3), a slight change, which is attributed to the presence of Ag and this change will lead us to propose that the material can be activated with solar light.

Once the material was characterized, the disinfection process took place. The treated water used contained 28/100 mL of more likely number of total fecal coliforms. A suspension of the treated wastewater and the catalyst pH 7 was recirculated in a tubular photoreactor and after 3 h under solar light exposure, the samples were taken out to incubate the presumptive test of total fecal coliforms.

In Fig. 3.5 is presented the presumptive media inoculated with the disinfected water. As can be seen, the presence of gas in tubes of the test of disinfection using pure TiO_2 shows that in three of three tubes of the 10^{-1} concentration were positive and two tubes of three on the 10^{-2} concentration also were positive. By converting these results with a more likely number table (Ervin et al. 2010; Gilbride 2014; Scaglia et al. 2014), the final concentration of total fecal coliforms was 14/100 mL, decreasing the concentration of these microorganisms a 50 % in comparison with the treated wastewater.

For the doped Ag-TiO_2 materials, only in two of the three tubes in the concentration of 10^{-1} for the 10 %w Ag were positive, meaning that with that catalyst, the more likely number of total fecal coliforms was <7.8/100 ml. The rest of the materials remove the 100 % of the total fecal coliforms after 3 h of solar light exposure.

To confirm the presence of fecal coliforms, especially the bacteria *Escherichia coli*, the confirmative and differential medium were prepared. With the pure TiO_2, the bacteria *E. coli* was present in a concentration <7.8/100 ml. From Fig. 3.6, it is possible to observe that there is no presence of gas in any of the Ag-TiO_2 materials tested, concluding that the doped material works as a disinfection agent and the obtained water can be reused for the food production free of pathogen agents.

3.3.3 Metal Removal

From infrared analysis, it is possible to recognize in most of the samples, and blank, representative bands detected in CTS spectrum are in agreement with other literature studies: at 3,400 cm^{-1} a wide and intense band appears as a result of the stretching vibrations of the –OH and –NH_2 groups. The double band that appears at 2,920 and 2,880 cm^{-1} are due to asymmetric and symmetric stretching vibrations of the –CH_2 groups. Bending vibrations of the –NH_2 groups and stretching vibrations of C=O groups cause the strong band at 1,657 cm^{-1} with a shoulder at

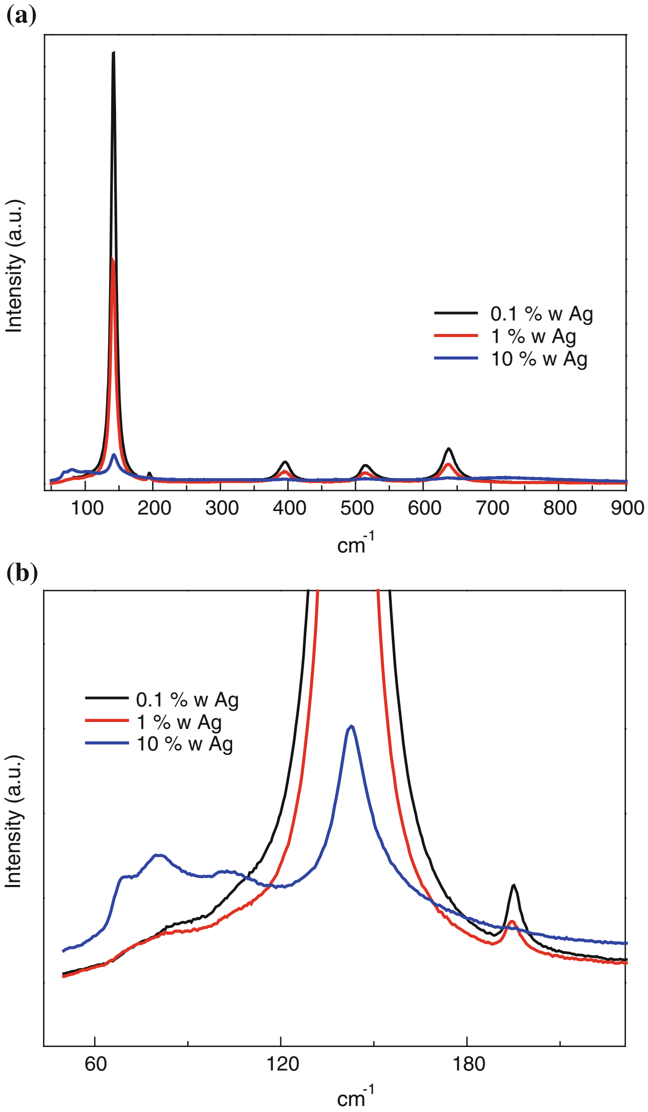

Fig. 3.4 Raman spectroscopy data to Ag-TiO$_2$ samples (**a**) from 900 to 100 cm^{-1} and (**b**) zoom from 250 to 60 cm^{-1}

Table 3.3 Bandgap values to the TiO$_2$ and Ag-TiO$_2$ samples

Material	Bandgap (eV)
TiO$_2$	3.2
0.1 %w Ag	3.15
1 %w Ag	3.15
10 %w Ag	3.15

Fig. 3.5 Presumptive media inoculated with the disinfected water

Fig. 3.6 Confirmative medium inoculated with the positive tubes from the presumptive tests

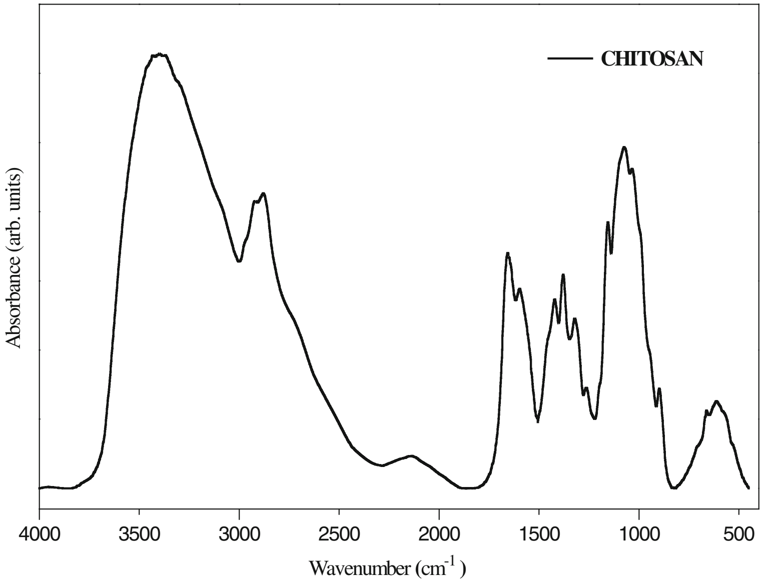

Fig. 3.7 Representative chitosan infrared spectra, blank

1,597 cm^{-1}. This band suggests CTS is a partially deacetylated product. The band at 1,421 cm^{-1} is caused by vibrations of the –CH_2–CO group (characteristic of a ring of the six members). The band at 1,379 cm^{-1} is attributed to the vibrations of the C–N bond, while the bending vibrations of –OH groups cause the band at 1,320 cm^{-1}. Finally, the strong band at 1,075 cm^{-1} is due to asymmetric stretching vibrations of the ether functionality (Fig. 3.7) (Flores-Ramírez et al. 2008).

The acetylation was computed using the Brugnerotto's Equation (3.1) integrating the bands at 1,320 and 1,420 cm^{-1}(Brugnerotto et al. 2001). The results obtained from Eq. (3.2) for DD are shown in Table 3.4, for both synthesis ways and alkali solutions.

These results shows that the deacetylation process was not effective for microwave method, due to short time and lower concentration compared with the traditional method, nevertheless the time and reagent quantity is really low and it is possible to reach highest values by modifying these parameters. Table 3.5 shows the results obtained for copper chelation obtained from Flame Atomic absorption Spectrophotometry; the concentration for the stock solution was determinate, after run calibration curve, in 4.9636 mgL^{-1} of copper.

It is possible to observe that the percentage of chelation decreases with the DD; this is because the samples have no enough free amino groups, decreasing the chelating capacity. It is also evident that the blank commercial chitosan is the best sample because this sample has around 80 % of deacetylation degree which indicates that the free amino groups' number is high compared with the samples obtained in the laboratory.

Table 3.4 Deacetylation degree computed for the samples

Traditional method		Microwave method	
NaOH	KOH	NaOH	KOH
73.57 ± 1.01	75.97 ± 0.70	62.93 ± 0.82	62.83 ± 0.37

Table 3.5 Chelate capacity from chitosan obtained for both ways

Sample	Concentration retained (mgL^{-1})	Percentage (%)
Blank	1.5857	31.94
Traditional with NaOH	0.9719	19.58
Traditional with KOH	0.9787	19.71
Microwave with NaOH	0.3589	7.23
Microwave with KOH	0.4136	8.33

The perspective is that the Chitosan modified with some anionic polyelectrolytes will increase the capacity chelating or natural polymers that provide better mechanical properties like, polyvinyl alcohol, mucilage, polyacrylamide, acrylic acid, etc.

3.4 Conclusion

This study has shown the results of wastewater treatment in purification plants within the UAQ, which is realized by several steps, biological treatment with activated sludge, disinfection process with a photocatalytic material, and metal removal with a natural polymer.

A growing volume from the activated sludge, which increases gradually as demand grew in the UAQ for using water from the sanitary services to the laboratory demands, helps to consume organic matter faster; after the process is evident the significant reduction in total solids, pH, and total fecal coliform become evident and necessary for a disinfection process to prevent disease spread.

The disinfection process was carried out with TiO_2 catalyst synthesized by the sol–gel method, and doped with 0.1, 1, and 10 %w of Ag, which after XRD analysis shows the presence of anatase phase and absence of rutile phase; with an average crystal size of 29 nm for pristine TiO_2 and for doped material average crystal size between 13 and 9 nm.

The material doped decreased the bandgap to 3.15 from 3.2, raw TiO_2, which is attributed to the presence of Ag and this significant change will lead us to propose that the material can be activated with solar light. Thus, in this process for pristine TiO2 there is a microorganism reduction of 50 % compared with the treated wastewater; while the doped material removes 100 % of total fecal coliforms after 3 h of solar light exposure, except for just one tube of 10 %w Ag in the concentration of 10^{-1}.

The confirmative medium shows that for the pure TiO_2, *E. coli* was present in a concentration <7.8/100 ml; and for doped material there is no presence of gas in any sample tested, which indicates that purification process has better performance with doped material and the obtained water can be reused for food production free of pathogen agents.

For the metal removal, the material obtained for traditional method shows high capacity of copper removal compared with material obtained by microwave method, indicating that amino group free number is the most important factor for metal chelation, and these materials could be enhanced with others of similar nature.

In general, the complete process of biological, disinfection, and metal removal, has a high potential to be used to obtain water with enough quality, free of pathogen agents (microorganisms and metal ions), to be reused either for food production or activities that require constant human contact.

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Chapter 4
Biosystems Engineering Applied to Greenhouse Pest Control

Enric Vila and Tomas Cabello

Abstract The recent development and adoption of IPM programs in the southeast of Spain, where the largest concentration of protected crops of the world has developed, excluding China, has become a reference model for other areas, specially with Mediterranean climatic conditions. At least four key driving forces have been involved, strong regulatory framework, subsidies to the growers, extension of knowledge, and Research and Innovation. The last has motivated a drastic change in the mind-set of growers since they recognize biocontrol as the best pest management after selection and development of suitable species and efficient biocontrol programs, which are mainly based on augmentative releases of commercially mass-reared populations. The quality and price of the beneficials, which has deserved less attention when analyzing the failure or success of biocontrol, has contributed significantly to this achievement. Biosystems engineering progress to mass rear the key species of insects and mites currently used in these augmentative programs are reviewed. Specifically, attention is paid to the predatory mites Phytoseidae and some species of the families Miridae and Anthocoridae, as well as some parasitoids. Changes in the productions and formulation of new release systems, such as sachets à la carte that have increased the affordable quantities of individuals, are discussed. Finally, trends and challenges on complementary and artificial diets, as well as automatisms, which may decrease production costs and open new opportunities for open field crops, are explored.

Contents

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4.1 Introduction

The recent development and adoption of IPM programs in Spain on horticultural protected crops is a good example of how well biological control can function and can contribute to transform the agriculture toward a more sustainable system. The plant protection system in the southeast Spanish greenhouses, where the highest concentration of protected crops of the world have developed in the last decades, has shifted from a traditional example of environmental and public health risk toward a reference model to be followed by other crop production areas. The analysis of this transformation, as a case study, may help to establish and discuss the critical factors that are needed worldwide for entering the ecology-based pest management era and the challenges that have to be faced for a more broad adoption of biological control strategies.

Biosystems engineering progress to mass rear beneficial insects and mites and the professional development of productions, although they have deserved little attention when discussing the success or failure of IPM, are some of the main factors that have contributed to improve the biocontrol programs. The biological control programs of pests in most of the greenhouses are based commonly not only in the natural control of the spontaneously colonizing natural enemies, but also on augmentative biological control. This means releases of entomophaga that have been mass reared by biomanufacturers in each crop and season. A recent review of the commercially available natural enemies describes 230 species (van Lenteren 2012), but this number may have increased, at least one species more, named *Trichogramma achaeae* Nagaraja and Nagarkatti (Hym.: Trichogrammatidae), produced and released in several countries by the authors of this chapter (Fig. 4.1) (Cabello et al. 2012a, b). During the last decades, it has moved from a cottage industry to professional production, which has identified many efficient species of natural enemies, developed quality control protocols, mass production, shipment and release methods, as well as adequate guidance for farmers (van Lenteren 2003). However, commercial augmentative biological control is applied on a very limited scale, i.e., on 0.4 % of cultivated land with crops of which this type of control could be used (Cock et al. 2010). Some of the crucial causes explaining the limited uptake are the attitude of the pesticide industry, the pesticides use customs

Fig. 4.1 *T. achaeae* adult female (native entomophagous) parasitizing eggs of *Tuta absoluta* (exotic species)

of the growers, attitude of governmental institutions, the influence of guidelines and regulations as well as a too stressed auto critical evaluation of the biological control community (van Lenteren 2012). But little attention has been paid to the recent developments about biosystems engineering applied to the mass rearing of beneficial arthropods, as a main factor that has contributed to build up the quality of the reared natural enemies and the economically affordable quantities to be released in the crops.

In this chapter, the causes that have contributed to successfully develop biocontrol programs in the Spanish greenhouses are analyzed, as a case study to discuss the main ingredients that are needed for a transition toward a sustainable agriculture. This review is then focused on augmentative biocontrol programs. There has been a change of attitude of farmers that recognize biological control as the best pest management system after several improvements provided by the current IPM programs. Although there are other involved factors, the last is the key driving force, and we explore why the previously pesticide addicted growers of vegetal and ornamental crops have suffered a drastic change in the mind-set. We focus special attention in this review in the improvement of mass rearing techniques, selection of species, quality and price of beneficials, and the releasing strategies developed by the biomanufacturers.

4.2 Chemical Era Decline in the Horticultural Protected Crops

The world greenhouse area is estimated, excluding China, at approximately 350,000 ha, and the protected area in Spain is around 53,800 ha, 88 % destined to vegetal production. Most of this area lies within the boundaries of Andalusia, southern Spain, which contains the highest known concentration of greenhouses within the EU community; an area of 28,576 ha's of protected crops, grown mostly under plastic structures along a narrow coastal strip in the Province of Almeria.

Pest control in greenhouse crops in northern Europe has shown an important evolution within the past 30 years through the replacement of chemical control by biological control, mainly due to pest resistance to insecticides (van Lenteren 2007). The same has recently been reported in greenhouse crops in Spain (van der Blom 2010). The causes have been the same: excessive use of chemical control (Cabello and Cañero 1994; Cabello 1996) and pest resistance levels to insecticides (Cabello 2009), what is overlapped with, paradoxically, the low technical efficiencies of pesticide application systems (Aguera et al. 1998; Garzón et al. 2000; Cabello et al. 2001). In contrast to the northern Europe, the implementation of biological control in Almeria was achieved in such a short time that some journals named it the 'green revolution of Almeria.' In the past, the pressure of pests in Almeria was very high, due to the high concentration of greenhouses, with overlapping of crops all year round, and the lack of hermetic structures, that together with the polyphagy of some pests promoted the high development of their populations (Cabello and Cañero 1994). Since many chemical active ingredients had been already banished in the EU, the high number of applications of the remaining legal ones gave the best scenario for development of resistances of pests, which were not controlled anymore, then promoting the use of illegal products in some cases, as an erroneously desperate alternative. The establishment of biological control completely reversed the situation, turning the province into a Mediterranean and worldwide reference about the successful application of IPM strategies.

Over the 2012 season, biological control has been applied to 70 % of the greenhouse crops of Almeria but the transition has not been accomplished at the same time in all vegetable crops. Sweet peppers were the first where biocontrol was fully established, changing from 2 % under IPM in 2006/2007 to more than 73 % in 2008. In the following years, almost 100 % of this crop was already produced under biocontrol, which means about 8,400 ha. In contrast, the shift was achieved a few years later in tomatoes, which is the other major crop of the province, probably because the chemical control was offering a reasonable pest solution in this crop. But once the growers realized that biological control was cheaper and offered a better control of the major pests, as well as a control of the exotic recently arrived pest *T. absoluta* (Lep.: Gelechiidae) (Fig. 4.2), the change was also fulfilled in only 2 years. By 2011, 50 % of the tomato crop had followed suit, and currently almost the total surface, approx. 10,200 ha, are protected by biological control systems.

Fig. 4.2 Adult female of the South American Tomato Moth, *T. absoluta*, introduced as exotic pest species in tomato crops in Spain

4.2.1 Overview of Biocontrol Programs in the Main Spanish Protected Vegetables

The fast transition toward a biological control pest management system was achieved in Almeria not as a merely opportunistic change but after years of research which began in public and private research centers already in the 1980s (see analysis of the model below). Several significant changes from previous IPM models used in the north European countries were developed in order to face the particular conditions of the area (landscape, climate…), both regarding the selection of new species adapted to the climate conditions and the shipment and release methods. The techniques and methods used in Almeria, not only the species released, but the timing, releasing systems and the ratios of introduction, have been discussed, for instance, by Cabello and Vila (2010), Cabello et al. (2011) and Vila and Cabello (2012). A short overview of some of the systems used in the main protected vegetables in Almeria and the most important facing problems is presented here (Table 4.1), with special attention to those factors interrelated with the improvements developed and provided by the biomanufacturers.

One of the major problems facing in Almeria is the high pressure of pest in summer and beginning of autumn, when the season of the main crops begin. This means that preventive methods and strategies to favor the early establishment of the natural enemies should primarily be used. A preventive method in biocontrol is the introduction of the beneficials before than infestation of pests occurs. The feasibility to build up early populations may depend on the dietary habits of the natural enemy. These can be either polyphagous (e.g., *N. pseudoferus*; Hem.: Nabidae), attacking a large number of phytophagous species, pests or not; oliphagous, killing pest species relatively close (e.g., *Aphidius* spp.; Hym.: Aphididae); or specialist, which only prey or parasitize a pest species (e.g., *Rodolia cardinalis*; Col.: Coccinelidae). Moreover, natural enemies might show more or less omnivorous habits, being able to feed upon a wide range of organic sources in addition to insects and/or mites (Lundgren 2009). Some species are omnivorous like the predatory bug

Table 4.1 Commercially available natural enemies mainly released in protected Spanish crops and their classification according to their type of use in biological control programs

Type	Natural enemy	Feeding strategy	Main pests controlled	Biological control method			Notes
				Preventive	Curative		
					Inoculative	Inundative	
Predatory mites	*Amblyseius swirskii*	True omnivorous (also feeding on pollen)	Whiteflies, thrips	✓ (1)	✓	✓	(1) Releases with factitious prey and pollen
	Neoseiulus cucumeris	True omnivorous (also feeding on pollen)	Thrips	✓ (1)	✓	✓	
	N. californicus	Zoophagous-oliphagous	Spider mites	✓ (1)	✓	✓	
	Phytoseius persimilis	Zoophagous-oliphagous	Spider mites	–	✓	✓	–
Predatory insects	*Aphidoletes aphidimiza*	Zoophagous-oliphagous	Aphids	–	✓	✓	–
	Adalia bipunctata	Zoophagous-oliphagous	Aphids	–	–	✓	–
	Chrysoperla carnea	Zoophagous-oliphagous	Aphids	–	✓	✓	–
	Orius laevigatus	True omnivorous (also feeding on pollen)	Thrips	✓ (1) (2)	✓	✓	(2) Intragremial predation on *A. swirskii*
	Nesidicocoris tenuis	True omnivorous (also feeding plant tissue)	Whiteflies, thrips	✓ (3)	✓	–	(3) Releases on seedlings with factitious preys
	Macrolophus pygmaeus	True omnivorous (also feeding plant tissue)	Whiteflies, thrips	✓ (3)	✓	–	
	Nabis pseudoferus	Zoophagous-polyphagous	Aphids, lepidoptera	–	–	✓ (4)	(4) Releases in hot-spots
	Feltiella acarisuga	Zoophagous-oliphagous	Spider mites	–	✓	✓	–

(continued)

Table 4.1 (continued)

Type	Natural enemy	Feeding strategy	Main pests controlled	Biological control method			Notes
				Preventive	Curative		
					Inoculative	Inundative	
Parasitoids	*Aphidius colemani*	Parasitism	Aphids	✓ (5)	✓	✓	(5) Banker plants
	A. ervi		Aphids	✓ (5)	✓	✓	
	A. matricariae		Aphids	✓ (5)	✓	✓	
	Dacnusa sibirica		Leaf miners	–	–	✓	–
	Diglyphus issaea		Leaf miners	–	✓	✓	–
	Encarsia formosa		Whiteflies	–	✓	✓	–
	Eretmocerus mundus		Whiteflies	–	✓	✓	–
	E. eremicus		Whiteflies	–	✓	✓	–
	T. achaeae		Lepidoptera	–	✓	✓	–

O. laevigatus (Hem.: Anthocoridae), since it feeds on different prey and also on pollen, or to a greater extent phytophagous, like *Nesidiocoris tenuis* (Hem.: Miridae), which also can feed on the plant (Urbaneja et al. 2005). This is an additional advantage that has contributed to the success achieved with this specie on tomato crops in all the Mediterranean area, despite that it also may eventually become a pest of the crop due to their damage (Wheeler 2000; Sánchez and Lacasa 2008).

The early setting up of the population of predators is sometimes also determined by the intraguild competition (IGP) (Brodeur and Boivin 2006). This is the case of *O. laevigatus* which can be established on sweet peppers feeding on predatory mites like *A. swirskii* Athias-Henriot and *Amblyseius* (*Neoseiulus*) *cucumeris* (Oudemans) (Acari: Phytoseiidae). Relying on releases of several species for the pest management is a common practice in greenhouses, both in Spain and in other areas. Not only different predators but also several parasitoids are released either at the same time or at different stages of the crop cycle (Cabello et al. 2011). This can lead to direct and indirect interactions, like apparent competition, resource competition, and intraguild predation (Janssen et al. 1998), with implications regarding the efficacy of those natural enemies as biological control agents (Yano 2005; Messelink et al. 2012). In some cases, like the mentioned *O. laevigatus* and *A. swirskii*, the final outcome regarding pest control is commonly favorable (Vila and Cabello 2012), but in others is negative. Intraguild predation is regarded to be widely present in nature as well as in biological control systems. As stated and comprehensively reviewed by Janssen et al. (2006), IGP in biocontrol systems occurs between predators when a natural enemy, the intraguild predator, engages in trophic interaction with another entomophagous, rendered as the intraguild prey, while both compete for the same pest, the extraguild prey. The competition between parasitoid natural enemies and its impact on biological control programs has also been reviewed by Brodeur and Boivin (2006), but IGP between predator and parasitoid natural enemies has been reviewed to a lesser extent (Roshenheim et al. 2005), with the exceptions of the interactions found in the biological control of aphids (Brodeur and Roshenheim 2000; Traugott et al. 2012) and between predatory mites (Montserrat et al. 2008).

Preventive introductions of natural enemies in the crop can be enhanced providing direct releases on seedlings, which has become the most extended practice in the Spanish tomatoes (see below), and also using refuge plants. Both methodologies can reduce the economic cost, and refuge plants have additional advantages that can mitigate several troubles. On Spanish sweet peppers the two major agents released, the predatory mite *A. swirskii* and the flower bug *O. laevigatus*, offer a good control of pests, thrips, and whiteflies (Vila and Cabello 2012). But two problems which are being addressed is that from time to time this crop is subjected to very high summer temperatures, accompanied by very low humidities which adversely affects the predatory mites in current use, and the lack of flowers with pollen when the flower bug is introduced. Refuge plants, as basil, can mitigate this situation, providing alternative food and enhancing the establishment of *O. laevigatus* (Cano

Fig. 4.3 Banker plant system used for the introduction of predatory mirids in greenhouse crops

et al. 2012). Also they can be moved out of the crop when chemical treatments are applied so that a part of the natural enemies can be recovered when reintroducing the plants. In the Spanish cucumbers, for instance, *A. cucumeris* and *A. swirskii* are the two phytoseid predators used to control the thrips, which are the main pest, but despite being effective the frequent use of fungicides to control powdery mildew in autumn can considerably reduce their populations (Vila and Cabello 2012).

There are few developments that have used refuge plants to favor conservation and development of predatory bugs in the greenhouses, as the mirid *M. pygmaeus* (Rambur) (Hem.: Miridae) (Arno et al. 2000), during longtime misidentified as *M. caliginosus* (Martínez-Cascales et al. 2006), *N. tenuis* (Cano et al. 2009) (Fig. 4.3) and *Dicyphus hesperus* (Sánchez et al. 2003; Lambert et al. 2005); or the predatory mites (Ramakers and Voet 1995, 1996). The most extended used of refuge plants is the control of aphids, using barley or wheat infested with aphids species that do not affect broadleaf crops, and which are used to multiply preventively populations of parasitoids, like *A. colemani* (Frank 2010; Huang et al. 2011). Recently, an improved system has been developed that offers a high capacity of multiplication of parasitoids in the Spanish protected crops (Vila et al. 2010).

Preventive methods as the introduction of refuge plants are not incompatible with curative releases when pest infestation occurs, neither with the development of conservation biological control. The former is a common practice to increase (augmentation) (Parrella et al. 1992) the populations of beneficials by supplying them from commercial rearing systems. It can be inundative, releasing high amounts to reduce pest populations in a short period of time (few days) (e.g., Stinner 1977; Sithanantham 1980; Cabello et al. 2012a), or inoculative, releasing low numbers so that the biocontrol will be performed by the following generations established in the crop (e.g., Hoffmann et al. 2002; Kuhar et al. 2004; Cabello et al. 2012a). The former is the preferred system since economic cost is lower if natural enemies can properly develop in the crop for the entire cycle. Application of complementary diets may help to develop the populations after inoculative introductions when preys are scarce (see below).

Finally, with regard to the techniques of conservation of natural enemies, it includes establishing agronomic practices that do not disturb, or even increase, the natural enemies that spontaneously appear in the farm plot (Barbosa 1998). One of the practices with higher disturbing effect over these natural enemies is pesticide treatment. However, nowadays, the active agents that appear in the market tend to be increasingly specific on target pest species with less detrimental effects on natural enemies.

4.2.2 Analysis of Almeria's Model

At least four of the major described ingredients to favor a transition from a pesticide-based pest control to IPM systems are clearly stated in the model of Almeria, i.e., a strong regulatory framework, extension of the knowledge, incentives for the farmers, and research and development of efficient biocontrol programs.

The governmental positions, which have recently showed a shift in their interest toward more sustainable agriculture, are essential to fulfill this transition. The attitude of the governmental institutions has been underlined as one of the reasons for the limited use of commercial biological control, since they have not clearly provided long-term support for research and implementation (van Lenteren 2012). This general statement was also applicable in Spain until the beginning of the green revolution in Almeria, when the government did react competently forced by the complete loss of efficiency of pesticides linked with the export problems after the scandal created by detections of illegal residues. Their first measure was to set up a deep surveillance program imposing high fines to the growers not fulfilling the MLRs. EU agricultural policy had already abolished at that time many chemical products in all the EU countries, but control had not always been properly guaranteed. Second, they participated on the extension of knowledge to establish non-chemical control alternatives, for instance with 30 advance classes about IPM, teaching more than 750 field technicians in only 2 years in Almeria, where both professors of public research centers and private sector participated. Finally, they also supported the process with subsidies of up to 50 % of the cost of the beneficials, granting more than €46 millions in 4 years. Importantly, this support was a critical point at the beginning but once the farmers realized that the biocontrol worked better than the chemicals it was not needed anymore. In tomato, for instance, where the biological control developed several years later, the shift to biological methods was fully accomplished without subsidies.

More recently, there has been a significant inflection point of the EU policy toward farming with minimal use of pesticides, clearly stated in the EU Directive 2009/128/EC; EC 2010). The new directive gathers up for the first time the basic philosophy of the IPM as the concept, originated already 50 years ago, described by the International Organization of Biological Control (IOBC) (Boller et al. 2004). This means, they give priority to all non-chemical crop protection methods, especially biological control. Each country has to set up a National Action Plan before 2014 establishing clear objectives in this sense and also indicators as tools to evaluate the accomplishment of the goals. Specifically, in Spain this NAP has been published and a National Commission Board to follow not only the application but also any other plant protection subject has been recently established, where for the first time representatives of the bioindustry, academics, and grower's organizations participate. Not only in EU, but also in various areas worldwide other sustainable approaches of pest control have been settled, as it was reviewed by Peshin et al. (2009).

From all the mentioned driving forces to stimulate the use of biological control, the recognition of the results of the plant protection alternatives by growers, which depends on previous years of research to fine settle IPM or organic successful programs, is the most relevant. This can be well exemplified in Almeria, where biocontrol programs adapted to the climatology and conditions of the area, after years of research, were able to be established when the green revolution began. Many trials had been attempted before without achieving a total success, because the released natural enemies were selected for north European countries with different environment. The proper selection of species well adapted to Mediterranean conditions changed the situation, namely the predatory mite *A. swirskii* Athias-Henriot (Acari: Phytoseidae) and the predatory bug *N. tenuis* Reuter (Het.: Miridae).

Researchers of the industry have collaborated with the public research sector in this process, not only in the suitable selection of the species as well as the shipment and release methods, which both were decisive, but also guarantying the availability of big quantities of mass-reared populations that were able to be supplied at the right time.

4.3 Role of Bioindustry

There are more than 500 companies selling natural enemies worldwide but only 30 are large producers (with more than 30 employees), of which 20 are located in Europe (Bolckmans 2008). The four biggest companies, which are the ones that employ more than 50 people, are the main suppliers of predators and parasitoids in all the major markets of Europe. It has been estimated a worldwide market of €300 millions at end user level (Cock et al. 2010), although this may have been reduced considerably because the prices of many natural enemies have decreased in the last years. For instance, the cost of beneficials released per hectare on sweet peppers in Almeria has decreased from more than €2,500 to less than 1,200 in the last 4 years. The commercial augmentative biological control is made mostly in protected crops, and even that the biological control in greenhouses is expected to expand considerably in the USA and also in Latin America (Bueno 2005) in the upcoming years, at present more than three quarters of worldwide market share is Europe. The major market in the last is Almería, estimating approx. €20 millions in 2012 (only natural enemies, bumblebees for pollination excluded).

All manufacturers, as well as some research organizations, consultants, extension services, and distributors involved on the development of biological control are organized since 1995 in Europe in the International Biocontrol Manufacturers Association (IBMA). They collaborate with other associations with similar aims to form a global federation, already with a working link with Biopesticides Industry Alliance in North America (BPIA). In the IBMA, a total of 98 companies dealing with production and promotion of macroorganisms are organized in a professional group, and collaborated with the Association of Natural Biocontrol Producers in North America, Australian Biological Control (ABC), and New Zealand.

One of the main tasks of the IBMA is to collaborate with the IOBC and the EU and national governments for the definition and implementation of quality standards, as well as prepare environmental-risk assessment methods necessary before introductions of exotic species. After more than 7,000 releases involving 2,700 species of exotic arthropods agents rarely have resulted in negative environmental effects, but there are a few exceptions (Jacas 2002; Cock et al. 2009, 2010). The establishment of a risk evaluation criteria as well as the harmonization of the beneficial insect and mites registration is needed not only to avoid negative environmental effects but also to keep a good image of the companies as well as to avoid excessive costs that would choke their growth and feasibility. Considering regulations for import and export of beneficials, the future of biocontrol could be seriously threatened by the recent plans concerning benefit sharing under the Convention of Biological Diversity, against which IOBC and IBMA have prepared a position paper (Cock et al. 2010).

Quality of predators and parasitoids supplied by manufacturers is undoubtedly a major issue to ensure the expansion of biocontrol methods as can be exemplified in the green revolution of Almeria, where the quality of the three main released predators, *O. laevigatus* (Fig. 4.4), *N. tenuis* (Fig. 4.5), and *A. swirskii* (Fig. 4.6), have suffered major improvements due to ameliorations of the mass rearing systems (see below). Inside the IOBC, the global Working Group named ‘Arthropod Mass Rearing and Quality Control’ is coordinating the common efforts of investigators and natural enemies producers to improve and guarantee quality of beneficials. One of the major driving forces that has resulted in significant improvements in quality is the heavy competition itself among producers. Then, development and reproduction parameters, as well as behavioral traits which need to be contrasted in field

Fig. 4.4 Adults of *Orius laevigatus* depredating eggs of Lepidoptera

Fig. 4.5 Adult of the omnivorous (zoo-phytophagous) *N. tenuis*

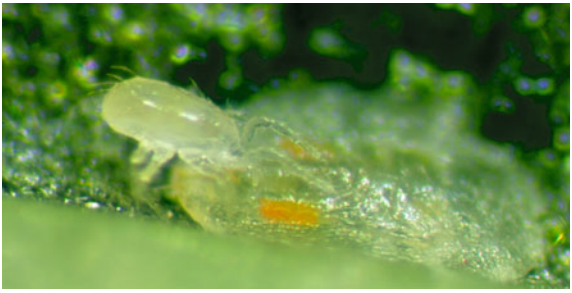

Fig. 4.6 Adult of the predatory mite *A. swirskii* feeding on whitefly nymph, *Bemisia tabaci*

conditions, like searching ability and pest control, have been improved among others. Changes in parameters related to rearing productions, as well as decreases in prices have also brought about improvements. A good example is the formulation of sachets that can multiply and release populations of predatory mites in excess of that indicated in the label.

Insect and mites rearing is not only a technical application but a true research field to settle at a front place (Cohen 2001). Development of cost-effective rearing techniques is needed so that augmentation can become competitive strategy for managing arthropod pests. Many described species by academic researchers as promising candidates for pest control have not yet been produced industrially since no adequate rearing systems have been developed. Even with the already commercialized species reducing the cost of production is one of the major issues, as an essential improvement to develop their use, for instance, outside-protected crops and the major companies are investing efforts in this direction (Table 4.2). It is remarkable that in spite of most biomanufacturers being small companies with not a strong R&D branch, serious advances have been achieved, especially if we consider that bioindustry has probably many hundred times lower budged than the biggest chemical companies.

No serious economical estimations have been made about the contribution of biological control in growing areas like Almeria, but main tomato producers stated already savings of more than 80 % of chemical treatments, while beneficials represent less than 1.5 % of the total investment made by the growers in the greenhouses per year (Cabrera and Uclés 2012). Not to mention the socioeconomic impact if pepper production would have disappeared due to inefficacy of chemicals since supermarkets were going to stop buying the product from Almeria (Pardo 2010), or the great environmental contamination reduction and public health improvement thanks to augmentative biocontrol. The ecosystem service provided by natural and inoculative biological control was estimated as a worldwide value of at least $400 billion per year (Costanza et al. 1997).

There are two main steps to reduce the cost of mass rearing arthropods (Fig. 4.7), the use of carrying materials together with factitious prey or host to eliminate the plants needed to grow the natural prey, and the utilization of totally artificial diets that obviates the use of any arthropod component (in vitro). Most of the productions in the past were based on a tritrophic system, and still some are, with a plant where the host or prey is developed, commonly a pest, and the parasitoid or predator. To produce some of the most used predators the plants have been eliminated by using alternative prey, mainly eggs of the moth *Ephestia kuehniella* Zeller (Lep.: Pyralidae) (Table 4.2), although some other alternatives are consumed in less amount, like eggs of *Sitotroga cerealella* (Olivier) (Lep.: Pyralidae), dipteral larvae of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), crustacean cysts of *Arthemia* sp. (Anostraca: Artimidae), coleopterous larvae of *Tenebrio molitor* L. (Col.: Tenebrionidae), and lepidopterous larvae of *Galleria mollonella* (L.) (Lep.: Pyralidae). To rear parasitoids of aphids it has also been developed the rearing system of several pests, as hosts, in artificial media, then using the same aphid host species, but eliminating the plants (Ance et al. 2002; van Emden 2009; Boivin et al. 2012).

The elimination of the plant to reduce costs is an important step, but a further problem remains due to the still high cost of the most used alternative prey, *E. kuehniella*. Development of artificial diets is an alternative solution. Artificial diets must satisfy the nutritional requirements of predators to ensure the continuous production of progeny of high quality (Cohen 2004). The *Ephestia* eggs can be bought in the market between 500 and 800 €/kg, depending on the desired

Table 4.2 Systems used for the commercial production of the main currently released natural enemies in greenhouses of Almeria, Spain

Type	Natural enemy	Factitious prey/hosts	Whole plant-free	Climatic controlled rooms	Climatic rooms pests on artificial diets	Pests on plants
Predatory mites	*A. swirskii*	*Carpoglyphus lactis* *Thyreophagus entomophagous* *Suidasia medanensis*	✔	✔	–	–
	N. cucumeris	*T. putrescentiae Tyreophagus entomophagus*	✔	✔	–	–
	N. californicus	*Glycyphagus sp.*	✔	✔	–	✔[a]
	P. persimilis	–	–	–	–	✔
Predatory insects	*A. aphidimiza*	–	–	–	–	✔
	A. bipunctata	*E. kuehniella*	✔	✔	–	✔
	C. carnea	*E. kuehniella*	✔	✔	–	✔[a]
	O. laevigatus	*E. kuehniella, C. capitata*	✔	✔	–	✔
	N. tenuis	*E. kuehniella, Artemia sp.*	✔	✔	–	✔[a]
	M. pygmaeus	*E. kuehniella, Artemia sp.*	✔	✔	–	✔[a]
	N. pseudoferus	*E. kuehniella*	✔	✔	–	✔[a]
Parasitoids	*A. colemani*	–	✔	✔	✔	✔[a]
	A. ervi	–	✔	✔	✔	✔[a]
	A.matricariae	–	✔	✔	✔	✔[a]
	D. issaea	–	–	✔	–	✔
	E. formosa	–	–	–	–	✔
	E. mundus	–	–	–	–	✔
	E. eremicus	–	–	–	–	✔
	T. achaeae	*Ephestia kuheniella*	✔	✔	–	–

[a] Several companies are still producing this natural enemies using pests reared on whole plants

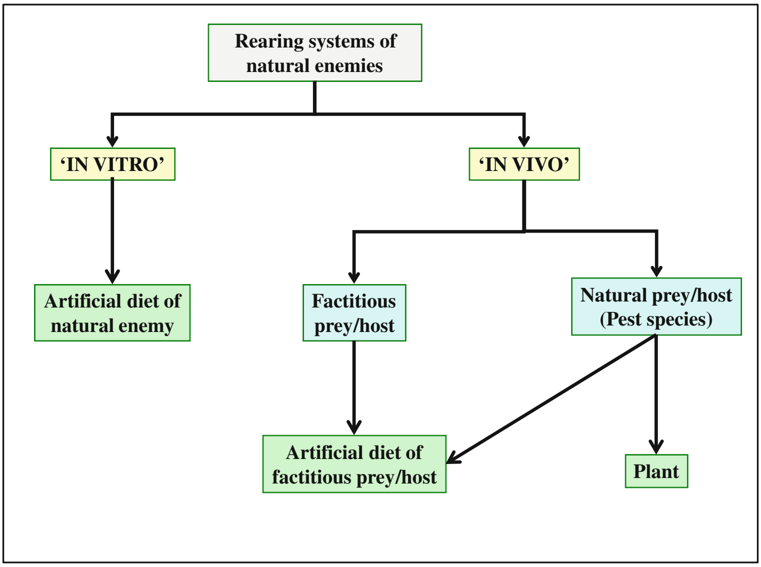

Fig. 4.7 Systems of rearing 'in vitro' and 'in vivo' used for the production of natural enemies in Spain

quantity, and for the production of several entomophaga this makes up the major investment. Since the biggest companies consume thousands of kilograms of this factitious prey/host (e.g.: Nagaraja 2013), their future depends on lowering their use in order to save money which is the only way to keep competitive in the market with the actual low prices of the predators. Some other advantages of using artificial diets eliminating part of the consummation of *Ephestia*, other than reducing costs, is a simplification of the production line, higher flexibility, and reduction of allergy problems generated by moth scales. One example of flexibility is related with the problem of overproductions, which is a common practice so that surges in demand can be met. Since both beneficials and most of the preys used to feed them are short life products that cannot be stored during longtime, the elimination of any arthropod food can contribute to a higher flexibility of productions.

Several reviews have been published about the scientific literature pertaining to factitious preys and artificial diets, to mention three fine ones: Thompson (1999), Thompson and Hagen (1999), both covering papers until 1998, and Riddick (2009), examining data from 1999 to 2007. Even that many works have been published by academic researchers, very few examples have been transferred to commercial industry. The most cited exception is the production of *Trichogramma* produced on artificial eggs. After several decades of work on the subject (Cabello et al. 1984; Cabello 1985), with only partial success in USA (Parra 2010), the production of this parasitoid was achieved efficiently using artificial diet in China (Liu et al. 1995; Parra 2010) and South America (Parra 2010; Consoli and Grenier 2010); in China the produced parasitoids have been released in thousands of hectares in different crops (Liu et al. 1995). One of the major limiting factors may be the still lower quality of most of the natural enemies reared with artificial diets when compared to the same ones reared with factitious preys, but a few exceptions have been published with high potential to be scaled up by the industry. The needed protection of results when companies are involved (secrecy, competition, patents, etc.) also delay the exchanges between researchers and slow down the improvements. Finally, automatism process, which has deserved less attention by academics, is a significant step for the industry.

An option for the biomanufacturers is the use of artificial diets not to completely substitute the factitious preys, but just a part of the diets, mixing it with the prey or just using it in specific stages of the predator. Not only important economical savings have been achieved without losing final quality of the insects, but also improvements of biological parameters of generalist predators have been accomplished with the mixtures of complementary non-arthropod diets together with *Ephestia* eggs on industrial productions, according to results obtained by the authors of this chapter. Other authors have also highlighted the benefits that feeding generalists predators with a mixed diet could have on reproduction (Evans et al. 1999; Leon-Beck and Coll 2007; Michaud and Jyoti 2008; Urbaneja-Bernat et al. 2012).

Generalist predators have been stated to be more suitable for being reared successfully using artificial diets, and certainly the best achievements obtained on industrial processes are with this generalist entomophaga. In this chapter attention will be focused on the latest improvements accomplished on rearing systems of the main generalist predators that are released on greenhouses in Almeria, as well as in other growing areas of protected vegetables in Europe. Specifically, attention is played to species belonging to the families Miridae, Anthocoridae, and the predatory mites Phytoseidae. The last ones have deserved little or no attention in the previous reviews published about factitious preys and artificial diets, even that they represent a significant number of the predators released by commercial producers. Moreover, Phytoseidae are the key natural enemies in several crops and after the latest mass rearing improvements they have a big potential as biocontrol agents for many crops, including open fields and, in general, crops that suffer of a low profit margin.

4.4 Predatory Mites

Currently, all commercially used in large quantities phytoseiid mite predators are reared ex-plant, not on their preferred pest species but with factitious preys, except *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) which is reared on plants with spider mites. The factitious preys are commonly a member of one of a few families of mites from the order Astigmata. These are small, soft-bodied individuals, known as ‘stored food mites’ or ‘stored product mites’ and as such live on a wide range of foods especially those of farinaceous origin (Hughes 1976). Using the right food materials and under the right physical conditions, these mites can be reared in large concentrations in containers held in temperature controlled rooms. The correct balance of ratio of predator to prey will provide a commercially acceptable sales product quite quickly.

There are well over 2,000 named species belonging to the family Phytoseiidae from which most of the commercial predators have been taken. Although many species have been described as promising candidates for biological control, at present only 30 species have been commercially produced worldwide, of which merely seven are produced and sold in large quantities in Europe (van Lenteren 2012; Gerson et al. 2003; Griffiths 1999). A reason for this paucity of candidates is that often the combination of a plant dwelling phytoseiid and a factitious stored product host is a new experience for both, frequently leading to incompatibility.

A good example of industrial mass rearing success is the predatory specie *A. swirskii*, that was first described as a promising candidate for biological control of greenhouse whiteflies and western flower thrips (Nomikou et al. 2001, 2002; Messelink et al. 2006, 2008) and later stated as a good predator of broad mites (van Maanen et al. 2010). In Almería the availability of commercial productions of this specie was a major step to successfully establish biocontrol programs. Before the green revolution many releases of the predatory mite *Amblyseius cucumeris* (Oudemans) had been completed without fully achievement of a good pest control, due to extremely high temperatures inside the greenhouses (up to 45 °C) and low humidities (<40 % RH) in summer, when the season of the main crops begins. *N. cucumeris* is a good predator of thrips and was the first predatory mite specie that was able to be mass reared indoors in very large quantities, already in the 1980s of the twentieth century, using the factitious prey mite *Tyrophagus putrescentiae* (Schrank) (Acari: Acaridae). But this predatory species was selected for north European greenhouse conditions and does not offers a good pest control at high temperatures.

The possibility of mass rearing high quantities so that big amounts of predators can be introduced in the crops with an affordable price is as important as the selection of a well-adapted specie to the local conditions. *A. swirskii* has become the largest production of predatory mites in Europe, and it is introduced in most of the protected vegetables except tomato, generally at more than 100 individuals/m^2. The rearing system of *A. swirskii* is based on the use of the factitious prey *C. lactis* (L.), (Acari: Carpoglyphidae) and the system was patented several years ago. After the success obtained with *A. swirskii*, and being award of the future potential of such large capacity to mass rear phytoseid predators, the main three or four biomanufacturers invested more effort to develop other candidates. This has brought already to six patents that have recently been granted by the European Office Patent, and some others may be in evaluation process. All of them are based on combinations of different species of Astigmatidae with Phytoseiidae. In Table 4.3, the description of the combinations protected in each granted patent is summarized.

Table 4.3 European patents developed by biomanufacturer companies of natural enemies in recent years

Patent number	Applicant	Factitious prey species	Predatory mite species	Granting date
EP 1686849 B1	Koppert BV	*C. lactis*	*A. swirskii*	October 8, 2008
EP 1830631 B1	Koppert BV	*C. lactis*	Different species other than *A. swirskii* from members of the subfamilies Amblyseiinae, Typhlodrominae, 25 spp. listed	May 5, 2010
EP 1965634 B1	Koppert BV	Large number of members belonging to the family Glyciphagidae	Different species other than *A. swirskii* from members of the subfamilies Amblyseiinae, Typhlodrominae, 29 spp. listed	August 4, 2010
EP 2042036 B1	Koppert BV	Species belonging to families: Pyroglyphidae (*Dermatophagoides pteronysius*, *D. farina*, *Euroglyphus longior*, *E. maynei*, *Pyroglyphus africanus*) & Glycyphagidae (*Glycyphagus destructor*, *G. domesticus*, *Lepidoglyphus destructor*)	*A. swirskii*	June 22, 2011
WO 2008/015393 A2	Syngenta bioline	*Thyreophagus entomophagus*	8 phytoseiidae genera *Amblyseius andersoni*, *A. californicus*, *A. cucumeris*, *A. limonicus*, *A. montdorensis*, *A. ovalis*, *A. stipulatus*, *A.* fallacis, *A. swirskii*, *A. womersleyi*	Under evaluation
EP 2124573 B1	Certis Europe BV	Members of Suidasiidae *Suidasia medanensis*	*A. swirskii*, *A. cucumeris*, *A. andersoni*, *A. californicus*, *Typhlodromus pyri*	May 4, 2011
EP 2380436 B1	Agrobío SL	Species from the order Astigmata not alive, fastfrozen members of the families Acaridae, Carpoglyphidae, Glycyphagidae, Chortoglyphagidae	Species of the subfamilies Amblyseiinae, 15 spp. listed	October 3, 2012

4.4.1 An Inert Diet

Frequently, the introduction of a new suitable predator is thwarted by the fact that when presented with a strange factitious host it refuses to predate. Two factors seem to be operating; first the astigmatid prey is often very active, using fast jerky movements as a defence against predators. Second, the Astigmata are known to possess an alarm pheromone defence system used to ward off attackers (Kuwara 1991). Under such circumstances it is impossible to produce a viable commercial product.

The R&D department of the SME's company Agrobio S.L. have recently overcome this problem by employing a new diet, which is based on the application of the well-developed fast frozen technologies for human consumption to rear predatory mites, then using dead Astigmatid preys (EP 2380436 B1). To date, in house trials, still on-going, have shown that by using this new system it is most likely possible to rear difficult predator candidates so that the high intrinsic rates of development needed to produce a viable sales product is attainable.

4.4.2 The Sachet á La Carte

The packaging method and releasing system is another issue improved by biomanufacturers that contribute significantly to the final outcome of the biocontrol programs. An initial problem was that the only practical way of applying the Phytoseiidae to the crop was to sprinkle loose material onto the plant leaves. Where the prey, which is susceptible to low humidities, below 70 % RH soon died or ran out of food. So that predators, in the absence or shortage of the pest species and pollen, failed to establish. An additional problem is the frequent application of fungicides that can prevent the development of the populations on the plants. This is the case in the protected cucumbers in Almeria, where weekly treatments of fungicides are made during autumn, since high incidence of phytopathogenic fungi occur during this period, partially due to unsatisfactory structures of the greenhouses with poor ventilation. Some active ingredients have been stated to kill more than 50 % of the populations, while others less aggressive may have a washing effect. The problem was partially solved by the amelioration of the sachet system used to distribute the predators in the crop, already developed many years ago, based on packaging quantities of the predator plus prey in a mixture of prey food and a filler substrate, often bran, in sachets made of a breathable chapter. A small hole pierced toward the top of the sachet permits the predator to exit, and the sachet can be attached to an individual plant thanks to a small hook (Sampson 1998). Recently, the enhanced system has been called Controlled/Quick Release System. The rate of emergence of the predator can be controlled by altering the initial balance of predator to prey and some other conditions of the packaged material. Basically, too little prey gives a quick release whereas a higher quantity can extend the emergence life up to 6–8 weeks, a controlled release. The first system is used when the pest is already present. The second provides a nucleus of predators ready to meet the first invaders. Then, packaging is made á la carte according to the target crop, the growing area and the period of the year. Recently, promising control of thrips on peach and nectarines orchards have been stated using improved sachets systems which can release up to 8,000 individuals of *A. andersoni* (Chant) per sachet (Vila et al. 2013).

Several artificial diets have been developed and tested to rear predatory mites but no commercial uptake as been fully accomplished due to very low fecundities of females when feeding with the alternative food (McMurtry and Scriven 1966; Kennett and Hamai 1980; Itagaki and Koyama 1986; Ochieng et al. 1987; Shih et al. 1993). Several promising achievements have been described, but also not used so far in a broad scale, adding to the crops complementary diets, in order to favor the establishment of the predators, using in some cases artificial diets (Ogawa and Osakabe 2008) but most commonly with pollen. The last can be offered using banker plants (Ramakers and Voet 1996; Messelink et al. 2005; Vantornhout et al. 2005; Nguyen and Shih 2010; Al-Shammery 2011; Huang et al. 2011), by introducing plastic cups with pollen sources (Nomikou et al. 2010) or by spraying or dusting pollen (Weintraub et al. 2009; Kasap 2005; González-Fernández et al. 2009; Nomikou et al. 2010; Gerson and Weintraub 2012). Decapsulated brine shrimps of *Artemia* sp. have also been stated as a potential food to be used in the mass rearing production of *A. swirskii* according to the performance of this predatory mite when feeding this food during six generations compared to other alternatives such as *Ephestia* eggs or artificial diets (Tung et al. 2013). Adding this *Artemia* cysts to ornamental greenhouse plants can significantly reduce the needed introductions of *A. swirskii* in order to control broad mites (Audenaert et al. 2013).

4.5 Predatory Bugs

After the mites the bugs are the largest productions of predators released in augmentative biocontrol programs in temperate and Mediterranean Europe. Bugs are interesting in protected cropping systems, because the zoo-phytophagy of some Mirids is favorable to a good survival of the insects in early season even in the absence of prey. Particularly in Almeria, the species *N. tenuis* has been the responsible of the adoption of biological programs in tomatoes, not only in almost 100 % of the protected crops but also in open fields. Sales in this area is estimated to reach 100,000 bottles of 1,000 individuals per year by the end of 2013. The successfully developed programs using this predatory bug are currently exported to other Mediterranean areas, like Middle East and North Africa.

Several improvements have been achieved by the manufacturers regarding the mass rearing of not only *N. tenuis* but also another largely produced mirid bug, as well as the anthocorid *O. laevigatus*, which are the main released bug species in the protected vegetables in Europe. Due to the secrecy of the productions systems developed by biomanufacturers is not possible to discuss the latest improvements, but at least two major advances have been uptake and further developed by the major companies. First, the production of bugs on greenhouses has been replaced by rearing in cages on climatic controlled rooms, without whole plants, where the different cohorts can be separated so that the age of the populations can be carefully controlled. This allows a perfect control of the climate throughout all the year, avoiding any temperature threshold problem and fluctuations due to changes with the number of hours with light. Then, not only reducing production costs, but also favoring a homogeneous product all year round. The populations can be packaged at the optimal age for laying eggs when released, for instance, with young mated adults after the pre-oviposition period, which will fasten the establishment of the first generation in the crop. This means that the crop is protected against pest from early stages and growers feel 'more confident' with biological control. This is an important subject since most growers and technical advisers over the world, who have been working for many years with chemical control, get anxious at the beginning of the crop when they see the first pest individuals, and very often they apply erroneously the so called preventive chemical treatments, which delays even more the growth of the predatory populations and compromise the biological control success.

A second major improvement by biomanufacturers that also guarantees the early establishment of the predatory populations is the release of the natural enemies in the seedlings before transplant into the greenhouse. This was already evaluated many years ago with *M. pygmaeus* and has recently been developed for introduction of *N. tenuis* in protected tomato crops (Vila et al. 2012). Major companies have participated not only with research trials but also with a strong extension effort, offering the first years many hectares per free to the main tomato cooperatives in Almeria, which have contributed to the fast adoption of the system to almost all the growers in only 2 years. Using this release system all the plants have eggs of the predator when transplanted so all the crop is protected from the beginning. Since *N. tenuis* is a zoophytophagous predator that can damage the plant when too high populations are built up and few prey is available (Wheeler 2000; Sánchez and Lacasa 2008) carefully adjustments have to be made of the ratio of introduction, according not only to the season but also to the cultivar (Cabello et al. 2012a, b).

The use of not only artificial living but also plant-free oviposition substrates and substitutive artificial food to eliminate the need of *Ephestia* eggs has been evaluated by several academic researchers. As it has already been stated in the field by the researchers and technicians of the private companies, Vandekerkhove et al. (2011) also did not find any lose of quality between populations of *M. pygmaeus* when reared for over 30 generations using artificial living and oviposition substrates compared with those of conventionally plant reared, both regarding biological parameters or predation capacity. Similar artificial living substrates were described for *O. laevigatus* (Bonte and De Clercq 2010). But the results when combining artificial food, as well as other alternatives to *Ephestia* eggs, plus the plant-free substrates, are not so clear and have not yet being adopted by commercial productions. Vandekerkhove et al. (2011) stated a similar predation rates when feeding with artificial diets from the first to the fourth instar nymphs, suggesting that at least initial stages of *M. pygmaeus* can be reared with this food without lose of quality. Castañe and Zapata (2005) reported that, despite their lower body weights and slower development, adults of *M. pygmaeus* reared for seven generations on an artificial diet were as effective at killing whiteflies and two-spotted spider mites as their peers reared on *E. kuehniella* eggs. Likewise, Castañe et al. (2002) found similar predation capacity of the mirid bug *Dicyphus tamaninii* Wagner (Hem.: Miridae) when feeding artificial diets, and Bonte and De Clercq (2010) found that both fifth instar and female adults of *O. laevigatus* feed artificial diet or pollen has lower weights but similar predation rates as those reared on *E. kuheniella* eggs. Another possibility is the incorporation of insect components other than *Ephestia* eggs in semiartificial diets. Almost all the semiartificial diets described to rear *Orius* contained insect tissue or related extracts (Ferkovich and Shapiro 2005, 2007).

Automatism and time-consuming for the production of the artificial diet is another major issue for the biomanufacturers. Portilla et al. (2011) obtained a modified semi solid artificial diet to rear the mirid pest specie *Lygus hesperus* Knight (Hem.: Miridae), which saved 20 % of previous used artificial diets and more than 75 % of time. In addition to the components of the diet, its state (liquid, solid, etc.) can influence the rearing success. On one hand, they can affect the acceptance of the food by the bugs which have sucking mouth. On another hand, they have to meet the commercial requirements in terms of acting as nutritious reserve during storage and transportation of biological control agents. Encapsulation devices for encapsulation of dome based on using stretching Parafilm® have been used (Vandekerkhove et al. 2011) and also Microencapsulation techniques has been developed for predatory bugs (Tan et al. 2013). Microencapsulation is an advanced packaging technique in widespread use for packaging microbial agents and chemical or food products in microcapsules in order to promote the quality of artificial diets. Although it has been a limited and complicated technique based on chemical properties, spray-drying process (Gharsallaoui et al. 2007), more recently new alternatives microfluidic-based synthesis of hydrogel particles, based on physical properties of liquids, have opened large possibilities (Kim et al. 2007). Tan et al. (2013) obtained significant improvements of an optimized artificial diet for rearing the anthocorid *Orius sauteri* (Poppius), an Asiatic predator, using microencapsulation. These authors showed great benefits for practical biological control application according to the predatory ability and population dispersion obtained with the mass reared populations using the microencapsulated artificial diets.

The described food alternatives in laboratory conditions need further research when applied in large productions. Sometimes researchers have defined alternative preys which are cheaper than eggs of *E. kuheniella* for rearing predatory bugs, but when scaling up the methodology on commercial productions decreases of fecundity of females and/or higher mortalities are obtained. For instance, brine shrimp cysts (*Arthemia* spp.) have been described as suitable for rearing the predatory mirid bug *M. pygmaeus* (Callebaut et al. 2004; Castañe et al. 2006) and the anthocorid bug *O. laevigatus* (De Clercq et al. 2005). Lower fecundities of the females have been stated by the authors when using this prey instead of *E. kuheniella* eggs in the commercial productions of this predatory bugs. Increases of cannibalism when rearing high populations using suboptimal diets may be one of the reasons. Another reason may be that commercial populations are developed up to the maximum carrying capacity, and introduction of suboptimal diets may stress the populations.

4.6 Conclusions

Currently, the global surface area under IPM is very limited but the possibility to develop and adopt it in a more broad scale is facing the best chances it never had. Two drastic changes in this direction in Europe, the current shift in attitude of politicians regarding the need to reduce chemical applications and increase the non-chemical alternative methods, as it is clearly stated in the EU Directive 2009/128/EC, and the often demand of products with a limit level of residues far below the legal ones, nationally or internationally regulated, by the supermarket chains. A good example of transition towards a more sustainable system, after failure of chemicals to control the pests and a strong market pressure to eliminate the residues, is the greenhouse vegetal production in the southeast of Spain. At present, almost 100 % of the two major protected crops in this area, tomato and peppers, are cultivated under IPM.

This achievement would have not been possible in the Spanish greenhouses without years of research to provide with appropriate natural enemies and efficient biocontrol programs. Biomanufacturer companies of natural enemies, despite being most of them small and medium companies without a strong R&D, have also participated in the successful implementation. Some of them have collaborated with the research institutions to select the needed species and to fine settle the IPM programs, but more significantly, they have developed the industrial mass rearing of these beneficials and improved the suitable releasing systems, as well as provided the needed quantities at the right time.

One of the major recent improvements on mass rearing techniques has been reached with the predatory mites, which as a result have become an essential element of augmentative programs on vegetables. Combinations of astigmatid mites as factitious preys to rear predatory species of the Phytoseiidae, using carrying materials in climatically controlled rooms, has made possible to increase the affordable quantities released per area of the largest five produced species. This has contributed to guarantee a satisfactory control of pests like whiteflies, thrips, spider mites and broad mites, not only in the Spanish but also other worldwide greenhouses. The application of fast frozen food technologies to provide dead astigmatid populations to predatory mites is a further promising development that have shown possibilities to rear difficult predator candidates, especially autochthonous species, that have not been able to be commercially produced until present. A further development to introduce predatory mites has been the formulation of sachets *á la carte*, with a quick or controlled release system. For instance, 5 years ago the producers were delivering sachets of *A. swirskii* that were able to release up to 800 individuals, while at present most of the commercial sachets produce approx. 2,500, and even some are able to multiply and release up to 8,000 individuals, delivering individuals into the crop during up to 8 weeks (Vila et al. 2013).

Another improvement provided by some of the major biomanufacturers is the rearing of the main released predatory bugs in climatic rooms, such as *M. pygmaeus*, *N. tenuis*, and *O. laevigatus*, using factitious foods without whole plants. This has contributed not only to save costs and keep companies competitive despite decreases of prices of biocontrol products but also to control more accurately the age of the populations released in the crops, supplying fertilized young females, which helps to guarantee an early establishment of the populations. Additional enhancements regarding the early establishment of the predators have been achieved releasing the natural enemies directly in the seedlings so that all plants have already eggs of the predators when transplanted in the crop.

Similar advances have been achieved with parasitoids of aphids that are produced in climatic controlled rooms, multiplying the host aphid species in artificial foods without plants.

More recent developments that may lead to a promising future of biological control are the incorporation of complementary diets that increase performance of populations, the development and use of alternative artificial diets to decrease the actual dependence to the expensive eggs of *Ephestia*, and the development of automatism processes. Despite that several successful results have been described by academics they have not yet being incorporated in the commercial productions, to some extent because some problems occur when scaling up to industrial processes. However, ongoing trials show significant improvements with mixtures of foods that decrease the levels of mortality of reared populations, and microfluidic-based synthesis of hydrogel particles offers solutions to supply food both in the productions and in the crops, as well as act as a nutritious reserve during storage and transportation of biological control agents. All this advances may help to develop and adopt biocontrol programs in low-profit margins crops, like open fields. Combination of augmentative programs, after selection and development of high quality industrial rearing of natural enemies, with conservation biological control strategies, is a future trend that will be decisive to get a successful transition to a productive and sustainable agriculture, the already named evergreen revolution by some authors.

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Chapter 5
Sugarcane as a Novel Biofactory: Potentialities and Challenges

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Abstract Sugarcane is the most productive crop plant to date, and its potential of becoming a crucial biofactory for generating high-value bioproducts is emerging only recently. Though it possesses one of the most complex genomes in the plant kingdom, important advances have been made in terms of transgenic approaches to generate new varieties, both by particle bombardment and *Agrobacterium*-mediated transformation. Nevertheless, crucial aspects in breeding programs and molecular technologies have to be developed or improved, before this crop consolidates as the highest productive biofactory. Social and biosafety issues also need to be addressed. Here, we highlight the most recent advances in the biotechnology of sugarcane to produce alternative products such as pharmaceutical proteins, biopolymers, and high-value carbohydrates, and strengthen opportunities and challenges of sugarcane as a biofactory of novel compounds. We conclude that the progress in molecular approaches to develop sugarcane into a sustainable biofactory demonstrates that this crop has tremendous potential and may play an important role in the growing bioeconomy through biopharming. Like no other contemporary crop, sugarcane is facing new paradigms and is expected to contribute at least partially to the development of new generation highly profitable biofactories.

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Contents

5.1 Introduction

Modern sugarcane, the main source of sucrose worldwide, belongs to the grass family (Poaceae) and was created about a century ago from the combination of *Saccharum* polyploid species. According to Daniels and Roach (1987), the genus *Saccharum* comprises six different species: *S. barberi*, *S. edule*, *S. officinarum*, *S. robustum*, *S. sinense*, and *S. spontaneum*. Of these, *S. officinarum* (the domesticated sugar-producing species) and *S. spontaneum* (a vigorous wild species with many aneuploidy forms) are thought to be the ancestors of cultivated sugarcane. *S. officinarum* originally derived from *S. robustum*, while *S. barberi* and *S. sinense* are thought to have been derived by crossing *S. officinarum* and *S. spontaneum* (Asano et al. 2004; Sandhu et al. 2012). However, Irvine (1999) suggested only two true species: *S. officinarum* and *S. spontaneum*, and therefore, current sugarcane commercial cultivars are thought to be hybrids with 80–90 % of the genome from *S. officinarum* and 10–20 % of the genome from *S. spontaneum* (Grivet et al. 1996; Hoarau et al. 2002).

The chromosome number of these species ranges from 36 to 200 (Asano et al. 2004; OGTR 2011). The polyploid and aneuploid nature of the genus *Saccharum* has made phylogenetic analyses and, as a result, breeding programs a tough task. Furthermore, the taxonomy and phylogeny of sugarcane is complicated as plants from five genera are thought to share common characteristics and form a closely related interbreeding group known as the "*Saccharum* complex". This complex comprises the genera *Saccharum*, *Erianthus* section *Ripidium*, *Miscanthus* section *Diandra*, *Narenga* and *Sclerostachya* (D'Hont et al. 1998; OGTR 2011), albeit controversial discussions still remain in the scientific community concerning the genetic relationships among genera in this complex and new hypotheses are being formulated. As a consequence, the assumption that *S. officinarum* is a result of a complex introgression between *S. spontaneum*, *Erianthus arundinaceus*, and *Miscanthus sinensis* (reviewed by Daniels and Roach 1987) is being analyzed in the light of new biochemical and molecular approaches. Accordingly, current extant species of the genera *Saccharum*, *Erianthus*, and *Miscanthus* are clearly distinct in their isozyme profiles, nuclear and cytoplasmic restriction fragment length polymorphisms (AFLPs), and simple sequence repeats (SSRs) and sequence data (reviewed by D'Hont et al. 2008). As a result of these analyses, it has been assumed that the genus *Saccharum* is a well-defined lineage that has diverged over a long period of evolution from the lineages leading to the *Erianthus* and *Miscanthus* genera (Grivet et al. 2006; D'Hont et al. 2008) and that cultivated sugarcanes probably emerged from wild *Saccharum* species, while secondary introgressions with other genera are not likely pathways (D'Hont et al. 2008).

The *Saccharum* species are not only polyploid, but also autopolyploid (hosting more than two sets of homologous chromosomes derived from a single species) and allopolyploid (possessing two or more unlike sets of chromosomes from different species) (Sreenivasan et al. 1987; Besse et al. 1997), which represent a tremendous challenge for breeders that normally base their statistical genetic approaches on models developed for diploid organisms.

A summary of the genetic characteristics of the *Saccharum* species and the "*Saccharum* complex" is shown in Table 5.1.

As a relatively recently domesticated species, sugarcane exhibits little of the available genetic diversity having been incorporated or actively analyzed for introgression into domesticated varieties (Dillon et al. 2007; OGTR 2011; Sreenivasan et al. 1987), and breeding programs in the early 1900s focused on hybridization of *S. officinarum* clones, but quickly progressed to interspecific crosses incorporating *S. spontaneum*. This resulted in improved agronomic traits, such as tilling, stand and trashiness abilities, ratooning and disease resistance, but required a backcrossing program to *S. officinarum*, called "nobilization," to elevate the sucrose content (Dillon et al. 2007; Edmé et al. 2005). Since then, the majority of breeding programs have focused on intercrossing between the hybrids, though in recent decades the larger increases in genetic gains have been made by incorporating more diverse germplasm into the cultivated backgrounds (Edmé et al. 2005; Dillon et al. 2007) not only to increase sucrose production, but also to diversify into other alternative products to regain profitability.

As a C4 carbohydrate metabolism plant having a perennial life cycle, sugarcane is one of the most productive cultivated plants. Apart from producing sugar, this crop has gained increased attention because of its importance as a biofuel source among other value-added products developed from sugarcane biopharming using molecular approaches. Nevertheless, sugarcane has one of the most complex genomes among cultivated plants, which has long hampered the development of crucial areas such as genetics to support breeding for crop improvement programs. With the advent of molecular techniques, the sugarcane genome has become less mysterious, although its complexity has still been confirmed in many aspects (D'Hont et al. 2008).

In this chapter, we review the current status of sugarcane as a potential biofactory focusing particularly on the production of pharmaceutical proteins, biopolymers, and alternative carbohydrates; topics related to the use of sugarcane for bioenergy generation have been thoroughly addressed elsewhere (Arruda 2012; de Siqueira et al. 2013; Kuan et al. 2013; Vermerris 2011) and therefore will not be discussed herein.

Table 5.1 Genetic characteristics of species from the *Saccharum* genus and *Saccharum* complex

Species or genus	Classification	Sugar content	Chromosome number (2n)	Monoploid genome size (Mbp)	References
Saccharum barberi	Ancient hybrid	Low	111–120	3,156–4,121	Asano et al. (2004), Bonnett and Henry (2011)
S. edule	Wild species	Nil	60–80		Asano et al. (2004), Bonnett and Henry (2011)
S. officinarun	Noble cane	High	70–140	985	Asano et al. (2004), Bonnett and Henry (2011), Zhang et al. (2012)
S. robustum	Wild species	Nil	60–200	1,195	Asano et al. (2004), Bonnett and Henry (2011)
S. sinense	Ancient hybrid	Low	80–124	4,183	Asano et al. (2004), Bonnett and Henry (2011)
S. spontaneum	Wild species	Nil	40–128	843	Asano et al. (2004), Bonnett and Henry (2011), Zhang et al. (2012)
Erianthus spp.	Wild sugarcane relative		20–60	980–1,205	Chae (2012)
Miscanthus spp.	Close sugarcane relative		38–76	2,150–2,650	Rayburn et al. (2009), Swaminathan et al. (2012)
Narenga spp.	Wild sugarcane relative		30–34		Sobral et al. (1994), Grivet et al. (2006)
Sclerostachya spp.	Wild sugarcane relative		30–96		Janaki-Ammal (1940), Butterfield et al. (2001), D'Hont et al. (2008)

5.2 Sugarcane Is More Than Sucrose

According to Hoarau et al. (2007), sugarcane converts the sun's energy into carbohydrates more efficiently than any other crop plant and has the unusual ability to store sucrose in stem cell vacuoles. This, along with its high biomass production and ease of cultivation, makes it one of the most interesting and productive agricultural crops. In fact, Waclawovsky et al. (2010) have established that the current world yield average for sugarcane is 80 t ha^{-1}, but the estimated theoretical yield potential is over 380 t ha^{-1}, while Moore (2009) calculated it over 472 t ha^{-1}, which supports the hypothesis of yield gains to be expected in the future.

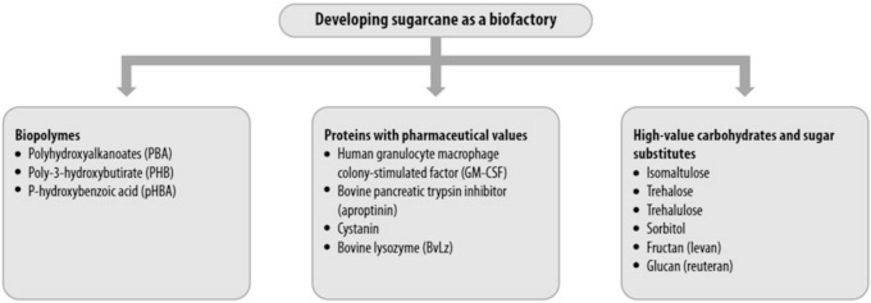

Fig. 5.1 Current main focus areas for developing sugarcane as a biofactory

Flourishing in humid and warm climates, sugarcane is mainly cultivated in tropical and subtropical regions on 25.4 million ha in more than 90 countries; its harvested biomass makes it the world's largest crop with nearly 1,800 million metric tons produced in 2011 as reported by FAO (http://faostat.fao.org). Mainly used to produce sugar, it accounts for approximately 75 % of the total world sugar production, while beet sugar is responsible for 25 %. By-products obtained from sugarcane include a wide range of derivatives (e.g., molasses, alcohol, fuel, livestock feed, paper, particle board) that can be used in the energy, food, chemical, pharmaceutical, and other industries (Hoarau et al. 2007; Tew and Cobill 2008).

Apart from sugar and bioethanol production, in the last few years, sugarcane has also turned into a target crop for biosynthesis of novel products such as proteins with pharmaceutical properties (Holland-Moritz 2003; Wang et al. 2005), biopolymers (Brumbley et al. 2004, 2007; McQualter et al. 2005a; Petrasovits et al. 2012), and high-value carbohydrates and sugar substitutes (Basnayake et al. 2012; Bauer et al. 2012; Chong et al. 2007, 2010; O'Neill 2011; Paterson et al. 2013) (Fig. 5.1).

Biopolymers are considered novel petrochemical substitutes that are environmentally friendly. Proteins with pharmaceutical value may contribute to the alleviation of important human diseases. Novel carbohydrates and sugar substitutes are crucial for developing nutraceutical products that can benefit consumers and other industrial processes.

The plant is well suited for such objectives due to some of its characteristics such as vegetative propagation, absence of flowering in most commercial varieties, production of a large biomass, large amount of carbon partitioned into sucrose (up to 42 % of the stalk dry weight), and the mobile pool of hexose sugars through most of its life cycle (D'Hont et al. 2008).

5.3 Sugarcane Biofactory for High-Value Biopolymers

Nowadays the production of plastics, polymers, surfactants, and other similar synthetic products is dominated by the petrochemical industry, although biotechnology, through metabolic engineering, may account for as much as 15 % of the US$250 billion polymer market by the year 2015 (Nielsen 2005; Endres et al. 2007). As a matter of fact, traditional chemical industries are already shifting from chemical to biological processes and new opportunities are continuously emerging in the pharmaceutical, food, and biomedical areas. As a consequence, using sugarcane to manufacture plastics has several potential advantages over the traditional methods of production, including higher yields, greater purity, lower energy use, and less waste production (Nielsen 2005).

Importantly, by using bioplastics it is likely to reduce toxic emissions in the environment and also diminish loads of industrial wastes on landfills (Fritz et al. 2001; Seinbüchel 2003). Besides, when plants are used as biofactories, the major limitations of organic synthesis, namely long product lead time and expensive plant design to handle toxic compounds at high pressure and temperature, are overcome (Nielsen 2005). As a consequence, various research groups worldwide have made important progress in the development of sugarcane as a novel biofactory in the last decade.

For instance, Brumbley et al. (2004) engineered the genetic pathway for poly-3-hydroxybutyrate (PHB) in sugarcane. In general, polyhydroxyalkanoates (PHA) which include PHB, have thermoplastic properties and are biodegradable. In a subsequent transgenic approach, McQualter et al. (2005a) reported that transgenic sugarcane plants harboring a chloroplast-targeted version of *Escherichia coli* chorismate pyruvate-lyase (CPL) (Siebert et al. 1996) and a 4-hydroxycinnamoyl-CoA hydratase/lyase from *Pseudomonas fluorescens* (HCHL) (Gasson et al. 1998) (both enzymes providing a one-enzyme pathway from a naturally occurring plant intermediate), were able to synthetize p-hydroxybenzoic acid (pHBA, an aromatic hydroxiacid which constitutes monomers of liquid crystal polymers used in the electrical and optical industries),which was quantitatively converted to glucose conjugates by endogenous uridine diphosphate (UDP)-glucosyl transferases and apparently stored in the vacuole. The largest amounts detected in leaf and stem tissue were 7.3 and 1.5 % dry weight, respectively, while there were no evident phenotypic defects. However, as a result of diverting carbon away from the phenylpropanoid pathway, there was a severe reduction in leaf chlorogenic acid, subtle changes in lignin composition, and an apparent compensatory upregulation of phenylalanine ammonia-lyase (McQualter et al. 2005b).

Brumbley et al. (2007) transformed sugarcane with three genes from the bacterium *Ralstonia eutropha* that encode the genetic pathway for the biosynthesis of PHB. In the best transformed line, PHB accumulated at 2.5 % of leaf dry weight. Furthermore, transgenic plants were evaluated as a production platform for pHBA using two different bacterial genes, one from *Escherichia coli* and the other from *Pseudomonas fluorescens*. Each of these genes modifies a different existing biochemical pathway in sugarcane. In the best line, a glycosylated form of pHBA accumulated in the leaf and stem tissue at 7.3 and 1.5 % dry weight, respectively.

Purnell et al. (2007) demonstrated that several transgenic sugarcane lines accumulating the bacterial PHB exhibited a vertical PHB concentration gradient, while the polymer concentration showed the lowest level in the youngest leaves and increased with leaf age. In addition, there was a horizontal gradient along the length of a leaf, with the PHB concentration increasing from the youngest part of the leaf (the base) to the oldest (the tip). The rank order of the lines did not change over time. Moreover, there was a uniform spatiotemporal pattern of relative PHB accumulation among the lines, despite the fact that they showed marked differences in absolute PHB concentration. Molecular analysis showed that the expression of the transgenes encoding the PHB biosynthesis enzymes was apparently coordinated, and that there were good correlations between PHB concentration and the abundance of the PHB biosynthesis enzymes. The maximum PHB concentration recorded (1.77 % of leaf dry weight) did not result in agronomic abnormalities. Although moderate PHB concentrations were achieved in leaves, the maximum total-plant PHB yield was only 0.79 % (11.9 g PHB in 1.51 kg dry weight).

As plant peroxisomes contain the substrate molecules and essential reducing power for PHB biosynthesis, Tilbrook et al. (2011) generated transgenic sugarcane with the three-enzyme *Ralstonia eutropha* PHA biosynthetic pathway targeted at these cell compartments. PHB accumulated in sugarcane leaves at levels up to 1.6 % dry weight, in both peroxisomes and vacuoles. A small percentage of total polymer was also identified as the copolymer poly (3-hydroxybutyrate-co-3-hydroxyvalerate). As a result of peroxisomal PHA biosynthesis, no obvious detrimental effect was observed on plants. This study highlights how using peroxisomal metabolism for PHA biosynthesis could significantly contribute to reaching commercial production levels of PHAs in crop plants.

Petrasovits et al. (2012) used different plant and viral promoters, in combination with multigene or single-gene constructs to increase PHB levels in sugarcane. Promoters tested included the maize and rice polyubiquitin promoters, the maize chlorophyll A/B-binding protein promoter, and a Cavendish banana streak badnavirus promoter. At the seedling stage, the highest levels of polymer were produced in sugarcane plants when the Cavendish banana streak badnavirus promoter was used. However, in all cases, this promoter underwent silencing as the plants matured. The rice Ubi promoter enabled the production of PHB at levels similar to the maize Ubi promoter. The maize chlorophyll A/B-binding protein promoter enabled the production of PHB to levels as high as 4.8 % of leaf dry weight, which is approximately 2.5 times higher than previously reported levels in sugarcane. However, the highest PHB-producing lines showed phenotypic differences to the wild-type parent, including reduced biomass and slight chlorosis.

5.4 Sugarcane Biofactory for Protein Products

Regarding pharmaceutical applications, one of the first approaches reported was done by Holland-Moritz (2003), who transformed sugarcane to produce pharmaceutical-grade human structural proteins for human therapeutics. Later, Wang et al. (2005) successfully produced the human granulocyte macrophage colony-stimulated factor (GM-CSF, used in clinical applications for the treatment of neutropenia and aplastic anemia) in transgenic sugarcane plants. Accumulation of GM-CSF protein ranged from undetectable to 0.02 of total soluble protein. Human bone marrow cells (TF-1), which require GM-CSF for cell division, proliferated when growth media was supplemented with transgenic sugarcane extracts. Comparison to purified commercially produced GM-CSF indicated that sugarcane-produced protein had essentially identical activity levels. In a 14-month field trial, accumulation levels remained stable.

Arvinth et al. (2010) transformed sugarcane cultivars Co 86032 and CoJ 64 with the *cry1Ab* gene driven by maize ubiquitin promoter through particle bombardment and *Agrobacterium*-mediated transformation systems. Gene pyramiding was also attempted by retransforming sugarcane plants carrying the bovine pancreatic trypsin inhibitor (aprotinin, which reduces bleeding during complex surgeries) gene, with *cry1Ab*. Aprotinin-expressing sugarcane pyramided with *cry1Ab* showed reduction in damage by the shoot borer *Chiloinfuscatellus*.

Henrique-Silva and Soares-Costa (2012) generated transgenic sugarcane expressing a His-tagged cystatin (a human protein used as biomarker for the identification and prevention of various diseases) under the control of the maize ubiquitin promoter. A transformed sugarcane plant presented high levels of protein expression and was selected for the purification of this protein through affinity chromatography in nickel columns. Therefore, it was demonstrated that sugarcane can be a viable expression system for recombinant protein production and that the His-tag purification strategy used to isolate the purified protein was effective.

Recently, Barros et al. (2013) generated transgenic sugarcane expressing recombinant bovine lysozyme (BvLz, used to control gram-negative pathogenic bacteria) in order to evaluate the feasibility of extraction and fractionation of recombinant proteins expressed in sugarcane stalks. Partial removal of native proteins was achieved using a 100 kDa membrane, but 20–30 % of the extracted BvLz was lost. Concentration of clarified extracts using a 3 kDa membrane resulted in twofold purification and 65 % recovery of BvLz. Loading of concentrated sugarcane extract on hydrophobic interaction chromatography (HIC) resulted in 50 % BvLz purity and 69 % recovery of BvLz.

5.5 Sugarcane Biofactory for High-Value Carbohydrates and Alternative Sugars

Other research groups have focused on developing sugarcane as a platform for the production of higher value isomers of sucrose such as isomaltulose and trehalose.

Isomaltulose is a natural isomer of sucrose. It is widely approved as a food with properties including slower digestion, a lower glycaemic index, and low cariogenicity, which can benefit consumers. Furthermore, isomaltulose displays reducing properties that make it attractive as industrial precursor for the manufacturing of biosurfactants and biopolymers (Lichtenthaler and Peters 2004;

Ravaud et al. 2007). In turn, trehalose is implicated in anhydrobiosis as a result of its high water retention capabilities, and is used in the food and cosmetic industries. The saccharide may form a gel phase as cells dehydrate, which prevents disruption of internal organelles and may function as an antioxidant as well (Reina-Bueno et al. 2012). Availability of these saccharides is currently limited by the cost of fermentative conversion from sucrose (Mudge et al. 2013).

Wu and Birch (2007) engineered an efficient sucrose invertase isolated from the bacterium *Pantoea dispersa*, with a monocot promoter and a vacuolar targeting sequence (Gnanasambandam and Birch 2004) and transformed sugarcane explants with this construct to produce isomaltulose. Isomaltulose accumulated in sugarcane stem storage tissues of transformed plants without any decrease in the stored sucrose concentration, resulting in nearly doubled total sugar concentrations in harvested juice. Transgenic plants also showed higher photosynthetic activity, sucrose transport, and sink strength, which indicates a possible feedback signal for sucrose biosynthesis, translocation, and storage (Wu and Birch 2007).

In order to develop an efficient *in planta* sugarcane-based production system by coupling the synthesis of alternative products to the metabolic intermediates of sucrose metabolism, Chong et al. (2007) evaluated the biosynthesis of sorbitol (a polyalcohol used as sugar substitute) in sugarcane using the *Malus domestica* sorbitol-6-phosphate dehydrogenase gene (*mds6pdh*). The average amounts of sorbitol detected in the most productive line were 120 mg g^{-1} dry weight (equivalent to 61 % of the soluble sugars) in the leaf lamina and 10 mg g^{-1} dry weight in the stalk pith. The levels of enzymes involved in sucrose synthesis and cleavage were elevated in the leaves of plants accumulating sorbitol, but this did not affect sucrose accumulation in the culm. Sorbitol-producing sugarcane generated 30–40 % less aerial biomass and was 10–30 % shorter than control lines. Leaves developed necrosis in a pattern characteristic of early senescence, and the severity was related to the relative quantity of sorbitol accumulated. When the *Zymomonas mobilis* glucokinase (*zmglk*) gene was coexpressed with *mds6pdh* to increase the production of glucose-6-phosphate, the plants were again smaller, indicating that glucose-6-phosphate deficiency was not responsible for the reduced growth. In conclusion, sorbitol hyperaccumulation affected sugarcane growth and metabolism, but the outcome was not deleterious for the plant.

Interested in the unusual development of the leaves in some transgenic sorbitol-producing sugarcane plants, Chong et al. (2010) compared the polar metabolite profiles in the leaves of those plants against a group of control sugarcane plants. Principal component analysis of the metabolites indicated that sorbitol, gentiobiose (a disaccharide), and gentiobiitol (a sugar alcohol) were strongly associated with sorbitol-producing canes. Gentiobiose and gentiobiitol were positively correlated with sorbitol accumulation.

Trehalulose is also a structural isomer of sucrose that has a sweet taste with similar physical and organoleptic properties to sucrose. Additionally, trehalulose is acariogenic and can be applied in diabetic and sports foods and drinks as its absorption reduces the rate at which monosaccharides and insulin are released into the bloodstream (Ravaud et al. 2007). Hamerli and Birch (2011) reported the transformation of sugarcane plants with a vacuolar-targeted trehalulose synthase gene modified from the gene in *Pseudomonas mesoacididophila* MX-45 and obtained transgenic lines reaching about 600 mM of trehalulose in mature stem juice. These plants retained vigor and trehalulose production over multiple generations under glasshouse and field conditions.

Sucrose is the translocated photosynthate and the largest soluble carbon store in sugarcane. The capacity to carry stored sucrose into pathways that provide substrates to produce alternative products would be highly advantageous in an efficient biofactory. Hence, a high-yielding sugarcane biofactory system would ideally contain culm tissues that function as a secondary source tissue rather than a sink in terms of sucrose balance (O'Neill 2011). To that end, O'Neill et al. (2012a) demonstrated that sucrose is mobilized from set storage parenchyma via phloem to the growing shoot tissue. Overall, metabolism in storage parenchyma shifts from futile cycling to a more quiescent state during sucrose mobilization. Subsequently, trehalose metabolism in sugarcane was engineered in an attempt to create a significant carbon drain of stored sucrose to impart value-added properties and enhance abiotic stress tolerance (O'Neill et al. 2012b). To that end, two transgenes were introduced into the sugarcane genome: trehalose-6-phosphate synthase-phosphatase (TPSP) to increase trehalose biosynthesis, and an RNAi transgene specific for trehalase to abrogate trehalose catabolism. In RNAi-expressing lines, trehalase expression was abrogated in many plants although no decrease in trehalase activity was observed. In TPSP lines trehalase activity was significantly higher. No events of co-integration of TPSP and RNAi transgenes were observed, suggesting that trehalase activity is essential to mitigate embryonic lethal effects of trehalose metabolism (O'Neill et al. 2012b).

Moreover, transgenic sugarcane plants expressing a vacuole-targeted isomaltulose synthase in seven recipient genotypes (elite cultivars) were evaluated over 3 years under commercial field conditions (Basnayake et al. 2012). Isomaltulose concentration typically increased with internode maturity and comprised up to 217 mm (33 % of total sugars) in whole-cane juice. There was generally a comparable decrease in sucrose concentration, with no overall decrease in total sugars. After several cycles of field propagation, selections were obtained with cane yields similar to the recipient genotypes. Sucrose isomerase activity was low in these transgenic lines, and the results indicate strong potential to develop sugarcane for commercial-scale production of isomaltulose if higher activity can be engineered in appropriate developmental patterns.

Bauer et al. (2012) reported the effect of high molecular weight bacterial fructan (levan) and glucan (reuteran) on growth and carbohydrate partitioning in transgenic sugarcane plants. These polysaccharides are products of bacterial glycosyltransferases, enzymes that catalyze the polymerization of glucose or fructose residues from sucrose. Heterologous expression resulted in reduced total carbohydrate assimilation rather than a simple diversion of biopolymers by competition for substrate.

Lately, transgenic sugarcane plants with developmentally controlled expression of a silencing-resistant gene encoding a vacuole-targeted isomaltulose synthase were tested under field conditions. High yields of isomaltulose were obtained, up to 81 % of total sugars in whole-cane juice from plants aged 13 months (Mudge et al. 2013). Using promoters from sugarcane to drive expression preferentially in the sugarcane stem, isomaltulose levels were consistent between stalks and stools within a transgenic line and across consecutive vegetative field generations of tested high-isomer lines. Importantly, these data represent the highest yields ever achieved of value-added materials through plant metabolic engineering. The sugarcane stem promoters are promising for strategies to achieve even higher isomaltulose levels and for other applications in sugarcane molecular improvement. Silencing-resistant transgenes are critical for delivering the potential of these promoters in practical sugarcane improvement. At the isomaltulose levels now achieved in field-grown sugarcane, direct production of this disaccharide in plants is feasible at a cost approaching that of sucrose, which should make the benefits of isomaltulose affordable on a much wider scale.

5.6 Potentialities and Challenges of Sugarcane as a Biofactory

Recently, sugarcane has become an important crop for food and energy production, and is emerging as a pivotal biofactory for high-value products. Its ability to accumulate high levels of sucrose in its stems and its distinctive high yield make it a unique crop, showing it to be the highest tonnage crop among cultivated plants. Though breeding programs have focused on improving sugar content, an evolving industry of biofuel and bio-based compounds such as biopolymers, pharmaceutical proteins, and novel carbohydrates may require vast amounts of biomass and, therefore, higher yields as well (Dal-Bianco et al. 2012).

Compared to other major crops, efforts to improve sugarcane are limited, as a consequence of its narrow gene pool, complex genome for molecular breeding, and the long breeding/selection cycle. These constraints, nonetheless, make sugarcane a good candidate for the application of molecular technologies. In recent years, considerable progress has been made in understanding the sugarcane genome, creating transgenic plants with improved agronomic, industrial, or other important traits, developing novel molecular markers, and understanding the molecular aspects of sucrose biosynthesis, transport, and accumulation in greater detail (Lakshmanan et al. 2005; Ming et al. 2006; Paterson et al. 2013). Accordingly, biotechnological routes for sugarcane improvement including technological data available and the use of marker-assisted breeding, genome sequencing, genetic engineering, and gene discovery for traits of interest are being addressed to reach higher productivity goals and develop sustainable molecular pharming.

Although a plethora of advantages of crop plants as biofactories are well documented (Ahmad et al. 2010; Becerra-Moreno et al. 2012; Jacobo-Velázquez et al. 2011; Jenkins et al. 2011; Rigano et al. 2013) as they are renewable resources of lower environmental impacts with balanced carbon emission, these systems also face technical constraints and have to compete functionally and economically with traditional petrochemical production methods (Nielsen 2005; Goldemberg et al. 2008). Consequently, low-cost raw materials, efficient biocatalysis, and product innovations are all key determinants of success. Accordingly, sugarcane juice is a readily fermentable low-cost feedstock, and the bagasse, representing outstanding sources of low-cost green process energy, and fermentable and aromatic compounds (Chandel et al. 2013; Cheng and Zhu 2013; Nielsen 2005). Moreover, sugarcane has several other traits that give it tremendous potential to become a critical crop for transition from petrochemical-based to bio-based economies (Paterson et al. 2013). Then, using sugarcane as a biofactory of novel environmentally friendly products may also offer possible diversification for cane-growers, as well as reducing the reliance by rural sectors on sugar prices (Nielsen 2005). Currently, important research groups are involved in this form of biopharming projects around the world.

Nevertheless, many impasses must be overcome before sugarcane biofactories can become a commercial fact. To contend with commercial protein production systems that use well-established molecular protocols in plants such as maize and tobacco, approaches will need to bring about much higher levels of protein expression in the transformed sugarcane plants, especially in the stalk. These challenges will demand the identification, isolation, and amplification of new promoter regions (both constitutive and inducible), development of novel vectors, and success with both transcriptional and post-transcriptional gene modification and silencing. Moreover, the protocols for protein extraction and purification at an industrial level from vegetative tissues represent a daunting task that has to be addressed with several innovative strategies. Practical knowledge and skills in this field are in their infancy, and especially for global industries such as sugarcane (Paterson et al. 2013).

Constraints related to the long time required for conventional breeding of sugarcane (i.e., it takes 12–15 years to carry out, test and launch a new variety to the market) and its highly complex genome (polyploidy and aneuploidy) may be overcome by using molecular approaches. However, sugarcane exhibits recalcitrance to genetic transformation and several parameters usually need optimization at the variety level to reach higher transformation efficiencies (Scortecci et al. 2012). Indeed, the first protocol developed for genetic transformation of sugarcane was particle bombardment (biolistic) of cell suspension, embryogenic callus or meristem (Bower and Birch 1992; Snyman et al. 2006), but the efficiency of this method depends on callus formation and plant regeneration, which varies with genotype and culture conditions (Kaeppler et al. 2000; Scortecci et al. 2012). Later, *Agrobacterium tumefaciens*-mediated transformation arose (Arencibia et al. 1998; Brumbley et al. 2008) and was more efficient than biolistics for its higher stability on transgene expression, which derives from the smaller number of transgene copies integrated into the genome (Dai et al. 2001; Scortecci et al. 2012). Nevertheless, *Agrobacterium*-mediated transformation has shown low efficiency and is highly genotype- dependent, so that some in vitro culture parameters resulted as key factors to improve this transformation method, as well as genotype screening, explant type and quality, selective agents, and *Agrobacterium* strains (Arencibia et al. 1998; Arencibia and Carmona, 2006; Manickavasagam et al. 2004). Importantly, Jackson et al. (2013), van der Vyver et al. (2013) and Mayavan et al. (2013) have recently reported successful results using both transformation methods for sugarcane.

Although no commercial transgenic sugarcane variety is available in the market so far, genes associated with sucrose content (Papini-Terzi et al. 2009), resistance to pests and pathogens, including constructs against insects, bacteria, and viruses (Arencibia et al. 1997, 1999; Falco and Silva-Filho 2003; Ingelbrecht et al. 1999; Weng et al. 2006, 2011; Zhu et al. 2011; Ismail 2013), herbicide-resistance genes as selective markers (Manickavasagam et al. 2004) and drought tolerance (Molinari et al. 2007) have been successfully cloned into some varieties. Besides, none of those reports refers to plastid transformation, even though this technology is considered a valuable tool for improving the containment of the transgene, and enhancing the biosafety of genetically modified (GM) plants (CBU 2007; Gottschamel et al. 2013; Ruf et al. 2007; Scortecci et al. 2012).

The inheritance of the chloroplasts in most plants is maternal, as these organelles are not carried by pollen. The manipulation of the chloroplast genome for crop improvement is therefore a highly promising technology for biosafety reasons. There are several examples of agronomical and biotechnological applications of plastid transformation with enhanced biosafety and higher transgene product yields in C4 plants and green microalgae (Wang et al. 2009; Chen and Melis 2013; Hanson et al. 2013) and new advances are being developed (Gottschamel et al. 2013). Although chloroplast genetic transformation is still very incipient in monocots like rice (Lee et al. 2006) and wheat (Cui et al. 2011; He 2012), and it has not been reported for sugarcane (Scortecci et al. 2012), the research avenue is widely open since the chloroplast genome of sugarcane has been completely sequenced (Calsa-Júnior et al. 2004), which enables recombination-based transformation with huge potential for basic and applied research in molecular pharming.

Importantly, a repertoire of gene promoters that work efficiently and precisely regarding level, timing, and location of expression is a critical element of transgenic cultivar development (Scortecci et al. 2012).

Public opinion currently appears to be biased against foods derived from GM organisms, and the cane industry faces a general community rejection of sugar produced by GM plants (Grice et al. 2003). In other industries, GM cultivars that are environmentally friendly and not designed for human consumption (e.g., *Bt*-cotton) have been accepted reasonably well. One of the main causes of public concern about genetic engineering has been the lack of information about the process and the types of products, particularly nonfood products that can be developed. As a consequence, in many countries GM sugarcane is facing release restrictions (Grice et al. 2003; Cheavegatti-Gianotto et al. 2011; Scortecci et al. 2012), which has to be taken into consideration when designing sugarcane programs aimed at developing biofactories using GM strategies. Due to the potential for new alternative uses of sugarcane other than food, such as supplying high-value niche markets with a variety of novel products, the need for further analyses into product diversification as a way of increasing industry returns has also been emphasized (Grice et al. 2003).

Thus, despite the convoluted genetic system present in sugarcane, which largely limits the use of traditional genetic markers in breeding programs, it is becoming clear that molecular genetics and genomics will play important roles in sugarcane breeding programs, as transformation techniques become more efficient and more molecular tools (characterization of genes of interest, transformation vectors, specific promoters) become available.

Of economical relevance, Hansen et al. (2011) describe a series of recent patents on methods and techniques involving genes coding for proteins and breeding techniques with agronomic applicability on economically important crops, including sugarcane.

The sequencing of the complete sugarcane genome led by an international research group from Australia, Brazil, China, France, South Africa, and the USA is underway, and will greatly contribute to deciphering vital genetic information controlling crucial desirable traits related to genomic organization, promoters, gene regulators, and gene networks controlling metabolic pathways (Hotta et al. 2010; Scortecci et al. 2012; Dal-Bianco et al. 2012).

Moreover, sugarcane plantations are often criticized as they occupy large field areas of fertile arable land that otherwise could be used for food production, for impacting the environment with deforestation and land degradation, monocultures, as well as pollution (run-off of fertilizers, pesticides and molasses; pre-harvest burning and air pollution) (Scortecci et al. 2012; Uriarte et al. 2009). As environmental and social responsibility issues are being addressed in agriculture more often, it is also criticized that sugarcane production systems rely heavily on low-paid seasonal jobs and labor abuses worldwide (child labor, slavery regimen, hazardous conditions, underpayment) (Martinelli and Filoso 2008; Miranda 2010; Scortecci et al. 2012). Therefore, a need for developing a sustainable sugarcane industry with social responsibility is demanded by society worldwide.

Till date, substantial efforts have been directed toward sugarcane as a biofabric for high-value products. While these achievements are commendable, a greater understanding of the sugarcane genome, cell, and whole plant biology will accelerate the implementation of commercially significant biotechnology outcomes (Lakshmanan et al. 2005; Ming et al. 2006). The rapid progress in molecular biology and emerging biotechnology innovations will play significant roles in future sugarcane crop improvement programs and will offer many new opportunities to develop it as a new generation industrial crop and a sustainable biofactory.

5.7 Conclusions and Future Perspectives

Sugarcane has become an ideal biofactory, as it converts sunlight and water into biomolecules such as sugar, fibers, and waxes in a very efficient manner, making it the most productive field crop among cultivated plants. However, theoretical and technical constraints are yet to be overcome. Accordingly, using sugarcane as a biofactory is an exciting but challenging area of research and innovation that could have a huge influence on the evolution of alternative sugarcane industries worldwide.

Its complex polyploidy and high level of heterozygocity make proper exploitation of sugarcane variability a tough task. Consequently, significant advances in traditional breeding of sugarcane are limited by its narrow gene pool, complex genome, and the long breeding cycle. However, these disadvantages make sugarcane a good candidate for the application of transgenic approaches. Indeed, examples of sugarcane transgenic lines exhibiting improved agronomic and industrial traits have been cited in this chapter.

Thus, the establishment of molecular approaches reviewed herein to develop sugarcane into a biofactory demonstrates that this crop has tremendous potential and may play an important role in the growing bioeconomy through biopharming. Like no other contemporary crop, sugarcane is facing new paradigms and is expected to contribute at least partially to the development of new generation highly profitable biofactories. Social and biosafety issues are expected to be considered in any program aimed at developing a sustainable novel sugarcane biofactory in the future.

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Chapter 6
Edible Ectomycorrhizal Mushrooms: Biofactories for Sustainable Development

Jesús Pérez-Moreno and Magdalena Martínez-Reyes

Abstract Ecologically, edible mushrooms can be: (i) parasites of plants or animals, (ii) saprotrophs, which live and feed on dead organisms, such as the industrially cultivated button, oyster or shiitake mushrooms, or (iii) ectomycorrhizal, which establish mutually beneficial symbiosis with the roots of host plants. Ectomycorrhizal wild mushrooms, which are the subject of this chapter, embrace the most expensive edible fungi, including truffles, porcini, matsutake, chanterelles, Caesar's mushrooms, or saffron milk caps. The international commerce of edible ectomycorrhizal mushrooms (EEMs) annually is worth billions of dollars. However, EEMs have been a largely unexplored source of bioactive compounds. Despite this fact, analgesic, antiallergic, anticarcinogenic, antibacterial, anticoagulant, antifungal, antihypertensive, anti-inflammatory, antinociceptive, antioxidant, antipyretic, antivenom, antiviral (including anti-HIV), cholesterol-lowering, hepatoprotective, and immune enhancement properties have been found in more than 100 species of EEMs. Some bioactive compounds including grifolin, polyozelyn, and novel lectins or ribonucleases produced by *Albatrellus, Boletopsis, Hygrophorus, Thelephora,* and *Polyozellus,* respectively, are exclusive of EEMs. Additionally, insecticide, nematicide, and allelophatic compounds with potential application to control agricultural pests and weeds have been found in EEM. Despite the fact that most EEMs have defied cultivation, some advances have been made in cultivation of truffles and broth cultures of species included in the genera *Hygrophorus*, *Lactarius*, *Morchella*, *Rhizopogon, Suillus,* and *Tuber* as a potential source of bioactive compounds with medical or nutraceutical importance. In addition, EEMs as a valuable non-timber forest product contributes to rural development and the establishment of truffle plantations contributes to rehabilitation of degraded areas and global carbon sequestration. In the future, advances in the cultivation of EEMs might produce bioactive compounds in industrial amounts.

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Contents

6.1 Introduction

Fungi are one of the most fascinating and understudied group of microorganisms worldwide. Since 1969, they have been recognized so different from other eukaryotes, in many essential structural and functional aspects, that they have been classified into their own kingdom (Whittaker 1969; Petersen 2012). More than 70,000 species of fungi have been currently described, and it has been conservatively estimated that there are 1.5 million species on Earth (Hawksworth 1991, 1994). Fungi have always played important roles in human life, as food or medicine. In early civilizations they were used as agents in the production of various food products including bread, wine, and beer. The Babylonians and Sumerians were brewing as early as 6,000 BC and reliefs on tombs dating from 2,400 BC document beer making in Egypt. Egyptians were also probably the first to bake leavened bread (Young 2013). From the 1940s fungi have permanently been used as a source of antibiotics, which has been considered a crucial factor that dramatically increased human longevity. More recently, they have also been used as a source of enzymes with industrial application. Members of the Association of Manufacturers and Formulators of Enzyme Products have commercialized more than 160 enzymes produced by 25 fungal genera (Ostergaard and Olsen 2010).

Fungi are also widely used in the industrial production of detergents, sweeteners, or personal care products. In agriculture, a number of fungal species have been used as biological agents to control weeds, pests, and pathogens. Fungi include yeasts, molds, and mushrooms. Mushrooms are defined according to the Oxford Dictionary (2013) as "… *fruiting bodies that produce spores, growing from the hyphae of fungi concealed in soil or wood…*". In a more practical definition, Hall et al. (2003 after Chang and Miles, 1992) defined a mushroom as "… *any fungus with a distinctive fruiting body that is large enough to be … picked by hand …*". Mushrooms constitute a component of paramount importance in the structure and function of all the known ecosystems of the planet, mainly in nutrient recycling. Mushrooms, as any fungi, are heterotrophic organisms, and therefore they have to use extracellular sources of energy for their maintenance, growth, and reproduction. Therefore, they have at least three different pathways, not mutually exclusive, to acquire their energy sources (from plants, animals, or other microorganisms), as carbon compounds, being therefore (i) saprotrophic, (ii) parasitic, or (iii) mutualistic.

One the most fascinating groups of mushrooms are those that are able to establish symbiotic relationships with the roots of plants, called mycorrhizae. The word mycorrhiza was coined at the end of the nineteenth century, by Anton Frank (1894), to define the structures formed between the roots of trees that were completely covered with hyphae of some edible underground fungi called truffles (*Tuber* spp.). Since that time, but particularly in the last two decades, due to their paramount ecophysiological and biotechnological importance, the study of mycorrhizal symbiosis established between the plant roots and fungi has blossomed in the scientific literature. Mushrooms and truffles establish mainly a type of mycorrhiza called ectomycorrhiza with the roots of Gymnosperm and Angiosperm trees, but also with some shrubs and herbs. It has been estimated that the number of fungal species that establish ectomycorrhizal symbiosis ranges between 20,000 and 25,000 species (Comandini et al. 2012; Rinaldi et al. 2008). Despite the fact that some of the ectomycorrhizal mushrooms (EEMs) are able to produce some intoxications to humans, called mycetisms, including mortal poisoning in a limited number of species including the "angels of death" such as *Amanita phalloides, Amanita verna, Amanita bisporigera,* and *Amanita virosa* (Pérez-Moreno and Ferrera-Cerrato 1995; Pérez-Moreno et al. 1994), there is a vast group of edible ectomycorrhizal mushroom (EEM) species (Fig. 6.1), and belowground truffles that are included among the most expensive edible mushrooms in the world (Table 6.1). Due to these high prices, the worldwide market of edible EEMs has been valued in billions of American dollars annually.

The cultivation of around 100 species of commercial mushrooms in large factories include only saprotrophic species (Chang 2008). In contrast, the highly priced edible EEMs can only be cultivated in tightly controlled plantations of trees and shrubs, at a relatively limited scale, due to the fact that they are obligate symbionts with living plants. Unlike the widely cultivated saprotrophic mushrooms, in the case of the ectomycorrhizal edible mushrooms, which are the subject

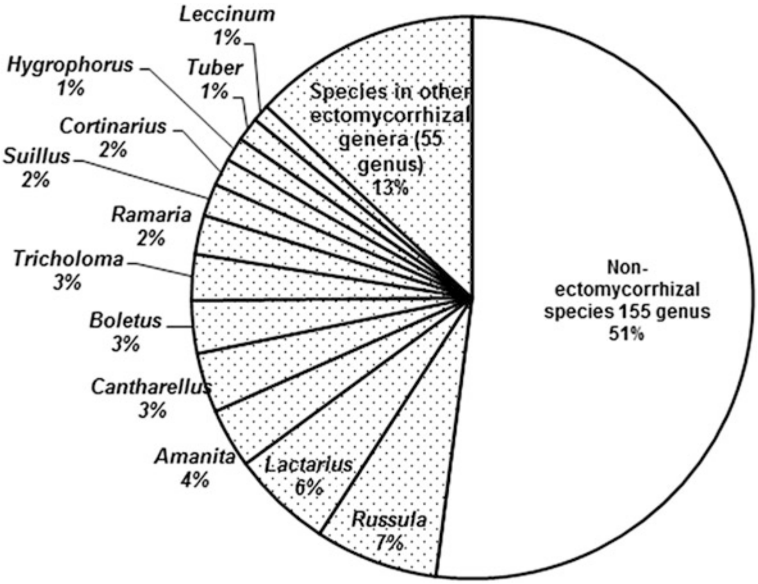

Fig. 6.1 Edible ectomycorrhizal genera and species known worldwide; from a total of 1018 known species of edible mushrooms, 488 species are included in ectomycorrhizal genera (*dotted segments*). Calculations of edible mushrooms were based on Boa (2004) and of ectomycorrhizal genera were based on Rinaldi et al. (2008)

of this chapter, they cannot be directly cultivated on organic substrates under controlled conditions.

The interest in edible EEMs has recently bloomed, so the literature related to species like Chanterelle (*Cantharellus cibarius* s.l.), porcini (*Boletus edulis* s.l.), matsutake (*Tricholoma matsutake*), Caesar's mushroom (*Amanita caesarea* s.l.), or truffles (*Tuber* spp.) counts thousands of scientific papers and books (Figs. 6.2, 6.3, 6.4). For example, entering *B. edulis* into Google will return about three-quarters a million hits and entering Truffle mushrooms will return 9.8 million hits. Mushrooms have also been highly appreciated for their medicinal properties; the first conclusive record being reported 5,300 years ago in what is undoubtedly a fascinating story.

In 1991, two German hikers discovered in the border of the Austrian and Italian Alps an extremely well-preserved mummy, which among other objects had two mushrooms (Fleckinger 2011). These mushrooms were identified by Peintner et al. (1998) as the birch polypore (*Piptoporus betulinus*) and the tinder mushroom (*Fomes fomentarius*). They constitute the first-aid kit recorded for humans. The birch polypore which is edible in early stages, has been considered to have in folk medicine antiparasitic and antimicrobial activities and has been used in the treatment of wounds, rectal cancer, and stomach diseases (Lemieszek et al. 2009; Capasso 1998). This species contains an antibacterial compound called polyporenic acid C (Pöder 1993). Tea obtained from this mushroom has antifatiguing,

Table 6.1 Main edible ectomycorrhizal mushrooms, their estimated world market value, in American dollars, and main countries of commercialization

Common name of mushroom	Scientific name	Approximate in-reason retail market	Approximate wholesale prices (per kilogram, grade one)	Main countries of commercialization[a]
Chanterelle	*Cantharellus cibarius* s.l.	$1.67 billion[b]	$8 to $22[b]	Canada, China, France, Germany, Mexico, United States of America
Desert truffle	*Terfezia* spp. and *Tirmania* spp.	Unknown	$27 to $334[c]	Algeria, Cyprus, Egypt, Iran, Iraq, Kuwait, Morocco, Syria and Tunisia.
Ganbajun	*Thelephora gambajun*	$350 million[d]	$20 to $50[d]	China
Italian white truffle	*Tuber magnatum*	>$150 million	$1000 to $30,000[e]	Italy
Matsutake	*Tricholoma matsutake* s.l.	$500 million	$30 to $1000[f]	Japan
Périgord black truffle	*Tuber melanosporum*	>$150 million	$250 to $1200	Spain, France, Italy, United States of America
Porcini	*Boletus edulis* s.l.	>$250 million	$13 to $198	Canada, China, France, Germany, Italy, Mexico, Spain, United States of North America

From Hall et al. (2003), with some data for [b] chanterelle from Watling (1997) and Arora and Dunham (2008); for [c] desert truffles from Morte et al. (2012) and for [f] matsutake price from Matsutani (2010); [a] mainly based on Pegler (2002), Hall et al. (2007; 2003); Pérez-Moreno and Ferrera-Cerrato (1996a) and Pérez-Moreno et al. (2010) [d] estimated in the production of 10,000 tons in Yunnan, China, in 2002, at an average price of USD35 per kg, based on He et al. (2011); [e] in international auctions in 2005, 2006 and 2007 one single Italian white truffle reached, in each occasion, prices of USD 330,000, USD 340,000 and USD 350,000, respectively (see text)

Fig. 6.2 The American matsutake (*Tricholoma magnivelare*) is one of the most valuable edible ectomycorrhizal mushrooms in North America

Fig. 6.3 The Caesar's mushroom (*Amanita caesarea* s.l.) has potent antioxidant and antimicrobial properties

immunoenhancing, and soothing properties (Stamets 1993). More recently, Lemieszek et al. (2009) showed that extracts of dried sporomes of this mushroom have anticancer activity against cell cultures of human lung (A549) and colon adenocarcinoma (HT 29). Additionally, an Australian podiatrician reported having

Fig. 6.4 The Chinese truffle (*Tuber sinense*) has antioxidant and antimmodulatory properties

packed it behind ingrowing toenails with "excellent results" (Pöder 1993, Hilton 1987). In the case of *F. fomentarius*, Peintner et al. (1998) based on different sources pointed out that apart from being a fire starter this mushroom has a range of medicinal properties including diuretic, laxative, styptic (and therefore called surgeon's agaric by surgeons, barbers and dentists) functions, and has been used as a remedy against dysmehorrhoea, hemorrhoids, and bladder disorders. Additionally, hemostatic properties were reported for this mushroom by Saar (1991) in Kanthy people form Western Siberia, and a cytotoxic effect from sterols (fomentarols A–D) extracted from this mushroom, against some lines of human cancer cells, has been recently proved by Zhang et al. (2013). Based on these evidences, the association of sporomes of these two fungi, with the mummy called Ötzi, constitutes the most ancient conclusive evidence of the use of mushrooms by humans. Meanwhile, the most ancient record of medicinal properties from edible EEMs is dated at the end of the ninth century and appears in the Quran, the central religious text of the Muslims. It is narrated in the *hadith* (which refers to reports of actions of prophet Mohammad) named "*Sahih Muslim*" in Book 23, Chapter 26 entitled "*Excellence of truffles and their use as a medicine for the eyes,*" that the prophet Mohammad said "*… Truffles are part of the 'manna' which Allah, sent to the people of Israel through Moses, and its juice is a medicine for the eyes…*" (Sahih 2013). The collector of this ancient report, Ibn al-Hajjaj, lived in Nishapur (currently located in Iran) between 817/818 and 874/875 CE. He widely traveled to gather his collection of reports to regions which are now in Iraq, the Arabian Peninsula, Syria, and Egypt (Sahih 2009). Interestingly, in the last decade different authors (Dib-Bellahouel and Fortas 2011; Mandeel and Al-Laith 2007; Janakat et al. 2004, 2005) have confirmed these statements by discovering the presence of potent antimicrobial agents in dessert truffles (in the edible mycorrhizal genera *Terfezia* and *Tirmania*) which are effective against bacteria, such as *Staphylococcus aureus, Pseudomonas aeruginosa,* or *Chlamydia trachomatis*, important agents of human eye infections such as bacterial conjunctivitis, blepharitis, dacryocystitis, or even trachoma.

In general, edible EEMs constitute a group that has been understudied in terms of their bioactive compounds, despite the fact that they can constitute an important source of novel bioactive compounds useful for human health and fitness. Some of these bioactive compounds have been only found in this particular group of mushrooms due to their peculiar ecophysiological symbiotic character with trees. The aim of this chapter is to present a review of the edible ectomycorrhizal species and the bioactive compounds that have been isolated from them, having analgesic, antiallergic, anticarcinogenic, antibacterial, anticoagulant, antifungal, antihypertensive, anti-inflammatory, antinociceptive, antioxidant, antipyretic, antivenom, antiviral (including anti-HIV), cholesterol-lowering, hepatoprotective, and immune enhancement properties. Additionally, some insecticide and allelophatic compounds with potential application to control agricultural plagues and weeds found in edible EEMs are also reviewed. Finally, some information related with the limitations and potential perspectives for industrial production of these bioactive compounds, including the initial advances of cultivation of edible EEMs, for example in commercial plantations of truffles or broth cultures, and the importance of commercial picking of these mushrooms worldwide, as a very important non-wood forest product, for sustainable development and alleviation of global change is analyzed.

6.2 Bioactive Compounds with Medical or Nutraceutical Importance

Traditionally, edible EEMs have been highly appreciated due to their well-known nutritional value attributed to high levels of protein, fiber, carbohydrates, vitamins and minerals, and low fat levels. Some of these mushrooms have also been used by ancient cultures to increase human longevity and life quality, due to their medicinal and nutraceutical properties (Chang and Buswell 1996; Lindequist et al. 2005; Yu-cheng et al. 2009; Ferreira et al. 2010; Wasser 2011; Patel and Goyal 2012; Rathee et al. 2012; Stachowiak 2012). Currently, there is an increasing industrial interest in them as potential biofabrics, because they are an important potential source of novel bioactive compounds used as medicine or nutraceuticals (Table 6.2). One of the most important properties of edible wild mushrooms, is their contents of antioxidant compounds which has received recently some attention because of their great importance to reduce the negative effects of degenerative diseases in general (Barros et al. 2007c; Ferreira et al. 2009; Heleno et al. 2010; Preeti et al. 2012; Fernandes et al. 2013).

6.2.1 *Antitumor and Immunostimulating*

Cancer is a disease caused by normal cells changing so that they grow in an uncontrolled way, forming tumors and invading nearby parts of the body. There are

Table 6.2 Species of edible ectomycorrhizal mushrooms and their reported medicinal properties and active principles

Species	Medicinal properties	Active principles or extracts	Source
Amanita caesarea (Scop.) Pers.	Antioxidant	Phenolic compounds (catechin, ferulic acid, *p*-coumaric acid and cinnamic acid), β-carotene	Sarikurkcu et al. (2010) , Valentao et al. (2005a , b)
	Antimicrobial	Acetone extracts effective against *Candida albicans*	Doğan and Akbas (2013)
Amanita ceciliae (Berk. & Broome) Bas	Antioxidant	Methanol extracts from sporomes	Akata et al. (2012)
Amanita rubescens Pers.	Antioxidant	Phenolic compounds (*p*-Hydrooxybenzoic acid), ascorbic acid	Ribeiro et al. (2006, 2007)
Albatrellus confluens (Alb. & Schwein) Kotl. & Pouzar	Antitumor	Grifolin andaurovertin B and the novel Aurovertin P showed cytotoxic activities on five human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7 and SW480)	Guo et al. (2013a, b), Luo et al. (2011a, b), Ye et al. (2005, 2007)
Albatrellus ovinus (Schaeff.) Kotl. and Pouzar	Antioxidant	Neogrifolin	Nukata et al. (2002)
Astraeus hygrometricus (Pers.) Morgan	Antitumor	Heteroglucan	Mallick et al. (2010)
	Immunostimulatory	Polysaccharide	Mallick et al. (2010)
Boletopsis grisea (Peck) Bondartsev & Bondartsev & Singer.	Antioxidant	*p*-terphenylderivatives	Liu et al. (2004)
Boletopsis leucomelaena (Pers.) Fayod	Antitumor, antileukemia	Novel lectin with apoptosis-inducing activity	Koyama et al. (2002, 2006)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
Boletus aestivalis (Paulet) Fr.	Antioxidant	Phenolic compounds	Kosanić et al. (2012)
Boletus badius (Fr.) Fr [= *Xerocomus badius* (Fr.) E.J. Gilbert]	Antitumor	Aminoacids (theanine)	Li et al. (2008)
	Antioxidant	Tocopherols (α-tocopherol)	Elmastas et al. (2007)
	Antihypertensive	Aminoacids (theanine)	Li et al. (2008)
Boletus edulis Bull.	Antioxidant	Phenolic compounds (protocatechuic acid, *p*-coumaric acid, caffeic acid, tannic acid), tocopherols (α- β- and γ-tocopherols), β-carotene	Li et al. (2013), Guo et al. (2012a, b), Vidović et al. (2010), Barros et al. (2008a, b), Tsai et al. (2007), Puttaraju et al. (2006)
	Antitumour	Extracts of sporomes, selenium	Lucas et al. (1957), Zaidman et al. (2005), Slejkovec et al. (2000)
	Antiviral	Lectins	Santoyo et al. (2012)
	Cholesterol-lowering and antihypertensive	Extracts from sporomes have significant amounts of Lovastatin and γ-aminobutyric acid	Chen et al. (2012)
Boletus regius Krombh	Antioxidant	Phenolic compounds	Guo et al. (2012a, b)
Boletus reticulatus Schaeff.	Antioxidant	Methanol extracts from sporomes	Akata et al. (2012)
Cantharellula umbonata (J.F. Gmel.) Singer [=*Cantharellus umbonatus* (J.F. Gmel) Pers.]	Antioxidant	Phenols, flavonoids, ascorbic acid and β-carotene	Kumari et al. (2011)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
Cantharellus appalachiensis R.H. Petersen	Antioxidant	Phenols, flavonoids, ascorbic acid, and β-carotene	Kumari et al. (2011)
Cantharellus cibarius Fr.	Antiflammatory	Methanolic extracts	Moro et al. (2012)
	Antioxidant	Phenolic compounds (Gallic acid, *p*- coumaric acid, Caffeic acid, Ferulic acid, rutin, Tannic acid) Tocopherols (α- β- and γ- tocopherols), flavonoids, ascorbic acid, β-carotene, flavonoids	Akata et al. (2012), Kumari et al. (2011), Chen et al. (2010a, b, c), Ebrahimzadeh et al. (2010), Barros et al. (2008a), Puttaraju et al. (2006), Valentao et al. (2005a, b); Agrahar-Murugkar and Subbulakshmi (2005), Mau et al. (2002)
	Antigenotoxic	Aqueous extracts from sporomes have antigenotoxic potential on human mononuclear cell cultures	Méndez-Espinoza et al. (2013)
Cantharellus isabellinus Heinem.	Antioxidant	Phenols, flavonoids, ascorbic acid and β-carotene	Kumari et al. (2011)
Cantharellus lateritius (Berk.) Singer.	Antioxidant	Phenols, flavonoids, ascorbic acid, and β-carotene	Kumari et al. (2011)
Cantharellus miniatescens Heinem.	Antioxidant	Phenols, flavonoids, ascorbic acid, and β-carotene	Kumari et al. (2011)
Cantharellus minor Peck	Antioxidant	Phenols, flavonoids, ascorbic acid, and β-carotene	Kumari et al. (2011)
Cantharellus pseudoformosus D. Kumari, Ram.	Antioxidant	Phenols, flavonoids, ascorbic acid, and β-carotene	Kumari et al. (2011)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
Cantharellus rhodophyllus Heinem.	Antioxidant	Phenols, flavonoids, ascorbic acid, and β-carotene	Kumari et al. (2011)
Chroogomphus rutilus (Schaeff.) O.K. Mill [=*Gomphidius rutilus* (Schaeff.) S. Lundell]	Antioxidant	Polysaccharide fractions	Guo et al. (2013a, b)
	Immunological activity	Polysaccharide fractions	Gao et al. (2012, 2013)
Clavulina cinerea (Bull) J. Schröt.	Antioxidant	Ascorbic acid	Agrahar-Murugkar and Subbulakshmi (2005)
Cortinarius anomalus (Fr) Fr.	Antioxidant	Phenolic compounds	Reis et al. (2011)
Cortinarius caperatus (Pers.) Fr. [= *Rozites caperatus* (Pers.) P. Karst.]	Antiviral	Proteins	Piraino and Brandt (1999), Piraino (2006)
Cortinarius purpurascens Fr.	Antioxidant	Polyketide-derived, anthraquinone pigments	Bai et al. (2013)
Cortinarius violaceus (L.) Gray	Antioxidant	Phenolic compounds (p-hydroxibenzoic- and cinnamic acid), α-, β-, γ-, and δ-tocopherols	Reis et al. (2011)
Craterellus cornucopioides (L) Pers.	Antiiflammatory	Methanolic extracts	Moro et al. (2012)
	Antioxidant	Tocopherols (α-, β- y γ-tocopherol), flavonoids, ascorbic acid, β-carotene	Palacios et al. (2011); Barros et al. (2008a)
	Antihyperglycemic	Ethanol and aqueous extracts from sporomes showed α-glucosidase and α-amylase inhibitory activity	Liu et al. (2012a, b)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
Gomphus clavatus (Pers.) Gray [= *Cantharellus clavatus* (Pers.Fr.)]	Antioxidant	Phenolic compounds (Protocatechuic acid, Gallic acid, Gentisic acid, Vanillic acid, Syringic acid, Cinnamic acid, Caffeic acid, Ferulic acid, Tannic acid)	Puttaraju et al. (2006)
Helvella crispa (Scop) Fr.	Antioxidant	Phenolic compounds (Protocatechuic acid, Gallic acid, Gentisic acid, Vanillic acid, Tannic acid)	Puttaraju et al. (2006)
Helvella lacunosa Afzel	Antioxidant	Organic acids (oxalic acid, malic acid,citric acid, fumaric acid), δ-tocopherol, lycopene	Leal et al. (2013)
Hydnum repandum L.	Antitumor	Polysaccharides	Chung et al. (1982)
	Antioxidant	Phenolic compounds (Protocatechuic acid, Gallic acid, Gentisic acid, Vanillic acid, Syringic acid, Cinnamic acid, Tannic acid)	Puttaraju et al. (2006)
Hygrophorus agathosmus (Fr) Fr.	Antibacterial and antifungal	Chloroform extracts from sporomes effective against *Enterobacter aerogenes, Salmonella typhimurium; Staphylococcus epidermidis* and *Bacillus subtilis*	Yamaç and Bilgili (2006)
Hygrophorus camarophyllus (Alb. & Schwein.) Dumeé, Grandjean & Maire [= *H. marzuolus* (Fr.) Bress.]	Antioxidant	Phenolic compounds, flavonoids	Palacios et al. (2011)
Hygrophorus russula (Schaeff.) kauffman	Anti-HIV	A novel ribonuclease isolated from sporomes showed inhibitory effect toward HIV-1 reverse transcriptase	Zhu et al. (2013)
Laccaria amethystina Cooke	Antitumor	Polysaccharides	Chung et al. (1982)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
Laccaria laccata (Scop.) Cooke [= *L. amethystea* (Bull.) Murrill]	Antioxidant	Ascorbic acid	Egwin et al. (2011)
	Antitumor	Polysaccharides	Chung et al. (1982)
Lactarius camphoratus (Bull) Fr.	Antioxidant	Phenolic compounds	Ozen et al. (2011)
	Antimicrobial	Methanol extracts effective against Escherichia coli	Ozen et al. (2011)
Lactarius deliciosus (L.) Gray	Antioxidant	Phenolic compounds (Protocatechuic acid, Gallic acid, Gentisic acid, Ferulic acid, *p*-Hydroxybenzoic acid, Tannic acid) Ascorbic Acid, *β*-Carotene	Kalogeropoulos et al. (2013), Akata et al. (2012), Barros et al. (2007a, 2008a, 2009), Puttaraju et al. (2006), Valentao et al. (2005a, b)
	Antiinflammatory	Methanolic extracts	Moro et al. (2012)
	Antibacterial	Mycelial methanolic extracts effective against *Mycobacterium smegmatis*, *Staphylococcus aureus and Bacillus subtilis*	Suay et al. (2000)
Lactarius flavidulus S. Imai	Antitumor	Lectin	Wu et al. (2011)
Lactarius hatsudake Nobuj. Tanaka	Antiiflammatory	Ergosterol peroxides	Gao et al. (2007)
	Antivenom (*Crotalus adamenteus* venom)	Ergosterol peroxides	Gao et al. (2007)
Lactarius lividatus Berk. & M.A. Curtis	Antitumor	Polysaccharide (fucoidan)	Tako et al. (2012)
	Anti-HIV	Polysaccharide (fucoidan)	Tako et al. (2012)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
	Anticoagulant	Polysaccharide (fucoidan)	Tako et al. (2012)
	Immune enhancement	Polysaccharide (fucoidan)	Tako et al. (2012)
Lactarius piperatus (L) Pers.	Antioxidant	Ascorbic acid, β-carotene	Barros et al. (2007a)
Lactarius rufus (Scop.) Fr.	Anti-inflammatory	Polysaccharide (β-glucans)	Ruthes et al. (2013)
	Antinociceptive	Polysaccharide (β-glucans)	Ruthes et al. (2013)
Lactarius salmonicolor R. Heim & Leclair	Antioxidant	Methanol extracts from sporomes	Akata et al. (2012)
Lactarius sanguifluus (Paulet) Fr.	Antioxidant	Phenolic compounds(Protocatechuic acid, Gallic acid, Gentisic acid, Vanillic acid, Syringic acid, Cinnamic acid, *p*-Coumaric acid, Caffeic acid, Ferulic acid, Tannic acid)	Kalogeropoulos et al. (2013), Puttaraju et al. (2006)
Lactarius semisanguifluus R. Heim & Leclair	Antioxidant	Phenolic compounds	Kalogeropoulos et al. (2013)
Lactarius vellereus (Fr). Fr.	Antibacterial	Acetone extracts of sporomes effective against *Bacillus subtilis*	Doğan et al. (2013)
Leccinum aurantiacum (Bull.) Gray	Antioxidant	Phenolic compounds	Vidović et al. (2010)
Leccinum pseudoscabrum (Kallenb. Šutara) [*L. carpini* (R. Schulz) M.M. Moser ex D.A. Reid]	Antioxidant	Phenolic compounds	Kosanić et al. (2012)
Leucopaxillus giganteus (Quél) Singer.	Antitumour	Galactomannoglucan	Mizuno et al. (1995a, b)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
Lyophyllum decastes (Fr.) Singer	Antitumour	Polysaccharides (homoglucans)	Ukawa et al. (2000)
Morchella angusticeps Peck	Antioxidant	Phenolic compounds (Protocatechuic acid, Syringic acid, Caffeic acid, Tannic acid)	Puttaraju et al. (2006)
Morchella conica Krombh.	Antioxidant	Phenolic compounds (Protocatechuic acid, Gallic acid, Gentisic acid, Vanillic acid, Syringic acid, Caffeic acid, Ferulic acid, Tannic acid)	Puttaraju et al. (2006)
Morchella elata Fr.	Antioxidant	Phenolic compounds	Zeng et al. (2012)
Morchella esculenta (L) Pers.	Antioxidant	Phenolic acids, tocopherols (α-, δ-and γ-tocopherol), ascorbic acid, polysaccharides	Meng et al. (2010a, b); Heleno et al. (2013), Gang et al. (2013), Mau et al. (2004)
	Antimicrobial	Extracts effective against 5 pathogenic bacteria more effective than streptomycin and ampicillin, with demelizing activity against 4 pathogenic strains of mycromycetes	Heleno et al. (2013)
	Anti-inflammatory	Ethanolic extracts of mycelium	Nitha et al. (2007)
	Hepatoprotective	Aqueous–ethanolic extracts of mycelium	Nitha et al. (2013)
Paxillus involutus (Batsch) Fr.	Antibacterial and antifungal	Mycelial methanolic extracts effective against *Enterococcus faecium*, *Staphylococcus aureus*, *Bacillus subtilis and Aspergillusfumigatus*	Suay et al. (2000)
Peziza vesiculosa Bull.	Antitumour	(beta-1,3-glucan),polysaccharides	Mimura et al. (1985)
Polyozellus multiplex (Underw.) Murrill	Antitumour	Polyozellin	Lee and Nishikawa (2003)
	Anti-inflammatory	Polyozellin	Lee et al. (2011), Jin et al. (2006a, b)
	Antiviral	Methanol extracts from sporomes function as α-glucosidase inhibitors	Lee et al. (2013)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
Ramaria botrytis (Pers.) Ricken	Antioxidant	Phenolic compounds (*p*-Hydroxybenzoic acid, Protocatechuic acid) Tocopherols (α- and β-tocopherol) Ascorbic acid, β-Carotene	Barros et al. (2009)
	Antibacterial	Aqueous methanolic extracts from sporomes have a bactericide effect against clinical isolates of *Pasteurellamultocida, Streptococcus agalactiae*, and *Streptococcus pyogenes* resistant to antibiotics	Alves et al. (2012)
Ramaria brevispora Corner, K.S. Thind & Dev	Antioxidant	Ascorbic acid	Agrahar-Murugkar and Subbulakshmi (2005)
Ramaria cystidiophora (Kauffman) Corner	Antimycobacterial	Four butenolides, the major one termed ramariolide showed in vitro antimicrobial activity against *Mycobacterium smegmatis*, and *Mycobacterium tuberculosis*	Centko et al. (2012)
Ramaria flava (Schaeff.) Quél.	Antibacterial	Ethanol extracts from sporomes inhibit the growth of *Staphylococcus aureus, Escherichia coli*, and *Bacillus subtilis*	Liu et al. (2013a, b)
	Antitumor	Ethanol extracts showed antitumor activity against three human cancer cell lines (BGC-803, NCI-H520, and MDAMB-231)	Liu et al. (2013a, b)
	Antioxidant	Phenolic compounds (quercetin, chrysin, and pinocembrin)	Liu et al. (2013a, b)
Rhizopogon luteolus Fr.	Antibacterial	Mycelial methanolic extracts effective against *Pseudomonas aeruginosa, Serratiamarcescens, Mycobacterium smegmatis*, and *Staphylococcus aureus*	Suay et al. (2000)
Rhizopogon roseolus (Corda) Th. Fr.	Antioxidant	Methanol extracts from sporomes	Akata et al. (2012)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
Russula anthracina Romagn.	Antioxidant	Methanol extracts from sporomes	Akata et al. (2012)
Russula brevipes Peck	Antioxidant	Phenolic compounds (Protocatechuic acid, Gallic acid, Gentisic acid, Syringic acid, Caffeic acid, Tannic acid)	Puttaraju et al. (2006)
Russula delica Fr.	Antioxidant	Phenolic compounds, tocopherols (α-tocopherol) β-carotene	Kalogeropoulos et al. (2013), Elmastas et al. (2007)
	Antibacterial	Aqueous methanolic extracts from sporomes have a bactericide effect against clinical isolates of *Pasteurella multocida, Streptococcus agalactiae*, and *Streptococcus pyogenes* resistant to antibiotics	Alves et al. (2012)
Russula cyanoxantha (Schaeff.) Fr.	Antitumour	Steroid (ergone)	Zhao et al. (2011)
Russula griseocarnosa X.H. Wang, Zhu L. Yang & Knudsen	Antioxidant	Phenolics, flavonoids, ergosterol, and β-carotene	Chen et al. (2010a, b, c)
Russula lepida Fr.	Antitumour	Lectins	Zhang et al. (2010)
Russula virescens (Schaeff.) Fr.	Antioxidant	Phenolics, flavonoids, β-carotene	Chen et al. (2010a, b, c)
Sarcodon imbricatus (L.) P. Karst. [= *S. aspratus* (Berk.) S. Ito]	Antioxidant	Phenolic compounds (p-Hydroxybenzoic acid), ascorbic acid, β-carotene	Barros et al. (2007a, b, 2008 a, b, 2013)
	Antitumor	Fucogalactan	Mizuno et al. (2000)
Scutiger pes-caprae (Pers) Bondartsev & Singer [= *Albatrellus pes-caprae* (Pers.) Pouzar)]	Antitumour	Selenium	Slejkovec et al. (2000)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
Suillus bellinii (Inzenga) Watling	Antioxidant	Phenolic compounds	Kalogeropoulos et al. (2013)
Suillus bovinus (L.) Roussel	Antioxidant	Phenolic compounds	Guo et al. (2012a, b)
Suillus collinitus (Fr.) Kuntze	Antitumor	Methanolic extracts from sporomes increased p53 expression and caused cell cycle arrest and apoptosis in a breast cancer cell line	Vaz et al. (2012)
	Antioxidant	Ascorbic acid, methanol extracts from sporomes	Akata et al. (2012), Valentao et al. (2005)
Suillus granulatus (L.) Roussel	Antioxidant	Phenolic compounds (*p*-Hydroxybenzoic acid, quercetin)	Ribeiro et al. (2006, 2007)
Suillus lakei (Murrill) A.H. Sm. & Thiers	Antioxidant	Extracts from mycelium showed a significant reduction of oxidative damage caused by xantineoxidase	Gonzalez-Barranco et al. (2010)
Suillus luteus (L.) Roussel	Antioxidant	Phenolic compounds (Quercetin), Tocopherols	Zeng et al. (2012), Reis et al. (2011), González-Barranco et al. (2010), Ribeiro et al. (2006)
	Antibacterial	Mycelial methanolic extracts effective against *Bacillus subtilis*	Suay et al. (2000)
Suillus placidus (Bonord.) Singer	Antitumor	Suillin acts as a potent apoptosis inducer in human hepatoma HepG2 cells	Liu et al. (2009)
Suillus variegatus (Sw.) Kuntze	Antibacterial	Mycelial methanolic extracts effective against *Staphylococcus aureus*, and *Bacillus subtilis*	Suay et al. (2000)
Terfezia boudieri Chatin	Antioxidant	Phenolic compounds, ethanolic extracts	Dundar et al. (2012)
	Antibacterial and antifungal	Acetone extracts of sporomes effective against *Bacillus subtilis and Candida albicans;* and antibacterial methyl alcohol extracts from spromes inhibit the growth of *Klebsiella pneumoniae*	Dogan et al. (2013), Akyuz et al. (2010)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
	Increase of sexual androgen hormones	Alcoholic extracts of sporomes significantly increased the levels of luteinizing hormone and testosterone.	Khojasteh et al. (2013)
	aphrodisiac		
Terfezia claveryi Chatin	Antibacterial	Aqueous extracts effective against *Pseudomonas aeruginosa*, and *Staphylococcus aureus* in vitro	Janakat et al. (2004); Gouzi et al. (2011)
Thelephora aurantiotincta Corner	Antitumor	Thelephantin O, vialinin A, (*p*-terphenyl derivatives) which suppress the proliferation of humanhepatocellular carcinoma cellsviaironchelation	Norikura et al. (2011; 2013)
	Antioxidant	*p*-terphenylderivatives	Liu et al. (2004)
Thelephora ganbajun M. Zang	Antioxidant	Phenolic compounds	Guo et al. (2012a, b)
	Analgesic and antipyretic	Aminophenol	Zhou et al. (1992); Mortimer et al. (2012)
Thelephora vialis Schwein.	Antioxidant	Vialinin A	Xie et al. (2005); Xie and Abe (2006); Xie et al. (2006)
	Antitumor	Vialinin B inhibitor of tumor necrosis factor-α production	Ye et al. (2009a, b)
	Anti-inflammatory	Vialinin A is more efficient than the widely used immunosuppressive drug tacrolimus (FK506).	Okada et al. (2013)
Tirmania nivea (Desf.) Trappe	Antibacterial	Aqueous extracts effective against *Pseudomonas aeruginosa*, and *Staphylococcus aureus* in vitro	Gouzi et al. (2011)
	Antioxidant	Phenolic compounds, ascorbic acid and carotenoids	Al-Laith (2010)
Tirmania pinoyi (Maire) Malençon	Antimutagenic	Ethanol extracts from sporomes inhibit mutagenic effects	Hannan et al. (1989)
Tricholoma equestre (L.) P. Kumm.	Antioxidant	Phenolic compounds (*p*-Hydroxybenzoic acid)	Ribeiro et al. (2006)
Tricholoma giganteum Massee	Antioxidant	Ethanol extracts from sporomes	Chatterjee et al. (2011b)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
	Antitumor	Ethanol extracts from sporomes	Mizuno et al. (1996); Chatterjee et al. (2013)
Tricholoma matsutake (S. Ito & S. Imai) Singer	Antioxidant	Alkali-extracted polysaccharide fractions	Tong et al. (2013)
	Antitumour	A novel polysaccharide named TMP-A extracted from sporomes originated inhibition of growth of S180 tumors in vivo. Previously hot water extracts proved to be effective against Sarcoma 180	Hou et al. (2013); Ikekawa et al. (1969)
	Antibacterial	A novel polysaccharide named TMP-A extracted from sporomes originated inhibition of *Micrococcus lysodeikticus* growth in vitro	Hou et al. (2013)
Tricholoma mongolicum S. Imai	Antitumour	Lectins, sugar-bindingproteins	Wang et al. (1996, 1998)
Tricholoma terreum (Schaeff.) P. Kumm. [=*T. myomyces* (Pers.) J.E. Lange]	Antioxidant	Methanol extracts from sporomes	Akata et al. (2012)
Tricholoma populinum J.E. Lange	Antiallergic	Ergosterol peroxide from whole mushrooms	Kreisel et al. (1990)
Tuber aestivum Vittad	Antioxidant	Ergosterol and phenolic compounds extracted from sporomes	Villares et al. (2012)
Tuber magnatum Picco	Antioxidant	Phenolic compounds and flavonoids	Segneanu et al. (2012)
Tuber melanosporum Vittad	Antioxidant	Ergosterol, flavonoids, and phenolic compounds extracted from sporomes	Segneanu et al. (2012); Villares et al. (2012)

(continued)

Table 6.2 (continued)

Species	Medicinal properties	Active principles or extracts	Source
Tuber indicum Cooke & Massee	Antioxidant	Ergosterol, phenolic compounds, and polysaccharides extracted from sporomes	Villares et al. (2012); Luo et al. (2011a)
	Potential precursor of steroid hormones	Steroidal glucoside	Jinming et al. (2001)
Tuber sinense X.L. Mao	Antioxidant	Water- and alkali-soluble polysaccharides extracted from sporomes	Zhao et al. (2012b)
	Immunomodulatory	Polysaccharides extracted from sporomes showed a significant increase of macrophage phagocytosis	Zhao et al. (2012a)
Verpa conica (O.F. Müll.) Sw.	Antioxidant	Tocopherols (α-tocopherol), and β-carotene	Elmastas et al. (2007)

Species nomenclature is based in Index fungorum (2013); edibility is based mainly in Boa (2004) or in more recent information from the specific cited references; and the ectomycorrhizal status of the fungal species is based in Rinaldi et al. (2008) and Comandini et al. (2012)

over 200 different types of cancer because there are over 200 different types of body cells (CRUK 2013). This disease is a leading cause for death worldwide. For example, in 2008 cancer originated nearly 7.6 million human deaths (Jemal et al. 2011). The anticancer drugs currently available are far to be target specific and have numerous side effects and complications in clinical management. For this reason, there is an increasing interest in the search for alternative metabolites useful in the treatment of cancer, including those existing in wild mushrooms. The traditional use of mushrooms as antitumor agents is known from ancient times in southeastern Asia (Dai et al. 2009; Sullivan et al. 2006). Anecdotally, an ancient Japanese legend mentions that wild monkeys almost never suffer cancer, hypertension, or diabetes, due to their habit to consume wild mushrooms (Daba and Ezeronye 2003). The first-known report of antitumor agents from mushrooms is that of Lucas et al. (1957) who found effective extracts in sporomes of the highly appreciated edible wild mushroom called "porcini" (*B. edulis* Bull. ex Fr.) for the treatment of the Sarcoma S-180 tumor cells in mice, interestingly based in Bavarian folklore. Although these authors were able to find an antitumor agent in extracts of sporomes of this mushroom, they were not able to extract it from mycelial cultures. Since then, a number of bioactive molecules including a number of antitumor agents, have been identified from different edible EEMs, including interestingly a lectin from the porcini mushroom (Bovi et al. 2011). Different pharmaceutical compounds with potential antitumor and immunostimulating properties have been isolated from sporomes or cultivated mycelium of this group of mushrooms (Table 6.2). These substances have included low-molecular weight compounds such as amino acids, triacylglycerols, or selenium and high-molecular weight compounds such as homo and heteroglucans, glycans, glycoproteins, glycopeptides, and proteins (including some lectins). Antitumor agents have been found in typical edible ectomycorrhizal genera including *Albatrellus, Astraeus, Boletus, Hydnum, Lactarius, Polyozellus, Russula, Suillus, Thelephota, Tricholoma,* and *Tuber.* Some of the antitumor compounds have been isolated exclusively from specific species or genera of edible EEMs including for example: polyozellin from *Polyozellus multiplex*, suillin from *Suillus placidus*, grifolin from *Albatrellus confluens, Albatrellus flettii,* and vialinin and thelephantin from *Thelephora aurantiotincta* and *Thelephora vialis* (Table 6.2).

The "blue chanterelle" (*P. multiplex)*, with a disjunct distribution in North America and Southeast Asia, is known to play a potential suppressive role in stomach cancer. Lee and Nishikawa (2003) suggested that extracts from *P. multiplex* may have suppressive effects on stomach cancer, one of the four most lethal types of cancer in humans. These authors showed that feeding rats with a low concentration (0.5 or 1 %) of water extract from this mushroom enhanced the activities of the enzymes glutathione S-transferase and superoxide dismutase, and increased the abundance of the molecule glutathione. The extract also increased the expression of the protein p53. All of these substances protect the human organism against stomach cancer. Kim et al. (2004) and Jin et al. (2006a, 2006b) attributed these antitumor properties to the bioactive compound polyozellin. Previously, Lee et al. (2000), Kim et al. (2002) and Song and Raskin (2002) have analyzed extracts from *P. multiplex* and found similar dibenzofuranyl derivatives than polyozellin, named kynapcin-12, -13, -28, and -24. This last compound has currently been already synthesized by palladium catalysis with a 12 % overall yield from commercially available 3,4-dihydroxybenzaldehyde (Yang et al. 2009). The Japanese "kurokawa mushroom" (*Boletus leucomelas*) is an edible species, from which a novel lectin was isolated by Koyama et al. (2002). This lectin inhibited the proliferation of human monoblastic leukemia U937, due to its apoptosis induction. Interestingly, this is the first mushroom lectin with apoptosis-inducing activity, and therefore with a potential as anticancer agent. Recently, Hou et al. (2013) reported that a novel polysaccharide named TMP-A with a molecular weight of 8.89×10^4 Da isolated from matsutake (*T. matsutake*), an edible mushroom used in traditional Chinese medicine for several thousand years, significantly promote the lymphocyte and macrophage cells in vitro and originated inhibition of growth of S180 tumors in vivo. Additionally to these antitumor and immune activities, these authors demonstrated that this polysaccharide also has antibacterial properties against *Micrococcus lysodeikticus.* The antitumor activity from matsutake confirms a previous report from Ikekawa et al. (1969) who demonstrated that aqueous extracts of this mushroom, possibly an unknown polysaccharide, from *T. matsutake* have antitumor activity against transplanted Sarcoma 180 in Swiss albino mice. Through submerged fermentation of the "Bay bolete" [*Boletus badius* (=*Xerocomus badius*)] it is possible to produce theanine (c-glutamylethylamide), which is an aminoacid with antitumor activity and L-theanine has synergistic effect on the antitumor activities of doxorubicin, anthracyclines, cisplatin, and irinotecan (Li et al. 2008; Wegiel et al. 2001). A galactomannan which enhanced macrophage activation was isolated from *Morchella esculenta* by Duncan et al. (2002). Suillin, a derivative of a phenolic acid (protocatechuic acid) belonging to the prenylphenol class isolated from the edible EEM *S. placidus*, has proven to have an antitumor activity, as a potent apoptosis inducer in human hepatoma HepG2 cells (Liu et al. 2009). More recently, Vaz et al. (2012) reported that methanolic extracts of another edible species of the same genus, *Suillus collinitus* increased p53 expression and caused cell cycle arrest and apoptosis in a breast cancer cell line. It is interesting to note that also some toxic EEMs (Quang et al. 2006), for example *Inocybe umbrinella,* have also been reported to produce antitumor activities against breast cancer cells. Zhao et al. (2009a, b) reported that proliferation of tumor cells including hepatoma HepG2 cells and breast cancer MCF7 cells were inhibited by a novel lectin extracted from dried sporomes of this toxic mushroom species. These last findings are particularly important because breast cancer is one the major health problems in women worldwide. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide, accounting for 23 % (1.38 million) of the total new cancer cases and 14 % (458,400) of the total cancer deaths in 2008. About half the breast cancer cases and 60 % of the deaths are estimated to occur in economically developing countries (Jemal et al. 2011). Edible ectomycorrhizal species of *Thelephora* including *T. vialis* and *T. aurantiotincta* are commercialized in great quantities in Southern China, in the Province of Yunnan. Different *p*-terphenyl derivatives including thelephatin O and vialinin A have been isolated from these mushrooms. The first total synthesis of natural, unsymmetrical 2′,3′-

diacyloxy-*p*-terphenyls, thelephantin O was carried out very recently (Fujiwara et al. 2012). These bioactive compounds, thelephantin and vialinin A, have shown to be potent cytotoxic agents against cancer cells, by decreased cell viability in human hepatocellular carcinoma cells (HepG2) via iron chelation, and human colonic carcinoma cells (Caco2) (Norikura et al. 2011, 2013). Grifolin is a secondary metabolite isolated from fresh sporomes of the EEM *A. confluens* which has the ability to inhibit tumor cell growth by inducing apoptosis (Ye et al. 2005). This process regulates the cell death through a strictly controlled program playing an important role in most human functions, from fetal development to adult homeostasis (Reed 2001). Defects in the regulation of apoptosis or programmed cell death make important contributions to the pathogenesis and progression of most cancers and leukemias (Reed 2004). For this reason, bioactive compounds, such as grifolin, which inhibit the proliferation of tumor cells by inducing apoptosis, are considered to have potential as antitumor agents. Ye et al. (2007) identified the novel targets of grifolin by studying its effect on the human nasopharyngeal carcinoma (CNE1) cell line, and Luo et al. (2011b) showed that grifolin inhibits the growth of cancer cell lines by upregulating death-associated protein kinase 1DAPK1 via p53 in these nasopharyngeal carcinoma cells. Jin et al. (2007) found that grifolin induces apoptosis via inhibition of PI3K/AKT signaling pathway in human osteosarcoma cells. Therefore, grifolin might represent a promising candidate in the prevention and intervention of cancer by targeting DAPK1 signaling to induce cell cycle G1 phase arrest (Luo et al. 2011b). Ergone is a bioactive steroid found in different mushrooms including the edible wild mushroom called “charcoal burner” (*Russula cyanoxantha*) (Gao et al. 2000). Zhao et al. (2011) found that ergone extracted from sporomes of *R. cyanoxantha* has a potent antitumor activity against hepatocellular carcinoma HepG2 cells by inducing G2/M phase arrest and apoptosis. They also found that ergone-induced apoptosis by activating caspases via both intrinsic and extrinsic pathways. In addition, the ratio of Bax/Bcl-2 markedly increased in cells treated with ergone. Previously, a ceramide potentially involved in the apoptosis process was extracted from this species by Gao et al. (2001). These results are significant as they provide an insight into the molecular mechanism of ergone which might be a potent chemotherapeutic agent for the treatment of liver cancer. This type of cancer is a worldwide major health problem, with an estimated incidence ranging from 500,000 to 1 million new cases annually (Lau and Lai 2008). Additionally, it has been found that ergones have other beneficial functions including cytotoxic (Lee et al. 2005) diuretic (Zhao et al. 2009a, b) and immunosuppresive activities (Fujimoto et al. 2004). Zhang et al. (2010) isolated for the first time a lectin from the important ectomycorrhizal genera *Russula*. As this novel lectin came from the edible mushroom, called in China “red mushroom” (*Russula lepida*) was named RLL. The protein was characterized and showed a potent antitumor activity, having an antiproliferative activity toward the hepatoma HepG2 cells and human breast cancer MCG-7. Daily intraperitoneal injections of RLL (5.0 mg/kg body weight/day for 20 days) brought about 67.6 % reduction in the weight of S-180 tumor. Similarly, Zhao et al. (2010) isolated another novel dimeric lectin, from *Russula delica*, with a molecular weight of 60 kDa and high hemagglutinating activity, which potently inhibited proliferation of HepG2 hepatoma and MCF 7 breast cancer cells, with an IC50 value of 0.88 and 0.52 μM, respectively. Both compounds isolated from *R. delica* and *R. lepida* have been recently purified using ion exchange chromatography and gel filtration (Zhang et al. 2012). The "milk-caps" (genus *Lactarius* spp.) are important medicinal mushrooms (Dai et al. 2009; Joshi et al. 2013). For example the "fleecy milk-cap" (*Lactarius vellereus)* is a natural source of antimutagens with potential pharmacological application in cancer prevention. Methanol extract of *L. vellereus* is highly protective against 2-amino-3-methylimidazo (4,5-f) quinoline mutagen-induced DNA damage in human derived cells (Mlinaric et al. 2004). Fujimoto et al. (1993) showed that genaryl phenols named flavidulols A, B and C, isolated from the Japanese edible mushroom *Lactarius flavidulus* showed an immunosuppressive effect. Wu et al. (2013) isolated from this Japanese mushroom a 14.6-kDa RNase which suppressed proliferation of HepG2 and L1210 cells with an IC50 of 3.19 and 6.52 μm, respectively. Previously, these authors (Wu et al. 2011) have isolated a dimeric 29.8 kDa lectin, named LFL, from the same mushroom species, which suppressed the proliferation of hepatoma (HepG2) and leukemic (L1210) cells with an IC50 of 8.90 and 6.81 μM, respectively, in the case of the lectin (Wu et al. 2011). Due to the fact that LFL exhibits potent antitumor activity in vitro, the possibility that eventually this lectin might be developed into an agent useful for cancer therapy is opened.

6.2.2 Antidiabetes

Diabetes mellitus is a chronic metabolism disease in which the amount of glucose in the blood is too high because the body cannot use it properly. If left untreated, it leads to heart disease, stroke, blindness, and kidney failure. It has been estimated that in 2011, 366 million people have diabetes (Diabetes UK 2012). For these reasons, it has been considered that diabetes is a major health risk in the world. This disease can be defined as a metabolic disturbance of carbohydrate, fat, and protein metabolism which leads to elevation of both fasting and postprandial blood glucose levels, which is called hyperglycemia. A therapeutic approach for treating it is through inhibition of the hydrolyzing enzymes like α-glucosidase and α-amylase in the digestive tract (Zhang et al. 2013; Palanisamy et al. 2011; Chiasson et al. 1998). Due to the high cost, and numerous side effects, of drugs used to treat diabetes, there is an increasing interest in the search of bioactive compounds having this inhibitory effect from natural sources including edible mushrooms (De Silva et al. 2012a, b). Liu et al. (2012a, b) studied the antihyperglycemic properties of some edible EEMs by evaluating the α-glucosidase inhibitory and α-amylase inhibitory activity of ethanolic and aqueous extracts of some EEMs. The aqueous extract of the common chinese medicinal mushroom, *Catathelasma ventricosum,* revealed the highest α-glucosidase inhibitory activity (EC50 value 2.74 μg/mL) of all of the evaluated mushrooms, while the aqueous extract of the cosmopolitan "deceiver mushroom" (so-called because of their high morphological variability)

Laccaria laccata (=*L. amethystea*) revealed the highest α-amylase inhibitory activity (EC50 value 4.37 μg/mL). Meanwhile, ethanolic extracts from other cosmopolitan mushroom called the “black trumpet” or the “black chanterelle” (*Craterellus cornucopioides*) showed also a high α-glucosidase inhibitory activity (EC50 value 8.28 μg/mL). In a similar way, previously it had been shown that extracts of the “red-banded *Cortinarius*” (*Cortinarius armillatus*), an edible rare species in North America but the most common *Cortinarius* in Europe (Niskanen et al. 2011), lowered raised blood glucose levels by inhibiting α-glucosidase (ASAK 2000, 2004). Similarly, Komai et al. (2006) found that extracts from mycelium of matsutake (*T. matsutake*), “fried chicken mushroom” (*Lyophyllum decastes*), “hon-shimeji,” (*Lyophyllum shimeji*) and the “charcoal burner” (*R. cyanoxantha*) have a strong α-glucosidase inhibitory activity. Methanol extracts from the “blue chanterelle” (*P. multiplex*) have also been reported to have an inhibitory effect on α-glucosidase (Lee et al. 2013). Ohuchi and Aoyagi (2010) carried out a broad screening of the inhibitory activity of α-amylase and α-glucosidase of water and ethanol extracts of 195 species of Japanese mushrooms. They found that some EEMs including *Tricholoma giganteum* (Nioushimeji), *Russula nigricans* (*Kurohatsu*), and *Morchella esculenta* (*Amigasatake*) have a conspicuos α-amylase inhibitory activity. Meanwhile, *Boletus pseudocalopus* (Niseashibeniiguchi), *Albatrellus dispansus* (Koumoritake), *Cortinarius alboviolaceus* (Usufujifuusentake), *Boletopsis leucomelas* (Kurokawa), and *C. armillatus* (Tsubafuusentake) presented a strong α-glucosidase inhibitory activity. They identified three bioactive compounds that originated the inhibition of α-glucosidase: (i) nojirimycin derivatives, (ii) α-homonojirimycin; and (iii) 7-*o*-*β*-D-glucopyranosyl-α-homonojirimycin. These bioactive compounds, particularly nojirimycin derivatives, were the main active components responsible for the α-glucosidase inhibitory activities in the EEMs *B. pseudocalopus, C. armillatus,* and *C. alboviolaceus*. Additionally, these authors reported also some α-amylase inhibitory effects from ethanol extracts of sporomes of the edible EEMs *Cantharellus luteocomus* (“Tokiirorappatake”), *Cortinarius triumphans* [=*Cortinarius crocolitus* (“Kiobihuusentake”)], *Lactarius subzonarius* (“Kesiroharumodoki”), *Lactarius torminosus* (“Karahatutake”), *R. cyanoxantha* (“Kawarihatu”), *Russula emetica* (“Dokubenitake”), *Russula foetens* (“Kusahatu”), *Tricholoma portentosum* (“Simohurisimeji”), and *Tricholoma vaccinum* (“Kudaakagesimeji”). More recently, some polysaccharides, with a molecular weight estimated between 3,700 and 1.7 × 107 Da were extracted from *C. ventricosum* and the antihyperglycemic activity of these polysaccharides named CVPs in induced diabetic mice was studied by Liu et al. (2013a, b). A significant decrease in the concentrations of blood glucose, total cholesterol, triglycerides, low-density lipoprotein-cholesterol (LDL-C), and maleic dialdehyde, and a significant increase in the concentrations of high density lipoprotein-cholesterol (HDL-C) were recorded by the authors in mice feed with the CVPs compared with untreated diabetic mice. Additionally, when normal mice were treated with CVPs, all detection indexes and pathologic morphologies of liver, kidney, and pancreas were similar to untreated normal mice, which indicated CVPs are safe for normal mice. As a potent antioxidant activity was also recorded, the authors considered that this could be a factor that might have influenced the hypoglycemic and hypolipodemic observed effect.

6.2.3 Antimicrobial

The discovery of penicillin derived from *Penicillium* fungi, formally attributed to Alexander Fleming in 1929, (Fleming 1929), and the first cure of nenonatal patients with eye gonorrheal infections with this compound by Cecil George Pain, in Sheffield, England in 1930 (Wainwright and Swan 1986), opened an increasing interest of the study of fungal compounds with antibiotic properties. Currently, there has been an increasing interest in the search of novel fungal antimicrobial agents, particularly due to: (i) the emergence and spread of antibiotic resistance of pathogenic microorganisms; (ii) the numerous side effects of some antibiotics; and (iii) the concern of appearance of multidrug resistance microbial infections (Alves et al. 2012; Alanis 2005; Barranco et al. 2010; Bala et al. 2011). From an ecological perspective, the production of antibiotic compounds is fundamental for the mycelium of EEMs to survive in natural conditions, due to the fact that they have to permanently compete with bacteria, fungi, virus, and other soil microorganisms. For this reason, it is not surprising that, in the last decades different antimicrobial compounds have been found in extracts of mycelium or sporomes of edible EEMs including bioactive compounds active against *Escherichia coli, Enterobacter aerogenes, Salmonella typhimurium, P. aeruginosa, S. aureus, S. epidermidis, Bacillus subtilis,* and *Candida albicans*. The source of these extracts has come from widely consumed EEMs included in the genera *Amanita, Cantharellus, Hydnum, Hygrophorus, Rhizopogon, Sarcodon, Suillus,* or *Tricholoma* (Table 6.2). It has been found that different phenolic compounds, including dihydroxybenzoic and protocatechuic acids, could be used as antimicrobial agents, specifically against microorganisms resistant to commercial antibiotics (Alves et al. 2013). Extracts from sporomes of the edible EEMs *Ramaria botrytis* and *R. delica* have shown for example a bactericide effect against clinical isolates of *Pasteurella multocida, Streptococcus agalactiae,* and *Streptococcus pyogenes* resistant to different conventional antibiotics (Alves et al. 2012). In addition, recently, it has been isolated from the sporomes of the edible ectomycorrhizal coral mushroom *Ramaria cystidiophora* a novel butenolide named ramariolide with antimicrobial activity against the pathogenic bacterial species *Mycobacterium tuberculosis*, considered the causative agent of most cases of tuberculosis (Centko et al. 2012), which shows the potential of this field of research. In relation to bioactive antiviral agents, from EEMs, it has been found that the truffle *Tuber borchii*, or whitish truffle mainly found and highly appreciated as food in Central Italy, contains a protein named cyanovirin-N, which has the ability to block virus entry into target cells, therefore having also potential use as inhibitor of human immunodeficiency virus (HIV) activity (Percudani et al. 2005) More recently, the solution structures for three recently discovered cyanovirin-N homolog family of lectines has been

Fig. 6.5 Mexican market stall selling a mixture of species of the ectomycorrhizal genera *Ramaria, Gomphus, Russula* and *Lactarius*, which have a variety of novel bioactive compounds

determined *(*Koharudin et al. 2008). It is frequent that EEMs have multifunctional health benefits, for example recently Liu et al. (2013a, b) found that the "broom mushroom" (*Ramaria flava*) a common species marketed in China and Mexico (Fig. 6.5), had antioxidant, antitumor, and a clear antibacterial activity against *E. coli, S. aureus* and *B. subtilis.* Some bioactive compounds with antibacterial activities are specific of EEMs for example a geranylphenol called flavidulol A has been found only in the "kihatsudake" (*L. flavidulus*), an edible mushroom which has a bitter taste (Takahashi et al. 1993; Wishart et al. 2012).

The flavidulol A has a potent antibacterial activity against *S. aureus* and *B. subtilis*, and also has antifungal activity against the dermatophytes *Trichophyton rubrum* and *T. mentagrophytes* (Takahashi et al. 1988). Another antibiotic called grifolin, only found in the ectomycorrhizal genus *Albatrellus*, was originally isolated en 1949 from *A. confluens* by Hirata and Nakanishin (Anke 1978) and posteriorly also isolated from *A. flettii.* It has potent inhibitory activity on *Bacillus cereus* and *Enterococcus faecalis* (Liu et al. 2010). The antibiotic lactaroviolin isolated from the "saffron milk cap" (*Lactarius deliciosus*) structurally described in 1954 (Karrer et al. 1945; Sorm et al. 1954; Bergendorff and Sterner 1988), inhibit the growth of *M. tuberculosis*, the bacteria, causative agent of tuberculosis (Anke et al. 1989; Anke 1978). In a very interesting discovery, Lovy et al. (2000) showed that the EEMs *Russula xerampelina* (known as the shrimp mushroom), *Boletus variipes,* and *Boletus queletii*, showed activity against *Plasmodium falciparum*, a protozoan parasite that causes malaria in humans. Antimicrobial activities originated by low molecular weight molecules, carbohydrates, or proteins have been studied in EEMs from Portugal (Alves et al. 2012; Barros et al. 2007a), China (Liu et al. 2013a, b; Dai et al. 2009), Turkey (Doğan and Akbas 2013; Doğan et al. 2013), Mexico (Ochoa-Zarzosa et al. 2011), Serbia (Heleno et al. 2013; Kosanić et al. 2012), Korea (Lee et al. 1999; Park et al. 1995), Macedonia (Nedelkoska et al. 2013), Algeria (Gouzi et al. 2011; Dib-Bellahouel and Fortas 2011) and Jordan (Janakat et al. 2005). It is interesting to note that

Fig. 6.6 Seller of the desert truffle (*Terfezia* spp.) in Morocco; incomes from this commercialization are very important for marginal rural populations in the North of Africa

phenolic compounds from wild mushrooms, including some ectomycorrhizal species, with antimicrobial activities have been recently reviewed by Alves et al. (2013). Some of the most highly apprecciated EEMs have shown antimicrobial activity, these includes for example the "Caesar's mushroom" (*A. caesarea* s.l.) against *C. albicans* (Doğan and Akbas 2013), the chanterelle (*C. cibarius* s.l.) against *E. coli* (Ozen et al. 2011), Porcini (*B. edulis* s.l.) against *C. albicans, Klebsiella pneumoniae, P. aeruginosa* and *S. aureus* (Kosanić et al. 2012), "saffron milk cap" (*L. deliciosus* s.l.), particularly the young sporome stages, against *B. cereus, B. subtilis, S. aureus* and *P. aeruginosa* (Barros et al. 2007b), Matsutke (*T. matsutake*) against *B. subtilis* (Tsuruta and Kawai 1979), "blue milk cap" (*Lactarius indigo*) against *E. coli* diarrheagenic strains, *P. aeruginosa, Enterobacter cloacae*, *S. aureus* and *Salmonella enterica* (Ochoa-Zarzosa et al. 2011), "hedgehog mushroom" (*Hydnum repandum*) against *E. coli* and *P. aeruginosa* (Ozen et al. 2011) and "*Japanese shoro*" (*Rhizopogon roseolus*), *against B. subtilis*, *E. aerogenes* and *E. coli* (Solak et al. 2006). A very interesting case is that of the desert truffles included in the genera *Terfezia* and *Tirmania* (Figs. 6.6, 6.7).

These mushrooms are distributed in Northern Africa, Southern Spain, and Middle Eastern countries. Traditionally, dessert truffles have been considered as a very important source of antibiotics. Recently, it has been discovered that *Terfezia claveryi, T. leonis, Tirmania nivea,* and *T. pinoyi* have potent antibiotic properties against pathogenic bacteria such as *P. aeruginosa, B. subtilis, C. trachomatis,* and *S. aureus*, which are the causal agents of widely distributed eye diseases such as conjunctivitis, blepharitis, or trachoma (Gouzi et al. 2011; Dib-Bellahouel and

Fig. 6.7 Moroccan "tajine" with dessert truffles (*Terfezi*a spp.); numerous antimicrobial, antioxidant and even aphrodisiac properties have been reported in this group of mushrooms

Fortas 2011; Mandeel and Al-Laith, 2007; Janakat et al. 2005). This examples show that despite their inherent cultivation difficulties, including slow growth, low strain isolation rate in vitro or difficulties to obtain cultivation of sporomes, it is worth the screening of extracts of sporomes or mycelium of edible EEMs due to the fact that they can be a very important source of novel antimicrobial metabolites active against new human pathogen targets.

6.2.4 Anti-HIV and Other Virucidal Compounds

Very recently, Zhu et al. (2013) extracted from *Hygrophorus russula* a common edible EEM in China (Liu et al. 2009) and Mexico (Pérez-Moreno et al. 2010), a novel ribonuclease with significant HIV-1 reverse transcriptase inhibitory activity. Previously, Suzuki et al. (2012) extracted from the same mushroom, a novel mannose-specific lectin which showed a mitogenic activity against spleen lymph cells of an F344 rat, activating the proliferation of both T and B cells responsible of the immune system. These authors also made another interesting discovery because this novel lectin, named HRL, strongly binds to the gp120 protein present on the surface of viruses and surface sugar chains on host cells, which plays an important role as one of the entry methods for virus infection. Compounds that can bind to the glycoprotein gp120 from HIV are attracting attention recently. In the specific case of the HIV, there are two kinds of glycoproteins in its envelope, gp120 and gp40, which bind to the receptors on the surface of host cell and lead to the infection of virus to the host. Then bioactive compounds that can specifically bind to these glycans might hinder the interactions between the proteins of the viral envelope and the cells of the host and prevent further interactions with the co-receptors (Botos and Wlodawer 2005; Balzarini 2006). The edible EEM *H. russula* from which this HRL lectin has been isolated is therefore a candidate for a functional food that can prevent HIV infection in future. Another protein, belonging to another novel family of lectins, collectively designated as cyanovirin homologous CVNHs proteins, a specifically cyanovirin named TbCVNH has been isolated from the whitish truffle (*T. borchii*) and specifically designated TbCVNH (Koharudin and Gronenbom 2013; Koharudin et al. 2008). Due to the fact that this family of lectins also interact with the glycoprotein gp120 and other mannosylated viral surface proteins, cyanovirins have the potential to inactivate a wide range of enveloped viruses in addition to HIV, including those of Ebola (Barrientos et al. 2003), H1N1 from influenza A (Smee et al. 2008), hepatitis C (Takebe et al. 2013), and HSV1 the Herpes simple virus (Yu et al. 2010). Currently due to its potent inhibitory action, cyanovirins are being evaluated as topical virucidal agents (Keeffe et al. 2011). *Lactarius hatsudake*, is an edible EEM, largely consumed in eastern Asia, which has long been used as an antitumor and antiviral agent in Chinese traditional medicine (Dai et al. 2009). Zhang et al. (2007a, b) isolated two novel ergosterol-type sterols which had a significant anti-HIV activity by inhibiting HIV replication in C8166 cells in vitro. Wang et al. (2007) isolated a peptide, from the edible mushroom *Russula paludosa*, with HIV-1 reverse transcriptase inhibitory activity. Zhao et al. (2010) isolated and characterized, from the edible mushroom *R. delica*, a novel lectin composed of two identical subunits, each with a molecular weight of 30 kDa, which potently inhibited HIV-1 reverse transcriptase activity with an IC50 of 0.26 μM. Meanwhile, Xu et al. (2010) isolated and characterized, from sporomes of this edible mushroom, two novel guaiane sesquiterpenes called lactariolines A and B, with modulatory effects on interferon-gamma production. The interferon-gamma is a dimerized soluble cytokine (Boehm et al. 1997), which is an important activator of macrophages and critical for innate and adaptive immunity against tumor control and intracellular bacterial and viral infections. Antiviral activities also have been recorded from other edible EEMs. For example, two novel proteins named RC183 and RC28 with anti-herpes, varicella zoster, influenza A, and respiratory syncytial viruses has been isolated, characterized, and molecularly cloned from *Cortinarius caperatus* (=*Rozites caperatus*) (Piraino 2006; Gong et al. 2009; Piraino and Brandt 1999). Wu et al. (2011) isolated from dried sporomes of the edible "kihatsudake mushroom" (*L. flavidulus*) a 14.6-kDa RNase which inhibited the activity of HIV-1 reverse transcriptase.

6.2.5 Antihypertensive and Hypocholesterolemic Effects

Due to the fact that currently cardiovascular disease is a major cause of mortality, wild edible mushrooms are currently investigated for their therapeutic properties. In in vitro and in vivo research, mushrooms have shown to reduce risk factors of cardiovascular disease, such as high total cholesterol and low-density lipoprotein (LDL) cholesterol, and high blood pressure (Guillamón et al. 2010). Several in vivo experiments have demonstrated the hypotensive effect of γ-aminobutyric acid or GABA. In spontaneously hypertensive rats, GABA has showed to have an antihypertensive effect (Hayakawa et al. 2005; Hayakawa et al. 2005). Additionally, the hypotensive effect of some food products containing GABA, without side effects, has been demonstrated in hypertensive patients (Tanaka et al., 2009; Shimada et al. 2009). GABA is an amino acid which has been reported in high amounts in some edible EEMs such as *B. edulis* (Chen et al. 2012). It also has been demonstrated that grifolin and neogrifolin, two bioactive compounds found in “Ningyotake” EEM (*A. confluens*) had a plasma cholesterol-lowering effect (Sugiyama et al. 1992, 1994). These authors showed that the hypocholesterolemic action of grifolin might be elicited by the augmented excretion of cholesterol into the feces, and as consequence there was a reduction in the cholesterol and the triglyceride plasma levels. Hypocholesteromic action, accompanied with fecal excretion of bile acids, has also been reported in mice feed with *L. decastes* (Ukawa et al. 2001). The bioactive compound neogrifolin has also been found in other edible EEMs such as *Tricholoma imbricatum* (Zhang et al. 2009).

Ergothioneine, a novel antioxidant, is present in edible mushrooms and is not synthesized, but is accumulated, by humans through diet. Martin (2010) found that the bioactive agent ergothioneine inhibits monocyte binding to endothelial cells characteristic of early cardiovascular disease. This amino acid has been found in different edible EEMs including *Leucopaxillus giganteus* (Harada et al. 2008), *Rozites caperata* (Tokuyama and Yamamoto 2008), *Tricholoma* sp. (Ito et al. 2011), and *L. decastes* (Nguyen et al. 2012). The regular consumption of mushrooms containing ergothioneine therefore should be a factor or reduction of risk of cardiovascular disease.

Lovastatin, a member of the statin family of compounds, inhibit the rate-limiting enzyme in the production of cholesterol, lowering total, and LDL cholesterol levels and therefore reducing the risk of coronary heart disease. This compound has shown to have anti-inflammatory, antioxidant, and pro-fibrinolytic properties and to prevent acute coronary syndromes and atherosclerotic disease (Aarons et al. 2007). Chen et al. (2012) and Lo et al. (2012) demonstrated that sporomes of *B. edulis* and broth culture of *M. esculenta*, respectively, have high contents of lovastatin, and therefore their regular consumption should be a factor of hypocholesterolemic activity, along with the other potential benefits associated with cardiovascular health.

In addition, it is important to mention that some edible EEMs, including *A. caesarea, Amanita ponderosa, B. edulis, C. cibarius and C. cornucopioides,* and

L. deliciosus, are an important source of bioactive compounds with potential hypocholesterolemic activity including ergosterol derivatives and *β*-glucans (Gil-Ramírez et al. 2011; Table 6.2).

6.2.6 Anti-Inflammatory

Another important function of some edible EEMs is their anti-inflammatory activity. Due to the fact that chronic inflammation is involved in the pathogenesis of important diseases including atherosclerosis, rheumatoid arthritis (Libby 2008), diabetes (Wellen and Hotamisligil 2005), neurodegenerative diseases (Amor et al. 2010), obesity (Skalicky et al. 2008), and some cancers (Kraus and Arber 2009: Shacter and Weitzman 2002), currently there is an increasing interest in the finding of natural products with anti-inflammatory effects. Some edible mushrooms have demonstrated to be an important source of bioactive compounds with anti-inflammatory activities (Lull et al. 2005). Nitha et al. (2007) reported an anti-inflammatory activity in extracts from culture mycelium of the "yellow morel" (*M. esculenta*). Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that regulates cell proliferation, differentiation, and apoptosis. Furthermore, it can induce production of other cytokines and immune responses. Dysregulated TNF-α production is implicated in many diseases such as rheumatoid arthritis, a disease in which TNF-α is present in higher concentration than normal. Ye et al. (2012) demonstrated that vialinin A is an extremely potent inhibitor against TNF-α, similarly than thelephantin G (Ye et al. 2009a, b). Previously, Xie et al. (2006) isolated a novel compound designated vialinin B, from sporomes of the edible mushroom *T. vialis*, and demonstrated that also potently inhibits TNF-α production in RBL-2H3 cells (IC50 = 0.02 nM) and is a promising antiallergic agent, similarly than vialinin A (Onose et al. 2008). Recently, it has been shown that vialinin A, isolated from the EEMs *Thelephora vialis* and *T. aurantiotincta*, also is a potent anti-inflammatory agents and that it exhibits more effective anti-inflammatory activity than the widely used immunosuppressive drug tacrolimus (FK506) (Okada et al. 2013). Moro et al. (2012) found that some popular EEMs worldwide including the "chanterelle" (*C. cibarius*), the "saffron milk cap" (*L. deliciosus*) and the "black chanterelle" (*C. cornucopioides*) showed a potent anti-inflammatory activities by inhibition of nitric oxide production and iNOS, IL-1b and IL6 mRNAs expression in response to lipopolysaccharide stimulation. Also, some *β*-D-glucans with potential anti-inflammatory activities were recently isolated and characterized from the EEM *Lactarius rufus* (Ruthes et al. 2013). A number of other EEMs have shown to have potent anti-inflammatory activities (Table 6.2).

6.2.7 Hepatoprotective

Liver is an important organ for detoxification. However, liver diseases are becoming serious health problems in worldwide. Most of the hepatotoxins induce tissue injury after having been metabolized to free radicals and cause subsequent cell damage through mechanism of covalent binding and lipid peroxidation (Knight et al. 2003). The hepatoprotective effect of mushrooms, mainly those widely cultivated has been recently reviewed (Soares et al. 2013). However, there are some recent reports showing that also some EEMs exhibit hepatoprotective activities. For example, Chen et al. (2012) evaluated the albumin and globulin levels in blood serum, and the alanine aminotransferase test in mice feed with, or without, different doses of dried sporomes of *Russula* spp. showing that the evaluated extracts have a positive effect by relieving liver injury and improving liver function. Similarly, Nitha et al. (2013) demonstrated that the aqueous–ethanolic extracts of the mycelium of the "yellow morel" (*M. esculenta*) possesses profound hepatoprotective activity. They considered that its hepatoprotective activity, elicited by the extracts, might be due to the strong free radical scavenging activity and the ability to activate antioxidant enzymes of the mycelium of this mushroom (Nitha et al. 2010; Meng et al. 2010a, b). The findings thus suggest the potential use of the aqueous–ethanolic extracts of cultured mycelia of *M. esculenta* as a novel therapeutically useful hepatoprotective agent.

6.2.8 Miscellaneous

Antivenom The eastern diamondback rattlesnake (*Crotalus adamenteus* Palisot de Beauvois) is the largest rattlesnake and the heaviest poisonous snake. According to the Guiness Book of World Records, adult individuals of this species average 1.52 to 1.83 m in length and weight from 5.5 to 7 kg; the heaviest known specimen, shot in 1946, measured 2.4 m in length, and weighted 15.3 kg (Wood 1982). It has a potent venom phospholipase A2 with a complex amino acid structure (Heinrikson et al. 1977). An antivenom compound against this potent venom has been isolated from the EEM *L. hatsudake*. Gao et al. (2007) discovered two novel ergosterol derivatives: 5α,8α-epidioxy-(22*E*,24R)-ergosta-6,22-dien-3β-ol (ergosterol peroxide) and 5α,8α-epidioxy-(24*S*)-ergosta-6-en-3β-ol). Additionally, they demonstrated a selective inhibitory activity against *C. adamenteus* venom phospholipase A_2 (PLA_2) enzyme. The authors reported this antiphospholipase A_2 activity for the first time showing that this group of sterols might be used as an antivenom bioactive compound.

Cosmetics The cosmetic industry is permanently seeking for new natural products, and currently mushrooms, due to their chemical composition or presence of bioactive compounds, are being used, or patented to be used, in some cosmeceuticals and nutricosmetics. Cosmeceuticals are products which are applied topically, including creams, lotions, and ointments, while nutricosmetics are those products that produce evident benefits but have to be ingested orally (Manela-Azulay and Bagatin 2009; Hyde et al. 2010). Currently, among the used mushrooms in the cosmetic industry are included mainly edible ectomycorrhizal truffles such as *Tuber uncinatum, Tuber melanosporm* Vitt., *Tuber magnatum* Pico Vitt., *Tuber aestivum, Choiromyces maeandriformis*, and *Tuber brumale* Vitt. A product called "rodarom white truffle" is a water soluble extract from the highly appreciated "whitish truffle" (*T. aestivum*) and is commercialized by the Company Croda Inc. The product is recommended according to the site Innovadex (2013) "…in use in mature skin care, eye serums, salon hair care, facial care for men and milk baths…". Also there is a patent describing a cosmetically acceptable gel containing an active complex, with a starting substance constituted by an aqueous extract from real truffles patented by Golz-Berner and Zastrow (2005), in the US Patent 6843995. The patent description claims that this cosmetic preparation improves stimulation of the immune system, has a regenerative effect and an improved balance of the skin and is also effective against hair loss. It is claimed that truffles are selected among different high-quality species of truffles in the genera *Tuber* or *Choiromyces* or mixtures of them. Ceramides are waxy lipids, which are one of the main components of the epidermis of human skin. Together with cholesterol and saturated fatty acids, ceramide creates a water-impermeable, protective organ to prevent excessive water loss due to evaporation. Topical ceramides have shown to improve barrier function of damaged skin, reducing skin sensitivity, and responsiveness to environmental stress, thus leading to reduction in skin appearance problems such as red blotchiness (Bissett 2009). For this reason they have been used in cosmetics as epidermal hydrating agents but they are expensive due to their scarcity. Ceramides have been found in some EEMs as the "Chinese truffle" (*Tuber indicum*) (Gao et al. 2004), the "scaly hedgehog" [*Sarcodon imbricatus* (=*Sarcodon aspratus*)]; the "osiroisimeji Japanese mushroom" (*Lyophyllum connatum)* (Yaoita et al. 2002, 2003); the "pink-tipped coral mushroom" or "*Houkitake* in Japanese" (*R. botrytis*) (Yaoita et al. 2007), and *R. cyanoxantha* (Gao et al. 2001). Novel ceramides have been also isolated from other EEMs such as *Cortinarius lucorum* (Fr.) Karst. (=*C. umidicola* Kauff.) (Liu et al. 2003). Other compound that has been used in the cosmetic industry is the lycopene, which is an antioxidant pigment, and has been used, in the cosmetic industry due to their potential natural sun protective benefits and collagen production, a natural anti-aging skin compound. Lycopene has been recorded in some EEMs such as *Suillus variegatus* and *Suillus bovinus* caps, but particularly in the stems of this latter species (Robaszkiewicz et al. 2010), thus potentially these species could be used in the cosmetic industry.

Natural pigments Eyal and Spencer (1990, 1992) reported that the cultivated mycelium through submerged fermentation of a mutant strain of the "yellow morel" [*M. esculenta* (=*Morchella rotunda*)] was able to produce blue (indigo) and a red-purple pigments. The bright blue pigment is claimed to be safe and nontoxic, useful in "… food … cosmetic and textile coloring…" and possible to extract with chloroform, ether, or ethyl acetate as a dark blue solution very stable,

Table 6.3 Species of edible ectomycorrhizal mushrooms and their reported potential agricultural use and active principles involved

Mushroom species	Activity	Active principle or extract	Reference
Albatrellus ovinus (Schaeff.) Kotl. & Pouzar	Antimicrobial	Ethyl acetate extracts of sporomes had antimicrobial effect against the plant pathogen *Nematospora coryli,* which originates stigmatomycosis	Stadler and Sterner (1998)
Amanita excelsa (Fr.) Bertill	Nematicidal	Fermentation filtration showed a strong toxicity against the plant pathogenic nematodes *Aphelenchoides basseyi* and *Meloidogyne incognita*	Chen et al. (2010a)
Amanita gemmata (Fr.) Bertill. [= *A. junquillea* (Quél.]	Nematicidal	Broth culture extracts, negatively influence the growth of the parasite pine nematode *Bursaphelenchus xylophilus*	Dong et al. (2006)
Amanita muscaria (L.) Lam.	Insecticidal	Thedioleine 1,3 17, a diester of glycerol, oleic and ibotenic acids	Michelot and Melendez-Howell (2003); Takemoto et al. (1964).
Boletus aereus Secr.	Insecticidal	Proteins (haemolysin and lectin) extracted from powdered sporomes toxic to *Drosophila melanogaster*	Wang et al. (2002)
Boletus badius Pers. [= *Xerocomus badius* (Fr.) E.J. Gilbert]	Insecticidal	Proteins (haemolysin and lectin) extracted from powdered sporomes toxic to *Drosophila melanogaster*	Wang et al. (2002), Mier et al. (1996), Besl et al. (1987)
Boletus edulis Bull.	Insecticidal	Extracts from powdered sporomes toxic to *Drosophila melanogaster*	Mier et al. (1996)
Boletus erythropus sensu Persoon (=*B. queletii* Schulzer)	Insecticidal	Extracts from powdered sporomes toxic to *Drosophila melanogaster*	Mier et al. (1996), Besl et al. (1987)
Boletus luridus Sowerby	Insecticidal	Extracts from powdered sporomes toxic to *Drosophila melanogaster*	Mier et al. (1996)
Boletus subtomentosus L. [=*Xerocomus subtomentosus* (L.) Quél.]	Insecticidal	Proteins (haemolysin and lectin) extracted from powdered sporomes toxic to *Drosophila melanogaster*	Wang et al. (2002), Mier et al. (1996), Besl et al. (1987)

(continued)

Table 6.3 (continued)

Mushroom species	Activity	Active principle or extract	Reference
Cantharellus tubaeformis Fr. (=*Craterellus tubaeformis* (Fr.) Quél.)	Insecticidal	Extracts from powdered sporomes toxic to *Drosophila melanogaster*	Mier et al. (1996)
Chroogomphus rutilus (Schaeff.) O.K. Mill.	Nematicidal	Ethyl acetate extracts of sporomes negatively affected *Caenorhabditis elegans*	Stadler and Sterner (1998)
Clavulina cinerea (Bull.) J. Schröt.	Insecticidal	Extracts from powdered sporomes toxic to *Drosophila melanogaster*	Mier et al. (1996)
Cortinarius purpurascens Fr.	Insecticidal	Extracts from powdered sporomes toxic to *Drosophila melanogaster*	Mier et al. (1996)
Cortinarius violaceus (L.) Gray	Allelopathic	Plant growth regulating compounds	Araya (2007)
Gomphidius glutinosus (Schaeff.) Fr.	Antimicrobial	Ethyl acetate extracts of sporomes showed a negative effect against the plant pathogen *Nematospora coryli,* which originates stigmatomycosis	Stadler and Sterner (1998)
Hebeloma mesophaeum (Pers.) Quél	Antimicrobial	Ethyl acetate extract of sporomes showed antimicrobial effect against the plant pathogen *Nematospora coryli,* which originates stigmatomycosis	Stadler and Sterner (1998)
Hydnum repandum L. [=*H. rufescens* Schaeff.]	Nematicidal	Ethyl acetate extracts of sporomes showed a negatie effect against *Caenorhabditis elegans*	Stadler and Sterner (1998)
Hygrophorus chrysodon (Batsch) Fr.	Insecticidal	Proteins (haemolysin and lectin) extracted from powdered sporomes toxic to *Drosophila melanogaster*	Wang et al. (2002)
Hygrophorus niveus (Scop.) Fr. (= *Hygrocybe virginea* (Wulfen) P.D. Orton & Watling)	Insecticidal	Extracts from powdered sporomes toxic to *Drosophila melanogaster*	Mier et al. (1996)
Laccaria amethystina Cooke	Nematicidal	Etil acetate extracts from sporomes negatively affected *Caenorhabditis elegans*	Stadler and Sterner (1998)
Lactarius deterrimus Gröger	Nematicidal	Ethyl acetates extracts of sporomes negatively affected against *Caenorhabditis elegans*	Stadler and Sterner (1998)

(continued)

Table 6.3 (continued)

Mushroom species	Activity	Active principle or extract	Reference
Lactarius gerardii Peck	Nematicidal	Broth culture extracts negatively affected the growth of the parasite pine nematode *Bursaphelenchus xylophilus*	Dong et al. (2006)
Lactarius necator (Bull.) Pers.	Nematicidal	Ethyl acetates extracts showed a negative effect against *Caenorhabditis elegans*	Stadler and Sterner (1998)
	Antimicrobial	Ethyl acetate extracts of sporomes showed a negative effect against the plant pathogen *Nematospora coryli,* which originates stigmatomycosis	Stadler and Sterner (1998)
Lactarius rufus (Scop.) Fr.	Antimicrobial	Ethyl acetate extracts of sporomes showed a negative effect against the plant pathogen *Nematospora coryli,* which originates stigmatomycosis	Stadler and Sterner (1998)
Lactarius torminosus (Schaeff.) Gray	Nematicidal	Ethyl acetate extracts of sporomes showed a negative effect against *Caenorhabditis elegans*	Stadler and Sterner (1998)
	Antimicrobial	Ethyl acetate extracts of sporomes showed a negative effect against the plant pathogen *Nematospora coryli,* which originates stigmatomycosis	Stadler and Sterner (1998)
Lactarius trivialis (Fr.) Fr.	Nematicidal	Ethyl acetates extracts of sporomes showed a negative effect against *Caenorhabditis elegans*	Stadler and Sterner (1998)
	Antimicrobial	Ethyl acetate extracts of sporomes showed a negative effect against the plant pathogen *Nematospora coryli,* which originates stigmatomycosis	Stadler and Sterner (1998)
Lactarius vellereus (Fr.) Fr.	Nematicidal	Ethyl acetate extracts of sporomes showed a negative effect against *Caenorhabditis elegans*	Stadler and Sterner (1998)
	Antimicrobial	Ethyl acetate extracts of sporomes showed a negative effect against the plant pathogen *Nematospora coryli,* which originates stigmatomycosis	Stadler and Sterner (1998)

(continued)

Table 6.3 (continued)

Mushroom species	Activity	Active principle or extract	Reference
Lyophyllum decastes (Fr.) Singer	Antibacterial	Water extracts from substrate where the mushroom was cultivated, significantly reduced powdery mildew, angular leaf spot, leaf spot, and scab of cucumber plants originated by *Podosphaera xanthii, Pseudomonas syringae pv. lachrymans, Corynespora cassiicola* and *Cladosporium cucumerinum*, respectively	Parada et al. (2012)
	Antifungal	Water extracts from substrate where the mushroom was cultivated, originated a reduction of *Colletotrichum orbiculare*, the causal agent of cucumber anthracnose	Parada et al. (2011)
	Allelopathic	Plant growth regulating compounds	Araya (2007)
Morchella esculenta (L.) Pers.	Allelopathic	Plant growth regulating compounds	Araya (2007)
Paxillus involutus (Batsch) Fr.	Effective antiphytoviral activity against tobacco mosaic virus	Novellectin	Wang et al. (2013b)
	Antimicrobial	Ethyl acetate extracts of sporomes showed a negative effect on the plant pathogen *Nematospora coryli,* which originates stigmatomycosis	Stadler and Sterner (1998)
Peziza badia Pers.	Antimicrobial	Ethyl acetate extracts of sporomes showed a negative effect on the plant pathogen *Nematospora coryli,* which originates stigmatomycosis	Stadler and Sterner (1998)
Pisolithus sp.	Anti-phytogenic	Phenolic compounds produced in culture broth had a potent inhibitory effect on the *Fusarium* sp.	Chari et al. (2012)
Ramaria eryuanensis R.H. Petersen & M. Zang	Insecticidal	An ether extract identified as ergosta-7,22-dien-3beta,5a, 6beta-triol showed activity against larvae of *Plutella xylostella* and another ether extract showed activity against *Mylhimna separate*	Wang et al. (2005, 2010)

(continued)

Table 6.3 (continued)

Mushroom species	Activity	Active principle or extract	Reference
Ramaria flava (Schaeff.) Quél.	Antifungal inhibition against the phytopathogens *Fusarium graminearum*, *Gibberella zeae*, and *Pseudocercosporella capsellae* (= *Cercospora albomaculans)*	Ethanol extracts	Liu et al. (2013a, b)
Ramaria formosa (Pers.) Quél	Allelopathic	Plant growth regulating compounds	Araya (2007)
Russula albonigra (Krombh.) Fr.	Nematicidal	Ethyl acetate extracts of sporomes showed a negative effect against *Caenorhabditis elegans*	Stadler and Sterner (1998)
Russula alutacea (Fr.) Fr.	Nematicidal	Fermentation filtrates showed a strong toxicity against the plant pathogenic nematodes *Aphelenchoides basseyi*, *Heterodera glycines*, and *Meloidogyne incognita*	Chen et al. (2010a)
Russula sanguinea Fr.	Nematicidal	Fermentation filtrates showed a strong toxicity against the plant pathogenic nematodes *Aphelenchoides basseyi*, *Heterodera glycines,* and *Meloidogyne incognita*	Chen et al. (2010a)
Strobilomyces strobilaceus (Scop.) Berk [=*S. floccopus* (Vahl) P. Karst.]	Nematicidal	Broth culture extracts negatively affect the growth of the parasite pine nematode *Bursaphelenchus xylophilus*	Dong et al. (2006)

(continued)

Table 6.3 (continued)

Mushroom species	Activity	Active principle or extract	Reference
Suillus bovinus (L.) Roussel	Insecticidal	Extracts from powdered sporomes toxic to *Drosophila melanogaster*	Mier et al. (1996)
	Inhibitor of a phytopathogen	Culture filtrates suppressed in vitro growth of *Heterobasidion annosum*	Mucha et al. (2009)
Tricholoma equestre (L.) P. Kumm. [=*T. flavovirens* S. Lundell]	Insecticidal	Extracts from powdered sporomes were toxic to *Drosophila melanogaster*	Mier et al. (1996)
	Nematicidal	Ethyl acetates extracts of sporomes showed a negative effect against *Caenorhabditis elegans*	Stadler and Sterner (1998)
Tricholoma saponaceum (Fr.) P. Kumm.	Insecticidal	Extracts from powdered sporomes were toxic to *Drosophila melanogaster*	Mier et al. (1996)
	Antimicrobial	Ethyl acetate extracts of sporomes showed a negative effect against the plant pathogen *Nematospora coryli,* which originates stigmatomycosis	Stadler and Sterner (1998)
Tricholoma sejunctum (Sowerby) Quél.	Insecticidal	Extracts from powdered sporomes were toxic to *Drosophila melanogaster*	Mier et al. (1996)
Tricholoma sulphureum (Bull.) P. Kumm.	Insecticidal	Proteins extracted from powdered sporomes were toxic to *Drosophila melanogaster*	Wang et al. (2002)
Tricholoma terreum (Schaeff.) P. Kumm	Nematicidal	Ethyl acetate extracts of sporomes negatively affected *Caenorhabditis elegans*	Stadler and Sterner (1998)
Tricholoma ustale (Fr.) P. Kumm.	Nematicidal	Ethyl acetate extracts of sporomes negatively affected *Caenorhabditis elegans*	Stadler and Sterner (1998)
Tuber aestivum Vittad.	Allelopathic	Coumarins, scopoletin, angelicin, and bergaptene	Tirillini and Stoppini (1996)

(continued)

Table 6.3 (continued)

Mushroom species	Activity	Active principle or extract	Reference
Tuber borchii Vittad.	Allelopathic	Coumarins, scopoletin, angelicin, and bergaptene	Tirillini and Stoppini (1996)
Tuber melanosporum Vittad.	Allelopathic	Volatile organic compounds and plant hormones produced by the mycelium	Splivallo et al. (2007, 2009, 2011)
Xerocomellus chrysenteron (Bull.) Šutara [=*Xerocomus chrysenteron* (Bull.) Quél.]	Insecticidal	Novel group of lectins	Trigueros et al. (2003), Birck et al. (2004), Wang et al. (2002), Besl et al. (1987)

Species nomenclature is based in Index fungorum (2013); edibility is based mainly in Boa (2004) or in more recent information from the specific cited references; and the ectomycorrhizal status of the fungal species is based in Rinaldi et al. (2008) and Comandini et al. (2012)

which can easily be concentrated and dried, according to the US Patents US5077201 A and US 5137826 A.

Food flavorings Mushrooms have been used as food flavoring materials for centuries. Despite the fact that commercial cultivation of sporomes of the "yellow morel" (*M. esculenta)* has not currently been successful, its cultivated mycelium is extensively used as a flavoring agent (Nitha et al. 2013; Litchfield 1967). Harvested mycelial biomass contains mushroom aroma compounds and good nutritious composition, including high amounts of proteins, vitamins, and mineral elements. The composition of mushrooms suggests two medicinal properties: nutritional benefit as food supplement and therapeutic advantages. Additionally, mushroom powder can be used as flavoring of soups and sauces (Sobieska et al. 2006). In the case of *M. esculenta*, it has been also demonstrated that the aqueous–ethanolic extracts of the cultivated mycelium of this mushroom act as strong hepatoprotective (Nitha et al. 2013) and nephroprotective agents without any side effects (Nitha and Janardhanan 2008), which are important incentives to continue using it in the food industry. Due to this, the conditions (including fermentation time, temperature, and broth content) to optimize the cultivation of mycelium of *M. esculenta* in submerged fermentation, and their effects on the production of different bioactive compounds such as extracellular polysaccharides content and mycelial growth is receiving a growing interest (Xu et al. 2008), providing a reference for large-scale extractions of bioactive compounds by industrial fermentation in the future (Meng et al. 2010a, b).

6.3 Bioactive Compounds of Agricultural Interest

More than 50 species of EEMs have shown different antagonistic effects against bacterial, fungal, or virus plant pathogens or against nematodes or insects representing, therefore, a potential source of bioactive compounds useful in a more sustainable agriculture (Table 6.3).

Insecticide Natural compounds with potential application in agriculture have been found in EEMs, these include mainly bioactive compounds with insecticide, nematicidal, and allelopathic effect against some insects or weeds. Mier et al. (1996) screened extracts of dried sporomes of edible and poisonous mushrooms in order to find potential bioactive compounds with insecticide properties. Among the 175 different species of Ascomycotina and Basidiomycotina evaluated, 79 species inhibit insect development. The model insect they use was the non-mycophagous *Drosophila melanogaste*r. Some of the most toxic extracts were originated from EEMs such as *B. badius, Boletus luridus, B. edulis, Boletus erythropus, Cantharellus tubaeformis, Clavulina cinerea, Cortinarius purpurascens, Hygrophorus chrysodon, Hygrophorus niveus, S. bovinus, Suillus subtomentosus, Tricholoma equestre, Tricholoma saponaceum, Tricholoma sejuctum, Tricholoma sulphureum*, and *Xerocomellus chrysodon.* Posteriorly, Wang et al. (2002) tried to identify the chemicals involved in this insecticidal activity, the toxicity of 14 species of mushrooms were studied for water solubility, thermolability, and dialysis. These authors suggested that proteins were responsible for most of the insecticidal activity of mushroom sporomes and they pointed out that these proteins might be an important source of plant protection against insects. Among proteins, lectins and hemolysins were good insecticide candidates because the toxicities were not affected by protease. Afterwards, Birck et al. (2004) described a new lectin family, with structure similar to actinoporins, extracted from the red cracking bolete (*Xerocomellus chrysenteron*). One member of this family, the lectin XCL from *Xerocomus chrysenteron* induced drastic changes in the actin cytoskeleton after sugar binding at the cell surface and internalization, and therefore has potent insecticidal activity. The model insects evaluated were *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and *D. melanogaster* (Miegen) (Diptera: Drosophilidae). Insecticidal effects have been also reported for the highly appreciated EEM *Cantharellus cibarius* (Cieniecka et al. 2007). Besl et al. (1987) studied the effect of dried sporomes of 127 species of edible and poisonous mushrooms on larvae of *D. melanogaster* and found that some EEMs such as *B. erythropus, Xerocomus badius, X. chrysenteron, X. subtomentosus*, and *Hygrocybe punicea* showed a strong inhibition in the growth of the larvae of this insect. Probably, the most well-known insecticide properties of an EEM are that of the fly agaric (*Amanita muscaria*). Michelot and Melendez-Howell (2003), based on Muto and Sugawara in 1970, considered that the putative fly attractant of this EEM is the dioleine 1,3**17**, a diester of glycerol and oleic acid and the "ibotenic acid" has been attributed as the insecticide bioactive compound by Takemoto et al. (1964). An ether extract from *Ramaria eryuanensis,* an EEM described from Yunan, China (Petersen and Zang 1989), identified as ergosta-7,22-dien-3beta,5a,6beta-triol showed insecticidal activity against larvae of *Plutella xylostella* (Wang et al. 2010). This insect known as the diamondback moth is one of the most widely distributed insects in the world, being recorded from more than 80 countries. Its control on cruciferous crops costs annually more than one billion dollars, mainly with insecticides (Mohan and Gujar 2003). Another ether extract from the edible mushroom *R. eryuanensis* has been reported to have insecticidal activity against *Mylhimna separate* (Wang et al. 2005). The larvae of this moth feed on a number of crops, including corn, rice, or sorghum,and therefore are considered as one of the most serious pests in Asia and Australia and have been recorded in 27 countries including China, Australia, New Zealand, and some Pacific islands (Sharma and Davies 1983).

Nematicidal Nematicidal properties have also been recorded from EEMs. Dong et al. (2006) found that mycelial extracts, in broth culture, from the EEMs *Amanita gemmata* (=*A. junqileia*), *Lactarius gerardii*, and *Strobilomyces floccopus* negatively affect the growth of the parasite pine nematode *Bursaphelenchus xylophilus.* This nematode has been devastating vast areas of pine forests in Asian countries since the beginning of the twentieth century and from 1999 have spread into European forest, causing worldwide concern, because once trees are infected with this disease, they die some weeks later (Kazuyoshi 2013). This nematode is additionally damaging the matsutake EEM production in Japan by killing *Pinus ponderosa* host trees, showing how complex ecological interactions can be (Faier

2011). Stadler and Sterner (1998) reported that ethyl acetates extracts of sporomes of the EEMs *Chroogomphus rutilus, Hydnum rufescens, Lactarius deterrimus, L. necator, L. torminosus, L. trivialis, L. vellereus, Russula albonigra, Tricholoma flavovirens*, and *T. ustale* had a nematicidal effect against the model roundworm *Caenorhabditis elegans*. The bioactivity of some of these extracts was increased as a response to physical injury of sporomes for some species, emerging the possibility that chemical defense systems, mediated by enzymatic conversions, might be activated by mechanical damage. These authors observed, for example, an increase in the production of linoleic acid and S-coriolic acid in the case of *T. terreum*, and of fatty acids in the case of *L. amethystina* as a consequence of physical damage to the sporomes, and in parallel, a more evident nematicide effect was recorded. Also, nematicide activities of EEMs were reported by Chen et al. (2010a, b, c). These authors recorded strong toxicity of fermentation filtrates of: (i) *Amanita excelsa* against *Aphelenchoides basseyi* and *Meloidogyne incognita*, and (ii) *Russula sanguinea* and *R. alutacea* against the plant pathogenic nematodes *A. basseyi, Heterodera glycines*, and *M. incognita.* In this last case, the authors carried out a purification of the fermentation filtrates due to their strong nematicide effect.

Anti-phytopathogenic Stadler and Sterner (1998) reported that ethyl acetates extracts of sporomes of the EEMs *Albatrellus ovinus*, *Gomphidius glutinosus*, *Hebeloma mesophaeum, L. necator, L. rufus, L. torminosus, L. trivialis, L. vellereus, Paxillus involutus*, *Peziza badia,* and *T. saponaceum* had antimicrobial effect against the plant pathogen *Nematospora coryli,* which originates stigmatomycosis, a fungal disease that occurs in crops, such as cotton, soybean, pecan, pomegranate, citrus, and pistachio. Similarly than in the case of the nematicidal effect reported by these authors, an increase in the antifungal activity was observed when there was a mechanical damage of the sporomes and this was associated with a higher bioactivity. Parada et al. (2011, 2012) demonstrated that water extract from substrate where the edible mushroom *L. decastes* was cultivated was effective in the reduction of fungal and bacterial diseases of cucumber plants, including mildew, angular leaf spot, scab, and anthracnose caused by *Podosphaera xanthii*, *Pseudomonas syringae* pv. l*achrymans* and *Cladosporium cucumerinum,* and *Colletotrichum orbiculare*, respectively. Chari et al. (2012) demonstrated that phenolic compounds produced in culture broth of the EEM *Pisolithus* sp., originally associated with the legume tree *Acacia mangium* had a potent inhibitory effect on the phytopathogen *Fusarium* sp. Recently, Wang et al. (2013a, b) carried out the purification and characterization of a novel lectin from the wild mushroom *P. involutus*, which manifested antiphytovirus activity toward tobacco mosaic virus (known as TMV) with a 70.61 % inhibition at a concentration of 200 μg/mL. Toyota and Hostettmann (1990) isolated and characterized a diterpenic ester with antifungal activity against the formation of spores of *C. cucumerinum,* a pathogen that affects cucumbers, from the EEM *Boletinus cavipes*. They called this bioactive compound as cavipetin. Mucha et al. (2009) demonstrated that culture filtrates of the EEM *S. bovinus* are able to suppress in vitro the growth of the most economically important forest pathogen in the Northern Hemisphere: *Heterobasidion annosum* which is responsible for the loss of one billion U.S. dollars annually. The culture filtrates of *S. bovinus* are able to affect the cytoskeleton and mytocondrial morphology of this important forest pathogen, and inhibit dramatically their growth.

Herbicide the allelopathic effects of mushrooms, and their potential implications in control of weeds of agricultural importance, have been analyzed by different authors such as Araya (2007) and Chaumont (1985). Araya (2007) evaluated the potential allelopathic effect of dried sporomes of 83 mushrooms by using as model plants lettuces (*Lactuca sativa* L.). This author found that some EEMs including *M. esculenta, Cortinarius violaceus, L. decastes*, and *Ramaria formosa* showed a very strong inhibitory plant growth effect, due to the production of allelochemical bioactive compounds. However, one of the most fascinating examples of allelopathy induced by the mycelium of EEMs is produced by truffles. In this case, the development of some herbaceous plants and most bushes around the trees colonized by these mushrooms (for example *Tuber melanosporum* Vitt) is inhibited by a number of volatile organic compounds secreted by the ectomycorrhizal mycelium (Splivallo et al. 2007) and the soil remains almost naked. This phenomenon forms characteristic "burnt areas" or "*brûlé*" in French (Streiblova et al. 2012). Some bioactive phytotoxic compounds like dimethyl sulfide, 2-butanone, 2-butanol, 2-methyl propanol, 2-methyl butanol, 3-methyl butanol, 2-methyl propanal, 2-methyl butanal, and 3-methyl butanal have already been identified (Lanza et al. 2004; Pacioni 1991). Also, it has been shown that some hormones including ethylene and indol acetic acid act as potent herbicides (Splivallo et al. 2009). Additionally, different coumarins, including scopoletin, angelicin, and bergaptene, have been found in *T. melanosporum* Vitt, *T. aestivum* Vitt, and *T. borchii* Vitt (Tirillini and Stoppini 1996). This is an interesting fact because it has been demonstrated that some coumarins have an evident allelopathic effect on some plants (Guo et al. 2013a, b; Abenavoli et al. 2003). These studies are potentially important in the search of bioactive compounds of mycological-origin biopesticides targeted against weeds.

It is important to highlight that in general, the process of production of secondary metabolites by EEMs is an extremely important coevolutionary process which regulate their ecological interactions with faunal, plant, and microbial components in natural ecosystems, and this is the main reason of their great complexity (Mello et al. 2013; Müller et al. 2013; Splivallo et al. 2011; Kempken and Rohlfs, 2010; Rohlfs and Churchill, 2011). Despite this, the findings of insecticidal, nematicidal, antifungal, and allelopathic properties from EEMs undoubtedly open the possibility to find novel bioactive natural compounds useful in the control of pests (or biogenic pesticides), pathogens and weeds in agriculture.

6.4 Biotechnology and Sustainability

The sustainable development approach was considered important since the 1970s, but it was not formalized until 1987 when the United Nations World Commission on Environment and Development proposed a definition in the report entitled

"Our Common Future." This organization defined sustainable development as "… *the development that meets the need of the present without compromising the ability of future generations to meet their own needs* …" (WCED 1987). Although valuable as a policy statement, this definition has proved to be too abstract for governments, businesses, and civil societies trying to design new production systems or applied new social and environmental practices. Only by the middle of the 1990s there were more than 70 definitions of sustainable development, each emphasizing different values, priorities, and goals (Pretty 1995). What is clear is that a single definition is impossible and sustainability must, therefore, be understood and applied in a wide sense (Pérez-Moreno and Ferrera-Cerrato 1996a, b). Currently, it has been generally accepted that sustainable development needs a convergence between economic development, social equity, and environmental protection (Drexhage and Murphy 2010).

In this context, it is important to analyze that an adequate use and management of EEMs can contribute to the sustainable forest development. The forests influence different environmental processes of paramount importance in the maintenance of natural systems on earth. For example, they have a conspicuous influence on hydrologic cycles, in the reduction of soil erosion, and in the conservation of enormous animal plant and microbial biodiversity. In this way, forests also provide a wide range of economic, social, and cultural benefits and services to humankind (GFR 2010). Additionally, forests play an important role in carbon storage and reduction of greenhouse gases like carbon dioxide (Read et al. 2009). According to FAO (2006), the global store of carbon in forests is around 638 Gt, in ecosystems, compared with 750 Gt of carbon stored in the atmosphere. Thus a key factor to control or reduce climate change is the maintenance of forests. Unfortunately, deforestation incorporates about 5.9 Gt of CO_2 annually, something like 60 % of the carbon that is absorbed by all the world's forests (Myers-Madeira 2008). Maintaining forests is a complex paradox which faces social, economic, and environmental challenges. Currently, one of the most important international trends to maintain forests masses is to improve the sustainable forest management of non-timber forest products, including among them the wild edible fungi. Edible ectomycorrhizal fungi can contribute to sustainable forest management, and therefore to sustainable development, because of: (i) their demonstrated paramount ecological importance in the structure, function, and maintenance of forests (Read and Perez-Moreno 2003); (ii) the high commercial value of some species whose international market is valued in billions of dollars annually (Yun and Hall 2004; Hall et al. 2003); (iii) the enormous social and cultural significance of hundreds of species of EEMs in developing and developed countries, where the harvest of wild EEMs is highly valued by local populations economically, culturally, and socially as food or by their medicinal or nutraceutical properties (Pérez-Moreno et al. 2010; Pérez-Moreno et al. 1993); (iv) the potential for cultivation of some species, especially the successful commercial establishment of truffle plantations (Hall et al. 2007; Olivier et al. 2012), and (v) the enormous genetic potential represented by EEMs as producers of bioactive compounds of great economic, ecologic, or social importance in human health, agriculture, or industry (presented in the previous sections of this chapter). The use of bioactive compounds from EEMs could be developed through two strategies: (i) massive cultivation of edible ectomycorrhizal mycelium in order to industrially extract useful bioactive compounds, as demonstrated in species in the genera *Morchella* or *Tuber* (see previous sections of this chapter); and (ii) wider establishment of commercial plantations, mainly, but not exclusively, of truffles, with their corresponding economic, social, and environmental benefits including its contribution to carbon sequestration, by keeping forest masses, and the subsequent mitigation of global change. Next, three aspects will be discussed more deeply: (i) the importance of edible ectomycorrhizal fungi as a non-timber forest products and their potential for sustainable forest management of forests, (ii) the progress in the establishment of truffle plantations; and the description of some initial attempts of cultivation of other ectomycorrhizal edible mushroom species in forest plantations, and (iii) the seminal studies in Mexico, related to the biotechnological development of inoculation with EEMs to native trees.

6.4.1 A Non-Timber Forest Product

Since 1991, the promotion and development of non-wood forest products is currently a priority area of FAO's Forestry Department (FAO 2013). The objective of this programme "… *is to improve the sustainable utilization of non-wood forest products in order to contribute to the wise management of the world's forests, to conserve their biodiversity, and to improve income generation and food security…*". EEMs constitute an important non-timber forest product of great environmental, social, and economic importance. Despite the fact that timber has been historically the major forest product with economic importance, there has been an increased interest in EEMs in the last decades. In some cases, it has been estimated that the value of edible mushrooms can be comparable or even higher than that of timber, with the additional environmental benefits (Palahi et al. 2009; Díaz-Balteiro et al. 2003).

From the environmental perspective, EEMs play an important ecological role in the structure, functioning, and maintenance of the natural ecosystems where they prosper, including boreal, temperate, subtropical, and some tropical biomes. They are of paramount importance in the following processes: (i) Nutrient ecosystem recycling, mainly carbon, nitrogen, and phosphorus (Pérez-Moreno and Read 2000, 2001a, b; Read and Perez-Moreno 2003); (ii) Control of pathogens; (iii) Maintenance of food webs for wild life species, for example as food source for wild animals; (iv) Increased resistance of stress factors including presence of metals, drought, or increased temperature (Smith and Read 2008); and (v) establishment of common mycelial networks, due to the fact that the ectomycorrhizal

Fig. 6.8 Commercialization of wild porcini (*Boletus edulis* s.l.) and coral mushrooms (*Ramaria* spp.) in Yunan, China; a source of a variety of bioactive compounds and important revenues for local rural communities

Fig. 6.9 Gourmet dish prepared by a French chef, containing matsutake (*Tricholoma matsutake*) and Chinese truffles (*Tuber indicum*), both important functional foods

fungi are able to connect their associated trees in nature (Pérez-Moreno and Read 2004), and influence the functioning of these connected trees through transfer of carbon, nutrients, water, and allelochemicals affecting therefore plant establishment, survival, and growth, and the community diversity and stability in response to environmental stress (Simard et al. 2012).

In addition to their ecological importance, the EEMs, or the processed or preserved products derived from them, have been an important factor of great social and economic importance, where they are a regular economic income, in different regions of the world (Figs. 6.8, 6.9). Here just some examples: (i) The international market of the EEMs is annually valued in billions of dollars. The worldwide demand of EEMs is huge, particularly in the so-called “mycophilic” countries. For example, it has been estimated that only Italy had an annual demand of 70,000 tons of wild EEMs, mainly porcini (*B. edulis* s.l.) (Salerni and Perini 2004); (ii) García-Montero et al. (2010) based on data from Wang Yun stated that the commerce of Chinese black truffles (*Tuber* spp.) is currently a multimillion dollar industry, that has brought an important income to rural population in China. For example, in 2006 the total export of native Chinese black truffles was estimated to be up to 800 tons worth more than US$20 million; (iii) In Finland, a local company which market EEMs, mainly porcini (*B. edulis*), and other species like chanterelle (*C. cibarius*) and black trumpet (*C. cornucopioides*), emerge recently, since 1997. At the peak of the mushroom production, the number of pickers from marginal rural areas, supplying this local company can reach between 15,000 and 20,000 people. In 2003, this local Finish company harvested 1,100 tons mainly of porcini with revenue of USD7.4 million (Cai et al. 2011); (iv) In Tanzania, the commercialization of more than 35 native edible ectomycorrhizal species from miombo woodland (in the genera *Amanita, Boletus, Cantharellus, Lactarius, Russula*, and *Suilllus*), can be an important income for marginal rural population. This commercialization process plays an important social function in the local ethnic groups, by reducing conflicts between community and forest conservers. The activity also provides alternative employment, improve food security to rural disadvantaged groups especially women and old people, conspicuously improving their livelihood (Tibuhwa 2013); (v) In Sweden, around 21,000 tons of edible wild mushrooms were picked in 1997 (Lindhagen and Hörnsten 2000). In the Czech Republic annual surveys carried out since 1994 to 2005 indicate that the total amount of wild edible mushrooms picked during these years ranged from 13,500 to 29,700 tons (Šišák 2006). Meanwhile, Turtiainen et al. (2012) reported that 42 % of all households were engaged in mushroom picking in a total harvest of 15,000 tons of wild edible mushrooms in Finland; (vi) The Pacific Northwest of the United States is probably the region where there has been a most accurately description of commercial picking of wild edible mushrooms. Widespread commercial harvesting of wild edible mushrooms started in the region in the 1980s. In the area, recent immigrants especially from Southeastern Asia, can harvest mushrooms profitable without the language skills and formal education required for other jobs (Pilz and Molina 2002). Economically, the most important species in the area are the American matsutake (*Tricholoma magnivelare*), chanterelle (*C. cibarius*), and morels (*Morchella* spp.). In 1992 a fresh weight of 590, 500, and 374 tons of morels, chanterelles, and American matsutake, respectively were harvested in the states of Idaho, Oregon, and Washington (Schlosser and Blatner 1995). In this process, 10,500 pickers participated and the estimated gross value was of USD 41 million (Rowe 1997); and (vii) In Mexico, one of the most important mycophilic countries, more than 400 species of wild edible mushrooms are consumed. A high proportion of this, around 65 % are ectomycorrhizal. In the country more than 200 species of wild mushrooms are also used currently in traditional medicine by local ethnic groups (Guzmán 2008; Bautista-González 2013). There are different levels of commercialization of this wild mushrooms including: (a) self-consumption; (b) commercialization by local pickers; (c) commercialization through chains of brokers; and (d) exportation to international markets. EEMs constitute an element of paramount cultural, social, and economic importance in a number of ethnic groups in the country, potentially linked to the conservation and sustainable management of forests in different regions of the country (Garibay-Orijel et al. 2009; Pérez-Moreno et al. 2008, 2010).

The recollection of natural production of EEMs has been an important factor which improve the socioeconomic conditions of rural population in different regions of the world, including for example southeastern China in the Province of Yunnan, Tibetan regions (Wang and Liu 2011), central Spain (Martínez et al. 2012), and the Pacific Northwestern of the United States (Pilz and Molina 2002) and Mexico (Pérez-Moreno et al. 2010). However, it is important to mention that despite the fact that there is no formal evidence that picking of mushrooms is harmful for subsequent fruiting, except in cases where mycelia are destroyed (Arnolds 1995), currently there is a concern related with decline of natural production of some edible ectomycorrhizal musrhooms related with: (i) loss of degradation of natural habitats; and (ii) undesirable harvest methods (Barron and Emery 2009; Mortimer et al. 2012). Therefore, there is an urgent need for regulatory issues to protect this important non-timber forest product. In this context, there has been an increased interest in the management of the forest, called mycosilviculture, in order to increase the production of highly priced EEMs (Savoie and Largeteau 2011). The knowledge and development of mycosylvicultural practices have mainly received attention in Southeastern Asia, Europe, and North America. The development of mycosilviculture has been particularly important due to: (i) the difficulties to commercially cultivate most of the EEMs; and (ii) the important incomes generated by the picking of EEMs for local populations. For this reason, different studies have been conducted either to carry out management guidelines in order to allow a more sustainable harvesting (Mortimer et al. 2012) or the influence of sylvicultural management to increase natural productions of EEMs (Salerni and Perini 2004). In Southern Tuscany in Italy Salerni and Perini (2004) showed that the medium thinning increased the productivity of *B. edulis* s.l. while litter removal had a negative effect. More recently, Bonet et al. (2012) recorded in Northeastern Spain, a significant increase of saffron milk caps (*L. deliciosus*) production to *Pinus pinaster* forest thinning. The productivity of *L. deliciosus* sporomes was five times greater in plots in the first year after thinning and two times greater in the second year, as compared to non-thinned plots. Finally, it is important to mention that due to the socioeconomic importance of harvesting natural productions of wild EEMs, rural populations might be encourage to maintain forest masses in different parts of the world, which are related with carbon sequestration, a major issue which could reduce the emission of greenhouse gases and to contribute to ameliorate the global warming.

6.4.2 Truffle Cultivation

Truffles are underground edible fungi, which belong mainly to the Ascomycete genus *Tuber*, and are included among the most expensive foods. Some of these species have been highly esteemed as food delicacies from ancient times in different European (France, Spain, Italy, and Greece), Northern Africa (Morocco, Algeria, Tunisia, and Egypt) and Middle Eastern countries (Iran, Siria, Bahrein, and Kuwait). The most highly priced truffles historically have been the European species as stated in the definition of the word truffle in the Oxford Dictionary (2013): …“ *any one of various underground fungi of the family Tuberaceae; spec. an edible fungus of the genus Tuber, a native of Central and Southern Europe, esteemed as a delicacy; esp. T. aestivum or cibarium, the Common (English) Truffle, and T. melanosporum, the French Truffle, which have a black, warty exterior, and vary in size between that of a walnut and that of a large potato, which they more or less resemble in shape*….”. European truffles have been called black gold or diamonds (Renowden 2005). Another important group of truffles are those called the “desert truffles” in the Ascomycete family Terfeziaceae. Historically, desert truffles of the genera *Terfezia* and *Tirmania* have been an important food, and medicinal resource, for Saharan nomads (Volpato et al. 2013) and Southern European and Middle eastern ethnic groups (Jamali and Banihashemi 2012; Al-Qarawi and Mau 2012). More recently, it has been discovered that other regions in the world for example those in southwestern China, mainly Yunnan, and Sichuan, have a high diversity of native truffle species. For example in 2010 and 2011, 10 new species of white truffles were described from these regions. Some of these species, for example the recently described *T. panzhihuanense* is a good edible white truffle with a great commercial potential (Deng et al. 2013). All of the truffle species are obligatory ectomycorrhizal fungi, and therefore they are associated with the roots of specific species of trees, including oaks (*Quercus*), pines (*Pinus*), beech trees (*Fagus*), lime trees (*Tilia*), and hazels (*Corylus*); or shrubs, as *Helianthemum* or *Cistus* (Stobbe et al. 2013; Turgeman et al. 2011). The ecological dispersal of truffle spores is carried out by fungivorous animals, which are attracted by volatile compounds, like dimethyl sulfide, produced by the fungi (Johnson 1996; Schickmann et al. 2012).

Truffles can reach extremely high prices in the markets of the world. In autumn 2004, a large Italian white truffle was bought by a London restaurant in a charity auction for US$52,000 (Renowden, 2005). Similarly, some years later in Rome, two white truffles were sold in charity auctions as well, in USD$330,000 and USD$200,000, in 2007 and 2008, weighing 1.5 and 2.8 kg, respectively (BBC 2007; Howell 2013; Pérez-Moreno et al. 2010). The cultivation of truffles started long before the scientific knowledge related with them was developed. The truffles establish an obligatory symbiotic relationship with the roots of trees or shrubs in order to obtain their carbon compounds, which as mentioned before in this chapter is called “mycorrhiza.” The formal study of this symbiosis started in 1887, when Anton B. Frank coined the term (Frank 1894) to describe the structures formed by the roots of trees completely covered with truffle mycelium. However, the first truffle cultivation trials were carried out a century earlier by the French Pierre Mauléon and Joseph Talon, between 1790 and 1810, in Beuxes and Saint Saturnin-les-Apt, two small towns located in central western and southeastern France, respectively (Hall and Zambonelli 2012). In what is now considered an extraordinary observation skill, these persons found that seedlings planted under trees producing truffles and transplanted to new areas, were able to produce truffles. This is currently called the Talon method of cultivation of truffles and amazingly is currently still used. Parallely to these discoveries in Tuscany, Italy, in 1807, Giovio described a method in which plants could be inoculated with truffle fragments. The pioneer truffle grower, Joseph Talon and his famous quote "… if you want to harvest truffles, plant acorns," was followed by Rousseau de Carpentras who grew a seven-acre plantation of truffles, which was awarded at the Universal Exhibition in Paris in 1855. By 1868, the Department of Vaucluse, located in southeastern France produced 380 tons of truffles (Truffes.org 2013). At the present time, truffle plantations are established mainly with three species: the white truffle (*T. magnatum),* the black truffle (*T. melanosporum*), and the Burgundy truffle [*T. aestivum* (=*T. uncinatum*)] (Stobbe et al. 2013; Hall et al. 1998a, b, 2003; Bertault et al. 1998).

Currently, the exceptionally high values of truffles have attracted the interest of growers all around the world. The biggest truffle plantation is located in Spain, around 170 km to the north of Madrid, it covers 607 ha with 150,000 inoculated trees of *Quercus ilex* (Black 2006), and it has been estimated that it produced around 3 tons of *T. melanosporum*, representing a multimillion dollar industry. Just to mention some other examples: (i) In Italy, Bencivenga (2009) estimated that annually about 120,000 trees colonized with truffles are planted in 300 ha of land. Most of these trees are colonized with the black truffle (*T. melanosporum*), and the Burgundy truffle (*T. aestivum*) with 80 and 15 % of the total, respectively. Only very few plantations in Italy, are inoculated with other species including the "white truffle" (*T. magnatum*) and other species such as *T. brumale* and *T. borchii*, with less than 5 % of the total. Truffle production starts in Italy approximately after 15 years, and can reach up to 100 kg per ha; (ii) In Central Italy in the region of Umbria, Donnini et al. (2013) reported that the establishment of truffle plantations has been an important factor to restore, reclaim, and rehabilitate marginal and derelict lands and to improve the socioeconomic conditions of rural populations in the area. In the 1980s the local government of this region promoted the establishment of 59 truffle plantations, comprising 115 hectares using seven species of truffles with different hosts. Additionally, to the economic profits derived from the sell of the produced truffles some derelict areas have rehabilitated through these programs and have contributed to protect the soil from erosion and to the regeneration of vegetation cover. The authors also describe that currently, as a consequence of this success, numerous projects that involve universities, truffle hunters and growers association, as well as public administrations are in progress; (iii) In eastern Spain, in the region of Sarrion, Samils et al. (2008) reported the existence of about 4,500 ha of truffle orchards of *T. melanosporum*, with 530 members organized in a local truffle association, in areas which were previously unproductive since 1987. Additional to the important economic incomes produced by for the local community the authors reported an increased local land prices, a tendency of expansion of truffle orchards, and therefore a conspicuous oak reforestation. Thus, the promotion of truffle cultivation carried out with the support of the local community, organized in a truffle association, government subsidies and local banks have achieved in this region of Spain mutual goals of biodiversity conservation while improving rural local economy. More recently, it also has been reported in Spain that the reforestation with *Quercus ilex* inoculated with *T. melanosporum* can be successful after forest fire, being an strategy with an economic incentive (Martínez de Aragón et al. 2012); (iv) Currently, the French nurseries have an annual production of around 400,000 trees inoculated mainly with *T. melanosporum* (Hall et al. 2007). This fact gives an idea of the great interest that exists of establishing new truffle orchards; (v) In the Swedish islands of Gotland and Öland, some plantations of *Quercus rubur* inoculated with *T. aestivum,* with a total of more than 2,000 trees were established from 1999 to 2001. Interestingly, the first plantation was harvested just 6 years after planting (Weden et al. 2009); (vi) In Tunisia, the cultivation of the desert truffle *Terfezia boudieri*, inoculating plants of the shrub *Helianthemum sessiliflorum* has recently started. The establishment of farms cultivating this mushroom might be of paramount regional importance for improving economic development of very-poor local communities (Slama et al. 2010); (vii) some initial attemps of black truffle introduction have also been started in South America (Pereira et al. 2013); and (viii) Currently, New Zealand has more than 100 truffle orchards of *T. melanosporum*. From the commercial point of view, this is particularly important due to the fact that these truffle plantations potentially might be able to produce truffles when there is no natural production in the Northern hemisphere (Guerin-Laguette et al. 2013).

The environmental and social benefits of the establishment of truffle plantations go beyond the economic ones because they represent an innovative and sustainable form of land use. Hall et al. (2007) pointed out that compared to conventional agriculture, truffle cultivation has a lower environmental impact and has very little use of machinery and chemicals. In addition, it promotes the development of well-adapted ecosystems with a rich biodiversity, as pointed out by Stobbe et al. (2013). This can be particularly important in countries, where deforestation is a great problem, because truffle cultivation might favor restoration programs along with making direct contributions to rural economies (Weden et al. 2009).

6.4.3 Other Edible Ectomycorrhizal Species

It is important to note that most of the species of EEMs highly appreciated worldwide have defied cultivation in commercial plantations. These have mainly included porcini, matsutake, chanterelle (see below), saffron milk cap (Guerin-Laguette et al. 2003), Japanese shoro (Himomura et al 2012), and Caesar mushroom (Endo et al. 2013). However some initial advances have been produced in the last decades, in relation to cultivation of the mycelium of these mushroom, or the inoculation of the associated hosts of these highly valuable mushrooms.

Porcini is one of the preferred edible wild mushrooms worldwide. Currently, it has been shown that the group of porcini mushrooms, in the past included under the name of *B. edulis* s.l., consists of at least 18 related species which constitute part of a monophyletic group grouped now in the genus *Boletus* section *Boletus*

(Dentinger et al. 2010). These species establish ectomycorrhizal relationships mainly with trees in Fagaceae, Pinaceae, and Betulaceae and also with some shrubs for example Cistaceae, throughout the Nortern Hemisphere, and also they have been introduced in other countries like Australia and New Zealand (Águeda et al. 2008; Hall et al. 1998a, b); Catcheside and Catcheside 2012).. It has been estimated that annual consumption of porcini species ranges from 20,000 to 100,000 tones and that the main international markets included France, Italy, Germany, and North America (Hall et al. 1998a, b). For example, from 2002 to 2006, Italy imported more than 17,000 metric tons of fresh or refrigerated porcini mushrooms (Sitta and Floriani 2008). The annual production of porcini in the Iberian Peninsula has been estimated to be in the order of 8,000 tons (Ortega and Martinez 2008). Porcini mushrooms have been a very important factor of forest conservation, in the autonomous community of Castilla and Leon in Spain, where they are commonly use in local and gourmet cuisine (Frutos et al. 2012). Production of plants colonized with some species of porcini has been currently achieved (Olivier et al. 1997; Poitou et al. 1982; Molina and Trappe 1982) which have opened some initial perspectives in the cultivation of this mushroom. Hall et al. (1998a, b) highlighted the fact that the biotic interactions, of the microbiome associated with the hymenium, could be of paramount importance in the cultivation of this species. Duñabeitia et al. (1996) failed to synthesize mycorrhizal plantas using mycelial inoculum of porcini, either in vitro or in unsterile containers, but they were able to produce ectomycorrhizas by using hymenial tissues, containing spores. Additional to the synthesis with trees, mycorrhizas with some shrubs, members of Cistaceae, have been successfully produced with three porcini species (*Boletus aereus, B. edulis*, and *Boletus reticulatus*), showing that their morphological characters were very similar, including white monopodial pinnate morphology, a three-layered plectenchymatous mantle on plan view and boletoid rhizomorphs (Águeda et al. 2008). Olivier et al. (1997) and Díaz et al. (2009) registered the production of primordia of porcini under axenic conditions, which could be an indicator of certain saprotrophic ability, associated with the ectomycorrhizal nature of these mushrooms. However, it is important to note that this is just an initial step in the domestication of the species involved, whose production currently depends exclusively on natural production and harvest.

The matsutake is the highest prized mushroom in Japan. Originally the concept of matsutake in Japan was circumscribed to one species [*T. matsutake* (S. Ito et S. Imai) Singer], but currently the term refers to a complex of related species highly valued as a gourmet luxury ingredient, which include mainly *T. matsutake, T. magnivelare* y *T. caligatum* (Yang et al. 2008). In 1941, Japan produced 12,000 tons of matsutake, however since then a dramatic decrease has been observed, with a production of 1,000 and only 39 tons in 1970 and 2006, respectively. Due to this dramatic declines, Japan has imported huge amounts of matsutake, which accounted more than 2,800 and 1,700 tons in 2005 and 2006, respectively, from China, North Korea, Canada, United States, Turkey, Mexico, Morocco, Bhutan, Ukraine, and Thailand (Saito and Mitsumata 2008). The wholesale value of this mushroom was USD 425 per kg in Kyoto in 2006 (Saito and Mitsumata 2008), and can reach up to USD 560 per kg, depending on the quality and country of origin (Wang et al. 1997). The annual consumption of matsutake in Japan has been estimated to be in the order of around 3,000 tons (Saito and Mitsumata 2008). Currently, it is not possible to cultivate the matsutake. However, some advances have been carried out, which include the production of mycorrhizal plants in vitro conditions, either as a consequence of inoculation with mycelium or, similarly than in the case of truffles, through the sowing of seedlings on adult colonized trees.

The chanterelle (*C. cibarius* s.l.) is probably the most popular edible wild mushroom in the world, the value of its international market has been estimated annually in more than USD$1.67 billion (Watling 1997). There are specialized companies that commercialized sporomes of chanterelles with specific sizes and particular presentations, either fresh, frozen, or salted, originated from specific areas for example from eastern Europe (Ecolink 2013). Despite the fact that chanterelle has defied cultivation until now, some advances have been produced (Danell and Fries 1990). Danell and Camacho (1997) produced experimentally sporomes of *C. cibarius* in 16 months old inoculated pines (*Pinus sylvestris*) in greenhouse. It has been considered that one of the reason related with the difficulties to cultivate mycelium, and to inoculate afterwards their associated tree hosts, is the abundant presence of bacteria which frequently overgrow the mycelium of chanterelle in culture media and include species of *Pseudomonas, Streptomyces, Xanthomonas*, and *Bacillus*, which population size can be up to 2000 times higher than in other mushrooms such as *Agaricus* (Rangel-Castro et al. 2002; Danell et al. 1993).

Yun and Hall (2004), based on information from Honrubia, pointed out that plantations of the shrub *Helianthemum* inoculated with *Terfezia* in Spain, are able to produce sporomes 2 years after inoculation, with yields ranging from 50 to 170 kg per ha, and in irrigated farms yield increases of up to 300 kg per ha could be achieved. The potential of development of cultivation of desert truffles on semidesert areas has currently a growing interest.

Trees inoculated with other important EEMs have produced sporomes under field conditions including *L. deliciosus* (Guerin-Laguette personal communication), *Suillus luteus* (Chapela et al. 2001), and *Suillus granulatus* (Poitou et al. 1989). Additionally, some advances in the cultivation of *Lyophyllum shimeji* have been carried out (Yamanaka 2008). However, the cultivation of EEMs in commercial plantations, except those of truffles, despite their enormous environmental, social, and economic importance is still in its infancy.

6.5 Advances in Mexico

Currently, one of the main environmental problems in third-world countries, including Mexico, is the high deforestation rate, with the consequent increased greenhouse gas emission and their contribution to global change. For example, in the period from 1990 to 2005 Mexico annually loosed an average of 318,000 ha of forest. This represents one of the highest deforestation rates in the world, surpassed for example from 1990 to 2010 only by Brazil, Indonesia, Sudan, Myanmar, Nigeria, and Tanzania (GFR 2010). As a result, Mexico currently has serious environmental problems in terms of soil erosion and additionally the country is an important emitter of greenhouse gases, ranking 13th worldwide (Myers-Madeira 2008). Reforestation is a complex issue, which includes among other technical limitations in the country, a low survival rate when trees are planted under field conditions. This is particularly dramatic for some forest species like pines (*Pinus* spp.), where the survival transplanting rate ranges from 0 to 20 % in Mexico. One reason which explains this low survival is the lack of ectomycorrhizal symbionts in the roots of most of the forest species produced in greenhouses, whose presence is obligate when they grow under natural conditions. However, inoculation of trees with EEMs has not been traditionally included in plant production in most of the countries. Therefore, the biotechnological development of inoculation of forest trees with ectomycorrhizal fungi is an urgent need in countries like Mexico. A criterion for selection of EEM that has recently gained importance is their edibility (Aggangan et al. 2013), due to their enormous social, economic, and environmental importance. In Mexico for example, more than 400 species of wild edible mushrooms are consumed, and the country is one of the most important cultural and genetic centers of diversity of edible mushrooms worldwide (Pérez-Moreno et al. 2010). Traditionally, organic layers, which include mainly fermentation horizons, A and O soil horizons are used as a source of ectomycorrhizal propagules to inoculate forest trees. However, this methodology has different disadvantages, which include (i) use of great quantities of forest organic material with the subsequent negative environmental and economic impact; (ii) inconsistency in the quality control of the inoculum due to the heterogeneity of distribution of ectomycorrhizal sources of inoculum in natural conditions; and (iii) probability of introduction of propagules of pathogens or weeds in the areas of tree production with their associated risks (Pérez-Moreno 2002). Taking into account this scenario, in the *Colegio de Postgraduados*, Texcoco, Mexico different efforts have been developed during the last two decades, led by the author of this chapter, in order to develop a biotechnology of inoculation of native trees, mainly in the genera *Pinus, Quercus*, and *Abies,* with EEMs.

Till date, some successful results have been generated by inoculating forest trees either with ectomycorrhizal mycelium or spores. Inoculation of strains of the ectomycorrhizal genera *Hebeloma, Suillus,* and *Pisolithus,* have shown to be beneficial on tree growth (García-Rodríguez et al. 2006; Pérez-Moreno et al. 2010). However a much cheaper, simpler, and efficient technology has been developed by using ground pilea of the sporomes containing enormous amounts of ectomycorrhizal spores. Conspicuous root colonization, reaching up to 97 %, has been successfully developed by inoculating ground pilea mainly with different fungal species (Fig. 6.10) included in the edible ectomycorrhizal genera *Laccaria, Hebeloma* and *Suillus* (Carrasco-Hernández et al. 2010; Méndez-Neri et al. 2011; Martínez-Reyes et al. 2012). All of the studied species are widely marketed in

Fig. 6.10 Conspicuous colonization of the roots of a native pine from Mexico (*Pinus montezumae*) inoculated with spores of the edible ectomycorrhizal mushroom *Hebeloma mesophaeum* s.l

central Mexico as human food (Pérez-Moreno et al. 1993, 2008). As a consequence of this root colonization by EEMs, beneficial effects in terms of plant growth and nutrient contents have been recorded in *Pinus hartwegii* Lindl. (Perea-Estrada et al. 2009), *Pinus patula* Schiede ex Schltdl. and Cham, *Pinus pseudostrobus* Lindl. (Carrasco-Hernández et al. 2011) and *Pinus greggii* Engelm. ex Parl. (Méndez-Neri et al. 2011; Martínez-Reyes et al. 2012).

Among the main findings it has been observed that: (i) there is an evident nutrient transfer of N, P, K, Ca, and Mg, to the forest trees inoculated with edible EEMs compared with non-inoculated plants. Of particular importance has been the finding that the external ectomycorrhizal mycelium is able, not only to transfer P and Mg to the roots of the associated trees, but also to the shoots of these plants (Martínez-Reyes et al. 2012); (ii) when multiple simultaneous inoculation, with up to six ectomycorrhizal fungi have been carried out, dominance of one of the inoculated species in terms of root colonizations has been recorded. But the beneficial effects reported to the host trees in these cases are comparable with those observed in plants inoculated exclusively with the dominant ectomycorrhizal

Fig. 6.11 Formation of sporomes of *L. laccata*, an edible mushroom with antioxidant and antitumor properties, in a 1-year-old inoculated Mexican pine (*Pinus montezumae*)

fungal species (Carrasco-Hernández et al. 2011); (iii) some EEMs could be a factor of drought tolerance to the associated hosts more efficiently than others, for example *Hebeloma* species can confer more resistance than *Laccaria* species (Perea-Estrada et al. 2009); and iv) In the last 4 years, it has been possible to record abundant initial production of edible sporomes (Fig. 6.11) of different species of *Laccaria*, including *L. laccata* and *L. bicolor*, and some species of *Hebelom*a, including *Hebeloma mesophaeum* s.l., *Hebeloma leucosarx,* and *H. alpinum* s.l.; currently the ontogeny of the sporomes of some of these species and the factors which influence the formation of these edible ectomycorrhizal sporomes are under study (Martínez-Reyes et al. 2012; Carrasco-Hernández 2011).

In addition to these basic results, it is interesting to note that the inoculation of *P. greggii* with species of *Suillus,* has improved the survival rate up to 57 %, after 1 year of transplanting trees of *P. greggii*, while in the case of non-inoculated plants, this has remained much lower, up to 19 % (Pérez-Moreno et al. 2009). After 7 years, the survival rate of trees inoculated with species of *Suillus* has remained up to 50 % while in the case of non-inoculated plants it has been only of 5 %. These results show that inoculation with EEMs in Mexico is a factor for increase in survival when transplanting trees from the greenhouse to field conditions. Along with this beneficial effects in terms of survival rate, this year (2013) more than 4 kg of *Suillus* were picked associated with the trees of *P. greggii* inoculated with this EEM (Carrasco-Hernández personal communication). Therefore, it is important to note that the development of this biotechnology is not only useful in the production of EEM for commercial purposes, but for the beneficial effects in terms of growth and survival under field conditions of the associated trees, with the consequent capture of greenhouse gases, carbon capture and storage, and potentially ameliorating the effects of global climate change. However, it is important to note that despite the initial successes and seminal research developed in *Colegio de Postgraduados*, there is still a huge challenge related to the biotechnological development of EEM use with forest application and sustainable management in Mexico.

6.6 Conclusions

EEMs represent an important understudied source of novel bioactive compounds useful for improving human health, as medicine or nutraceuticals. Analgesic, antiallergic, anticarcinogenic, antibacterial, anticoagulant, antifungal, antihypertensive, anti-inflammatory, antinociceptive, antioxidant, antipyretic, antivenom, antiviral (including anti-HIV), cholesterol-lowering, hepatoprotective, and immune enhancement properties have been found in more than 100 species of EEMs distributed in more than 35 genera of wild fungi among Ascomycotina and Basidiomycotina. In addition to this importance as medicinal and functional food, this group of mushrooms represents an attractive source of bioactive compounds useful in a more sustainable agriculture, due to the fact that antagonistic effect against pests, plant pathogenic bacteria, fungi, nematodes, or viruses have been detected in more than 50 species in 25 ectomycorrhizal genera. Currently, the ecophysiological function of these bioactive compounds for most of the ectomycorrhizal species, under natural conditions, has not been very well understood. The characterization of the bioactive compounds, from EEMs, with therapeutic effects of human health, and agricultural application is in its infancy. Despite this fact, it is quite clear that the potential characterization and purification of bioactive compounds from EEMs might lead to the formulation of novel therapeutic compounds. Additionally, more basic and applied research related to the massive cultivation of mycelium, or plantations inoculated with EEMs, of a wider range of edible ectomycorrhizal species, where useful bioactive compounds have been detected would be highly desirable to industrially produce these useful bioactive compounds. Advances in the cultivation of EEMs can contribute to multiple problems currently faced by humans, including production of nutritive and healthy food, production of nutraceuticals, contribution to sustainable rural development, and through keeping forests, to contribute to carbon sequestration and therefore to global change.

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Chapter 7
Integral Management of Lignocellulosic Biomass by Biorefining

Sergio de Jesús Romero-Gómez

Abstract The constant rise in oil prices, along with the projected end to the world's oil reserves, forces to find a substitute feedstock for fuel and chemical commodities production. Lignocellulosic biomass is the material that can substitute oil at least partially as it can be produced in high quantities in a sustainable way as a by-product of many agricultural and industrial processes, contains precursors of many chemicals, and can even be converted into fuel by relatively easy processes. Biorefineries are industrial facilities that can transform feedstock into fine chemicals of biofuels. This work reviews the existing advances, especially in the ability to process lignocellulosic material in a fast and economically sustainable manner, and what is necessary to do in several fields to make sustainable biorefining a reality.

Contents

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7.1 Introduction

Constant increase in oil prices, possible depletion of oil reserves, and climate change concerns are the main reasons to search for sustainable oil substitutes. In most parts of the world, oil is the leading source of energy and provides the main part of chemical precursors used to manufacture a large variety of products, from plastics and composites used in almost all aspects of our lives to food and fine chemicals (Howard et al. 2003; Waltz 2008). While energy requirements may be covered at least partially by alternative raw sources as solar, wind power, hydrogen, and tidal energy, chemical commodities require a renewable source of chemical precursors that can be produced in a sustainable way (Lynd et al. 2003).

Lignocellulosic biomass as agriculture, forestry, paper pulp, and food wastes is the best potential alternative source for the production of both biofuels and fine chemicals with the potential to cover our most important needs (Kerr and Service 2005; Kumar et al. 2008). Energy or chemical precursors obtained from lignocellulosic biomass by-products or agricultural waste and forest activities cannot compete with food production, as is the case of biofuel production from sugarcane or maize starch, or even indirectly for crop lands and resources, as is the case of oils used for biodiesel production (US Department of Energy; Turner et al. 2007; Bhatia et al. 2012).

Some crops are more suitable for certain uses than others, for example, banana and pineapple are two fruit crops whose wastes are very suitable for biofuel production, but many more agricultural wastes may be used for the production of fine chemicals or energy, and those potentially useful materials are treated as waste in most countries, increasing the chance of litter accumulation with the potential to produce environmental harm (Isitua and Ibeh 2010; Bahtia and Paliwal 2010; Bahtia et al. 2012).

Lignocellulosic biomass is a heterogeneous material formed mainly by long chains of cellulose; any fiber is made of thousands of beta 1,4 linked glucose residues, every two residues forms a cellobiose-repeating unit. Cellulose chains are produced in tightly packed crystalline and amorphous regions. Smaller molecules of hemicellulose formed by D-mannose, D-galactose, D-xylose, and D-arabinose as well as 4-O-methyl-D-glucoronic acid are scattered in the cellulose fibers; while lignin, a complex three-dimensional structure formed by aromatic monomers forms a matrix around the fibers (Lynd et al. 2002). Cellulose can be hydrolyzed to release D-glucose that can be used to produce biofuels or chemical feedstock by fermentation or chemical transformation. However, the heterogeneous quality of the material, the presence of crystalline insoluble cellule and lignin molecules, makes the degradation of this material difficult (Turner et al. 2007).

Although biomass degradation is an efficient natural process, it is not fast enough to provide energy or chemicals at a sustainable pace. Inspite of pretreatments of chemical and physical nature proposed to disrupt lignin which is the main obstacle in lignocellulose degradation, the degradation of each one of the components of Lignocellulosic biomass remains a slow and expensive process, and many of the monomers released cannot be used efficiently by microorganisms or easily transformed in some other economically interesting molecules. Hence, lignocellulosic biomass conversion to energy and fine chemicals requires intensive research effort if it is to really substitute oil as the main source of energy and precursors to the world. Many advances have to be done in several fields such as lignocellulose pretreatment, microbial strain improvement, enzyme activity modification and enhancement, wastes management, and a new understanding of the whole process.

A promising approach to overcome many limitations in the integral management of lignocellulosic biomass is the productive systems known as biorefineries. A biorefinery is an industrial facility that integrates biomass conversion processes to produce fuels, power, and value-added chemicals from biomass (Kamm and Kamm 2004).

7.2 Lignocellulosic Biomass and Degradation

Lignocellulosic biomass is a heterogeneous material formed by cellulose, that is, a linear polymer formed by D glucose connected by beta-1,4 glycosidic bonds; every two glucose residues are arranged in opposite directions and form a cellobiose unit, and these units are repeated thousand of times creating cellulose chains that are packed by hydrogen bonds to form elementary microfibrils. These microfibrils are attached to each other by hemicelluloses that are heterogeneous polymer of pentoses, hexoses, and sugar acids as pectins, and are covered by lignin, a polymer formed mainly by conferyl, sinapyl, and p-coumaryl alcohols. Lignin gives the material its structural strength and serves as a barrier to the penetration of solutions or enzymes to the interior of the lignocellulose structure; this is the hardest component of the biomass to degrade (Ha et al. 1998; Balan et al. 2009; Hamelinck et al. 2005).

The proposed idea about lignocellulose degradation is that in which cellulose fibers are attacked initially by endoglucanases that break the bonds in the chains at random sites to reveal free and nonfree reducing ends. The ends are then attacked by cellobiohydrolases (exoglucanases) that move along both chain threads releasing cellobiose units. The cellobiose units are hydrolyzed by beta-glucosidases to render glucose; this step is essential to preserve the reaction rate as it avoids the build up of cellobiose that would inhibit the cellobiohydrolases activity. Thus the entire system has to operate in a well-timed paced manner in order to get an effective cellulose degradation process (Lynd et al. 2002). The lignocellulose degradation process is much more complicated in reality, mainly due to presence of lignin that surrounds the cellulose chains and may be the reason why the most effective cellulose degrading organisms possess several enzymes for each activity, for example, *Cellulomonas thermocellum* has at least nine endoglucanases, four exoglucanases, and five hemicellulases (Kumar et al. 2008).

7.3 Lignocellulose Degrading Organisms

Bacteria and fungi are able to degrade the amorphous soluble cellulose in a rapid and efficient way (Lynd et al. 2002), but the ability to degrade crystalline cellulose is restricted to very specialized cellulose degrading microorganisms (Coughlan and Mayer 1992). Cellulose degrading bacteria belong mainly to the anaerobic *Clostridium* genus or aerobic *Cellulomonas* genus of Gram-positive bacteria and some Gram-negative anaerobic *Fibrobacteria* and *Mixobacteria* genus (Glazer and Nikaido 1995).

The best lignocellulose degraders are fungi that belong to soft rot fungi (*Ascomycetes*) and brown or white rot fungi (*Basidiomycetes*) groups. Each group produces a collection of dedicated enzymes for the degradation of specific plant polysaccharides; along with this there are many families of glycoside hydrolases, esterases, and lyases. Many of these families can have multiple catalytic activities, and many of these activities show complementary activity over the same substrates (Dias et al. 2004; Cantarel et al. 2009; Coutinho et al. 2009).

Filamentous fungi that has an ecological niche as saprobes has the enzymatic tools to degrade lignocellulosic biomass but show some specialization; for example, *A. niger* has a large number of enzymes for pectin degradation, while *Phanerochaete chrysosporium* shows a very specialized enzymatic kit for lignin degradation. This specialization can be explored to discover new enzymes with novel activities or more suitable reaction conditions as resistance to higher temperatures or product accumulation, as is required to the efficient degradation of the complex polysaccharides of plant biomass. Degradation of crude biomass is a very complex to achieve, enzyme mix necessary for the complete degradation of plant biomass depends on the type of biomass, growth conditions, and pretreatment used in the said material. In order to improve the efficiency of both lignocellulose degrading system and organisms it is necessary to develop several strategies that go from a better understanding of the lignocellulose degradation to the improvement of known strains or the discovery and use of new lignocellulose degrading organisms (Saha and Cotta 2010; Van den Brink and de Vries 2011).

7.4 Pretreatment of Lignocellulose Material

To overcome the resistance of crystalline cellulose to degradation, many treatment processes have been developed. These processes may include exposition to physical, chemical, and thermal treatments (Martínez et al. 2005). Even when all treatments are effective to degrade cellulose in some grade, the use of those pretreatments may represent a major ecological inconvenience and can increase degradation price to make it economically unsustainable (Kumar et al. 2008).

7.4.1 Physical Pretreatments

Physical pretreatments are based mainly on reducing particle size of lignocellulosic material to increase interchange surface area and availability of the fibers to enzyme degradation; this can be accomplished using different technology levels from milling, micromilling, or ball milling to steam or ammonia explosion. Steam explosion in one of the most commonly used pretreatment techniques; in this treatment, material is subjected to high pressure and temperature for a short period of time after which it is rapidly depressurized causing an explosion of the fibers of cellulose. The main disadvantage of this treatment is the need to use diluted acids to increase yields, which causes the accumulation of by-products that can inhibit the culture of many organisms; factors as pressure, residence time, and temperature have been studied and recent studies indicate that longer residence time at lower temperatures are better for lignocellulose degradation (McMillan 1994; Duff and Murray 1996; Wright 1998; Bhatia et al. 2012). Another interesting treatment is ammonia fiber explosion where biomass is treated with liquid ammonia at high pressure and after a short residence time it is rapidly depressurized. The temperature used for this treatment is about 60 °C lower than the temperature of near to 160 °C necessary for steam explosion; this results in a much lower energy expenditure for the process. This process has advantages such as lower moisture content, less sugar degradation, and there is no loss of biomass. The main disadvantage is higher cost compared to other treatments (Mosier et al. 2005; Chundawat et al. 2007).

7.4.2 Chemical Treatment

Chemical treatment can be divided into alkaline or acid treatments; in alkaline treatment, basic salts as sodium, potassium, or ammonium hydroxide are used to cause the degradation of glycosidic side chains, cellulose swelling, partial decrystallization of cellulose, and partial solvation of hemicelluloses, an advantage of this treatment is alkaline treatment can be performed at lower temperatures and bases can be used in a much more diluted concentration than acids, but much longer residence times are required. Normally this kind of treatment results in the complete removal of lignin, improving the reactivity of cellulose and hemicellulose in further degradation processes (McIntosh and Vancov 2010; Bhatia et al. 2012). Acid treatment uses diluted or concentrated acids to break lignocellulosic material; the most commonly used acid is sulfuric acid that has demonstrated to be effective in the treatment of many different materials. Acids can be used in a concentrated or diluted form, and though both treatments are effective, they have different drawbacks. Concentrated acids require large quantities of acid and may cause the production inhibitors of further degradation, while the use of diluted acids requires a much longer residence time and higher temperatures to be effective, and can also result in the formation of inhibitory by-products. The main advantage of acid treatments is that it can render fermentable sugars that require no further treatment to be used (Martínez et al. 2005; Zhu et al. 2009).

7.4.3 Solvents

Ionic liquids are salts composed of a large organic cationic part along a small anion that remain liquid at room temperature and have a very low vapor pressure. These liquids can be adjusted to dissolve cellulose directly from different plant biomass sources (Swatloski et al. 2002). Binder and Raines (2009) reported the direct production of 5-hydroxymethylfurfural (HMF) from untreated corn stover using *N*,*N*-dimethylacetamide (DMA) containing lithium chloride (LiCl) as solvent, this allows the synthesis of HMF in a single step with a yield between 32 and 47 % against 50 % obtained from pure cellulose; conversion of cellulose into HMF was not affected by lignin or protein content. HMF is a six-carbon molecule that can be used as an analog of terephthalic acid and hexamethylenediamine and therefore can be converted into an assortment of acids, aldehydes, alcohols, and amines, as well as 2,5-dimethylfuran (DMF), a chemical with potential as gasoline substitute. The process reported is fast and simple and, more important, it allows the use of raw material to produce fuel and chemicals in simple steps. A common limitation to ionic liquid treatment is cellulose inactivation; the necessary use of high-pressure equipment and the high cost of ionic liquids even when near to 99 % of it can be recovered by evaporation due to low vapor pressure (Perepelkin 2007).

7.4.4 Biological Delignification

Fungi degrade lignin through a family of extracellular enzymes collectively called lignases. These can be divided into two main families, laccases (phenol oxidases) and peroxidases (lignin and manganese peroxidases), but it is known that there are many other enzymes whose role in lignin degradation has not been clarified. In biological treatment, microorganisms and their enzymes are used for the selective delignification of lignocellulose materials; this treatment has high yields with low cost, low energy requirements, and generates nonpolluting by-products; on the other hand, biodelignification takes much longer than chemical or thermal processes, usually 8–12 weeks (Yu et al. 2010). Even when white, brown, and soft rot fungi have been used to degrade lignocellulose, white rot fungi remains the most effective for delignification of lignocellulosic materials, and it has been demonstrated that white rot fungi are able to selectively degrade lignin in wheat straw and are good choices for delignification of lignocellulosic residues (Fan et al. 1987; Arora and Chander 2002; Yu et al. 2010; Sánchez 2011). Lignin degrading enzymes have been extensively expressed in homologous organisms, most in filamentous fungi with variable results; a special case is the expression of laccase gene from *Cyathus bulleri* that has been expressed in *E. coli* with very good results making it the first laccase successfully expressed in a bacterial host (Salony et al. 2006).

7.5 Solid-State Fermentation

Solid-state fermentation (SSF) is the growing of microorganisms over solid substrate in near absence of liquid free water; the water necessary for microbial growth is present in the culture absorbed into substrate or associated to other components of the culture medium. SSF is a good system for fungi cultivation as culture conditions are similar to the conditions that fungi meet in the natural habitat. As a result, fungi produce higher enzyme and protein yields, and can even synthetize some metabolites that are produced in very low yields or are not produced at all in submerged fermentation (Raimbault 1998; Viniegra-González et al. 2003). SSF is a practical treatment for lignocellulose degradation from complex substrates as agricultural, forestry and food-processing wastes, and SSF may be used with fast growing fungi capable to degrade lignin in an exclusive way, so it may be possible to have a lignin-degrading system that may leave cellulose and hemicellulose ready for further use or engineered microbes can be used to perform lignocellulose degradation and compound production at the same time. If that is accomplished, then SSF can be much more efficient than the typical two steps of lignocellulose degradation and fermentation normally planned for biorefining, as in SSF enzymes activities produced, are coordinated for the degrading substrate which results in both less enzyme requirement and shorter process times. There is an increase in hydrolysis rate by conversion of sugars that can inhibit enzymatic activity and less water content results in smaller volume reactors and less contamination chances (Sun and Cheng 2002; Malherbe and Cloete 2003). Problems associated with SSF are due to its nature; heat build up, heterogeneity, possible contamination, difficult scaling-up, biomass growth estimation, but these have not avoided the production of many enzymes and microbial products. Solving these problems may prove difficult as many of the said problems are responsible for the advantages, for example, formation of gradients is given as the cause for heterogeneity along the culture which has been pointed as responsible for higher enzyme production, and the low content of water that promotes heat build up keeps enzymes from being diluted. Working in the engineering aspects and the scale-up will require further research on bioreactor design and operation (Lonsane et al. 1992).

7.6 Improving Systems

7.6.1 Co-Cultivation

The conversion of lignocellulosic material in precursor products requires the synergic action of different enzymes and all the known microbial species show deficiencies in some or several of the enzymatic activities required to degrade this material, for example, as discussed previously, *A. niger* is a good degrader of pectic materials, while *Trichoderma reesei* is a good producer of cellulose degrading enzymes. This has given rise to the idea of a co-cultivation of both organisms for the production of lignocellulose degradation enzymes used in the treatment of cellulose waste (Maheshwari et al. 1994). Kumar and Singh (2001) reported the co-cultivation of *Aspergillus niger* and *Trichoderma reesei* for the degradation of aquatic weed. In that work, they reported an increase of 20–24 % in endo and exoglucanase activities and about a 13 % increase in the beta-glycosidase activity compared to the maximum enzymatic activities under single culture conditions.

7.6.2 Mutagenesis

It is known that production of biomass degrading enzymes is controlled by induction, catabolite repression, and end-product inhibition, so it is normal that at the beginning, search for hyper-producing lignocellulose degrading organisms was attempted by random mutagenesis looking for improvements in cellulase production and mutants insensible to catabolite repression. Successful cases of higher productivity of cellulases producing organisms obtained by mutagenesis are quite rare, a 4x higher production of cellulose was obtained by mutagenesis of a *Bacillus* strain free of catabolite repression (Kotchoni et al. 2003). While Kuhad et al. (1994) reported a *Fusarium oxysporum* cellulose hyperproducer mutant obtained by UV treatment followed by chemical mutagenesis with nitrosoguanidine showed an increase of 80 % in cellulose production (Kuhad et al. 1994), many *Trichoderma reesei* mutants used for commercial production of cellulose are derived from the same wild strain QM6A isolated for the USA army laboratory (Esterbauer et al. 1991). In general, random mutagenesis has been incapable of bringing overproducer lignocellulose degrading organisms perhaps because cellulose degradation is a very coordinated genetic and biochemical process.

7.6.3 Molecular Biology

Although some progress has been made using random mutagenesis methods, molecular biology along with protein engineering techniques seems more suitable to allow the organized improvement in enzyme production, and enzyme activity along with the pH and temperature resistance necessary to get a real increase in lignocellulotic degradation needed to reach sustainable process from lignocellulosic biomass. It is possible to improve genes by directed mutagenesis, get catalytic sites with a better substrate access, simpler protein folding patters, or synthesize new enzymes genes engineered for a given substrate by computer assisted design. In silico experiments will take relevance to test any enzyme in simulated environments in order to improve its characteristics before using it in real life. Another interesting possibility is multifunctional enzymes, in which several different or even sequential enzymatic activities can be integrated in a single protein molecule; there are many interesting reported cases as a bifunctional exoglucanase–endoglucanase (Warren et al. 1986), a chimeric endoglucanases–xylanase (Tomme et al. 1994), and special mention is deserved for the endoglucanase, cellobiohidrolase, and xylanase activity enzymes reported by Aylward et al. (1999). Another interesting enhancement is the improved thermostability of recombinant manganese peroxidase by engineering of disulfide bridge stability (Reading and Aust 2000).

Combination of genetic and recombinant DNA has allowed the construction of improved strains as *T. reesie* RUT-P 37 that has double cellulase specific activity compared to QM6 the original wild strain (Montenecourt et al. 1983) or *T. reesei* CL 847 strain resistant to catabolite repression that has a fourfold increase in cellulose productivity compared to wild strain. (Durand and Clanet 1988). It is necessary to include all techniques in a coordinated way to overcome the bounds to lignocellulose degradation that is a result of the complex structural nature of biomass.

7.7 Biorefineries

7.7.1 Introduction

Oil has been the main source of energy and fine chemicals of the world for the last 70 years, but prices are increasing due to the higher demand, depletion of reserves, and political instability. The use of oil is related to pollution and climate change, so there is a movement toward the substitution of oil for renewable sources, but the actual production of biofuels from sugarcane, vegetable oils, and maize starch creates a competition with food production both by diverting food grade materials or by absorbing crop land, and is considered to be an indirect cause for the rise in food prices (Sachs 2008). Therefore, the best candidate to substitute oil is lignocellulosic biomass, as it is renewable, cheap, readily available, and is produced in high quantities as a by-product of forest, agriculture activities, and even some industries (U.S. Department of Energy; Turner et al. 2007).

Lignocellulosic biomass is the most abundant source of biomass in the world and is readily available as it is obtained as a by-product of any agriculture or timber activity and even some industrial processes such as paper recycling render various forms of lignocellulosic biomass as a waste. FAO estimates the production of 2.9×10^6 tons of lignocellulosic wastes just from cereal crops and 1.08×10^8 metric tons of lignocellulosic waste from sugarcane; moreover, in some products such as palm oil and coffee, the waste generated is higher at 90 % of the total biomass produced. Hence lignocellulosic biomass is practically an inexhaustible source of energy and precursors (FAOSTATS 2011).

7.7.2 Biorefineries

A biorefinery is an industrial facility capable to use the activity of microbial cells to transform biomass into chemicals, commodities, and fuels. In order to make this a promising idea achieving success is necessary to change the production systems and even complete economies from oil-based materials to biological products in a gradual approach. Meanwhile, bigger and better biorefineries have to be developed and established; this requires a new approach in which biology, chemistry physics, and engineering will have to work together to develop new technologies (National Research Council 2000). Moreover, to make the substitution of oil as a source of energy and chemical precursors in the world a reality, it will be a necessary much more than creating biorefineries, to process fast and efficiently very high quantities of biomass. The main goal for biorefineries is the ability to receive several biomass sources and implement several processes that confluence in a mixture of very well-defined products (Turner et al. 2007; Kumar et al. 2008; Bhatia and Paliwal 2010).

The United States Department of Energy has published a list of valuable products based on petrochemical refineries, but biorefineries have to develop a product assortment including not only petroleum-based products but a new variety of products not available from oil refineries (Kamm and Kamm 2004; National research council 2000). These refineries are yet to be developed. Actually there are Phase I refineries where food grade grains are converted into ethanol in a fixed processing system, however, they have no flexibility and cannot be used for other purposes; Phase II refineries use feedstock grain and are capable to produce diverse end-products according to demand, as bioplastics, glucose, oil, and ethanol (Nonato et al. 2001). A Phase III biorefinery must be able to produce several end-products on demand, but much more important, it must be able to process several different feedstocks predominantly lignocellulose wastes of many sources (Kamm and Kamm 2004).

The complications that hinder the successful achievement of this idea are presented in the following example. In the initial process to obtain ethanol from cellulose biomass, a lignin disrupted biomass is treated with fungal cellulases to release glucose. The glucose is then fermented conventionally by *Saccharomyces cerevisiae* to yield ethanol (Lynd et al. 2002); this process is complex and presents several problems, the most important of which is the inhibition of cellulose degradation by accumulation of end-products. To overcome these problems, simultaneous saccharification and fermentation has been developed. In this process, cellulose degrading enzymes are added to the fermenting bioreactor, so glucose is consumed as fast as it is produced. This improves the process in two ways; first, avoiding cellulose degrading end-product inhibition, and at the same time catalytic rate of all enzymes is improved as there is no buildup of by-products on the media, but as a result other challenges arise in the new process such as the accumulation of pentoses released in the culture media that wild yeast is incapable to use as fermentable sugars from these two solutions has been presented a recombinant *S. cerevisiae* able to use pentoses and *Klebsiella* strain that is naturally able to use xylose and arabinose developed as a high yield alcohol producer (Zhou et al. 2001; Hughes et al. 2009).

This example illustrates two additional points, an organism able to degrade lignocellulosic biomass may be unable to grow in an efficient well-known production system or produce a valuable molecule in a cost effective process. On the other hand it is possible to have a very good producing organism incapable to use lignocellulosic degradation material proficiently. Therefore, the requirement to find what French (2009) calls the Ideal Biofuel Producing Microorganism (IBPM) is according to this a suitable organism must be able to: (1) hydrolyze cellulosic material effectively with minimal preprocessing, (2) it must be able to convert sugar released by lignocellulosic degradation into molecules useful as fuels, or chemical industry feedstocks, (3) it must be able to produce those molecules at a high concentration without poisoning itself, and (4) it must be capable of rapid growth in a bioreactor and suitable to be used in an industrial context (French 2009).

New technologies are making possible the discovery and development of improved enzymes, with novel enzymatic activities, multiple catalytic sites, and better suited to act in the harsh environments of industrial process. Searching for new enzymes has never been so widespread both in new environments and in so many previously unexplored new species, including noncultivable or symbiotic organisms. Many of the these findings can be finally be exploited thanks to new technologies that allow the creation of designed/engineered enzymes isolated from never seen microorganisms and adaptation of microorganisms that can cope with the requirements of new process in biorefineries still to be conceived (Turner et al. 2007; Bastien et al. 2013; Lee et al. 2013; Weiman et al. Weimann et al. 2013).

Synthetic Biology
In the search for the IBPM, many reports described the search for organisms with a vigorous growth and the ability of expression of cellulases to degrade biomass in an effective way (Lynd et al. 2002, 2005); while the degradation of amorfous cellulose has been attained, degradation of crystalline cellulose as sole carbon source remains elusive for recombinant organisms. A recurrent problem is the low expression level of the recombinant cellulases along the low specificity of cellulases caused by the heterogeneity of the lignocellulosic biomass (Lynd et al. 2005), this combination turns into a very low yield of ATP for the organism and therefore the failure of a vigorous growth (van Walsum and Lynd 1998). It is clear that many issues have to be solved in order to develop an efficient IBPM; here synthetic biology can play a very important role.

7.7.3 Synthetic Biology

Synthetic biology intents the creation of novel genes and organisms using synthetic DNA and despite being relatively new, it has proved to be a practical and useful method to resolve biotechnological problems (Na et al. 2010). Synthetic biology is based on the use of modular pieces of DNA in which modular components are synthesized to order and are used for the expression of any known or designed gene sequence. This allows the use of new coding sequences obtained from massive sequencing or metagenomic studies along with designed or engineered gene sequences among libraries of promoters, enhancers, and secretion signals to determine by experimentation which combination gives the best results for a given culture condition or substrate combination (Prather and Martin 2008). Synthetic biology has contributed to the expansion of hosting strains making feasible the heterologous production of several proteins and metabolites from host organisms. Moreover, improved metabolic chains can be produced by the use of concatenated active sites where each enzymatic reaction happens on the same protein structure. Thus each reaction proceeds faster along the enzymatic support, and biological circuits that react to the microenvironment in culture can be integrated inside the cells, so that they can adapt to the changing conditions inside the bioreactors in a smart way (Dueber et al. 2009; Lu et al. 2009).

Use of synthetic biology is focused on the construction of biosynthetic paths for the production of nonnatural chemicals in cells and modification of genetic expression. In this case, the synthetic genes are assembled in a host organism suitable for industrial production of the said chemical, but a more used approach is adaptation of enzymes by modifying substrate specificity in a given metabolic path to promote the formation or consumption of a metabolite of interest. One of the most demonstrative works in the development of an L-homoalanine producing strain developed by introducing a synthetic pathway with a modified glutamate dehydrogenase, this enzyme has been modified to convert 2 ketobutyrate into the nonnatural amino acid L-homoalanine (Leonard et al. 2010; Zhang et al. 2010).

Synthetic biology makes feasible the expression of several cellulases, lipases, and esterases that can be regulated in a coordinated way in any recombinant well-known organism as *Saccharomyces* o *E. coli*. This approach is very similar to nature, where a single organism expresses several combinations of cellulases that wok in a synergic system. Production of several enzymes normally results in a much higher level of degrading activity. The modular nature of synthetic biology allows the combinatorial assay of enzyme genes of different origin and activity, which can then be screened against several lignocellulose substrates for the most effective degradation ability for a given substrate. These experiments can be used to generate databases of heterologous gene degrading abilities against any given substrate (Fench 2009). Decreasing costs of synthetic DNA results in a cheaper and simpler way to assemble complex multigene systems. This flexibility has the potential to allow the generation of useful organisms from different origins to obtain an IBPM, thus making the conversion of biomass into chemicals or biofuels a reality.

7.7.4 Conversion of Lignocellulosic Biomass into Value-Added Products

Conversion of biomass into valuable products may begin with biofuels as ethanol, biodiesel methane, and hydrogen. All these molecules already have a large market since they are used as chemical feedstock and are used extensively by Brazil and USA as primary fuel or additive (Kerr and Service 2005). Production of ethanol from food grade materials is an expensive substitute for oil-based fuels, and one of the most urgent issues that need to be addressed by new technologies and production from lignocellulose wastes is the best alternative (Kumar et al. 2008).

Methane and hydrogen as biofuels exemplify the best opportunity to show how a integrated process may work, both molecules can be produced as a by-product of cellulose waste or municipal sewage water treatment. There are advances in production of methane from a wide range of lignocellulose biomass. Maheshwari et al. (2000) and Wang et al. (2011) demonstrate the feasibility of production of hydrogen in an integrated process that includes lignocellulose degradation and hydrogen production by microbial fuel cells.

Linocellulose biomass is a good consistent material for hydrogen production. In an integrated process pretreated lignocellulose biomass as barley malt by-product may be used to produce hydrogen by bacterial fermentation in fuel cell or can be co-produced along with other amino products to yield electricity when used with sewage sludge fermentation and fuel cell fermentation (Kapdan and Kargi 2006; Ni et al. 2006). In an advanced biorefinery, it may be suitable to use any cellulose or municipal wastes and use them to produce methane or hydrogen with a high yield.

Degradation and conversion of lignocellulosic biomass may used for the production of many organic molecules of economic value; biomass-released sugars can be fermented to fuels and commodity chemicals by the appropriate microbes both natural and engineered (Elmekawy et al. 2013). It is reported that primary base chemicals ethylene, propylene, benzene, toluene, and xylene can be used to synthesize more than 75 % of organic chemicals (Morris and Ahmed 1992). Vanillin and gallic acids are two interesting monomers for the pharmaceutical industry that can be obtained from lignocellulose biomass. Vanillin is used as a flavoring molecule in the food industry and has antimicrobial and antioxidant properties and used as an intermediate in the production of herbicides, drugs, and household products (Davidson and Naidu 2000; Walton et al. 2003). Vanillin is synthesized from ferulic acid that can be released from corn cob alkaline treatment (Torres et al. 2009). Hemicellulose is an interesting part of plant biomass and is a good source of xylose that can be converted into xylitol and furfural. Xylitol has properties as teeth hardener, remineralizer agent, and food sweetener, so it is used in toothpaste and chewing gum industries (Roberto et al. 2003), and furfural is used in the production of furfural phenol plastics and pesticides (Montane et al. 2002; Rahman et al. 2007). Lignin can represent up to 35 % of lignocellulos biomass and is formed by guayacil, syringyl, and coumaryl alcohols that can be separated by solvent dissolution in alkali or organosolv and the insoluble lignin can be separated by acid hydrolysis of cellulose and hemicellulose and composting. Together, these treatments release high quantities of highly valuable sulfur-free phenolic groups (Argyropoulos and Menachem 1997; Bridgwater 2004; Buranov and Mazza 2008).

7.8 Conclusion

Many advances have been made in lignocellulose degradation and many more are needed before an economy based in this material substitute oil-based economy. Biorefineries must be able to use almost any feedstock to produce almost any material the society demands. A big part of that technology does not exist currently, but needs to be developed before lignocellulose can be managed to supply our needs of fuels and chemicals.

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Chapter 8
Microalgae and Cyanobacteria Production for Feed and Food Supplements

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Abstract Sustainable alternative sources of protein and lipids have become a priority due to asymptotic limitations of production of traditional crops under the burden of exponential population growth, climate change, and energy resource limits. Microalgae and cyanobacteria contain substances that have high biological value, such as polyunsaturated fatty acids, proteins, pigments, antioxidants, vitamins and minerals, as well as carbohydrates. Moreover, they have higher productivity than traditional crops and can be grown in places where other crops cannot be grown, such as deserts or high-salinity environments. Microalgae in the past have been used in the diet of humans and animals but mainly as supplements. Biotechnological use of the entire biomass or protein/lipids of microalgae for feedstock in human and animal consumption was reviewed. It was found that microalgae have great potential to replace standard food crops for animal and human consumption, and ongoing biotechnological advances in cultivation techniques and process technology will likely place new microalgae biomass products in the world commercial stage in the near future.

Keywords Microalgae · Cyanobacteria · Food · Protein · Lipids · Human consumption · Animal consumption

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Contents

8.1 Introduction

Globally, the demand for proteins and lipids is rising due to an exponentially growing population; however, production with traditional crops has been lagging with only limited production improvements possible in the near horizon. Therefore, it is becoming crucial to find sustainable alternative sources of proteins and lipids. The energy and food market demands not only large quantities but high-quality products. Presently, the major sources for lipids and proteins are palm oil and soybeans and soybean cake (FAO 2012). These sources are not particularly attractive in terms of sustainability since they lead to deforestation in the region of origin (Lima et al. 2011; Wicke et al. 2011), thus further increasing production of these crops is highly undesirable. Even alternative sources of vegetable oils or proteins derived from traditional terrestrial crops, e.g., maize, sugarcane, rapeseed, has reached maximum peak landmass utilization areas while also contributing to water scarcity, forest devastation, as well as imposing price pressures on the foodmarket (Timilsina et al. 2012).

Ever since the publication of a future protein gap in the 1930s there has been an ongoing search for alternative protein sources for human consumption. Microalgae has been considered since then as a possible candidate of protein and lipids, but over the years popularity has fluctuated due to technical difficulties and productivity gains with traditional crops. However, in the last decade, microalgae have garnered renewed interest as an alternative sustainable food or protein and lipid source. This increase in popularity has been caused by the worldwide decrease in available petroleum, thereby driving price increases of virtually all commodities, decreases in arable land due to sodic conditions, climate changes, and productivity limits being reached for traditional crops.

Microalgae and cyanobacteria are particularly attractive as possible food sources for animal and human consumption since they grow fast, can use nonarable land, brackish, marine or wastewater, and the quality of the protein is of equal or better quality than standard vegetable or even some meat-based proteins. In comparison to traditional crops, microalgae have a much higher productivity per unit area and therefore use less landmass to produce similar quantities (Mata et al. 2010). Moreover, proteins and lipids are the largest fractions in most microalgae. Proteins can be used as a food, feed, health, and bulk chemical source. Lipids can be used as a source for biofuels, chemicals, and edible oils. Moreover, microalgae fabricate many other nutritional products such as vitamins, lipids, carbohydrates, pigments, and minerals.

Algae, microalgae, and cyanobacteria have been documented to be used in animal and human diets since very early times in at least Mexico and China. Despite various successes, the most widespread production of microalgal culture has been as a higher value health food (*Spirulina* powder, and *β*-carotene derived from *Dunaliella*) for human consumption, and in artificial food chains providing crucial feedstock for aquaculture husbandry of crustaceans, mollusks, and finfish. Hence to date, other than in aquaculture and in certain stages of animal development, microalgae have not been used as a complete biomass replacement alternative in human food or animal feedstock. The purpose of this chapter is to review the state of the art of the technology and to the explore the knowledge gaps necessary to close in order to use microalgae biomass or its primary constituents of proteins/lipids for animal and human consumption.

8.2 Microalgae for Consumption

8.2.1 Nutritional Value

Proteins and edible oils have additional functional properties that need to be considered when evaluating as a food source such as taste, structure, stability, and toxicity levels. These properties as well as their nutritional value should be evaluated when considering microalgae as an alternative food source.

In terms of proteins, the type and quantity of amino acids are the determining quality factors for use as a staple food product for human and animal consumption. It is interesting to note that there are about 500 amino acids identified, but only 20 amino acids—just 4 % of the known amino acids, are necessary for human growth and nutrition. Of these 20 amino acids, 8 are considered essential. Humans and animals must consume these essential amino acids in their diet in sufficient quantities in order to maintain homeostasis, growth, and good nutrition. For animal feedstock, soya and maize are mostly used and these crops do contain the complete range of amino acids, however not always in the right ratio. Experiments adding specific amino acids, such as methionine and lysine with broiler chickens, have

Table 8.1 Typical amino acid profile of different microalgae as compared with conventional protein sources and the WHO/FAO (1973) (g per 100 protein)

Source	Ile	Leu	Val	Lys	Phe	Tyr	Met	Cys	Try	Thr	Ala	Arg	Asp	Glu	Gly	His	Pro	Ser
WHO/FAO	4.0	7.0	5.0	5.5	6.0	–	3.5	1.0										
Egg	6.6	8.8	7.2	5.3	5.8	4.2	3.2	2.3	1.7	5.0	–	6.2	11.0	12.6	4.2	2.4	4.2	6.9
Soybean	5.3	7.7	5.3	6.4	5.0	3.7	1.3	1.9	1.4	4.0	5.0	7.4	1.3	19.0	4.5	2.6	5.3	5.8
Maize[a]	3.7	13.6	4.3	2.1	5.3	3.5	1.7	1.2	0.4	3.1	10.8	3.6	5.8	12.4	2.8	2.0	8.3	5.0
Chlorella vulgaris	3.8	8.8	5.5	8.4	5.0	3.4	2.2	1.4	2.1	4.8	7.9	6.4	9.0	11.6	5.8	2.0	4.8	4.1
Dunaliella bardawil	4.2	11.0	5.8	7.0	5.8	3.7	2.3	1.2	0.7	5.4	7.3	7.3	10.4	12.7	5.5	1.8	3.3	4.6
Scenedesmus obliquus	3.6	7.3	6.0	5.6	4.8	3.2	1.5	0.6	0.3	5.1	9.0	7.1	8.4	10.7	7.1	2.1	3.9	3.8
Arthrospira maxima	6.0	8.0	6.5	4.6	4.9	3.9	1.4	0.4	1.4	4.6	6.8	6.5	8.6	12.6	4.8	1.8	3.9	4.2
Arthrospira platensis	6.7	9.8	7.1	4.8	5.3	5.3	2.5	0.9	0.3	6.2	9.5	7.3	11.8	10.3	5.7	2.2	4.2	5.1
Aphanizomenon sp.	2.9	5.2	3.2	3.5	2.5	–	0.7	0.2	0.7	3.3	4.7	3.8	4.7	7.8	2.9	0.9	2.9	2.9

Sources [a] Maize amino acid profile data from Mertz (1970). Rest of data from Becker (2007) been found to increase growth rate (Lima et al. 2008); however, adding these additional specific amino acid supplements is usually costly. Different types of microalgae contain different amounts of the essential amino acids and, therefore, it is desirable to select the right species for specific applications that lower costs in animal production. Comparing amino acid composition among microalgae species, soya, and maize indicates that microalgae can be satisfactory as a protein source for animal and humans (Table 8.1).

For most food applications, oil present as triacylglycerides (TAGs) is preferred. Fortunately in microalgae, fatty acids are primarily stored as TAGs which increase in quantity depending on stress conditions (Hu et al. 2008). When microalgae are placed under a variety of stress conditions, e.g., nutrient depletion, pH changes, high salinity, accumulation of fatty acids found in TAGs reached levels of 50 % of the total biomass (Hu et al. 2008). Moreover, many microalgae contain the two essential fatty acids, EPA (C20:5) and DHA (C22:6), and other $\omega 3$ fatty acids that most other crops do not contain. EPA and DHA fatty acids are necessary for correct functioning of human and animal metabolisms and with synthesized routes nonexistent, consumption is required from other food sources in order to obtain these essential fatty acids. Palm oil is used extensively as a food source and comparing the fatty acid profile of different species of microalgae with palm oil shows that microalgae contain the same fatty acids as palm oil and even have a more diverse range of fatty acids (Table 8.2; Vanthoor-Koopmans et al. 2013).

8.2.2 Current Commercial Use of Microalgae

Several microalgal species are used nowadays commercially, in this group the green algae Chlorophyceae *Chlorella vulgaris*, *Haematococcus pluvialis, Dunaliella salina*, Haptophyceae *Isochrysis galbana*, and the cyanobacteria *Arthrospira maxima*, *Arthrospira platensis, Spirulina subsalsa* stand out. These species are mainly used as nutritional supplements for humans (Fig. 8.1), as animal feed additives, and as neutraceuticals (Gouveia et al. 2008). Until now only high-value products are made using microalgae.

Some species of the genus *Chlorella*, and particularly *C. vulgaris*, has been used as alternative medicine since ancient times and it is known for being a traditional food in the Orient. It is widely produced and marketed as a food supplement in many countries, including China, Japan, the United States, and Europe. *Chlorella* is considered as a potential source of a wide spectrum of nutrients, carotenoids, vitamins, minerals. Yamaguchi (1997) reports its importance as a health promoting factor in many kinds of disorders such as gastric ulcers, wounds, constipation, anemia, hypertension, diabetes, and infant malnutrition. In relation to chemical composition *Chlorella* contains about 50 % protein (Lee 1995; Becker 1994); however; the most important substance in *Chlorella* is β-1,3-glucan, which is an active immunostimulator, a free-radical scavenger, and a reducer of blood lipids (Spolaore et al. 2006; Gouveia et al. 2008).

Table 8.2 Comparison of the major FA composition of different species of microalgae against data published previously and data from the Microalgal Biotechnology Laboratory at CIBNOR

Species	Percentage of fatty acids of the total													
	14:0	16:0	16:1	16:2	16:3	16:4	18:0	18:1	18:2	18:3	18:4	20:4	20:5	22:6
Nannochloris sp.[a]	1.8	15.1	16.6		0.2		1.0	57.7	0.6	0.8	0.3	5.9		
Phaeodactylum[b]	9.2	26.8	45.4				0.7	4.6					12.3	1.1
Nostoc commune[c]	0.3	43.5	11.3	0.4			1.5	6.9	19.3	16.3				
Pavlova lutheri[a]	11.8	23.6	28.3				2.0	12.4					12.1	9.2
Arthrospira maxima[d]	0.5	30.9	10.0				1.5	3.6	21.7	29.3	0.1			
Arthrospira platensis[d]	0.2	48.7	7.5					4.4	21.2	13.9	0.1			

[a] Viso et al. (1993)
[b] Tonon et al. (2002)
[c] Temina et al. (2007)
[d] Unpublished data from the Microalgal Biotechnology Laboratory at CIBNOR

Fig. 8.1 Nutritional supplements for humans

H. pluvialis can accumulate the highest level of astaxanthin in nature (1.5–3.0 % dry weight). The carotenoid pigment is a potent radical scavenger and singlet oxygen quencher, and surpasses the antioxidant benefits of β-carotene, vitamin C, and vitamin E. *Haematococcus* is currently the prime natural source of this pigment for commercial exploitation, particularly in aquaculture salmon and trout farming (Lorenz and Cysewski 2000). In fact, there are extensive culture zones in the north of Chile, near Atacama desert. Production is basically directed to human consumption.

D. salina is a halotolerant microalga which can be found in the sea, salty lakes, and salt reservoirs. This species is able to accumulate large amounts of glycerol and β-carotene, both with high value in the chemical industry. β-catorene is a valuable chemical mainly used as natural food coloring and provitamin A (retinol). In culture conditions, some strains of *Dunaliella* can contain up to 10 % and great amounts of β-carotene under stress conditions (Ben-Amotz and Avron 1980; Oren 2005).

The marine microalga *I. galbana* is considered an important source for food production, a valuable source of polyunsaturated fatty acids (LC-PUFA), pigments, and other nutraceuticals (Bandarra et al. 2003), mainly eicosapentaenoic acid (EPA, 20:5ω3) and also docosahexaenoic acid (DHA, 22:6ω3) that are accumulated as oil droplets in the cell (Liu and Lin 2001). These microalgae have been used as a feed species for commercial rearing of many aquatic animals, vertebrate and invertebrate, particularly larval and juvenile mollusks (Fig. 8.2), crustaceans, and fish species (Fidalgo et al. 1998), as well as an alternative source for human feeding.

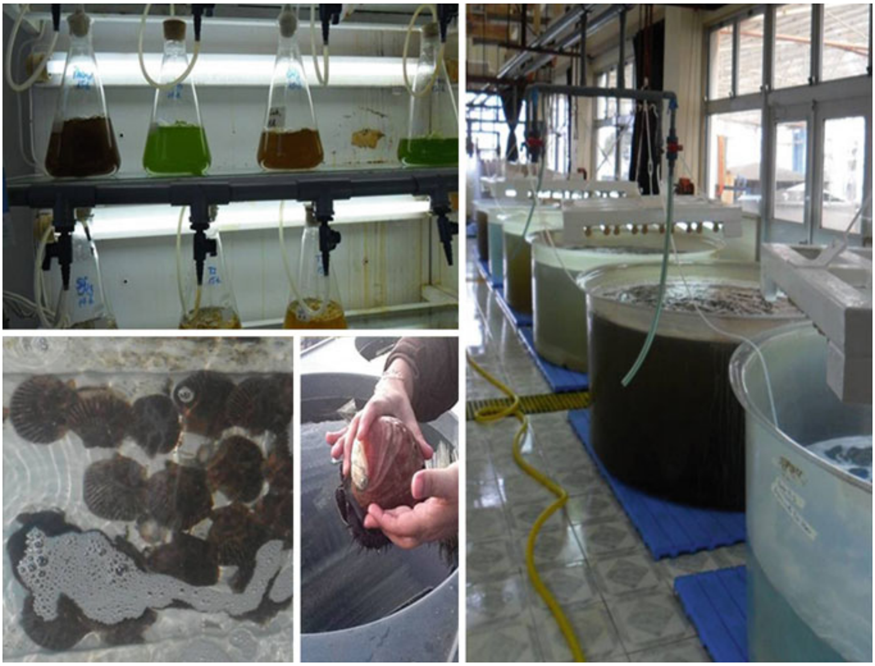

Fig. 8.2 Microalgae cultures as food for larval and juvenile mollusks. (Aquaculture Center of the North Catholic University of Chile and Company producing of mollusks-IV Coquimbo Region-Chile)

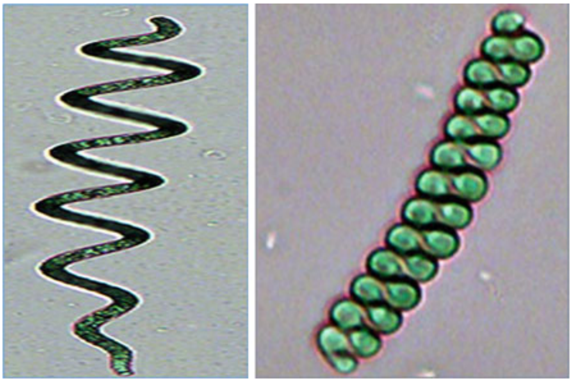

Fig. 8.3 *Arthrospira maxima* (*left*) and *Spirulina subsalsa* (*right*). Fotos: Laboratorio Biotecnología de Microalgas del CIBNOR, La Paz, Baja California Sur, México

Two of the most interesting and studied cyanobacteria are *Arthrospira* and *Spirulina* (Fig. 8.3). They contain high amounts of protein. Particularly, *Arthospira* reach 50–70 % protein in its composition and a low-nucleic acid content (4 %), fatty polyunsaturated acids (1.5–2 %), lipids (5–6 %) as well as thiamine (B1),

Fig. 8.4 *Arthrospira maxima* cultures in Ecuador. **a** Pilot plant in Nayon, Ecuador, to produce the inoculum. **b** Inoculum of *A. maxima*. **c** Rustic ponds in construction

nicotinamide/niacin (B3), pirodoxin (B6), folic acid (B9), cyanocolamin (B12), vitamins C, D, and E. Some pigments found in *Arthrospira* are chlorophyll *a*, xanthophyll,
β-carotene, myxoxanthophyll, zeaxanthin, canthaxanthin, 3-hydroxy echinenone, β-cryptoxanthin, among others, as well as phycobiliproteins, c-phycocyanin, and allophycocyanin (Vonshak 1997; Phang et al. 2000; Basurto-Peña 2009).

Arthrospira is a well-known cyanobacterium that was consumed long back in Chad Lake in Africa and by the Aztecs in Central Mexico (Fig. 8.5). The Aztecs picked it from Texcoco Lake to use it for cooking "tamales," a little corn cake enveloped with corn leaves or as food additive. The Aztecs kept this tradition until the sixteenth century (Farrer 1966; González 1987; Basurto-Peña 2009). Actually, *Arthrospira* is produced in many countries such as Japan, USA, Italy, India, China, Chile, Thailand, Singapore, where it is used as a food supplement (Basurto-Peña 2009). Ecuador has set up a governmental program dedicated to the rustic *Arthrospira* cultures developed by poor people for self-consumption using indigenous and resistant strains (Fig. 8.4).

8.2.3 Microalgae for Human Consumption

It was not until the 1950s that microalgal biotechnology began to emerge as a viable technology; since then it has had numerous successful commercial applications as mentioned above. Microalgal products are now used to enhance the nutritional value of human food owing to their chemical composition. However, microalgae to date are still not a major food group replacement. This is mostly due to its green color and fishy smell of the microalgae powder. Nowadays, more effort is placed in searching for new ways for using microalgae in prepared foods like bread, soup, noodles, and more, in order to gain public interest and acceptance for human use.

Feeding microalgae to humans as a major dietary item has been a virtually unexplored field of investigation. However, there have been limited studies carried out in the past with prisoners. Taste experiments have been conducted where they found that consumers accept green algae powder in their food when it was hidden in already green foods like spinach noodles and mint ice cream (Morimura and Tamiya 1954). Earlier workers (Hayami and Shino 1958) have reported the "true digestibility" of decolorized *Chlorella* to be 83.0 %. They also reported that the residual smell and taste of the decolorized *Chlorella* were intolerable and that it impaired appetite (Hayami et al. 1961). In later studies, "true digestibilities" of 75 % for blanched microalgae and 86 % for methanol extracted microalgae were reported. In these two studies, approximately 30 g of microalgae were fed per day. This supplied about one-third of the total protein. Only one study (Powell et al. 1961) reports feeding amounts of microalgae in excess of 35 g (dry weight) per day. Subjects tolerated amounts of up to 100 g/day quite well, but acute toxicity symptoms developed at 200 and 500 g levels.

8.2.4 Microalgae for Animal Consumption

Microalgae can be used to enhance the nutritional value of animal feed, due to their well-balanced chemical composition. In addition, microalgae can be incorporated into the feed for a wide variety of animals ranging from fish (aquaculture) to pets and farm animals (Spolaore et al. 2006). In fact, 30 % of the current world algal production is sold as animal feed applications (Becker 2004). Microalgae are utilized in aquaculture as live feeds for all growth stages of bivalve molluscs, juvenile fish, crustaceans, and for zooplankton used in aquaculture food chains. Several microalgae species have been tested as feed, but probably less than 20 have gained widespread use in aquaculture. Microalgae as feed source must possess a number of key attributes to be useful, mainly: (1) an appropriate size for ingestion, (2) readily digestible, (3) rapid growth rates, (4) high tolerance to environmental conditions, and (5) good quality as a nutrient (no toxins).

The survival, growth, development, productivity, and fertility of animals are a reflection of their health. Feed quality is the most important exogenous factor influencing animal health, especially in connection with intensive breeding conditions and the recent trend to avoid "chemicals," like antibiotics. Today, there is evidence that very small amounts of microalgal biomass, almost exclusively of the genera *Chlorella*, *Scenedesmus*, and *Spirulina*, can positively affect the physiology of animals. In particular, a nonspecific immune response and a boosting of the immune system of the animals was observed (Richmond 2004). Microalgae or its products have recently been used as probiotics in the aquaculture industry in order to increase production. The research on probiotics for aquatic animals is increasing with the demand for environment friendly aquaculture. A wide range of microalgae have been evaluated, although the mode of action of the probiotics is rarely investigated, but possibilities include competitive exclusion, i.e., the probiotics actively inhibit the colonization of potential pathogens in the digestive tract by antibiosis or by competition for nutrients or space, alteration of microbial metabolism, and by the stimulation of host immunity. Probiotics may stimulate appetite and improve nutrition by the production of vitamins, detoxification of compounds in the diet, and by the breakdown of indigestible components. On the other hand, microalgae could be necessary to improve quality or growth characteristics in aquaculture. For example, copepods are recognized as excellent feeds for fish larvae, but they have proven difficult to produce in intensive systems due to the lack of feed (microalgae), so it is necessary to know the biology of microalgae species, which could improve their production rates and support fish production in a friendly manner.

8.3 Microalgae Selection

For the successful set up of a microalgal culture, it is necessary to select appropriate strains taking into account goals and available resources. First of all it is necessary to keep in mind for what purpose the production will be used; microalgae have been used for food, feed, biofuels, vitamins, pigments, or a combination of these. Different species contain different amounts of the various possible products. Another important factor for selection is growth rate of the microalgae under the circumstances available in order to get sufficient production. The following considerations need to be taken into account:

Diurnal temperature fluctuations Working with large-scale microalgae cultures requires care with temperature fluctuations. Temperature fluctuation can decrease culture production efficiency, or may increase production depending on the species (Renaud et al. 2002). In order to solve this undesirable situation on growth culture, there are two options; first consider strains that are adapted to wide temperature fluctuations (Vonshak 1987; Becker 1994; Abalde et al. 1995). The second option is temperature control to prevent large fluctuations.

Photoinhibition Light is a necessary factor for microalgae, however, it can also be a limiting factor. Often, the upper layers in a microalgae culture are exposed to a high irradiance so that cell photo-oxidation can occur resulting in a state of nongrowth, known as photoinhibition. Selection of a light resistant strain will provide generally higher yields or production microalgae cultures. To prevent high irradiance in the upper layers of the microalgae culture mixing is an important culture parameter. Good mixing can prevent photo inhibition due to lower average light intensities per cell.

Dark respiration It is known that dark respiration can be responsible for more than 35 % of biomass loss produced during luminous daytime (Vonshak and Richmond 1988). It is desirable to use low dark respiration rate strains for large-scale cultures (Vonshak 1987; Becker 1994; Abalde et al. 1995).

High O_2 concentration High oxygen concentration can be deleterious for the photosynthesis process. The carbon intake decreases when oxygen is highly concentrated in a culture, sometimes leading to cell death and total collapse of the culture (Becker 1994; Albalde et al. 1995). It is interesting to note that even in this area research is needed since heated debates are ongoing on how microalgae die, either by apoptosis or necrosis, or some other mechanism(s) (Jiménez et al. 2009). A good solution for maintaining the culture alive and healthy is by mixing thus homogenizing nutrients and reducing high oxygen gradients. It is also generally desirable to choose a high oxygen-tolerant strain.

Osmotic stress In open pond large-scale cultures (See Chap. 4 for further explanation) high evaporation rates occur (more than 10 L m^{-2} d^{-1}) (Albalde et al. 1995). Addition of new nutritive medium can cause an increase in salts in the system and microalgae can undergo osmotic stress with deleterious results. It is recommendable to select salt-resistant strains. Some microalgae species prosper in a natural way in mixohaline environments; these are a good alternative since they are already adapted to these conditions.

Culture media It is desirable that the selected strain is not a highly selective organism in relation to its metabolic necessities, so it can possibly be fed by a wide variety of alternative media like plant fertilizers, agricultural fertilizers, worm slurry, or modified commercial media in order to get an economic system.

Competitiveness The selected strain preferably should be highly competitive if an open production system is used in order to avoid contamination. Ecological displacement is a common problem in outdoor cultures at large scale.

Indigenous strains An indigenous strain is an alga population occurring naturally near the place of the culture installation. Indigenous strains are already adapted to the particular local environmental and fluctuations. This provides a serendipitous ecological benefit since the species is adapted to survive in its niche.

Co-products Although one of the most important goals is to obtain biomass, a good strategy is also to obtain high-value biological co-products like polyunsaturated fatty acids, steroids, pigments, carotenoids, antioxidants, antibiotics, antifouling substances, among several others. The production of these substances, besides bulk production of proteins, can make the culture system that is economically feasible or more profitable.

A good strain selection is a key process for microalgae culture success. The strains that have been collected and isolated from extreme natural environments are the ones that will give us better results to isolate and grow them in mass cultures. Depending on the culture system chosen to culture the microalgae more or less control of the environmental conditions is possible. When culture conditions can be well controlled the selected microalgae will be less vulnerable to changes. Moreover, in controlled environments product quality can be guaranteed better. However, every manner of control has its price. It is preferable to optimize production by limiting the number of artificial controls, thereby reducing production costs.

8.4 Production Systems

Another factor that has effected the mainstream adoption of microalgae as a possible food staple or as a biofuel is the cost of production. Microalgae are expensive to produce, although efforts are under way addressing cost-efficient mass cultivation of these organisms. Presently, many different systems for microalgal biomass production have been evaluated (Richmond 2004; Tredici 2004; Carvalho et al. 2006; Ugwu et al. 2008). All the cultivation systems must take into account (1) light utilization, (2) CO_2/O_2 balance and gas exchange, (3) temperature, (4) pH, (5) sterility, and perhaps most important for evaluating the economic feasibility of a cultivation system, (6) the net energy ratio. Net energy ratio (*NER*) of a system is defined as the ratio of the total energy produced (E_p) (which is the energy content of biomass, in some papers just lipids are considered since it is convertible to biodiesel) over the total energy required for cultivation (E_c), occasionally also referred to energy return on energy invested (EROI).

$$NER = EROI = E_p / E_c \tag{8.1}$$

Some definitions in the literature also include summing the energy expenditures of materials and construction with energy of cultivation (E_c); however, this energy expenditure can be amortized over the life span of system and hence is variable. Leaving this out provides a result that is more direct, indicating how much biomass energy is acquired versus the energy expended to create it. As a reference, petroleum NER is today about 10 (Gupta and Hall 2011) although in the 1970s it was believed to be around 100, and is widely considered to be one of the driving forces of economic growth. For any cultivation production system to be economically favorable NER must be greater than one, although for food production this is not necessarily a barrier condition as for biofuel production. Present schemes for microalgae cultivation can be generally classified as open, closed, and semi-closed. For tubular bioreactors systems, apparently NER is < 1 and slightly higher than one for flat panel and open raceway pond systems (Jorquera et al. 2010). In the future more sophisticated designs will be needed to reduce costs.

Fig. 8.5 Cultures in open pond *Haematococcus pluvialis* in the Atacama Desert, Chile

8.4.1 Open System

Currently, most industrial microalgal cultivation systems are open ponds (Fig. 8.5). These systems are favored for their low capital and operative costs. Nevertheless, one of their major disadvantages is the lack of control on environmental conditions and therefore low biomass productivity. Furthermore, difficulties in species control tends to limit open ponds to microalgal cultivation in highly selective environments such as high salinity and extreme pH conditions that exclude contaminating organisms (Suh and Lee 2003). In general these systems are limited to use microalgae strains that are extremophiles that have the ability to withstand hostile growing conditions such as *Chlorella*, *Spirulina* or *Dunaliella*. This type of system requires the microalgae to outcompete other microorganisms in this culture medium since it is directly exposed to the environment. Not only do these systems limit which microalgae strains can be grown, but growth is also limited to the surface of ponds resulting in low biomass concentration.

Within the open systems, the best choice seems to be the open shallow pond made of leveled raceways 2–10 m wide and 10–50 cm deep at most to allow suitable illumination (Jorquera et al. 2010); running as simple loops they use low-energy consuming paddlewheels for gas/liquid mixing and circulation. Systems cover areas of hundreds to a few thousand square meters. Flow rate is approximately 0.2–0.5 m s^{-1}. In many cases, in these systems acetate is generally added continuously or semi-continuously (dripping) to promote the microalgal production (Lee 1997).

These systems are typically used in open commercial systems for the cultivation of microalgae and cyanobacteria, such as *Chlorella* sp., *Nannochloropsis, Arthrospira platensis, D. salina, Anabaena* sp., *Phaeodactylum tricornutum,* and *Pleurochrysis carterae,* among others, (Moheimani and Borowitzka 2006; Pushparaj et al. 1997;

Moreno et al. 2003; Jiménez et al. 2003; Garcia-Gonzalez et al. 2003; Richmond 1992; Richmond and Cheng-Wu 2001; Laws et al. 1983; Radmann et al. 2007).

8.4.2 Closed System

The more recently developed and technologically advanced closed systems provide better choices for growing almost every microalgal strain, since the culture is protected from invasion of contaminating organisms and hence extremophile microalgae are unnecessary unlike open systems.

Many different PBRs have been developed in the last three decades and different designs were recently reviewed by Wang et al. (2012). Photobioreactors are closed systems that can be designed in a variety of configurations, e.g., tubular and flat-plate reactors. They have been used in the cultivation of *Chlorella sp., Porphyridium cruentum, P. tricornutum, A. platensis, Nannochloropsis* sp., *Chlorella sorokiniana, H. pluvialis,* and *Tetraselmis suecica*, among others (Fuentes et al. 1999; Acién et al. 2001; Tredici and Zitelli 1988; Chini Zittelli et al. 2006; Ugwu et al. 2002; García-Malea et al. 2006a, b; Cheng-Wu et al. 2001; Degen et al. 2001; Gouveia and Oliveira 2009; Rodolfi et al. 2009; Yoo et al. 2010).

Rigid vertical column or tubular photoreactors are usually cylinders with heights of up to 4 m and radii of 0.2 m and smaller, to allow for light penetration to increase surface–volume ratio. The height limit is related to the gas transfer limitations and the strength of materials used to build the columns. In high columns, varying CO_2 gradients may be established which limit CO_2 to some of the microalgae, thereby creating pH gradients (Xu et al. 2009). Furthermore, increased column length increases O_2 concentration in the photoreactor due to photosynthesis, which can reach a level that can inhibit growth. Vertical column PBRs are characterized by their high volumetric gas transfer coefficients. Gas is usually bubbled from the bottom of the column and usually enables optimal O_2 removal as well as effectual CO_2 utilization.

Two other types PBRs used are vertical flat panels and horizontal tubular reactors. The vertical flat panels have advantages of higher luminosity of column reactors since they are typically thinner than column reactors, thus decreasing light path. While horizontal column PBRs, as the name implies, are usually placed horizontally close to the ground. This is done to attain uniform solar radiation along the entire columns. However, generally the vertical column PBR is preferred because productivity per unit area of ground is greater by placing the photobioreactors vertically (Cuaresma et al. 2011).

In most photoreactors microalgal growth is limited by light penetration as the microalgae culture grows, due to self-shading. In comparison to open pond, generally photobioreactors sustain higher volumetric productivity than open ponds, with better capture of radiant energy, more optimal use of the cultivation area, and variable energy consumption values for mixing and gas/liquid mass transfer (depending on the type of photobioreactor).

Nevertheless, photoreactors require much more energy for mixing typical values of 55 W m^{-3} for flat plates and up to 2,000–3,000 W m^{-3} for horizontal tubular reactors have been reported (Carvalho et al. 2006; Sierra et al. 2008). Raceway ponds are made of less expensive materials, their construction involves lower costs, and they require less energy for mixing, of the order of only 3–4 W m^{-3}.

8.4.3 Semi-Closed Systems

These systems are similar to open system but are covered by a physical structure to protect culture from environmental changes. For example, in China most production systems have adopted a semi-closed culture system, where raceway culture ponds are covered by glass houses or transparent plastic sheets, thereby protecting culture from frequent summer rains, storms, and/or cold winters. Although investment costs increase, this system allows for year-round production.

8.5 Microalgae Harvest

Microalgae cultures have enormous water quantities, it is necessary to remove this water. There are chemical, physical, and biological processes available to do this (Fig. 8.6). Mainly, these processes are reduced to separate the solid and liquid components. According to Molina-Grima et al. (2003), the harvesting process is energy-dependent and represents the main percentage of total production cost (20–30 %) being still considered as a major limiting factor in this activity. The harvest is not always easy, mostly due to the cell size of most microalgae, which makes it necessary to use large forces for separation. However, it is possible to develop an economic and efficient harvest method according to microalgae specie.

The most common harvesting methods are gravity sedimentation, centrifugation, filtration and microscreening, ultrafiltration, flotation, sometimes with an additional flocculation step or with a combination of flocculation–flotation, and electrolytic separation techniques (Uduman et al. 2010; Gouveia 2011). The selection of harvesting method is dependent on the properties of microalgae, such as density, size, and value of the desired products (Brennan and Owende 2010).

A clear example of lower cost harvesting are the cyanobacteria of *Arthrospira* and *Spirulina* genera that thrive forming floccules that build thick floating layers; this situation makes the harvest easy using filtration methods. This is a rapid and energy efficient way of getting biomass (Benneman and Oswald 1996). A different situation is related to the chlorophyta *H. pluvialis* that presents the tendency to sinking through water column when the agitation process is interrupted; this makes harvesting easy by collecting the cells from the bottom.

The process of harvest can be divided into two steps (Brennan and Owende 2010): (1) Bulk harvesting, separate microalgal biomass from the bulk suspension. By this method, the total solid matter can reach 2–7 % using flocculation, flotation,

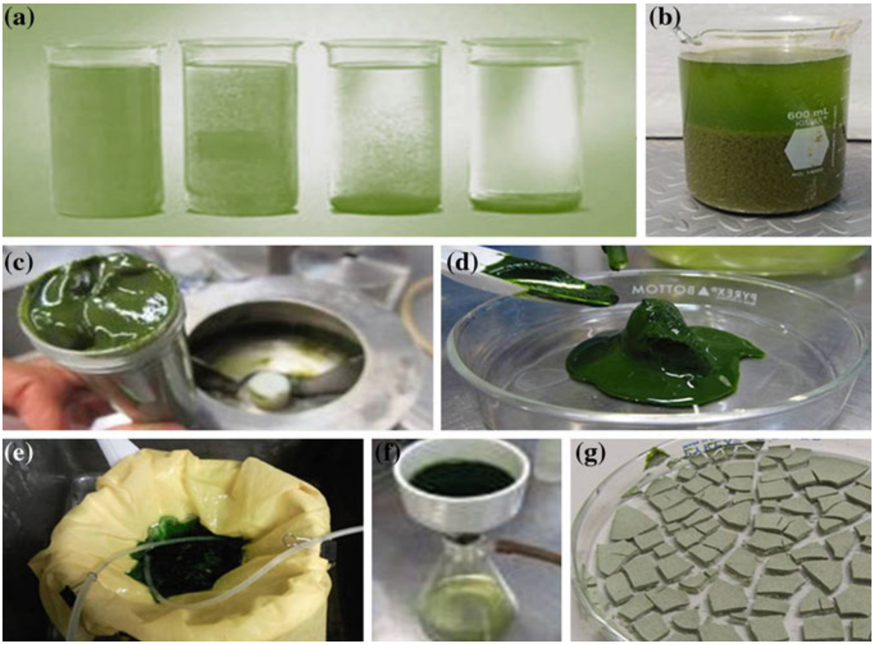

Fig. 8.6 Harvesting microalgal biomass. **a** Flocculation, **b** autoflocculation of the species *Desmodesmus quadricauda*, **c** biomass harvested by continuous flow centrifugation, **d** biomass centrifuged, **e** passive filtration with screen meshes of the cyanobacterium *Arthrospira maxima*, **f** vacuum pump filtration using Millipore filters, **g** dry biomass

or gravity sedimentation. (2) Thickening, concentration of the slurry using filtration and centrifugation. This step needs more energy than bulk harvesting. The possible techniques for harvesting are discussed below.

Centrifugation Centrifugation is a common method for microalgae harvest in laboratories, however, it is recommended principally for small volumes because of its high energy demand. The energy input alone has been estimated at 3,000 kW/ton (Benemann and Oswald 1996). However, this technique is very efficient as a secondary harvesting method to concentrate initial slurry to an algal paste (Gouveia 2011).

Gravity Sedimentation Sedimentation is a simple process, commonly applied for separating microalgae. Density and size of microalgae cells determine the sedimentation velocity, but mostly this process is slow (0.1–2.6 m/h) and in high-temperature environments, the biomass could be deteriorated (Gouveia 2011).

Flocculation and Autoflocculation Coagulation and flocculation are used to increase sedimentation velocity of cells. Microalgae cells are stabilized by negative electric charges on its surface; this condition drives back the surrounding neighbor particles in a similar way as two magnetic poles with the same charge. In this situation bigger masses called floccules are formed. These masses do not sink. Coagulation destabilizes floccules by neutralization of separating forces. This goal is achieved by adding chemical coagulating substances and energy to the system. The most common coagulating substances are aluminum and iron salts. Recent research has shown that pH alterations can increase the flocculation process speed (Kemmer and McCallion 1995), other investigations have worked on cationic starch obtaining good results with freshwater species but not with marine species (Vandamme et al. 2009, 2011).

Nevertheless, not all microalgae species need a flocculant agent, some can form floccules in a natural and spontaneous way, others form floccules under nitrogen stress, pH, and dissolved oxygen. This is known as autoflocculation (Gouveia 2011).

Filtration Is a method applied successfully in the laboratory, but on a major scale some problems that appear are membrane clogging, formation of compressible filter cakes, and in particular high maintenance costs. On a large scale, the energy consumption of the filtering process is equivalent to the centrifugation process so this is only recommended for filamentous microalgae or colony forming microalgae (Molina-Grima et al. 2003; Gouveia 2011).

Two of the primary screening devices in microalgae harvesting are microstrainer and vibrating screen filters, excellent due to their mechanical simplicity and availability in large unit sizes. Microstrainers are rotative filters with a very thin net submitted to frequent washings in order to avoid clogging of the filter. Microstrainers are simple, economic, easy-to-handle devices. However, for small-sized microalgae flocculation is recommended before microstraining (Molina-Grima et al. 2003; Gouveia 2011). Some variables involved in the process like type of filtering membrane can increase costs but can optimize the process.

Flotation The process of flotation is a gravity separation in which air or gas bubbles are attached to solid particles and then carry them to the liquid surface. It is a commonly used approach to remove microalgae from reservoir water prior to its use as drinking water. The water is first ozonated, after which the sensitized cells are treated with about 10 ppm polyelectrolyte salts, prior to being subjected to flotation. Flotation is more beneficial and effective than sedimentation with regard to harvesting microalgae (Chen et al. 1998).

Electrolytic separation An electric field drives charged microalgae to move out of the solution. Water electrolysis generates hydrogen that adheres to the microalgal floccules and carries them to the surface. The mechanism involves consecutive stages: Generation of coagulants by electrolytic oxidation of the electrode, destabilization of particulate suspension, and breaking of emulsion and aggregation of the destabilized to form floccules (Gouveia 2011).

Microwave-synthesized magnetic microparticles Prochazkova et al. (2013) report some interesting results related to a microwave system where a low-cost oxidized iron suspension is used to harvest *C. vulgaris*. The separation efficiency was tested against several variables as culture media, pH, among others. This study shows that iron oxide amount, pH level, and ions present in the culture medium are important elements for good harvesting results. Phosphorous ions were identified as the element that interferes with iron oxide during the separation of magnetized cells in the system.

The efficiency of harvesting *C. vulgaris* with iron oxide magnetic microparticles is comparable with other magnetic microalgae separation procedures. From the highly diluted culture broth, thick-magnetized microalgal slurry was obtained within minutes, while a complete demagnetization of cells was achieved within 1 h, if necessary. Consequently, the separation efficiencies depend upon culture medium composition and microalgae species (Prochazkova et al. 2013).

Ultrasound This separation process is based on gentle acoustically induced aggregation followed by enhanced sedimentation. Testing the microalgae *Monodus subterraneus*, Bosma et al. (2003) got a harvest efficiency of more than 92 %. On the industrial scale, centrifuges can be better used to harvest microalgae because of lower power consumption, better efficiency, and higher concentration factors. In laboratory or pilot plant scale, the ultrasonic harvesting process has the advantages of being operated continuously, evokes no shear stress, and the occupation space is very small. Also, it never gets blocked with cells; cells are still viable, because no shear stress is involved. This means the harvested biomass can be used as inoculums or can still be investigated by analytical techniques. When an organism excretes a high valuable secondary metabolite, this technique can be used as a retention system. The resonation chamber acts as a biological filter by rejecting the organisms and allowing the solubilized product to pass. Higher biomass concentrations can be reached inside the bioreactor and the concentration of the desired product will rise (Bosma et al. 2003).

8.6 Future Prospects

Microalgae are an excellent biological resource and one of the most promising sources for new products and applications (Pulz and Gross 2004). They can be used to enhance the nutritional value of food and animal feed due to their well-balanced chemical composition. Microalgae cultures are better in relation to agricultural crops as they do not need enormous extensions of fertile ground, present low dependence of climatic swaying, are a good possibility for biofuels production, are a good atmospheric carbon tramp, and are good alternatives for high-value biological co-products.

Although work with microalgae and production systems has increased and is running successfully in many parts of the world, especially in Asia, further research work is needed to close the gap between possible low-cost bulk production like food, feed, or biofuels using microalgae. Till now, only high-value products are produced using microalgae. Partly due to this, development in low-cost production systems is slow. For producing high-value products there is no need to lower production costs significantly. However, to really take advantage of all benefits that algae have it is necessary to create lower cost production plants and also be able to obtain all available products. Lower cost production can be achieved by producing microalgae in controlled environments where production efficiency can be increased and less man-hours are needed; higher efficiency will mean also lower harvesting costs. Moreover, lower investment costs in bioreactor material will be necessary and the use of renewable energy can help to lower energy usage and costs.

As mentioned above, microalgae contain a broad range of products, and it would benefit the economical cost calculation marvellously when more or all of these possible products can be obtained. To be able to do this, new techniques for extraction are necessary. Currently, the focus has been on obtaining a single product, therefore the extraction techniques used were only focused on this one product and hence damaging the other possible products inside the cell. Development is necessary in the field of biorefinery, for which a proposition for a biorefinery process is made based on mild techniques (Vanthoor-Koopmans et al. 2013).

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Chapter 9
Aquatic Biosystems: Applications in Aquacultural Engineering as a Sustainable Technology

Genaro M. Soto-Zarazúa, J. Fernando García-Trejo, Manuel Toledano-Ayala and Edgar Rivas-Araiza

Abstract Aquaculture has been supporting human demands for fish products for centuries and is an important industry worldwide. Global production from aquaculture has been increasing steadily, having more than doubled in the last decade; aquaculture now supplies one-third of seafood consumed worldwide. However, the expansion of aquaculture has been accompanied by degradation of the natural environment, especially on marine aquaculture. Direct impacts of fisheries and aquaculture are habitat modification, collection of wild seedstock, changes of food webs, introduction of nonnative fish species, and diseases that harm wild fish populations, and nutrient pollution. According to the FAO, major issues that need to be addressed are problems with access to proper technology and financial resources, together with environmental impacts and diseases. Some others argue that further increases in aquaculture production will come mainly from further investment in biotechnology or nanotechnology ranging from protein expression and DNA vaccines, water filtration and remediation, nanoparticles, gene delivery (and chips) to transgenic technologies. The purpose of this chapter is to present the progress in the research about to get a sustainable aquaculture.

Keywords Aquaculture technology · Sustainable aquaculture · Aquatic biosystem

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Contents

9.1 How the Aquaculture Could Be Sustainable

Aquaculture is an activity practiced by Chinese people since 2,800 B.C. The first culture species were the carp (*Cyprinus carpio*) and mullet (*Mugil cephalus*), the first documented knowledge about fish culture in captivity belong to the carp. This activity has been supporting human demands for fish products for centuries and now is an important worldwide industry. Over the years, this practice has become more technical with the objective to make the work easily but mainly in order to increase production. Nowadays this activity has grown to an entire industry that handles both supplements as the product itself. Global production from aquaculture now supplies one-third of seafood consumed worldwide. With this massive increase in world production, the current aquaculture industry is one of the fastest growing sectors in food production (FAO 2011). However, this rapid growth in the aquaculture industry has generated ecological damage due to a huge extractive use of water, land, and feeds. Besides, it is important to consider that these requirements are associated to another impacts such as polluting, salinization of soils, nutrient-loading, clearing of natural habitat, overexploit of ground water reserves, introduction, and transmission of diseases (Pillay 2004). At this respect, Bailey (1985) establishes a new term "blue revolution," which describes the expansion of fish farming in tropical regions, according to this idea aquaculture must provide huge quantities of fish and help to solving problems of world food security and alleviating poverty. However, production increase in aquaculture demands feeds, energy for the cultured species and almost always is obtained through catch, so the fastest growing sectors in food production threatens its ability to continue to provide increasing yields in a sustainable manner, and concerns with the resulting from fish farming have led to calls for the "greening of the blue revolution" (Costa-Pierce 2002).

The situation in México is not an exception; the expansion of aquaculture has been accompanied by degradation of the natural environment, especially on marine aquaculture (Saborio Coze and Flores Nava 2009). As it happens in the world directly impacts of fisheries and aquaculture are:

- Introduction of nonnative fish species
- Introduction of exogenous parasites
- Nutrient pollution
- Habitat modification
- Overcollection of wild seed stock
- Changes of food webs
- Increase of interspecific competition.

It is clear that current food productions techniques in aquaculture are good just under financial point of view but always leave aside the environmental aspects. The relationship between aquaculture and environment is complex specifically the biodiversity topic. Many examples of positive and negative impacts have been documented, however until now there is no solution which allows the development of a relationship between food production and the environment. This solution must be adopting a new paradigm based on ecological concepts of extreme resource efficiency and the closing of nutrient and waste cycles, resource-use optimization (García-Trejo and Soto-Zarazúa 2012). As can be seen, this is not an easy task since it requires the creation of multidisciplinary teams which can see the problem holistically and try to give a solution that benefits all parties involved in the process.

9.2 Aquatic Biosystems from a Biology View Point

The outlook for food production and its consumption, in the early twenty-first century, is looming unsustainable, due to the overpopulation (Ehrlich and Ehrlich 1990). The current human population already exceeds 7,000 million, with an annual growth rate of 77 million per year, it is estimated that by 2025 will reach 8,000 million (CONAPO 2010). Nowadays food production cannot be clearly estimated because of variation on weather conditions. This can be added to the lack of knowledge, unsuitable technology, and lack of fertile land. Study on the effects of limiting factors for production process and weather conditions is already started (Parry et al. 2004). The problem is complex and is not an easy task; every effort should be in the same way in order to reach the minimum knowledge about production in this century. Scientific society has been trying to form multidisciplinary groups that address the problem from different perspectives. This is the case of the Cuerpo Academico de Ingenieria de Biosistemas (CAIB), which is a multidisciplinary group of engineers from areas such as; chemistry, biology, molecular biology, control and automation, plant physiology, and agronomy. This group has generated a novel working model, based on transdisciplinarity, Fig. 9.1.

The main objective is to solve three questions:

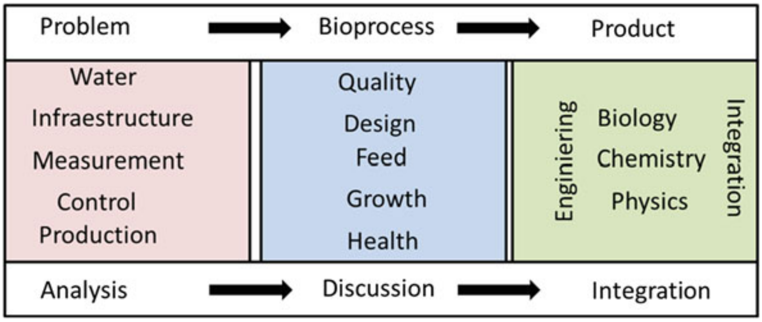

Fig. 9.1 Transdisciplinarity working model to integrate different areas in order to establish research on sustainable aquaculture

1. How basic needs, aspirations, and desires of today's society without compromising to future generations can be satisfied?
2. How a healthy environment, physically attractive and biologically productive, can be maintained perpetually?
3. How can we get the balance among social sustainability, environmental, and economic?

This chapter shows a perspective and progress on aquatic biosystems, its strategy to get the knowledge in order to achieve sustainable technologies.

9.3 A Definition to the Aquatic Biosystems

The definition of aquatic biosytem is critical to understand its scope and function. The word comes from the Greek *bios* meaning life and therefore refers to everything that has life. The word "system" comes from the Greek "syn" meaning together, and "histemi" which means to establish. So the definition of "biosystem" is understood as the analysis, design, and control of an entity or biological process which can be oriented to production, processing materials, and food in a sustainable manner in order to improve human health and environment. The integration of the aquatic environment restricts the entity or biological process to all that live in the water.

The study of aquatic biosystems has presented an incredible development in recent years, mainly because its areas aim to achieve optimal use of water resources. This area traditionally had its scientific basis in aquaculture. Considering this way of working, the group has defined as a priority the production of aquatic organisms in a way that is cost-effective and environmentally-friendly. The topics that have been addressed concerning aquatic production are:

9.4 Aquaculture Wastewater Recycling

Aquaculture activity requires huge quantities of water with an adequate physicochemical quality (Alatorre-Jácome et al. 2011). Research in Amazcala campus, from the Universidad Autónoma de Querétaro, shows that is possible to have fish production with a low waste of water at least with Tilapia (Soto-Zarazúa et al. 2010). To solve water quality problems, various systems for replacement, filtration and recirculation of water have been proposed in various scientific studies (Piedrahita 2003; Shnel et al. 2002; van Rijn et al. 2006). In these systems, mechanical and biological clarification and ultraviolet sterilization processes were applied in different configurations to keep the water properties within the acceptable ranges for fish survival and growth. Regarding dissolved oxygen, some controllers have been developed (Bergheim et al. 2005). On the other hand, fish behavior has been analyzed to evaluate the optimal ranges for fish production (Xu et al. 2006). A recirculation system has been tested in order to probe the time for water exchange. Results showed that the system allows keeping recirculation water during 4 months with desirable physicochemical parameters. This fact represents a significant saving for farmers and environment. Even more, when the water is delivering from the system could be used in other systems such as aquaponics, microalgae culture, lombriculture among others (Rico-García et al. 2009).

9.5 Control and Environment for Aquaculture

In recent years, consumers around the world have increased the consumption of fish due to the recognition of their nutritional value (Tingman et al. 2010); for this reason, intensive aquaculture aims to increase fish production capacity per cubic meter of water. However, when fish densities are large, the influence of temperature, dissolved oxygen and water quality on fish survival, and growing rates increases. Aquaculture facilities therefore require an automated method of keeping these parameters within acceptable ranges to optimize their efficiency by reducing labor and operational costs (Avnimelech 2006); when these parameters are outside of the optimal ranges, stress and slow fish growth is fostered, which has a direct effect on quality, quantity, and harvest time. According to Burel et al. (1996), the control of these factors is complicated, and they can only be monitored and kept within tolerable ranges.

Fish culture facilities inside greenhouses represent an important alternative method of maintaining water temperatures within the acceptable range for fish survival. Thereby obtaining high growth rates with low feed conversion rates, due to the effect of temperature on fish metabolism and consequently on food consumption. However, the temperature control can be improved by the design of new strategies for water distribution among the tanks, taking into account the tanks' position inside the greenhouse and the temperature effect of air and soil on the water.

9.6 Organically Farmed Fish

Intensive aquaculture systems are aimed to produce high fish densities in order to increase the productivity. However, reduction on feed intake and fish growth is caused by earlier fish reproduction. This is true when we are talking about Tilapia (*Oreochromis niloticus* L.). This affects the final yield and therefore the farmer economic income. This problem can be reduced via male sexual induction immediately after the eggs eclosion. Steroid hormones, steroid analogs, or non-steroid compounds have been widely used in male sex reversal of various tilapia species. The percentage of sex-reversed males depends mainly on the type of hormones dose, timing and duration of administration, fish species, and size/age of larvae. These practices have big advantages because the fast fish growth allows us to obtain a more profitable production and increase the farmer profit (El-Sayed 2006). Aquaculture activity recently has adopted a new perspective called "Responsible Aquaculture," which states that farmers should know the quality of wastewater discharged into rivers or seas. However, in México this is not possible, because most of the farmers work in rural conditions, so they ignore the levels in water and its effect in other species (Soto-Zarazúa et al. 2012). Elimination of hormones, organically fish production, has generated some disadvantages on production such as; low rates of specific growth, mixed culture (male:female), and as the final result, increase in cost. The low rate of specific growth is a big problem for aquaculture because it implies a long time for harvest, 3 or 4 months adds. During this time, farmers need to invest in fishmeal, water, space. Besides, organically farmed fish have nutritional fishmeal requirements for which acceptable organic alternatives are not currently available. Then, a lot of research needs to be made on this topic. Fish culture under greenhouse has been probed in order to increase production without hormones. The result showed that it is possible to increase the specific growth of fish on greenhouse culture but two problems were founded: (1) High production on algae and (2) alteration on metabolism.

9.6.1 High Production on Algae

In an experiment conducted under greenhouse conditions environmental temperature variation was between 5 and 40 °C, and temperature inside the tanks shows a variation of 10 °C, between 20 and 30 °C. Those are recommended range for Tilapia culture (Timmons et al. 2002). So it can be said that cultivation under greenhouse diminish the effect of this factor and is helpful tool for water temperature control. However, this range of temperature is a nice condition for algae; as a consequence, a high growth was found inside the tanks. During the day, a saturation of dissolved oxygen is reached, but during the night the oxygen decreases leaving fish in hypoxia conditions, Fig. 9.2 shows some days in the production cycle mortality of 100 % was found in many tanks.

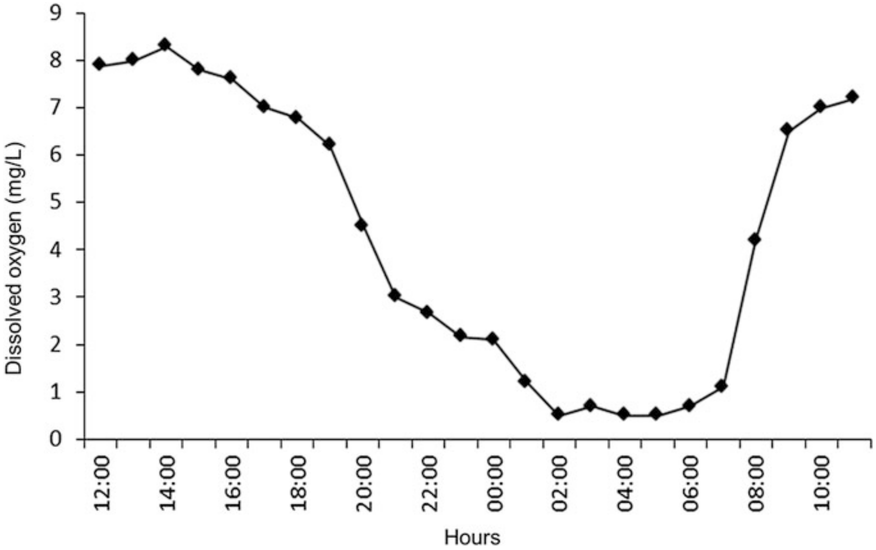

Fig. 9.2 Average dissolved oxygen behavior during a 24 h cycle inside treatments

9.6.2 *Metabolism Alteration*

As can be seen, Fig. 9.3 shows the results from an experiment in which metabolism were measured. This experiment consists in three different densities and its effect on tilapia oxygen consumption. It is possible to see that a maximum peak of consumption was found during the dark period, 02:00 h. Studies on tilapia metabolism show contrary results compared with results obtained under greenhouse culture. According to Ross and McKinney (1988), during the light period oxygen consumption is higher than dark period. However, under hypoxic conditions Tilapia acquires the oxygen gasping at the water surface, which leads to spend extra energy. Also this behavior could be supported with the results of (Mishrigi and Kubo 1978) in an experiment where the main objective was to test the effect of territoriality. According to this author, intraspecific competition modifies fish activity and oxygen consumption, so it can be said that increasing density affects metabolism and the recommendation would be to keep low densities.

9.7 Biotechnological Approaches on Native Species with Potential for Production

The efforts and the perception of the environment are different between countries, but ultimately the problem to be addressed holistically. Studies on fish typically focus on species that currently have commercial value, causing species that lack

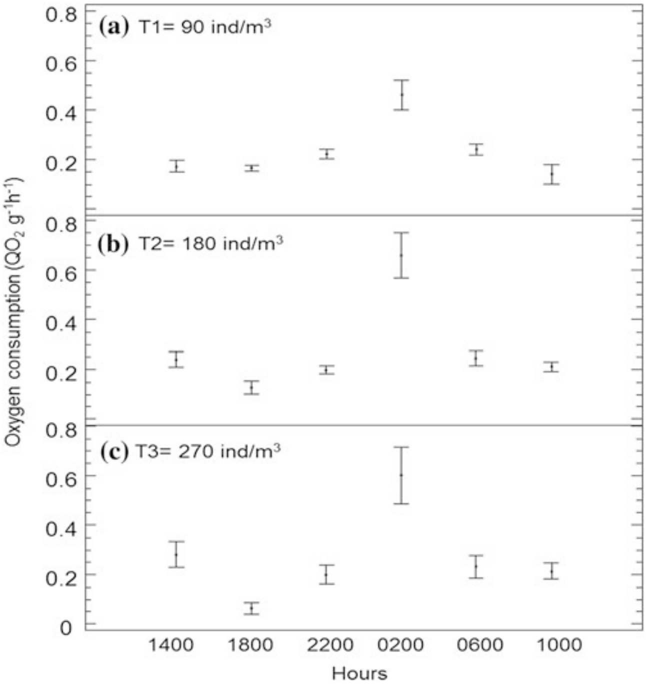

Fig. 9.3 Medium values of oxygen consumption (QO2 g^{-1} h^{-1}) for each hour during a 24 h cycle, *whisker* shows standard error (SE) for each measurement in the treatments, **a** T1 = 90, **b** T2 = 180, **c** T3 = 270

such market value to be ignored. This is the case of several freshwater native species, which can be founded in central and South America. Some attempts to cultivate native species have occurred mainly in areas or rural communities, where in addition to enhance the conservation of species protein contributes to the diet of the community (Ross and Beveridge 1995). One of the most interesting case studies in Mexico is growing "white fish" (*Chirostoma estor*) with the aim of repopulating some areas where the introduction of their populations has been declined (Martínez-Palacios et al. 2001). Most documented is that of the native Central American cichlid (*Cichlasoma urophthalmus*), of which there have been many studies to support its culture (Ross et al. 2008). In southern region some attempts to grow some silversides in Argentina (*Odontestes Basilichthys*), some *Characida*e family members have been grown in Brazil and more recently three species of carnivorous cichlids aquarium purposes. In Peru it has favored the cultivation of called pirarucu (*Arapaima gigas*), one of the largest fish of fresh water. Possibly the *Cichlidae* family members are those that show the greatest potential for cultivation (Bardach et al. 1986).

To successfully achieve the cultivation of a native species, compared to the technological advantages offered by more exotic trading requires knowledge of the biology, ecology, and aquaculture potential (ability to live at high densities, accept food encapsulation, and withstand high environmental variations) of each species. If aquaculture potential studies are performed with ecophysiological and bioenergetics approach may be developed predictive models of how to develop a population under different environmental factors, and even develop experimentally testable hypotheses (Gutiérrez-Yurrita and Montes 1998).

Main purpose of this chapter is to show the experience of three studies with native species; one refers to a small native species located in the state of Querétaro and with a great ecological importance, *Girardinichthys multiradiatus* (Garcia-Trejo et al. 2013). The study of this fish focused on the description of its habitat throughout a hydrologic cycle in which ecophysiological responses were determined in order to establish guidelines for its management and to preserve its population. In this work population structure and dynamics were getting, so trophic and ecophysiological responses to fluctuations in environmental factors were also identified.

On the other hand, a very interesting species were studied, native mojarra *Herichthys cyanoguttatus* founded on the basin of the Pánuco river. In this case the purpose was to evaluate its useful in fishery and later in the aquaculture. The work consisted of two stages: First, the characterization of their environment in order to locate stable populations of the mojarra and to characterize ecologically its habitat. Second, the mojarra was moved to the laboratory to try different forms of acclimatization for its future use by giving them tried food. Once acclimated, the stock was used to carry out density studies of individuals for culture (capacity of load), as well as of ideal thermal for its production.

9.8 Overview of the Future Trend in Aquaculture Development

Aquaculture has been supporting human demands for fish products for centuries and is an important industry worldwide. Global production from aquaculture has been increasing steadily, having more than doubled in the last decade; aquaculture now supplies one third of seafood consumed worldwide. With the massive increase in world aquaculture production in 1990s, the current aquaculture industry is one of the fastest growing sectors in world food production (FAO 2011). However, the expansion of aquaculture has been accompanied by degradation of the natural environment, especially on marine aquaculture. Direct impacts of fisheries and aquaculture are habitat modification, collection of wild seedstock, changes of food webs, introduction of nonnative fish species and diseases that harm wild fish populations, and nutrient pollution. According to the FAO, major issues that need to be addressed are problems with access to proper technology and financial resources, together with environmental impacts and diseases. Another argues that further increases in aquaculture production will come mainly from further investment in biotechnology with specific goals such as:

(a) Minimum use of nonrenewable energies.
(b) Cultural context.
(c) No environmental impact.
(d) Recirculation and reutilization.

The development of new strategies or technologies does not imply that the old one disappears; to the contrary, the main idea is to use the experience and improve existing technology.

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Chapter 10
Natural Foods as Biosystems to Face Noncommunicable Chronic Diseases: An Overview

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Abstract The maintenance of health and prevention of chronic diseases is influenced by naturally occurring phytochemicals in foods. Plants display an immense diversity of specialized metabolites, many of which have been important to humans. In the last few years, the use of plants to produce chemical compounds with health-promoting properties has been extensively studied. In addition to supplying the substrates for producing energy, many dietary chemicals are bioactive, directly or indirectly altering the regulation of biosystem processes and the expression of genetic information. The study of gene–nutrient interactions in the management of chronic diseases, such as coronary heart disease, hypertension, cancer, diabetes, and obesity and the role of nutrients in gene expression started two decades ago. Success in these areas requires investigators working on large population studies designed to adequately investigate gene–diet interactions. Knowledge of genetic susceptibility to disease will help identify those at higher risk for disease, as well as their response to diet. The prospect of targeting specific dietary treatment to those predicted to gain the most therapeutic benefit clearly has important clinical and economic consequences, particularly in high prevalence diseases, establishing how gene expression can be modified using combined dietary strategies.

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Contents

10.1 Introduction

Plants as autotroph are able to acquire the basic elements (minerals) and synthesize the full spectrum of organic molecules required to support their growth and propagation. Humans require the same basic elements as plants, but lack the ability to synthesize many organic molecules (i.e., so-called essential micronutrients [certain amino acids and vitamins]), for which plants are the main dietary source. Therefore, human nutritional health is dependent on plant food directly or indirectly (through feeding on animals that feed on plants). In contrast with the three major nutrients (carbohydrates, proteins, and lipids), micronutrients by definition do not provide energy and are needed in relatively small amounts by organisms. Micronutrient deficiency has been directly linked to human disease for well over a century (Fitzpatrick et al. 2012).

The importance of diet to health has become even more obvious with the realization that many of life's modern diseases are the result of subtle but chronic metabolic imbalances related partly to diet. One of the major issues in the twenty-first century facing humankind is the prescription to stay healthy and delay the onset of chronic metabolic diseases. Chronic metabolic diseases still afflict a substantial percentage of modern human population despite advances in medical and health care technologies (Sarmidi and Enshasy 2012).

Despite these exciting development in the healthcare products and services, humans are increasingly overwhelm by problems ranging from malnutrition, chronic metabolic diseases, iatropic in medical care (Lazarou et al. 1998), environmental degradation, water contamination, and increasing cost of living. Chronic metabolic diseases such as diabetes type 2, cancer, cardiovascular disease, hypertension, and autoimmune disorder are on the rise globally (Moussavi et al. 2007). As a result, there is a major paradigm shift in the healthcare industry from sickness centered to wellness focus.

Plants and plant products play very important role in human life. As a food source, plants directly constitute 93 % of the human diet, with the remaining 7 % being indirectly derived from plants via animal products. Beside food application, plants have been utilized as medicines for thousands of years and still used as source of important traditional medicines in both developed and developing countries. In general, plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavors, colors, food additives, and bioinsecticides. More than 100,000 plant secondary metabolites have already been identified, which represent only 10 % of the actual total in nature and only half the structures have been fully elucidated. In spite of large growth of chemical industries, plants will continue to provide novel products as well as chemical models for new drugs in coming centuries. In the USA, where chemical synthesis dominates the pharmaceutical industries, 25 % of the pharmaceuticals are based on plant-derived chemicals (Sarmidi and Enshasy 2012).

Nutrition research has highlighted the role of several nutrients in regulating the genome machinery. Many vitamins and micronutrients are substrates and/or cofactors in the metabolic pathways that regulate DNA synthesis and/or repair and gene expression (Fenech and Ferguson 2001). Nutrigenomics employs high-throughput genomics technologies to provide a genetic understanding of the mechanism affecting the balance between health and disease by common dietary chemicals (i.e., nutrition) that alters the expression and/or structure of an individual's genetic makeup. The conceptual basis for this branch of genomic research can best be summarized with the following five tenets: (a) Common dietary chemicals act on the human genome to alter gene expression or structure directly or indirectly; (b) Under certain circumstances and in some individuals, improper diet can be a serious risk factor for several diseases; (c) Some diet-regulated genes (and their normal, common variants) are likely to play a role in the onset, incidence, progression, and/or severity of chronic diseases; (d) The degree to which diet influences the balance between healthy and disease states may depend on an individual's genetic makeup; (e) Dietary intervention based on knowledge of nutritional requirement, status, and genotype (i.e., "individualized nutrition") can be used to prevent, mitigate, or cure chronic disease (Kaput and Rodriguez 2004). Dietary chemicals can affect gene expression directly or indirectly. At the cellular level, nutrients may act as ligands for transcription factor receptors (Dauncey et al. 2001; Jacobs and Lewis 2002); can be metabolized by primary or secondary metabolic pathways, thereby altering concentrations of substrates or intermediates; or, positively or negatively affect signal pathways (Clarke 1999; Eastwood 2001).

Currently, nutrition-related health issues remain commonplace and account for many of the leading causes of death around the world. Reciprocally, a reasonable good fraction of cancer deaths maybe prevented, by modifying the diet composition (i.e., content of fiber, polyphenols, fat/oil, protein, spices, cereals, etc.) and regular physical exercise (Vanden Berghe 2012). The relationship between nutrition and health–economic outcomes is important at both the individual and the societal level. While personal nutritional choices affect an individual's health condition, thus influencing productivity and economic contribution to society, nutrition interventions carried out by the state also have the potential to affect economic output in significant ways (Gyles et al. 2012).

10.2 Cancer Influenced by Dietary Compounds

Cancer is set to become a major cause of morbidity and mortality in the coming decades in every region of the world. The United Nations (UN) has forecast that the global population will reach 8 billion by 2030. The effect of population aging and growth will be greatest in low-income and middle-income countries. These changes translate to a predicted global burden of 20.3 million new cancer cases by 2030 compared with an estimated 12.7 million cases in 2008, and a predicted 13.2 million cancer-related deaths worldwide by 2030, from 7.6 million in 2008 (Bray et al. 2012).

Natural products are potential source of chemical constituents with antitumor and cytotoxic activities owing to their enormous metabolism, which synthesize various structurally diverse bioactive compounds. Medicinal plants can reduce or minimize the toxic side effect of chemotherapy and radiation treatment by reinforcing their cancer abating action. In addition, medicinal plants have significant roles in the treatment of cancer and most new clinical applications of plant secondary metabolites and their derivatives have been applied toward fighting cancer (Nema et al. 2013).

Recently, Melchini et al. (2013) reported the antiproliferative activity of the dietary isothiocyanate erucin, a bioactive compound from cruciferous vegetables, on human prostate cancer cells. Erucin increased p21 protein expression and ERK1/2 phosphorylation significantly ($P \leq 0.01$) in a dose-dependent manner inhibiting PC3 cell proliferation. A microarray gene expression analysis identified the curcumin-regulated genes in a highly invasive human breast carcinoma cell line (MDA-MB 231) (Cine et al. 2013). Gene set enrichment analyses on our whole genome expression data revealed downregulation of the epidermal grow factor (EGF) pathway elements following curcumin treatment. Furthermore, gene network analysis revealed a significantly relevant network among the differentially expressed genes, centered on the EGR1 and FOS genes. The members of these pathways and networks play an essential role in the regulation of cancer cell growth and development. According to a genome-wide transcriptomics analysis, rosemary polyphenols alter the expression of ~ 4 % of the genes covered by the Affymetrix Human Gene 1.0ST chip in human SW480 and HT29 colon cancer cells (Valdés et al. 2013). However, only ~ 18 % of the differentially expressed genes were common to both cell lines, indicating markedly different expression profiles in response to the treatment. Differences in induction of G2/M arrest cycle observed after rosemary polyphenols treatment in the two colon adenocarcinoma cell lines suggest that the extract may be differentially effective against tumors with specific mutational pattern. Furthermore, recent findings revealed that polyphenols could interact with cellular signaling cascades regulating the activity of transcription factors and consequently modulate gene expression. Together with this classical regulatory pathway, polyphenols affect the expression of microRNAs (miRNA). miRNAs are small, noncoding RNAs implicated in the regulation of

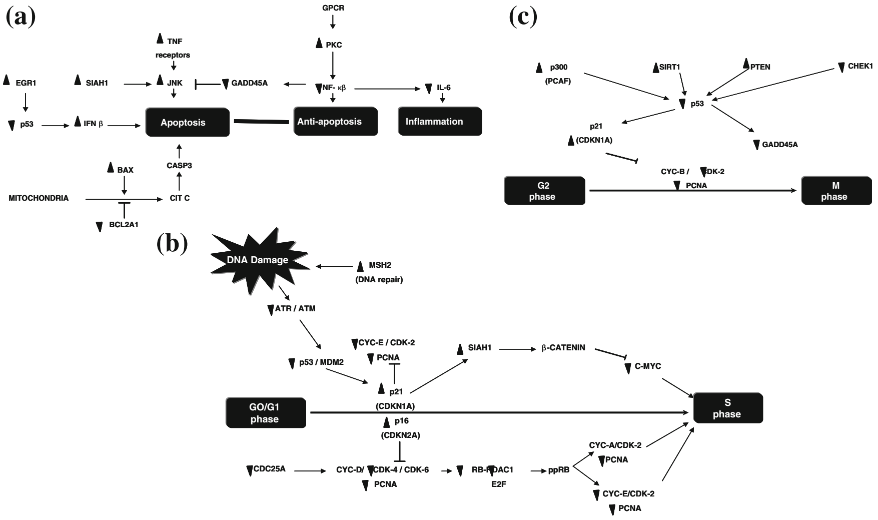

Fig. 10.1 Changes in gene expression in (**a**) apoptosis and inflammatory pathways, **b** G1/S and **c** G2/M cell cycle phase, respectively. Symbols indicate upregulation (▲) or downregulation (▼) in mRNA expression as derived from array analysis. Adapted from Campos-Vega et al. (2010)

gene expression that control both physiological and pathological processes such as development and cancer (Milenkovic et al. 2013).

Other dietary components that regulate cancer cell proliferation and survival include dietary fiber (Campos-Vega et al. 2013). The transcriptional effects of the non-digestible fraction (NDF) from common bean cv. Bayo Madero on the gene expression profile regulated by Tp53 signal transduction in an in vivo model of early stage colon cancer were investigated to elucidate the molecular mechanism involved in prevention (Vergara-Castañeda et al. 2012). Significant differences were detected in 72 genes of the Tp53-mediated signaling pathway involved in apoptosis, cell cycle regulation and arrest, inhibition of proliferation and inflammation, and DNA repair. Campos-Vega et al. (2009) demonstrated that common bean is an excellent source of NDF that can be fermented in the colon and produce Short Chain Fatty Acids (SCFAs), compounds previously reported to exert health benefits. Later, this research group investigated the molecular changes of p53 pathway in HT-29 cells after 24 h exposure to in vitro (human gut flora, FE-hgf) Fermented NDF (cv. Bayo Madero) (Campos-Vega et al. 2010). Significant differences were detected in 72 of 84 p53-mediated signal transduction response genes involved in apoptosis, cell cycle, and cell proliferation. It was demonstrated that common beans and/or its NDF modulate gene expression profiles in HT-29 cells (Fig. 10.1), providing insight into the mechanism underlying its overall protective function against colon carcinogenesis. Recently, Campos-Vega et al. (2012), suggested that human gut in vitro Fermented Non-Digestible Fraction (FNDF) from common beans can elicit beneficial protective effects in colon cancer by modulating protein expression in HT-29 cells. FNDF inhibited HT-29 cell growth and modulated protein expression associated with apoptosis, cell cycle arrest, and proliferation, as well as morphological changes linked to apoptosis evaluated by TUNEL and hematoxylin and eosin stains, confirming previous results on gene expression.

Diet that inhibits one or more neoplastic events and reduces cancer risk has been suggested as a preventive approach to control breast cancer. Ray et al. (2010) used human breast cancer cells and primary human mammary epithelial cells as an in vitro model to assess the efficacy of Bitter Melon (*Momordica charantia*) Extract (BME) as an anticancer agent. The BME treatment enhanced p53, p21, and pChk1/2 and inhibited cyclin B1 and cyclin D1 protein expressions, suggesting a mechanism involving cell cycle regulation. Sesamin, a lipid-soluble lignan, a class of phytoestrogen, isolated from sesame (*Sesamum indicum*), inhibited the proliferation of a wide variety of tumor cells including leukemia, multiple myeloma, and cancers of the colon, prostate, breast, pancreas, and lung (Harikumar et al. 2010). In this study, sesamin also potentiated tumor necrosis factor-α–induced apoptosis and this correlated with the suppression of gene products linked to cell survival, proliferation, inflammation, invasion, and angiogenesis. Sesamin down-regulated constitutive and inducible NF-κB activation induced by various inflammatory stimuli and carcinogens, and inhibited the degradation of IκBα, thereby indicating its potential against cancer and other chronic diseases through the suppression of NF-κB signaling pathway. Dark chocolate protects against colon cancer by inhibiting Aberrant Crypt Foci (ACF) formation by down-regulating cyclooxygenase 2 and RelA (inflammatory mediators) gene expression (DeIulio and Hong 2010); whereas blueberry phytochemicals inhibit growth and metastatic potential of MDA-MB-231 breast cancer cells through modulation of the phosphatidylinositol 3-kinase pathway (Adams et al. 2010). Intake of grape-derived polyphenols effectively reduced the development of colon carcinoma tumors in vivo by blunting tumor vascularization and by inhibiting proliferation and promoting apoptosis of tumor cells subsequent to an upregulation of tumor suppressor genes (Walter et al. 2010).

The above observations may indicate that whole food products rather than single bioactive components are important in mediating physiological effects in the body. However, other studies support the idea “of a single compound.” For example, Caffeic Acid Phenethyl Ester (CAPE, a phenolic compound) in a single dose administered before carcinogenic initiation induced by Diethylnitrosamine (DEN) prevents the appearance of preneoplastic lesions of hepatocarcinogenesis initiation (Beltrán-Ramírez et al. 2010). Comparison of gene expression profiles induced by DEN with a previously administered single dose of CAPE showed that DEN treatment increased gene expression associated with oxidative stress, genes cell cycle regulation, and cytochrome P450 modification. CAPE plus DEN reduced the expression of cytochrome involved in DEN bioactivation as well as the expression of oxidative stress and cell cycle regulators. In other study, dietary omega-3 (n-3) Polyunsaturated Fatty Acids (PUFA) suppressed expression of

EZH2 (associated with metastasis and poor prognosis in cancer patients) in breast cancer cells (Dimri et al. 2010).

The cross-resistance profile of 60 tumor cell lines was analyzed using inhibitori 50 % concentration (IC_{50}) of eight different withanolides (the major secondary metabolites of *Withania somnifera*) and 19 established anticancer drugs (Efferth and Greten 2012). The cell lines revealed cross-resistance between the eight withanolides. Consistent cross-resistance between withanolides and nitrosoureas was also observed. Then the authors performed transcriptomic microarray-based COMPARE and hierarchical cluster analyses of mRNA expression to identify mRNA expression profiles predicting sensitivity or resistance toward withanolides. Genes from diverse functional groups were significantly associated with response of tumor cells to withaferin A diacetate, e.g., genes functioning in DNA damage and repair, stress response, cell growth regulation, extracellular matrix.

Another compound related with beneficial health in cancer is iodine, since it induces apoptosis by regulating MAPKs (Mitogen-Activated Protein Kinase)-related p53, p21, and Bcl-xL in thyroid cancer cells (Liu et al. 2010). One study comparing the proliferative properties of genistein and daidzein, demonstrated modulations of cyclin-dependent kinase-related pathway genes, DNA damage signaling pathway and a downregulation of EGF and IGF on human prostate cancer cell leading to cell cycle arrest (Rabiau et al. 2010).

Only few dietary components among hundreds that have been proposed as potential cancer preventive agents have been used in clinical trials. The impact of lycopene supplementation has been studied in a crossover design with 30 healthy men. Talvas et al. (2010) showed that cancer prostatic cell incubation with sera from men who consumed purified lycopene (from tomato), led to significant upregulation of *IGFBP-3*, *c-fos*, *uPAR*, and *Bax*:*Bcl-2* ratio, and downregulation of *cyclin-D1*, *p53*, and *Nrf-2* compared with sera collected after placebo consumption, inducing apoptosis and cell cycle arrest. Multivitamins, folate, and green vegetables have been suggested to protect against gene promoter methylation in the aerodigestive tract of smokers (Stidley et al. 2010). Published population-based cases of rectal cancer (Slattery et al. 2010) concluded that fat-rich dairy products might be associated with an increased risk of CIMP+ (CpG Island Methylator Phenotype) tumors, while low-fat dairy products reduced risk of CIMP+ . Omega-3 fatty acids were associated with an increased risk of a CIMP+ tumor. High levels of vegetable intake reduced risk of both TP53 and KRAS2 mutations. High intake of whole grains reduced the likelihood of a TP53 mutation, whereas high intake of refined grains increased the likelihood of a TP53 mutation. Dietary fiber was also associated with reduced risk of TP53 and KRAS2 rectal tumor mutations. These data suggest that diet is associated with specific types of rectal tumor mutations and epigenetic changes.

Epidemiological and preclinical evidence continues to highlight dietary components ranging from macro- to micro-constituents as likely modifiers of cancer processes.

10.3 Diabetes: Sweet Genes

Diabetes is a worldwide growing problem. There are 25.8 million adults and children in the US that have diabetes that is 8.3 % of the population. Throughout the world, 347 million people are diagnosed with diabetes and an estimated 3.4 million deaths in 2004 were related to high blood sugar. By the year 2030, the WHO predicts that worldwide twice as many deaths will be a direct result of diabetes as in 2005. Diabetes is a major contributor to heart disease and stroke as well as blindness, kidney failure, and lower limb amputation and is the seventh leading cause of death in the United States (Dombrowski et al. 2013).

It is well established that diet, even independent of body weight, can influence the risk of T2D (Esposito et al. 2010; Salas-Salvadó et al. 2011; Dämon et al. 2011). Vegetables and fruits are notable components of diets associated with lower risk of T2D (Esposito et al. 2010; Salas-Salvadó et al. 2011; Dämon et al. 2011; McEvoy et al. 2012; Carter et al. 2010). Plant foods are good sources for nutrients associated with lower T2D risk, such as fiber (Ye et al. 2012), potassium (Chatterjee et al. 2011), and magnesium (Dong et al. 2011), among others.
A recent study demonstrates the chemo-protective effect of a flavanol-containing foodstuff such as cocoa, which likely plays a role in the protection afforded by fruits, vegetables, and plant-derived beverages against diseases such as TD2, for which excess production of reactive oxygen species (ROS) has been implicated as a causal or contributory factor (Martín et al. 2013). Ins-1E cells, pancreatic beta cells, treated with a cocoa phenolic extract showed remarkable attenuation of cell damage after being submitted to stress. Other results suggest that quercetin or naringenin may protect β-cells from cytokines toxicity by enhancing cell survival through PI3-kinase pathway, independent of p-p38 MAPK or Inducible Nitric Oxide (NO) Synthase (iNOS) (Lin et al. 2012).

On the other hand, a polysaccharide extract derived from *Triticum Aestivum* Sprout (TASP) stimulates insulin secretion and production in vitro via K^+ channel closure and calcium influx on the RIN-5F pancreatic β-cell line and rat pancreatic islets (Lee et al. 2012). TASP significantly increased glucose-induced $45Ca^{2+}$ uptake and proinsulin mRNA expression in rat islets. Apoptosis is the main form of β-cell death in diabetes. Ginsenoside Rg1 and Rb1 protect β-cells from high glucose/cytokine-induced pancreatic β-cell apoptosis by inhibiting NO production and regulating apoptosis-related genes (Chen et al. 2012). Among these genes, *Bax*, *Fas*, and *Caspase-3* gene expressions are upregulated by high glucose, whereas only *Bax* and *Caspase-3* gene expression are elevated by cytokines.

A study showed that diet-induced gene expression of isolated pancreatic islets from a polygenic mouse model of the metabolic syndrome (Dreja et al. 2010). In the prediabetic state, 2,109 islet transcripts were differentially regulated (>1.5-fold) between diabetogenic high-fat diet (HF) and a diabetes-protective carbohydrate-Free (CHF) diet. Thirty-nine of the identified genes correlated with data from the Diabetes Genetics Initiative and Wellcome Trust Case Control Consortium genome-wide scans for TD2. HF diet-induced early changes in gene expression associated with increased cell cycle progression, proliferation, and differentiation of islet cells, and oxidative stress (Table 10.1). In addition, pathway analysis identified oxidative phosphorylation as the predominant gene set that was significantly upregulated in response to the diabetogenic HF diet. Acacia Polyphenol (AP) significantly suppressed increases in body weight, plasma glucose, and insulin in obese diabetic KKAy mice fed HF diet (Ikarashi et al. 2011). AP increased mRNA expression of adiponectin and adiponectin secretion, suppressed TNF-α secretion and mRNA expression by white adipocytes, and elevated GLUT4 (mRNA) expression in skeletal muscle in addition to reducing obesity. Another potential antidiabetic and liver-protective agent, CAPE suppressed hepatic glucose output by inducing mRNA expression of Glucokinase (Glk) and Pyruvate Kinase (Pyk) in streptozotocin-induced diabetic rats, while inhibiting phoshoenolpyruvate carboxykinase in diabetes. CAPE also decreases the harmful effects of diabetes on the liver of rats (Celik et al. 2009).

A Ginkgo Biloba Extract (GBE) has been associated with diabetes control. GBE improves glucose metabolism in diabetic rats and reduces the diabetes-induced diaphragm damage. The mechanism of action of the extract may be related to the promotion of the mRNA expression of GLUT4 in diaphragm and improvement in the uptake and metabolism of blood glucose (Li et al. 2010). Berberine, a quaternary ammonium salt found in plants such as Berberis, goldenseal (*Hydrastis canadensis*), and Coptis chinensis, restores diabetic endothelial dysfunction through enhanced NO bioavailability by upregulating eNOS (NO synthase) and suppressing NADPH oxidase expressions in diabetic rats induced by high-fat diet and streptozotocin (Wang et al. 2009). Also, dietary quercetin (a flavonoid), alleviates diabetic symptoms and reduces streptozotocin-induced disturbance of hepatic gene expression in mice. Quercetin may improve liver and pancreas functions by enabling the recovery of cell proliferation through the inhibition of *Cdkn1a* expression (Kobori et al. 2009) (Table 10.1). Other study showed that emodin, bioactive compound extracted from *R. palmatum L,* one of the most widely used herbs in Chinese medicine, significantly elevated the mRNA expression level of *PPAR* γ and regulated the mRNA expressions of *LPL*, *FAT/CD36*, *resistin*, and *FABPs* (*ap2*) in liver and adipocyte tissues (Xue et al. 2010).

A systematic review showed that the improvement of β-cell function and insulin secretion is possible with antioxidant compounds from antidiabetic plants (Abdollahi et al. 2012). The most important pathways for the improvement were: oxidative stress suppression, cytokine-induced impairment, suppression of nuclear factor NF-κB- a key regulator of endothelial activation, Uncoupling Protein 2 (UCP2), activation, insulin-like activity, and intracellular calcium expression.

Only a limited number of studies report unbiased, genome-wide expression profiling of pancreatic islets in the course of a diabetogenic dietary intervention in rodents. Even fewer studies have investigated diabetes-associated global gene expression profiles in human samples from donors with TD2. However, the overlap of genes implicated in both adaptation to diabetes-inducing nutritional factors in rodent studies and genes differentially regulated in TD2 is striking. In islets, overload of glucose and long-chain fatty acids results in induction of genes

Table 10.1 Mechanisms of action of selected diet compounds in cancer, diabetes, obesity, coronary, and other diseases from cell, animal and human studies

Associated compound or diet type	Modulated genes	Function	Model	Cancer type	Reference
Cancer					
Sulforaphane	Phase 2 enzymes↑, G2/M phase of the cell cycle↑ Other phases of the cell cycle ↓	Cellular defences Cell cycle Cell cycle	In vitro (cell culture)	Prostate	Bhamre et al. (2009)
Multivitamins, folate, and green vegetables			Human	Lung	Stidley et al. (2010)
Dietary fiber	SIAH1↑, PRKCA↑ MSH2↑ CHEK1↓ GADD45A↓	Induction apoptosis Repair DNA Cell cycle check point Cell cycle arrest and DNA repair gene	In vitro (cell culture)	Colon	Campos-Vega et al. (2010)
Momordica charantia extract	p53↑, p21↑, pChk1/2↑ cyclin B1↓ cyclin D1↓	Cell cycle	In vitro (cell culture)	Breast	Ray et al. (2010)
Sesamin	Bcl-2↓, survivin↓ Cyclin D1↓ COX-2↓ Matrix metalloproteinase-9↓ Intercellular adhesion molecule 1↓ Cascular endothelial growth factor↓	Cell survival Proliferation Inflamation Invasion Angiogénesis	In vitro (cell culture)	Leukemia, multiple myeloma, and cancers of the colon, prostate, breast, pancreas, and lung	Harikumar et al. (2010)

(continued)

Table 10.1 (continued)

Associated compound or diet type	Modulated genes	Function	Model	Cancer type	Reference
Caffeic acid phenethyl ester	glutathione reductase↓ p53↓ P450↓ CYP2B1 GST-κ ↓ GST-θ↓	Oxidative stress Cell cycle regulation Detoxification	In vivo (rats)	Liver	Beltrán-Ramírez et al. (2010)
Omega-3 fatty acid	EZH2↓	Metastasis	In vitro (cell culture)	Breast	Dimri et al. (2010)
Dark chocolate	COX2↓ RelA↓	Inflammation	In vivo (rats)	Colon	DeIulio and Hong (2010)
Grape-derived polyphenols	VEGF↓ MMP-2↓ MMP-9↓ COX-2↓ p16↑ p53↑ p73↑	Tumor vascularization Tumor suppressor genes	In vivo (rats)	Colon	Walter et al. (2010)
Blueberry Phytochemicals	PI3 K/AKT and NFκB↓	Growth and metastatic	In vivo (rats) and in vitro (cell culture)	Breast	Adams et al. (2010)
Lycopene	IGFBP-3↑ c-fos↑ uPAR ↑ Bax:Bcl-2 ratio↑ cyclin-D1↓ p53↓ Nrf-2↓	Proliferation and apoptosis Carcinogenesis Apoptosis Cell cycle regulation	Ex vivo (human and cell culture)	Prostate	Talvas et al. (2010)

(continued)

Table 10.1 (continued)

Associated compound or diet type	Modulated genes	Function	Model	Cancer type	Reference
Erucin	P21↑ ERK1/2↑	Proliferation	In vitro (cell culture)	Prostate	Melchini et al. (2013)
Curcumin	EGF pathway↓	Proliferation/survival	In vitro (cell culture)	Breast	Cine et al. (2013)
Non-digestible fraction common beans	P53 pathway	Apoptosis, cell cycle arrest and cell cycle proliferation	In vitro (cell culture)	Colon	Campos-Vega et al. (2010)

Associated compound or diet type	Modulated genes	Function	Model	Reference
Diabetes				
Cocoa phenolic extract	PI3, pathway	Survival	In vitro (cells)	Lin et al. (2012)
Ginsenoside	Bax Fas Caspase-3	Apoptosis	In vitro (cell culture)	Chen et al. (2012)
High-fat diet	*Cacna1d*, *Chd2* *Clip2*, *Igf2bp2*, *Dach1*, *Tspan8* *Cdkn1b*, *Tmem27*, *Pax6*, *Cat*, *Prdx4* and *Txnip*	Increased cell cycle progression, proliferation and differentiation of islet cells, and oxidative stress	In vivo (rats)	Dreja et al. (2010)
Acacia polyphenols	Adiponectin↑ TNF-α ↓ Glut-4↑	Body weight and glucose output	In vivo (mice)	Ikarashi et al. (2011)
Caffeic acid	Glk↑ Pyk↑	Hepatic glucose output	In vivo (Rats)	Celik et al. (2009)
Ginkgo biloba extract	GLUT4↑	Glucose transporters	In vivo (rats)	Li et al. (2010)
eNOS	eNOS↑	Nitric oxide synthase	In vivo (rats)	Wang et al. (2009)
Quercetin	*Cdkn1a*↓	Cell cycle regulation	In vivo (rats)	Kobori et al. (2009)

(continued)

Table 10.1 (continued)

Associated compound or diet type	Modulated genes	Function	Model	Reference
Emodin	*PPARγ* *LPL*, *FAT/CD36* *FABPs* (*ap2*)	Proliferator-activated receptor-γ Lipoprotein lipase Fatty acid translocase Adipocyte fatty acid binding protein	In vivo (rats)	Xue et al. (2010)
Obesity				
Adenophora triphylla extract	LDL receptor↑ CYP7A1↑ HMG–CoA reductase↓	Low-density lipoprotein receptor, lipid metabolism- cholesterol 7α-hydroxylase, lipid metabolism-Cholesterol synthesis	In vivo (rats) and In vitro (cells)	Choi et al. (2010)
Wasabia japonica Matsum, leaves	PPARγ↓ C/EBPα↓ aP2↓	Peroxisome proliferator-activated receptor CCAAT/enhancer-binding proteín Adipocyte fatty acid binding protein 2	In vitro (cell culture)	Ogawa et al. (2010)
Safflower oil	Orexin↓ Ghrelin↓ PPARα↑	Feeding regulation Stimulates food intake and promotes energy storage Nuclear hormone receptors that control lipid oxidation, adipocyte differentiation, glucose and lipid storage	In vitro (mices)	Zhang et al. (2010)

(continued)

Table 10.1 (continued)

Associated compound or diet type	Modulated genes	Function	Model	Reference
Anthocyanin-rich tart cherries	PPAR-α PPAR-γ IL-6 TNF-α	Nuclear hormone receptors that control lipid oxidation, adipocyte differentiation, glucose and lipid storage Inflammation process	In vivo (rats)	Seymour et al. (2009)
Long-term dietary restriction	Proglucagon↓ Cholecystokinin↓ Ghrelin↓ GOAT↓	Insulin secretagogue Responsible for stimulating the digestion of fat and protein Stimulates food intake and promotes energy storage	In vivo (rats)	Reimer et al. (2010)
Propionic acid	Leptin↑	Regulates adipose tissue mass	In vivo (humans)	Al-Lahham et al. (2010)
Dietary mix [(resveratrol, green tea extract, α-tocopherol, vitamin C, n-3 (omega-3) polyunsaturated fatty acids, and tomato extract]	Genes, transcriptome adipose tissue (197 genes)	Overweight	In vivo (humans)	Bakker et al. (2010)
Coronary diseases				
Whole grape	PPAR-α↑ PPAR-γ↑ Coactivator-1α↑ PPAR-γ↑ Inhibitor-κBα↑	Cardiac inflammation and fibrosis NF-κB inhibitor	In vivo (rats)	Seymour et al. (2010)
Soy protein	PPARα↑ Protein-1↓	Cardiac inflammation and fibrosis Sterol regulatory element binding	In vivo (rats)	Torre-Villalvazo et al. (2009)

(continued)

Table 10.1 (continued)

Associated compound or diet type	Modulated genes	Function	Model	Reference
Mediterranean diet	COX-2↓ MCP-1↓ LRP1↓ CD36↑ TFPI ↑ (tissue factor pathway inhibitor)	Vascular inflammation Foam cell formation Involved in thrombosis	In vivo (humans)	Llorente-Cortés et al. (2010)
Other diseases				
β-carotene, vitamin D2, and linoleic acid	↓Hepatitis C virus	Hepatitis C virus replication	In vitro (cell culture)	Liu et al. (2010)
Lauric, myristic, and palmitic acids	↑Hepatitis C virus	Hepatitis C virus replication	In vitro (cell culture)	Liu et al. (2010)
Chinese propolis	IgE, iNOS, TGF-β1, TNF-α↓	Transcriptional activity (allergic diseases)	In vitro (cell culture)	Ammar et al. (2013)

involved in the defense of oxidative stress, ER stress, proinflammatory cytokines and chemokines, cell cycle control and growth, as well as metabolic pathways for lipid formation and storage. Moreover, several genes are differentially expressed in the prediabetic and diabetic states, while being genetically associated with diabetes-related traits in human genome-wide association studies (Chadt et al. 2012). Several (but not mutually exclusive) pathophysiological pathways involved in nutrition-induced impairments of β-cells have now been established (Fig. 10.2), and novel aspects such as epigenetic modifications of DNA and associated proteins are currently under investigation (Chadt et al. 2012).

10.4 Obesity: Feed Your Genes Right

Obesity can be defined as a condition of abnormal or excess fat accumulation in adipose tissue, to the extent that health may be impaired. Body Mass Index (BMI), calculated as [(weight in kg)/(height in m)^2], is considered to be the most useful population-level measure of obesity, and a simple index to classify underweight, overweight, and obesity in adults (WHO 2000).

Obesity is a major public health concern worldwide, contributing to increased morbidity and mortality. It has emerged as a significant cause of diabetes, cardiovascular disease (CVD), and renal insufficiency. The accelerated worldwide increase of obesity prevalence has been named “globesity,” with considerable variations observed between and within countries. Coincidentally, epidemiological patterns, food habits, and lifestyles are changing very fast. Obesity affects both sexes and all ages, and, in Latin America, it increases with age and is always more prevalent in women, especially those from lower social strata (Aballay et al. 2013).

Many national and international agencies recommend an increased intake of fruits and vegetables in order to decrease the risk of developing lifestyle related diseases including overweight and obesity (WHO 2003). Different examples of dynamic changes in DNA methylation patterns due to the restriction or supplementation with different nutrients related to obesity have been reported. In neonatal exposure to a DNA-hypomethylating compound such as bisphenol A has been associated with higher body weight, although its effects were prevented by diet supplemented with different methyl donors such as folic acid or genistein. Genistein-induced hypermethylation of the Agouti gene decreasing its expression and protecting offspring from obesity. In fact, transgenerational amplification of body weight has been prevented by a promethylating dietary supplement. In this sense, maternal supraphysiological methyl group (folate, cobalamin, choline, and betaine) supply, and a low-protein diet in rodents throughout pregnancy modify DNA methylation of some key metabolic genes (agouti, glucocorticoid receptor, and PPAR-α). All these data strongly suggest that epigenetic mechanisms may be boosted or impaired by dietary factors in the mother and could be involved in obesity susceptibility in the offspring (Campión et al. 2009).

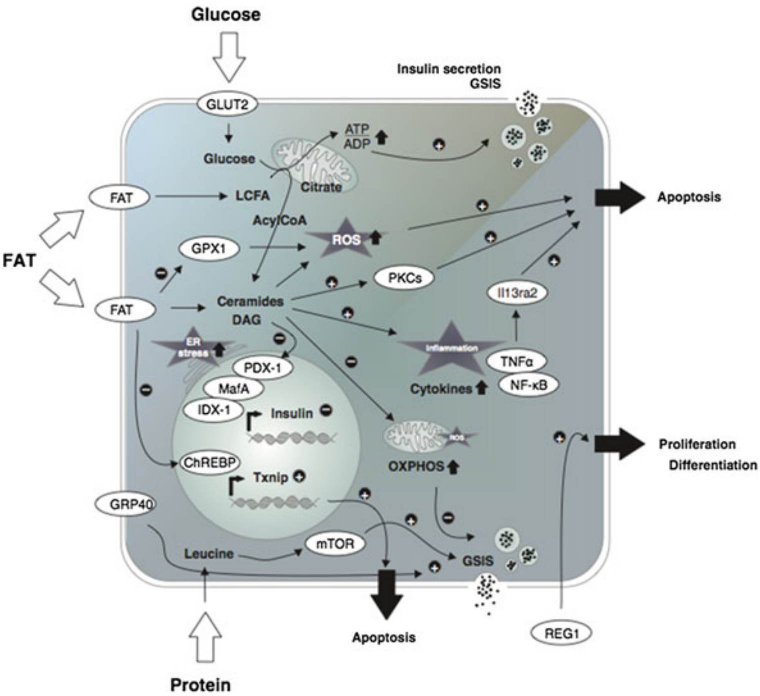

Fig. 10.2 Impact of different macronutrients on β-cells—glucose enters the β-cell via the facilitative glucose transporter GLUT2 (rodents) and is rapidly metabolized. The subsequent increase in the ATP/ADP ratio induces transport of insulin-containing storage vesicles to the plasma membrane and secretion of insulin into the extracellular space. In the presence of dietary carbohydrates in combination with high amounts of lipids, the flux of fatty acid-derived Acyl-CoA toward mitochondrial oxidation is severely decreased due to impaired import of Acyl-CoA into mitochondria (Prentki et al. 2002), despite upregulation of OXPHOS genes and enhanced oxidative metabolism. Non-oxidized long-chain fatty acid (LCFA) might be converted to toxic lipid metabolites (ceramides, DAG) interfering with multiple cellular pathways, thereby increasing inflammatory processes and activating protein kinase C (PKCs). As a consequence, all these changes result in ROS production and enhanced apoptosis of β-cells. Moreover, transcription of genes crucial for maintenance of β-cell function and integrity is affected by these toxic LCFA byproducts by influencing the activity of various transcription factors involved in insulin production or cell maintenance and survival (e.g., PDX-1, MafA, IDX-1, and FOXO1). In succession, these disturbances lead to reduced insulin production as well as secretion and increased apoptosis of β-cells. Defined protein diets, especially those containing high amounts of branched chain amino acids (leucine) as well as acute stimulation of GPR40 with free fatty acids have beneficial effects on insulin secretion and β-cell survival, presumably involving the mTOR pathway (Adapted from Chadt et al. 2012)

Some examples of diet-regulated obesity-related genes have also been reported. For instance, *W. japonica* Matsum, leaves (WLE), a native Japanese plant, suppressed the differentiation of 3T3-L1 preadipocytes, mediated, partly through modulation of PPARγ, C/EBPα, and other specific genes, such as aP2. These results suggest that WLE may prevent obesity and insulin resistance by inhibiting the differentiation of preadipocytes (Ogawa et al. 2010). In addition, in vivo and in vitro study on HepG2 cells and HF diet-induced obese mice showed that *A. triphylla* extract (oriental medicinal) has antioxidant, antiobese, and hypocholesterolaemic effects, and that these hypocholesterolaemic effects may be achieved by multiple mechanisms, including cholesterol reduction, degradation and biosynthesis, and increase in LDL uptake (Table 10.1) (Choi et al. 2010).

Other dietary factors such as safflower oil are likely to participate in obesity prevention by modulating mRNA levels of PPARα, Orexin, and Ghrelin gene expression of adipocytes in mice (Zhang et al. 2010). The *CLOCK* gene has been implicated in weight reduction in obese patients participating in a Mediterranean dietary program based on a relationship between *CLOCK* gene polymorphisms and obesity (Garaulet et al. 2010). Also, the mediterranean diet protected against waist circumference enlargement in 12Ala (pro 12Ala polymorphism) carriers for the PPARγ gene in a 2-year follow-up study of 774 subjects at high cardiovascular risk (Razquin et al. 2009).

Recent data show that hot water soluble solids, rich complement of polyphenols, from fermented rooibos (*Aspalathus linearis*) inhibit adipogenesis and affect adipocyte metabolism (Sanderson et al. 2013). Treatment of 3T3-L1 adipocytes with the rooibos soluble solids inhibited intracellular lipid accumulation. Inhibition of adipogenesis was accompanied by decreased messenger RNA (mRNA) expression of PPARγ, PPARγ, Sterol Regulatory Element Binding Transcription Factor 1 (SREBF1), and FASN, suggesting its potential in preventing obesity. Furthermore, the administration of Apple Polyphenols (AP) protects against body weight gain and fat deposition and improves glucose tolerance in rats, leading to reduced *Lep*, *Plin*, and SREBF1 mRNA levels and increased Aquaporin 7 (*Aqp7*), Adipocyte Enhancer-Binding Protein 1 (*Aebp1*), and Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha (*Ppargc1a*) mRNA levels in epididymal adipocytes (Boqué et al. 2013). Oils rich in phenols, whether natural (Virgin Olive Oil, VOO) or artificially added (SOP), (compared with sunflower seed oil), reduce postprandial inflammation of Peripheral Blood Mononuclear Cells (PBMCs) by activating nuclear NF-κB increasing cytosolic concentration of the NF-κB inhibitor (IκB-α), the mRNA levels of NF-κB subunits and activators (p65, IKKβ, and IKKα) and other inflammatory molecules (TNF-α, IL-1β, IL-6, MIF, and JNK), and lipopolysaccharide levels (Perez-Herrera et al. 2012).

On the other hand, a dose-dependent effect of oat cereal β-glucan on improving metabolic indexes of obesity in mice has been reported (Lin et al. 2013). Dose-dependent relation was observed on oat β-glucan and body weight change, average energy intake, total cholesterol, HDL cholesterol, plasma neural peptide Y, arcuate neural peptide Y mRNA, and Y receptor 2 mRNA levels.

Long-term dietary restriction (5 months) influences plasma ghrelin and Ghrelin-O-Acyltransferase (GOAT) mRNA, a satiety hormone, level in rats (Reimer et al. 2010). The adult dietary restriction (DR) rats gained less weight over 5 months and had lower fat mass than adult Ad Libitum (AL) rats. DR down-regulated proglucagon and cholecystokinin mRNA in the duodenum and ghrelin mRNA in the stomach of adult rats compared to AL; (GOAT) mRNA in the stomach was upregulated 14-fold compared to adult DR rats.

Gut bacteria may influence obesity through metabolites (mainly, SCFA) produced by dietary fiber fermentation. Obese microbiota elevates propionate production compared with that of normal weight from rye grain samples due to the lowest propionate production and highest butyrate production during fermentation (Yang et al. 2013). Thus, Al-Lahham et al. (2010) demonstrated that propionic acid (PA, SCFA) stimulates the anorexigenic hormone leptin expression and reduces the proinflammatory factor resistin in human adipose tissues. This suggests that PA is involved in the regulation of human energy metabolism and inflammation, and may thus influence the development of obesity and TD2.

Additionally, continuous Hawthorn Pectin Pentaoligosaccharide (HPP) ingestion may be used as dietary therapy to prevent obesity and CVDs. HPP significantly increased the hepatic fatty acid oxidation-related enzyme activities of acyl-CoA oxidase, carnitine palmitoyltransferase I, 3-ketoacyl-CoA thiolase, and 2,4-dienoyl-CoA reductase and their corresponding mRNAs (Li et al. 2013).

Other study showed the effects of dietary compounds [resveratrol, green tea extract, α-tocopherol, vitamin C, n-3 PUFAs, and tomato extract], by using a nutrigenomic approach by large-scale profiling of genes, proteins, and metabolites in blood, urine, and fat tissue in overweight men (Bakker et al. 2010). A multitude of subtle changes were detected by an integrated analysis of the "omics" data, which indicated that dietary mix modulates inflammation of adipose tissue, improved endothelial function, affected oxidative stress, and increased liver fatty acid oxidation in overweight men (Table 10.1).

High-protein diets favor weight loss and its maintenance. In this regard, an interesting study showed that leucine supplementation modulates fuel substrate utilization and glucose metabolism in previously obese mice by modulating several genes involved in fatty acid metabolism and mitochondrial activity in the epididymal white and the brown adipose tissues, but not muscle (Binder et al. 2013). Also, parts of vegetables' health benefits are mediated by changes in energy metabolism, inflammatory processes, and oxidative stress, according to Pasman et al. (2013).

10.5 Coronary Diseases: Gene-Nutrient Connection

CVD is caused by disorders of the heart and blood vessels, and includes coronary heart disease (heart attacks), cerebrovascular disease (stroke), elevated blood pressure (hypertension), peripheral arterial disease, rheumatic heart disease, congenital heart disease, and heart failure. The major causes of CVD are consumption of tobacco, physical inactivity, and an unhealthy diet. An estimated 17.1 million people died from CVDs in 2004, representing 29 % of all global deaths. By 2030, almost 23.6 million people will die from CVDs, mainly from heart disease and stroke (Williams et al. 2004; WHO 2009).

The relationship between dietary factors and coronary heart disease (CHD) has been a major focus of health research for almost half a century. The pioneering work of Keys and Aravanis (1980) stimulated many subsequent studies of diet and CHD, which have evaluated the effects of numerous dietary nutrients, foods, and dietary patterns on CHD risk. It has been suggested that broader adherence to recommendations for daily intake of fruits, vegetables, fish, and fatty acids may take away as much as 20–30 % of the burden of CVD and result in approximately 1 extra life year for a 40-year-old individual (Engelfrieta et al. 2010). Also, a Spanish cohort study showed an inverse association between adherence to the Mediterranean diet and the incidence of fatal and nonfatal CVD in initially healthy middle-aged adults (Martínez-González et al. 2011).

In another study, whole-grain and bran intakes were associated with reduced all-cause and CVD-specific mortality in women with diabetes mellitus suggesting a potential benefit of whole-grain intake in reducing mortality and cardiovascular risk in diabetic patients (He et al. 2010). Certain plant-derived foods and drinks, including chocolate, wine, berry juices, different types of tea, among others, have been targeted for different studies in the last decades for their potential use or benefit on cardiovascular health, both in vitro and in vivo. These foods contain a wide range of phenolic compounds, including flavonols, flavanols (catechins), procyanidins, and/or anthocyanins (Pascual-Teresa et al. 2010).

A randomized, parallel, controlled clinical trial in healthy volunteers suggests that benefits associated with the traditional mediterranean diet (TMD) and VOO consumption could be mediated through changes in atherosclerosis-related gene expression (Konstantinidou et al. 2010). TMD consumption decreased plasma oxidative and inflammatory status and gene expression related with both inflammation [INF-γ (INFγ), Rho GTPase-activating protein15 (ARHGAP15), and interleukin-7 receptor (IL7R)] and oxidative stress [adrenergic β_2-receptor (ADRB2) and polymerase (DNA-directed) κ (POLK)] in PBMC. All effects, with the exception of the decrease in POLK expression, were particularly observed when VOO, rich in polyphenols, was present in the TMD dietary pattern. Intake of another phenolic rich food, whole grape, impacts cardiac peroxisome proliferator-activated receptor, nuclear factor κB activity and cytokine expression in rats with diastolic dysfunction that may be significant to the observed diet-associated cardioprotection (Seymour et al. 2010).

Li et al. (2013) reported that quercetin and rutin exhibit cardioprotective effects on cardiac fibrosis induced by isoproterenol in rat's heart, moreover, the effects of rutin are weaker than quercetin in the same dose. The mechanism of these effects may be related to anti-oxidative stress, the renin-angiotensin-aldosterone system inhibition, decrease expression of transforming growth factor β-1 TGF-β1 and CTGF, and subsequent reduction in the deposition of the extracellular matrix. While an onion peel extract may have anti-thrombotic effects by downregulating MAPK activation upon coagulation stimulus, leading to prolong time for arterial thrombosis (Lee et al. 2013). Enzymatic extract of rice bran restores endothelial function and vascular contractility in obese rats by reducing vascular inflammation and oxidative stress by suppressing aortic inducible NOS (iNOS) and tumor necrosis factor (TNF)-α expression (Justo et al. 2013).

Dietary soy protein has been suggested to reduce cardiac lipid accumulation and the ceramide concentration in high-fat diet-fed rats and ob/ob mice (Torre-Villalvazo et al. 2009). Soy protein intake led to lower cholesterol and triglyceride concentrations in the hearts of rats and ob/ob mice in association with a greater PPAR**α** mRNA concentration and a low level of sterol regulatory element binding protein-1 mRNA. A study in a high cardiovascular risk population shows that the MTD influences expression of key genes involved in vascular inflammation [cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and monocyte chemoattractant protein (MCP-1)], foam cell formation [low-density lipoprotein receptor-related protein (LRP1), LDL receptor and CD36], and thrombosis [tissue factor (TF) and tissue factor pathway inhibitor (TFPI)] (Llorente-Cortés et al. 2010) (Table 10.1).

The very long-chain n-3 fatty acids, especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are believed to be particularly important in the prevention of CVD (Russo 2009). Preformed EPA and DHA are found predominantly in fish and fish oils. α-Linolenic acid (ALA, 18:3n-3), a shorter-chain n-3 fatty acid, is present in various plant-based foods such as flaxseed, walnut, soybean, and canola, can be metabolically converted to EPA and DHA and is also of interest for CVD prevention (Jung et al. 2008). The exact mechanisms by which n-3 fatty acids exert a cardioprotective effect are unclear but are being better defined. n-3 fatty acids can influence many aspects of CVD pathogenesis, including arrhythmias, lipid concentrations, blood pressure, platelet aggregation, vascular relaxation, inflammation, and likely arterial cholesterol delivery. For example, n-3 fatty acids affect the expression of several key proteins as modulators of many genes involved in lipid metabolism, inflammation, and smooth muscle cell proliferation, genes that can play a pivotal role in prevention and treatment of CVD and atherosclerosis (Fig. 10.3) (Jung et al. 2008).

Additionally, epidemiological studies suggest a protective effect of olive oil (monounsaturated fatty acids rich) consumption on CVD (Covas et al. 2007). In agreement with this, genes related with atherosclerosis processes were modulated after olive oil ingestion in humans (Konstantinidou et al. 2009). Also, olive oil polyphenols enhance the expression of cholesterol efflux related genes in vivo in humans, according to a randomized controlled trial (Farrás et al. 2013).

Other study explored the overall nutrigenomic effect associated with a TMD (Castañer et al. 2013). Of 18 cardiovascular canonical pathway analyses, 12 pathways were differentially expressed, and 43 % of pathways were modulated by TMD supplemented with VOO or nuts; the most prevalent pathways were related to atherosclerosis and hypertension.

However, a recent systematic review and meta-analysis of randomized controlled trials showed no evidence to support the use of vitamin and antioxidant supplements for prevention of CVDs (Myung et al. 2013).

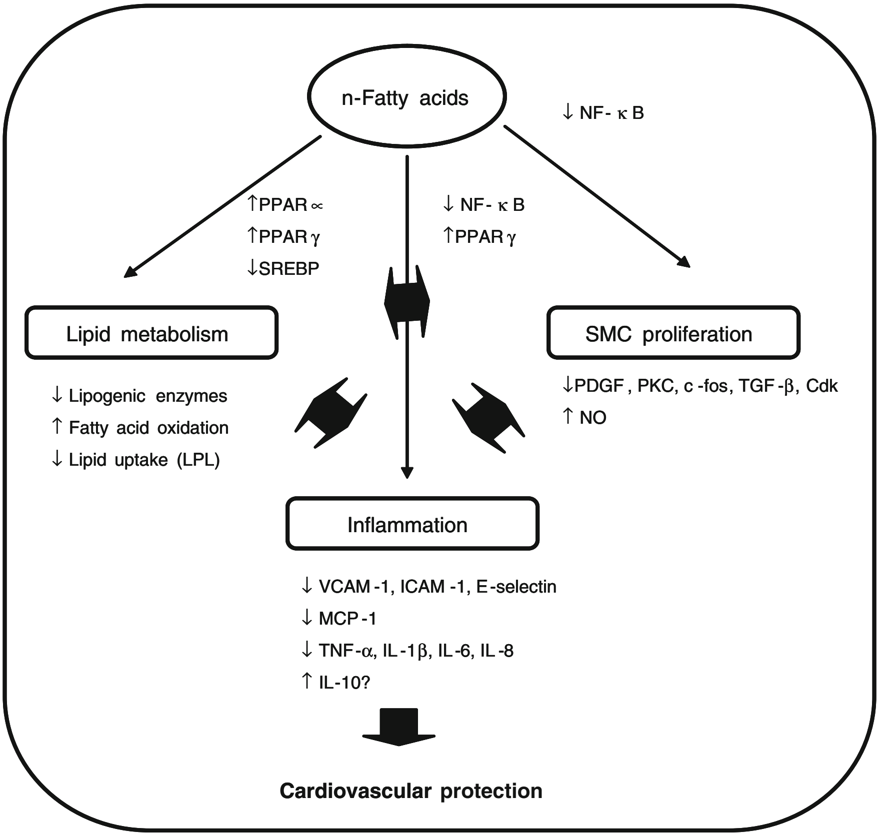

Fig. 10.3 Possible mechanisms for cardioprotective effect of n-3 fatty acids. *Cdk* cyclin-dependent kinase; *ICAM-1* intracellular adhesion molecule 1; *LPL* lipoprotein lipase; *MCP-1* monocyte chemoattractant protein 1; *NF-κB*; *NO* nitric oxide; *PDGF* platelet-derived growth factor; *PKC* protein kinase C; *PPAR* peroxisome proliferators-actived receptor; *SREBP* sterol-responsive-element binding protein; *TGF-β* transforming growth factor-*β*; *TNF-α*; and *VCAM-1*, vascular cell adhesion molecule-1 (Adapted form Jung et al. 2008)

10.6 Other Diseases

Alzheimer's disease (AD) is the main (40–60 %) type of dementia. Over 25 million people suffer dementia worldwide, and this number may escalate to 75 million in the next 20–30 years. Main risk factors for dementia include genetic factors, age, cerebrovascular disorders, and environmental factors (Cacabelos et al. 2003). Nutritional factors may also contribute to deteriorate cognition and brain function in dementia. Some genes potentially involved in nutrigenetics may also participate in lipid metabolism, brain function, and longevity. Novel nutraceutical products obtained from marine sources with biotechnological procedures have demonstrated atheroprotective properties and lipid-lowering effects, devoid of hepatotoxic activity. Some of these nutraceuticals exhibit a genotype dependent therapeutic effect, reflecting a nutrigenomic profile (Cacabelos et al. 2008). Some examples of genes relevant for both nutrigenomics and dementia are methylene tetrahydrofolate reductase (MTHFR), apolipoprotein E (APOE) and APOA1, leptin, interleukin-1 (IL1), tumor necrosis factor (TNF), and peroxisome proliferator-activated receptors (PPARs). Among these genes, APOE gene (19q13.2) is the most prevalent risk factor for AD, especially in subjects harboring the APOE-4 allele, whereas carriers of the APOE-2 allele might be protected against dementia (Cacabelos et al. 2005). Other study shows that orange juice has possesed the significant ability to increase significantly mRNA expression levels of tumor necrosis factor receptor (tnfr) gene in the brain neuronal tissues of male rats (Ebuehi et al. 2013). An interesting study reported that zinc and docosahexaenoic acid (DHA) affect expression levels of histones H3 and H4 in human neuronal M17 cells (Sidli et al. 2012). Zinc reduced the levels of the anti-apoptotic marker Bcl-2, while increasing the apoptotic marker caspase-3 levels correlating with cell viability assays. Conversely, DHA treatment resulted in increased acetylation of H3 and Bcl-2 levels and decrease in deacetylation, methylation, phosphorylation of H3 and caspase-3 levels, suggesting that DHA promotes gene expression and neuroprotection.

Another condition considered a major global health problem is *Hepatitis C virus* (HCV) that infects approximately 170 million people worldwide. The combination of pegylated interferon (IFN) with ribavirin is currently the most effective therapy for chronic HCV hepatitis, and long-term treatment has been shown to improve the sustained virological response (SVR) rate. However, the SVR rate still remains at approximately 55 % and patients with HCV genotype 1 infection combined with steatosis are significantly less likely to achieve a week-12 early virological response (EVR) or a SVR. Nutrigenomics offer hope in the search for novel therapeutic and nutritional management options. Dietary free fatty acids (FFA) composition can inhibit both HCV replication and HCV induced- lipogenesis genes. Three nutrients, β-carotene, vitamin D2, and linoleic acid inhibit HCV RNA replication and their combination has additive and/or synergistic effects on HCV RNA replication. PUFAs including arachidonic acid (AA), DHA, and EPA also inhibit HCV RNA replication, while saturated (lauric, myristic, and palmitic) and monounsaturated (oleic) fatty acids induce HCV RNA replication. PPAR and SREBP-1c genes, known to induce fatty acid oxidation and synthesis, respectively, are key targets for PUFA control of hepatic gene expression. n-3 PUFAs are weak agonists of PPARs compared with pharmacological agonists, but has significant effect on insulin sensitivity in various tissues, particularly skeletal muscle. These results provide useful information for improvement of the SVR rates of patients receiving the currently standard IFN therapy. In addition, these findings may contribute to the development of nutritional supplements of use in the treatment of people with chronic hepatitis C (Liu et al. 2010). More recently, Azocar and Diaz et al. (2013) evaluated the safety and efficacy of Chlorella in 18 patients chronically infected with HCV genotype 1. The HCV RNA levels showed a decrease in 69.23 % of the patients associated with a significant decrease in alanine aminotransferase liver enzyme levels.

HIV-infected individuals present a cluster of conditions that activate or injure the vascular endothelium. The administration of folates may exert beneficial effects on endothelial function in different populations at risk for CVD, according to the study of Grigoletti et al. (2013) where short-term folinic acid supplementation improved vascular reactivity in HIV-infected individuals.

An interesting review found that lentinan, a polysaccharides from shiitake mushrooms, is able to increase host resistance against various kinds of cancer and infectious diseases including acquired immuno deficiency syndrome (AIDS) (Rahman and Choudhury 2013). Lentinan is able to inhibit replication of Adenovirus type 12, Abelson virus and VSV- encephalitis virus. Although lentinan itself has no ability to block HIV infection, concomitant treatment with 3′-azido-3′- deoxythymidine (AZT) suppresses the surface expression of HIV antigens more than AZT alone. Additionally, phenolic compounds represent an important natural source of antiretrovirals for AIDS therapy due to their significant anti-HIV-1 activity and low toxicity. In a new search for potent anti-HIV-1 agents from plants, phenolic compounds isolated from methanol (70 %) extract of *Diospyros lotus* fruits were tested for anti-HIV-1 activity (Rashed et al. 2012). Gallic acid was the most active compound against HIV-1 with Therapeutic Index (TI) value of >32.84 and the other compounds were less potent.

Other study provides evidence that Rhamnogalacturonan II (RG-II), from Panax ginseng C. A. Meyer, plays a critical role in ameliorating the pathogenic process of asthmatic inflammation in mice (Jung et al. 2012). In this regard, Chinese propolis attenuates in vivo and in vitro asthmatic reactions (Ammar et al. 2013). This extract significantly reduced aggregation of inflammatory cells in bronchoalveolar lavage (BAL) fluid and in lung tissues with marked dilated bronchia. Also, ethanolic extract of propolis markedly reduced serum IgE and lung mRNA levels of inducible nitric oxide synthase (iNOS), transforming growth factor-β1 (TGF-β1), and tumor necrosis factor-α (TNF-α) in mice.

Table 10.1 summarizes recent studies on diet-gene expression on cancer, diabetes, obesity, and coronary and other diseases from cell, animal, and human studies.

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Chapter 11
New Horizons for the Production of Industrial Enzymes by Solid-State Fermentation

Gustavo Viniegra-González

Abstract This review tries to shed some light on the basic principles that might help to improve industrial production of enzymes by SSF, mainly in the research subjects of strain improvement, online process control, and the use of crude enzyme preparations in food, feed, and biopharmaceutical industries.

Contents

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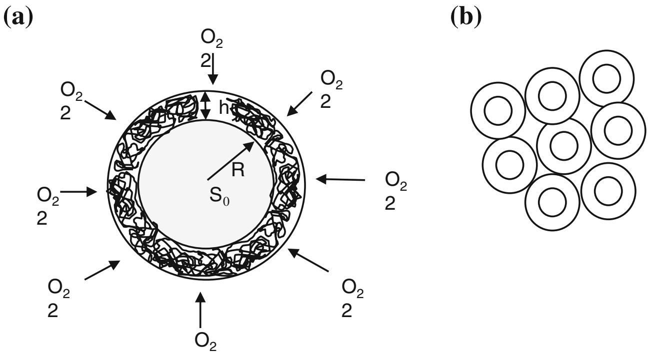

Fig. 11.1 **a** Diagram of a spherical particle with radius, *R*, imbibed with soluble substrate at bulk concentration, S_0, and supplied with oxygen at partial pressure, $[O_2]$ in the surrounding atmosphere. Biomass is growing on the solid surface with thickness, *h*. Specific area, $\alpha = 3/R$. Substrate availability, $\sigma_0 = S_0\alpha^{-1} = S_0(R/3)$. Oxygen and substrate flow toward the biomass layer in opposite directions. **b** Diagram of a packed bed of spherical particles with oxygen flowing-in and carbon dioxide flowing-out, through the interstitial space

11.1 Introduction

Enzymes are mass produced by submerged fermentation (SmF) using genetically engineered strains. For example, production of subtilisin, a heat-resistant protease added to household detergents or α-amylase and glucoamylases in the starch industry. However, at the beginning of the twentieth century, industrial enzymes were produced by solid-state fermentation (SSF) and were seen as an alternative to traditional malting of grains for the production of ethanol. After the Second World War, the use of conventional chemical engineering technology made it easier to scale-up SmF processes as compared to the more difficult scale-up of SSF. Later, the advent of genetic engineering of microbial strains selected gave impetus to SmF as the major choice for large-scale enzyme production. Notwithstanding such technological trends, there are new market signals to watch, mainly in the fields of saving water and energy and also for the new trends for "organic" foods and the management of complex fermentation systems. Thus, there are new horizons for upgrading old fashioned SSF systems because they require lower power inputs, generate ready-to-use crude enzyme preparations, reduce wastewater treatment and sometimes enzyme mixtures are more effective than pure enzyme

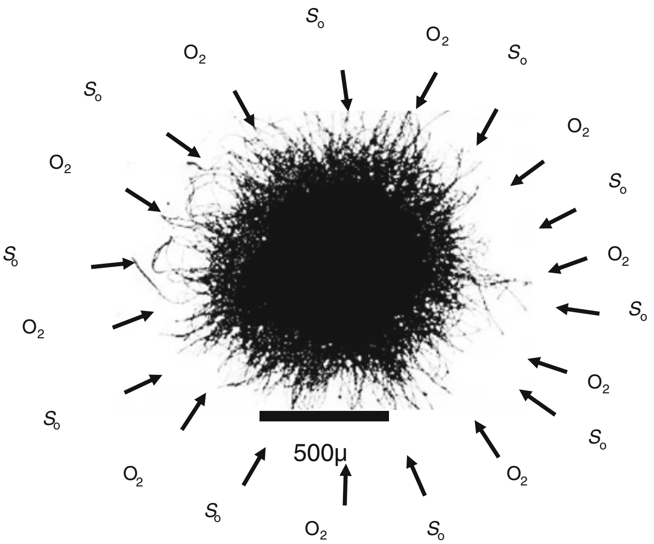

Fig. 11.2 Fungal pellet of *Aspergillus niger* grown by SmF in a shake flask. Oxygen (O_2) and sugar (S_0) flow from the bulk of the liquid toward the interior of the pellet

concentrates. Also, SSF has remained as the choice for important food processes such as koji-like industries in Asia.

11.2 Definition of Solid-State Fermentation

Solid-state fermentation can be defined as a culture of aerobic microorganisms made of filamentous or single cells growing preferentially on beds of moist solid particles (Fig. 11.1a), where interstitial air occupies more than 50 % of the total volume to allow passive gas diffusion (Fig. 11.1b), and the surface area to volume ratio (α = specific area) of the fermentation inter-phase is higher than $\alpha = 10\ \text{cm}^{-1}$. For example, wheat bran particles having a diameter smaller than $d = 0.1$ cm.

Such definition includes biodegradable supports such as wheat bran itself, cereal particles, and lignocellulosic fibers imbibed with nutritional broths. It also involves the use of non-biodegradable solid supports such as polyurethane foam or amberlite beads. However, it does not include the conventional surface cultures, either solid (agar plates) or liquid (shallow trays), where the specific area is typically lower than $\alpha = 10\ \text{cm}^{-1}$. This is supported by recent results of Ortega-Sánchez et al. (2012) showing that biomass yield is maximal when the specific area of the solid support is high, especially when the bulk substrate concentration is elevated ($S_0 \geq 0.1\ \text{g cm}^{-3}$).

Table 11.1 Oxygen diffusivity (D_{O_2}), oxygen concentration [O_2], and thermal conductivities in air and water ($T = 20^{\circ}$ C and $P = 1$ atm)

Medium	D_{O_2} ($cm^2 s^{-1}$)	[O_2] ($g\ cm^{-3}$)	κ ($Wm^{-1}K^{-1}$)
Air	1.50×10^{-1}[a]	3×10^{-4}	0.0257[c]
Water	1.97×10^{-5}[b]	6×10^{-6}	0.609[d]

[a] http://compost.css.cornell.edu/oxygen/oxygen.diff.air.html

[b] http://compost.css.cornell.edu/oxygen/oxygen.diff.water.html

[c] http://www.engineeringtoolbox.com/air-properties-d_156.html

[d] http://www.engineeringtoolbox.com/thermal-conductivity-liquids-d_1260.html

On the other hand, SmF are defined as stirred and aerated suspensions of cells where the gas phase (air) is mechanically dispersed in very fine bubbles ($d < 0.1$ cm) in order to have adequate oxygen mass transfer. In SmF processes, biomass is grown either as a dispersion of single cells or as clumps of cells (Fig. 11.2), with solid bulk concentration density lower than $S = 0.1\ g\ cm^{-3}$, i.e., substrate concentrations with biochemical oxygen demand (BOD) lower than 1,000 ppm ($1 \times 10^{-3}\ g\ cm^{-3}$).

Physical and physiological constraints for SSF cultures

Data shown in Table 11.1 indicate that diffusivities (D) and concentrations (C) of oxygen are higher in air than in water. Therefore, oxygen mass transfer in SSF is faster and requires less power inputs than in SmF. However, thermal conductivities (κ) are much higher in water than in air (Table 11.1) making heat transfer more difficult in SSF than in SSF processes.

Experimental data and modeling of SSF cultures of *A. niger* grown on packed bed reactor found that the dimensionless coefficient of heat generation was 10,000 times higher than the dimensionless coefficient of heat removal (Saucedo-Castañeda et al. 1990). This supports the notion that non-stirred fermentation beds should be thinner than 3 cm (Underkofler et al. 1947; Rathbun and Shuler 1983; Saucedo-Castañeda et al. 1990; Ridder et al. 1998). Otherwise, overheating will inhibit fungal growth.

Oxygen mass balance and oxygen profile measurements across fungal layers show that oxygen penetration depth within the fungal mat is smaller than $h = 0.01$ cm (Oostra et al. 2001; Rahardjo et al. 2002). This implies that aerobic SSF happens only in very thin biomass layers. Such notion was proposed longtime ago by Hill (1928) and discussed by Pirt (1966, 1967). In turn this justifies the need to design SSF processes with specific area larger than $\alpha = 10\ cm^{-1}$, to assure that the fungal mats are very thin.

Data published by Favela-Torres et al. (1998) and Ortega-Sánchez et al. (2012) using SSF cultures of *Aspergillus niger*, grown on Amberlite beads or agar plates, show that the inverse of biomass yield, $1/Y_{X/S} = gSgX^{-1}$, is proportional to initial surface substrate availability, $\sigma_0 = S_0\alpha^{-1}$ ($gScm^{-2}$) as indicated in Fig. 11.3 and is followed by Eq. (11.1)

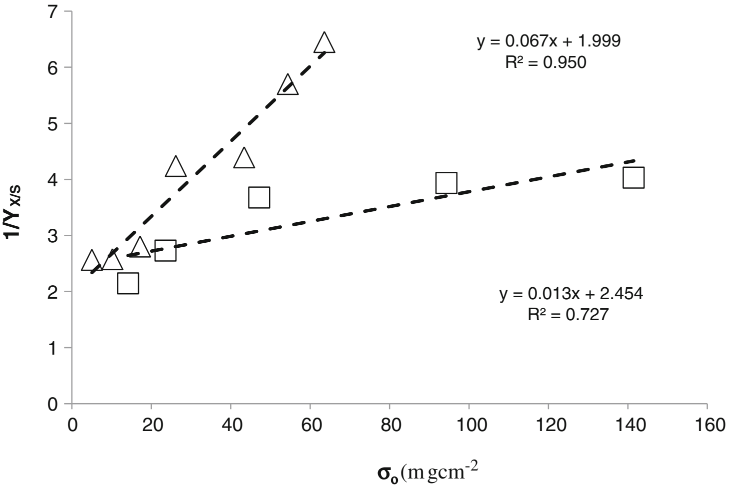

Fig. 11.3 Plot of inverse biomass yield ($1/Y_{X/S}$ = gS gX^{-1}) of *Aspergillus niger* grown with various glucose concentrations and two different solid support. (☐) Amberlite beads ($d = 0.06$ cm; $\alpha = 100\ \text{cm}^{-1}$) and (Δ) agar plates. Data from Favela-Torres et al. (1998) and Ortega-Sánchez et al. (2012), respectively. Initial substrate availability was calculated as $\sigma_0 = S_0\alpha^{-1}$ (mg cm^{-2}). In the case of agar plates, S_0 values, and α values, changed independently of each other. For Amberlite beads, the values of α were fixed and S_0 had different values

$$\frac{1}{Y_{x/s}} = \frac{1}{Y_0} + \frac{\sigma_0}{\varepsilon} \tag{11.1}$$

where, Y_0, is the maximal biomass yield and ε = (g cm^{-2}) is an efficiency factor inversely proportional to the ratio between maintenance (m) and growth (μ) coefficients. For example, $\sigma_0/\varepsilon \propto m/\mu$ (Ortega-Sánchez et al. 2012). Thus, ε corresponds to the maximal biomass surface density for a given culture. Correlations between Eq. (11.1) and data for two separate reports (Favela-Torres et al. 1998 on Amberlite beads, Ortega-Sánchez et al. 2012, on agar plates) are shown in Fig. 11.3. The maximal yield was similar for both correlations ($Y_0 \approx 0.5$) and was obtained when σ_0 was very small. Whereas $\varepsilon = 0.015$ g cm^{-2} for agar plates and $\varepsilon = 0.075$ g cm^{-2} for Amberlite beads, supporting the idea that coefficient ε depends on the culture conditions (strain, support, medium composition, etc.). On the other hand, $Y_0 = 0.5$ is the accepted maximal yield for aerobic cultures grown on glucose (Heijnen and Roels 1981).

Ortega-Sánchez et al. (2012) developed a mass balance model on the spherical shell of biomass with depth, h, covering spherical particles of radius R and having biomass volumetric density ρ_v (see Fig. 11.1a) as shown in Eq. (11.2)

$$M = (\Lambda + 1)^3 - 1 \tag{11.2}$$

where, $M = Y_0 S_0 / \rho_v$ and $\Lambda = h/R$. The right-hand side of Eq. (11.2) is the ratio $\phi = [(h + R)^3 - R^3] R^{-3}$, between, the volume of biomass shell over the volume of the spherical particle (see Fig. 11.1b). The dimensionless mass M is the ratio between biomass production (Y_0S_0) over the packing density of the mycelium (ρ_v). It should be noted that, after complete depletion of the substrate, the mass balance holds if $Y_0 S_0 = \phi \rho_v$. Solving for $S_0 = \phi \rho_v Y_0^{-1}$, Eq. (11.3) is obtained

$$S_0 = \frac{\rho V [\Lambda + 1]^3 - 1}{Y_0} \tag{11.3}$$

Assuming $h = 0.01$ cm, $R = 0.03$ cm, $\rho_v = 0.05$ g cm^{-3}, and $Y_0 = 0.5$ gXgS^{-1}, the value of $S_0 = 0.137$ g cm^{-3} is obtained, showing that it is possible to obtain high biomass yields with strong substrate concentrations ($S_0 > 0.1$ g cm^{-3}) if the value of the specific area of the solid support is high, $\alpha = 100$ cm^{-3}.

Lauckevics et al. (1985) suggested that SSF systems are limited by “steric hindrances” of the fungal mycelia growing in the interstitial space of the fermentation bed (Fig. 11.1b) but they did not clarify the nature of such hindrances. The average solid density of biomass measured with *A. niger* by Ortega-Sánchez et al. (2012) was $\rho_v = 0.05$ g cm^{-3}. Auria et al. (1990, 1993) obtained a value $\rho_v = 0.04$ g cm^{-3} with *A. niger* grown on Amberlite beads packed in slender columns and Nopharatana et al. (2003) reported a maximal value $\rho_v = 0.035$ g cm^{-3} for a surface culture of *Rhizopus oligosporus*. Assuming that solid content in the biomass is 20 %, wet biomass occupies 25 % of the fungal mat volume. Furthermore, the biomass densities obtained by Ortega-Sánchez et al. (2012) and Nopharatana et al. (2003) were measured on agar plates without any physical hindrances above the solid surface. Auria et al. (1993) measured the pressure drop of SSF culture in the interstitial space of a column packed with Amberlite beads having $R = 0.03$ cm and found a pressure drop to 5 % of the initial value. Perhaps the major physiological problem of SSF is not a geometrical hindrance limiting fungal growth but an increase in the tortuosity of the complex biomass network that limits adequate oxygen supply and creates frictional resistance when air is forced to flow through the interstitial volume of a fermentation bed. Anyway, the existence of a maximum value $\rho_v < 0.05$ g cm^{-3} is an important constraint for SSF when it is compared to yeast SmF cultures with densities up to $\rho_v \approx 0.20$ g cm^{-3} (DW) as indicated by Heyland et al. (2010).

It should be noted that the accepted solid content of microbial cells is 20 % and this very high value of cell densities should be viewed with caution because it implies that the cell suspension is a very thick slurry equivalent to the cream obtained in yeast factories after centrifugation of the fermentation beer. A value $\rho_v \approx 0.10$ g cm^{-3} for high-cell density cultures is more reasonable and undoubtedly higher than the measured value for ρ_v in SSF systems.

11.2.1 Scale-up of SSF Trays Packed with Solid Particles

Packing density of solid particles is a function of the particle geometry. According to Kyrylyuk and Philipse (2011), random packing of spheres yields a packing fraction around 0.63 (37 % porosity) and a maximal random packing of rods of 0.7 (30 % porosity). These estimates are higher than the experimental result of 0.49 packing density of 51 % moist wheat bran particles (Underkofler et al. 1947) in fermentation trays or 0.45 packing density for cylindrical columns packed with Amberlite beads (Favela-Torres et al. 1998).

Underkofler et al. (1947) reported the commercial operation of a moldy bran factory with a throughput daily capacity of 10 tons using mechanically loaded trays, 125 kg each (dry basis). Fermentation beds were, 3.8 cm thick, 1.5 m (5 ft) wide, and 4.3 m (14 ft) long. Hence, each daily batch involved the loading of 80 trays (10 trays h^{-1}). This shows that it is feasible to scale-up SSF tray factories if the loading and unloading of trays is made mechanically. For that purpose, the use of external mechanical vibrators has been patented (Virtanen et al. 2008). Upgrades of the tray SSF process are currently operated by a proprietary design of Altech, Co. implemented in Ciudad Serdán, Puebla (México) and by Plafractor® technology of Biocon in Bangalore, India (Mazumdar and Suryanarayan 1999).

11.3 A Brief Background of Enzyme Production by SSF

11.3.1 SSF in Koji-Based Processes

Production of enzymes by SSF is necessary for the production of Far East fermented foods such as *shoyu* (soybean sauce), *misó* (mold fermented paste), *natto* (Bacillus fermented soybeans), *saké* (rice wine), and vinegar (Hesseltine 1983). He mentioned that in 1979, the volume of fermented grains (rice or soybeans) in Japan and Korea was around 800,000 tons. They all require the production of a crude enzyme preparation called *koji* made of rice-fermented mashes inoculated with *Aspergillus oryzae.* processes used at industrial scale for more than a century in Japan and China (Machida et al. 2008).

11.3.2 Adaptation of Koji Industry to Starch Industry in USA

Takamine (1894a) adapted the koji process, using wheat bran for the production taka-koji or "*moyashi*" obtained by drying the spent moldy bran. He later patented the making of an alcoholic precipitate of a water extract of taka-koji (Takamine 1894b) called "diastatic enzyme." This precipitate was sold as a digestive aid called “taka-diastase” by Sankyo Co and was the pioneer of crude amylolytic extracts produced by SSF. Such developments were reviewed by Takamine (1914).

Underkofler (1938) patented the use of rotating drums in order to produce amylase by “moldy brans” or wheat bran fermented by *A. oryzae*. Underkofler et al. (1939) suggested the advantage of moldy bran over malting or acid hydrolysis in order to improve the conversion of starch into ethanol. As indicated in the preceding section, Underkofler et al. (1947) reported the successful industrial scale-up of the moldy bran process but using trays instead of rotating drums because of the attrition problems of fungal biomass by mechanical stirring. The process was used to produce a crude amylolytic preparation blended with cooked cereal grains that was the input for ethanol production. The final ethanol yields were around 80 % of the theoretical yield. Hence, Takamine and Underkofler planted the seeds of present bio-ethanol industries.

11.4 General Criteria for Developing Enzyme Production by SSF

Basic research on the production of enzymes by SSF processes started around 1980, and was published in more than 1,000 research papers but only a handful of enterprises have used this technology outside of China and Japan (Pandey et al. 1999; Viniegra-González et al. 2003). Here, we discuss the need to develop and apply three separate sets of criteria for the production of enzymes by SSF technology

(a) Improvement of microbial strains;
(b) Process control with special attention to changes in temperature and respiratory rates;
(c) Minimization of product recovery cost.

11.5 Improvement of Microbial Strains

11.5.1 Comparison of Enzyme Titers Between SSF and SmF Processes

It is worth stressing that comparisons between SSF and SmF processes should be done between industrial processes of similar throughput capacity. In order to illustrate this matter, a practical calculation is presented.

Enzyme titers are reported using the International Unit (amount of enzyme with activity $= 1 \times 10^{-6}$ mole P min^{-1}) produced on the basis of initial dry weight of a solid substrate ($E_S = \text{IU kg}^{-1}$) for SSF processes or on the basis of fermentation volume ($E_L = \text{IU L}^{-1}$) for SmF processes.

Machida et al. (2008) stated, “The ability of secretory production of proteins is further enhanced in solid-state culture compared with submerged culture. For example, *A. oryzae* can produce ~50 g of α-amylase from 1 kg of wheat bran, which is roughly equivalent to 1 L of liquid culture medium.” Terebiznik et al. (1996) reported the purification of α-amylase from *A. oryzae* with specific activity $\eta = 2\ 000$ IU mg^{-1}. This amounts to say that the enzyme titers reported by SSF Machida et al. (2008) in Japan would be $E_\mathrm{S} = 100$ million IU kg^{-1}.

According to van Brunt (1986) industrial SmF production of gluco-amylase using *A. niger* has reached levels in the order of $E_\mathrm{L} = 20$ g L^{-1} and the reported specific activity is $\eta = 1.4 \times 10^4$ IU g^{-1} (McDaniel et al. 2008). Hence industrial enzyme titers for SmF process would be around $E_\mathrm{L} = 2.8 \times 10^5$ IU L^{-1}. These reports indicate that SSF and SmF processes have reached the same of order of magnitude ($\approx$10 g) of active enzyme per unit of weight or volume, respectively, and are industrial benchmarks to take into account for strain improvement.

It should be noted that industrial production of amylases by SmF is done in fed-batch fomenters with throughput capacities higher than 10 m^3 per batch. On the other hand, the simplest bioreactor design for SSF processes is the shallow tray system with a throughput capacity of 10 ton per day. Thus, a fair comparison between both systems ought to be done with comparable working loads and taking into account the differences in specific activities. For example, the SmF factory with *A. niger* would produce 100 kg of active enzyme with $\Delta E = 140$ million units IU. The SSF factory with *A. oryzae* would produce 200 kg of active enzyme with $\Delta E = 400$ million IU. This comparison based on published data suggest that both SmF and SSF industrial systems have the same order of magnitude of productivity of enzymes used for starch hydrolysis. Apparently, the key to such productivity is in the high quality of the microbial strains adapted to the specific kind of industrial fermentation.

11.5.2 General Considerations on Strain Improvement

It seems important to recall that “improvement of the microbial production strain offers the greatest opportunity for cost reduction without significant capital outlay” (Stanbury et al. 1995) and it is important to identify the traditional and updated technologies for strain improvement.

Random mutation is the classical approach for strain improvement. This procedure requires the analysis of billions of mutants using manual or automated phenotypic screens scoring phenotypes on agar plates, test tubes, and shake flasks. Manual screening was, for many years, the choice for commercial operations. Now, this technology is put into practice in Asian countries where laboratory skills are available and labor costs are low. For more than 20 years Chinese laboratories were the main source for new improved strains but their increasing labor cost is forcing them to shift to mechanical screening. To put this situation into perspective it is worth pointing out that development of a superior industrial strain may require the work of scores of technicians doing repetitive work over a period of several years in order to isolate a superior microbial strain (Demain and Davis 1998). In Western countries with much higher labor costs, screening robots were developed called High Throughput Screens (HTS). They are effective and require high capital investments in the range of millions of dollars, since robot systems are not mass produced but tailor-made for that purpose (Parekh et al. 2000).

An interesting alternative is a combination of genetic engineering with random mutation. It usually takes two steps (a) A chosen gene, coding for an enzyme with interesting commercial applications is cloned from an organism with undesirable production features to a microbial strain with desired fermentation ability, and (b) the transformed strain with low potency is random mutated and screened by an HTS until a desired industrial strain is obtained.

A landmark development was the cloning of calf chymosin traditionally used for curd making but available in very small quantities. The gene was transferred and expressed by an improved strain of *A. oryzae*. Dunncoleman et al. (1991) reported the achievement of industrial fermentations with titers higher than $E_L = 1$ g L^{-1} and claimed to have used "a mutagenesis protocol with a novel robotic screening program." Such development was the basis of a series of patents assigned by the US Patent Office in 1992 to International Genencor Inc. Such breakthroughs paved the way to mass production of chymosin without the problem of a limited supply of slaughtered calves. It is worth recalling that glucoamylase, one of the most important bulk enzymes, is produced from an amplified copy of the corresponding gene of *A. niger* (van Brunt 1986). Thus, enzyme production by SmF processes has reached the level of commodity production.

An interesting possibility is to link a biochemical screen, such as resistance toward a toxic chemical, or a pleiotropic (multiple action) mutation that enhances the performance of a complex regulatory network. One interesting case is the discovery that microbial mutants resistant to 2-deoxyglucose (2-DG) very often become enzyme overproducers. This is related to the discovery that catabolite repression in yeast is associated to hexokinase PII (Entian 1980) and resistance to 2-deoxyglucose (2-DG) of many microbial organisms yield complex phenotypes, including de-repression of many hydrolases and phosphorylases and also, according to Carlson M (1999) *dgr* mutants are related to Snf1 protein kinase that is a "central component of the signaling pathway for glucose repression in yeast." Apparently this happens because *dgr* mutants, often have a pletiotropic effect on phosphorylases genes that reduce the toxicity of phosporylated DG and, at the same time, they de-repress regulatory networks (Ashokkumar et al. 2004). This technique has been applied to the selection of improved *A. niger* strains producing higher titers of pectinases as compared to the wild type (Antier et al. 1993 Similar results have been applied to *A. niger* invertase production (Montiel-González et al. 2002; Ashokkumar et al. 2004; Rajoka and Yasmeen 2005), xylanases (Bokhari et al. 2010), xylosidase (Rajoka and Khan 2005), beta-glucosidase (Bokhari et al. 2008), and cellulases (Rajoka 2005). But to the best of our knowledge none of those reported *dgr* strains have overcome the benchmark of $E_S > 10^4$ IU g^{-1}. Perhaps, a mixed approach of 2-DG resistance and THS is required to obtain strains comparable to such benchmark.

11.5.3 Improvement of Fungal Strains Adapted to SSF or SmF Processes

Shankaranand et al. (1992) wrote about “the need for an extensive screening program for the selection of a potent culture most suited for SSF system.” Antier et al. (1993) obtained DG-resistant mutants of *A. niger* in two culture media one with low water activity ($a_w = 0.96$) and other with high water activity ($a_w = 0.99$). The former were better adapted to produce pectinase in coffee pulp than the latter which were better for pectinase production in shake flasks suggesting that different genes were coding for the production of enzymes adapted to SmF or to SSF. Ishida et al. (1998) showed that the gluco-amylase gene, *glaB*, is specifically induced in SSF and not in SmF. In a similar way, Biesebeke et al. (2002) found that expression of gene *brlA*, necessary for sporulation, is specifically expressed by SSF of *A. oryzae.* Furthermore, Machida et al. (2008) indicated that the genome of *A. oryzae* used in *koji* processes “contains 12,074 genes” and Wang et al. (2010) published the transcriptome of *A. oryzae*, showing that “4,628 genes were differentially expressed between LC (liquid culture) and SC (solid culture), including 2,355 and 2,273 genes up- and downregulated on SC, respectively.” Altogether, such information is conclusive that nearly 40 % of the genome of *A. oryzae* is specialized for SSF and, consequently, the techniques for strain improvement of enzyme producers should be different whether their industrial use is for SSF or for SmF technologies. For example, the gene *glaB* is selectively activated when *A. oryzae* is grown on agar plates provided with a Millipore membrane opposing hyphal extension, whereas gene *glaB* is not (Ishida et al. 1998). These authors simulated the actual SSF environment on agar plates provided with starch, low a_w, high temperature, and Membrane Millipore, and obtained 6,300 IU/mg protein that considered equivalent to koji SSF. Biesebeke et al. (2005) also found differences on the expressions of glucoamylases and proteases between SSF and SmF and atributed to differences in carbohydrate metabolism.

Furthermore, Tsuchiya et al. (1994) made a fusion between pro-chymosin and *glaA* genes in *A. oryzae* and found that chymosin was expressed in SmF with levels on wheat bran 500-fold higher in SSF as compared to SmF, later, Ishida et al. (2006) suggested that the *glaB* promoter also can be used for recombinant protein production by SSF. Thus, a combination of empirical, physiological, and molecular knowledge seems to be quite relevant for new areas of SSF in the production of conventional enzymes and recombinant proteins.

11.5.4 Combinatorial Analysis of Regulatory Mutants in Complex Genetic Networks

Recent work indicates that improvement of fungal strains requires multiple mutations in domains such as, gene dosage, protein folding, glycosylation, protein transportation within the endoplasmic reticulum, and protein excretion at the hyphal tips (Geysens et al. 2009). This makes it necessary to consider multiple mutations for the isolation of fungal strains with very high potency in enzyme production. For example, the selection of a particular combination of k mutations in a domain of N mutations sites (codons), assuming that each mutation is non-synonymous (non-neutral), the number of possible combinations, C_R, is given by Eq. (11.4)

$$C_R = \frac{N!}{(N-K)k!} \tag{11.4}$$

After some manipulations and using the Stirling approximation, $\log(N!) \approx N\ln(N)$, with $k = N/2$, when C_R is maximal as a function of k. Equation (11.4) yields Eq. (11.5)

$$C_R \approx Ae^{N\ln(2)} = A10^{bN} \tag{11.5}$$

It seems necessary to compare the number C_R to the capacity of HTS working systems with a screening frequency, v, during a period of time, Δt, as follows:

$$v\Delta t = A10^{bN} \tag{11.6}$$

Solving for, N, the following relationship is obtained:

$$N = \frac{1}{b}\log(\frac{v\Delta t}{A}) \tag{11.7}$$

Taking, $A = 0.3216$, $v = 10^9$ strains/week, $\Delta t = 50$ weeks/year, $1/b = \ln(10)/\ln(2)$, it is concluded than in 1 year, the HTS could examine a maximum number of combinations at only, $N = 37$ mutation sites. Equation (11.7) shows that given the finite capacity of HTS, it is practically impossible to screen all the possible combinations of mutants obtained in a large genetic network, posing the question of using alternative ways to improve the screening for superior microbial strains. Obviously the number, C_R, will approach, N, if $h \to 1$. Therefore it seems quite important to use strong constraints for the experimental screening of superior strains as well as in the search of optimal choices of parameters of genetic and metabolic in silico networks. In other words, to reach significant results in a finite period of time it is necessary to screen for very few simultaneous mutations, k, in a rather small domain of mutation sites, N. This opens the question on how to put evolutionary constraints in the selection of superior mutants.

11.5.5 Adaptive Evolution In Silico and In Vivo of Microbial Systems

In the current scientific literature, special attention is paid to adaptive evolution, where fast growing strains are enriched in the population after various hundreds of generations. In this context, Price et al. (2003) indicated the need to use physical, metabolic, and physiological constraints when modeling in silico adaptive experiments. Along such line of thought, Ibarra et al. (2002) grew an *E. coli* K-12 strain for 700 generations on glycerol as a single carbon source. They observed that the evolved population increased their growth rate from a suboptimal to near optimal level as calculated previously by in silico. This result proved the usefulness of constrained metabolic models in the search for opportunities to improve strains using adaptive evolution. Furthermore, Dykhuizen and Dean (1990) showed that the relative fitness of two strains of *E. coli* growing in a mixed culture with lactose as carbon source was explained by differences in two kinetic steps: lactose permease and lactase. They emphasized the importance of finding the metabolic bottlenecks to optimize adaptive evolution experiments.

Therefore, it seems quite possible to improve microbial strains, after few hundred generations of adaptive selection, if the objective function (the phenotype) is related to the growth process. For example, if certain enzyme activities are related to the intake and utilization of a given carbon source. The problem is how to put constraints forcing the evolving organism to improve specific functions. For example, related to the improvement of a small set of enzymes. This is a promising approach because it is leading toward the identification of few mutation sites related to strategic points of the metabolic network, instead of searching throughout the complete genetic network. Such a new approach could be the way to combine random mutation during adaptive evolution, with optimal in silico design of culture conditions.

11.5.6 The Use of Image Analysis for Automated Screens of Microbial Strains

An interesting possibility for scoring superior mutants is the use of automated image analysis of small colonies grown on agar plates (Loera and Viniegra-González 1998) or directly on wheat bran particles (Couri et al. 2006, Dutra et al. 2008).

Loera and Viniegra-González (1998) used image analysis to estimate the growth rate and pectinase potency of a collection of *A. niger* mutants. They found that potency was inversely correlated to growth rate, in high or low levels of water activities. This result can be related to the observation of Wösten et al. (1991) that glucoamylase secretion is located at the tips of leading hyphae of *A. niger*. Apparently, enzyme secretion and fungal growth compete for the same space at the hyphal tips. As a consequence, more potent strains would have lower hyphal extension rate.

Couri et al. (2006) and Dutra et al. (2008) developed an automated procedure of image analysis for direct measurement of fungal biomass growing on the surface of wheat bran particles. They found a linear correlation between the enzyme activities and mycelial surface area. This is an important result because it provides a non-destructive method to measure biomass grown on irregular solid particles that are intended to be used for actual SSF processes. A possible future development is the use of HTS with image analysis using small samples of solid substrates together with colored enzyme reactions in such a way that strain improvement is done in very similar conditions to their practical application.

11.6 Online Process Control of Enzyme Production by SSF

Bulock et al. (1965) observed that *Penicillium* cultures had "two successive physiological phases … the first, or trophophase, (and) the second, … or idiophase." In the first phase, exponential growth occurred. In the second phase, secondary metabolism was dominant and gave rise to the formation of conidia. Ikasari and Mitchell (2000) found that those two phases can be related to an initial exponential increase of the respiration rate, followed by an exponential decay. This was confirmed by Lareo et al. (2006). Thus, the transition from trophophase to idiophase can be followed by respiratory curves. Also, enzymes produced during the first phase are often destroyed to be recycled as nitrogen sources in the second phase and this fact makes important to control the physiological state of an SSF process by the oxygen uptake rate (OUR) and the carbon dioxide production rate (CPR). Therefore, online process control of SSF process helps to assess the quality and extent of enzyme production. Furthermore, it has been shown that online respirometry is a good way to estimate the heat balance of the whole culture, because substrate oxidation is necessarily associated to heat dissipation (Saucedo-Castañeda et al. 1990). This approach of process control was established more than 30 years ago in SmF processes. But it took some years to adapt online respirometry to SSF processes. Saucedo-Castañeda et al. (1994) stated "the lack of such facilities (online respiratory monitoring) has been, in part, responsible for the neglect of SSF processes up to 1980 in Western and European countries." Online respirometry has been applied to monitor the production of, *β*-*N*-acetylhexosaminidase of *Verticillium lecanii* (Matsumoto et al. 2004), lipase of *Rhizopus homothallicus* (Rodríguez et al. 2006) and lactase of *Kluyveromyces marxianus* (Tovar-Castro et al. 2008). Furthermore, the data acquisition system has been improved (Chinn et al. 2003) together with advanced programming analytic techniques (Araya et al. 2007) and the use of sensors and alternative measurements has been reviewed and discussed by Bellon-Maurel et al. (2003). The use of NMR imaging and respirometry of *A. oryzae* cultured on wheat kernels, showed the complex diffusional and physiological processes involved in SSF cultures (Hoogschagen et al. 2001). Altogether the instrumentation and analytical past limitations of SSF processes have been overcome with alternatives for advanced process control.

11.7 Using SSF Solids as Crude Enzyme Preparations

Reduction of downstream processing in SSF operations include the use of fermented solids, either wet or dry, in a variety of applications. This way, expensive treatment of fermentation waste waters is avoided and energy expenditures are minimized. Having this in mind, it seems useful to consider that

(a) Enzyme application should not require pure enzymes, or even more, should be benefited by the use of crude enzyme preparations.
(b) Enzyme recovery should include biological stabilization of the material without affecting the desired catalytic activities.
(c) Crude preparations should be free from toxic or other undesirable properties.
(d) Possible recycling of final residues in a profitable and environmental friendly way.
(e) There should be a significant reduction of the carbon foot print of integrated processes.
(f) Integration between the production of SSF solids and their use should be cost-effective.

11.7.1 The Use of Wet SSF Solids in the Production of Foods and Feedstuffs

As mentioned in preceding sections traditional soybean and rice fermentations are very important in food industries of several Asian countries. In Japan, *koji* (wet fermented rice) is used as a biocatalyst to transform soybean paste into a bouillon base called *miso* (Ebine 2004). The process involves the blending of *koji* with a paste of steamed and salted soybeans and is incubated in closed vats until the desired reaction point is reached (Hesseltine 1967, 1983). In *saké* (rice wine) production, *koji* is blended with steamed rice and inoculated with yeast, letting the fungal enzymes supply sugar to yeast in order to produce alcohol (Akita 2007).

An interesting adaptation of Japanese SSF technology was developed in Spain by Murado et al. (2008). They found a way to produce *shochu*, a liquor distilled from *moromi* (a traditional alcoholic bagasse), which in turn is produced from a chestnut porridge by a combined action of *A. oryzae* and *Saccharomyces cerevisiae.* The liquor was produced in Galicia to be exported to Japan. A similar approach was followed by Schmidt et al. (2001) by blending the SSF fermented mash with alfalfa silage and improving roughage digestibility. This approach is amenable for biorefinery operations in order to transform starch, cellulose, and hemicelluloses into fermentable substrates with significant reduction of energy costs.

The practical importance of *koji* approach is related to important downstream savings because product recovery is the most expensive part of enzyme production. To illustrate this point is worth noticing that SmF beers never surpass the level of $E_S = 50\ \mathrm{g\ L^{-1}}$ and protein recovery involves time and energy consuming operations such as: ultra filtration, fractionated precipitation, or affinity chromatography. Also, the remaining liquid effluents require expensive operations because of their high BOD loads in such a way that the investment on the treatment plant is as high as the capital cost of the main SmF facility. Therefore, the use of wet SSF solids as catalysts is a very efficient way to reduce downstream expenses.

11.7.2 The Use of Dry SSF Products as Environmental Catalysts

Cammarota et al. (2001) found that dry SSF solids produced as fermented cakes of babassu (*Orbignya oleifera*) by *Penicillium restrictum* improved the anaerobic digestion of dairy waste waters by UASB (Up-flow Anaerobic Sludge Blanket) reactors fed with organic loads having nearly 1 g L^{-1} of fat. Untreated effluent had a Chemical Oxygen Demand (COD) removal of only 50 % but pre-treated effluents had 90 % COD removal. Same procedure was applied successfully for the anaerobic treatment of poultry slaughterhouse effluents (Valladão et al. 2007), waste waters from swine meat (Rigo et al. 2008) and waste waters from dairy industries (Damasceno et al. 2008). This use of SSF solids was also useful for the pre-treatment of oily effluents digested by active sludge systems (Jung et al. 2002; Rosa et al. 2006).

Those are examples of how dry SSF solids can be used as catalysts to solve environmental problems. Again, downstream costs are lower than recovering enzymes from SmF beers because the final moisture content is lower than 50 % and the use of industrial oven dryers is straightforward.

11.7.3 The Use of SSF Solids as Industrial Biocatalysts

Nagy et al. (2006) found that dry SSF cultures of *Mucor hiemalis*, “without special enzyme isolation processes” were able to resolve enantiomeric mixtures of secondary alcohols. Fernandes et al. (2007) and Salum et al. (2010) have proven the concept of using SSF dry solids as biocatalysts in the production of biodiesel with ethanol instead of the conventional use of methanol and without a strong base dissolved in water. Martínez-Ruiz et al. (2008) have used as biocatalyst the cultures of *Rhizopus* sp. grown on perlite particles mixed with olive oil. This biocatalyst was able to produce ethyl-oleate from a mixture of oleic acid, ethanol and hexane. In this case, oil cakes will be used as solid substrates for the production of lipases, oils will be transformed in biodiesel and the final solid residues can be recycled as feedstuffs or compost.

Hoskins and Lyons (2009) have used dry SSF solids obtained from cultures of *A. oryzae* grown on distillery grains, as an enzyme complex to be mixed with the corn mash in a similar way that Underkofler et al. (1947) used moldy bran but now in the context of biofuel production. Also, Hölker (2007) has patented the production SSF catalysts by a continuous fermentor. He mixed such catalyst with grass silage to improve biofuel production (methane or ethanol) by a subsequent anaerobic fermentation of the agricultural residues. These examples indicate that SSF dry solids can be quite helpful in the design and operation of biorefineries.

11.7.4 SSF Solids as Feed Additives

Castor beans (*Ricinus communis*) are being considered in Brazil by PETROBRAS as an alternative raw material for biodiesel production but the cake, obtained after oil extraction, has toxic compounds that forbids its use as a feedstuff. Godoy et al. (2011) showed that SSF with *Penicillium simplissimus* detoxified this cake and produced lipases that can be used in the formulation of a variety of animal diets. So it seems possible to integrate SSF lipase production, with a final disposal of detoxified castor bean cake as a feedstuff additive.

SSF solids rich in phytases are used as poultry feed additive in order to reduce the load of phytates that hinder phosphate absorption in cereals (Wu et al. 2004). This additive is produced in Ciudad Serdán (Puebla State, Mexico) by Alltech de México SA de CV (2013) and uses proprietary tray technology. They claim that their product called Allzyme SSF® is a mixture of seven enzymes that help to increase feed efficiencies in poultry or pigs.

Therefore, the use of SSF, initiated at the beginning of the twentieth century as an adaptation of koji fermentation to starch processing industry, is being reconsidered in the fields of fine chemicals, biofuel, and feed industries.

11.7.5 Mass Balance Between Production and Use of SSF Solids

An interesting industrial problem is the mass balance between a fermentation unit producing crude enzyme extracts and the reaction unit transforming raw materials into final products. For example, SSF solids (wet or dry) blended with cereal mashes for the production of sugars.

The analysis of this problem requires a few definitions such as the number of annual batches of the fermentation unit, n_F, and the reaction unit, n_R. Each fermentation batch is loaded with, Q, kilograms of biodegradable dry mass (starch, cellulose or hemicellulose), and is run through an average operational time of t_F (hours). The resulting crude extract (SSF solids) has an enzyme titer, E_S (IU kg M^{-1}), and is used in the reaction unit for the production of ΔP kilograms (sugars) for an average reaction time, t_F (h). The mass balance has a frequency factor $1/\tau = t_F/(t_R)^2$, and is given by Eq. (11.8).

$$QE_S\tau = \Delta P \tag{11.8}$$

The time factor, τ, includes the ratio, t_R/t_F, in order to balance the timetable of fermentation and reaction units and is multiplied by t_R which is the reaction time to produce ΔP by the action of E_S enzyme units produced by the fermentation of Q kg of substrate.

Solving for E_S in Eq. (11.8) yields Eq. (11.9)

$$E_S = \frac{\Delta P}{Q\tau} \tag{11.9}$$

where, Q and ΔP, are expressed in consistent units (moles or kilograms). In many practical applications, $t_F \approx (t_R)^2$. For example, the fermentation period, t_F, is often close to 4 days ($t_F = 96$ h) with a reaction period, t_R, of nearly 10 h. Now, let us assume a fermentation unit with $Q = 10^3$ kg per batch, having 75 fermentation batches in 300 days and producing small molecules (MW $\sim$ 200 g/mol) in 600 reaction batches. It would require an enzyme titer $E_S = 10^6$ IU gM^{-1} in order to yield 7,200 tons of product per year (96 tons of product per ton of solid substrate load). This example illustrates that the benchmark of $E_S = 10^6$ IU Kg^{-1} given in the Introduction is a reasonable goal for industrial SSF fermentation units. Unfortunately, most of the published papers from academic laboratories have enzyme titers quite below this benchmark and this may explain why only few new enterprises have been created outside Far Eastern countries, because of the scarcity of superior fungal strains that emulate the operational levels achieved by Asian SSF industries. Perhaps the advent of cheaper HTS robots, constrained by in silico models of metabolic networks, and aided by automated analysis of mutant strains will help to develop new industrial applications for SSF systems.

11.8 Conclusions

Production of enzymes by SSF have new horizons for industrial development in the fields of food fermentations, transformation of organic residues, new feed additives, and biofuels. But important improvements should be made in the areas of mechanical loading and unloading of trays, high-throughput screens of potent microbial strains, new conceptual approaches toward integrated biorefineries made of fermentation and reaction units followed by online process control.

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Chapter 12
Advances in Computational Fluid Dynamics Applied to Biosystems

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Contents

12.1 Introduction

The study of the environmental conditions in the biological process is complicated, as in many cases it is necessary to use models based on energy transfer. Mathematical models based on mass and energy balances assume a homogeneous environment. These models generate a set of nonlinear ordinary differential equations without an analytical solution. However, a more detailed monitoring of the environment inside these processes reflects a two-dimensional and three-dimensional variability of environment variables. Recently, this problem has been

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tackled using the fundamental equations of fluid dynamics. The set of numerical methods applied in order to solve those equations are called Computational Fluid Dynamics (CFD). This technique provides a numerical solution from an energy balance of a controlled volume, which in comparison with other methods and expensive technologies allows an efficient study of the environment conditions (Rico-Garcia et al. 2008). CFD considers the values of the independent variables as primary unknowns in a finite number of places inside the domain, and then a set of algebraic equations are derived from the fundamental equations applied to the domain and can be solved by pre-established algorithms.

The study of complex biosystems, in which there are several physical, chemical, and biological interacting processes and phenomena, has been favored by the development of computer simulation tools during the last decade and with the increase in computational processing power it is possible to develop numerical models such as more accurate simulations for transport phenomena and energy exchange (Norton et al. 2007). As a consequence, these studies have led to improvements in the design of buildings and equipment such as greenhouses, broilers facilities, stables, and bio-digesters.

According to Boulard et al. (2002), CFD is a branch of fluid mechanics that use numerical methods and algorithms to solve and analyze problems involving fluids flow. Therefore, it is possible with the use of computers to perform millions of calculations to simulate the interaction of liquids and gases with surfaces defined by the boundary conditions. In recent studies of modeling of airflow, CFD has deepened to test its effectiveness in relationships of climatic factors (Bournet and Boulard 2010). Computational parametric studies on greenhouse structures can help in the identification of design factors that affect greenhouse ventilation under specific climatic conditions (Rico-García et al. 2011).

In the past years, many studies have used CFD to investigate the climate conditions inside buildings. CFD has been able to increase the degree of realism by taking into account climatic conditions and simulation of biological processes, considering temperature, humidity, CO_2, and solar radiation, among others in 3D models. The results have been able to improve our understanding of the phenomenon of ventilation. Therefore, this chapter discusses significant recent studies to understand how the use of CFD has evolved.

12.2 Fundamental CFD Equations

CFD is based on the governing fluid dynamics equations (continuity, momentum, and energy). The equations obtained directly from the volume or fixed element in space is known as “conservative form.” The equations obtained directly from the volume or movement with the fluid element are called “non-conservative form” (Anderson 1995).

12.2.1 Substantial Derivative

The substantial derivative is physically the exchange rate of any substance that moves with a fluid element. It consists of two parts, where the first part is called the local derivative, which is physically the rate of change over time in a fixed point. The second part is called the convective derivative, which is physically the exchange rate due to movement of the fluid from one point to another in the field of fluid, where the fluid properties are spatially different. The resulting material can be applied to any field variable fluid, for example: pressure (p) or temperature (T) (Anderson 1995).

$$\nabla \equiv i\frac{\partial}{\partial x} + j\frac{\partial}{\partial y} + k\frac{\partial}{\partial z} \tag{12.1}$$

$$V \equiv (u, v, w)$$

$$\frac{\partial}{\partial t}\iiint_V \rho \mathrm{d}V + \iint_S \rho V \cdot \mathrm{d}S = 0 \tag{12.2}$$

$$\begin{aligned}
\rho\frac{\mathrm{D}u}{\mathrm{D}t} &= -\frac{\partial p}{\partial x} + \frac{\partial t_{xx}}{\partial x} + \frac{\partial t_{yx}}{\partial x} + \frac{\partial t_{zx}}{\partial x} + \rho f_x \\
\rho\frac{\mathrm{D}v}{\mathrm{D}t} &= -\frac{\partial p}{\partial x} + \frac{\partial t_{xy}}{\partial x} + \frac{\partial t_{yy}}{\partial x} + \frac{\partial t_{zy}}{\partial x} + \rho f_y \\
\rho\frac{\mathrm{D}w}{\mathrm{D}t} &= -\frac{\partial p}{\partial x} + \frac{\partial t_{xz}}{\partial x} + \frac{\partial t_{yz}}{\partial x} + \frac{\partial t_{zz}}{\partial x} + \rho f_z
\end{aligned} \tag{12.3}$$

$$\begin{aligned}
\rho\frac{\mathrm{D}w}{\mathrm{D}t}\left(e + \frac{V^2}{2}\right) = \rho q &+ \frac{\partial}{}\left(k + \frac{\partial T}{\partial x}\right) + \frac{\partial}{\partial y}\left(k + \frac{\partial T}{\partial y}\right) + \frac{\partial}{\partial z}\left(k + \frac{\partial T}{\partial z}\right) - \frac{\partial(up)}{\partial x} \\
&- \frac{\partial(vp)}{\partial y} - \frac{\partial(wp)}{} + \frac{\partial(ut_{xx})}{\partial x} + \frac{\partial(ut_{yx})}{\partial y} + \frac{\partial(ut_{zx})}{\partial z} + \frac{\partial(vt_{xy})}{\partial x} + \frac{\partial(vt_{yy})}{\partial y} \\
&+ \frac{\partial(vt_{zy})}{\partial z} + \frac{\partial(wt_{xz})}{\partial x} + \frac{\partial(wt_{yz})}{\partial y} + \frac{\partial(wt_{zz})}{\partial z} + pf \cdot V
\end{aligned} \tag{12.4}$$

(1) Continuity equation
(2) Momentum equation (a nonconservative)
(3) Components in x, y, and z
(4) Energy equation (a nonconservative)

The equations form a coupled system of partial differential nonlinear equations. So far no analytical solution has been found. It is commonly assumed that the fluid is an ideal gas where the intermolecular forces can be neglected. For an ideal gas equation of state is:

$$p = \rho RT \tag{12.5}$$

where R is the specific gas constant. For a calorically ideal gas we have:

$$e = CvT \tag{12.6}$$

where Cv is the specific heat at constant volume (Norton et al. 2007).

12.3 CFD Procedure

Domain setting and grid generation are the first steps in the modeling process. This implies the choice of a computational domain that is large enough to correctly assess the main mechanisms that occur in the system and to avoid interference with artificial boundaries (Bournet and Boulard 2010). CFD modeling process comprises three stages, preprocessing, solution, and post-processing.

Preprocessing is the most time-consuming activity because mesh generation is the basis for a good simulation; therefore keeping an adequate strategy should allow reliability in the calculation and physically consistent results. The meshing process is based on a series of activities that can be summarized as follows:

(a) Geometry generation (Fig. 12.1)
(b) Geometry decomposition
(c) Computational mesh generation (Fig. 12.2)
(d) Functional and quality meshing
(e) Defining the boundary conditions and
(f) Export mesh

One of the most important problems currently in CFD is that it is time-consuming for a simulation to converge due to the high number of cells that come from the process of meshing. Thus, for many years it was chosen to model in two-dimensional (2D) simulations. However, air distribution inner greenhouse has a third-dimensional (3D) pattern, for that nowadays 3D CFD models are more commonly developed. According to Bournet and Boulard (2010) the calculation process is an iterative one that requires the definition of convergence criteria, and the user of the CFD codes must decide on an appropriate level of convergence, generally 10^{-4} of the value of a variable at all of the nodes. Faster convergence may also be reached by optimizing the grid shape or by assuming the Boussinesq model rather than by setting up the problem on the basis of the ideal gas theory. However, this choice may not be applicable in the case of large thermal gradients and may also overlook the influence of temperature on air viscosity.

The presence of turbulence in a fluid is indicated by the fluctuating velocity components and the quantities carried out by the flow, even when the boundary conditions for the problem under study are kept constant. These fluctuations determine the difference between laminar flow and turbulent flow (Fig. 12.3). For most situations ventilation (effect of temperature, wind or both) measurements and visualization experiments have demonstrated the turbulent airflow inside and

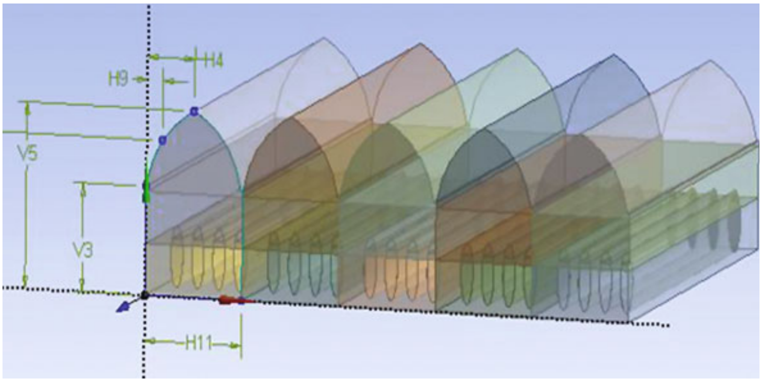

Fig. 12.1 Geometry generation for a greenhouse

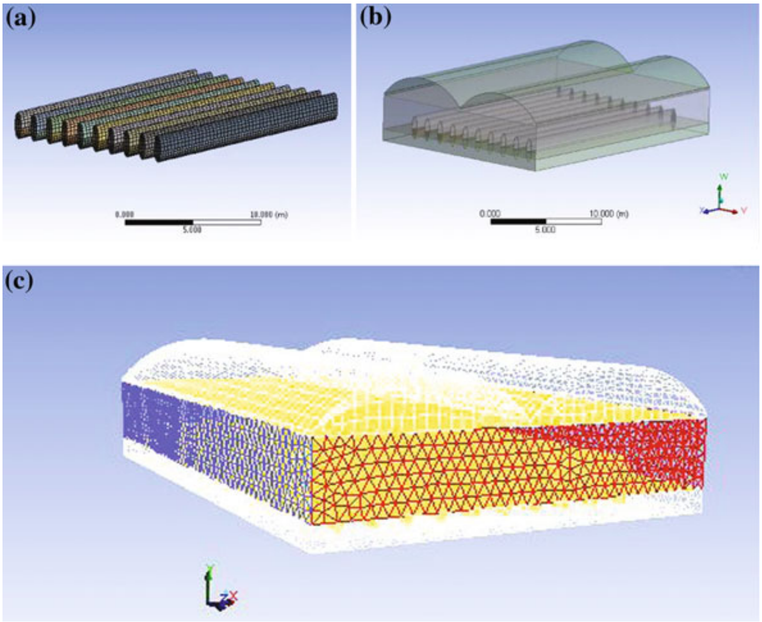

Fig. 12.2 Computational mesh generation for a greenhouse (De la Torre-Gea and Rico-García 2012)

outside the greenhouse. Therefore, the phenomenon of turbulence must be taken into account (Norton et al. 2007).

If inertial effects are large enough with respect to viscous effects, then the flow can be turbulent. Turbulence means that the instantaneous velocity varies at each point in the flow field. The turbulent nature of the velocity can be explained considering that the rate consists of the sum of two components, a main component

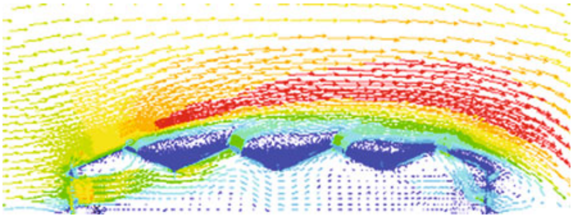

Fig. 12.3 The velocity vectors on a multi-hood greenhouse of four ships. CFD model considers anti-insect mesh vents (Ríco-García 2008)

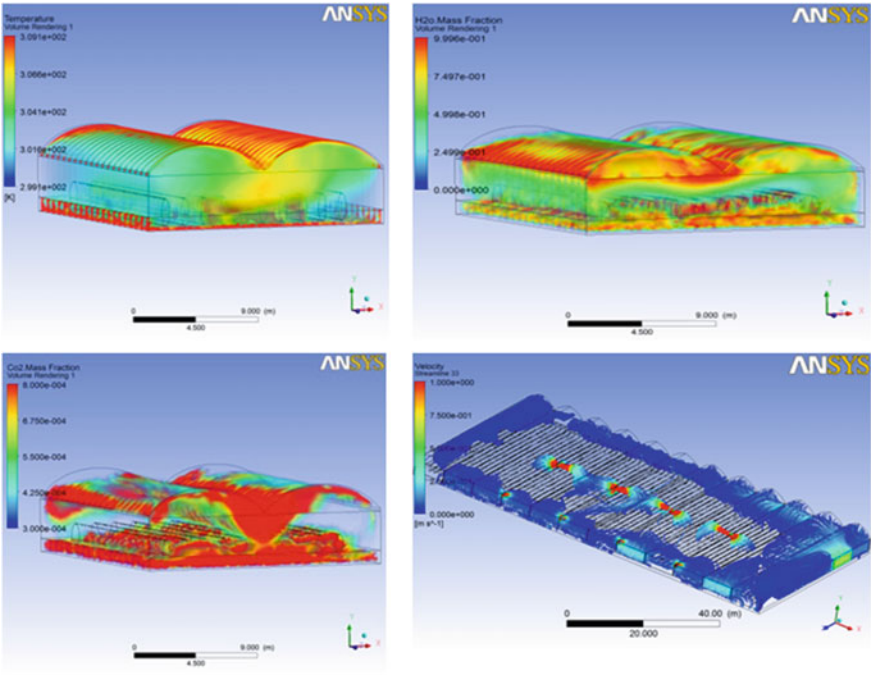

Fig. 12.4 Wind velocity (m s^{-1}) CO_2 concentration (kg m^{-3}), humidity (kg m^{-3}) and temperature (°C) representative postprocessing characteristics in different scenarios (De la Torre-Gea and Rico-García 2012)

(stable) and a fluctuating component. Most turbulence models, standard k-ε Model, Re-Normalized Group Turbulence Model (RNG), to name a few, increase the turbulence model flow viscosity (Rico-García 2008).

The postprocessing stage allows the user to visualize and search for the solution. Figure contours, vectors, and graphs can be obtained by analyzing the solution. It is remarkable that figures allow us to observe the full distribution of temperatures, speeds, pressures, etc., on the whole flow field (Fig. 12.4).

As soon as the CDF model has been tested, the greenhouse environment computational can become a powerful climate analysis tool. Currently, it is possible to see, for instance, the wind distribution along the greenhouse when the income windows are up or down, and the consequent temperature profiles, among many other possibilities. Although in the last decade research on wind behavior inside the greenhouse has been enormous, yet, as a fundamental part of the greenhouse environment modeling process it is necessary to take into consideration the physical verification in order to provide certainty about the results obtained by numerical simulation. Scale models, water and wind tunnels, and measurements of the climatic variables are some of the main options for verification of the CFD models of the greenhouse climate.

12.4 CFD Approaches

CFD modeling is used to design facilities that provide suitable climatic conditions for crops. According to Sase (2006), within a mild climate, appropriate design and control of ventilation is required to ensure effective cooling and uniformity of the environment. It is possible to design an optimal greenhouse by calculating the area, volume, and vents area as well as the material properties of the roof (Impron et al. 2007).

Rico-García et al. (2006), comparing two different greenhouses, showed the importance of its geometry and found that the ventilation rate for a greenhouse with larger vertical roof and windows was better than a multi-span greenhouse. Omer (2009) describes several designs of low energy greenhouses. In agreement with Baeza et al. (2008), design changes in the greenhouse, such as size and shape of vents, can improve air movement in the area of crops. Bakker et al. (2008) investigated energy balance, determining that the amount of energy used per unit of output is defined by improvements in energy conversion, environmental control to reduce energy consumption, and efficiency of agricultural production.

In a study of outdoor areas using the turbulence model Reynolds-averaged Navier–Stokes equations (RANS), van Hoff (2010) found that small geometric modifications can increase the ventilation rate by up to 43 %. The performance of ventilation in enclosed spaces is affected by the flow of outside air, type of cover, height of the installation, and the ventilation opening (Kim et al. 2010). Computational parametric studies on greenhouse structures can help to identify design factors that affect greenhouse ventilation under specific climatic conditions (De la Torre-Gea et al. 2011a).

12.4.1 Wind Directions

The wind direction outside the greenhouse is an important factor in defining the flow of air and climate inside the greenhouse system. The boundary conditions of wind speed distribution are deduced from experimental data and wind direction with respect to the longitudinal axis of the greenhouse, which can range from 0 to 90°. Roy and Boulard (2005) simulated the impact of wind at 45 and 90°, showing the influence of wind direction in the air velocity, temperature, and humidity distributions inside the greenhouse; a similar result was found by Campen (2008). Rico-García et al. (2006) also showed that a greenhouse with larger vertical roof windows works better with a windward condition, whereas the multi-span greenhouse works better with a leeward condition. Therefore, wind direction affects the degree of ventilation. In an experiment carried out by Khaoua et al. (2006), four different openings of roof vents obtained ventilation rates from 9 to 26.5 air exchanges per hour for the windward and 3.7–12.5 on the leeward wind condition, respectively, which can maintain acceptable and uniform climate conditions for particular cases where the wind is perpendicular to the main axis of the greenhouse. Overhead ventilation to the windward and leeward directions represents a reduction in the ventilation rate by 25–45 %, compared with only opening to the windward direction (Bournet and Ould Khaoua 2007). Openings to the windward direction generate the highest rate of ventilation; however, the greatest homogeneity of the temperature and wind speed arises from combining windward and leeward roof vents (Bournet and Ould Khaoua 2007).

Kacira et al. (2008) showed that the air temperature inside the greenhouse was higher on the windward side than on the leeward side when roof vents were used. Wind speed had a linear influence on air exchange rates, while the wind direction did not affect them. Majdoubi et al. (2009) observed a strong wind air current above a tomato canopy that was fed by a windward side vent and a slow air stream flowing within the tomato canopy space. The first third of the greenhouse, until the end of the leeward side, was characterized by a combination of wind and buoyancy forces, with warmer and more humid inside air that was removed through upper roof vents. There may be a conflict between increasing ventilation and improving uniformity because there is little information on air movement affecting the cooling efficiency and the uniformity of the environment (Sase 2006). According to Rico-García (2008) the relationship between the thermal gradient and ventilation of gases shows a linear behavior, while the relationship between the combined effect of temperature and wind greenhouse ventilation presents a piecewise linear behavior. The wind pattern in a greenhouse is strongly affected not only by the outside wind velocity but also by the number of greenhouse spans. In case the greenhouse has three or four span roof windows orientation is independent, however, when the greenhouse has five or more spans, side ventilation is dominant over the roof ventilation (Fig. 12.5).

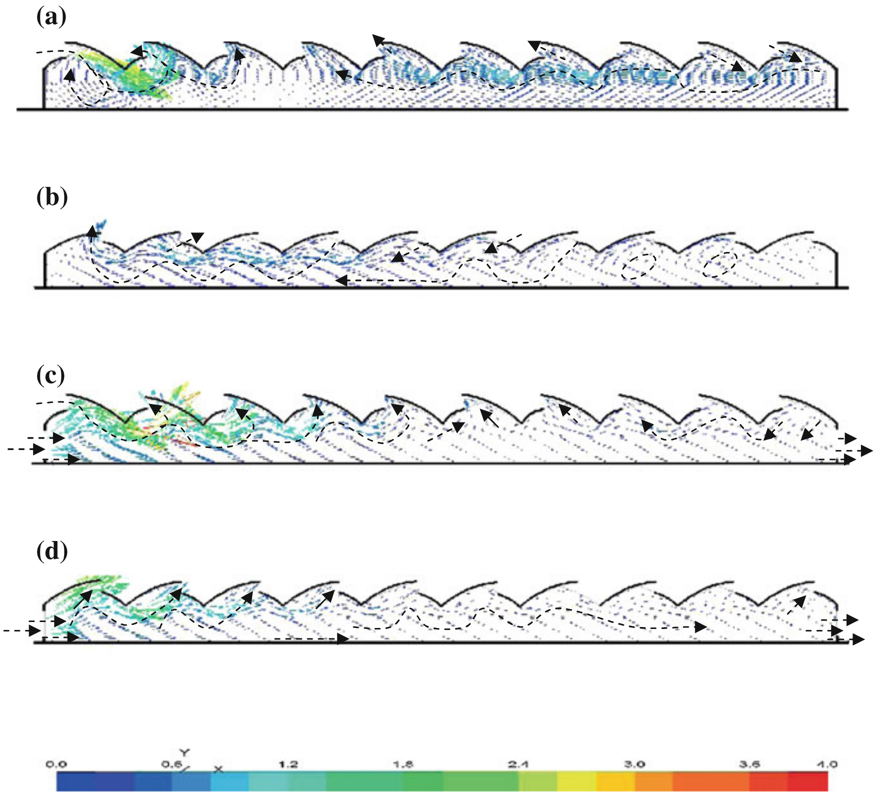

Fig. 12.5 Wind velocity interior vectors with 5 ms^{-1} wind velocity outside the greenhouse, on four open windows sceneries tested. **a** Roof windows windward open, **b** roof windows leeward open, **c** roof and side windows windward open, **d** roof and side windows leeward open

12.4.2 Heat Exchange

Rouboa and Monteiro (2007) simulated, by using a CFD model, the effects on temperature and wind speed on the introduction of hot water pipes along a greenhouse at nighttime conditions, under three shapes: natural convection heating (case A), artificial heat pipes (case B), and natural ventilation (case C) by using the turbulence model. Re-Normalization Group (RNG) observed an average increase in air temperature to 2.2, 6.7, and 3.5 °C; the turbulence was lower for case A, slightly increasing with the heating system for case B, and higher for case C, due to the effect of natural ventilation.

12.4.3 Solar Radiation and Temperature

Some studies have used solar radiation and transpiration models based on the heat and water balances of the crop, to investigate the distributions of air temperature and humidity and also the interactions between the crop and the air, in addition to the airflow distribution (Sase 2006). According to Tablada et al. (2005), the factor of solar protection plays a crucial role in maintaining stable thermal conditions indoors, even if the outside air temperature is higher. The slightly higher air speed on the top floor is insignificant in view of reducing the negative effect of the solar radiation over the roof and facade. The temperature of the greenhouse cover is an essential parameter needed for any analysis of energy transfer in the greenhouse. A sub-model developed by Impron et al. (2007) calculated the transmission of radiation through the greenhouse, including the reduction of NIR transmission through the roof. Tong et al. (2009) developed a numerical model to determine time-dependent temperature distributions based on hourly measured data for solar radiation, indoor air, soil, and outside temperature, taking into account variable solar radiation and natural convection inside the greenhouse during the winter in northern China.

12.4.4 Temperature and Air Exchange

The effect of solar and thermal radiation is often taken into account by setting specific wall or heat fluxes at the physical boundaries of the greenhouse. Radiation transfer within the crop itself is still the major concern since it determines the two main physiological crop processes: transpiration and photosynthesis. This challenge is now launched and will probably receive more attention within the next few years (Bournet and Boulard 2010). Pontikakos et al. (2006) analyzed data obtained from a CFD model, showing that the external boundary temperature is a critical parameter in the pattern of internal greenhouse temperatures and that for specific external temperatures and wind directions, airspeed becomes the crucial parameter. According to Molina-Aiz et al. (2006), opening vents affect the airflow, the ventilation rate, and the air temperature distribution in a greenhouse, where the mean air temperature at the middle varied from 28.2 to 32.9 °C with an outside air temperature of 26 °C, there were regions inside the greenhouse that were 13 °C warmer than the outside air. Nebbali et al. (2006) used a semi-analytical method to determine the ground temperature profile from weather parameters and other characteristics, to help in evaluating heat flux exchange between the surface and the air. Rico-García et al. (2008) showed that ventilation in greenhouses due to the temperature effect produces high air exchange rates; however, those air patterns occur near the openings, causing almost no air exchange in the central zone of the greenhouse due to a stagnant effect that reduces the wind effect throughout the greenhouse. In agreement with the results of Majdoubi et al. (2009), convection and radiation are the dominant forms of heat transfer. The measurements show that the difference between the air temperature inside and outside the greenhouse is strongly linked to solar radiation and secondly to wind speed. However, Chow and Hold (2010) obtained the following conclusions from studying buoyancy forces from thermal gradients:

(a) Thermal radiation without air involvement changes air temperature distribution by radiating upper zone thermal energy in the wall toward the lower zone wall, which then affects air temperature through conduction and convection;
(b) The inclusion of air absorption increases the effect of radioactive thermal redistribution by allowing air to absorb and radiate heat, reducing temperature gradients further; and
(c) Thermal boundary conditions and heat loads affect the predicted absolute temperature bounds, but do not affect the temperature distribution.

Radiation conditions play an important role in redistributing heat. Atmospheric conditions, especially relative humidity, are important for the calculation of radiation and heat transfer.

12.4.5 Turbulence and Buoyancy

As computing power has increased, the complexity and sophistication of CFD models have also increased. According to Norton and Sun (2006), the standard k-ε turbulence model commonly used in CFD models for greenhouses, in some cases provides inadequate results, and the choice of turbulence models must be based on the phenomena involved in the simulation. Different turbulence models give rise to differences in speed, temperature, and humidity patterns, confirming the importance of choosing the model that most closely matches the actual conditions of turbulence (Roy and Boulard 2005). Teitel et al. (2005) showed that the output of the turbulent heat flux is mainly due to cold air entering the greenhouse, which produces hot and cold eddies coming in and out of the greenhouse. Roy and Boulard (2005) showed that the effects of wind direction on climate parameters inside the greenhouse are usually simulated by using different turbulence models available, to determine the energy balance between the flow of perspiration and the flow of radiation. Under ventilation parameters based on Bernoulli's theorem, Majdoubi et al. (2007), showed that bad ventilation performance is not a result of the low value of the greenhouse wind-related ventilation efficiency coefficient, but rather, that the low rate of discharge due to pressure drop in airflow is generated both by the use of anti-insect screens with small openings as an obstruction due to the orientation of the rows of crops. Moreover, Rouboa and Monteiro (2007) note that the RNG turbulence model is best suited to simulate microclimates in arc-shaped greenhouses.

According to Baxevanou et al. (2008), the circulation of air buoyancy effect shows the importance of internal temperature gradients, forced convection resulting from natural ventilation predominates. Rico-García (2008) found that applying temperatures as the main driving forces for the buoyancy effect provides a simple way to study ventilation and inner air patterns. Vera et al. (2010a) observed that differences in temperature and ventilation rates strongly influence the movement of air, pushing it through openings where space is colder, while creating rising air currents when it is hot. Majdoubi et al. (2009) showed that the buoyancy forces induced by air temperature and increased humidity result in loops of air between the crop and the roof windows, which in turn tend to accelerate the pace of evacuation of heat and water vapor, enhancing indoor climate. Fidaros et al. (2010) studied turbulence in Greek greenhouses and found that external temperature variation is very important because internal temperature is determined by convection induced by the input current. The housing area had a higher circulation in the center of the greenhouse near the deck and in the corners of the ground, where the effect of the input current is weak. Defraeye et al. (2010) used a RANS turbulence model in CFD simulations to evaluate heat transfer by forced convection at the surface of a cube immersed in a turbulent boundary layer for applications in the atmospheric boundary layer (ABL), where wind speed is not disturbed at a height of 10 m. In a study of airfoil wakes, three turbulence models were simulated by Roberts and Cui (2010); the Reynolds Stress Model (RSM) is superior to the k-ε model, and when a time-dependent solution is necessary, LES is the desired option. However, LES does require the airfoil geometry to be included in the domain because it performs poorly when given only inlet velocities, turbulence kinetic energy, and eddy dissipation at the trailing edge of the airfoil. According to Bournet and Boulard (2010), although they have been used for a long time in both agriculture and environmental studies, less empirical approaches to turbulence based on the use of LES have never been applied to greenhouse climate modeling and might perhaps be used to look for a solution to this complex situation.

Inside buildings, it is difficult to maintain a thermally stratified space with low ceilings, such as in offices and houses. Vera et al. (2010b) studied buoyancy in enclosed spaces, drawing the following conclusions:

(a) Rising air currents and the exchange of humidity are closely related to the temperature difference between the lower and upper spaces. Low temperature in the upper space promotes the exchange of humidity and airflow through the opening; the hotter you are, the greater the restriction of air and humidity transport.

(b) The existence of upward air currents when the space is warmer than the bottom is caused by local conditions such as nonuniform temperature distributions in the upper space and convective warm currents of the base and humidity source.

(c) Compared with conditions without mechanical ventilation, ventilation severely restricts the flow of air through the opening.

The main difficulty in the choice of the model is that greenhouse systems cover a range of length and velocity scales that generally require different modeling approaches (Bournet and Boulard 2010).

12.4.6 Humidity

Roy and Boulard (2005) simulated wind directions of 0, 45, and 90° with respect to the orientation of the greenhouse to determine wind speed, temperature, and humidity distributions inside the greenhouse, getting a good approximation for the humidity. In agreement with Demrati et al. (2001), models allow estimation, with better accuracy of water requirements for a banana crop under cover and improved water saving in regions where water is the main limiting factor for agriculture. Roy et al. (2008) studied moisture on the surface of leaves at low light levels; crop transpiration and airflow were integrated into a single parameter model of leaf stomatal response to airflow and radiation. Campen (2008) showed that climate through a ventilation system is more homogeneous and the control is more efficient than with the conventional method of steam extraction. Dehumidifiers and cooling reduce the overall difference in humidity between the middle and lower areas of a greenhouse, as demonstrated by Kim et al. (2008) who used a 3D model to identify the heterogeneous distribution of relative humidity in a greenhouse. According to Majdobi et al. (2009), an increase in air temperature precedes a more moderate increase in specific humidity.

12.4.7 Incorporation Crop Effects and Crop Modeling

The effect of plants on greenhouse ventilation has also been studied in the past. For instance, Bournet and Ould Khaoua (2007), assumed that a crop of 90 cm high and low density decreases between 12 and 15 % greenhouse ventilation. Dayan et al. (2004) built a representative model of a greenhouse for three vertical segments, horizontally oriented to the directions of energy and vapor transfer between the segments containing plants, considering the external environment. They concluded that Representative Plant Temperatures (RPTs) can be calculated instead of measured. Roy and Boulard (2005) developed a 3D model for the characterization of climatic conditions in a greenhouse, incorporating five rows of ripe tomatoes as a porous medium where the buoyancy, heat, and moisture transfer between the crop and airflow inside were considered. The heat and moisture transfer coefficients are deduced from the characteristics of the laminar boundary layer of the leaf, which are calculated with the velocity of flow in the crop. Khaoua et al. (2006) found that under external conditions of 1 m s^{-1} air velocity and 30° of temperature, wind speed at crops' height vary according to the modalities of ventilation from the windward 0.1 and 0.5 m s^{-1} for the leeward side, while temperature differences range from 2.0 to 6.1 °C. In a study with tomatoes, Majdoubi et al. (2007) found that crop rows oriented perpendicular to air movement reduce the rate of airflow through the cultivation in a greenhouse by 50 %. According to Baeza et al. (2008), a greenhouse with natural ventilation efficiency must combine a sufficient number of air changes to remove excess heat, with good circulation of air through the crop. The effect of the crop was evaluated by Impron et al. (2007) by using a sub-model to determine the effects of ventilation, the properties of the cover, and crop transpiration. In agreement with Kruger and Pretorius (2007), the temperature and velocity at the plant level are influenced by the arrangement and number of windows. A study carried out by Sapounas et al. (2007) simulated a tomato crop as a porous medium, taking into account the addition of buoyancy to develop a model of the pressure drop of airflow due to the crop. The model depended on the area of leaf stage growth and cultivation, under the RANS turbulence model in conjunction with the RNG k-ε turbulence model. The results, validated with experimental measurements obtained at 1.2 m in the canopy, show that the evaporative cooling system is effective with numerical parameters, providing a useful tool to improve system efficiency. A study by Roy et al. (2008) on leaf level through an experimental setup based on Münger cells measured the temperature, relative humidity, and different heat flows to the leaves of soybeans, obtaining minimum stomatal resistance values ranging from 66 to 200 sm^{-1}.

Teitel et al. (2008) built a small-scale model and found that wind direction significantly affects the ventilation rate and temperature distribution in crops. A study by von Elsner et al. (2000) on the effect of near-infrared (NIR) reflecting pigments in microclimate and plant growth found that a temperature drop up to 4 °C in a young crop is the result of a 18 % reduction in the transmission of global radiation in spring. At the same time, during the rainy season, minimizing transpiration differences in temperature and shading reduces water requirements in the plants, and they observed parthenocarpic fruit rot and yield-reducing crop. In a tunnel-type greenhouse, a tomato crop was modeled by Bartzanas et al. (2008) by designing a porous medium, where they emphasize the influence of the heating system on greenhouse microclimate. The climatic behavior of the rows of the tomato crop is taken into account using external user defined functions (Baxevanou et al. 2008). According to Majdoubi et al. (2009), reorienting crop rows in simple ways improved climatic conditions. Endalew et al. (2009) performed CFD modeling of a plant with leaves and branches of the canopy, using turbulent energy equations in porous sub-domains created around the branches. Fidaros et al. (2010) simulated a greenhouse tomato crop as a porous medium to model radiation transport by discrete ordinates (DO). According to Teittel (2010), when applying the porous medium approach, the Forchheimer equation is often used, which can cause erroneous results with respect to the pressure drop through screens. An alternative way to calculate it through several panels of porous media used to simulate screens with realistic geometries. Moreover, the crop exerts a mechanical strain (drag force) on the flow just above but also interacts through the transpiration process with the temperature and humidity distributions (Bournet and

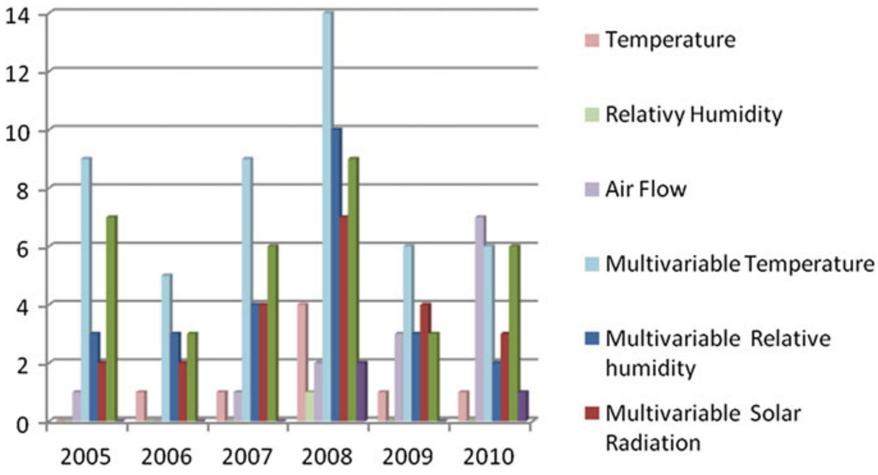

Fig. 12.6 Frequency of climatic variables analyzed by CFD models applied to greenhouses

Boulard 2010). A simple model of transpiration of a crop was developed by Sun and Wang (2010), who related it to the characteristics of ventilation in a greenhouse in eastern China, obtaining a good approximation. In general, there have been enormous efforts devoted to the analysis of ventilation in greenhouses (Norton et al. 2007); each new study provides new elements not only in the movement of air in the greenhouse but also in the forms it takes due to interactions occurring in the environment, such as position, shape, and size of windows, and (one of the most important), the presence of a crop (De la Torre-Gea et al. 2011b).

12.5 CFD Models in Greenhouses

Many CFD studies are focused on defining the conditions for a suitable environment. There has been less work on automation and control variables. Investigations that seek for a greater understanding of the interactions among climatic variables are increasing. Studies such as those of Teittel (2010), and Fidaros et al. (2010), evaluating geometries, have increased in the last year. Figure 12.6 shows the frequency of climatic variables studied during the period from the year 2005 to 2009 in studies of CFD models in greenhouses.

Most studies show multi-variable relationships, of which temperature and airflow are more common. Humidity has been linked to temperature, while there are still few CO_2 distribution models. Solar radiation is the subject of investigations that evaluate housing, and is also related to the temperature in simulations with a greater degree of realism.

Several studies have investigated the influence of solar radiation on temperature and relative humidity (Tablada et al. 2005; Impron et al. 2007; Tong et al. 2009),

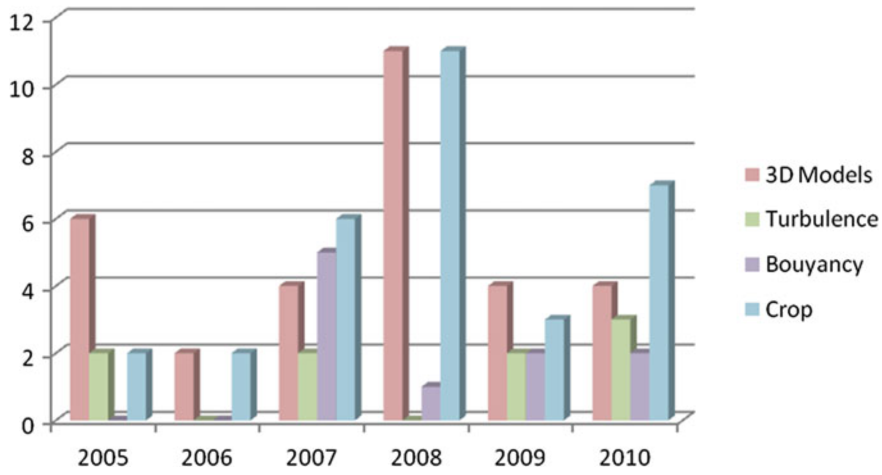

Fig. 12.7 Degree of realism and accuracy in the CFD models for greenhouse climate

and the result in crop response (Baxevanou et al. 2008). Other studies evaluated the incorporation of pigments (von Elsner et al. 2000) taking into account the convection and thermal gradients.

Most of the studies developed 3D models, some of which reported the use of models of turbulence and buoyancy, which have appeared more often during the past 2 years (Fidaros et al. 2010; Defraeye et al. 2010; Norton et al. 2010; Majdoubi et al. 2009). Incorporating turbulence models can make simulations more accurate, in turn increasing the processing and memory requirements for computing resources. Norton and Sun (2006) and Roy and Boulard (2005) discuss the importance of choosing the turbulence model that best meets the conditions of the study. Moreover, the concept of buoyancy appears frequently in order to incorporate the effects of growing space on the airflow and temperature gradients into the models.

Many studies consider the growing space, some of which are designed to measure phenomena based on their influence on the development and crop yield. Other studies are focused on the influence of crops on the other elements, such as temperature, relative humidity, CO_2 concentration, and airflow, where it is necessary to model the space occupied by the crop by using porous media (Fidaros et al. 2010). Other investigations measure biological phenomena such as evapotranspiration and PAR (Baxevanou et al. 2008) using indirect measures of climatic variables. However, some studies do not mention an experimental phase aimed at validating the numerical model. In studies of airflow, the experimental methods mostly used are scaled models and unidirectional anemometry; the tracer gas technique is used less often, as well as three-dimensional anemometry, which is considerably more expensive. Studies that have used new methods to assess ventilation systems, such as those by Molina et al. (2010), Endalew et al. (2009), Mikulka et al. (2010), and Defraeye et al. (2010) have been increasing in the past 3 years. The main question is the validation of these studies because they mainly concern with real scale greenhouses, whereas the measurements and characterizations have merely been done on scale models (Fig. 12.7).

CFD modeling is an area of knowledge that in recent years has developed enormously through the development of software and hardware, which has contributed to research on natural ventilation a greater understanding of the interactions between the variables that make up the climate inside greenhouses. In the past 5 years, CFD simulation has become increasingly realistic and detailed, obtaining more accurate solutions. However, their use requires depth and extensive knowledge of climatic variables, fluid dynamics, and turbulence. Simulating more accurately requires more processing power, so research tends to use CFD in conjunction with other tools. Further studies are required to incorporate more realistic crops beyond a porous medium, taking into account the role of gas exchange, which is necessary for an understanding of the physiology and phenology of crops. There is still a need to develop high-precision systems in greenhouses, and CFD is a powerful tool for defining parameters with high precision.

12.6 CFD in Bioreactors

CFD provides the ability to determine the circulation time based on position of the particle, therefore eliminating circulation times due to multiple triggering. When this was taken into account, CFD simulations resulted in similar unimodal CTDs for bioreactor tanks with similar geometries (Davidson et al. 2003).

Bioreactor designing is too complex but is a fundamental count on computer simulations which aid to direct the way in which to lessen the time spent on the developments. A generalized approach to predict oxygen transfer for bioreactors was developed by Dhanasekharan et al. (2005). The model predictions show good agreement with experimental data. The developed methodology was applied to stirred tank and airlift bioreactors at different scales of operation. Thus the approach was used for scale-up of bioprocesses. The model was improved further by solving for more number of discrete bubble size equations to resolve the bubble size distribution more accurately. Different bubble breakup and coalescence mechanisms have been investigated to account for non-Newtonian media and the presence of surfactants and impurities.

Most of the models proposed so far deviate from ideal mixing behavior, without considering the mixing mechanism within the bioreactor. This could lead to severe loss in yield and changes in microbial physiology. Thus, a kinetics multiscale model was proposed by Wan-Teng et al. (2011) in order to describe the non-ideally mixing mechanism of the bioreactor. Aeration rate and stirrer speed are implemented into the proposed model to study the effect of both parameters in the mixing mechanism of the bioreactor. Results suggested that yield predictions from CFD simulations gave rise to approximately 5.00 % error compared to yield results obtained from experiment. On the other hand, around 14.00 % error is observed for predictions of productivity. Thus, these suggest that the kinetics multi-scale model is suitable and precise to be utilized for yield and productivity predictions within a certain range of aeration rate and stirrer speed. With the implementation of the proposed kinetics multi-scale model into CFD simulations, the non-ideally mixed mechanism of the bioreactor could be observed and could enhance the physiology of the fermentation process.

A computational fluid dynamic simulation study has been performed by Krühne et al. (2010), in which the interaction between the fluid dynamic conditions and the growth of cells has been studied. A simple Michaelis–Menten type kinetic model has been selected in which a shear stimulation factor was incorporated. With this model it was possible to study the complex interaction between the cells and the flow through a model pore. It could be confirmed that the growth of cells under shear stimulation follow a different qualitative growth pattern than a non-shear stimulated growth of cells. The cells again influence the distribution of the flow through a pore and will result in a different local shear stress pattern.

The fluid flow inside and around a scaffold in a bioreactor is complex. Yan et al. (2011) present an investigation into such a flow within scaffolds cultured in both perfusion and non-fusion bioreactors, respectively. The simulation results demonstrate that the perfusion bioreactor provides a strong flow within the tissue scaffold, thus increasing the shear stress on the scaffold surface compared to the non-perfusion bioreactor. The results show that the value of the strand diameter and horizontal span can affect the shear stress generated on the scaffold. The effect of flow rate, a controllable parameter in the cell culture process, was also investigated and it was found that the average shear stress level increased linearly with the flow rate. The knowledge obtained from this research provides a quantitative insight into the velocity field and the shear stresses distribution within the scaffold cultured in bioreactors. The effects of the controllable factors identified by this study can be used to guide future scaffold design as well as experimental studies.

A co-current gas–liquid up-flow bubble column (10 cm ID and 1.88 m height) has been simulated by Mahajan (2011) using Fluent. The various parameter studied include bed dynamics such as phase holdups, pressure drop, velocity profile, and gas–liquid mass transfer. The eulerian–eulerian approach with mixture multiphase model and segregated solver was used. The standard k-ε model has been used to model turbulence. The species model was used to simulate the oxygen transfer from gas to liquid as species in the two phases.

Microchannels have demonstrated to be potential to CFD application. Primarily, the study has been carried in order to know the behavior of spread of two different viscous materials within each other through a microscopic needle. This flow understanding is part of the global concept of the irrigation dripping tripled perfusion bioreactor. One of the next steps after this current work is to integrate many needles in parallel and analyze the bioreactor internal flow (David et al. 2012).

Liovic et al. (2012) present a CFD model that is shown to be a realistic model of the hydrodynamics within a Corning stirrer-flask. The CFD models feature room for improvement through targeted improvements to turbulence modeling, stirrer motion modeling, and free surface modeling. The work shows CFD to be useful in identifying possibilities for microcarrier exposure to regions of stress levels that could cause cell damage and stem cell differentiation issues. As such, CFD shows promise for use as risk-mitigation tool prior to commissioning protocols used in stem-cell bioreactors.

A numerical simulation of the turbulent flow of a vortex-ingesting dual impeller stirred tank designed for the production of H_2 by biological fermentation of waste organics has been presented by Montantea et al. (2012). In this work, the fluid dynamic and operational features of the bioreactor are predicted. This leads to conclude that the CFD strategy already developed for simpler gas–liquid stirred vessels can be usefully applied to the design of bioreactors for hydrogen fermentation, provided that further developments of the model for including fermentation and mass transfer are performed. The overall process of H_2 production by fermentation has been already implemented in the hydrodynamic model by defining a volumetric reaction kinetic on the bags volumes, defined as porous regions with the same pressure drops as the real support adopted for the biomass growth. As for the gas flow rate, after stripping the hydrogen is allowed to escape from the reactor with the inert recirculated gas previously predicted by the fluid dynamic simulation.

12.7 Looking into the Future

Advances in telecommunications, such as wireless networking and Internet technology (TCP/IP), facilitate the monitoring of environmental conditions in greenhouses. Pontikakos et al. (2005) designed a Web-based application for real-time predictive modeling of temperature and air velocity patterns, which consist of a user interface, interpolation process data generated by CFD and an output interface.

A lighting systems model with different optical properties was developed by Mikulka et al. (2010) who show various settings for the R-FEM method in the CFX environment.

CFD ventilation space still tends to be a slow process today, while the computation time for the ventilation system and control simulation strategy is negligible. Sun and Wang (2010) found that the test method is more effective than the simplified numerical models, which require more powerful computers. Stavrakakis et al. (2010) concluded that Artificial Neural Networks coupled with CFD models are a powerful computational tool to evaluate the energy savings of various architectural designs.

Currently, CFD studies mainly considered natural ventilation, increasing the realism of simulations by adding features such as the modeling of the crop inside greenhouse. However, it is still necessary to model plant physiological processes such as respiration and transpiration that define the terms of CO_2 and relative humidity mainly in crop space (De la Torre-Gea and Rico-García 2010).

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Chapter 13
Instrumentation and Control to Improve the Crop Yield

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13.1 Introduction

Nowadays, there exist several threats around the world that cause catastrophic damages in the planet. One of the most important menaces is the lack of food that affects the health of people around the world. Animals and plants need food to survive, the survival insurance of both will help to ensure the food production and by consequence the aliment for each person around the world, which is a priority issue in all countries. At the present time, the food production has been affected more drastically by several biotic and abiotic factors that day by day diminish the quantity and quality of the worldwide food; this menaces the food security, principally in less developed countries. According to FAO on 2013, there exist 868 millions of persons suffering malnutrition around the world and the most of them millions are found in Asian and African countries; nonetheless, the highest percentage is concentrated in Africa (FAO 2013), where exists countries that has 65 % with nutrition problems. In order to battle this factors that decrease the quality and quantity of aliment around the world, the agriculture has incorporated technology that goes from employing simple sensors such as temperature sensors to sophisticated instruments such as phytomonitor. The use of technology in agriculture is commonly called precision agriculture and its goal is, by monitoring variables affecting the final production and quality of the plants, to increase the quantity and quality of the food production and by consequence decrease the affection provoked by the abiotic and biotic factors. The process going from sowing to having a product ready to be sold, has several stages that must be carefully monitored to ensure a product of high quality ready to be consumed; to reach this, the plant need to be monitored during the sow-to-ripening and post-harvesting processes; to do this, several kinds of technology and techniques has been used, one of them is the image processing by employing vision systems.

There exist several physiological phenomena which are manifested by visible changes in the plants such as: yellowing, deformation, darkness, or white-spotting of the plant leaves due to nutritional deficiencies or due to viral, bacterial of fungal pathogens principally (Contreras-Medina et al. 2012). Several physiological variables of the plant could be monitored by image processing such as estomatic conductance, photosynthesis, chlorophyll content, which could be related to Nitrogen deficiencies and by consequence to photosynthetic rate (Jones 1999; Jones et al. 2002; Netto et al. 2005). In the food industry, the image processing has been employed to determine the quality of a product based in several variables such as texture, size, shape, and color principally, these variables could be measure by experts but, the assessments will be subjective because of the judgments variation. Other application of image processing in the agriculture is the identification of weeds, which is other factor that provoke looseness in cultivated crops, and several techniques have been developed with the objective of segmenting automatically the weed, soils, and plants. Visible phenomena in plants and fruit have the possibility of being monitoring by using image processing.

13.2 Image Acquisition Process

There exists several algorithms to segment the image into a region of interest and region of no interest; they could be simple or difficult depending of the complexity degree of the image. The features of interest in agriculture and food industry generally are those related to texture, color, shape, and size (Jackman and Sun 2013; Gomes and Leta 2012). In the agriculture, the techniques employed are focused principally to physiological variables, symptoms detection and weeds segmentation, among others. In food industry, the objective is majorly focused to the quality of product and analyzes several techniques.

13.2.1 Image Processing in Food Industry

In the last decade, the use of technology in food industry has been increased greatly, principally due to the high demanding market that day by day requires and demand products of high quality. Also, the market restriction in the last few years has pursued the use of technology in food production (Gomes and Leta 2012). The systems of visual inspections normally consist of a light source, a camera, commonly a couple charge device (CCD) for capturing the image and a computational system for extracting features of images. Commonly, these kind of systems are used in production lines, where human activity is repetitive and the products need to be manufactured very rapidly, so that, decision making must be based on fast and accurate assessments during the overall process. The advantage of having this systems is that they offers repeatability and accuracy by eliminating subjectivity, tiredness, slowness and the absorbance of the cost related to human inspection (Gomes and Leta 2012). The system captures the image by using a camera, scanner, videos, etc. Subsequently, it converts the image into digital format and after this, a pre-processing stage is generally required to highlight the region of interest and to remove noise that could interfere at the time of extracting important features; the principal objective of a pre-processing stage is to segment the region of interest to finally apply a processing stage that is in charge of recognizing and interpreting the image, always seeking to make sense to the object of interest of the images. In addition, having optimal conditions to acquire a good quality image, permit having a less complex pre-processing algorithm.

According to Davies (2009), several types of food products have been analyzed through computational vision techniques including cereals and particularly wheat that has an important role in the food industry. The wheat (*Triticum aestivum* L.; Triticum durum), has been one of the main foods for thousands of years, and today it has the most worldwide economical importance; European countries, India and China are the main countries that contribute in more proportion to the world wheat production with 21.28, 17.62, and 12.36 %, respectively (Hawkesford et al. 2013). At following, a case of study of artificial vision system applied to grain inspections is presented.

13.2.2 Artificial Vision Systems Applied Grains

In Mexico, irrigated crops are mainly used for wheat growing, which 95 % of the annual harvest is produced in the autumn–winter season, such graminaceous represents the 21 % of the national basic grains consumption, second place just after the corn grain, with a capita consumption per year of 52 kg and industrial sales volume that will be increasing between 1 and 2 %. The Mexican national production in 2009 was 4.01 MT with a total value of more than 15 billion USD (Agrosintesis 2013).

The proper conservation of stored grains and seeds in any worldwide location is affected by various factors: regional ecology; barn, silo, or warehouse availability; type and conditions of grain or seed to be stored; storage climate conditions; and duration of storage, being the latter difficult to be controlled for high volume crops. A worldwide loss of stored wheat grain between 10 and 15 % is estimated by Neethirajan et al. (2007); where main causes are: harvesting machines, water permeability on grain gluten, bugs, spores, fungus among others. One of the most important factors to be considered in wheat grain storage is the water permeation on the grain gluten when temperature gradients happen, encouraging the presence of bugs, mainly Coleoptera, Lepidopterous and microorganisms like *Penicillium, Aspergillus, Alternaria, Fusarium, Cladosporium, and Rhizopus*. Serious types of damages on stored grains are caused by such bugs populations, from its devaluation up to the total loss, from an agricultural, economical, and nutritional point of view (Oliveira et al. 2013).

Two kinds of grain damage are caused by bugs, being the grain destruction and consumption by either adult bugs or bugs' larval states through feeding and oviposition of (Huang et al. 2013a, b). Besides, the economic and nutritional values, as well as the germination potential of the grain or seeds are decreased due to the contamination caused by the bug's excrements and dead bodies. On the other hand, the acquisition between 3,000 and 57,000 spores, fungus and other microorganisms in the harvesting process, generate toxins and consequently with harmful consequences (Bodroza-Solarov et al. 2012). Among the most common fungus located inside the grain are *Helminthosporium* spp.; *Giberella* spp.; *Diplodia* spp.; and *Colletotrichum* sp.

The objective of group algorithms in image processing is to find pixels groups having similar characteristics (intensity texture, shape, etc.), and that the groups represent homogenous regions in the image, González and Woods (2008). This kind of image processing has been widely used in many image segmentation applications in different areas such as medicine, food processing, chemistry, edaphologic, mechanics, among others (Quintanilla-Domínguez 2009, 2011; Ojeda-Magaña 2010; Cortina-Januchs et al. 2011); being such group determinations important to achieve characterizations in different fields of study through the generation of important data for calibrating predictive models and acquisition of morphological data (Baptista et al. 2012). The use of digital tools in image processing algorithms has many industrial applications in areas such as harvest, postharvest, and raw material processing. In these areas, the morphological dimensions of the objects (such as fruits, grains, cereals, and oilseeds) with defined or atypical geometries are required to be known (Peregrina-Barreto et al. 2013). The descriptor determination of a specie or genotype is an easy task; such descriptor makes reference to the structure and shape of a surface (López et al. 2008; Baptista et al. 2012). The use of these tools in the food area allows to make technical processes and to have a more strict control. However, there is little information available about implementation of monitoring systems based on image processing techniques for evaluating the effect of grain storage systems and their behavior on the matrix of silos and warehouses, where the general conditions of the grain may be affected and difficult to achieve a constant monitoring (Brosnan and Da-Wen 2002).

The study described in this chapter is based on the hypothesis that it is possible to quantify the morphological characteristic of wheat grains in the postharvest processing, this is done by using a digital image processing system in monitoring factors related with humidity that may have an effect in the grain quality during the storage stage.

The research objectives are: (i) to monitor the behavior of different types of wheat grains after different conditioning days have been applied, (ii) to evaluate the possible relationship between variety and water diffusivity capacity in wheat grains from its morphometry.

Our study is based on the fact that wheat grains humidity is a variable to be taken into account in the grain storage stage, and it is an important factor to be controlled in a specific values range along the grain reception, storage, and conditioning stages. In the case of hard and soft wheat grains, the maximum recommended humidity in storage stage is less than 14 %. The humidity variable control is a determinant factor to keep the best conditions of the grain, for higher humidity and temperatures above 25 °C a microbacterial proliferation may be present in the environment, since the grain has the capacity of water absorption, then it implies its combination with the solid and dry gluten material (Sokhansanj

Table 13.1 Classification of wheat grains

Variety	Characteristics	Flour yield (%)	Protein (%)	Properties	Uses	Group[a]
Soft red winter (GP), Jal (JA), Salamanca (SA)	Semi-long grain, brown-orange color	69.7	10.2	Soft wheat, soft gluten	Cookies	III
Rayón (RA)	Long grain, red and brown color range	70.2	10.5	Robust flour and little extensible, short and robust gluten	Breads	IV
Dark Northeast Spring, Canadian (RC), red	Slim and short, oscure color	68.7	12	Hard gluten and robust	Breads	I
Hard red winter (HR), Arandas (AR)	Slim and short, copper color	68.5	12.4–14	Hard gluten, robust and extensible	Breads	II

[a] *Source* Modified from www.sagarpa.gob.mx/Pronase/productos/trigo2.html

and Lang 1996). However, once the conditioning process has been applied, where the grains humidity is increased in soft wheat grains up to 15–15.5 and 16.5 % for hard grains, the wheat grains are kept at rest for 12–36 h long with a controlled temperature smaller than 45 °C to avoid microbacterial growth. This process stage is difficult to be monitored due to the silo or warehouse geometry.

The water contained inside the wheat grain acts as a hydration element on the tissues; the cells colloids take the form of an elastic gelatin allowing a higher speed diffusion of oxygen and carbon dioxide (Petra Hogy and Fangmeier 2008). The gas diffusion phenomenon is directly proportional to the elasticity of the jelly substances Huang et al. (2013a, b), and since decreasing the water contents decreases such elasticity, then the respiration gas interchange is decreased, hence a smaller vital activity of the organism is achieved. The biochemistry oxidation of carbohydrates, fat, and other nutritional materials allows that energy be released through the respiration (Bailey 1940).

The groups of the wheat grain varieties based on their properties and characteristics are presented in Table 13.1, the characteristics of same variety may be different; it is observed that those types of group IV have a higher flour yield (70.2 %) than the group II; but the last one has a higher protein content (12.4–14 %). There is another seeds report indicating that the production of certain compounds is affected by the crop place, weather, maturity degree, planting season, irrigation and production processes (Vinha et al. 2005; Kalua et al. 2005; Gómez-Alonso et al. 2002; Tovar et al. 2001).

13.3 Materials and Methods

13.3.1 Sample Preparation

The analyzed wheat grain varieties (SA, JA, HR, AR, RA, RC, GP) were donated by the factory named Molinera de México S.A de C.V, before the application of any grain treatment or processing. The wheat grains were weighted and conditioned to reach a humidity value of 15–15.5 and 16.5 % for soft and hard wheat grains, respectively. The grain samples were sealed and monitored every 24 h for first 7 days long and later every third day to complete a whole monitoring period of 21 days (504 h) days, and the environment temperature was controlled to <25 °C.

13.3.2 Image Processing

The block diagram of the vision system for capturing the sample images of all considered wheat grain varieties is depicted in Fig. 13.1. In order to have better contrasted images, a white background was used. The images of grains were

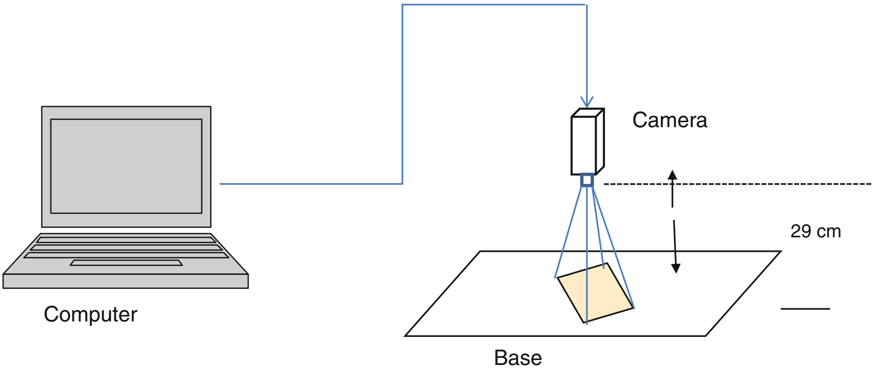


Fig. 13.1 Block diagram of vision system for image capturing of wheat grains

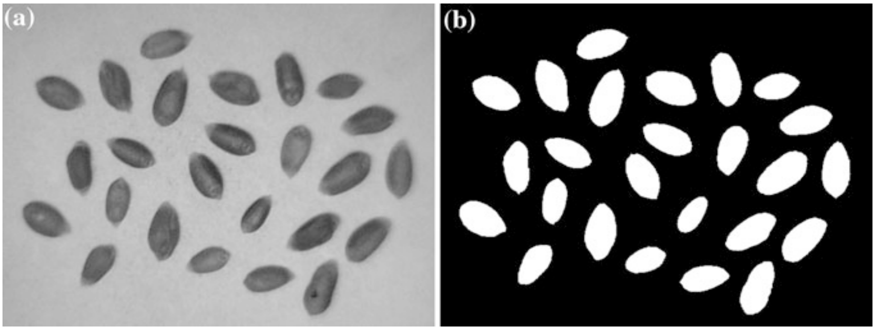


Fig. 13.2 **a** Wheat grains image captured by microscope, **b** preprocessed image

Fig. 13.3 Ellipsoidal grain shapeapproximation; where largest and smallest axes, and thickness are represented by a, b and c, respectively

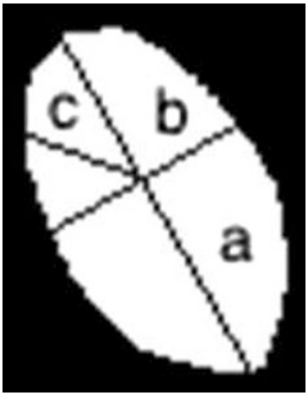


Table 13.2 Description of each essay

Wheat grain variety	Conditioning time (days)												
	1	2	3	4	5	6	7	9	10	13	15	18	21
JA	JA-1	JA-2	JA-3	JA-4	JA-5	JA-6	JA-7	JA-9	JA-10	JA-13	JA-15	JA-18	JA-21
HR	HR-1	HR-2	HR-3	HR-4	HR-5	HR-6	HR-7	HR-9	HR-10	HR-13	HR-15	HR-18	HR-21
AR	AR-1	AR-2	AR-3	AR-4	AR-5	AR-6	AR-7	AR-9	AR-10	AR-13	AR-15	AR-18	AR-21
RA	RA-1	RA-2	RA-3	RA-4	RA-5	RA-6	RA-7	RA-9	RA-10	RA-13	RA-15	RA-18	RA-21
RC	RC-1	RC-2	RC-3	RC-4	RC-5	RC-6	RC-7	RC-9	RC-10	RC-13	RC-15	RC-18	RC-21
GP	GP-1	GP-2	GP-3	GP-4	GP-5	GP-6	GP-7	GP-9	GP-10	GP-13	GP-15	GP-18	GP-21
SA	SA-1	SA-2	SA-3	SA-4	SA-5	SA-6	SA-7	SA-9	SA-10	SA-13	SA-15	SA-18	AS-21

captured with a resolution of 640 × 480 pixels using the digital microscope Dino-lite with a USB port connection to a personal computer and located at a distance of 29 cm.

The pixel/length and pixel/area ratios for the optical system were determined considering a geometric object with known length and area, based on an area reference of 1 cm^2 the ratios of 21/220 mm/pixel and 3.46/38013 cm^2/pixel were obtained.

The digital image processing and parameter computing algorithms were developed in MATLAB 13.2a (R2011b). A captured image sample by the used microscope is shown in Fig. 13.2b, as well as the pre-processed image in Fig. 13.2b, which is used to evaluate the considered parameters for estimating the morphometric of the wheat grains varieties.

The grains sphericity parameter was computed assuming an ellipsoidal shape for each wheat grain; this implies the computing of the largest and smallest axes, as well as the seed thickness, see Fig. 13.3.

13.3.3 Experiments Design and Statistics Analysis

The statistics analysis was done by setting two factors, the first was each one of the wheat grain varieties (SA, JA, HR, AR, RA, RC, and GP) and the second one the conditioning time (1–7, 9, 10, 13, 15, 18 and 21) with seven levels as it is given in Table 13.2.

The image resulting parameters were analyzed through media comparison by the Tuckey-Kramer method (Montgomery 2006), using JMP v5.0 as statistics tool with a reliability value of 99 % ($\alpha = 0.01$).

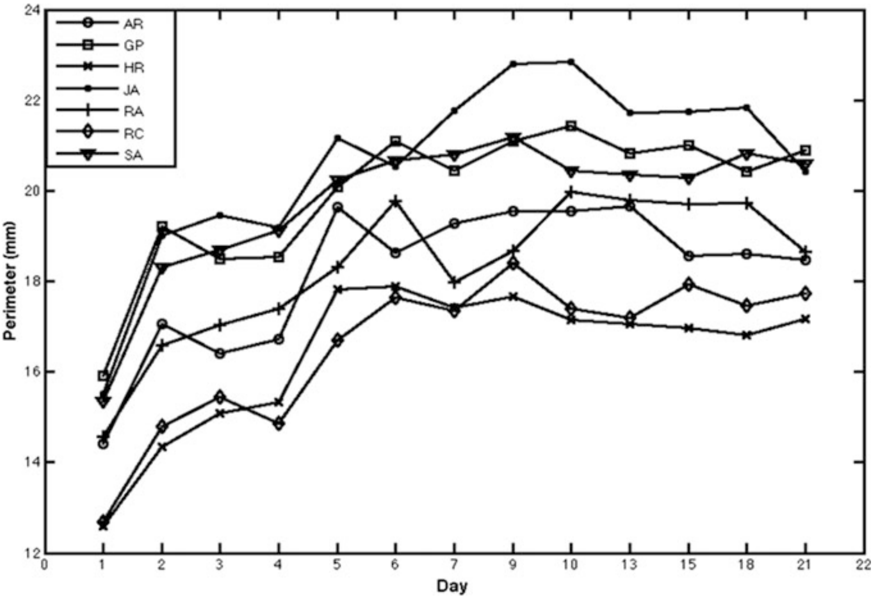


Fig. 13.4 Dimensional kinetics on studied wheat grain varieties

13.4 Results

A diffusive behavior of the wheat grain was detected along the conditioning time period; this behavior is shown in Fig. 13.4, where a continuing increase on grain perimeter is observed because the water content shifts into the grain endosperm. A greater perimeter increase is obtained by soft varieties (JA, SA, and GP), this is due to its hydration along the early conditioning hours, its humidity has been incremented as well as the temperature (An-zhen et al. 2013); the porosity nature of the grain allows an air diffusion through its mass, the respiration speed on the grains is closed related with the oxygen availability and it is a function of temperature (Dongena et al. 2011; Huang et al. 2013a, b); so moist grains get more warm than dry ones; most of the wheat varieties achieved a diffusivity balance in 5 days (120 h) of conditioning. However, soft grain varieties are more susceptible to trigger a metabolic change sequence, including respiration, protein synthesis, and reserve moving, beginning with the inhibition process through the cell division and lengthening in the germ, that produce the radicle emerging in comparison with the hard grain varieties (HR, RA y RC). In the same sense, the hydric potential benefits growing of bugs, fungus, and bacteria inside and outside the grain.

In wheat grains RC variety (group I), see Table 13.3, it is possible to distinguish that in conditioning days 2 and 3 there is no significant difference neither in area nor perimeter (14.03 ± 2.71 and 15.74 ± 2.55; 14.70 ± 1.31 and 15.50 ± 1.25,

Table 13.3 Wheat grain morphometrics for group I

Conditioning days	RC		
	Area (mm^2)	Perimeter (mm)	Sphericity index
1	10.55 ± 1.62c	13.03 ± 0.94c	0.82 ± 0.02a
2	14.03 ± 2.71b	14.70 ± 1.31b	0.79 ± 0.04ab
3	15.74 ± 2.55b	15.50 ± 1.25b	0.77 ± 0.05ab
21	20.77 ± 2.98a	17.80 ± 1.32a	0.76 ± 0.05b

Average ± standard deviation with media comparison by Tukey (<0.01), for same letters no significant difference

Table 13.4 Wheat grain morphometrics for group II

Conditioning days	AR		
	Area (mm^2)	Perimeter (mm)	Sphericity index
1	12.32 ± 0.93c	14.76 ± 0.93c	0.80 ± 0.021ab
2	18.24 ± 1.59b	17.18 ± 1.59ab	0.80 ± 0.05b
3	18.19 ± 1.42b	16.70 ± 1.42b	0.78 ± 0.04b
21	21.66 ± 1.19a	18.45 ± 1.19a	0.83 ± 0.04a

Average ± standard deviation with media comparison by Tukey (<0.01), for same letters no significant difference

Table 13.5 Wheat grain morphometrics for group III

Conditioning days	SA		
	Area (mm^2)	Perimeter (mm)	Sphericity index
1	15.50 ± 1.29c	0.85 ± 0.01a	0.83 ± 0.02ab
2	21.22 ± 3.05b	18.34 ± 1.35b	0.82 ± 0.01ab
3	21.91 ± 2.59b	18.65 ± 1.28b	0.81 ± 0.02b
21	27.16 ± 4.20a	20.80 ± 1.47a	0.80 ± 0.03b

Average ± standard deviation with media comparison by Tukey (<0.01), for same letters no significant difference

respectively). However, there is statistics difference in area and perimeter dimensions in days 1, 2, 3 and 21, area and perimeter value vary from 10.55 to 20.77 and 13.03 to 17.8, respectively. Concerning to the sphericity index, it is possible to establish that grain hydration is not uniform because it is moving away from the particle roundness as the conditioning days pass by.

A similar behavior for area and perimeter was obtained for wheat grain of group II (AR and HR), see Table 13.4. Regarding to sphericity index for AR variety no significant difference was found neither in days 1, 2, and 3 nor between day 1 and 21, from which we can deduce that the endosperm hydration is quite similar in each one of the grain sides, keeping in this way its original shape.

For wheat grain of group III (GP, JA, and SA), significant statistics difference in grain area and perimeter were observed between days 1 and 2, see Table 13.5.

Table 13.6 Wheat grain morphometrics for group III

Conditioning days	RA		
	Area (mm^2)	Perimeter (mm)	Sphericity index
1	12.34 ± 1.73c	14.56 ± 1.07c	0.85 ± 0.02a
2	17.34 ± 2.10b	16.62 ± 0.98b	0.81 ± 0.02b
3	17.64 ± 2.48b	16.75 ± 1.20b	0.80 ± 0.02b
21	23.15 ± 3.42a	19.01 ± 1.31a	0.80 ± 0.02b

Average ± standard deviation with media comparison by Tukey (<0.01), for same letters no significant difference

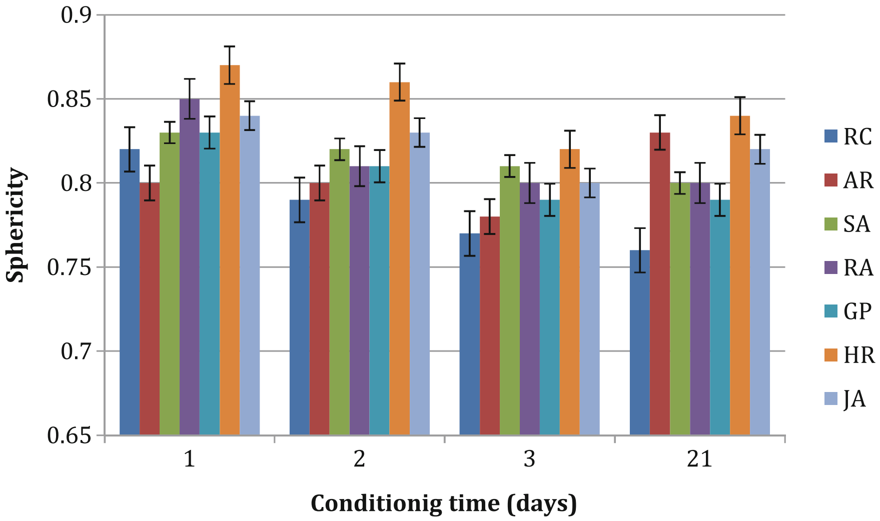


Fig. 13.5 Averages of sphericity indexes with typical error

Regarding to sphericity index, no significant difference among the three evaluated varieties was found.

The obtained dimensions values for wheat grain variety RA classified in group IV are presented in Table 13.6. Since the group characteristics and rheological qualities like extensibility, robustness and force, like the other groups statistics differences in area, perimeter and sphericity were obtained between days 1 and 2. However, no significant difference was observed in any of the evaluated parameters between days 2 and 3. The grain shape was modified in day 21 because of the significant increment in area and perimeter parameters.

Data dispersion for sphericity index parameter is depicted in Fig. 13.5, where first day is compared with the others (days 2, 3 and 21). As it is shown, a sphericity index loss in the grain shape begins at day 2 for all varieties except for RC variety, which sphericity loss begins until day 3; the reason might be the gluten composition on the endosperm and the behavior of the flour considered as an ideal flour with ratio of robustness/extensibility equal to one, which allows that flour from this wheat variety may be used either for traditional or mechanical bakery products.

13.5 Conclusions

Even all wheat grains are composed of three main parts (germ, endosperm, and teguments), a significant difference in porosity and diffusivity on the pericarp of the evaluated wheat grains varieties was observed. According to the obtained results, the pericarp permeability allows a better diffusivity on soft grain varieties than on hard ones, and consequently an easier moisture flow to the endosperm.

The hydric potential for soft wheat grains varieties is lower than for the hard ones, hence, it is an important issue to use all the required resources in order to keep low wheat grain humidity, ensuring in this way the right environment conditions for grain storage (Ramirez 1984). The humidity parameter measurement is a difficult task due to the geometrical shapes of warehouses and silos.

The wheat grains of group II (HR) resulted in a more spherical geometry because their sphericity indexes were the greatest compared with other varieties groups grains.

The obtained sphericity index in first conditioning days was greater for grain varieties SA, AR, HR, AR than for grain varieties GP, RC, and RA; this is because of the own characteristics of the grain. However, based on the grain area and perimeter values as conditioning days passed by, it was possible to identify that the adequate conditioning time could be between one and two days (24 and 48 h).

Even the given application advantages of the used artificial vision system limits were observed, such as that grain group classification is based on just dimensional parameters and it is not taking into account more grain specifications such as color, strength, and texture among others. Nevertheless, the obtained wheat grain data are helpful to know the processing conditions in manufacturing wheat grain flour, because the flour obtained from soft wheat grains requires less water and smaller kneading time than hard wheat grains flour. Among the rheology properties of wheat flours, it has been proved through farinograma that soft wheat grains varieties require four times less kneading time than hard wheat grains varieties like crystalline. On the other hand, the behavior of the dough stability for soft and hard wheat grains flours is quite similar. However, the stability time for dough with rayon wheat grains flours is greater; this is because of the quality of gluten, which is composed by proteins giving the viscoelastic characteristics of wheat (Acosta-Navarrete 2003).

13.6 Gas Exchange and Photosynthesis Sensors

The photosynthesis process is considered the most important biochemical reaction because it allows plants to transform sunlight energy into chemical energy (Millan-Almaraz et al. 2013). Because of this, plants fix carbon dioxide (CO_2) and release oxygen (O_2). This process is considered the most important biochemical reaction in the world because it produces 90 % of the planet biomass (Taiz and Zeiger 2006). On the other hand, photo-inhibition is a term utilized to describe all the environmental conditions that can negatively affect the photosynthetic activity. The most common stress factor is light. Consequently, accurate photosynthesis measurements are necessary to establish comparisons and understand plant productivity or biomass accumulation at the leaf, plant, canopy, or community levels as well as their interaction and response to environmental (Bakker et al. 2001), chemical (Marschner 1995), or biological (Stout et al. 1999) factors that generate stress conditions. Here, it can be inferred that it is very important the photosynthesis measurement because it reflect the whole crop biomass production which where it is very important to maintain high yields at the new generation plant factories.

There are many methods to perform photosynthesis measurements. However, the gas exchange method is currently the most commonly utilized technique to achieve this by measuring carbon dioxide exchange. Because of this, this method is widely utilized in commercial equipment and experimental setups in order to measure individual leaves, whole plants, plant canopy, and even forests (Millan-Almaraz et al. 2009; Schulze 1972; Bassow and Bazzaz 1998). This methodology is based on isolating the specimen or sample under analysis in a closed chamber to measure the initial gas concentration when the chamber is closed. After a few minutes, the chamber has been closed, recording changes in the proportions of gases from the air inside the chamber produced by the plant is also carried out. Consequently, it is possible to measure the gas exchange by analyzing O_2 or CO_2 concentration (Schulze 1972; Takahashi et al. 2001). There are two types of gas exchange designs: closed chambers where the sample is completely enclosed to measure the difference in gas without contact with outside air and the open chambers where air can freely enter and leave the chamber flowing through the sample (Hunt 2003).

Infrared sensors for gas analysis (IRGA) are the most utilized for CO_2 measurement and are based on an infrared emitter-photo detector par whose light beam is used to measure the concentration of gas molecules in the air. This is based on the absorption phenomenon of the light beam by molecules in a gaseous state (Hunt 2003). This phenomenon of absorption occurs because the heteroatomic gas molecules with odd number of atoms such as CO_2, CH_4, NH_3, to name a few, absorb a portion of the infrared light while the homoatomic gas molecules such as N_2 and O_2 do not. The CO_2 has a maximum detection at a wavelength of 4.25 μm, with peaks side of 2.66, 2.77 and 14.99 μm (Hill and Powell 1968). The calibration of these sensors to zero adjustment requires air free of CO_2 and other heteroatomic gases; therefore N_2 is most often used for calibration purposes. Also, the

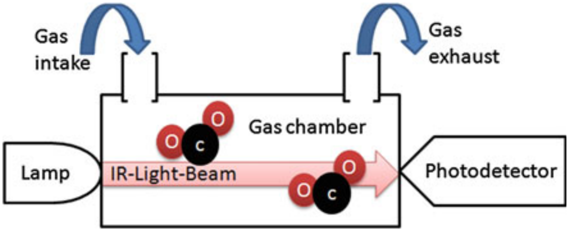


Fig. 13.6 Infrared CO_2 analyzer

adjustment requires a span of known concentration of CO_2 to be carried out with precision pumps (Hunt 2003). In Fig. 13.6, it can be observed a diagram that shows the emitter-photo detector pair and the infrared beam which is partially absorbed by CO_2 molecules.

As was aforementioned, the CO_2 exchange is the most commonly used method for building commercial and experimental photosynthesis monitoring systems. The design of these kinds of systems should include several considerations, including the operating range of CO_2 of the phenomenon being studied to ensure the selection of an appropriate IRGA according to the application. It has been reported that the range of physiological importance is 50–800 ppm (Hanstein et al. 2001). Electrochemical sensors for CO_2 are not appropriate because of their poor sensitivity to low CO_2 concentrations. Therefore, non-dispersive infrared sensors (NDIR) are the most appropriate. Another important aspect to consider is the air flow. It has previously been found that the range more appropriate for minor variations in the accuracy of a photosynthetic rate is 0.3–1.0 m s^{-1} (Kitaya et al. 2000). Another aspect to analyze is the design of the chamber seal. Earlier, different systems were designed with a seal of black neoprene and a transparent surface so that light falls on the leaf for photosynthesis process. This is problematic as the black surface of the seal obscures a portion of the road tested and causes dark respiration. This, in turn produces CO_2 that seeps into the leaf chamber of the leaves and produces miscalculation of the photosynthetic rate based on the CO_2 exchange in the chamber without taking into account the parasitic dark respiration (Pons and Welschen 2002; Long and Bernacchi 2003). The design of the shape of the leaf chamber should be selected according to the needs of the morphological species to be studied. There are chambers ranging from those used for small leaves all the way up to soil analysis chambers (LI-COR Corporation, Lincoln, NE, USA). Finally, the technological platform that allows electronic control for electrical and mechanical systems that are needed to make an entire photosynthesis monitoring system and the export and storage of data to a computer for future offline analysis on a personal computer (PC) or a microcontroller (μC) be carried out. In Fig. 13.7, it can be observed the most common gas exchange configuration for photosynthesis measurement.

This method provides an alternative to the CO_2 exchange, which can be used as an additional tool combined with CO_2 exchange in order to observe these phenomena. The procedure utilized in this method is basically the same as using CO_2. Nevertheless, this method has serious disadvantages. The first is that the O_2

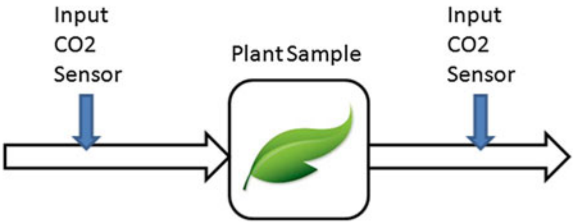


Fig. 13.7 Basic CO_2 gas exchange configuration for photosynthesis measurement

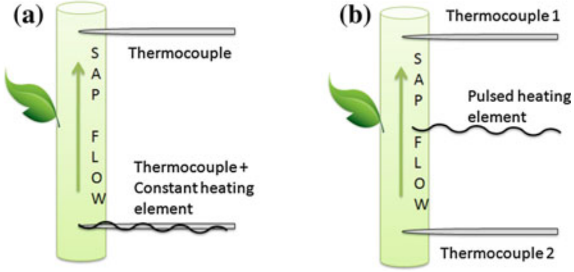


Fig. 13.8 Most utilized sap flow measurement methods, **a** TDM and **b** HRM setups

exchange technique is the difference between the initial and final concentrations, this is, it is smaller compared to CO_2 exchange systems and by this reason, the O_2 exchange systems require high precision sensors and expensive data acquisition devices (Hunt 2003). Another disadvantage is that the oxygen gas is more unstable than CO_2 and has to be maintained at a high and very stable temperature (around 700 °C) to maintain a stable molar concentration.

13.7 Sap Flow Sensors

In plants, there are two main vascular tissues at stems which are responsible of water and nutrition transport in the sap. Xylem is the first one and the responsible of water transport at the inner radius of a plant stem. In contrast, phloem is located in the outer radius of plant stem and is responsible of transporting photosynthesis products and other nutrients such as sugars (Taiz and Zeiger 2006). Sap flow measurement is an instrumentation technique which consists on introducing sensors in the plant xylem at stems or trunk to measure temperature differences that indicates sap flow information such as direction and density (Granier 1987).

Sap flow measurement has been utilized for many years to analyze different kinds of plant and its relations to water, soil and atmosphere conditions (Herzog 1995). In plants, sap flow has a specific trend to increase at middle day hours and starts to decrease at evening to finally reach its minimum value during night (Herzog 1995). Transpiration is another useful variable which allows detecting water related stress phenomena in plants (Millan-Almaraz et al. 2010). However, it results complex and costly when it is compared to sap flow technique that only requires temperature sensors and a heater element which are cheap and easy to be implemented (Davis et al. 2012). In order to perform sap flow measurements, there are three main methodologies: heat ratio method (HRM) (Burgess et al. 2001), heat balance (Sakurtani 1981), and thermal dissipation method (TDM) (Granier 1985).

TDM consists on using continuous heating in the stem and measuring the thermal dissipation capability when the sensor and stem are in steady state or thermal equilibrium (Allen and Grime 1995; Granier 1985). The variations in the heat dissipation that occurs during day are in function of sap flow. First, a heater element with an attached thermocouple is introduced in the stem by drilling a few millimeters of depth and the stem is heated electrically at a constant ratio and another thermocouple is introduced a few centimeters below the heater probe position in order to measure the amount of heat taken by the sap flow stream at xylem (Allen and Grime 1995; Davis et al. 2012). As observed at Fig. 13.8.

The HRM consists on sending short heat pulses of 1–2 s of duration to the plant stem. Consequently, the mass flow of sap is obtained from the speed measurement of the heat pulses moving along the stem where two thermocouples are introduced at equal distance but one below the heater and the other one at an upper distance from the heater (Marshall 1958; Swanson and Whitfield 1981; Davis et al. 2012). The main difference between TDM and HRM in terms of hardware is that HRM do not use a thermocouple attached to the heater but uses two thermocouples to measure the heat movement across sap stream. Furthermore, both temperature sensors return to same reading after 60 s of sending the heat pulse (Sakurtani 1981; Baker and van Bavel 1987).

13.8 Plant Morphological Sensors

As was aforementioned in the sap flow section, plants have a sap flow system where the xylem transport water, phloem transport sugars, and other nutrients and transpiration is a water loss mechanism which occurs during CO_2 exchange process during photosynthesis. As a consequence of water and nutrient flow across the plant, stems, and trunks produce diameter variations which are completely related to sap flow. Therefore, during day hours occurs a steam diameter decrease while it increases during nighttime (Berger and Selles 1993; Garnier and Berger 1986). Consequently, this stem diameter information result very useful for plant physiology research purposes and plant production systems where drought and other water-related stress conditions need to be monitored with accuracy (Morandi et al. 2007).

There are two main types of sensing elements which are utilized for morphological sensors: strain gauges and linear-variable-displacement-transducer (LVDT) (Morandi et al. 2007). Strain gauges consist in a variable resistor which appears in different shapes, this devices produces electrical resistance variations due to mechanical displacement. Strain gauge-based sensors are widely utilized as stem/trunk diameter sensors and fruit growth sensors (Ortuño et al. 2010). The theory of

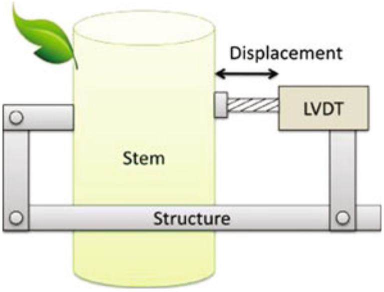


Fig. 13.9 Stem diameter sensor based on LVDT transducer

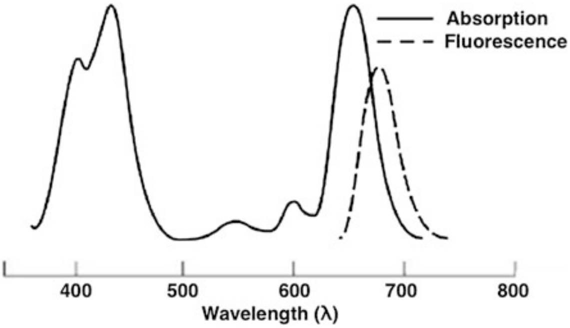


Fig. 13.10 Light absorption and chlorophyll fluorescence emission (modified by Taiz and Zeiger 2006)

operation consists in designing a mechanical arrangement which be capable of being attached to a trunk or a fruit and linked to the variable displacement section of the strain gauge. By this way, plant mechanical variations are converted to electrical resistance variations and consequently to a voltage signal often through a Wheatstone bridge circuit which is connected to a data logger (Morandi et al. 2007). On the other hand, LVDT sensors are more utilized to build point dendrometers which are a variable displacement bar connected to a transformer nucleus. The LVDT variable displacement bar is installed on the side of the plant trunk. Hence, any variations produced by the plant trunk diameter are sensed by LVDT nucleus (Korpela et al. 2010). Also there are some less common technologies to build stem diameter and other morphological sensors such as optical fibers to build point dendrometers for tree growth monitoring (Uchida et al. 2010).

The design of morphological sensors varies and depends on plant characteristics such as size, shape, or location. For example, there are fruit growth sensors which are designed to measure microvariations on apples, tomato, or other fruits size. Also, there are stem diameter sensors for different types of plants stems or trunks (Korpela et al. 2010). In Fig. 13.9, it can be observed some examples of morphological sensors based on strain gauges or LVDT sensors for different applications such as wine industry (Montoro et al. 2012), orchards (Bonet et al. 2010) among others.

13.9 Chlorophyll Fluorescence

As mentioned above, there are many ways to improve the agricultural production, chlorophyll fluorescence is a widely used technique with the aim to monitor the physiological state of the plant, therefore strongly related with the photosynthetic process.

The photosynthesis is the main process in the plant, being of great interest because the carbon, hydrogen, and oxygen assimilated make up ~96 % of dry matter of a typical plant (Taub 2010), and this is reflected on the crop yield.

First, in order to understand the chlorophyll fluorescence, it is necessary to know that photosynthetic organisms majorly are green, because only the blue and the red light are absorbed, as we can see in Fig. 13.10, resulting that the human eye perceive as the photosynthetic organisms on green. Insomuch as the fluorescence in the plant is a re-emitting energy process with increased wavelength of the photons previously absorbed. That is why in Fig. 13.10 the fluorescence response is a little move to the right, because it is consequently of the loss of energy in the re-emitting (Taiz and Zeiger 2006).

Photosystems (PS) are in charge of this re-emit of photons, there are two photosystems: PSI and PSII that are containing in chloroplast with thylakoid membranes. PSI has a very low fluorescence contribution under standard conditions, that is why can be neglected. On the other hand, the PSII is the larger fluorescence contributor, being the photosystem which the commercial chlorophyll fluorescence systems commonly perform (Johnson et al. 2009; Stirbet 2011).

The important of the PSII in order to improve the yield crop is constituted by pigments and proteins in the thylakoid membrane of chloroplast which is closely related with the stress conditions of the plant (Kautsky and Hirsch 1931). Being modified the re-emitting of photons by plant in red and far-red of the electromagnetic spectra varying comfort conditions of the plant (Lichtenthaler et al. 2013).

The chlorophyll fluorescence produced by PSII and PSI (last one with a small contribution) can be used as analytical tool (Belyaeva et al. 2011). It is necessary to use a fluorometer to measure and analyze the parameter that instrument provide us.

The fluorometers are constituted mainly by two stages. First stage is the way in which the photosynthetic sample is excited; this is reached by two different methods: passive and active methods. Passive methods are when the sample is excited by sunlight; the main advantage of this technique is that you can save electronically light source devices but the acquisition of the chlorophyll response is more complicated due to the environmental noise and lack of control of the light source (Fernandez-Jaramillo et al. 2012).

On the other hand, the active methods consists in the utilization of light emitter devices, among which are the Light Emitter Diode (LED) (Faraloni et al. 2011; Johnson et al. 2009; Kissinger and Wilson 2011; Ji et al. 2010). Lamps such as halogen (Van Gaalen et al. 2007), xenon that normally it is used on combine of

Table 13.7 Summary of fluorescence parameters

F_o, F, F_m	Minimum, steady state and maximum values of chlorophyll fluorescence in dark-adapted
F_v	Variable fluorescence (F_m–F_o)
F_v/F_m	Maximum quantum yield of photochemistry in PSII (dark-adapted)
F_o', F', F'_m	Minimum, steady-state and maximum values of chlorophyll fluorescence in light-adapted
$\Delta F'/F_m'$	Quantum yield of photochemistry in PSII (light-adapted)
F_v'/F_m'	Quantum efficiency of photochemistry in open reaction center of PSII (light-adapted)
F_q'	Difference between F_m' and F
F_q'/F_m'	PSII operating efficiency

another light sources (Lichtenthaler et al. 2013; Röttgers 2007) or even lasers and laser diodes (Thoren and Schmidhalter 2009; Kolber et al. 2005; Schächtl et al. 2005). In this stage, it is advisable to add an optical filter with the aim to avoid noise that light source can emit in chlorophyll fluorescence area.

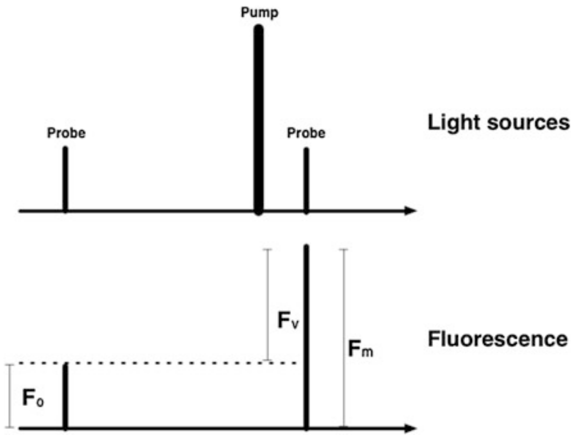
After the chlorophyll excitation on the second stage, it is necessary to acquire fluorescence response, there are different devices to apply in this stage according with the applications, commonly it is used the photodiode, because it could be easier to instrument and inexpensive option (Fedack et al. 2005; Kissinger and Wilson 2011). Other option is the employment of photomultipliers for finer and more sensitive applications (Bürling et al. 2011; Hunsche et al. 2011). In this stage, it is critical the use of optical filters in order to avoid the light source or environmental noise and have, as a result, a fluorometer capable of measuring the fluorescence lifetime of the sample.

13.9.1 Types of Fluorometers

There are many fluorometers in the market to meet the different needs and applications. The Pump and Probe Fluorometer (P&P) (Falkowski et al. 1986) works with a methodology to obtain the main chlorophyll fluorescence values contained in Table 13.7. The P&P basically consists on a chamber with two xenon lamps that are related by Pump and Probe. First it is necessary a weak probe flash to measure F_o, then after a delay the actinic flash allows obtain the F_m value to with these two previous values estimate F_v as shown in Fig. 13.11.

The problem with the utilization of Xenon flashes is that they need delay period to give a new flash again. The Fast Repetition Rate fluorometer (FRR) is rather similar than P&P. However, on FRR solve the delay problem, reaching even 100 Hz with the utilization a second lamp, nevertheless even at this frequency the flash rate is too slow to effectively measure the faster photosynthetic process (Kolber and Falkowski 1995). The FRR that builds from a earlier P&P resulted on a rapid chain of flashes ($\sim$27,500 μmol photons m^{-2} s^{-1}) over a period of 150–400 μs with the aim to obtain F_m (Suggett et al. 2000).

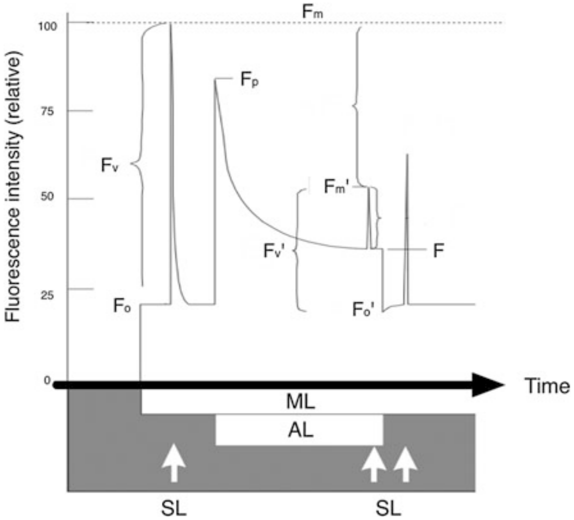
Fig. 13.11 Pump and Probe Fluorometer light source and fluorescence response



In awake of to improve the FRR and decrease the cost of fluorometer, it was created a new methodology named the Fluorescence Induction and Relaxation (FIRe). A multicolor excitation light source was implemented, and basically the protocol consists on four phases: first, a short strong pulse of 100 ms of duration called Single Turnover Flash (STF) with the aim to cumulative saturate PSII and measure the F_o to F_m. Second, it is necessary to apply a weak modulate light to acquire the relaxation kinetic of chlorophyll fluorescence yield on the time scale of 500 ms. Then, A strong long pulse with 50 ms duration, called Multiple Turnover Flash (MTF) in order to saturate PSII and the PQ pool. Finally, a weak modulate light is necessary to apply to record the kinetics of the PQ pool re-oxidation with duration of 1 s. Generating a sturdy fluorometer capable even to measure large time lapses under the water (Gorbunov and Falkowski 2004).

One of the most utilized and complete chlorophyll fluorescence measurement system is the Pulse-Amplitude Modulation (PAM) fluorometer. This measurement system consists in stages of sample excitations even with different light source as we can see in Fig. 13.12. First with this technique it is necessary to keep the photosynthetic sample isolate in the dark, usually with a chamber in order to do not subtract portability and do field measure. This dark-adapted time period can be between 10 and 20 min. Then a pulse measuring low red light ML is used to determine F_o of dark-adapted sample, it is important that ML must be low enough in order to avoid chlorophyll fluorescence emitting (Fig. 13.12). With the aim to determine F_m is applying a white light saturation pulse SL (e.g., 3,000 μmol (photon) $m^{-2}s^{-1}$). When the fluorescence decrease again to F_o level a continuous nonsaturating red actinic light is started AL, this induces a maximum fluorescence at level F_p and subsequent a slow decline between 3 and 4 min until reach F level again. Then in order to obtain F_m', a saturating flashes SL is necessary apply; this must be during AL. After several minutes of AL, when the constant low F is reached with the aim to activating only PS1 during a few seconds, a SL is given and then AL is turned off (Lichtenthaler et al. 2005).

Fig. 13.12 PAM chlorophyll fluorescence kinetics, modified by Lichtenthaler et al. (2005)



13.9.2 *Applications of the Fluorometers to Detect Stress*

Until now, the reader has been guided through the operation of fluorometers, in this section we will make a brief the application of the fluorometers in the stress detection. It is well known that the photosynthetic processes are quiet related with the comfort status of the plant, because the photosystems are the major targets of many environmental stress modifying the crop yield, hence the chlorophyll fluorescence kinetics (Lin et al. 2007). However, there are stresses that are not directly related with the chlorophyll fluorescence. For example, in drought stress, the decrease of the relative water content of leaves results in a stomatal closure and decreasing the supply of CO_2 to the mesophyll cells and then decreasing the rate of the photosynthesis. Mentioned above, there is not a direct impact on the efficiency of the primary photochemical events of the PSII or affect the chlorophyll fluorescence parameters (Table 13.7), such as maximum quantum yield of photochemistry in PSII in dark-adapted (F_v/F_m) (Massacci and Jones 1990). On the case of the salinity stress is very similar to drought, and it is provoke where large amounts of fertilization and irrigation are employed in climates with high evaporation, which results in changes in F_q'/F_m'.

In the case of the temperature, for example low temperatures, the metabolism is severely affected and the damage of the PSII that is high affecting also the measurement of the maximum quantum yield of the photochemistry in PSII. On the other hand, with the high temperatures the inactivation of the PSII and the disorganization of the thylakoid are taken into account causing a monitoring the rise of F_o as well as the F_v/F_m (Baker and Rosenqvist 2004).

The nutrition is critical in yield of the crops, being Nitrogen the main mineral nutrient needed for chlorophyll production (Muñoz-Huerta et al. 2013).

Decreasing the Nitrogen content in the case of the apple leaves decrease F_q'/F_m', F_q'/F_v', and F_v'/F_m' (Cheng et al. 2000). However, the F_v/F_m is not clearly affected unless the nutrition stress is critical (Baker and Rosenqvist 2004).

13.10 Conclusion

As has been noted in above-mentioned, some stresses have similar affectations on the chlorophyll fluorescence kinetics. However, it is necessary to pay attention in the modifications of the data produced by the fluorometers, because in the literature there are many ranges of the plant comfort, but the plants are living beings and such as us, are different in the thresholds of comfort at the moment of feel the environmental, nutrition, among others factors that can affect the photosynthetic process, and here lies the importance to apply a analytic tool such as the chlorophyll fluorescence measurement systems and benefit that is a noninvasive tool, being a good and remarkable attribute because its congruence to make a measurement without a stress such as measurement invasive do.

13.11 Climate Control in the Twenty-First Century, the Reliable Way the Guarantee the Food Supply

Food security is one of main global challenges in the current century. Word population will increase up, at least, the middle of the twenty-first century, and demand food will rise. Moreover, problems such as climate change and pests have a profound impact on food production. Then, it is easy to notice that traditional agricultural scheme cannot deal with these problems and an alternative solution has to be implemented (García-Mier et al. 2013).

Greenhouses are building structures that allow the creation of an indoor microclimate for crop development, protecting plants from severe external climate conditions or pest invasion. The greenhouse microclimate can be manipulated by control actions, such as heating, ventilation, CO_2 enrichment to name a few; in order to provide appropriate environmental conditions. These modifications imply additional use of energy in the production process. Furthermore, it requires a control system that minimizes the energy consumption while keeping the state variables as close as possible to the optimum crop physiological reference. Horticulture in greenhouse conditions is a rapidly expanding interest and is consequently increasing in its economic and social importance (Kolokotsa et al. 2010).

The greenhouse climate control problem consists in the creation of a favorable environment for crop development, looking for high yield, quality and low operative costs. Along the time, researches have shown that this is a very difficult task, due to the complexity of the greenhouse environment. Even small changes in the structure or location could drastically redefine the problem. For example, microclimate variables such as temperature and relative humidity (RH) are highly nonlinear and strongly coupled, and the greenhouse is largely perturbed by the outside weather. Also the aforementioned objectives (yield, quality, low cost) presents conflict of interest because generally, high control precision agriculture requires extra energy consumption (Haigen et al. 2010).

13.11.1 The Last Decade

Despite many technological limitations, greenhouse engineering developments during 1990s shows significant progress in different fields. There have been significant advances in the modeling of thermal behavior of the greenhouse and much effort was directed toward understanding ventilation. Analysis has shown that different sources exist (thermal and wind induced forces) and these have been analyzed. The existence of internal circulating air patterns has also been established. Considerable progress has been made in developing methods to provide CO_2 setpoint trajectories that maximize the margin between the financial benefit of enrichment and the cost of the CO_2 used (Critten and Bailey 2002).

Notwithstanding the impressive progress in control theory and the understanding about phenomena related with the greenhouse microclimate, conventional control techniques were almost exclusively used during last decade. Although reached solutions were barely reliable, attempts to use modern techniques to improve the quality of the control was limited by the high complexity of these techniques and the low computational power available, making its application on current hardware impossible (Sigrimis and King 2000).

13.11.2 Greenhouse

Different research has been conducted regarding climate control for protected agriculture applications. The main objective of these investigations is to find an accurate model that represents the greenhouse environmental dynamics and an efficient and a flexible controller that adjusts the microclimate variables of interest. This problem has been the focus of many re-searchers worldwide who have analyzed, experimented, and proposed many climate control systems in order to manipulate variables such as temperature, RH, CO_2 enrichment, radiation, and many others that are necessary to generate the fundamental conditions for successful protected agriculture (Van Straten et al. 2000).

The greenhouse-crop system can be considered as a solar collector involving sensible and latent heat exchanges. It is a complex system that can be divided into three main components, which presents a high mutual interaction: internal atmosphere, the crop, and the soil. The behavior of the whole system not only depends on these interactions, but also on the outside weather and on the actions of

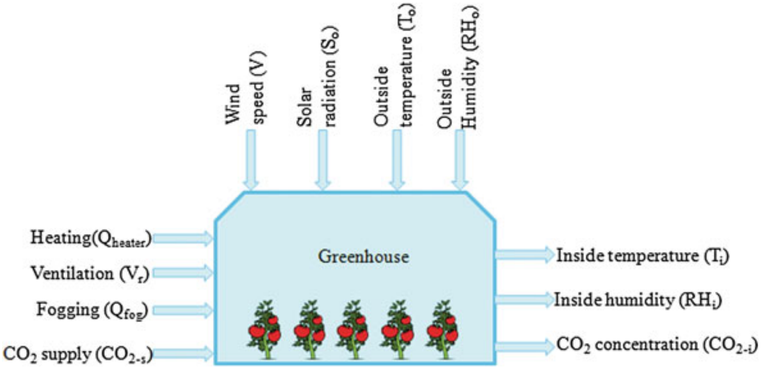


Fig. 13.13 Greenhouse climate model

actuators via the control system. A simple model from the greenhouse system can be formulated by energy and mass balance principle (Boulard and Wang 2000). Therefore, the simplest way to represent the greenhouse system, including external perturbations, microclimate variables, and even possible control inputs are presented in Fig. 13.13.

The output variables are: inside relative humidity HR_i in (%), inside temperature T_i (°C) and $\mathrm{CO_2}$ concentration CO_{2-i} (ppm). The input variables that can be manipulated are ventilation V_r ($\mathrm{m^3\ s^{-1}}$), heating control Q_heat (W), water capacity of the fog system Q_fog ($\mathrm{gH_2Os^{-1}}$) and $\mathrm{CO_2}$ dosage flux $\mathrm{CO}_{2-\mathrm{s}}$ (ppm). Measurable perturbations are: solar radiation S_o ($\mathrm{W\ m^{-2}}$), outside temperature T_o (°C), outside relative humidity $\mathrm{RH_o}$ (%) and wind speed and direction V_r ($\mathrm{m\ s^{-1}}$).

The importance of aforementioned greenhouse climate model lies in most control theories require the mathematical model of the system for tuning and simulating the proposed algorithms. Then, different mathematical greenhouse models have developed based on this scheme (Fig. 13.13). It includes from simple models that only describe air temperature or relative humidity to detailed models that even involve crop response (Setiawan et al. 2000). A simple model of the temperature changes in a greenhouse can be described by the differential equation 13.1.

$$\frac{dT_G}{dt} = \frac{1}{C}\left[K_{\mathrm{out,\,air}}(T_o - T_G) + q_h\right] \tag{13.1}$$

where the T_G is the greenhouse internal air temperature, C the greenhouse thermal capacity, $K_{\mathrm{out,\,air}}$ is the heat loss coefficient from greenhouse air to outside air. T_o is the external air temperature and q_h is the heating power (Arvanitis et al. 2000). Despite easiness of the model, is widely accepted and provides a quick, inexpensive, flexible and repeatable way to compare how the greenhouse temperature responses to certain control methodology. These characteristics are not achievable by using experiments.

However, model of Eq. 13.1 do not take into consideration other important factors and their interactions. Therefore, in an effort to reach a more accurate

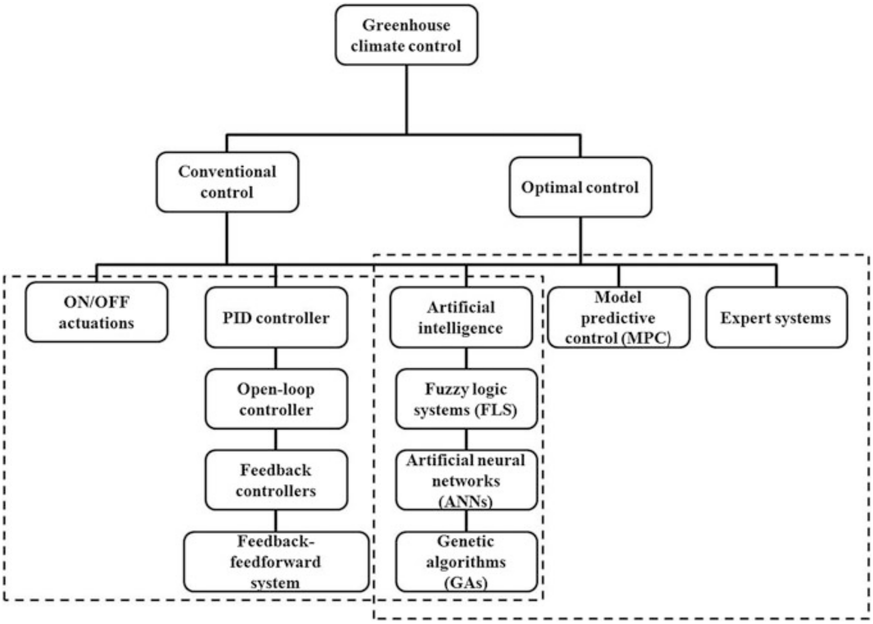


Fig. 13.14 Greenhouse control theories classification

model, variables such us absolute humidity, short-wave solar radiation, leaf area index, ventilation rate, and interaction rates between the main greenhouse components (crop, soil, internal atmosphere) were addressed in the greenhouse climate model by Castaneda-Miranda et al. (2006). Nevertheless, the performance of developed models is limited by the lack of crop response within the equation, because crops strongly influence the surrounding environment a in a dynamic way, according to their phonological development. This problematic have not been completely solved, but promising approaches that include greenhouse-crop interaction and complex processes such us photosynthesis or transpiration have been developed (Van Straten et al. 2000).

Is easy to notice how has evolved the way to addressed the problem, going from models which just describe the thermal behavior of the greenhouse by using energy and mass balance. To unconventional descriptions that consider the crop influence and also take account how this influence change over time. New tendencies move on this direction, giving the deserved importance on models and control theories to the main factor in the greenhouse, “the plat”.

In order to understand how control theories for protected agriculture have involved addressing the requirements of modern society, a classification that helps to understand the global panorama is required. However, establishing a division between controllers presented in the current literature is complicated, due to the variety and integration of diverse techniques used to solve the same problem. Figure 13.14 shows a classification which divides the most control techniques into two main fields (Duarte-Galvan et al. 2012). The first one is usually called conventional control which consists on climate controllers which try to control the greenhouse microclimate just by reducing the deviation between setpoint of the interest variables and measured values to zero. As examples of conventional controllers are ON/OFF, PID, other classical controllers and also artificial intelligent (AI) paradigms such as artificial neural networks (ANNs), fuzzy logic systems (FLS), genetic algorithms (GAs), among others. The other field is optimal control, in which factors such as greenhouse dynamic behavior, actuator capabilities, water and energy consumption and meanly the crop response are taken into account. Expert systems and model predictive control (MPC) are widely accepted for optimal control purposes. However, aforementioned AI-based techniques can be also considered like optimal production controllers when they reached objectives such us optimal crop growth, reduction of the associate costs, reduction of residues and the improvement of energy and water use efficiency (Ramírez-Arias et al. 2012).

Conventional controllers were widely used since computational tools were introduced in protected agriculture until the end of twentieth century. Nevertheless, the increase in power computational capabilities with cost reductions in the next decade allowed the application of more complex algorithms which deals with the optimal control scheme. In the rest of this chapter, the review of protected agriculture techniques will be focus on optimal control taking into consideration both modeling and controllers.

13.11.3 Optimal Control

The objective of modern greenhouse industry is a sustainable crop production system by reducing water and energy consumption and biocide use while maintaining a high crop quality and yield. In addition to new materials and advance building methodologies, this can be achieved by modifying the microclimate control strategy. However, the optimal control scheme is only slowly accepted in practice due to the lack of reliable crop development models for the wide variety of crops. Also, experimental probes that show advantages and clear assessment of the risks involved and the theoretical limitation is required. In order to effectively validate the performance of proposed optimal control algorithms, greenhouse models which include parameters related with crop response are required.

13.11.4 Modeling

A good dynamic model of the process is essential in order to achieve a good performance of the controller. Two different methods for computing greenhouse models can be found in the literature. One is based in terms of the physical laws involved in the process, and the other is based on an analysis of the input–output data of the process. In the first method, the process behavior is incompletely known, and physical modeling is not easy considering the complexity of the system that translates high order models with lot of parameters which are difficult to adjust due to the nonlinear behavior of the greenhouse model (Herrero et al. 2008). The second method consists in approximate the behavior without a priori information, for instance, polynomial fitting, ANNs, FLS, etc. However, they do not have physical meaning but are easier to obtain.

How was previously mentioned, modeling by physic laws is complicated because in order to achieve a reliable model, lot of parameters have to be included in the model. But, it has proved that physic-laws models had a better goodness-of-fit that black-box models (Blasco et al. 2007). However, adjusting these parameters is complicated. An alternative was purposed by Guzmán-Cruz et al. (2009), which used and compared different evolutionary algorithms (EA) such as GAs, Evolutionary Strategies (ES) and Evolutionary Programming (EP) to calibrate the parameters that defines the greenhouse inside temperature and relative humidity within a greenhouse with tomato crop. The calibration consisted in an optimization problem which works altering model parameters until getting a better fit between estimated and measured data. Results show a better performance of EP to predict the air temperature and RH behavior; however, least squares (LSQ) and sequential quadratic programming (SQP) methods slightly improve the estimation of temperature, but present lot of inaccuracy for RH. This assertion is supported by (Speetjens et al. 2010), which also indentified the key parameters of a physic-based model using a controlled random search (CRS). The model is composed by a limited number of states, which keep the computational load in an acceptable level. This model enhances practical applicability for in situ purposes.

However, in all aforementioned works, important sets of data are required. Measurements in the greenhouse during 2 years or more were necessary to calibrate the models; otherwise the risk to get trapped in a local minimum is highly probable. Other problematic relies in controlling greenhouses are strongly dependent on the geographical area; and solution that are valid in some regions must be adapted in order to fit others (Herrero et al. 2007). This problematic was partially solved developing a reduced thermal and mass (water vapor) model of the greenhouse by an on-line estimation of its parameters. This estimation was carry out using the particle swarm optimization (PSO), which present a better performance than GAs in some cases (Coelho et al. 2005; Hasni et al. 2011). Also, several advantages were reached, such as energy savings, soft optimal control effort a low computational burden (El Ghoumari et al. 2005). Automatic, on-lined estimation and adaptation of parameters in physic-based models were proposed by Speetjens et al. (2009) using Extended Kalman Filtering (EKF). This technique could deal with changing circumstances like plant growth, changes in material properties and modification in greenhouse design and layout. Then, EKF improves the model fit over a longer period. After tuning of the filter, the parameters were automatically adapted, so that changes in system were dealt with. The unique restriction of this technique is that the number of parameters to be estimated has to be not to large compared with available measurement data.

All the aforementioned models deal with the nonlinearities of the greenhouse system by using unconventional techniques to adjust the parameters of the model. However, many researches choose linear model techniques, attempting to formulate an easier model, easily programmable in current greenhouse climate controllers. This choice deals with many problems, taking into consideration several restrictions have prove to work properly (Singh et al. 2006).

Many parametric models have been proposed, they usually are polynomial equations which involve the main variables of phenomena (temperature, humidity, among others) and many coefficients which are obtained by different identification methods. The well-known off-line least squares method was used by Bennis et al. (2008), input–output signals sampled every minute during a day were used to build the model. The model also involves perturbation such as external humidity and temperature, radiation, and wind speed. Previous works opt for on-line identification, arguing that changing the operation conditions in the greenhouse requires re-tuning of the controller parameters in order to achieve optimum performance. However, fewer variables are taking into account in this methodology because the computational power required increases according with the number of variables to estimate (Arvanitis et al. 2000).

Despite the favorable results obtained by the aforementioned models, many authors argue that a more detailed knowledge about phenomena is required in order to increase the performance of controller and thus to meet objectives of modern agriculture (Katsoulas et al. 2007). Some works deal the problem of model plant behavior and it influence on the greenhouse microclimate. These behaviors could be related with plant growth, transpiration, or photosynthesis (Boulard and Wang 2000).

Other works focus in order to understand de ventilation phenomena inside the greenhouse by models with consider the heterogeneity of the system and how the different segments of the atmosphere interact with plan canopy or external environment (Abdel-Ghany and Al-Helal 2011; Dayan et al. 2004). Computational fluid dynamics (CFD) also serves to study ventilation, its effects on inside temperature and humidity, and how it is affected by external wind direction (Bournet and Boulard 2010; Kittas and Bartzanas 2007). Experimental work focused in understanding how the ventilation rate modifies the physiological state of the plan was conducted. Finally based on these studies, different methodologies were purposed to design the greenhouse geometry, the correct orientation, and even the required equipment in order to achieve a desirable yield and quality (Nebbali et al. 2012; Vanthoor et al. 2011). Also, software which use all the aforementioned models were developed in order to provide a tool that could predict the greenhouse behavior under certain weather characteristics previously its installation, equipment available, and greenhouse materials could also be included in this web-base application (Fitz-Rodríguez et al. 2010).

13.11.5 Controllers

The advantages of using optimal instead of conventional greenhouse climate control can be summarized as follows. An optimal control approach to greenhouse climate control fully exploits scientific quantitative knowledge concerning the greenhouse atmosphere, the soil, the equipment, the crop, and their interactions. All of them captured in a mathematical dynamic model that deals with the problem of maximizing the profit, achieving welfare of the crop through practices that minimizes production costs (Van Straten et al. 2010).

Robust controllers were applied in protected agriculture because its ability to deal with uncertain parameters, disturbances, or modeling mistakes (Linker et al. 2011). They were applied focusing on managing the high correlation between air temperature and hygrometry (Bennis et al. 2008). This decoupling allows the use of two control loops, one for each variable. Despite the modeling uncertainties and strong disturbances, the controller presented an acceptable performance.

Horticultural research has indicated that for the majority of plants, crop growth responds to long-term average temperatures rather than specific day and night temperature profiles. This principle is used in the well-known temperature integration technique (TI). Where is possible to adjust the setpoint temperature in a flexible way to obtain a desired average temperature instead at fixed value over the time. Therefore, energy savings could be obtained decreasing flexibility to the heating set points when conditions are favorable and lowering it when they are not. This knowledge was applied by Sigrimis et al. (2000) in the design of a tool available to exploit the interaction between photosynthesis and growth according to empiric knowledge. The method is based on varying heating set points using previously recorded information in order to achieve the desired average for any user-defined period. Meanwhile, (Körner and Challa 2003) decoupled process with fast temperature response (e.g., photosynthesis or stress) from a process with a slow response time. The objective was to improve the temperature integration concept by introducing dynamic temperature constrains; these flexible boundaries depend on the underlying crop process while increasing the potential for energy saving in greenhouses. Despite the promising energy savings obtained with temperature integration, the potential of this technique is limited by humidity if usual set points are maintained, because the high relation between those variables counteracts the TI. A promising solution is the use of a process-based humidity regimen. In this regimen, relative humidity set points can freely move within a range, avoiding affect the TI objectives. However, as humidity changes could highly affect the crop quality and yield, the set points its duration period were calculated in order to avoid plant-affecting processes such as Ca-deficiency, plant water stress, crop growth, crop development and airborne fungal diseases (Körner and Challa 2004).

Another way to improved TI in cold lands was addressed by (Aaslyng et al. 2003), which used mathematical models for estimating the absorption of irradiance, leaf photosynthesis and respiration. Then, the temperature which was controlled according to natural irradiance was allowed to vary more than usual. When light conditions were no appropriate for crop development, heating energy was reduced. On the other hand, when irradiance rises, plants are able to utilize higher temperatures and CO_2 concentrations more efficiently. Applying this TI modified techniques; energy saving per season between 20 and 38 % were achieved.

MPC is an advanced control technique commonly used for industrial application but recently applied in the field of protected agriculture. The objective is predicting the greenhouse variables behavior. A contribution to this scheme was offered by Van Straten et al. (2000), where information about crop growth simplifies the design of greenhouse control strategies to obtain a truly economical control strategy. This approach leads to the concept of selecting processes by time response where the short-term effects like photosynthesis and evapotranspiration are dealt with by an automated model predictive optimal controller, while the long-term effects are left to the grower. Energy savings are also achieving with this methodology, but in comparison with TI which offers flexible set points, the predictions of MPC allow the use of a soft optimal control effort (Piñón et al. 2005). Another important characteristic of MPC is its multivariable constraint handling capacity and the ability to set cost for each prediction. These characteristics were exploited including restrictions to energy and water consumption. For example, error and control signals are weighted differently during the day and the night, the errors weights are much higher during the day that at night and the constrains are relaxed during the night (Blasco et al. 2007).

As was previously mentioned, the grower intervention had not been completely avoided in the crop management. Then, decision support tools that assists the grower to choose the most appropriate climate regimen was proposed (Gupta et al. 2010). These regimens choose the most appropriate climate for plants according with its phonologic state in order to obtain the optimal gains of sustainability and plant quality. The greenhouse climate and crop model are studied separately and jointly considering the effects of six different regimes with increasing degrees of freedom for various climate variables which include: crop model, temperature integration, dynamic humidity control, and negative DIF regimes (DIF = the difference between average day temperature and average night temperature, and therefore reduces the use of chemical growth regulators) (Körner and Van Straten 2008).

Phytocontrol is the new theory which proposes the use of the plant physiological responses as input signal to establish the set points in the climate controller (Ton et al. 2001). Also, this has not proved to be a stable and reliable method, because it is necessary to gather a lot of information to prove the reliability of this theory (Linker and Seginer 2003). Nevertheless, different types of controllers have emerged demonstrating advantages and disadvantages between them, better performance for some actions among other characteristics.

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Chapter 14
Control Strategies of Greenhouse Climate for Vegetables Production

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Contents

14.1 PID Control

The most extended control in the greenhouse industry across the world is the classical Proportional-Integral-Derivative control (PID) (Ogata 2002; Kamp and Timmerman 1996; Bakker et al. 1995; Hanan 1998). The greenhouse environment is a complex system with large lag, multiple-inputs and multiple-outputs (MIMO), nonlinear and difficult to model mathematically, and therefore even the implementation of classical control methods has been found difficult (Wang et al. 2012). The greenhouse climate control is challenging due to highly nonlinear interactions

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between the biological and physical subsystems and also to the strong coupling of the two main control variables: temperature and humidity (Gurban and Andreescu 2012). However, PID controllers have mainly been applied to control air temperature, (Zhou et al. 2013) humidity, irrigation, and nutrients supply (Jaimes-Ponce et al. 2013). In several research works, PI/PID controllers are used as references (Cheng et al. 2013) because they are well known and also because researchers are looking for improvement in PID controllers performance by combining them with modern control approaches such as neural networks (Qu et al. 2011; Zuo et al. 2012) and genetic algorithms (Bounaama and Draoui 2011).

14.2 Optimal Control

14.2.1 Problem Statement

A greenhouse system is a complex biosystem with biological and climatic components that make it a mathematical challenge for its modeling and control. The dynamic mathematical models that describe the behavior of the greenhouse crop and microclimate generally are nonlinear and non-convex (Chalabi and Zhou 1996). In addition they are stiff because of the existence of several different timescales in the system. The time constants of the climatic variables and plant physiological processes such as photosynthesis and transpiration are too small in comparison to the crop growth (van Straten and van Henten 2010; van Straten et al. 2011). Research carried out during the last two decades (van Straten et al. 2011) has proved that time constants in the greenhouse vary from 1 to 2 months in case of the crop, 1–2 days for the soil and greenhouse climate, to 10–20 min in case of light (van Henten 1994; Tap 2000). Furthermore, there is a high uncertainty related to the input variables of the models such as external weather, but also to the initial conditions of the states and model parameters. The optimal control of the greenhouse climate implies the manipulation of the control variables in such a way that the profit of the grower is maximized. According to the optimal control theory (Kirk 1998) in order to state an optimal control problem it is required to define a dynamic mathematical model of the system, a set of physical constraints, and also a performance measure. Furthermore, due to the model complexity and nonlinearities, numerical algorithms are needed to solve the optimal control problem. In the greenhouse the main state variables are air temperature (T_g), humidity (V_i), and carbon dioxide concentration (C_i), but also for instance structural (W_s) and non-structural (W_n) biomass of the crop. The crop behavior in time inside the greenhouse is influenced by the external weather and control actions. On one hand, the most relevant disturbances are solar radiation (G), temperature (T_o), wind velocity (w), CO_2 concentration (C_o), and humidity (R_o) outside the greenhouse. On the other hand, most important control variables are manipulated variables that allow modifying the greenhouse environment such as windows opening (r_w), heating system (H), fogging system, ventilation system, cooling system, or CO_2 injection (ϕ_i). Formalizing the main ideas aforementioned (van Henten 1994; Tap 2000) a dynamic mathematical model of the greenhouse environment is given in state-space form as follows:

$$\dot{x} = f(x, u, v, p, t),\ x(t_0) = x_0,$$

where $x(t) \in R^n$ is the vector of n state variables:

$$x = \begin{bmatrix} T_g & T_s & C_i & V & W_n & W_s \end{bmatrix}^T$$

The vector of control variables $u(t) \in R^m$ may be given by $u = \begin{bmatrix} H & r_w & \varphi_i \end{bmatrix}^T$.

The disturbances vector $v(t) \in R^q$ might be specified by $v = \begin{bmatrix} T_o & G & w & C_o & R_o \end{bmatrix}^T$.

The vector of model parameters $p \in R^s$ includes physical coefficients associated with the climatic variables and also physiological coefficients appearing in the processes connected to crop growth.

The control variables usually have magnitude constraints. These constraints are represented by the equation:

$$u_{i,\min}(t) \le u_i(t) \le u_{i,\max}(t);\ i = 1, \ldots m$$

where $u_{i,\min}(t)$ and $u_{i,\max}(t)$ represent the lower and upper limits of the control inputs. The state variables also could have constraints since in general they are approximately known critical values for different crops. Then, it is important to specify constraints for the states:

$$x_{i,\min}(t) \le x_i(t) \le x_{i,\max}(t);\ i \in I_{xc}$$

where $x_{i,\min}(t)$ and $x_{i,\max}(t)$ mean the lower and upper limits for the states and I_{xc} is the corresponding index for the states constraints.

The performance measure can be formulated as the net income obtained during the growing period $[t_0, t_f]$ of the crop using the functional ($J(u)$):

$$J(u) = \Phi(x(t_f), t_f) - \int_{t_0}^{t_f} L(x, u, v, p, t)\mathrm{d}t$$

where the terminal function $\Phi(x(t_f), t_f)$ is profit obtained from the selling of the product at harvest time (t_f). The final time can be defined as fixed or also as a variable and therefore subjected to an optimization as well. The function $L(x, u, v, p, t)$ represents the running costs associated to the control systems applied to the system during the cultivation period. Thus, the open-loop optimal control problem consists of finding the optimal control strategies ($u^*(t)$) that optimize the performance measure ($J(u)$) subjected to the dynamic state equations and that satisfy the constraints given some predictions of the disturbance variables ($v(t)$) during the growing period ($[t_0, t_f]$) of the greenhouse crop.

14.2.2 Solution Methods

The solution methods applied to the optimal control of a greenhouse system are numerical and they are classified in indirect methods (Bryson 1999) which use the Pontryagin minimum principle (Seginer et al. 1991; Seginer and McClendon 1992; Seginer 1996; van Henten 1994; van Henten et al. 1997a, b; Trigui et al. 2001a, b; Ioslovich and Seginer 2002; Ioslovich et al. 2009) and direct methods (Bryson 1999) which transform the original optimal control problem in a nonlinear programming (NLP) problem (Chalabi et al. 1996, 2002a, b). According to the literature the first approach has been applied mostly to the greenhouse system. According to Kirk (1998); Bryson (1999); Lewis and Syrmos (1995) the Pontryagin minimum principle (PMP) is applied as follows:

By introducing the Hamiltonian function

$$H(x, u, \lambda, t) = L(x, u, v, p, t) + \lambda^T(t)[f(x, u, v, p, t)]$$

where $\lambda^T(t)$is the vector of co-states. The necessary conditions of optimality for a stationary trajectory which can be derived from the calculus of variations or differential of a functional (Bryson 1999; Kirk 1998; Lewis and Syrmos 1995; Athans and Falb 2007; Stengel 1994) are:

$$\dot{x} = \frac{\partial H}{\partial \lambda} = f(x, u, v, p, t)$$

$$\dot{\lambda} = -\frac{\partial H}{\partial x} = -\frac{\partial L(x, u, v, p, t)}{\partial x} - \lambda^T(t)\frac{\partial f(x, u, v, p, t)}{\partial x}$$

$$H(x^*, u^*, \lambda^*, t) \le H(x^*, u, \lambda^*, t)$$

$$\lambda^*(t_f) = \frac{\partial \Phi(x^*(t_f), t_f)}{\partial x}$$

This means that a two-point boundary value problem has to be solved by integrating the state equations forward in time and integrating backward in time the co-states equations. The stated optimal control problem has several boundary conditions and in the greenhouse system context at least four subproblems can be identified: (i) as the harvest time t_f of the crop is fixed and the state variables $x(t_f)$ are free at the final time, given that the aim is to maximize the amount of crop biomass (fruits, stems, leaves, etc.). (ii) Both the harvest time t_f and some or all the state variables at the final time $x(t_f) = x_f$ are fixed. (iii) The harvest time t_f is free and all or several state variables are specified at the final time. (iv) Both the harvest time and some or all the state variables at the final time are free and need to be optimized. Because of the stiffness of the greenhouse system, the application of optimal control theory is not straightforward and it has been found that two or three timescales decomposition are required in order to prevent a loss of optimality of the optimal greenhouse climate control (van Straten et al. 2011). In case of two timescales (van Henten and Bontsema 2009), it is necessary to state and solve an optimal control problem with slow dynamics to solve the long-term seasonal optimization taking into consideration the crop model and also a problem with fast dynamics to solve the short-term optimization problem using the greenhouse climate model. Researchers such as Seginer and Ioslovich (1998); Chalabi et al. (1996); Ioslovich and Seginer (1996) calculated suboptimal solutions given that their studies were focused only on the long-term optimization. On the other hand, van Henten (1994) proposed, the first time, the use of singularly perturbation systems theory stating and solving the open-loop optimal control problem in greenhouses. Tap (2000) proposed two timescales decomposition for the greenhouse optimal control problem. Also, Van Willigenburg et al. (2000) used three timescales. The numerical methods used to solve the dynamic optimization problem of the greenhouse climate have been mainly gradient-based methods (Bryson 1999). The solution of the optimal control problem of greenhouse climate using two timescales proceeds as follows (van Henten and Bontsema 1996, 2009): by separating the state variables with slow dynamics $x(t)$ such as dry weight of the crop, from those that have fast dynamics $z(t)$ such as air temperature and humidity, the system greenhouse climate-crop is given by the dynamic equation s:

$$\dot{x} = f(x, z, u, v, p, t); x(t_0) = x_0$$

$$\varepsilon \dot{z} = g(x, z, u, v, p, t); z(t_0) = z_0$$

where ε is a timescaling parameter. The control and state variable constraints are the aforementioned. The performance measure is defined now with the functional:

$$J(u(t)) = \Phi(x(t_f), t_f) + \int_{t_0}^{t_f} L(x, z, u, v, p, t)\mathrm{d}t$$

According to the approach proposed by the Dutch school, first, the subproblem with small dynamics or seasonal optimization need to be solved by assuming $\varepsilon = 0$. Then the dynamic equations corresponding to the slow dynamics of the system (s) are obtained:

$$\dot{x}_s = f(x_s, z_s, u_s, v, p, t); x_s(t_0) = x_0$$

$$0 = g(x_s, z_s, u_s, v, p, t)$$

Using these equations the aim is to control efficiently the slow dynamics of the whole greenhouse system. Therefore, the optimal control problem consists of the calculation of the optimal controls $u_s^*(t) \in [t_0, t_f]$ that maximize the performance criterion:

$$J_s(u_s(t)) = \Phi(x_s(t_f), t_f) - \int_{t_0}^{t_f} L(x_s, z_s, u_s, v, p, t)\mathrm{d}t$$

subjected to the slow dynamics equations and states and controls constraints.

In order to solve the subproblem with fast dynamics the dynamics of the slow-state variables is neglected and both the calculated optimal trajectories of the states $x_s^*(t)$ and the co-states $\lambda_s^*(t)$ coming from the solution of the slow subproblem are used as a reference. Now, the optimal control problem consists of finding the optimal controls $u_f^*(t) \in [t_0, t_f]$ that maximize the performance index:

$$J_f(u_f(t)) = \int_{t_0}^{t_f} [-L(x_s^*, z_f, u_f, v, p, t) + \lambda_s^{*T}(t) f(x_s^*, z_f, u_f, v, p, t)] \mathrm{d}t$$

subjected to the dynamic equations with fast dynamics:

$$\dot{z}_f = g(x_s^*, z_f, u_f, v, p, t);\ z_f(t_0) = z_0$$

where $x_s^*(t)$ and $\lambda_s^*(t)$ are the optimal states and co-states trajectories obtained from the solution of the subproblem with slow dynamics.

14.2.3 Receding Horizon Optimal Control

Because the optimal control of the greenhouse system described until now is an open-loop optimization that allows an offline solution it cannot cope with the uncertainties associated to the initial conditions, model structure, and weather predictions. Therefore, in order to implement optimal control strategies online, some feedback is required. A receding horizon optimal control has been proposed and applied to the dynamic optimization of the greenhouse system (Tap et al. 1996; Tap 2000) because a receding horizon optimal controller (RHOC) has several advantages in comparison with a Linear Quadratic Gaussian (LQG) regulator and a General Predictive Controller (GPC) in the context of the greenhouse system: the goal function is nonlinear and of economic nature instead of quadratic, the system is nonlinear in the controls and the disturbances such as solar radiation are exploited instead of suppressed (van Straten et al. 2002, 2011). In an RHOC the optimal control problem consists in finding the optimal controllers $u^*(t)$ that minimize the functional $J(u(t))$

$$J(u(t)) = \int_{t_0}^{(t_0+t_h)} \left[-L(x, z, u, v_f, p, \tau) + \lambda_s^{*T}(t) f(x, z, u, v_f, p, \tau)\right] \mathrm{d}\tau$$

subjected to the dynamic equations:

$$\dot{x} = f(x, z, u, v, t);\ x(t_0) = \hat{x}(s_{i+1})$$

$$\dot{z} = g(x, z, u, v, t);\ z(t_0) = \hat{z}(s_{i+1})$$

where t_h is a short-term finite horizon and v_f are the short-term weather predictions, $\hat{x}(s_{i+1})$, $i = 0, 1, 2, \ldots$ is the state estimate from the model and the measurements at each sampling instant s_i in which all the measurements and control are updated and a new optimal control problem is solved. It is apparent that $t_0 = s_{i+1}$ is required for $i = 0, 1, 2\ldots$ and also the final time is updated using $t_f = s_{i+1} + t_h$. Therefore, a new optimal control problem is solved at each time period $T = s_{i+1} - s_i$. Tap (2000) applied a gradient-based algorithm to solve this RHOC each minute. More recently, an RHOC has been applied to control a solar greenhouse (van Ooteghem 2007) and the nutrients supply to greenhouse tomatoes (van Straten et al. 2006). An extensive discussion on the so-called closed-loop optimal control in greenhouse has been elaborated recently (van Straten et al. 2011).

14.2.4 Model Predictive Control

The model predictive control (MPC) approach has been applied to the greenhouse system recently. According to the theory of model predictive control (Camacho and Bordons 1998), the three main components of an MPC required to derive an optimal control law of the system are a prediction model, an objective or cost function, and an optimization algorithm. There are several options for the components of an MPC. In the case of the greenhouse system some researchers have applied state-space models similar to the ones required in optimal control, for the greenhouse climate (Blasco et al. 2007; Piñon et al. 2005; El Ghoumari et al. 2005), and autoregressive models (ARX) with external inputs (Coelho et al. 2005). Regarding the cost function it has included the optimization of energy and water consumption in the greenhouse (Blasco et al. 2007). Objective functions including only the greenhouse climate have been used by other researchers. However, no one has so far used a model of the crop growth and development. Several optimization algorithms such as gradient-based methods (Piñon et al. 2005; El Ghoumari et al. 2005), but also global optimization approaches such as genetic algorithms (Blasco et al. 2007) and particle swarm optimization (Coelho et al. 2005) have been applied to solve the MPC in the greenhouse context. A model-based predictive control was tested in order to optimize the efficiency of two heating systems in a greenhouse: aerial pipes with hot water and air-fan heater. It was found that using linearized models of the system around the predefined set points and a generalized predictive control strategy, the performance was improved (Ramírez-Arias et al. 2005). Recently, a nonlinear model predictive control strategy was designed for temperature control in a greenhouse using natural ventilation (Gruber et al. 2011). The control strategy is based on a second-order Volterra series model coming from experimental input/output data of a greenhouse.

14.2.5 Robust Control

Robust control is an approach that attempts to design stable controllers in spite of the uncertainties and disturbances generally acting on the system (Morari and Zafiriou 1989). The control system specifications are given in time-domain and then translated to the frequency-domain using a second- or third-order model for the closed-loop system. The model uncertainties are also considered in the design procedure using Nichols charts. The design method allows for closed-loop stability and meeting the specifications of the system. This approach has only rarely been applied to control the greenhouse system. Linker et al. (1999) applied robust controllers for simultaneous control of temperature and CO_2 concentration in greenhouses. They applied the Horowitz's method to their design. Moreno et al. (2002) also designed a robust controller for temperature inside a greenhouse using the Horowitz's method. The system was modeled as a first-order system with uncertainty. More recently, the approach of H_2 robust control has been applied to control temperature and humidity of a greenhouse (Bennis et al. 2008). Their research work looks for closed-loop stability and variations of the controlled variables within acceptable ranges and proportions given by the operating conditions of the greenhouse system. They used the Linear Matrix Inequality (LMI) formalism to synthesize the controller. Recently, Linker et al. (2011) designed robust controllers using quantitative feedback theory (QFT) in order to guarantee good performance of the system in spite of modeling uncertainties and disturbances. The model considered air enthalpy and humidity ratio. The method starts with a nominal model and the computation of value-sets that describe the model uncertainties. These values are combined with the design specifications to calculate bounds that the nominal open-loop have to satisfy in such a way that the closed-loop system will be stable and meet the requirements. At the end a pre-filter is obtained.

14.2.6 Adaptive Control

In order to deal with uncertainties of the greenhouse system, the approach of adaptive control (Astrom and Wittenmark 1994) has been proposed by several researchers. For instance, multirate-output controllers were proposed to control greenhouse temperature. The multirate-sampled data of the system coming from a given sampling period is used to control the closed-loop system (Arvanitis et al. 2000). A space-state model for greenhouse air temperature was approximated with a first-order transfer function with dead time. Two design methods were tested: a multirate-output control based on adaptive pole placement and a multirate-output control based on an LQ optimal regulator. Other authors have used hybrid approaches to control temperature and humidity inside a greenhouse. For example, an adaptive hierarchical control has been proposed (Rodríguez et al. 2008). Both adaptive and predictive controllers were designed as well as hybrid models. An adaptive-feedforward controller was developed to control temperature by a ventilation system. A generalized predictive control using a CARIMA model of air temperature was used. A hybrid system including both a continuous and discrete set dynamics was used also in designing hierarchical control architecture of the greenhouse climate control problem. Also, a combination of feedforward and adaptive controllers were designed to control the temperature inside greenhouses (Berenguel et al. 2003). In this case, at each sampling time the adaptive controller estimates the parameters of a linear model using recursive least squares identification, adapts parameters and calculates the signal of a PI controller, and supervises the control system behavior. In a new direction an extended Kalman filter (EKF) for adaptation of the parameters of a complex physical greenhouse model was used (Speetjens et al. 2009) the first time. Researchers found better model predictions for the online parameter estimation using the EKF than those model predictions with fixed parameters. Also, Hameed and Sorensen (2010) performed a comparison of the performance of the extended Kalman filter and the unscented Kalman filter (UKF) so as to improve the air temperature and humidity predictions of a greenhouse climate model. A nonlinear physical model for temperature and humidity ratio inside a greenhouse was linearized in order to develop the EKF. The UKF used instead of linearization unscented transformations. Better performance was observed in case of UKF taking into account the estimation error and signal-to-noise ratio. The adaptive controller reduced the operating cost of the greenhouse system. Luan et al. (2012) developed a stochastic unscented Kalman filter to estimate the state variables of a greenhouse dynamic model in case of missing measured data. A two-states model including air temperature and humidity was used to evaluate the performance of the UKF. Better accuracy was reached by the UKF without considering missing measurements than the one with measurement loss.

14.2.7 Feedback and Feedforward Control

Setiawan et al. (2000) performed a comparison of pseudo-derivative-feedback (PDF) controller against proportional-integral (PI) controller using a dynamic thermal model of a greenhouse. Results showed that PDF control has a better capacity for load handling than a PI controller. PDF control was better in comparison to PI for system with and without time-delay. Using a fifth-order ARX model and also state-space models for nutrients supply to a greenhouse crop, both a feedback and feedforward control actions were implemented (Gieling et al. 2000). Water uptake was considered as a disturbance and several growing systems were tested. Results showed that the designed controller indeed compensates changes in water uptake by the crop as a response to variations in global radiation. An optimization methodology for irrigation and nutrients supply based on measurements of the greenhouse climate was developed (Sigrimis et al. 2001). A feedback loop and a feedforward control loop are coupled in order to minimize the control error. This optimization method was designed as a tool for real-time applications for both linear and nonlinear systems. A feedback–feedforward approach to system linearization and decoupling for climate control of greenhouses has been tested for ventilation/cooling and humidity proposed (Pasgianos et al. 2003). The method has three components. First, a model-based feedback–feedforward compensation for system disturbances based on an input–output linearization and decoupling. Second, the transformation of user-defined desired settings for temperature and humidity into feasible controller specifications, taking into consideration system's constraints. Third, PI control loops to compensate for model uncertainties and deviations from the disturbances predictions.

14.2.8 Multiobjective Optimization

Recently, the approach of multiobjective optimization (Miettinen 1998) has been applied to the optimization and control of the greenhouse environment. A generic multiobjective design optimization problem can be formulated as follows (de Weck 2004):

$$\begin{aligned}
&\min \mathbf{J}(\mathbf{x},\mathbf{p}) \text{ where } \mathbf{J} = [J_1(\mathbf{x}),\ldots,J_z(\mathbf{x})]^T\\
&\text{s.t. } \mathbf{g}(\mathbf{x},\mathbf{p}) \le 0 \ \mathbf{x} = [x_1,\ldots,x_n)]^T\\
&\mathbf{h}(\mathbf{x},\mathbf{p}) = 0 \ \mathbf{g} = [g_1(\mathbf{x}),\ldots,g_{m_1}(\mathbf{x})]^T\\
&x_{i,LB} \le x_i \le x_{i,UB},\ (i = 1,\ldots,n),\ \mathbf{h} = [h_1(\mathbf{x}),\ldots,h_{m_2}(\mathbf{x})]^T,\ \mathbf{x} \in S
\end{aligned}$$

where $\mathbf{J}$ is a vector of z objectives, $J_i \in \Re$. The objectives depend on a vector $\mathbf{x}$ of n design variables and a vector of parameters $\mathbf{p}$. The design variables are continuous and have domain upper $\mathbf{x_{UB}}$ and lower $\mathbf{x_{LB}}$ boundaries. For a feasible design $\mathbf{x}$ in the domain S a vector of m_1 inequality constraints $\mathbf{g}$ and other of m_2 equality constraints h need to be satisfied. The problem is to minimize simultaneously all elements of the objective vector.

A multiobjective optimization immune algorithm was proposed to control the environment of a greenhouse (Zhang 2008) manipulating heating, ventilation, and carbon dioxide concentration. The greenhouse control problem is conceptualized as a dynamic multiobjective programming problem. A discrete dynamic model including the air temperature, CO_2 concentration, and accumulated biomass was used. The maximization of profit was considered in the optimization. Given that conventional multiobjective control methods usually select the optimal value of each performance as the point control objective and minimize the deviation of each performance criteria, a multiobjective compatible control strategy has been proposed (Hu et al. 2010). A kind of hierarchical control architecture including two levels is proposed. The method uses suboptimal or region control objectives instead of point control objectives (Hu et al. 2011a). In the so-called compatible optimization level, climatic conditions and control constraints are specified according to requirements of the crop. The Pareto optimal points are reached using multiobjective evolutionary algorithms. Then, in the so-called compatible control level the control variables from the Pareto optimal fronts are chosen as the input vector of the control system (Hu et al. 2011b). In this way an optimal control of the greenhouse system is implemented using a feedback control architecture. A multiobjective controller optimization by means of evolutionary algorithms was performed for the greenhouse environment (Hu et al. 2011b). A physical model including air temperature and humidity ratio of the greenhouse climate was considered. A Proportional-Integral-Derivative (PID) controller was tuned by stating and solving a multiobjective optimization problem using a multiobjective evolutionary algorithm. More recently, a multiobjective hierarchical control architecture for greenhouse crop growth has been proposed (Ramírez-Arias, et al. 2012).These researchers defined the multiobjective optimization problem as a summation of the maximization of profits (J_1), crop quality (J_2), and water-use efficiency (J_3) during the optimization interval. The variables present in the objectives are functions of the air temperature and/or electric conductivity (EC), the measurable disturbances: photosynthetically active radiation and CO_2 concentration. The solution provides both diurnal and nocturnal set point trajectories of EC and indoor air temperature for the control horizon. In order to solve the problem a goal attainment algorithm, based on a sequential quadratic programming algorithm, was used. It is possible to specify priorities for the objectives using weights that can be modified at each iteration. The constraints for the design variables are defined by maximum and minimum values of temperature and EC coming from experts' knowledge. The results showed acceptable trajectories for short- and long-crop cycles. Regarding the control architecture it has three layers: a layer with a timescale of days or months solves the multiobjective optimization problem proving setpoints for the greenhouse crop. A layer with a timescale of days could modify the set points coming from the long-term layer according to short-term weather predictions in order to deal with uncertainties associated to the long-term layer. A third layer with a timescale of minutes or seconds provides the controllers that minimize the error between the calculated set points and the actual measured variables in the greenhouse system.

14.3 Intelligent and Soft Computing Control

Artificial intelligence (AI) techniques, intelligent control methodologies, and soft computing approaches such as fuzzy systems, neurocomputing, and evolutionary algorithms have been applied recently to design control algorithms for the greenhouse environment (Occhipinti and Nunnari 1996; Caponeto et al. 2000). In this section, the main achievements regarding these AI techniques to control the greenhouse environment are summarized.

14.3.1 Fuzzy Control

A fuzzy logic controller (FLC) has four main components: a fuzzification interface, a knowledge base, a decision-making logic or kernel, and a defuzzification interface (Lee 1990a, b; Passino and Yurkovich 1997). Currently, using fuzzy logic has become popular in many areas mainly in the area of controlled agriculture. Fuzzy logic is used for system modeling when the information is abundant; some typical variables are in-house and out-house temperature, humidity for both inside and outside the greenhouse, and wind direction (Lafont and Balmat 2002; Hurtado et al. 2010). The solutions proposed are generally based on the linearization of the process behavior around the operating points. Fuzzy logic makes possible to solve this problem thanks to its properties as a universal approximator. Among these techniques are found those based on the fuzzy clustering methods (Trabelsi et al. 2007; Errahmani et al. 2009). The proposed method uses in a first stage the fuzzy clustering technique to determine both the premises and the consequent parameters of the fuzzy Takagi–Sugeno rules. This approach has been applied to model the air temperature and humidity inside the greenhouse. Takagi–Sugeno fuzzy rules have been used to design a fuzzy controller for nocturnal temperature of a greenhouse (Nachidi, et al. 2011). Also they have been used in a reasonable greenhouse crop transpiration rate model for irrigation scheduling (Ren et al. 2011) and thereby to achieve the best effect. Some fuzzy logic model for estimation of greenhouse crop transpiration rate was developed (Deng and Wang 2006), this is commonly estimated from weather parameters. But also fuzzy logic has been used to model crop behavior such as photosynthesis processes (Center and Verma 1997). Another application in the area of greenhouses is the implementation of a control system or control strategy using LABVIEW software based on a Takagi–Sugeno (Cepeda, et al. 2011) control type. Another application is to control temperature inside the greenhouse to reduce cracking in tomato fruits (Hahn 2011), relative humidity inside the greenhouse, to adjust thermal systems, humidification, ventilation (Gates et al. 2001; Sriraman and Mayorga 2007), air temperature (Salgado and Boaventura Cunha 2005; Ehrlich et al.1996), or environment variables of the greenhouse climate (Bernardo et al. 2006). Some studies have used Mandani type controllers (Javadikia et al. 2009) using MATLAB to implement controllers on/off type, SIMULINK for temperature fuzzy control (Xu et al. 2006), others to control carbon dioxide concentrations (Shuying et al. 2010) using gas sensors and obtaining the desired responses, to name a few applications. Fuzzy logic controllers have been used in combination with classical P, PD (Kolokotsa, et al. 2010), or PID controllers in order to optimize the climate control of greenhouses (Alghannan 2000) and also to develop an intelligent indoor environment and management system for greenhouses. A fuzzy logic controller based on a Field Programmable Gate Array for greenhouse climate control was proposed recently (Castañeda-Miranda et al. 2006). Also, a type-2 FLC was designed for the greenhouse environment (Abdel 2010). A type-2 FLC is a fuzzy system that can handle rule uncertainties (Karnik et al. 1999). The advantages of FLC on traditional feedback control systems in greenhouses automation have been compared (Ödük and Allahverdi 2011). A zigBee wireless sensor network and a fuzzy controller has been proposed recently in order to control temperature and humidity of the greenhouse environment (Yang et al. 2013).

14.3.2 Neural Control

Artificial neural networks (ANN) have proved to be a powerful tool to solve several types of problems in different fields where approximation of nonlinear functions, classification, identification, and pattern recognition are required. Neural networks are mathematical representations of biological neurons in the way they process information as parallel computing units. In general there are two types of neural network architectures: (1) static (feedforward), where no feedback or time delays exists and (2) dynamic neural networks, whose outputs depend on the current, or previous inputs, outputs, or states of the network (Demuth et al. 2007).

One of the most widely used neural network architectures is the multilayer perceptron (MLP), which has been proved to approximate almost any continuous function over a compact subset of R^n, if given enough hidden layers and neurons within them (Master 1993). Its mathematical proof, based on the Stone-Weierstrass theorem, can be found in Gupta et al. (2003), and it has also been studied by many other authors such as Blum and Li (1991) and Hornik (1991). An MLP is a structure mapping an input space R^n into an output space R^m, by adjusting the connection weights that link the inputs to each of the neurons in the hidden layers and up to the output layer. Finding the appropriate number of hidden layers, neurons within each layer and weight adjusting methods, constitute the neural network design procedure. Determining a combination of these that best approximates the problem solution is a heuristic process that depends on the problem and type of data being processed.

Due to the complexity of the crop-greenhouse biosystem, some emphasis has been put into implementing artificial intelligence techniques that offer the advantage of modeling nonlinear relationships with a reduced number of input parameters, in contrast to analytical models that are too complex to be practical and are difficult to implement. Although artificial intelligent methods, including neural networks, fuzzy logic, neuro-fuzzy, evolutionary algorithms, or some combination among them, do not offer an explanation of the physical principles, they do allow for a prediction of the system dynamics provided enough data are presented in the training process.

In this way, hybrid artificial intelligent techniques such as neuro-fuzzy are finding more applicability on complex systems such as the crop-greenhouse biosystem (Elfelly et al. 2010). Neural networks and fuzzy logic offer the advantage of modeling nonlinear system relationships and add robustness in situations of imprecise and incomplete data, where uncertainty exists. Some application examples are in the areas of forecasting, control, optimization, and pattern recognition. Intelligence is added to the process by computing the degree of uncertainty, and computing with linguistic terms (fuzzy variables). More accuracy is obtained compared to mechanistic models. Furthermore, the number of parameters used to describe the system behavior is reduced.

Most of the studies implementing neural networks on greenhouses have focused on modeling the dynamics of the greenhouse environment, targeting only air temperature (Ferreira et al. 2002; Frausto and Pieters 2004; Linker and Seginer 2004; Lopez-Cruz et al. 2007; Patil et al. 2008; Seginer 1997) or both air temperature and relative humidity (He and Ma 2010; Salazar et al. 2008), or a combination of air temperature for CO_2 control (Linker et al. 1998; Salazar et al. 2010). Recent developments include hybrid systems based on neuro-fuzzy models (Lopez-Cruz and Hernández-Larragoiti 2010; Qiuying et al. 2010; Yousefi et al. 2010) for modeling both air temperature and relative humidity. Recent techniques have included modeling the greenhouse environment with hierarchical fuzzy modeling (Salgado and Cunha 2005), or controlling the environment with optimized fuzzy control (Ehrlich et al. 1996; Lafont and Balmat 2002).

However, very few studies have focused on the development of climate controllers using neural networks. For example, Pucheta el al. (2006) implemented a neurocontroller based on Neuro-dynamic programming on a lab-scale greenhouse for the optimal production of tomato seedlings. The neurocontroller drives the microclimate that controls the growth and development of the crop, while minimizing the operative costs and the final state errors on process variables and actuator signals. Fourati and Chtorurou (2007) implemented an Elman neural network to emulate the direct dynamics of the greenhouse, and trained a multilayer feedforward neural network (MLFFNN) to learn the inverse dynamics of the control process. The latter was placed in cascade with the Elman network to drive the system output to desired values. In a similar way Fitz-Rodríguez et al. (2012) proposed a Neural Network Predictive Controller to control the greenhouse climate of a naturally ventilated and fog-cooled greenhouse. Under this architecture an MLFFNN was trained to predict the system dynamics. The system controls fogging rates (given by pump pressure and the ON-time) and vent openings to drive the inside climate to the set points while achieving a uniform environment.

Fewer studies implement control strategies that model the whole biosystem, focusing either on the environment or on the crop component. In this way Tien (1997) implemented a hybrid Neuro-Fuzzy approach for the system identification and modeling of the total dry weight yield of tomato and lettuce. Center and Verma (1997) developed a fuzzy model to predict net photosynthesis of tomato crop canopies, and the results were highly correlated to the outputs of the mechanistic model TOMGRO to model crop processes. Ehret et al. (2008), used artificial neural networks to predict cuticle cracking in greenhouse peppers and tomatoes. Fitz-Rodríguez and Giacomelli (2009) implemented a dynamic neural network for modeling and predicting weekly production rates of greenhouse tomatoes as a function of environmental parameters and current and past crop conditions.

14.3.3 Evolutionary and Bio-Inspired Control

Evolutionary algorithms (Genetic algorithms, Evolution strategies, Evolutionary programming, and Differential evolution) are heuristic global search methods inspired by natural evolution and genetics (Eiben and Smith 2003). These algorithms have been applied recently to optimize and control the greenhouse environment. Guzmán-Cruz et al. (2009) used Genetic Algorithms (GAs), Evolutionary Strategies (ES), and Evolutionary Programming (EP) to calibrate parameters of a climatic model that describes the behavior of air temperature and relative humidity inside a greenhouse. Other authors have used genetic algorithms to carry out the parameter estimation for greenhouse crop growth model (Dai et al. 2009). Herrero et al. (2007) performed a nonlinear robust identification of a greenhouse model using a multi-objective genetic algorithm (MOGA). A MOGA to estimate and optimize the parameters of a pipe heating system inside a greenhouse was presented by Braga (2006). This algorithm is focused on heat radiation transfer. A robust identification of a nonlinear greenhouse model has been carried out using an evolutionary algorithm to solve a complex multimodal optimization problem (Herrero et al. 2008). An optimal control problem of the greenhouse climate aiming maximization of the profit was stated and solved using two evolutionary algorithms (Pohlheim and Hei*β*ner 1997). Both a multi-strategy competition and an evolution strategy evolutionary algorithm were evaluated (Pohlheim and Hei*β*ner 1999). A classical genetic algorithm was used as the optimizer in an MPC problem to optimize energy and water consumption (Blasco et al. 2007). A differential evolution algorithm was used to solve an optimal control problem of lettuce cultivation in a greenhouse (López-Cruz et al. 2003a, b). Evolutionary algorithms were applied recently to solve multiobjective optimization problems of the greenhouse environment (Hu et al. 2011b). Bio-inspired algorithms (Particle Swarm, Ant colony, and Artificial Bee colony optimization) are also heuristic global search methods inspired by the complex behavior of biological systems (Yang 2008) that can be used in the control of the greenhouse system. A particle swarm optimization algorithm was used in the control of greenhouse temperature using MPC approach (Coelho et al. 2005).

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Chapter 15
Plants as Bioreactors for Human Health Nutrients

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Abstract One of the biggest challenges in the coming years is to reduce the impact of chronic diseases. Unhealthy eating is an increasing problem and underlies much of the increase in mortality from chronic diseases that are occurring worldwide. Diets based on plant foods are strongly associated with reduced risks in major chronic diseases, but the constituents in plants that promote health have proved difficult to identify. Plant cells have been used, in the past two decades, as efficient host systems with great potential for the expression of foreign proteins and for the production of nonprotein bioactive small molecules. Till date, the systems implemented include a variety of approaches using whole plants, plant tissue cultures, and cell cultures for the synthesis of a myriad of compounds that include foreign protein expression for the production of antibodies and vaccines. Plant cells have also been used as bioreactors for the massive production on plant-derived bioactive small molecules which are important dietary components or used in the industry as pharmaceuticals, pesticides, flavors, and fragrances. Plant genetics and metabolic engineering can be used to make foods that differ only in their content of specific phytonutrients.

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Contents

15.1 Plant Secondary Metabolites and Production Routes

15.1.1 Carotenoids

The carotenoid biosynthetic pathway (Cunningham et al. 1993) takes place within plastids; the first committed step is the condensation of two geranylgeranyl diphosphate (GGDP) molecules to produce phytoene, catalyzed by the enzyme phytoene synthase (PSY). Two enzymes, phytoene desaturase (PDS) and ξ-carotene desaturase (ZDS), introduce four double bonds that convert phytoene into lycopene, the first coloring carotenoid, via phytofluene, ξ-carotene, and neurosporene. Then, lycopene may undergo cyclization by two different cyclases, one branch leads to β-carotene (β,β-carotene) and its derivative xanthophylls zeaxanthin, violaxanthin, and neoxanthin (Cunningham et al. 1993). The alternative branch leads to carotenoids with one β and one ε ring, such as α-carotene and lutein; the latter is a major xanthophyll in the light harvesting system of higher plants (Cunningham and Gantt 2001).

Carotenoid formation is a highly regulated process; the concentration and composition of leaf xanthophylls are affected by light intensity (Ruban et al. 1994), and the accumulation of specific carotenoids in chromoplasts of fruits and flowers is developmentally regulated (Giuliano et al. 1993).

15.1.2 Anthocyanins

The flavonoid biosynthesis pathway has been well characterized in an endless number of higher plants. The biosynthesis of flavonoids is initiated by the enzymatic step catalyzed by chalcone synthase (CHS); in the majority of plants chalcones are not the end-products, but the pathway proceeds with several enzymatic steps to other classes of flavonoids such as flavanones, dihydroflavonols, and finally anthocyanins.

Other flavonoids classes, isoflavones, aurones, flavones, flavonoles, and proanthocyanidins (tannins), represent side branches of the flavonoid pathway and are derived from intermediates in anthocyanin formation (Schijlen et al. 2004). When CHS alone is present in a plant, $6'$-hydroxychalcones are produced. The first deoxy flavonoid, isoliquiritigenin is synthesized by the co-action of CHS and polyketide reductase (Welle and Grisbach 1988) later called chalcone reductase (CHR) (Forkmann and Martens 2001). The isoliquiritigenin is the precursor of daidzein (5-deoxy-isoflavonid); this isoflavonoid is synthesized in two subsequent steps by the action of chalcone isomerase (CHI) and isoflavone synthase (IFS). IFS is a key enzyme responsible for the migration of the β-ring, resulting in the formation of isoflavonoids daidzein and genistein (Yu et al. 2003). CHI is able to isomerize $6'$-deoxychalcone and isoliquiritigenin, to the $5'$-deoxyflavanone liquiritigenin (Joung et al. 2003). The hydroxylation in C3 for the conversion of flavanones into dihydroflavonols is carried out by flavanone-3-hydroxylase (F3H). Consecutively, the enzyme dihydroflavonol-4-reductase (DFR) catalyzes the stereospecific reduction of dihydroflavonols to leucoanthocyanidins (flavan-3,4-diols). This enzyme converts a dihydroflavonol into leucoanthocyanidins via the reduction of ketone in the central ring of the flavonoids (Petit et al. 2007). The leucoanthocyanidins formed are the immediate precursors for the synthesis of anthocyanins. They are also precursors for the production of catechins and proanthocyanidins (Schijlen et al. 2004).

15.2 Methods to Increase the Yields of Secondary Metabolites in Plant Cell Culture

Plant cell culture (PCC) is one choice for producing secondary metabolites such as pharmaceuticals, agrochemicals, flavors, colorants, fragrances, and food additives. Under specific conditions and plant hormone balances, plant cell can become totipotent and are able to produce all compounds found in the whole plant (Ramachandra and Ravishankar 2002). PCC has some advantages over conventional agricultural production, like independence of geographical variables and the environment, the possibility of define production, quality and yield, production of new compounds not found in the original plant or with specific stereochemical requirements, and manufacture them in easy and efficient way.

In PCC there are several strategies to increase production of secondary metabolites, like production of fast growing cell lines, immobilization of cells in polymers, use of elicitors to accelerate the production of a given metabolite membrane permeation to facilitate diffusion of metabolites, scaling of cell culture bioreactors, and genetic engineering for production of specific metabolites (Oksman-Caldentey and Inzé 2004).

15.2.1 Plant Cell Culture (Somaclonal Variation)

Somaclonal variation is generated by genetic modification of in vitro culture, which manifests as inheritable mutations in regenerated seedlings (Larkin and Scowcroft 1981). The term somaclonal variation is used universally for all variants of PCC (Bajaj 1990), and is described as one of the major problems in the regeneration of plants grown in vitro. Plant cell growth in vitro is an asexual process that involves only mitotic cell division and theoretically should not cause any mutation (Larkin 1998). However, these mutational effects have utility in improving the crop through the creation of novel plant varieties with characteristics that could increase their commercial value such as disease resistance, improved biomass yield, and increased production of secondary metabolites (Karp 1994).

15.2.1.1 Causes of Somaclonal Variation

Unlike in vivo mutations, in vitro mutations occur more frequently and are more easily detected observing variants found in the regenerated plant in a short period of time. Mutations may occur because of exposure of plant tissue to the chemical compounds of the culture medium, the habituation to a new hormone balance (like auxin and cytokinin ratios), and the process in which the plant material is rearranged to form a new organ. Even some plantlets could express the natural variation already present in somatic cells before the PCC modifies the cell cycle pathways and causes transposon insertions and chromosomal aberrations (Vasil 1990). For PCC-based approaches it has to be considered that somaclonal variation may be present in the nuclear, mitochondrial, and chloroplast DNA (Aversano et al. 2009).

15.2.1.2 Methods for Detecting Somaclonal Variations

Somaclonal variation could be a major problem in operating large-scale micropropagation, whereby early detection and elimination of variants is essential to reduce losses to the producers. Detection of variations is also used to find lines with useful agronomic treats and production of compounds with commercial interest (Karp 1995). Several types of techniques for detecting somaclonal variations are from molecular, biochemical, and morphological characteristics. Morphological detection techniques depend on recognizing characteristics like height, leaf area, and pigmentation abnormalities (Israeli et al. 1995). Biochemical detection identifies various plant responses to physiological factors such as hormones, light, carbon dioxide assimilation, and alteration of pigment synthesis (Shahijram et al. 2003; Peyvandi et al. 2009) and they can do it in early stages of the plant, reducing economic losses. Molecular detection techniques are used to determine the genetic fidelity of micropropagated species. Some of these techniques are the karyotypes and molecular markers like RFLP, RAPD, AFLP, and SSR (Bairu et al. 2011).

15.3 Genetic Transformation of Plant Cells

Genetic transformation involves the introduction and integration of DNA into the nuclear, mitochondrial, and chloroplast genomes. Genetic transformation methods are classified into two types: the direct method when DNA is introduced in the plant cells or tissues by imbibition, microinjection, electroporation, or ballistic and the indirect method using the soil bacterium *Agrobacterium tumefaciens* to transfer foreign DNA to the plant material (Naqvi et al. 2010; Barampuram and Zhang 2011). Strategies on secondary metabolite production using genetic transformation; to transfer and integrate foreign DNA into de plant genetic material, increased expression of precursors of secondary metabolites, increased gene expression limited by the metabolic pathway of interest, creation of a new metabolic pathway from an existing route, inhibit competitive routes or catalytic steps of the metabolite of interest using antisense DNA or iRNA, handling regulatory genes like transcriptional activators or repressors, selection of mutants for production of secondary metabolites, and reorientation of secondary metabolite production to organs or tissues of interest by using specific promoters.

15.3.1 Methods for Detecting Genetically Transformed Cells

15.3.1.1 Marker Genes

Once transformed, it is necessary to select cell lines that express the integrated DNA. There are a number of marker genes that have been used in plant selection, such as neomycin phosphotransferase (*NPT*II) that confers kanamycin resistance and is the most used as a selectable marker gene for dicots. There are also other systems based on glyphosate resistance or spectinomycin and the bar gene (Phosphinothricin acetyltransferase, PAT) that confers resistance to Phosphinothricin, which is the active component of the herbicide Basta (Goldschmidt and Day 2011).

15.3.1.2 Reporter Genes

Reporter genes are used to demonstrate the transient or stable transformation of plant material. Some reporter genes encode enzymes whose substrate is not normally found in plants, such as the *uid*A gene coding for *β*-glucuronidase enzyme which produces a blue color to degrade the substrate X-gluc in the transformant tissue (Jefferson et al. 1987). Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has also been used as a selection gene and allows visualization of cells and transformed plastids by light excitation and without supplying the plant substrate (Chiu et al. 1996). This category also has Southern blot analyses to determine how many copies of the gene of interest have been inserted into the plant genome (Birch 1997).

15.3.2 Transfer of Multiple Genes Through Improved Vectors

The transformation with multiple genes simultaneously allows researchers to study and manipulate an entire metabolic pathway to produce secondary metabolites and proteins of interest. However, there are several barriers for transformation with multiple genes because the first plant transformation methods were developed to introduce only one or two genes. As more genes are introduced is lower the probability that all of them will be integrated and expressed (Naqvi et al. 2010) in plantlets.

The transfer of multiple genes can be achieved using conventional methods like crossing homozygous transgenic lines or through sequential transformation of a unique transgenic line; however, these methods are time-consuming and labor-intensive and the transgenic plants produced can segregate independently and lose their phenotype. This problem has been partially resolved with co-transformation and design vectors that enable the insertion of multiple genes in a single transformation event (Naqvi et al. 2010).

The transfer of multiple genes can be mediated by *A. tumefaciens* using binary vectors, wherein a vector has the T-DNA region with the gene of interest and the second vector contains the *vir* region. Ti binary vectors have a replication origin for *E. coli* that allows its multiplication, and the other has a replication origin for *A. tumefaciens.* This method is effective when the size of the DNA to be transferred is less than 50 kb (Valderrama et al. 2005). With conventional transfer vectors the introduction of multiple genes in a single DNA segment is possible, and it is efficient to introduce segments of 50–80 kb.

Another transformation system of higher potential is the use of artificial chromosomes with *A. tumefaciens* (BIBACs) as transformation vector. This system is similar to the bacterial artificial chromosomes or BACs. This vector was developed based on the knowledge generated from BACs, but it also contains an origin of replication for *E. coli* and one for *A. tumefaciens* allowing the introduction of multiple genes of interest and selection. This system is useful for transferring DNA segments of 150–200 kb (Hamilton 1997; Liu et al. 1999).

There are also methods of direct transfer of high capacity that use protective structures such as calcium alginate particles for encapsulating the DNA (Sone et al. 2002). This method enables introduction of DNA fragments up to 200 kb; however, it is not compatible with biolistic transformation, its efficiency is low, and it is not applicable in large-scale experiments (Wada et al. 2009).

The use of biolistic and *A. tumefaciens* has allowed the genetic manipulation of different plants of commercial interest. However, these methods have several limitations with the insertion of multiple genes, which occurs in an uncoordinated way, disrupting the integrity of the host genome. The limitations stimulated the development of plant mini-chromosomes which enable transfer of genetic complexes and multiple genes, along with regulatory elements for safe expression. These mini-chromosomes must contain artificial telomeres, centromeres, a replication origin, and an insertion site for genes of interest and regulatory ones (Yu et al. 2007). This technology allows the transfer of DNA of the order of Mb but has only been successfully applied to maize.

The most important application for the transfer of multiple genes is that they can create or modify metabolic pathways, which may include one or multiple genes to produce a specific metabolite. One of the most ambitious studies in this area was the production of β-carotene in rice. Carotenogenic genes with a marker gene were introduced in a single transformation event to produce what is now known as the “Golden rice” (Ye et al. 2000). Another study highlights the production of polyunsaturated fatty acids of long chain in mustard, using nine microbial genes encoding desaturases and elongases, through co-transformation with *A. tumefaciens* (Wu et al. 2005).

15.4 Metabolic Engineering

Metabolic engineering, defined as the manipulation of enzymatic processes, that enables the production of new compounds in an organism, improves their production, or prevents its degradation (DellaPenna 2001). The goal of metabolic engineering is overproduction of specific compounds; however, the interconnection of metabolic pathways is huge and the number of possible ways for metabolite manipulation is enormous (Woolston et al. 2013). The requisites for metabolic engineering process are manipulation of a target compound, make it thermodynamically favorable and not toxic, and have the enzymes required for expression of those metabolites in the host organism (Pickens et al. 2011).

Metabolic engineering uses Biochemistry and molecular biology, to alter the metabolic pathways, and statistical tools for data analysis along with bioinformatics for designing, analyzing, and predicting the behavior of metabolic fluxes in a new route. Thanks to this technology, it is known that at least 100,000 secondary metabolites are produced by plants. These metabolites can be used for therapeutic purposes, fragrances, flavors, and pesticides, among others (Goossens et al. 2003).

However, the plant extracts from which secondary metabolites come are insufficient to satisfy the market demand because plants grow slowly and with difficulty. Initially, chemical synthesis was able to satisfy the demand of secondary metabolites; however, the complexity of the molecular structures and stereochemical requirements of some secondary metabolites limit the use of this technology (Miralpeix et al. 2013).

Metabolic engineering provides tools that enable large-scale production of secondary metabolites with high-added value, in heterologous systems such as microorganisms, cell cultures, and plant organs. Genomics, transcriptomics, proteomics, and metabolomics can be used to determine the function of a gene at the transcript, protein, or metabolite level and establish new metabolic pathways in organisms. The genomic sequence contains the code of proteins, enzymes, and transcription factors involved in the biosynthetic pathway. Until now 25 plant genomes have been published, but only the genomes of *Arabidopsis thaliana* and *Oryza sativa* are almost identified. The genetic information of these model plants can be used to associate physiological processes in related plants, like *Medicago truncatula* and its pathway for nitrogen fixation in legumes, tomato as a model of development of fruit, and rice as a development model for grains (Moon-Yoon et al. 2013). The sequencing of model plants can help to establish new metabolic pathways and complete the existing ones. These developments provide more efficient methods to increase yields of secondary metabolites with high-added value (Hirsch and Robin-Buell 2013).

Microarray analysis allows obtaining a transcriptome profile of a plant under certain conditions and can identify many candidate genes involved in the production of secondary metabolites, based on their expression. However, this technology is being displaced by transcriptome sequencing (RNA-seq) which is more versatile for determining metabolic pathways in plants. This type of analysis provides the function of a gene but not its functionality. To implement this it requires further analyses, which include the isolation of cDNA, the construction of vectors for genetic transformation, determination of their metabolic profile, and quantitative analysis of the desired compound (Yonekura-Sakakibara et al. 2013).

The general problem for characterizing a plant metabolome is the complexity and chemical diversity of compounds. Analytical techniques like GC–MS can detect only a portion of a wide variety of compounds. It is estimated that the approximate number of metabolites in a plant can vary from 5,000 to 25,000 different compounds according to the species (Hegeman 2010). Only in *A. thaliana* 2,613 different compounds (PMN 2013) were detected. As no analytical technology is able to detect all the massive catalog of secondary metabolites in plant species, it is advisable to combine multiple analytical technologies to obtain a more accurate metabolomic profile, for example, the combination of chromatography-mass spectrometry (MS) and nuclear magnetic resonance (NMR). Currently, the MS technique is usually the choice for metabolomic studies when the sample volume is too limited for using NMR (Kueger et al. 2012).

Mass spectrometers of ultra-high definition, such as Orbitrap and Fourier Transform Ion Cyclotron Resonance-Mass Spectrometry (FT-ICR-MS), allow an extremely precise metabolomic profile of a sample. The resolving power and sensitivity of these techniques in molecules that contain abundant natural isotopes (e.g., ^{13}C, ^{41}K, ^{15}N, ^{17}S, and ^{37}Cl) is visible in their results. Its precision allows accurate calculation in the elemental composition of unknown compounds and can define which of these secondary metabolites are new (Allwood et al. 2012; Pollier and Goossens 2013).

Complex metabolic regulation by the gene expression and enzymatic activity is manifested in the metabolic flux analysis (MFA). MFA is the quantification of the flow of a metabolite and the rate at which the enzymes act in the biosynthetic pathway. Traditionally, this analysis is made with mass balance; however, it presents some disadvantages such as not distinguishing a cyclic reaction, parallel metabolic cycles that are not measurable coupled flows cannot be solved, it is possible to solve reversible stages in a cycle, it is not possible to determine for certain the flow of energy metabolites such as ATP, NADH, and NADPH (Wiechert 2001), also shows no information on metabolite biosynthesis site of interest in the cell or in a particular tissue, which is a constraint to understand how there is a metabolite spreading and for the design of extraction methods.

Fusion proteins provide us with a simple approach but is limited to the compartmentalization of enzymatic interactions in a metabolic pathway. It involves the generation of chimeric proteins attached via a linker region. This approach has been widely used and is implemented by the fusion of two or more genes with a linker region, which generates a single polypeptide with two or more features. Linker region reduces interference in the folding, which allows proteins to retain their native activity and spatially separate domains allow them to interact as necessary. This methodology has been applied to proteins which are used to increase the local concentration of intermediate products, and has also been applied in systems where the protein–protein interaction is necessary for their functionality (Woolston et al. 2013).

Transcriptomic data can define the possible reactions that occur in a particular physiological condition and together with the data provided by genomic sequencing and stoichiometry of metabolic reactions, genome-scale models can be designed. These models have been successfully established for many microorganisms, but are more complicated in plants because of their cellular and tissue structure. Currently, scale modeling has been established for leaves and cells of *A. thaliana* in suspension, and also for barley, *Brassica*, and C4 plants such as maize, sorghum, and sugarcane. There are many challenges to modeling plant genome in scale, as many genes and proteins have unknown functions. Central metabolism has been completely elucidated, but not the secondary metabolism because a lack of genetic sequence data of medicinal plants, few information of metabolite production at tissue level, and limited ability to identify the interactions of large numbers of plant species with infinite numbers of internal and external conditions (Collakova et al. 2012).

Other bioinformatic tools have been developed to support a wide range of experimental techniques and analyses. These tools are used to extract and interpret relevant information from large data sets, presenting complex models in a more manageable way and to propose efficient design metabolic networks. These applications allow the design, reconstruction, and visualization of metabolic networks in silico; transformation vector design, protein engineering, metabolic flux analysis, optimization, and automation of the culture conditions, and meanwhile, creating databases that allow massive collection of information generated by omics sciences (Copeland et al. 2012).

15.4.1 Strategies for Nutrient Production in Metabolic Engineering

Human health depends entirely on plant foods. Plants are critical components of the food chain providing nearly all essential mineral and organic nutrients in the human diet directly or indirectly. However, plant foods do not contain all the essential nutrients and do not meet sufficient amounts of daily food needs (Grusak and Dellapenna 1999; Farré et al. 2011). The plant metabolites are important because they change the quality, performance, resistance, and stress tolerance of crops (Dixon 2005). These agronomic features of plants are controlled by complex regulatory networks at multiple levels, where there is an interaction between genes, such as in the case of synthesis of organic compounds in plants, mainly primary and secondary metabolites, that represent an extensive group of interest, due to characteristics and applications in the field of biotechnology. Based on the previously described facts, a new era in plant biotechnology was reached by the first successful genetic transformation of plants by *A. tumefaciens* (Herrera-Estrella et al. 1983), metabolic engineering used genetic engineering and molecular biology tools to modify metabolic pathways in order to increase the concentration of metabolites of interest (DellaPenna 2001). Because of the importance of food with higher nutrient content, strategies began to be developed to increase vitamins, lipids, and proteins in plants. Also steps are being taken to increase crop yield and resistance to biotic and abiotic stresses. Unique gene introduction was the first strategy for manipulating metabolic pathways. However, the results obtained were not as expected. Multiple experiments dropped negative results and served to highlight the complexity of metabolic pathways and their regulatory mechanisms. Metabolic pathways are modulated in multiple levels and any disturbance may affect the entire system, resulting in unexpected metabolite production (Verpoorte and Memelink 2002). Because of this, the development of more complex and sophisticated strategies involving multiple changes in the metabolic pathway have come about. The strategies tried to insert more than one gene in the metabolic pathway to carry out the production of a metabolite of interest (Capell and Christou 2004). The negative regulation of gene expression was observed in petunia, where the overexpressed chalcone synthase, which is important enzyme in flavonoid synthesis, was expected to increase the concentration of anthocyanins. But the result was the inhibition in the pigments synthesis; such regulatory phenomenon was called cosuppression (Napoli et al. 1990). The previously described studies led to the discovery of gene silencing by iRNA, since it was elucidated that cosuppression was regulated by this mechanism; overexpression of mRNA activates the machinery responsible for degrading the molecules of RNA and prevents protein translation. The iRNA was adopted as a tool for the manipulation of metabolic pathways suppressing the expression of target genes (Fire et al. 1998) so far only single genes were used in the plant improvement; these experiments and advances in the methods of transformation allow the modification of multiple genes to control a metabolic pathway, showing better results. This technology allows the modification of a single transgene and thus itself controls the entire biosynthetic pathway. These proteins called transcription factors (TFs), are used as regulators of gene expression through DNA–protein interactions and provide a new tool for metabolic engineering, because of their ability to control multiple steps in a synthesis pathway. Transcription factors represent useful tools for manipulating the metabolism in specific organs or tissues (Grotewold 2008). After it was discovered that epigenetics regulate gene expression by DNA methylation and histone acetylation multiple studies have demonstrated that these mechanisms play a key role in gene expression during development of the plant, or under conditions of stress, generating an epigenetic memory that allows plants to inherit these changes through mitosis and even by meiosis (Chinnusamy and Zhu 2009). The analysis of biological systems with bioinformatics allowed the prediction of the metabolism by mathematical models and the use of databases, and thus the *in silico* design of strategies for metabolic engineering. Analyses of the metabolic fluxes provide tools for theoretical prediction for the performance of specific metabolic pathways (Schwender 2008). In recent years, tools have been developed that improve the understanding and increase the metabolic engineering capacity, through the interaction of omics sciences (genomic, metabolomic, and transcriptomic) increasing the chances of success (Riechmann et al. 2000; Wiechert 2001).

15.4.2 Steps for the Modification of Metabolic Pathways

The process for the modification of a given metabolic pathway is divided into four stages. First it is important to know and understand the metabolic pathway of interest, the precursor compounds, the intermediates and enzymes that participate their interaction with other routes of synthesis, control points and metabolic flux, the transcription factors involved, the mechanisms of cellular transport, and tissues where it develops the metabolic pathway. In the second stage is determined the metabolic manipulation tools. Examples include the introduction of a gene that express an enzyme that promotes the production of the metabolite of interest, the introduction of two or more genes that regulate the multi-step route, gene silencing mediated by iRNA, manipulation by expression of transcription factors, and transformation systems stable or transient (Capell and Christou 2004). The third step is the evaluation of modified organisms. This involves measuring the copy number of the gene of interest inserted, transcription, and metabolic profiles. At this stage, there could appear some technical problems. For example, the lack of expression of the gene of interest, the degradation or toxicity of an intermediary product, and in many cases it presents the existence of a limiting factor. At this stage the final product concentration is also evaluated (Dixon 2005).

15.4.3 Classification of Strategies for Modification of Metabolic Pathways

The strategies for increasing the production of nutrients are classified into three groups, the metabolic engineering initially used single genes insertion to obtain a final product of interest; these initial experiments yielded the first generation of transgenic crops and the discovery of the mechanism of cosuppression as negative regulation allowed the development of iRNA strategy after having understood the operation mechanism. Later, the strategy used in metabolic engineering was transformation with multiples genes which include several methods for this object such as crossing transgenic lines (Ma et al. 1995), sequential transformation (Qi et al. 2004), cotransformation with standard binary vectors (Wu et al. 2005), conventional vectors (Twyman et al. 2002), high-capacity binary vectors (BIBAC or TAC) (Liu et al. 1999), assisted direct transfer (high-capacity transfer) (Wada et al. 2009), artificial plant chromosome (Carlson et al. 2007), and operon systems (De Cosa et al. 2001; Naqvi et al. 2010). The techniques mentioned above comprise the first strategy used by metabolic engineering. In recent years with the appearance of TFs, it was possible to control entire metabolic pathways; these master regulators have yielded better results in crop improvement. So it established a new strategy in the field of metabolic engineering which controls multiple steps by a single transgene introduction (Yokotani et al. 2013). There are other regulatory mechanisms at the epigenetic level that are attractive for metabolic engineering in the potential applications for improving plants (Saze et al. 2003). Below we show the classification of the strategies used by the metabolic engineering in plant breeding.

15.4.3.1 Modification of Metabolic Pathways

Metabolic engineering in plants involves the modification of endogenous pathways to increase flux toward particular desirable molecules. In some cases the aim is to enhance the production of a natural product, whereas in others it is to synthesize a novel compound or molecule (Capell and Christou 2004). The manipulation of single genes was first used in metabolic engineering to modify biosynthetic pathways; it was performed by introducing single genes, multiple genes, and gene silencing by iRNA.

15.4.3.2 Modification of Single Genes

The modification of a metabolic pathway to increase the concentration of a product of interest using a single gene was the first strategy to create transgenic organisms, with the goal to have superior characteristics in crops (Capell and Christou 2004). Molecular biology and genetic engineering in conjunction with transformation and plant regeneration allow to obtain genetically modified organisms. The manipulation of single genes is of only limited value in metabolic engineering, and attention has shifted toward more complex and sophisticated strategies in which several steps in a given pathway are modified simultaneously to achieve optimal flux (Schwender 2008). Emerging in the early metabolic engineering, the first generation of transgenic plants, such as Bt cotton, express a toxin of *Bacillus thuringiensis* that makes the corn resistant to pests (Qaim 2010). Another example is the modification of *A. thaliana* with a gene from *Pseudomonas stutzeri* PTxd WM88. This gene encodes the enzyme phosphite oxide reductase which catalyzes the oxidation of phosphite. It allows the transformed plants to take advantage of a phosphorous source which is not metabolized by and results toxic for wild type plants (Lopez-Arredondo and Herrera-Estrella 2012).

15.4.3.3 Modification of Multiple Genes

The simultaneous transfer of multiple genes into plants enables researchers to study and manipulate entire metabolic pathways, express multimeric proteins or protein complexes, and study complex genetic control circuits and regulatory hierarchies (Halpin 2005). Early transformation methods for plants were developed with the implicit intention to introduce one or two genes, usually a transgene of interest and a selectable or screenable marker, and have been optimized on that basis (Twyman et al. 2002). This strategy of metabolic engineering is observed in the modifications made to the golden rice to express *β*-carotene (Ye et al. 2000). In this experiment were introduced three enzymes, phytoene synthase encoded by *psy* gene of *Narcissus pseudonarcissus,* phytoene desaturase encoded by *crt1* gene of *Erwinia uredovora* and *β*-Lycopene cyclase encoded in *lcy* gene of *N. pseudonarcissus*. Enzymes take as substrate geranyl diphosphate, an early intermediate in carotenoid synthesis pathway, which is transformed into lycopene and beta-carotene and finally deposited on rice endosperm giving it a strong yellow color (Ye et al. 2001). After the evaluation of the transformed lines it was possible to establish one of the limiting factors in the synthesis of *β*-carotene from golden rice, the enzyme phytoene synthase (psy) of *N. pseudonarsissus.*

A new strategy was established in order to increase the concentration of provitamin A in the endosperm of rice, various *psy* genes from different plant sources

(*A. thaliana, Daucus carota, Zea mays*, *Capsicum annuum*, and *Lycopersicon esculentum*) were tested. Phytoene synthase genes were introduced into *Z. mays* cell cultures, in order to determine the most active enzyme. The experiment showed that *psy* enzyme of maize achieves the highest increment in *β*-carotene expression. The second generation of golden rice was established; it used maize phytoene synthase and was able to increase the *β*-carotene content 16–37 mg/g (Paine et al. 2005).

15.4.3.4 Silencing by iRNA

The introduction of genes to increase the content of nutrients in plants involves the overexpression of the enzymes that synthesize them. Unfortunately, this strategy has been restricted by two main obstacles. In the first place, plants can respond to increased nutrient levels activating degradation pathways and avoiding the accumulation of these metabolites recognized as foreign (Dixon 2005). On the other hand, introducing gene copies can induce the phenomenon of cosuppression, where excessive expression of these genes activate negative regulatory mechanisms (Napoli et al. 1990). This tool has allowed the production of "Knock down" organisms with a decrease in gene expression. The silencing mechanism is activated by double-stranded RNA (dsRNA), with the target mRNA complementary strands. The dsRNA induction methods have been highly successful in reducing target gene expression. Manipulating of specific tissues can be used for gene expression and for gene silencing that degrades the desired product. The strategy could be used constitutively, but it would cause side effects to the plant, the strategies are somewhat inclined to the regulation of specific promoters (Tang et al. 2007).

15.5 Transcription factors Manipulation

In plants, like in most of eukaryotes organisms, the genetic expression is mostly handled at the transcription level. This regulation is mediated by a diverse group of proteins known collectively as transcription factors (TFs). These molecules recognize specific sequences of DNA in the promoters. Through protein–protein interactions, the TFs mediate the construction of the basal machinery for transcription that results in the RNA polymerase II activation and the mRNA synthesis. The TFs can be classified in families, based on the presence of conserved domains for DNA binding. The regulation of multiple genes is carried out for the combinatory action of transcription factors, like the union of TFs-binding protein–DNA or by TF and a cis-regulatory element (Riechmann et al. 2000). The transcription factors are considered a viable alternative in the manipulation of metabolic pathways in plants. Because of its capacity to control multiple steps in a synthetic pathway, the discovery of this master regulators was shown as a new strategy for metabolic manipulation (Table 15.1).

Table 15.1 Transcription factors used for nutrient manipulation in plants

Transcription factor	Metabolite	Plant	Reference
R and Cl	Antocyanins	*Z. mays*	Grotewold et al. (1998)
ORCA2	Alcaloids	*Catharanthus roseus*	Menke et al. (1999)
TT2 and TT8	Condensed tanins	*A. thaliana*	Nesi et al. (2000, 2001)
Rosea 1, Rosea 2 and Venosa	Anthocyanins (magenta)	*Antirrhinum majus*	Schwinn et al. (2006)
OR	Carotenoids	*Brassica oleracea*	Lu et al. (2006)
MYB75/PAP1	Anthocyanins	*Solanum lycopersicum*	Zuluaga et al. (2007)
DELILA (Del) and ROSEA (Ros1)	Anthocyanins	*Solanum lycopersicum*	Butelli et al. (2008)
Purple (Pr)	Anthocianins	*Brassica oleracea*	Chiu et al. (2010)
PcMYBlO	Anthocyanins	*Pyrus communis*	Pierantoni et al. (2010)
MYB12	Flavonoids	*Solanum lycopersicum*	Ballester et al. (2010)
DELILA (Del) and ROSEAL (Ros1)	Anthocyanins	*Brassica napus*	Nie et al. (2013)

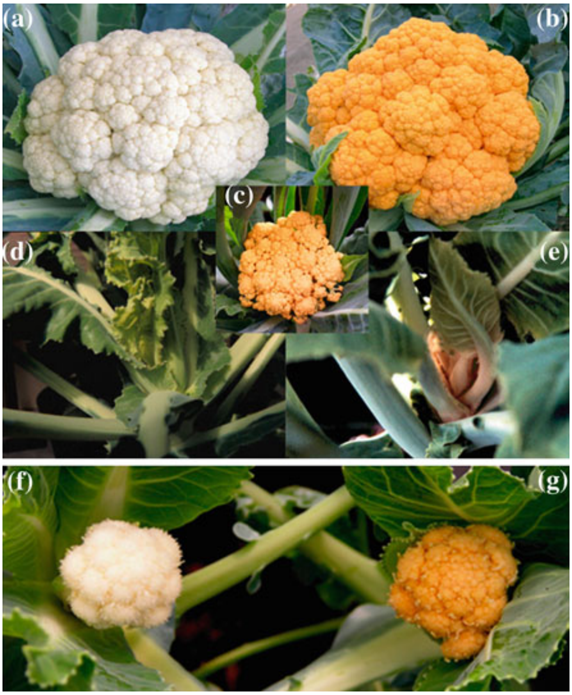

Fig. 15.1 Phenotype of the Or mutant and complementation of the orange phenotype. **a** Curd of wild-type cauliflower plant grown in the field. **b** Curd of commercial orange cauliflower (Or/or) grown in the field. The Or heterozygous plants exhibit normal growth like the wild type with general curd sizes of 15–20 cm in diameter. **c** Curd of Or homozygous mutant grown in the field. The homozygous mutant plants exhibit stunted growth with small curd sizes of 3–5 cm in diameter. **d** Apical shoot of a 3-month-old wild-type cauliflower plant grown in a greenhouse (from Lu et al. 2006)

The TFs represent a new group of strategies that allow to overcome the bottleneck in a multienzymatic reaction, controlling series of genes in specific tissues, organs, or plants (Grotewold 2008). Until recent years the knowledge about TFs was limited, because it was just possible to identify those that have a visible phenotype, for example flower pigments; those TFs were evaluated in accordance to the gain or loss of that trait. The integration of genomic, metabolomic, and bioinformatic technologies change the picture resulting in the identification of new transcription factors (Grotewold 2008).

15.5.1 Transcription Factor Expression for the Control of a Metabolic Pathway

The regulation of metabolic pathways through ectopic expression of TFs is a tool that allows the control of multiple genes with the introduction of a unique gene that represents a powerful strategy in metabolic engineering (Fig. 15.1). The sequencing of *A. thaliana* genome and the subsequent bioinformatic analyses suggest that more than 5 % of the genes of this plant code for TFs; however, less than 10 % of them have been characterized (Riechmann et al. 2000). This technology has allowed to identify more than 50 families of TFs, like GmERF3 transcription factor in soybean plants that belongs to AP2/ERF family. The analysis of these sequences shows that this TF contains an AP2/ERF domain of 58 amino acids and two nuclear localization signals (NLS). The expression of this gene was detected when the plants were submitted to treatments with high salinity, drought, abscisic acid, salicylic acid, jasmonic acid, ethylene, and soybean mosaic virus, but not in cold conditions (Chen et al. 2012). The ectopic expression of GmERF3 in tobacco plants under the control of 35 s CaMV promoter shows excellent results increasing the expression of PR genes, improving resistance to infections caused by *Ralstonia solanacearum*, *Alternaria alternata*, and Tobacco mosaic virus (TMV), besides increasing the tolerance to salinity and hydric stress (Zhang et al. 2009).

15.5.2 Design of Artificial Transcription Factors

15.5.2.1 Silencing Through Chimeric Repressor Gene Silencing Technology

Artificial transcription factors are winning impulse like metabolic engineering tools. The fusion between a transcriptional activator with a dominant repressor produces a repressive molecule. This process has various advantages over iRNA silencing; in this strategy we do not control the negative expression of a single gene, but a complete battery of genes that interfere in a specific process (Grotewold 2008). If *A. thaliana* were silenced by this technology, the TFs APETALA 3, AGAMOUS, LEAFY, and AtMYB26 related with development of petals, stamens, floral meristems, and anthers, would be placed under constitutive 35 s CaMV promoter, which merge with SRDX sequence. The transgenic plants that express every chimeric repressor exhibit high sterility. The ortholog gene of AP3 was used in rice, known as Super Woman (SPW1), and it was silenced by obtaining a sterile masculine phenotype with a high efficiency. These results indicate a powerful tool for the control of fertility in monocots and dicots (Mitsuda et al. 2006).

15.6 Epigenetic Strategies for Pathway Modification

Gene expression driven by developmental and stress cues often depends on post-translational modifications of histone and sometimes on DNA methylation. Most of these stress-induced modifications are reset to the basal level once the stress is relieved, while some of the modifications may be stable, that is, may be carried forward as "stress memory" and may be inherited across mitotic or even meiotic cell divisions (Chinnusamy and Zhu 2009; De Groote et al. 2012). Epigenetic stress memory may help plants for confront of with subsequent stresses more effectively; comparative studies on stress-responsive epigenomes and transcriptomes will enhance our understanding of stress adaptation of plants. As sessile organisms, plants need to continuously adjust their responses to external stimuli for changing growth conditions (Zhang et al. 2012). Since the seed dispersal range is often rather limited, exposure of progeny to the growth conditions of parents is very probable. The plasticity of plant phenotypes cannot be simply explained by genetic changes such as point mutations, deletions, insertions, and gross chromosomal rearrangements (Hauser et al. 2011). Since many environmental stresses persist in one or several plant generations, other adaptation mechanisms must exist. The inheritability of reversible epigenetic modifications, that regulate gene expression without changing DNA sequence, makes them an attractive alternative mechanism (Boyko and Kovalchuk 2011). Despite significant advances made in epigenetic research in recent decades, many questions remain unsolved, especially concerning gene expression modulation (GEM). Epigenetic marks are enzyme-mediated chemical modifications of DNA and its associated chromatin proteins. Although they do not alter the primary sequence of DNA, they also contain inheritable information and play key roles in regulating genome function. Such modifications including cytosine methylation, posttranslational modifications of histone tails and the histone core, and the positioning of nucleosomes (histone octamers wrapped with DNA). These constructs influence the transcriptional state and other functional aspects of chromatin (Feng and Jacobsen 2011). Adverse environmental phenomena distort the growth, development, and productivity of crop plants. As a defense, plants have sophisticated mechanisms to respond and acclimatize to these stresses conditions by prompt changes at transcriptional and posttranscriptional levels of whole gene complexes. Various genes belonging to diverse transcription factor families have been shown to regulate stress-responsive genes, thus playing an important role in stress signaling. Recently, epigenetic mechanisms have been implicated in the regulation of the expression of genes related to abiotic or biotic stresses (Chinnusamy and Zhu 2009). These mechanisms regulate almost all the genetic functions including transcription, replication, DNA repair, gene transposition, and cell differentiation. Also, modifications in chromatin and generation of small RNAs have been shown to be involved in transcriptional and posttranscriptional control of gene expression, critical for stress responses. The expression of genes can also be altered by transgenerational or heritable modifications in chromatin. DNA methylation is controlled by hormonal fluxes, which are in turn influenced by various abiotic and biotic factors (Zhang et al. 2012) resulting in plant adaptation. Thus, deciphering how the methylation and functions of epigenetic machinery will be based. It will also provide a valuable platform for potential applications including genetic manipulation of plants toward enhanced tolerance to environmental stresses. The mechanism of establishment of DNA methylation is the covalent transfer of methyl group from S-adenosyl methionine to the 50 position of cytosine, thus result in converting cytosine into 5-methylcytosine (5 mC). Plants have high levels of 5 mC, ranging from 6 to 25 % of total cytosine, depending on the species. This epigenetic memory is accumulated by plants during their vegetative phase under the influence of the environment; it is passed on to the next generation by germline cells, which are later established during development. DNA is methylated at promoter as well as gene body regions, thus allowing the gene to be in a repressed state (Saze et al. 2003). Thus, a decline in the level of methylation is likely to lead to an increase in gene expression. Further, enzymes participating in cytosine methylation are grouped into three distinct categories: methyltransferase1 (MET1), chromomethylase3 (CMT3), and domains rearranged methylase (DRM). Transgenerational epigenetic inheritance (TEI) is referred to the inheritance of expression states and the traits not determined by the DNA sequence, the molecular mechanisms involved in the process are only rarely verified. This especially applies to the heritability of environmentally induced traits, which have gained interest over the last years. The decision about which epigenetic marks are reset and which ones are not crucial for the understanding of TEI. We will consider examples of epialleles induced by genetic and/or environmental factors used in experimental setups. For example, the progenies of stress-treated plants showed increased global hypermethylation even in the absence of stress, but these transgenerational effects did not persist in successive generations during the absence of stress. In Arabidopsis, the level of cytosine methylated DNA was measured in progenies of treated and untreated plants for two generations (Boyko et al. 2010). Higher 5mC levels were maintained in the progenies of treated plants in response to stressed as well as unstressed conditions, relative to the progenies of untreated plants of the same generation, suggesting that DNA methylation decreases during the absence of stress. Viral infection in tobacco and exposure of UV-C and flagella in Arabidopsis have shown to stimulate the transgenerational inheritance of stress tolerance even to the untreated progeny via increased homologous recombination frequency and global genome methylation (Boyko et al. 2010).

15.6.1 Genetically Induced Epialleles

The molecular mechanisms and factors involved in the creation, maintenance, and stability of epialleles, in general, responsible for the continuity in the propagation of epialleles during these various developmental transitions are poorly understood, with the exception of cytosine methylation (Jullien et al. 2008). Therefore, the formation and transmission of heritable epialleles is expected to correlate with the different levels and/or distribution of CG methylation and such marks should be resistant to transgenerational resetting. Indeed, MET1 seems to be essential for the precision of TEI not only during vegetative growth but also during post-meiotic development of the gametophytes (Saze et al. 2003). The first classical example of an epiallele is the gene variant causing the radial symmetry in peloric phenotype of Linaria flowers, which are usually bilaterally symmetric, has been attributed to hypermethylation of the CYCLOIDEA gene (Cubas et al. 1999). The above-mentioned examples of epialleles, controlled through a genetic element in *cis* or *trans*, provide the most convincing examples of TEI. However, these specific examples do not address the molecular mechanisms and factors involved in the creation, maintenance, and stability of epialleles in general. It is also not clear whether there is a broader spectrum of loci that are subjected to similar mechanisms of epiallelic regulation within the genome (Paszkowski and Grossniklaus 2011). Experiments addressing these issues exploit mutations in genes encoding various epigenetic regulators. Such studies show that the chief components involved in the formation of epialleles, which can be propagated over many generations in the absence of the mutation initially triggering the epigenetic changes, are linked to the maintenance of CG methylation (Johannes et al. 2009). The two best studied factors required for the maintenance of CG methylation are DDM1 and MET1, such that ddm1 and met1 mutants were used to establish plant strains with genome-wide alterations of epigenetic marks (Kakutani et al. 1999). Recently, the role of DNA demethylation in Arabidopsis antibacterial defense has been reported (Yu et al. 2013). *Pseudomonas syringae* pv. tomato DC3000 infection in Arabidopsis revealed that mutants met1 and ddc were unable to develop the typical infection symptoms and showed the resistant phenotype because of their loss cytosine methylation capability. Moreover, several pathogen-responsive genes were differentially regulated in these mutants, signifying the role of DNA methylation in establishing plant defense against bacterial pathogen. A gene named elongator complex subunit2 (ELP2) regulated the genomic DNA methylation pattern in Arabidopsis, and subsequently stimulated the pathogen-induced DNA methylation alterations (Saze et al. 2003). Hypomethylation of DNA during pathogen infection has been shown to influence the defense-related gene expression. The rice R gene Xa21G, which was demethylated chemically exhibited inherited resistance to *Xanthomonas oryzae pv. Oryzae*. Thus, epigenetic processes may be a defense mechanism that allows plants to safeguard their offspring against repetitive biotic stresses without stable inherent trait fixation. An enhanced knowledge on this phenomenon will generate possibilities to moderate disease susceptibility and other agronomic characteristics in plants.

15.7 Plant-Based Factories of Bioactive Molecules (Alternative Models)

The production of the previously described molecules in plant bioreactors involve the use of whole plants and plant cell cultures from different species of mono and dicots as well as the use of specific moss species. In this section we describe and discuss the current status, the advantages and disadvantages, and the overall potential of the use of the diverse plant-based bioreactor systems (Prakash et al. 2008; Schawb et al. 2008).

15.7.1 What to Produce and How?

15.7.1.1 Plant-Derived Bioactive Molecules

Plants are sessile organisms which have evolved a myriad of responses to their land-based, freshwater, and marine environments to rapidly overcome and adapt in function of specific biotic and abiotic stresses. The refined developmental and biochemical plasticity reached by plants along the time involves molecular diversity. An organism which is able to face a myriad of external stimulus needs a vast variety of metabolites to deal with the changing environment.

Therefore, plants have evolved diverse pathways of plant secondary metabolism, several of which have been the focus of study during decades and small molecules derived from these pathways have shown to be bioactive and functional for health and nutrition of humans and other animals. Several others have been recognized as important in diverse industrial sectors as sweeteners, flavors, fragrances, and insect repellents. Although plants produce many of these molecules, they are not produced in the required amounts for industrial requirements in order to be competitive in a specific market. This is a challenge that has led to the design of new strategies for plant bioreactors to increase the production of a given molecule. In the subsequent paragraphs, we provide examples of the current research strategies and applications of bioreactors devoted for the production of these nonprotein bioactive molecules.

In order to improve the production of native or non-native molecules which are the result of a metabolic pathway, several approaches have been used in the recent years. In theory, engineering the metabolic pathway of a native molecule is a straightforward strategy to increase the yields of the metabolite. However, in practice this is not so easy, it has to be considered that plants have also evolved metabolic cross talk between pathways and mechanisms to modulate the homeostasis of a given molecule which in excess can alter important developmental and biochemical processes of the cell and the whole plant. Nevertheless, there has been success in approaches that can increase the production of a metabolite by overexpressing genes that encode for key enzymes that directly influence the production of the metabolite or by transcriptionally silencing genes encoding for enzymes that use the precursor of the desired bioactive molecule into a different route and final metabolic product. In most of the approaches described, stable transgenic plants are generated by introducing an engineered DNA that contains the gene encoding for the protein of interest to be produced (or the RNAi construct for the silencing of a target gene), whose transcription is under the control of a "strong" regulatory region or promoter region that makes sure the overexpression (or the silencing) is efficient. In both cases, transformants are generated by *Agrobacterium*-mediated transfer of the engineered DNA into wild-type plants, as previously described.

In the last decade a variety of studies targeting plant metabolic pathways have been described, most of them with reasonable success in their attempt to improve the synthesis of nutritional molecules such as carbohydrates, polyamines, fatty acids, vitamins A and E, carotenoids, flavonoids, and mineral nutrients as iron and zinc. Several examples of these are reviewed in Sharma and Sharma (2009). Here we describe in detail the most recent ones.

An interesting recent example, using the overexpression approach in flax plants (*Linum usitatissimum*), increases the levels of flavonoids. Zuk et al. (2012) generated transgenic flax plants overexpressing key genes of flavonoid pathway; the resulting plants stably inherited and expressed the transgenes through generations cultivated in field. Analyses of these plants showed an increase not only in the production of flavonoid compounds such as kaempferol, but also in phenolic acids, lignan, and fatty acids accumulation in oil from transgenic seeds. The authors suggest that fatty acids accumulation is due to protection from oxidation during fatty acid synthesis and seed maturation. Moreover, fibers of flax plants had an increased level of catechine and acetovanillone. These results point to a potential use of this type of approach as an important source of flavonoids, phenolic acids, and lignan for biomedical application.

Producing oils with novel traits in crop plants has been a goal for diverse research laboratories, which claim that oils with unusual fatty acids could replace in the future petroleum-derived compounds and, in this way, promote green chemistry. With these ideas in mind, Van Erp et al. (2011), engineered *A. thaliana* plants with castor bean (*Ricinus communis*) fatty acid hydroxylase (RcFAH) and phospholipid: diacylglycerol acyltransferase (RcPDAT). Plants which express only RcFAH increased castor-like hydroxy fatty acid (HFA) content to only 17 % of the total seed oil. Plants co-overexpressing both FAH12 and PDAT1A proteins increased HFAs up to 30 % in seed oil.

A remarkable study by Naqvi et al. (2009), which combines the overexpression of plant and bacterial genes in *Z. Mays* seeds, resulted in transgenic corn plants with increased levels of vitamins. The experimental design used four cDNAs (two of them from plants and two of them from bacteria) encoding enzymes in metabolic pathways for the vitamins β-carotene, ascorbate, and folate. Constructs using these cDNAs were transferred to *Z. mays*. Analyses of stable transgenic pants showed that β-carotene in the transgenic kernels was increased to 169-fold, ascorbate levels up to 6-fold, while folate levels were doubled. This approach provides a potential use of engineered plants to deal with vitamin deficiency in the world's poorest regions (Ye et al. 2000).

Changing the expression of genes using RNA silencing has also proved to be a successful strategy to modulate metabolic pathways. This type of strategy has been recently used to alter the expression of MYB-type transcription factors to activate the anthocyanin pathway in salvia by silencing PAP1 expression (Zhang et al. 2010) and Medicago by downregulating LAP1. In both cases the results show that silencing these MYB transcription factors generate transgenic plants with high anthocyanin contents.

Another interesting application of RNAi in metabolic engineering is to knock out a native pathway and then use the transgenic cells as biotransformation factories; Runguphan et al. (2009) used a *Catharanthus roseus* hairy root culture system in which the tryptamine biosynthesis pathway silenced. Under such conditions if the culture is provided with a synthetic tryptamine analog, the mutated system generates a variety of non-natural products. This novel approach can be used not only for the successful synthesis of non-natural alkaloids, as in this case, but also to synthesize modified metabolites of diverse molecular nature with potential applications.

15.7.1.2 Production of Heterologous Proteins and Nonprotein Molecules Using Plant-Based Systems

Either using whole-plant stable transgenics or transient expression systems, involving cell cultures and hairy roots, several strategies have succeeded in the production of nonplant proteins and other exogenous molecules.

Diverse therapeutic products have been produced using plant-based systems; an interesting and successful case is the production of antibodies in plants, the "plantibodies" approach (De Jaeger et al. 2000; Ma et al. 2005).

It has been a challenge for diverse research groups to produce antibodies in plants since these molecules need to be assembled in the proper manner and folded correctly in order to be recognized by antigens. Another challenge to overcome is the glycosylation pattern of the proteins, which is different depending on the biological system used for their production and the glycosylation pattern of a given peptide is a major characteristic for the function and efficiency of the antibody (Sethuraman and Stadheim 2006). A compendium of the first plantibodies and other pharmaceuticals generated in plants is part of the excellent review by Ma et al. (2005). Here we mention in chronological order a few of the most recent examples: human serum albumin (Sijmons et al. 1990), human interferon alpha (Zhu et al. 1994), antimalaria epitope vaccine (Turpen et al. 1995), secretory antibody (IgA) (Ma et al. 1995), Clostridium xylanase (Herbers et al. 1995), Glucocerebrosidase (Cramer et al. 1996), anticancer therapeutic protein (McCormick et al. 1999), human growth hormone (Leite et al. 2000), industrial polypeptide (Guda et al. 2000), lactoferrin (Nandi et al. 2002), wound-healing agent: human factor XIII (Gao et al. 2004), vaccine anticanine parvovirus (Molina et al. 2004), antithreat agent (anthrax), antigen-type vaccine (Koya et al. 2005), human insulin (Nykiforuk et al. 2006), bovine aprotinin (Tissot et al. 2008), human tpa (Hahn et al. 2009), collagen (Stein et al. 2009), and tissue repair agent elastin (Floss et al. 2010). The former examples are, mostly whole-plant approaches for the production of proteins used as pharmaceuticals and other plant-made industrial products. They show clearly that the use of plant systems as bioreactors for heterologous proteins has a great potential for industrial, therapeutic, and nutritional applications.

Other plant-based systems used as bioreactors have been developed, e.g., tissue cell cultures and suspended cell cultures which have been transformed with stable or transient expression constructs for protein production; the latter avoids the integration of the transgene to the genome of the plant system used as protein factory. *In vitro* transient expression of foreign proteins in plant cell and tissue cultures represents an advantage to avoid restrictions to use stable transgenic plants in the open field.

The bottleneck for both stable and transient assays, using viral vectors or T-DNA constructs, is that the accumulation of the transgene in callus and suspended cell cultures is generally low. Nevertheless, the recent work by Shadwick and Doran (2007) showed that hairy roots provide a better system to increase plant virus replication, pointing to hairy root cultures as a potential system for in vitro production system of foreign proteins encoded in plant viral vectors.

Using these systems it has been possible to produce diverse bioactive metabolites, of which we list some of the most recent: The anticancer metabolite taxane (Syklowska-Baranek et al. 2009), antioxidant betalains (Georgiev et al. 2010), ginsenosides (Mathur et al. 2010), the analgesic harpagoside (Grabkowska et al. 2010), and the antimicrobial phytoalexin (Kawauchi et al. 2010) are some examples.

More recently, hairy roots have been proposed as a system for vaccine production and delivery (Skarjinskaia et al. 2013) and for the synthesis of novel and potent sweeteners of protein molecular nature (Pham et al. 2012).

The explosive increase in the use of microalgae deserves special mention. These organisms have become one of the favorite bioreactor systems for the synthesis of diverse products; recently there has been a boom in the production of biofuels using microalgae bioreactors (Chisti 2007; Wijffels and Barbosa 2010). However, algae-based systems have been used in the production of several exogenous molecules, including products that have importance in the cosmetic or food industry, and they have also been used for the massive production of endogenous bioactive molecules such as vitamins, pigments, and lipids; a detailed description of several approaches can be consulted from several excellent reviews (Walker et al. 2005; Raja et al. 2008; Potvin and Zhang 2010).

More recently, microalgae-based bioreactors started to be used as an alternative for the production of foreign-recombinant proteins on a large scale (Mayfield and Franklin 2005). Using such approaches it has been possible to produce a full-length IgG antibody using *Chlamydomonas reinhardtii* chloroplast, (Tran et al.

2009). Recently, Hempel et al. (2011) were able to produce a fully assembled and functional monoclonal human IgG antibody against the Hepatitis B surface protein and the respective antigen using the diatom *Phaeodactylum tricornutum*. These technological accomplishments potentiate even more the use of algal-based bioreactors for the production of other antibodies and therapeutic proteins.

Another interesting system that has been recently used for bioactive molecules production is based on the use of moss as bioreactors, specifically the Moss model system *Physcomitrella patens*, whose genome is fully sequenced and has been developed in recent years as a system for recombinant protein production in photobioreactors. The achievements done in this Moss species include the creation of strains with nonimmunogenic humanized glycan patterns, a necessary characteristic to use the system for functional antibodies. The group of Ralf Reski has shown that humanized antibodies produced in Physcomitrella display a superior enhanced effectiveness compared to the current ones available from other sytems. The use of Moss for the synthesis of pharmaceutical proteins is well described in two interesting reviews from the Refski group (Decker and Refski 2004, 2007).

15.8 Concluding Remarks

Plants that have traditionally served as food and fiber are now being engineered as novel biomanufacturing systems, with particular attention focused on the creation of plants for producing compounds of nutraceutical, medical, and pharmaceutical use. Transgenic plants containing immunogenic proteins have been created by introducing coding sequences from human pathogenic viruses or bacteria linked to plant regulatory sequences into transformation vectors. Stepwise progress has been achieved in these studies. Plant biochemistry can make significant contributions to human health through the identification and measurement of the many metabolites in plant-based foods, particularly those known to promote health (phytonutrients) (Cathie et al. 2011).

The development of new tools for genome manipulation in plants will help in the development of new crops of higher health value. An important consideration is plants with potential for manipulation for improvement. Several materials from the wild must be useful as candidates for nutrient content modification through the new strategies developed. These crops also will help to feed and heal people suffering from chronic diseases in the countries where they are grown.

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Chapter 16
When Modernity is Not Enough: Towards the Construction of a Glocal Model of Agri-Food Production

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Abstract Based on a review and a reflection upon development policies and programs for the production of food in Mexico, this chapter proposes a model that identifies the problematic dichotomies created by modernity between the local and the global, and the traditional and the modern; the unification of which, for rural change, is presented as a possible utopia capable of combining local practices and knowledge with innovations from other traditions of thought such as the scientific tradition. To this end, we propose that the resolution of the issue begin with a "dialog of knowledges" that would allow for integration and synergy, and also work as a strategy for the construction of alternative development scenarios. We call this model the Via UAQ.

Contents

"Glocal" is a term that suggests equal, dialectical, and reflective attention to the "localization of the global and the globalization of the local" (Escobar 2004, p. 129). This concept was developed by Arif Dirlik (2000) in order to demonstrate the implications of globalization on local processes and vice versa.

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16.1 Introduction

According to the 2013–2018 National Development Plan, “in Mexico, 46.2 % of the population lives in poverty and 10 % in extreme poverty. According to the CONEVAL [Mexico’s National Council for the Evaluation of Social Development Policy], between 2008 and 2010, in a scenario of economic crisis and volatility of international food prices, the only social deficiency that increased in incidence was that of food” (PND 2013, p. 44). One of the biggest challenges in the construction of a just, equitable, and developed nation lies in the ability of the Government and of its institutions, as well as of society and its various sectors, to create multiple proposals that are innovative and diverse, for the promotion of comprehensive processes for well-being that are relevant to development at all levels of society.

Since 1950, discourse regarding well-being and development has promoted various programs sponsored by the nation states, in order to boost economic development and a sense of unity in all regions of their territory. In Latin America, the development agenda has worked for, among other goals, the creation of the nation as an entity capable of generating unity, stability, order, and progress, while also functioning as an economic strategy for the initiation of the expansion of markets on a global scale.

Many of the intervention processes related to policies for the well-being and development of societies considered impoverished or marginal have been linked to the promotion of productivity and economic activity. In our country, the majority of these policies were aimed at rural sectors, both peasant and indigenous; hence, the institution of the tradition/modernity dichotomy.

Through programs such as the Green Revolution, the Government created an ideology in which knowledge driven by science and technology became real, effective, and productive knowledge. These ideas permeated the core of society and became an argument that discredited traditional knowledge and technologies, promoting the belief “that peasant knowledge of production is a set of understandings, practices, and beliefs that do not change […] and as it is traditional, it is static and therefore stagnant” (Díaz et al. 2011, p. 235).

In the case of Mexico, there are various experiences documented (Chontalpa in Tabasco, Chacc in Yucatán, Pujal Coy in San Luis Potosí) that resulted in asymmetric economic relations, technological and corporate cronyism, indebtedness and definitive migration, the devastation of ecosystems, and deterioration in the biophysical relationships had by water, soil, and topsoil.

The years have passed and the country’s rural sector remains an important topic of discussion in terms of the ways in which it is possible to achieve development and equity. Facing this scenario, it is worth asking: Is it possible to generate a model of rural development in order to create processes that would revert and attenuate poverty, inequality, and the desertification of ecosystems? How should this model be built and what are the social conditions and participants essential for its correct functioning? Is it possible that universities and academia could be added to this effort? What are the challenges regarding participation and management in the creation of a new means for the construction of local progress and development?

The objective of this Chapter is to provide some possible reflections on the construction of an alternative means of local development, which we call the Via UAQ using as a starting point, the various experiences that have been implemented in recent decades in Mexico as well as the numerous strategies that are currently being realized in the country and in other parts of the world. We know that in the lessons learned there is a set of knowledge that is relevant due to its innovation and strategy, from which we can make our hypotheses and propose a model that goes beyond social and technological organization, and overrides the dichotomies between the local and the global, and the traditional and the modern. We believe that the joining of the global and the local—glocality—for rural change, at its different levels, is a possible utopia capable of combining local practices and knowledge with innovations from other traditions of thought such as the scientific tradition, based on a "dialog of knowledges" that would allow for integration and synergy while also functioning as a strategy for the implementation of differential scenarios of development and well-being in the short, medium, and long term.

This article analyzes various stages of the rural world in Mexico, in order to present the important global changes in agriculture and their impacts at the local level in the country, from the beginning of the twentieth century until the agricultural crisis in Mexico in the mid-1990s. The purpose of all this is to contextualize an intervention strategy for the construction of an agri-food production model that meets the demands of the market and is within the capacity of the production sites.

16.2 (R)Evolutions in the Countryside

At a pace set by a people with a declared passion for speed appeared the excessive growth of their social and cultural domains, in which cement increasingly drowns the land, and where sustainable food, water, and energy are no longer possible in many parts of the world. The scientific, political, economic, and symbolic changes of the past 50 years in the history of mankind have generated an endless number of modifications among the systems of coexistence of the species that inhabit planet Earth. Economistic order, progress, and unilinear development have progressively taken a heavy toll on both biological and cultural diversity. However, along with an excessive abuse of natural resources, which are essential to the reproduction of the human species, arose concern from the various disciplines of scientific knowledge about how to find ways to stop and reverse those processes which are so destructive to nature.

Thus, understanding the society in which we live allows us to understand the appearance of a social, environmental, and institutional situation in our country. For this reason, it is extremely important to reflect upon development policies, mainly those intended for rural areas, and upon their social implications and environmental consequences.

According to various scholars (Warman 1984; Bartra 2011; Lazos 2011; among others), the territorial and demographic composition of the country has changed structurally; "in 1900 almost three-fourths of the population lived and worked in rural areas, 72 %. In the year 2000, rural Mexicans represent 25.3 % of the population" (Warman 1984, p. 9). This transformation leads us to devote our attention to the conditions of the current Mexican countryside.

In the context of a postrevolutionary period, when the Government needed to establish a new administration of the territory, it conducted various "operational (construction of infrastructure for production, distribution, and means of collective consumption) and regulatory (financial, agricultural, industrial, and environmental policy) interventions" (Aguilar-Robledo 1995, p. 9) in order to regionalize, organize, and sectorize the production activities of the population.

Between 1934 and 1940, Lázaro Cárdenas, through his agrarian policy effected "a major relocation of populations and economic activities such as the lower part of the River Yaqui, La Laguna, the Mexicali Valley, among others" (Aguilar-Robledo 1995, p. 14). Along with various legal and administrative measures, in this Administration numerous agricultural activities were promoted; "Agrarian policy instruments included credit resources through specialized institutions, the establishment of guaranteed prices, and in particular, investments in irrigation and communications in rural areas. By the end of the Cárdenas Administration, agricultural investment accounted for almost 30 % of the total public investment" (Gollás 2003, p. 10).

Starting with the presidency of Ávila Camacho (1940–1946), the issue of rural Mexico became a means for procurement of the nation's economic development. In his 6-year term, for example, tropical agriculture was promoted, generating the mobilization of populations to such territories in order to carry out agricultural activities aimed at marketing and the opening of new markets. Thus, in Mexico, from this administration on structural reforms at the level of the national economy generated various strategies and policies for the sector in order to give rise to foreign investment by encouraging the entry of technologies, industries, and knowledge related to the efficiency of the farms. It was conceived as a priority that the country's demographic growth should become comparable to its economic growth; and, to this end, the continuity of agricultural activities in conjunction with the generation of government policies for the promotion of their implementation became a strategy to encourage centralized growth and social control.

However, the decade of the 1950s structurally transformed the various social and economic policies found throughout the world. With the ascension of the capitalist model, the discourse of modernity based on development and scientific knowledge was positioned internationally, as stated in Harry Truman's inaugural address as President of United States in 1949:

"More than half the people of the world are living in conditions approaching misery. Their food is inadequate. They are victims of disease. Their economic life is primitive and stagnant… What we envisage is a program of development based on the concepts of democratic fair-dealing… Greater production is the key to prosperity and peace. And the key to greater production is a wider and more vigorous application of modern scientific and technical knowledge" (Truman 1964).

As suggested by several authors (Escobar 2012; Frenkel 1953; Cardoso and Faletto 1979), Truman's speech defined the path and the logic of the economic policy of the United States and of the world, while also determining the characteristics linked with backwardness, underdevelopment, and the *invention of the third world* (Escobar 1999) as territorialized problems, the amelioration of which was feasibly done from the capital. From that moment on, concepts such as poverty and hunger were seen as social ills, the modification and transformation of which were based on knowledge, planning, and design, and also the economic interventions of global development aid entities such as the World Bank and later the International Monetary Fund.

Thus, the Latin American Governments, given the need to expand their markets, accepted the model of economic development promoted by the World Bank and they also accepted loans from the World Bank so that they could implant the idea of modernity and development, understood as the increase in the gross domestic product and increased foreign investment in the country. In general terms, this constituted a need for planning and administration of the territory with a development-based approach; "perhaps the most important antecedent in the documentation of the territorial intervention by the Mexican Government is the regional focus on water basins that began in 1947" (Aguilar-Robledo 1995, p. 15). The Tennessee Valley Authority model was exported from the North to the South of the continent, with a vision of modernization, industrialization, production, and foreign investment in development; "in the 1950s, agricultural development policies sought to transform indigenous rural societies into competitively productive and commercial agricultural societies, in order to supply the cities and nascent industry" (Lazos 2011, p. 256).

In the case of the peasant populations, it was thought that once they had become integrated into market society, and at the instant they set aside their conditions of cultural origin, they would no longer demonstrate the *barbarism and savagery* attributed to their origin and community activities. It was thought that the peasants and the rural sector would then be able to enter into *the great civilization offered by the modern nation and the free market.*

Based on the above, the institutions of the Government constructed a system of ideas, practices, and logics that defined the components "to be developed" within national territory. Hence, throughout the various administrations, different "development" strategies were built in order to encourage economic growth in the various regions of the country, especially those with rural characteristics; in addition to the basin planning approach, there were "declarations of national parks and industrial parks (1953), the national border program (1961), the national commission for arid zones (1970), and the comprehensive program for rural development (1973)" (Aguilar-Robledo 1995, p. 15). Also, starting in the 1950s, "government institutions were created for scientific research, for agricultural credit, for the development of seeds and fertilizers, and also so that they might act as channels forthe marketing and distribution of agricultural products" (Lazos 2011, p. 265).

Government institutions related to agricultural credit, agriculture companies, introduction of agricultural technologies, and the transformation of production were all strengthened. All this led, over 20 years, to a period in Mexico called stabilizing development or the Mexican miracle, which, according to different authors (Gollás 2003; Garrido 2003) occurred between the 1950s and the 1970s. "In general, "stabilizing development" shared the dynamics and limitations of the strategies focused on industrialization, under conditions of protectionism and guided by the dynamics of the economic action of the Government" (Garrido 2003, p. 256).

One of the intervention programs that was most representative of this ideal was the Green Revolution.[1] The Government and its institutions involved in agricultural development established a wide range of activities, strategies, and methods in order to *establish modernity* in different regions of the Mexican countryside. This intervention established that technology (the use of chemical fertilizers and genetically modified seeds), new machinery, and the knowledge of experts (mainly agronomists, agricultural-livestock technicians, and veterinarians) were the most effective ways to bring rural Mexico and its inhabitants out of the backwardness and poverty in which they were immersed.

In this *Revolution*, technology created the imaginary of the triumph of reason and science over the local knowledge and techniques of both mestizo peasants and indigenous people. The modernity model began to change the relationship of the peasants with nature, introducing into their language ideas such as productivity, supplies, consumption, appreciation, market, competition, investment, profit, and loss. Descola points out in his numerous studies[2] how the agricultural societies change their modes of relationship with nature, some of them passing from a reciprocal connection involving the reintegration of biopsychosocial balances to one that is characterized by the rapaciousness typical of the extractive postcapitalist agricultural societies.

The establishment of these languages of development and modernity as a structural means to achieve an economic benefit began to create a *standard* regarding the use of the environment: nature as a means of production and marketing as well as a form of monetary interaction.[3] In this scenario, one of the characters who, with the passing of the years, became a central figure within the processes of social intervention was the technician or the extension agent. This was a person sent by institutions, whose immediate responsibilities included the *extension of the light* of science and techniques in the pueblos and communities where *the empire of customs and traditions had ruled.*

This person would focus his or her efforts, during visits and in field work, on the transformation of all ideas and activities that were outside the parameters of

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[1] Barkin (1998), Boege (1996), Leff (2004), Díaz-Cerecer (1989), and Warman (2001), among others.

[2] See Descola (1994, 1996).

[3] The next section elaborates on the relationship between nature and poverty.

development and productivity, such as those relating to nature and reciprocity. In this logical scheme, the subject intervened upon was seen as a passive recipient who was obligated to reproduce the *wisdom* that was being imparted thereto.

The [Green Revolution] RV programs made progress by means of analogies that held development as a synonym of modernity, and modernity meant economic improvement, thus generating the *perfect formula* for the solution of economic problems and the achievement of a *better quality of life*. In the mid-1950s and the 1960s, it was possible to see economic results; for example, in the production of "corn, since it contributed significantly to the generation of foreign currency for the country, due to the availability of exportable balances that allowed for technological innovations" (Garrido 2003, p. 235).

It is important to note that during the first years of the application of the RV, favorable results were seen in production growth. There was an increase of the yield per hectare sown, as well as the entry of new crops and their successful distribution and sale in markets. What in the end might seem to have been an increase in productivity and an improvement in the quality of life for the peasants, resulted in indebtedness on loans for the acquisition of new technologies, in the division and dissolution of community networks, in the progressive erosion of land due to the use of fertilizers, and in a sharp plunge in corn prices. These are factors which currently make it impossible for Mexico to achieve food self-sufficiency as regards corn.

One of the important issues raised by proponents of this program was the reason for the failures. If there had been availability of financial, human, and material resources, along with agents specializing in technical knowledge, how could the pre-established goals of the intervention not have been met? Numerous answers were posed in multiple forms and with various subtleties; those that were heard most often held the farmers responsible for the failure, placing the blame for the situation on them and their cultural peculiarities. Hence the creation of the following institutional discourse, a symbolic construction with respect to the people of the Mexican rural sector: *field workers are poor because they want to be and because they cling to their customs which do not let them move forward.*

In subsequent decades and under these kinds of arguments, there was a severe crisis in the Mexican countryside, which translated directly into the increasing poverty of its inhabitants. In many rural regions of the country, the situation became extremely alarming, generating national and international migration as a means of subsistence.

What we want to highlight with this experience is that this "revolutionary" process of the modernization of rural Mexico served as one of the main bastions for the justification in the social imaginary[4] of the Government's suggestion that farmers are dependent because they have no intellectual and cultural capacity to

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[4] The social imaginary is a concept created by the Greek philosopher Cornelius Castoriadis, commonly used in the social sciences to designate social representations embodied within institutions. In Castoriadis' work (1975), it has a precise meaning, since it implies a conceptual effort by materialism to relativize the influence that *materiality* has on social life.

succeed and therefore must be guided by the institutions of the State and the market in order to achieve development.

When the Government is associated with the ideas of modernity and development, the theology of social intervention is constructed, which is based on reason and scientific knowledge as the only ways to combat *social needs and evils*. Thus, institutions are positioned as the only vehicle capable of creating possible solutions, because they have the means to implement these processes.

In short, we can see how the Government and its institutional arms have taken charge of transmitting to society these ideologies about groups in situations of disadvantage, thus legitimizing their own forms of intervention. However, it is important to point out what we call the dual character of the Mexican Government, which on the one hand generates social disenchantment about the ineffectiveness of its agricultural policies, and on the other hand is understood as the only body capable of redeeming all those wishing to pursue the course of development.

16.3 Poverty and Nature: The Crisis of the Mexican Countryside

In reaction to this desire for the creation, in local contexts, of structural and functional conditions that might lead to conditions such as well-being or human and social development, various methodologies and theoretical models have been developed that support such conditions; thus, there exists a wide range of possibilities when we speak about experiences related to the topic of development, in which participants, discourses, implications, goals, and destinations have found, in the domain of *doing* many possibilities for experimentation.

In these experiences and experiments, nature has been taken into account as one of the central factors in the implementation of interventions. The management that has existed for decades in the rural sector in Mexico reveals the different processes of understanding of nature that have existed; from the pre-Hispanic societies which combined agricultural knowledge with the ritual cycle of their vision of the cosmos, to the modern farmer who, in addition to possessing ancestral knowledge, successfully uses fertilizers and agrochemicals.

As we mentioned previously, the vision that is held regarding nature responds to a rationality in which the human species understands its place within its environment; this rationality leads humankind to position itself through a set of discourses and practices that establishes the type of use and management to be employed, produces very specific results; from units of domestic production intended for personal consumption, to units of intensive management where production is designated for national and international marketing.

Between these two settings, there is a remarkable distance in terms of technological resources, human resources, risk factors, uncertainty regarding the process of production, and contingencies pertaining to sales. However, far from occupying ourselves now with a detailed description of the diverse and emerging scenarios that exist between one position and another, in this section we intend to observe the transformation of the use and management of natural resources and its implications for the rural areas of the country.

From that imperious need to achieve rural development through the planned exploitation of water and soil, until to the assessment of the damages caused by this desire, a wide range of various works has been implemented throughout the Republic of Mexico. In the case of water, the construction of dams, levees, and irrigation canals is the overwhelming evidence that speaks to us of the type of action taken by institutions in regard to the countryside; it was possible to provide water to the vast rural landscape of the country. This is understandable, since most farming in Mexico depends on rainfall, and the production of this system tends to fail in economic terms due to the uncertainty that exists regarding the weather and the amount of precipitation that falls during the production cycle. And if to this we add the effects that climate change has had on the country, we are able to perceive that droughts, hurricanes, and storms are a product of the extractive and erosive relationship that humans have established with nature.

Water has been used as the means to achieve an increase in productivity; however, the use of soil and vegetative cover also has its effects on the determination of productive success or failure. During the years of the modernization of the countryside, the use of agrochemicals and pesticides was a recurring element in the stimulation of the mineral composition of the soil and thus in the generation of plants capable of producing good results from harvest and better dividends in the market. In the early 1970s in some places of the country, such as the Comarca Lagunera region in the North of the Republic, this was true; there was a flourishing production that indicated a healthy condition of the Earth. There was an exceptional production corn and beans, but no good bid prices. From within this disjuncture between the production and the market arose one of the first fundamental water management breakdowns. How was it possible that, despite all the water and the technology, the rural sector in Mexico could fail to develop as planned? Another consequence of this situation was the formation and emergence of various groups of political bosses who, in addition to governing the control of resources, instituted a form of colonization through paid work involving most of the population of the rural areas.

In the end, these practices for the modernization of the countryside interrupted the endogenous process of each soil according to its characteristics. The breakdown of this process generated a codependency of technological supplies that had been acquired largely from government subsidies, and when the technological supplies were released on the market, their prices were too far above the purchasing power of the producers, which resulted in eroded soils. It is also important to mention the impact of the loss of traditional knowledge and practices related to the endogenous processes of restoration, the addition of which deepened poverty.

Along with these differences seen from an economic perspective, there was the establishment of a social and environmental project that saw the market as its central factor. This situation generated a transition, in that the inhabitants were

Table 16.1 Garrido (2003, p. 16) calculated with data provided by the Bank of Mexico, SECOFI (Secretariat of Commerce and Industrial Development)

President	Term in office	Per capita income in thousands of 1993 Mexican pesos	Average number of hours worked needed to acquire a basic food basket
M. Ávila Camacho	40–46	21	13
M. Alemán Valdés	46–52	25	15
A. Ruiz Cortines	52–58	29	12
A. López Mateos	58–64	35	8
G. Díaz Ordaz	64–70	43	6
L. Echeverría	70–76	54	5
J. López Portillo	76–82	64	5
M. de la Madrid	82–88	64	9
C. Salinas de G.	88–94	67	16
E. Zedillo	94–00	69	25

now perceived as workers; this implied that work was a commodity and that nature was a product. Therefore, the development of the nation (in this case Mexico) began to focus on the purchasing power of workers and on the potential that they had to generate capital by means of the transformation of nature.

As for the relationship among the Government, capital, and the free market, along with the impoverishment of society, it is clear that various amendments to the Constitution giving freedom and concessions to foreign economies were generated in the political sphere, and the adjustments and exchange rates that would enable financial fluency in all dimensions were progressively established. Subsequently, many of the industries and companies *managed* by the Government began to collapse, creating a propitious scenario for the generation of policies related to the privatization of the production and distribution of food.

In Mexico, for example, with the progressive proletarianization generated during the 1970s along with the decrease in Government subsidies that had supported farmers and producers in rural areas, the proletariat began to suffer the effects of such processes. Mass layoffs by companies and Government institutions, and a national migration that began to head toward the large cities, along with progressive international migration, emerged as social phenomena resulting from these reforms, as the market and its price adjustment on the basic diet of Mexicans began to surpass the salaries and purchasing power of the people, as given in the following table (Table 16.1).

As a result, throughout the 1980s, social and economic gaps appeared, caused by national and international financial reform. However, during this same decade, there were important subsidies for corn cultivation; “Between 1979 and 1982, it was 49 %, which was reduced to 21 % between 1983 and 1988” (OECD 1997). It is important to note that financial movements in international markets generated strong political implications for the regulation of this crop. Between 1989 and 1993, “corn production grew by almost 80 %, from 10.9 million tons to 18.2 million tons, remaining at this level for approximately 3 years more. Thus Mexico achieved corn self-sufficiency by temporarily canceling or reducing imports" (Fritscher 1999, p. 148).

In addition, Government protection of this crop in the international arena had positive effects, since initially corn was kept away from the policies of price liberalization. Within the country, guaranteed prices were maintained in institutions such as CONASUPO [National Company for Popular Subsistence], which made large acquisitions "purchasing 45 % of the total production by the beginning of the 1990s" (Fritscher 1999, p. 149).

However, it is important to note that the support provided to the country's corn growth was designed specifically for agriculture companies or producers that possessed large quantities of hectares of farmland. This support consisted of technological packages, business plans, and a very important element: water. For small producers that had less than 5 ha and whose agriculture was seasonal, other support was created, but it was limited to the provision of few resources justified by *lost harvest*, which encouraged low productivity, mostly destined for personal consumption.

In 1994, legislative conditions and market conditions were categorically transformed. The corn market was liberalized, subsidies were reduced, tariff protection on imports was withdrawn, and the United States (the leading producer and distributor of corn worldwide) created a foundation for the debacle of the cultivation of this crop in many parts of the world.

The sum of these facts has resulted in the fact that, today, the Mexican Government has increased the amount of corn it imports that is produced by the United States. This, according to various authors (Fritscher 1999; Rubio 1999; Bartra 1999; among others) and various social movements (Sin maíz no hay país [Without corn there is no country], Unión Nacional de Productores de Maíz [National Union of Corn Producers], La Vía Campesina [The Peasants' Way],[5] among others), is considered one of the major failures of the Mexican Government's agricultural policy. Paradoxically, this occurred in a country in which corn had been, for generations and in numerous regions, the main source of energy (carbohydrates and proteins), as well as a food that provided a strong sense of identity.

From the mid-1990s to the early years of the third millennium, the trade reforms continued at the global level, the transnational companies and especially those related to the field of foods (Cargill and Continental)[6] being the main mobilizers. One of the reforms took place in 1994 (in addition to Mexico's signing of the North American Free Trade Agreement): the creation of the World Trade Organization (WTO) which "is not only an agent of trade liberalization, but also a tribunal for enforcing corporate rights to manage consumption" (McMichael 1999, p. 23). The national implications of the creation of the WTO can be understood on multiple levels and in various dimensions. Some implications, for example, were the generation of various agri-food products, the use of high productivity technologies, the

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[5] For more information see Gasparello and Quintana (2009).

[6] Kehman and Krebs (1996, pp. 122–130).

establishment of geopolitical relations linked to food production, and the pauperization of all producers located outside the agribusiness scheme.

Since then, within government policies a differential understanding of what the "Mexican Countryside" means can be observed. On the one hand it is understood that there are regions and producers with the technological, financial, organizational, productive, and market capacity to generate substantial economic returns in order to increase the Gross Domestic Product; and set apart from these regions and producers, there is a Mexican countryside characterized by marginalization, poverty, backwardness, inconsistent production, and environmental deterioration. This differentiated understanding created two strategic trends in the agricultural sector; one was the establishment of incentives for investment of private capital in the production of food and raw materials for industry; possibilities were opened for agribusinessmen to obtain credit, advisory services, and other support in order to improve production quality and establish better positioning than that of the competition in the national and international markets.

The other trend created in the agricultural sector was the creation of the policy of the fight against poverty, combining various elements essential to the reduction of the regional inequality in which millions of Mexicans live daily. For this strategy, several central issues were defined, based on which it was intended to achieve a balance among the conditions of economic inequality, inequality of access to and use of natural resources, and differential access to social welfare services.

In this sense, we believe that it is necessary to devote attention to what we call the *semantic relationships* surrounding modernity and development. These relationships build ideas that identify, standardize, and hierarchize various interpretations made by society. For example, the word *poverty* is used by government economic policy as a stigma in order to define the conditions and capabilities of certain populations as compared to the rest of society. The word *poverty* today brings to mind faces, settings, and discourses; we imagine people from the countryside or from the outlying parts of the cities, who, paradoxically, have abundant natural resources but are not able to exploit them or to see how others are progressively destroying them.

In the 6-year presidential term of 2000–2006, programs were implemented in order to eradicate poverty. This was one of the guiding strategies for the activation of and support for the development of impoverished populations. It was used in such a way that the definition of poverty created an enormous debate; its political, factual, social, and descriptive use generated an enormous ambiguity as to the social group that it represented. Scholars such as Boltvinic and Hernández (2000) have reported that poverty as a metaphor encapsulates diverse possibilities and circumstances for use, in addition to having a strong semantic charge and prescribing treatment based on power relationships.

Today we can say that this metaphor is used to describe those groups in society that are in *atypical* situations regarding the political and economic logic that the contemporary nation state promotes and that the market makes possible. The word "poverty," then, has supported the attempt to objectify and define that which is strange to and different from the majority of society, and its semantic charge implies a relationship of rejection and discrimination.

Another point related to the above is what we call the dual character of the State; in this regard, we have examined situations like those indicated by Casillas: "The procurement of high rates of return, in the short term, in some agricultural activities, by means of modern technologies that are unsuitable for the local potential of the resources, has led to the salinization, alkalinization, and compaction of the soils. This has resulted in the loss of the soils' productive capacities and in turn, in many cases, the abandonment of the agricultural activities that had previously been carried out there. Rural poverty and food insecurity, which prevail in hundreds of marginalized rural communities, are unavoidable consequences of this situation" (Casillas 2006, p. 259).

Along with this perception, we have also noted how the Government represents itself as a strategy to overcome poverty. This is reflected in the 2013–2018 National Development Plan: "The countryside is a strategic sector, due to its potential for poverty reduction and impact on regional development. Facing the twenty-first century, the agricultural sector presents many opportunities that must be strengthened. It is necessary to promote a strategy for the construction of the new face of the countryside and the agri-food sector, with a focus on productivity, profitability, and competitiveness, that is also inclusive and incorporates the sustainable use of natural resources" (PND 2013, p. 82).

However, to the disappointment of the first perception, the second seems to open possibilities for interventions other than those already known and tried; we have observed that the tendency of national rural sector policy will follow the trajectory of the past 20 years; "restructuring agro industry's control over agriculture and preparing the conditions for the strengthening of national food production" (Rubio 1999, p. 58). Thus, we can see that the Mexican countryside is today deprived of its ability to reverse or at least mitigate the effects of poverty, and faces unfair competition from international markets, especially the United States. Neoliberal Government policies are directed toward the agro-industrialization of the countryside and therefore toward the generation of a mass of agricultural wage workers stripped of their knowledge and practices related to the local use and management of nature.

16.4 Towards the Construction of a Model of Glocal Intervention

A long time ago my grandfather used to tell this story, and it is said that it is very old, like time, and that it comes from a time on Earth before there were men like us; there were only animals and God, but the difference is that the animals had word and thought; they could speak, sing, and even become angry. In that era there was a mouse who stole the corn that was kept in the loft of a God; it was easy *for the mouse to steal the corn, take it to an island, and be the sole owner of the ears of corn. Time passed and the mouse always had his corn. One day a crow that was passing by asked the mouse: "What is your occupation?" The mouse replied that he was eating corn. The Crow then knew that the mouse had stolen the corn from God, and he also know that this was the reason why God did not have enough corn for some newly-created humans whom he had been sustaining with tortillas only. Then the crow said to the mouse, "If you like to travel, I can take you wherever you want. Just give me a few ears of corn so that I might take them to a land where there is nothing to eat and which is inhabited by some animals called humans". The mouse thought it over before responding to and accepting the proposal, but he was also tired of living alone on his land and so he replied to the crow, "I will give you the corn, but you must take me to that place of which you have spoken". The crow accepted and the mouse loaded a few ears of corn onto his back, and then they returned to the place where the humans lived. Since that day, humans have been eating corn, and mice have been stealing sown corn* (Van der Fliert 1988, p. 212).

Don Ernesto, the teller of this story, like many of his neighbors in San Ildefonso, is an indigenous peasant, owner of less than 5 ha of farmland, which for several generations has been used for planting corn, beans, and squash, but he also knows how to grow sorghum, barley, and onions. He irrigates his fields with the water that falls from the sky; production is 2.5 tons and the vast majority of it is used for personal consumption, exchange, with a bit left over to sell to his neighbors.

Farmers like him don't clearly understand macroeconomics, price liberalization, or importation of seeds. What they do know is that, progressively, during the last 20 years, young people have become less engaged in this activity, prices of the supplies needed for production have been rising, and that this activity today represents a huge risk, if not a certain financial loss. The predominant situation of the majority of small agricultural producers in Mexico is that the characteristics of their production units do not coincide with the conditions established by the market.

Nearly 20 years after the signing of the North American Free Trade Agreement, with nearly half the population of Mexico in poverty and a rural sector divided by the policies of privilege for agribusiness, facing national and international migration, and the sale and/or rent of land due to market speculation, we can ask ourselves: is there a model designed to stop or reduce the phenomenon of devastation caused by macroeconomic decisions made for local contexts? Is it possible to generate a process of restoration of nature based on food production such as the one employed by rural society? How can we create local conditions that favor global development but are relevant to the collectives on various territorial and generational levels? Is it possible to change the future of a Mexican countryside that, after nearly 20 years of free trade, continues to resist the ravages of market liberalization?

For the last 30 years throughout Latin America, peasant and indigenous societies have been the sectors with the most intense expression of the impoverishment that is so common in various cultural contexts. It is also in those contexts where numerous social, political, and religious initiatives have flourished, with very diverse concerns and very different strategies aimed at assisting in the generation of programs and projects for the construction of endogenous well-being and development.[7]

Now it is our intention to explore the ways in which the various participants in an intervention project are related, starting with two poles, and then we will consider the nuances, hybridizations, and combinations in the various stages involved in these processes of work. On the one hand there is the classical relationship in which external agents espouse a predesigned justification, generate a local need, and are usually the figures who coordinate, direct, plan, and finance the project. This scheme involves a vertical power relationship, since the participating population acquires a passive role in the construction and design of the actions—receiving, participating in a limited manner, or being consulted occasionally as to their experience in the process.

However, we have seen cases in which a local organization or collective poses a need and then searches for intervention groups that might be able to satisfy that need. This strategy also involves a model of vertical interaction but in the opposite direction; that is, it is centered on the local initiative, which presents its intentions and interests, and validates and allows the entry of other party into its context, while also evaluating, sanctioning, and monitoring the various targets of the intervention process. The notable features in this scenario include the authority of the local groups, their level of organization for the establishment of actions, and their active approach to making decisions.

Both models have different advantages and disadvantages in terms of their methods, processes, and outcomes at the time of implementation, but what we want to do now is experiment with the nuances, hybridizations, and combinations, by including models of endogenous development as well as processes for the strengthening of intellectual abilities (pedagogical process), for making connections (organizational process), and for participation (self-managed process) among the stakeholders. In order to begin, it is necessary for the intervening agency to change, through dialog, its objectives, intentions, and ways of planning and financing, so that the relationship between the parties involved may be able to build new challenges and set new destinations (Fig. 16.1).

In cases of interventions promoted by local collectives, we have seen that it is essential that, over time, an ethic of knowledge be constructed, through a dialog of knowledge, which implies that the complementarity of ignorance for the construction of a collaborative logic is possible.[8] These kinds of experiences promote the creation and design of possible alternatives for the collective reflection upon

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[7] For example: community development, rural development, research, participatory action, and ethno-development (Fals 1991, Toledo and Barrera-Bassols 2008).

[8] For more information see Díaz et al. (2011), Lazos (2011), Leff (2011) on the processes of production-based innovation in peasant farming units, technological linkage in local contexts, the transnationalization of agriculture, and multiple environmental rationalities. They all agree that peasant farming systems are able to assimilate knowledge that is different, academic, and relevant to their local needs, and also to use it on a global level.

Fig. 16.1 Hybrid model part one. Own authorship

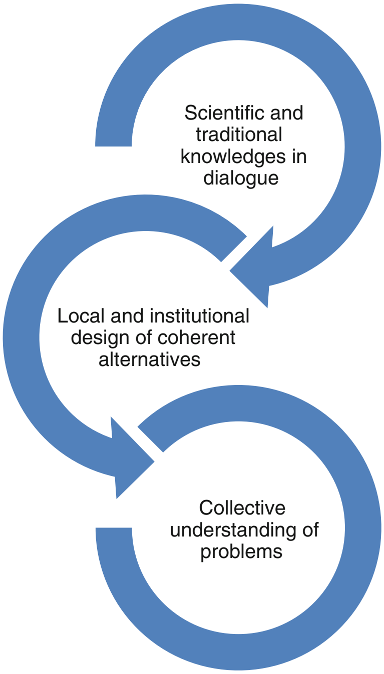

Fig. 16.2 Hybrid model part two. Own authorship

and the understanding of problems. The idea is to foster a social relationship able to question its hierarchies in order to search for other forms of problem solving, favoring a linkage of understandings and knowledge in order to solve cultural and production-based dilemmas (Fig. 16.2).

One other aspect that is important to keep in mind is that, in the last 15 years in Latin America, there has been the popular idea that community groups running intervention projects are made up of women. This is due to two major factors; the first has to do with the economic crises of the 1980s, when men migrated to other regions in search of better wages for the maintenance of their family unit. This generated a restructuring of family relationships, placing women at the head of the household. The second factor refers to the work of government institutions, social organizations, and international bodies that have integrated into their operating rules that they ought to work with women, for gender mainstreaming, among other reasons. Today, there are women who have outstanding experience in the field of project participation, whose main success has been the formation of new generations associated with the experience of working in intervention processes.

Finally, another one of the participants in these processes of intervention in rural contexts is the university system. According to multiple authors (Bonfil 1991; Morin 2007; Argueta et al. 2011; Bocco et al. 2000; among others), today, universities play one of the most important roles in the construction of alternatives aimed at the reflection upon and transformation of social problems. As institutions for the creation of new knowledge and the training of professional workers, universities must, as a necessary condition for social relevance, directly engage in the construction of innovative intervention processes.

Before discussing a model that could qualify as a Via UAQ, the subject of the following and final section, we must first consider some challenges related to the initiation of a socially and culturally relevant process of intervention: the knowledge of the participants as to their characteristics, values, sensitivities, and possessions, which are the intellectual, symbolic, and material elements with which they build their daily lives. This phase is widely known as a diagnosis, which is aimed at recognizing the experiences of previous interventions. Usually there are two poles of action; one of them is the conduct of an external investigation in which the researcher establishes a set of questions and techniques in order to document the aforementioned features of the place and its population. The other option has to do with a so-called self-diagnosis, which is done by the inhabitants themselves, based on a self-reflection upon their surroundings, identifying those aspects that they consider necessary in order to understand their surroundings and the intervention thereupon.

Based on the experiences analyzed and implemented, we are able to suggest that both schemes are very useful if over time procedural stages can be established therefor. We suggest that a diagnosis initially be done from an external perspective, to subsequently be analyzed by an exchange of perspectives (the external with the internal), which can complement each other and create greater dialog and discussion for the generation of information and specific actions.

In consideration of the points mentioned thus far, we also suggest that everyday practices can become a pathway for discussion on and incorporation of new technologies. This synergy requires a transformation in the old methodological schemes established in order to understand the inhabitants, which implies that the knowledge of the representatives of the institutions, the academics, and politicians, must defeat the a priori hierarchies that generate conditions of inequality, marginalization, or the exclusion of the inhabitants' views. What we propose is a paradigm shift in which the voice of the people would be essential for the planning, execution, and management of actions.

Based on these premises for the design of interventions, can we speak of an intervention in the agri-food domain that is able to reduce poverty, promote production, and be sensitive toward the preservation of nature? Can we speak of one that is able to look at the frontiers of market policy as possible options for the exchange of economic and political methodologies? How can we enhance local knowledge so that it might function as an important component of the incorporation of new technologies such as biosystems engineering? And, to what extent is it possible to create from this union a pedagogical process that is able to generate a different vision of modernity? As we are now aware of the nature and the purpose of the creation of a new model, it is now important to analyze the way in which it should be designed, where it should be applied, and what are the social and ethical conditions necessary for its implementation in the short and medium term.

16.5 The Via UAQ: An Alternative Modernity is Possible (Conclusions)

Based on a review and analysis of intervention experiences in the Mexican countryside, as well as of the ideologies that have given rise thereto, and the types of social relationships that they have established, and considering the importance of and the contributions made by both science and local knowledge, as a first step we propose a methodology of work that involves a reassessment of the knowledge and practices of indigenous people and peasants. With this step we seek to reverse the historical processes that have led this population to think that it is ignorant, and it is our aim that the indigenous people and peasants recognize that they are also owners and generators of knowledge; i.e., that they have something to contribute, in this case, regarding agri-food in their environment.[9]

Thus, the first step is a diagnostic phase that includes actions related to the above, and, at the same time, the formation of a multidisciplinary team (biologists, anthropologists, geographers, rural sociologists, agronomists, engineers, and veterinarians). This team would be able to describe in situ both the biophysical conditions of nature and the cultural and spatial conditions necessary for the use and management of the same, as regards food-oriented activities, and the work of the team would be performed in constant dialog with the local population. This diagnosis is intended to generate a baseline that allows for a detailed and comprehensive reading, for the understanding of the processes of production and conservation, while also unifying relevant scientific and traditional knowledge. At this early stage of diagnosis, it is also important to understand the various experiences that local population is facing or has faced, in order to comprehend its disposition and its expectations regarding a process of intervention.

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[9] In an interesting study, Elena Lazos reports that the debacle of the Mexican countryside, mainly associated with a lack of effort and knowledge in the peasant sector or the existence of small properties, was due to factors more structural in nature, including: "(a) the disintegration of their territories and social institutions for the regulation of land and nature; (b) cronyism in the relationship between large agriculture companies and development policies; (c) economic inequality caused by free trade; (d) competitive disadvantages due to price fixing; (e) the control over earnings by agro-industrial corporations; (f) major failures of development projects that swept away the original ecosystems" (Lazos 2011, p. 257). All this leads us to think that, in order to build a process that is comprehensive and relevant to social relations, these structural causes must be taken into account in the construction of alternatives thereto, and they must not be reproduced in ways that are even more complex and difficult.

This diagnosis should also be used for the social integration of the presence of outsiders in the rural area, and, above all, in order to generate a space for dialog, which we believe should be situated in local decision-making practices and be based on the locals' normative forms of participation. This is one of the actions we propose for the reversal of the pervasive relationships of domination. In this regard, it is important to locate the local authorities, the formal and informal collectives that make decisions, and the community leaders. It is also essential to learn about their forms of negotiation and conflict resolution, in addition to locating the spaces, times, and participants that are relevant for the presentation and discussion of intervention projects.

A second phase involves a process of consultation that facilitates open and explicit consent given by the inhabitants, in which the steps of introduction, generation of a working group, determination of methods, and discussion of responsibilities/authority are clear and widely known. The process of open consent is essential in order for there to be a committed and sustained participation by the local population. It helps to place the intervention within the population by establishing potential participants who could make up the working group.

It should be noted that the selection of the users will depend on the objectives of the project; however, preferably there should be women or representatives of some previously established committee present, who are associated with the agricultural sector and have some kind of experience in intervention project management. Other individuals who should be included are the male heads of household who have experience in collective farm work (*ejidatarios* [rights holders of communal lands] or those who have experience as members of social collectives in conservation and production-related projects), and hold the rights to the property on which they live, since in many cases intervention processes develop infrastructure that must be established and placed somewhere, and it is important to determine ownership. When the works are implemented on communal property rather than on family-owned small property, it is essential to explore the decision making and organizational processes that exist in the collective, in order to create forms of consensus building based on the objectives of the intervention and local political needs.

It is also very important that within the local working collective there be young people who have received high school instruction, in addition to having some experience with digital literacy. Due to the participation of young people, the working team will be able to communicate in a language mediated by digital technologies.

Once the results of the diagnostic activity have been obtained, the consent of the population, obtained by a process of consultation and the establishment of horizontal working relationships, continues with the ordering and the systematization of information and experience. At this stage, it is necessary implement actions that include the members of the community team, through a pedagogical process of strengthening abilities and skills in local research, in which their systems of communication are necessary for the understanding and, where appropriate, translation of the information collected. The task of ordering will generate a hierarchization, differentiation, and selection of information, which will involve decision making regarding which information is important to associate with the objectives of the intervention. In this process, the local working group must be involved so that it can represent the community and at the same time create a level of identity that leads to a better sense of ownership of the intervention.

It is necessary to emphasize the fact that much of the success or the failure of an intervention process will depend on the pedagogical process and the process of prestige that are built with local users. The formation of a unified group, associated with identity, together with the construction of a model for a dialog of knowledge, must be systematic and consecutive, based on the needs of the group, and reaffirm the features of membership at each step. In intervention processes involving multiple regions, visits by participants from other regions can function as a local incentive; the users hear in their own language a narration of the experiences that others have had. In addition to establishing affinity and horizontality for knowledge transfer, this generates networks that link common experiences in broader processes of management and processes of coverage that go beyond the community. Processes of knowledge transfer that are successful in terms of establishing ownership are those that clearly show each of the stages of the implementation process, indicating how the stages will be executed. This implies instruction not only in abstract terms, but the manner of instruction and teaching must also be based on local codes and needs.

The results of the diagnostic process regarding local knowledge, beliefs, and practices in the agri-food domain will allow us to observe the level of experience that the local population has in terms of food production. As we might expect, rural communities have deep and extensive experience regarding food conservation and production. This means that from their own bio-cultural heritage there is a set of traditions passed down regarding uses and management, ranging from the selection of native seeds to the domestication and control of exotic and native species. It is also critical to learn about the local perception of food production, whether it is an activity related to tradition, poverty, prestige, need, daily life, or trade.

Once this is understood, there must be a densification of the meaning of the intervention, which should integrate various dimensions as to why food is produced. Thus, those local knowledge and practices can be used in a constructive way, generating a framework of different values and attitudes, within which an image of prestige and recognition can be created. It is important to note that the vast majority of the rural population, due to national and international conditions, understands that growing and producing food is a risky activity that is related to poverty and ignorance. This perception is a major obstacle for the implementation of a technology transfer process related to agro-food activity, since local values such as these limit the process of acceptance of ideological change. It is for this reason that we propose the Via UAQ begin with the search for new forms of communication and the generation of new technological scenarios that are able to transform social imaginaries, values, and perceptions, in order to become the driving force behind a different modernity that is aimed at sustainable food systems and respectful of cultural diversity and nature.

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