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Agricultural, Forestry and Bioindustry Biotechnology and Biodiscovery

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To Adriana, Abigail, Emily, Daynet, Paloma, David, Douglas, and the entire CIBE-ESPOL team
and
In memory of
Wilmer Omar Moposita Llundo

Preface

Food security, crop protection, biodiversity, and human and environmental health are among the main needs and concerns of modern society. Biotechnology and life sciences represent constantly evolving areas that are key for the rational use of natural resources. These resources are indispensable for the development of society. This book presents memoirs of the IV International Biotechnology and Biodiversity Congress held in Guayaquil, Ecuador, 2018, as extent works and reviews on the trend of agricultural and forestry biotechnology, molecules and materials biodiscovery, ethnomedicine, environmental impact, and bioindustry research. Many of these topics are described from the Latin American perspective and show how the biodiversity and ancient knowledge of its countries are key for worldwide sustainable development. This book aims to present the latest scientific research on topics such as food security, crop protection, biodiversity, bioindustry, and human and environmental health solutions.

This book departs from traditional biotechnology or a congress proceeding book. It is the first that integrates the knowledge gained from analyses of the works presented at the Congress, by having expert present their ideas as to the trend involved. Latin America has unique importance for the world. Most Latin American countries are rich in biodiversity that encompasses huge genetic resources and invaluable knowledge for the future welfare of society. The book allows this unique knowledge to be available to a broader audience. Since most of Latin America's scientific research stays hidden from the global scientific community because of linguistic barriers and shortage of funds, this book permits this knowledge to be disseminated and known. The book also provides insights into the scientific bases of traditional knowledge recovered from Latin America's ancient cultures. Ancient knowledge had guided us into discovering promising new materials and drugs and will continue to do so. Finally, this compilation incorporates the shared vision between industry needs and academia, closing the link between the main actors in countries' development.

Guayaquil, Ecuador — Pablo A. Chong

Acknowledgments

Biotechnology is a valuable tool for the study of various plant processes in the twenty-first century. It is expected that biotechnology, in combination with other disciplines such as physiology and biochemistry, provides extraordinary solutions in classical plants genetic improvement programs. The integration of all these disciplines will make it possible to develop genotypes with better development and growth and tolerance to various biotic and abiotic stresses derived from the already visible scenery of climate change. In addition, thanks to biotechnology, we can expand the products obtained from plant species, increasing the economic and sustainable opportunities in the era of bioeconomy.

Thanks to all the chapter authors of this book for generously sharing the results of their research. Thanks also to ESPOL University, Biotechnological Research Centre of Ecuador (CIBE), the scientific committee of the IV International Congress of Biotechnology and Biodiversity, and to BIOALI-CYTED (P117RT0522) for creating the framework so that the authors of this book could work together in the area of biotechnology to provide solutions that ultimately result in economic and, of course, social benefits.

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Part I
Trends on Forestry and Agricultural Biotechnology

Trends on Forestry and Agricultural Biotechnology will cover diverse topics and latest technologies applied to forestry and cultivation of banana and cocoa, two very important commodities worldwide, and important crops for food security in developing countries of Latin America. The part begins by discussing the polemic adoption of biotechnological (as GMOs and CRISPR-Cas) crops and products as viewed by different Latin American markets and countries.

Chapter 1
Genetically Modified Organism's (GMO's) Impact and Current Status in Latin America: Technological Sovereignty or Dependence?

Sandra Sharry and M. Valentina Briones

Abstract The debate on the adoption and development of GMOs continues today. The polarizations on their use have been established and do not seem to change. This polarization has also been established in Latin America, although two countries permit the cultivation of GMOs (Brazil and Argentina). After 30 years of the first GMO plant, what happened in Latin America? What position have your countries taken on the adoption, adaptation, and development of GM crops? It seems that the struggle for the development of these crops originated on other continents, but their consequences had an impact in Latin America. This debate has meant rising revenues in some countries and the delay of others in the use of this powerful technology. Is it ethical? This debate has left some countries in Latin America and the Caribbean in a technological unit, and others have been able to close the gap between the developer countries and them. GMO technology continues to be surrounded by controversial debates involving different actors. This chapter draws attention to the conflicts generated in polarized contexts and shows how, in situations of a wanted polarization, strategies are used only to defend themselves and maintain control of the situation in both positions. The point of view is from the scientific and technological development.

Keywords GMO · Biodiversity · Biosafety · Technological sovereignty · Latin American

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Introduction

Agricultural biotechnology, or green biotechnology, has allowed the improvement of crops through genetic engineering techniques. These types of crops, also referred to as genetically modified or transgenic (GMOs), are based on 13–15 years of research and an investment of millions. It takes a huge investment of time and resources to bring a new biotech crop to the market. A 2011 survey found that the cost of discovering, developing, and authorizing a new plant biotechnology trait introduced between 2008 and 2012 was $136 million. The longest product development phase is the regulatory and registration phase. On average, about 26% of those costs ($35.1 million) were incurred in part of the regulatory testing and registration process. A recent study published in the International Journal of Biotechnology demystifies the belief that releasing a GM crop costs hundreds of millions of US dollars. The study assessed the cost and time of developing a GM late blight-resistant (LBr) potato variety for deregulation and release as a public good, in a specific developing country, and the cost would be between US$1.3 and 1.5 million, within a period of 8 to 9 years. Such costs are not far from a conventionally bred variety, although the two should not be compared since GM produces products unachievable by conventional breeding. Publicly funded institutions have been deterred from developing biotech crops because of the cost implications attached to the process of developing and releasing a GM variety (Schieck et al. 2016). These findings therefore could suggest that public institutions in developing countries can make significant contribution to crop improvement through DNA recombination. However, costs remain high for some countries. Therefore, they comply with rigorous tests relating to human, animal, and environmental safety. The main characteristics of the biotechnological crops currently on the market are tolerance to insects and herbicides in crops such as soybean, cotton, corn, and canola, tolerance to water stress (drought) in corn and soybeans, color modification in carnations and roses, and resistance to viruses in papaya and beans. It is expected that in the next years, biotechnological seeds currently in the process of research and development will be released to the market and will be useful in the combating of climate change, including corn, soybean, cotton, and rice that make better use of nitrogen, tolerate droughts, and provide higher yields (Otero and Lapegna 2016). In any case, this will be influenced by the emergence of a new tool such as gene editing.

Many of the advanced developments that are already undergoing biosafety assessments could reach the market and consumers. Several of them will never reach the market, and many characters will be incorporated or improved by the new breading techniques (NBTs). The GMOs are widely cropped in an increasing number of countries. Each biotech crop is analyzed on a case-by-case basis, rigorously evaluated before reaching the market. In 2018, 26 countries grew 191.7 million hectares of biotech crops. The GMO's growing area was 93.3% in the USA, Brazil (93%), Argentina (~100%), Canada (92.5%), and India (95%). In these countries were approved and commercialized new biotech crops and traits to target problems related to climate change and the emergence of new pests and diseases. A total of 70

countries have adopted biotech crops. From 1996 to 2016, a total of US$186.1 billion economic benefits was gained by countries planting biotech crops (ISAAA 2018). The highest gain was obtained by the USA (US$80.3 billion), Argentina (US$23.7 billion), India (US$21.1 billion), Brazil (US$19.8 billion), China (US$19.6 billion), Canada (US$8 billion), and others (US$13.6 billion). Despite the relatively high adoption rates, the GM crops are far from being widely accepted. In many countries, attempts to introduce and establish GM food crops led to disputes among different stakeholders, but the main question is… why?

Transgenic Crops in Latin America

There is no doubt South America is an agricultural continent: accounting for more than 30% of the agriculture harvested area in the world, this region is responsible for over 12% of all the food exports. With a promise to boost this market and bring greater facilities to farmers, biotech-derived genetically modified (GM) crops were introduced into South America, and their adoption is rapidly increasing since they were first approved for cultivation in Argentina. In recent years, Brazil and Argentina have sustained their position among the world's top five countries growing biotech crops, while Paraguay and Uruguay are among the top 10. Genetically modified (GM) crops were introduced in Latin America in 1996, when the Argentine government approved the commercialization of herbicide-resistant soybeans. From then on, the production of GM soybeans (and other transgenic crops such as corn and cotton) expanded throughout the region. This expansion took off in the early 2000s, when transgenic soybeans were adopted in Uruguay, and GM seeds were smuggled from Argentina and illegally planted in Paraguay and southern Brazil in 1999 and legalized in 2004 and 2005, respectively (Hetherington 2013). Ten countries in Latin America planted biotech crops in 2018 including Brazil (51.3 million hectares), Argentina (23.9 million hectares), Paraguay (3.8 million hectares), Uruguay (1.3 million hectares), Bolivia (1.3 million hectares), Mexico (218,000 hectares), Colombia (88,000 hectares), Honduras (35,500 hectares), Chile (10,454 hectares), and Costa Rica (139 hectares) for a total of 81.93 million hectares. Latin America covered 42.7% of the global biotech area of 191.7 million hectares in 2018. Argentina and Brazil have a long history of integration with global commodity markets. Their large landowners and capitalized farmers have an important weight in the agrarian political economy.

New and innovative traits and plants are being developed in South America. In Brazil, IR sugarcane has been planted in 400 hectares for the first time (ISAAA 2018). The Brazilian research entity EMBRAPA has developed a viral disease-resistant bean with RNA interference (RNAi) mode of action. The same strategy was used to develop GM potato for virus Y resistance in Argentina. More recently, in 2015, a eucalyptus event with increased wood productivity was approved in Brazil, and it is the first GM tree approved for cultivation in the region. The Argentinian government through the Argentine National Advisory Committee on

Agricultural Biotechnology (CONABIA) approved eight biotech crop applications in 2018: seven full approvals comprised of four maize IR/HT stacked events, two HT soybeans, and alfalfa event, plus one soybean event for food, feed, and processing only. However, the use of transgenic crops has been hotly debated and contested in Mexico and Central America, where GM crops are perceived to represent a threat to domestic food sovereignty and biodiversity and have triggered grassroots mobilizations, and campaigns and judiciaries have set up moratoria for their deployment (Klepek 2012; Otero 2014).

Debate

For many people in developing countries, genetically modified crops have become evil, causing problems to humans and the environment and only increasing the income of multinationals. On one side of the heated discourse are people who firmly believe that GM crops pose a threat to human health and biodiversity. On the other hand, there are mainly scientists who are convinced that genetic engineering of plants represents a technology with enormous potential to increase food production in an environmentally sustainable way. This controversy has generated a polarized debate where important facts are largely ignored and where relatively few new ideas are introduced in order to find ways to use this technology in the safest way possible. So, genetic modification of organisms is the subject of strong controversy. On the one hand, environmental organizations around the world warn possible gene transfer from GMOs to other organisms that may get out of control as they expand, polluting "natural" crops. There is strong opposition to the possible consequences of the spread of such crops, which has led some countries to establish moratoriums or ban outright and has in some cases led to riots, such as the burning of GMO fields in some areas of Europe. Often its proponents point out that this type of technology can serve to alleviate hunger in the world and to reduce the action of a number of diseases (e.g., golden rice, or the development of molecular farms). Scientists, however, have an intermediate stance, stressing that so far none have found adverse consequences of the use of GMOs on health and the environment (Sharry 2012). Moreover, many critics do not trust the industry or regulatory bodies, which they consider to be allies of biotech companies. The propaganda of some nongovernmental groups, which is usually exercised through irresponsible journalism, has led to a serious deterioration of public confidence in scientists and government regulatory institutions. The destruction of test sites by the most radical environmental groups, the proposed moratoriums on GMO crops, and food retailers that refuse to sell GMO food products are just a few of the manifestations that have arisen from strong opposition to the GMs. Unfortunately, this has happened without an open, sensible, and in-depth discussion of scientific, economic, and political facts that may have consequences for the development and use of this technology in poorer and more vulnerable countries. Therefore, the central debate has been reduced to the

socioeconomic impact of the use of GMOs and the access of this technology by poor countries and small farmers.

Public opposition has largely been based on arguments of ethics. Genetic modification was looked upon as a particularly invasive technology that would change nature in unprecedented ways (Sharry 2012). Another important point is often left out of the debate is how to make sure that new technologies help people in nondeveloping countries. Some argue that GM technology is controlled by large multinational companies and will therefore never be used by smallholder farmers. Consequently, instead of condemning and blocking GM crop technology, government-funded institutions and nongovernmental organizations should find ways to ensure that knowledge is transferred to these countries. Nongovernmental organizations insist that the voice of the general public, unlike only scientists, must be heard and taken into account. Certainly, everyone agrees with this position. However, one wonders which audience these organizations refer to. Do you really know the problems and needs of smallholder farmers in nondeveloping countries? Many people assume that GM technology is meant to replace traditional breeding and that it will solve all current agricultural problems. It is important to understand that solving the problem of food production for a growing population without harming the environment will require the concerted use of traditional breeding as well as GM crop technology, each being used to solve specific problems and need.

Until now, the development of GM crops has been driven by their potential market value, determined by farmers in the USA and Western Europe, or in the case of Argentina and Brazil because of large planting organizations that manage much of the agricultural territory of these countries. How will we cope with the increasing demand for food if a few major companies control the technology and small farmers in poor countries do not fall into the category of potential consumers? The high costs of releasing GMOs through biosecurity frameworks that delay crop release have not allowed developments in public LAC institutions to put in the market their own developments. Who has benefited from the biosafety system reinforcements? These biosafety systems and intellectual property can only be paid for by multinationals. That is, the campaigns that polarized the debate ended up encouraging the concentration of power and money in a few hands. So, at this point the reflection should be on *access*. GMOs will not eliminate the problems of wealth distribution and therefore food. Nor will they solve the problem of access to food. However, the inclusion of novel traits offers a potential increase in agricultural productivity, or better quality and characteristics of nutrition and processing, which can directly contribute to improving health and human development. GMOs can also have indirect consequences, through detrimental impacts on economic (including trade), social, and ethical factors. These impacts need to be assessed in relation to the benefits and risks they may bring to society (Sharry 2012). In this sense, we must use all the tools and technologies available today to be able to remain as specie on planet Earth, in a sustainable way. This includes recombinant DNA technology and thus GMOs. The use of this powerful technology should be analyzed on a case-by-case basis, in a broader framework, including the different situations and problems to be solved, in the context of each country. That is, not for everything or everyone,

only when it is necessary and there are no cheaper or more viable options. Many people are not properly informed about these technologies and are deliberately confused by stories from some media outlets or by anti-biotechnology activists. In any case, these beliefs should be considered when it comes to developing GMOs, primarily for food (Sonnino and Sharry 2017).

Technological Sovereignty or Monopoly and Dependence?

Poor countries face the challenge of achieving competitive and sustainable development of agriculture and agribusiness that is compatible with the conservation and proper management of natural resources and the reduction of hunger and urban and rural poverty. This challenge entails at the same time the search for a higher quality, safety, and nutritional value of food. There is consensus in the global scientific community that conventional technology will not, on its own, increase or diversify food production in sufficient quantity and quality to feed a population that will almost double in 50 years. This will directly influence the food security of several countries, especially those developing countries, where demands will be higher. Addressing these challenges means that poor countries are more actively incorporating the latest developments and scientific and technological results. Increasingly, the performance and competitiveness of agriculture and food trade are impacted by the emergence of new knowledge, such as advanced biotechnology and its products, such as GMOs. This is influencing the industry in several ways. For example, in addition to improving productivity, they are making it possible to study alternative uses of agriculture, in the pharmaceutical industry and in the bioenergy and biofuels industry. Initially, any invention related to biotechnology and GMOs, in particular, would have to go through ethical reflection. Collaboration in the midst of ethical criteria can become the way to protect traditional knowledge, foster new learning processes, and promote shared benefit models between North and South, East, and West, in order to socialize long-term benefits and ensure health, food security, a healthy environment, and the well-being of life for all the inhabitants of the planet. In short, the complexity of global problems leads to bioethical reflections that open new paths. They should link sustainable development to universal values of equity, justice, and beneficence. Loss of sovereignty and markets and south-north dependence are the issues that anti-GMO groups argue. However, biotech crops can become development opportunities, as evidenced by policies and decisions made by countries such as Argentina, Cuba, Colombia, and Brazil, which have decided to invest in the development of biotech products. As the importance of the private sector grows, so do the transaction costs faced by developing countries in accessing and using technologies. Therefore, the development of their own biotechnology, mainly in the public sector, or in private-public consortia, would allow developing countries to have and use high-end products that make them competitive. These countries must adopt, adapt, and develop DNA technologies, i.e., they must be part of the new knowledge and information society. It should be understood as a knowledge society

that uses as much biological information (i.e., genes and genetic code) as information and communications technologies (ICTs). A technology circulation system that preserves incentives for innovation in the private sector is urgently needed, while addressing the needs of poor farmers in the developing world. However, modern biotechnology itself, which results in the improvement of plants and animals, is not enough to solve food production problems. It should be part of an efficient agricultural production system, where required inputs and management practices are favorable for the complete expression of genetic potential (FAO).

Twenty years of GMO crops in the field clearly show that political and legal conditions in poor and developing countries for assessing biotechnologies that can alleviate poverty and increase food security are not present. There is an urgent need to develop capacities in these countries, both for research and to generate adequate legal frameworks. Conflicting opinion on GMOs in developing countries is a mixture of ideologies, policies, science, and ignorance that demonstrate that these new technologies are sometimes little, or not understood. In these countries, the focus is on the risks (as in the EU) rather than putting it on the benefits. Therefore, the potential for use of these biotechnologies is lost (Sharry 2010; Sonnino and Sharry 2017). Poor countries can achieve technological sovereignty by taking their own choices based on informed debate and analysis of their own risk-benefits. In the case of development for small producers in poor countries, it is absolutely necessary that technologies be royalty-free and that it is possible to harvest the GM seeds and sow them, so that they are distributed without paying a return to the industry. This can be achieved through investments in the public sector or with patent-free use agreements. As IFPRI experts put it: "The usual prediction is that the poor in developing countries will not be able to reap benefits from modern biotechnology in the near future. This may be true, not because technology has little to offer, but because it does NOT have a chance to be USED." Some developers, e.g., universities and small seed breeders, have started developing GMOs which are of relevance to the handling of serious societal problems, including the climate challenge and the biodiversity challenge. However, several things have changed in these 30 years, the techniques have improved, and especially the CRISPR technology, developed in 2012, has made it far simpler to quickly and more accurately alter genes without inserting genetic material from other species. In addition, it is possible to make small changes like turning genes on and off. GMOs are not agriculture's future? Today's science is very different and enables us to precisely target and direct a plant's natural gene-editing process. Will Latin American countries allow the circulation of foreign discourse to prevent the use and development of this new technology? Will it be the same thing that happened with the GMOs? It is an open debate. Some countries have already decided to invest in these new developments and implement policies for the safe implementation of the new NBTs, as is the case in Argentina and Brazil. No country should stay out of scientific development, and the use of this development should respond to its sovereign interests and as far as possible the interests of humanity as a whole.

Conclusions

Almost 30 years after the introduction of the first genetically modified (GM) food into the market, the debate over genetically modified organisms (GMOs) remains contentious. Far from having reached a consensual stage, the discussion seems to be polarized, intense, and conflictive. Pro- and anti-GM arguments have multiplied and are often framed as a mix of economic, agricultural, ethical, environmental, political, ecological, and cultural issues. Indeed, consumers, farmers, NGOs, national governments, international regulatory bodies, scientists, retailers, and the biotech industry are some of the parties involved in the controversy – and each of them has assembled a complex web of positions, arguments, and facts (Salazar et al. 2019).

The development of a GMO is a complex, long, and costly process that requires efficient coordination of multiple phases and activities, with the support of a highly trained interdisciplinary team and an appropriate regulatory plan. The lack of global harmonization in regulatory requirements can generate asynchronicity in GMO approval times and create trade barriers. Therefore, the country/company must have good infrastructure to support development, qualified personnel maintenance, intellectual property law and biotechnology and biosecurity legislation access, and needs/capacity-intensive scan to increase domestic production of GM crops. Transgenic crops have been progressively adopted in LAC, with impacts perceived by some as negative and by others as positive, in relation to sustainability, poverty reduction, and equity goals (IAASTD 2009). The key issue here is to ensure that producers and scientists have access to new technologies. The keyword is ACCESS. Who decides what we will do with the new technologies available to improve crops, us or others? Biotechnologies generated in highly industrialized countries meet the fundamental objectives of their sociopolitical systems. It is thus a full expression of their culture. Access to technology is key and must be ensured because it represents one more opportunity for the smallholder farmer to break the vicious cycle of rural poverty (Melo Araujo, 1995).

Today, unjustifiably restrictive rules designed to meet the needs of industrialized and wealthy countries deny developing countries the opportunity to access these new biotechnologies. Without access to new biotechnologies, countries can face the loss of sovereignty and the detriment of their markets and increase their South-North dependence. A system of circulation of biotechnologies is therefore urgently needed to meet the needs of poor farmers in the developing world. There is an urgent need to develop local capacities, both for research and to generate adequate legal frameworks. Developing countries can achieve technological independence by making their own decisions based on informed debate and analysis of their own risks-benefits.

> There is a universal principle about agriculture, nothing should be done too late, things must be done in due course, missed opportunities never recover. Gaius Plinius Secundus

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Chapter 2
New Biotechnology Promising Approaches for Disease Control on Bananas and Plantains

Pablo A. Chong and Efrén Santos-Ordoñez

Abstract Bananas and plantain are very important cash crops and staple food worldwide. Many diseases, especially the ones caused by fungi, are the main threat for a sustainable production of the fruits. So far, conventional disease control is performed mainly by extensive fungicide applications, threatening occupational health, and the environment. In most banana-producing countries, fungicide applications accumulate over 50 applications per year in conducive environments, placing pathogens under a strong selective pressure and favoring the appearance of fungicide-resistant strains. In some cases, emerging diseases like TR4 have no known treatment threatening the whole banana industry. In view of the above, it is necessary to search for new alternatives for disease control and crop management. New biotechnology pest control methods such as RNAi technologies, biomolecules, and nanoparticles arise as promising applications for environmentally friendly disease controls. Furthermore, Banana's genetic improvement through new breeding techniques could improve the sustainability of the banana and plantain production. The development and application of these technologies to improve banana and plantain management will reduce the need for fungicide applications and decreasing their negative health and environmental effects in the population and ecosystems surrounding farms.

Keywords Biotechnology · Control diseases · RNAi · Nanoparticles · *Musa*

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Introduction

Bananas are one of the most important crops worldwide (Ploetz et al. 2015). Global export reached 20.2 million tons in 2019 (FAO 2020). Nonetheless, global export of bananas represents only 15.5% of the total banana production. The remaining 84.5% represents the production of different *Musa* varieties for local consumption. This underscores the importance of these fruits as a staple food in many tropical and subtropical developing countries. It is believed that banana is a starchy staple food for approximately 500 million people (Collins 2014). Many banana varieties for local consumption are relatively cheap and easy to produce. On the other hand, most of these varieties are susceptible to a plethora of diseases that causes substantial direct and indirect losses (Ploetz 2000). The main control management involves frequent fungicide applications with a very high environmental and economic burden (Risède et al. 2010; Díaz et al. 2018; Chong et al. 2019). As such, control of diseases has a major effect on subsistence production of banana and plantain since most of the smallholders are unable to afford the costs of these fungicides (Ploetz 2000). Contemporary management problems underscore the need for alternative disease management practices and strategic decisions toward sustainable and environmentally friendly banana production.

Main Disease in Banana

Despite the regional differences worldwide, the most important diseases in banana crops are caused by fungi followed by bacteria, virus, nematode, and insect, respectively (Ploetz et al. 2015). The most important re-emerging banana disease is *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *cubense* (FOC). The new FOC disease is caused by the genetic lineage vegetative compatibility group 01213, colloquially denominated Tropical Race 4 (TR4) (Ordoñez et al. 2015). The wilting caused by TR4 is the biggest and most serious threat that especially commercial banana production faces. This disease is one of the most grave and lethal diseases because the banana commercial variety Cavendish is highly susceptible to the pathogen, there is no available control treatment, and it has multiple dispersal pathways (Martínez and Pérez 2019; Ordoñez et al. 2015; Ploetz et al. 2015). Once the pathogen is introduced in an area, the spores could remain viable for more than 20 years (Martínez and Pérez 2019; Ploetz 2015; Ploetz et al. 2015). This makes the Cavendish banana production unfeasible in the infected areas. This same scenario had already occurred in the past with the same pathosystems. Fusarium wilt caused by FOC Race 1 eliminated worldwide the banana industry based on "Gros Michel" variety. Tropical race 4 threatens to make the same with the current industry based on the Cavendish varieties (Ploetz et al. 2015). The disease has been reported in many countries of Southeast Asia, Middle East, the Indian subcontinent, Africa, even Europe (United Kingdom) (Zheng et al. 2018), and recently in Colombia and

Turkey in 2019 (García-Bastidas et al. 2019; Özarslandan and Akgül 2019). So far, no effective control for the disease has been established.

Another important fungal disease in banana is black Sigatoka, or black leaf streak disease (BLSD), caused by *Pseudocercospora fijiensis* (Morelet) Deighton (1976), previously *Mycosphaerella fijiensis* Morelet (1969) (Churchill 2011; Marín et al. 2003; Crous et al. 2013). The disease affects the leaves, destroying the foliage if control measures are not applied (Marín et al. 2003). As a result, there is a reduction in the photosynthetic activity, causing up to 50% of yield production losses and a premature ripening of the fruit (Espinal, Martínez, and Peña 2005; Rodríguez-Gaviria and Cayón 2008; Rodríguez 2009). The conventional control measure for BLSD is the chemical control mainly performed through aerial spraying of fungicides. This type of control is expensive and requires a substantial infrastructure and technical expertise. Moreover, it has been observed in many countries that uncontrolled use of fungicides causes negative impacts on human, animal, and environmental health (de Lapeyre et al. 2009; Marín et al. 2003; Snelders et al. 2012; van Wendel de Joode et al. 2016). One main problem with the chemical efficacy control is the appearance of resistant fungal strains to systemic fungicide groups (Diaz-Trujillo et al. 2018; Chong et al. 2019; Sierotzki et al. 2000; Ma and Michailides 2005). This phenomenon is favored by continued fungicide application, which as a result produces a rise in the fungicide spray cycles (Romero and Sutton 1996; Marín et al. 2003; de Lapeyre et al. 2009). The intensive fungicide usage enforces selection pressures in the pathogen population which have great variability and adaptability, allowing the selection of genotypes capable of thrived under fungicide activity (Latin 2011; Marín et al. 2003; Chong et al. 2019; Diaz-Trujillo et al. 2018; Amil et al. 2007; Canas-Gutierrez et al. 2009).

Other important established banana diseases include yellow Sigatoka disease caused by *Pseudocercospora musae*, anthracnose caused by *Colletotrichum musae*, Moko caused by *Ralstonia solanacearum*, banana streak virus (BSV), and the burrowing nematode *Radopholus similis* (Ploetz et al. 2015; Blomme et al. 2017). The relatively rapid, long-distance dissemination of diseases is thought to be associated with anthropogenic movement of infested material, especially by suckers (Ploetz et al. 2015). Banana suckers are lateral shoots developing from the rhizome of the mother plant that are used for vegetative propagation of the plant by the growers. Before the era of tissue culture, this was the one and only way to reproduce the plant, and it greatly contributed to the global dissemination of diseases like FOC, BLD (Ordonez et al. 2015; Ploetz 2015). Among important emerging banana diseases are *Xanthomonas* wilt (BXW) caused by *Xanthomonas campestris* pv. *musacearum* and blood disease caused by *Ralstonia syzygii* subspecies *celebensis* (Blomme et al. 2017; Ploetz et al. 2015). These emerging diseases and some other important diseases like eumusae leaf spot (*Mycosphaerella eumusae*), freckle (*Phyllosticta maculata* and associated species) (Wong et al. 2012), banana lesion nematode (*Pratylenchus goodeyi*), and banana bunchy top virus (BBTV) have relative narrow geographical distributions, but may incur major losses (Ploetz et al. 2015). For most of these diseases, effective quarantine and the use of clean seed material are the only measures available to reduce their dispersal (Blomme et al.

2017; Ploetz et al. 2015). In the case of the foliar blights, including the Sigatoka complex (Crous et al. 2013), insect and nematode pests could be managed by using chemical control (Ploetz et al. 2015).

Promising Pathogens Control Strategies and Their Potential Use in Banana

RNAi Technologies

New technologies are emerging as candidates for next-generation plant disease management. RNA interference (RNAi) technology has recently emerged as a powerful strategy for new biological pesticides, although improvements are still needed (Girard et al. 2016; Wang et al. 2017; Wang et al. 2016). RNAi is a conserved gene regulation (silencing) mechanism in most eukaryotic species including fungi (Dang et al. 2011). The vascular system of plants naturally translocates RNAs; therefore, sprays on leaves, injection in trunks, or soil application of double-stranded RNA (dsRNA) could translocate within the plant and through plant vessels, and control pathogen infections. This molecular mechanism could be exploited for the development of new pathogen control strategies (Koch et al. 2016; Li et al. 2015).

It has been demonstrated that application of a long noncoding dsRNA (791 nt CYP3-dsRNA), which targets three fungal cytochrome P450 lanosterol C-14α-demethylases on barley leaves, inhibits the pathogen *Fusarium graminearum* growth in both local and distal parts of detached leaves in contact with the molecule (dsRNA was applied by spray in one point of the leaf) (Koch et al. 2016). The efficient spray-induced control of fungal infections at distal tissues shows that dsRNA is systematically translocated within the plant (Koch et al. 2016). Other studies indicate significant inhibition of gray mold disease when external applications of sRNAs or dsRNAs, targeting *Botrytis cinerea* DCL1 and DCL2 genes, were applied on the surface of fruits, vegetables, and flowers (the effect was measured after the *B. cinerea* infection) (Wang et al. 2016).

RNA silencing has been also used in *P. fijiensis* and *F. oxysporum* studies to suppress the expression of endogenous genes. Mumbanza et al. (2013) reported that exogenous synthetic dsRNA molecules targeting adenylate cyclase, DNA polymerase α subunit, and DNA polymerase Δ subunit genes reduced conidia germination of banana pathogens *F. oxysporum* and *P. fijiensis* in in vitro tests (Mumbanza et al. 2013). Silencing of PfSlt2, PfFus3, and PfHog1 in *P. fijiensis* through *Agrobacterium tumefaciens*-mediated transformation showed significantly lower gene expression and reduced virulence, invasive growth, and lower biomass in infected leaf tissues with transformants (Onyilo et al. 2017; Onyilo et al. 2018). These early results show the potential of this technology as a source of innovative tools for the control of most banana pathogens. As RNAi technologies are very specific for the target specie's molecule/gene, the unwanted effect on nonrelated

species will be low. This promises pathogen control strategies which are more health and environmentally friendly.

Biosurfactants

Some of the most promising biomolecules are surfactants of microbial origin. The versatility, antimicrobial properties, and low toxicity of these compounds have allowed their use in many industrial applications including food, cosmetic, chemical, and pharmaceutical industries (Ivankovic et al. 2009; Deepak and Jayapradha 2015). In this context, new generation of microbial surfactant molecules is currently being developed (Deepak and Jayapradha 2015). Biosurfactants are molecules with both hydrophobic and hydrophilic groups in them having the property to be soluble in both polar and nonpolar solvents (Deepak and Jayapradha 2015). This give them the capability to have surface activity and improve the solubility and bioavailability of hydrophobic organic compounds. Among the different types of biosurfactant molecules with important effects on microorganisms' control are the lipopeptides.

Lipopeptides are biosurfactants that, in addition to having high surface activity, could also act as antimicrobial agents (Deepak and Jayapradha 2015). Lipopeptides could be applied in processes related to the management of plant pathogens in agricultural activities (Mnif et al. 2016). In fact, lipopeptides are the basis of many commercial organic products designed for pathogen control. For example, Deepak et al. (2014) tested the efficacy of lipopeptide biosurfactant extracts from *Bacillus thuringiensis* pak2310 for the inhibition of *F. oxysporum* spores (Deepak and Jayapradha 2015). Deepak in vitro studies showed promising results for the use of *B. thuringiensis* lipopeptides to control banana pathogens like FOC, especially as surface spore disinfectants. Nonetheless, for the control of the disease, application strategies must be explored to define adequate forms of molecule delivery on planta, especially in the case of soil-borne pathogens as FOC.

Metal-Based Nanoparticles

Advances in nanoparticle technologies had led to its incorporation in multiple biological applications. Based on its antimicrobial and antiviral properties, nanoparticles (NPs) have been widely used in many applications for agriculture, food, cosmetic, textile, chemical, and pharmaceutical industries (Bello-Bello et al. 2017). One advantage of the use of NPs for pathogen control treatments is their low-dose effectiveness for plant protection and even for plant growth stimulation (Bello-Bello et al. 2017). Silver, copper, and zinc and their derivatives have been well known for their antimicrobial properties (Mahdizadeh et al. 2015; Malandrakis et al. 2019). In this context, silver (AgNPs), copper (CuNPs), copper oxide (CuONPs), and zinc oxide (ZnONPs) nanoparticles prove effective against *Botrytis cinerea*, *Alternaria*

alternata, *Monilinia fruticola*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* fsp. radicis *lycopercisi*, *Fusarium solani*, and *Verticillium dahliae* on both in vitro and in vivo tests (Malandrakis et al. 2019). In particular, AgNPs have been proved to be especially toxic to many pathogenic fungi (Haroon et al. 2019). AgNPs have many modes of action including the disruption of the microbe's cell membrane potential, causing cell death (Mahdizadeh et al. 2015). This in turn shows that although AgNPs have low toxicity for humans (Malandrakis et al. 2019), more studies and proper actions should be made to avoid undesirable effect over the environment, especially in the case of microbiomes.

Mahdizadeh et al. (2015) tested AgNPs against *Rhizoctonia solani*, *Rhizoctonia solani*, *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, *Trichoderma harzianum*, and *Pythium aphanidermatum* showing a differential dose effect for each species with *T. harzianum* the least affected. In this case, Mahdizadeh et al. (2015) argue that the beneficial fungi *T. harzianum* could be protected using a proper dose that will control the pathogens and at the same time will have a little effect on the beneficial fungi growth (Mahdizadeh et al. 2015). More studies should be implemented to assess the effect of the AgNPs on other microbial beneficial species related to each plant species and their environment. Since physiochemical synthetic methods to generate AgNPs are expensive and environmentally dangerous, new biological methods or "green synthesis" based on microbes and plants are being developed (Bello-Bello et al. 2017). Biological synthetized AgNPs have been tested against *Penicillium* sp., *Fusarium* sp., *Aspergillus* sp., and *Ralstonia solanacearum* proving to be effective at very low concentration (Haroon et al. 2019). NP properties make them good candidates' tools for future plant pathogen control. Nonetheless, more studies are needed to guarantee that NP application is safe for the environment.

Genetic Improvement of Disease Resistance in Banana and Plantains

Genetic improvement for BLSD disease resistance in banana has been explored in the past by conventional breeding methods (cross pollination). However, the process has been difficult due to different factors including (i) high levels of sterility in male and female gametes, (ii) the polyploidy that exists in most of the cultivars, (iii) and the long periods of the growing cycle (Swennen et al. 2003). Furthermore, genetic improvement by conventional methods is very difficult in the Cavendish subgroup cultivars due to the almost complete sterility of the female gamete, including the main cultivars used in the dessert banana production worldwide (Sági et al. 1998; Aguilar Morán 2013). Even though several institutes had developed BLSD-resistant hybrids, their acceptance by growers has not been established, mainly by their low organoleptic quality that hinders the interest of the global market.

Biotechnological approaches to genetically improve crops have been applied successfully worldwide (BCH, ISAAA, GM Crop databases). Therefore, through

genetic engineering, it is possible to insert only the genes necessary to provide desired characteristics such as disease resistance. Thus, the organoleptic properties or characteristics of post-harvest would continue as in the original cultivar. Additionally, these genes could be confined in the plant because of the sterility characteristics of the *Musa* cultivars. Candidate genes for resistance to black Sigatoka and Fusarium wilt disease identified in wild bananas or resistant cultivars could be introduced in susceptible banana (and plantain cultivars) for generation of intragenic banana and plantain plants. Durable resistance to both pathogens should be accomplished using the strategies proposed. Furthermore, risk analysis should be performed using the closest banana (in terms of genetics) as a comparator. According to established analysis and reports in banana, results revealed that the risk is comparable to the non-genetically modified bananas under proper risk management conditions (Kabuye et al. 2011; Nimusiima et al. 2015; Dale et al. 2017b). Additionally, reduction of pesticides should be accomplished in *Musa* cultivars genetically modified for pathogen resistance (Dale et al. 2017a).

The identification, isolation, and characterization of promoters are necessary for use for specific expression in genetically modified crops. The use of native banana promoters in the generation of cisgenic or intragenic plants should be better accepted by consumers and facilitate risk analysis for the establishment of a commercial crop. Once the promoter is identified, characterized, and isolated, it is necessary to choose the gene to be used in a crop improvement program through genetic engineering. To identify native genes for the development of intragenic or cisgenic plants, different techniques could be used to determine the expression of specific genes that their final product is responsible for improving the desired character. These genes could be fused to regulatory expression sequences as promoters and terminators and used in genetic transformation for crop improvement. Gene discovery and function elucidation could be accomplished through several techniques including differential display, suppression subtractive hybridization, and RNA-seq. Several gene candidates have been identified using these approaches and could be used to generate a cisgenic or intragenic banana plant.

New breeding technologies (NBT), including genome editing, are recently being used for genetic improvement of crops. Therefore, NBT are promising tools for genetic improvement of banana and plantain's resistance to pest and diseases. While genetic transformation is mainly performed by the introduction of foreign DNA for transgene expression, or even for native gene silencing, genome editing consists, mainly as the modification (edition) of host DNA. Three main technologies for genome editing are available including transcription activator-like effector nuclease (TALEN), zinc finger domains (ZFN), and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/CAS9). The main strategy used is CRIPSCR/CAS9 by knocking out genes resulting in improved traits including pathogen resistance (Tripathi et al. 2019). Therefore, the identification of susceptible genes could be the main target using NBT for pathogen resistance. In banana, CRISPR/CAS9 have been preferred. Furthermore, three different ways for genome editing with CRISPR/CAS9 could be performed including protoplast transformation, particle bombardment, and *Agrobacterium tumefaciens* (reviewed by

Rojas-Vásquez and Gatica-Arias 2020). *Agrobacterium tumefaciens* is used to transform host genome using plasmids harboring the T-DNA containing the CAS9 and gRNA sequences to be expressed in the host. Therefore, the ribonucleoprotein (Cas9 and gRNA) will assemble in the host and cause the edition of the native gene according to the gRNA. Genome editing in banana has been accomplished through a genetic transformation approach (Hu et al. 2017; Kaur et al. 2018; Naim et al. 2018; Shao et al. 2019; Ntui et al. 2019; Tripathi et al. 2019); however, and depending on the legislation for each country, the edited plants (which are transformed) could follow tedious processes for risk assessment. Therefore, the main challenge is to make the genome editing of banana without the genetic transformation step.

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Chapter 3
Complementation of Bananas Conventional Breeding Programs Through Biotechnological Genetic Improvement

Jorge López, Efrén Santos-Ordoñez, and Lianet González

Abstract Bananas (*Musa* spp.) are among the world's most important crops. In terms of gross value of production, they are the fourth most important global food crop. Hence, bananas and plantains are important for food security in developing countries where they form an integral component of the farming systems. However, due to the prevailing low yields attributed principally to diseases including black leaf streak (caused by *Pseudocercospora fijiensis*) and Fusarium wilt (caused by *Fusarium oxysporum f. sp. cubense*, different races), and stress from climatic conditions, it is necessary to complement classic breeding with biotechnology induced in vitro mutation breeding by using gamma irradiation and genetic transformation. These techniques aim to increase productivity and to improve the resilience of the crop to climatic change. Conventional breeding has had a dramatic impact, and as a result, several *Pseudocercospora fijiensis*-resistant cultivars have been released. Mutation induction resulting by the induced mutation through gamma ray irradiation is an alternative technique in developing/generating bananas with improved traits. Furthermore, genetic transformation is an alternative for crop improvement with several advantages over other methods; however, biosafety regulations may hinder the application in several countries. The induced mutation and genetic transformation techniques are very useful for banana improvement.

Keywords Cell suspensions · Genetic transformation · *Agrobacterium tumefaciens* · Mutation induction · Zygotic embryos

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Introduction

Bananas and plantains from the genus *Musa* spp. (denominated bananas onwards) are the fourth most important food crop after rice, wheat, and maize and among the top ten food commodities for South-East Asia, Africa, and Latin America (Chandra et al. 2018). In Cuba, this is a high-priority crop as part of the national food system because of its ability to produce all year round and high per capita consumption rate (López et al. 2017). In addition, banana fruits are rich in carbohydrates, certain minerals like potassium, and vitamins A, B, and C (Singh et al. 2018). Hence, they are important for food security in developing countries where they form an integral component of the farming systems and provide income to the farmers (Suprasanna et al. 2012). However, low yields are common which is attributed to affectations by diseases (Alakonya et al. 2018; Bubici et al. 2019) and disorders caused by abiotic stress like drought, salinity, and heat (Ravi y Vaganan 2016). Major issues in banana and plantain production in tropical regions worldwide are diseases. The most important are black leaf streak (BLS) and Fusarium wilt caused by *Pseudocercospora fijiensis* and *Fusarium oxysporum* f. sp. *cubense* (*Foc*), respectively. The latter is a major alert in banana production countries due to recent appearance of Foc tropical race 4 (FocTR4) in different regions worldwide, including in Latin America in 2019, specifically in the Guajira Department in Colombia (García-Bastidas et al. 2019). Furthermore, the FocTR4 affects the *Musa* cultivars grown in exporting countries of dessert banana, which are resistant to the *Foc* race 1.

The main strategy for controlling BLS is through conventional agrochemicals leading to a critical problem for the environment and the health of the people. However, constant agrochemical applications lead to the development of pathogen resistance. Therefore, several fungicides have been discontinued or are applied in rotation due to resistance in the pathogen. Usage of different fungicides has been responsible for the development of fungicide resistance in pathogens because they target one specific gene product in most of the cases. On the other hand, targeting multiple gene products may decrease the probability for developing resistance.

Initially, progress in banana conventional breeding has been slow because of very limited research on this crop in the past. For other inherent problems, the process is difficult due to different factors, including (i) high levels of sterility in male and female gametes, (ii) the polyploidy that exists in most of the cultivars, (iii) and the long periods of the growing cycle (Swennen et al. 2003). Furthermore, genetic improvement by conventional methods is very difficult in the Cavendish subgroup cultivars due to the almost complete sterility of female gamete, including the cultivar 'Williams' (AAA genotype), which is one of the main cultivars used in the dessert banana production in Ecuador and main exporter countries (Ságі et al. 1998; Aguilar 2013). Even though several institutes had developed hybrids, worldwide cultivation is not established in the majority of the cultivation area and much less in the export industry.

Additional difficulties in conventional breeding include a prolonged life cycle, the low in vivo rate of propagation, narrow range of genetic variability, and retention

of seedless cultivars by breeders related to the preference of consumers for seedless fruit (Bakry et al. 2009; Brown et al. 2017). In spite of these difficulties inherent in the banana crop, notable progress has been made over the last years to develop artificial hybrids.

Several challenges need to be overcome, including organoleptic properties of the fruit (taste, aroma, absence of seeds) and to design a durable resistance to pathogens (Swennen et al. 2003). With respect to biotechnological approaches for genetic improvement of banana and plantains, some of the issues encountered in conventional breeding could be overcomed.

Tissue culture is an excellent mechanism for promoting banana propagation, conservation, and germplasm distribution since most cultivars are seedless. Embryo rescue, involving the excision and culture of developing zygotes, represents the technology that most easily assists conventional breeding (Ortiz 2013). These techniques offer the opportunity for variation induction, handling of large plant populations using established selection methods, and rapidly cloning selected variants. All these strategies could increase the efficiency of mutagenic treatments and subsequent screening of mutant materials. Somatic embryogenesis is also an excellent system for clonal propagation and mutation induction (López et al. 2017).

Nowadays, biotechnological approaches for genetically improved crops have been applied successfully worldwide. For instance, through genetic engineering, it is possible to insert only the genes necessary to provide desired characteristics such as disease resistance. Thus, the organoleptic properties or postharvest characteristics should continue as in the original cultivar.

Additionally, these genes would be confined in the plant because of the sterility characteristics for most of the *Musa* cultivars. Candidate genes for resistance to diseases like BLS and Fusarium wilt from wild bananas or resistant varieties could be introduced in susceptible banana cultivars for generation of cisgenic/intragenic banana and plantain plants. The introduction of protein-coding genes with their native regulatory sequences (promoter and terminator) from a compatible plant for cross pollination or the crop plant itself through genetic transformation is denominated as cisgenesis (Schouten et al. 2006). Furthermore, new breeding techniques including genome editing (e.g., for knockout of susceptible genes) in susceptible *Musa* cultivars may result in pathogen-resistance edited plants.

Conventional Breeding

The conventional plant breeding by means of hybridization is based on the plant reproduction through seeds addressed by plant breeders. Pollen from male parents always is collected around 07.00 hours from flowers. After pollinization, emerging inflorescences from female parents are covered with transparent plastic bags, to avoid natural crossing with external pollen until the last flower would be pollinated. Hand pollinations are performed daily between 07.30 and 10.30 hours on freshly exposed female flowers by rubbing a cluster of male flowers containing pollen onto

the female flowers. Pollinated bunches are labeled with tags. Bunches are harvested at maturity – when the first fruit started yellowing – and ripened in a storeroom until all fruits became yellow and the pulp was soft. Seeds are extracted subsequently and immediately sent to the tissue culture laboratory for embryo culture. This is performed because the seeds produced by bananas and plantains are reported to have a high degree of dormancy; therefore, direct germination in soil is unfeasible (Ortiz and Vuylsteke 1995; Batte et al. 2019). Embryos are extracted and germinated in vitro according to the described procedures to rescue zygotic embryos. In vitro seedlings are transferred to the greenhouse nursery for weaning, and ploidy level is determined for each genotype by flow cytometry. Aneuploids or hyperploids (pentaploids and above) are discarded because these hybrids exhibited gross abnormal foliage or stunted growth (Vuylsteke et al. 1990).

Most cultivated bananas and plantain are triploids. Although triploidy confers a certain vigor to the plant, it also contributes to the sterility that greatly limits the use of hybridization and constitutes a challenge to conventional breeding methods. In spite of these difficulties inherent in the banana crop, notable progress has been made over the last years (Ortiz 2013, 2015; Ortiz and Swennen 2014).

Ploidy level in *Musa* could be diploid, triploid, or tetraploid. The 3x and 4x bananas are often more vigorous and give larger fruits than diploids. Most banana productions in the world rely on triploids, while tetraploidy is considered as the maximum ploidy level giving usually viable plants with overall higher water content, poor fruit post-harvest qualities, and dropped leaves. Thus, triploidy is generally considered as the optimum ploidy level to have good agronomic behavior as it guarantees the highest gamete sterility in production conditions. In natural conditions, triploid varieties have resulted from cross-pollinations between diploid clones producing 2n gametes and diploid clone producing n gamete. The appearance of some tetraploids must have followed the same process ensuing the production of 2n gametes by triploid clones (Tomekpé et al. 2004; Perrier et al. 2018).

Edible diploids are of fundamental importance for genetic improvement, as many of them still produce more or less fertile pollen and/or, when artificially pollinated, could produce hybrids with parthenocarpic fruits. While the diversity of edible diploids is subject to the same constraints as that of the triploids, these diploid derivatives of wild taxa have the potential to introduce the desired traits (e.g., abiotic and biotic stress resistance in wild sources) into new hybrids as well as qualities linked to edibility and agronomic performance. Breeding schemes are using this advantage in different combinations for the eventual production of improved triploids. The construction of synthetic diploids in breeding programs should not only catch the sources of disease/pest resistance but also include fruit quality in its broadest sense. Diploid improvement has almost exclusively been through the use of *Musa acuminata* varieties such as 'Calcutta 4'(*Musa acuminata*), a source of resistance to the Sigatoka complex, yellow Sigatoka, Fusarium wilt, banana weevil, and burrowing nematodes (Ortiz 2015).

Improvement for pest and disease resistance or tolerance is the primary objective of banana breeding. Secondary objectives are linked to the diversity of cultivar growing environments and include tolerance to cold and drought stresses, short

plant size, and strong root system to avoid wind damage and optimize nutritional uptake, among others (Brown et al. 2017).

The main strategy for genetic improvement is to breed resistant triploid hybrids as final products. Triploid cultivars were proved to give a selective advantage over other ploidy levels: diploid cultivars are usually less productive and less vigorous although some diploid clones, AAcv Pisang Mas of ABcv Kunnan, are of some significant value but produced for small or niche markets with high added value. Tetraploid hybrids were the first resistant cultivars bred and may actually be satisfying in terms of yield, bunch, and fruit sizes. However, tetraploids occasionally contain seeds, and their poorer fruit quality has never met the requirements for large-scale adoption by markets and consumers (MusaNet 2016).

This strategy was taken up for other dessert and cooking bananas to confer resistance to black leaf streak and to nematodes. Dessert tetraploid hybrids were developed at *Fundación Hondureña de Investigación Agrícola* (FHIA, Honduras), from crosses between dwarf mutants of 'Gros Michel' and 'Prata' with improved diploids resistant to Sigatoka diseases and nematodes. Cooking bananas were developed from crosses between plantains and resistant diploids at International Institute of Tropical Agriculture (IITA, Nigeria), *Centre de coopération internationale en recherche agronomique pour le développement* (CIRAD, Guadeloupe), *Centre africain de recherchessur les bananiers et plantains* (CARBAP, Cameroon), *Empresa Brasileira de Pesquisa Agropecuaria* (EMBRAPA, Brasil), and FHIA to confer resistance to black leaf streak (Tenkouano et al. 2011).

Some outstanding hybrids were obtained by this strategy. 'FHIA 21', a cooking tetraploid hybrid released by FHIA, is now being cultivated for local markets in some countries in West Africa, in Central and South America, and in the Caribbean, as a substitute to black leaf streak susceptible plantains (Bakry et al. 2009). A major outbreak of this approach is the discovery of endogenous integrated sequences of the endogenous banana streak virus (eBSV) in the plantain genome, releasing infectious viral particles in the progenies issued from crosses. However, these viral sequences have been shown to behave as pseudo-genes and could in some cases segregate as heterozygous locus (Gayral et al. 2008), paving the way for the elimination of infectious eBSV sequences. Tetraploid hybrids AAAB, AAAA, and AABB (primary tetraploid hybrids) are often much more fertile than the triploid parent and could produce seeds when crossed with a diploid accession. At CIRAD, where the "reconstructive breeding" approach has been prioritized for several years, progress has been made in developing AAA dessert bananas. Several hybrids recently obtained ('CIRAD 916' and 'CIRAD 918') have been released for large-scale evaluation to banana growers in the Caribbean (Dominique, St Vincent, St Lucia, Cuba) and in Australia to supply domestic markets. 'CIRAD 925' is to date the most promising hybrid created, combining nearly all the qualities to respond to the export industry requirements. The elite hybrid is presently under large-scale evaluation in grower's fields in Guadeloupe and Martinique to validate its adaptation within the different steps of the export industry sector.

Musa crossbreeding relies on the fertilization of female-fertile parents by pollen of male parents to generate hybrid seed. Diploid parents produce more pollen than

polyploid cultivars or hybrids (Dumpe and Ortiz 1996; Fortescue and Turner 2004; Ssesuliba et al. 2008), which suggests the need for using the former as male parents. The descendants obtained from these 4x x 2x crosses are predominantly triploids (secondary triploid hybrids). Thanks to redistributions and recombinations between A and B chromosomes during meiosis, triploid hybrids free from infectious eBSV could be obtained from these crosses the initial 3x x 2x cross also produces diploid hybrids, whose genetic background comes from the triploid mother plant. These primary diploid hybrids are eventually used in the 4x x 2x cross to bring additional mother-plant background to the secondary triploid hybrids. In this breeding scheme, selected resistant primary tetraploid hybrids could be either released as new improved cultivars or subsequently crossed with other improved diploid selections to obtain secondary triploid hybrids. In this approach, the genetic diversity used on the triploid side is very limited, due to the low fertility of triploid cultivars (De Oliveira et al. 2001; Perrier et al. 2018).

The choice of the diploid parents to be crossed in the first or second step is therefore crucial. Considering that the unreduced triploid eggs of the maternal parent are genetically homogenous, the breeding effort is only based on the improvement of the diploid parent. Within the great diversity of the diploid pool, wild and edible diploids have been selected for pest and disease resistance in triploid cultivars. However, wild relatives are highly fertile but have very few of the outward appearances of cultivated bananas, while edible diploids, even if they are more attractive in terms of bunch appearance and fruit qualities, are at most moderately fertile and often not resistant to diseases (Perrier et al. 2018).

Contrary to the 3x/2x strategy, 4x/2x does not attempt to improve existing varieties but rather to create new improved varieties, from ancestral varieties. These hybrids must, therefore, bring together all the classic characteristics of bananas that are intended for improvement and, in addition, the improved characters that the strategy pursues. This strategy, particularly used by CIRAD, has been possible, thanks to a better knowledge of the evolution of bananas based on their morphological and molecular characteristics. In this way, the importance of genetic variability within the genome *acuminata* was related to the variability in the quality of the fruits of the large cultivated groups. The most surprising example is probably the relationship between the subspecies *Musa acuminata* spp. *banksii* and cooking bananas, which has allowed the production of triploid cooking banana hybrids of purely cumulative origin (Tomekpé et al. 2004).

Other constraints in banana breeding are related to the occurrence of banana streak disease (BSD) in progenies caused by several strains of BSV (a plant pararetrovirus, genus *Badnavirus* from the Caulimoviridae family). Many interspecific *acuminata/balbisiana* hybrids derived from parents free of BSV have been found infected with one or several BSV strains that may lead to complete death. This infection is thought to arise from viral integrated sequences (EPRV, endogenous pararetrovirus) in the nuclear genome of *M. balbisiana* (B genome) (Harper et al. 1999). Some of the plants are immediately infected by virus after crosses (Lheureux et al. 2003). Other hybrids showed a later expression of the disease under stress conditions (e.g., in vitro propagation (Dallot et al. 2001) or unfavorable conditions

such as cold temperature. The mechanism by which the EPRV-BSV is activated in interspecific hybrids is under investigation. Some investigators decided not to use donors of B genome as they were shown to increase the probability of triggering the activation of EPRV-BSV. On the other hand, *M. balbisiana* confers rusticity, hardiness, good ratooning, and ability to produce strong root system in hybrids. Till now, no *M. balbisiana* accession free of EPRV-BSV has been found in the most important ex situ *Musa* collections around the world. Therefore, there is a huge necessity to prospect new endemic *M. balbisiana* accessions in the center of origin of the species (Uma et al. 2005) and to determine their status regarding BSV integrated sequences. At the same time, it is necessary to initiate a breeding program to free the B genome from EPRV-BSV susceptibility.

In Cuba, the banana breeding program is performed by *Instituto de Investigaciones de Viandas Tropicales* (INIVIT); the cross breeding between 'INIVIT PB-2003' (cultivate obtained by selection) × SH-3362 (improved diploid from FHIA) has resulted in the tetraploid hybrid 'INIVIT b-2006' with tolerance to *Mycosphaerella musicola* and nematodes, with an average bunch yield of 18 kg (Ramírez 2003). Another hybrid tetraploid obtained was 'INIVIT PB-2012' type Bluggoe exhibiting tolerance to *M. musicola* and *P. fijiensis*, with an average yield of 50 t/ha for high density plantings with 12 months of crop cycle and drought resistance. This cultivar is distributed along the country (González and Rodríguez 2018).

Mutation Breeding

Mutation induction system based on in vitro techniques to obtain mutant plants and propagate desirable mutants offers an alternative approach in widening the genetic variability and producing novel traits by increasing the frequency of mutations (over the spontaneous rates) and inducing desired genetic mutations (such as unmasking recessive traits) that lead to superior performance and the development of a new variety (Sarsu et al. 2018a). Since the 1970s, in vitro mutagenesis has gained increased popularity as it has overcome major limitations of conventional mutagenesis, especially in the production and handling of large mutant populations and the establishment of reliable and easy screening methods (Maluszynski et al. 1995; Suprasanna et al. 2012).

Physical as well as chemical mutagens have been applied successfully on in vitro derived plant materials. However, among the physical agents, gamma- and X-rays and also UV radiation are the most popular choices. More than 90 percent of released in vitro mutant varieties are derived from physical irradiation (http://mvgs.iaea.org/Search.aspx). The success of any in vitro mutagenesis program depends on the establishment of reproducible in vitro plant regeneration procedures, optimization of mutagenic treatments, and efficient screening of the mutagenized populations for desired variations (Xu et al. 2012; Sarsu et al. 2018b). The use of plant cells and tissues in culture offers exciting applications in crop mutation breeding and increases the overall efficiency of the mutagenic treatments because it provides

ways for rapid and mass propagation of initial target material for irradiation, and rapid, and mass propagation of any mutant populations (Pathirana 2011; Spencer-Lopes et al. 2018). A preliminary key step toward the successful use of micropropagation for mutation breeding is the choice of the mother plant, since the starting material should provide a reliable genetic basis and high phytosanitary levels, thus maintaining healthy (pathogen-free) plant material in a clean, well-controlled, and protected environment, avoiding contamination with biotics, including latent microbial contaminants, and improving surface sterilization of the initial explants (Jain 2010).

Explants Used for Mutation Induction

In banana and plantain, shoot tip culture is the most common practice for plant regeneration, as it is a suitable material for mutation induction. This explant is regularly used for large-scale clonal propagation of plants in commercial laboratories due to rapid multiplication, providing availability of genetic material all year round, which is ideal for true-to-type plantlet production by direct shoot induction. The principal disadvantage of shoot tip culture for mutation induction is chimera formation in a treated plant population that requires dissociating by plant multiplication up to the M1V4 generation (Jain 2010). In vitro mutagenesis of multicellular meristems could lead to a high degree of chimerism. The regenerated mutant plants will be potentially unstable with regard to cellular genotypes and expressed phenotypes due to segregation in subsequent vegetative propagated generations (López et al. 2017).

Somatic embryogenesis (SE) is an excellent system for clonal propagation and mutation induction; using this plant regeneration system could increase the efficiency of mutagenic treatments (López et al. 2017). The methodology for somatic embryogenesis involves a series of sequential staged passes through four consecutive phases: (1) induction of SE, (2) somatic embryo formation, (3) maturation of somatic embryo, and (4) somatic embryo germination and conversion into viable plantlets (Parrott 1993).

In Cuba, the plant regeneration through SE in *Musa* has been scaled up for commercial production of planting materials, which have been evaluated by farmers under field conditions. The genetic stability of regenerated plants and high yields obtained under field conditions demonstrate the feasibility of scaling up this biotechnological protocol and adapting it to commercial production of planting materials to mitigate a critical bottleneck in the value chain of this important crop (Orellana et al. 2010; López et al. 2013).

SE originates from a single cell and therefore prevents chimeras among regenerated plants and makes them an ideal subject for mutagenesis (Jain 2010). The main advantage of using the embryogenic cell suspension for in vitro mutagenesis treatment is the instant production of non-chimeric populations, or the quick dissociation of the chimeric sectors if they are found. In addition, somatic embryogenesis

allows rapid dissolution of chimeras and facilitates the development of homohistant mutants (Jankowicz-Cieslak and Till 2017).

Mutagen Dose

The dose increase causes drastic mutations, such as chromosomal aberrations, and could cause cell damage in apical meristem; therefore, lower doses are usually preferred. Thus, it is recommended to conduct preliminary assays to determine the appropriate doses for each plant material. Radiation and chemical sensitivity tests should always be performed to determine the mutagen dose that results in a 50 percent reduction in plant height, root initiation, survival rate, propagation rate, and fresh weight, among others. This value is known as RD50 or efficient mutation dose (EMD) and is widely used to predict the most effective and most efficient mutagen dose. Theoretically, LD50 will cause the highest frequency of mutations (Van Harten 1998).

In practice, a breeder applying irradiation treatment on vegetatively propagated crops may decide to settle for a growth reduction of 30–50 percent (RD30–50) for M1V1 plants or a survival rate of 40–60 percent (LD40–60) depending on the sensitivity of the plant material (Sarsu et al. 2018b). When searching for a desired trait, mutation induction protocols should aim to increase the degree of plant's genetic diversity within the resulting plant population. In order to accomplish this goal, it is necessary to continue integrating the gamma ray mutagenesis processes with selection of in vitro cell lines or tissue cultures using a selection agent (e.g., a medium with a high concentration of salt or phytotoxin).

Mutation Induction in *Musa*

The *Musa* mutation induction system based on in vitro techniques (shoot tip culture) to obtain mutant plants and micropropagate desirable mutants was developed by Novak et al. (1989); later Roux (2004) standardized the methodology to provide guidelines for mutation induction programs in this culture. The different *Musa* accessions showed different responses depending on the ploidy level and genomic constitution. The following ranges of doses were recommended: 10–20 Gy for diploid cultivars (AA and BB), 30–40 Gy for triploid cultivars (AAA and AAB), and 40–50 Gy for triploid cultivars (ABB) (Roux 2004).

The irradiated embryogenic cell suspension (ECS) survival has been too high and variable. Roux et al. (2004) stated that the ECS of cultivars 'Williams' and 'Three Hand Planty' grew even at a very high dose of 250 Gy. Kulkarni et al. (2004) observed that a 40 Gy dose in cv. 'Gran Nain' was completely lethal. Due to the higher hydration levels, the ECS were more radiosensitive. However, previous

reports indicate that gamma irradiation at approximately 70 Gy was completely lethal to the shoot-tips.

The time at which the cells are irradiated is very critical. According to Roux et al. (2004) and Kulkarni et al. (2007), the optimal timing for irradiation is considered to be 4–6 days after subculture because at this time the majority of cells are in the G1 phase. Studies on irradiated embryogenic cell suspension from cultivars 'CEMSA ¾' (AAB) and 'Calcutta 4' (AA) showed that the LD50 should be calculated during the embryo germination stage (sprouts and roots emission) (López et al. 2017; Sales et al. 2013).

In the book "Biotechnologies for Plant Mutation Breeding, Protocols", edited by International Atomic Energy Agency (2017), Chap. 4 describes a protocol on how to use embryogenic cell suspensions (ECS) in plantain (*Musa* spp.) using both in vitro gamma irradiation and plant regeneration to achieve genetic improvement. The process involves a series of steps to select ECS for irradiation properly and the posttreatment handling for plant regeneration, as well as mutant selection during acclimatization phase and under field conditions (Fig. 3.1) (López et al. (2017).

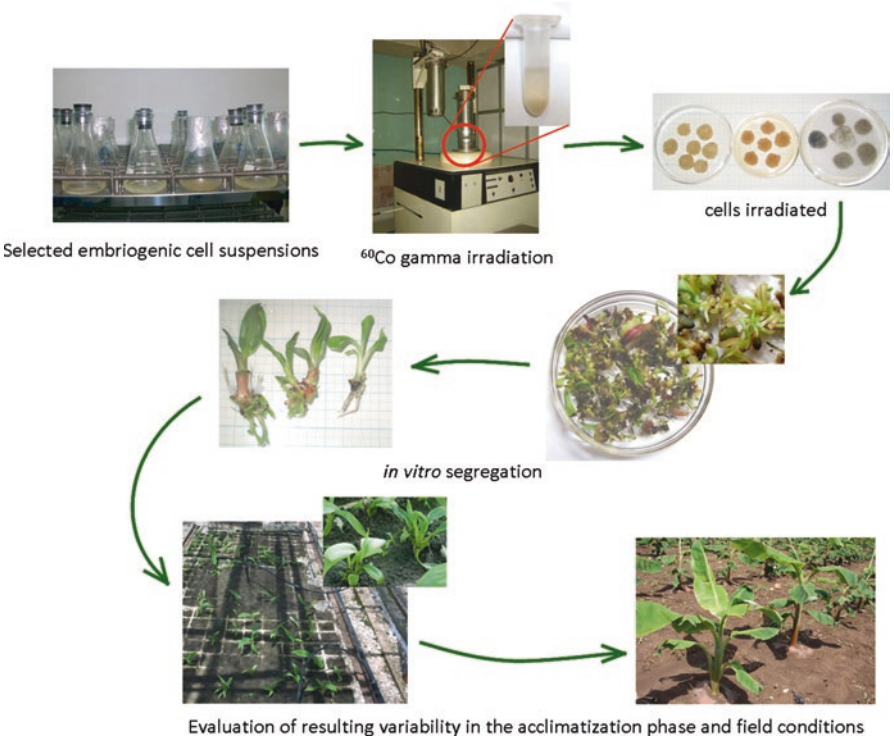

Fig. 3.1 The use of embryogenic cell suspensions (*Musa* AAB) for in vitro mutagenesis. (Taken from López et al. (2017))

Among the works published on the induction of mutations in bananas and plantains, the mutants 'Novaria' (with early flowering), cultivated in Malaysia (Chai et al. 2004); 'INIVIT PV- 0630', which is a small banana plant (AAB) with good agricultural yield, generalized in Cuba (Ventura 2010); and the banana 'Klue Hom Thong KU1' (with larger bunch size) in Thailand (Smith et al. 2005) stand out as results at the production scale. Another group of cultivars obtained by this technique could be found on the IAEA/FAO Mutant Varieties Database (2019).[1] Therefore, mutation induction is a laudable alternative to complement *Musa* spp. breeding by integrating classical breeding and other auxiliary techniques, such as molecular biology and genetic engineering (Wilde 2015). This would facilitate the use of the strengths offered by each method or technology to be applied (Sipen et al. 2011; Xu et al. 2012; Forster et al. 2014; Babu et al. 2015; Bhalang et al. 2018). Advances in the field of molecular biology make an important contribution to the selection of mutants, which helps to facilitate a rapid and accurate identification of changes at the genomic level (Jankowicz-Cieslak et al. 2017).

Genetic Transformation

Explants Used for Genetic Modification

Different plant tissues have been used for genetic modifications. However, in banana and plantains, ECS is the ideal explant for genetic transformation, because it avoids the risk of chimera formation (Sowmya et al. 2016). Strategies to develop ECS in banana and plantains include the use of male inflorescence (Cote et al. 1996) and scalps (Strosse et al. 2003). Other application of ECS is their use in germplasm banks for cryoconservation (Panis et al. 2007) or coupled to a temporary immersion system (TIS) to improve micropropagation (Korneva et al. 2013). Most genetic transformation reports in bananas involved the use of ECS (reviewed by Sowmya et al. 2016). The major disadvantage in the development of ECS is the time required from explants (including immature inflorescence or scalps) to obtain ECS and for the regeneration into in vitro plants after genetic modification (Liu et al. 2017). Furthermore, generation of ECS is genotype dependent (Tripathi et al. 2012), indicating that protocols need to be adjusted according to the genotype. Therefore, other explants have been used including meristematic regions from in vitro plants (Tripathi et al. 2005; Rustagi et al. 2015; Villao et al. 2019; Villao et al. submitted) or sturdy shoots induced from floral apices slices (Liu et al. 2017).

[1] http://mvd.iaea.org

Methods of Genetic Transformation for Banana

The first reports of genetic transformation of bananas were published in the mid-90s (Sági et al. 1992; Sági et al. 1995a, b, c; May et al. 1995). Different methodologies were tested including electroporation (Sági et al. 1992, 1995b, c), microprojectile bombardment (Sági et al. 1995a, b, c), and *Agrobacterium tumefaciens* (May et al. 1995; Pérez et al. 1998, 2000, 2006a, b; Ganapathi et al. 2001; Khanna et al. 2004; Arinaitwe 2008). The preferred methods for genetic transformation in banana include microprojectile bombardment and *Agrobacterium tumefaciens* (reviewed by Khanna and Deo 2016). Furthermore, *Agrobacterium*-mediated transformation has been used preferably, due to the low copy number of integrated transgenes and higher efficiency of transgenic lines obtained (Khanna and Deo 2016). Different strains of *A. tumefaciens* were used including AGL1 (Khanna et al. 2004; Tripathi et al. 2010; Paul et al. 2011; Magambo 2012; Sunisha et al. 2019), C58C1 (Yip et al. 2011), EHA105 (Chakrabarti et al. 2003; Pei et al. 2005; Yip et al. 2011; Betty 2011; Shekhawat et al. 2011; Subramanyam et al. 2011; Tripathi et al. 2012; Ghag et al. 2012; Namukwaya et al. 2012; Shekhawat and Ganapathi 2013; Hu et al. 2013; Kovács et al. 2013; Ghag et al. 2014a, b, c), and LBA4404 (Khanna et al. 2004; Sreeramanan et al. 2006; Maziah et al. 2007; Subramanyam et al. 2011; Elayabalan et al. 2013; Mohandas et al. 2013; Namuddu et al. 2013; Rao et al. 2014), among others. Mainly genetically modified bananas and plantains have been developed for the resistance of pathogens, including Fusarium wilt, black Sigatoka, and bacterial wilt (Table 3.1). Most *Musa* genotypes improved by genetic transformation include AAA and AAB (Table 3.1). Furthermore, genetic improvement has been accomplished for abiotic stress including salinity and drought (Table 3.1).

Biosafety Concerns

One of the major issues in GMO is the use of selectable marker genes (SMG), because the SMG are only used for the selection of GM events. However, several studies related to the use of SMG in genetically modified crops have indicated their safe use for the environment and health of animals and humans. For instance, toxicity or allergenic effect is absent for the NPTII and HPH selectable marker proteins (Flavell et al. 1992; Fuchs et al. 1993; Lu et al. 2007; Zhuo et al. 2009), and bioinformatic analysis revealed no homology to any known allergen (Fuchs et al. 1993; Lu et al. 2007; EFSA 2009). The risk to the environment of genetically modified banana or plantains is comparable to the risk of non-transgenic bananas or plantains.

Table 3.1 Genetic transformation for resistance to biotic and/or abiotic stress in *Musa* spp.

Cultivar (genotype) Name		Genes introduced	Trait improved/ phenotype	Reference
'Matti'	AA	Peanut salinity-induced pathogenesis-related class 10 protein	Drought and salinity tolerance	Rustagi et al. (2015)
'Furenzhi'	AA	chit42	Resistance to Fusarium wilt, FOC race 4	Hu et al. (2013)
'Grand Nain'	AAA	Bar	Resistance to herbicide Basta	Becker et al. (2000)
	AAA	Protein-engineered rice	Resistance to nematode *Radopholus similis*	Atkinson et al. (2004)
	AAA	P5CS	Salt stress tolerance	Ismail et al. (2005)
	AAA	Saccharomyces cerevisiae tps, tpp	Salt stress tolerance	Santamaria et al. (2009)
'Williams'	AAA	Bar	Resistance to herbicide Basta	Becker et al. (2000)
'Gros Michel'	AAA	rcc2, Rcg3	Black Sigatoka	Kovács et al. (2013)
'Pei Chiao'	AAA	Plant ferredoxin-like, protein (pfl p)	Resistance to Fusarium wilt, FOC race 4	Yip et al. (2011)
'Taijiao'	AAA	Human lysozyme gene	Resistance to Fusarium wilt, FOC race 4	Pei et al. (2005)
'Nakinyika'	AAA	Plant ferredoxin-like protein (Pfl p)	Resistance to bacterial wilt (*Xanthomonas campestris* pv. *musacearum*)	Namukwaya et al. (2012)
	EA – AAA	Plant ferredoxin-like protein (Pfl p) gene	Banana Xanthomonas wilt (BXW)	Namukwaya et al. (2012)
'Mpologoma'	AAA-EAHB	Hrap	Banana Xanthomonas wilt (BXW)	Tripathi et al. (2010)
'Rasthali'	AAB	MSI-99	Resistance to Fusarium wilt FOC race 2, and Mycosphaerella musicola	Chakrabarti et al. (2003)
	AAB	Antimicrobial peptide (Ace-AMP1)	Resistance to Fusarium oxysporum f.sp. cubense race 1	Mohandas et al. (2013)
	AAB	ntron-hairpin-RNA (ihpRNA) transcripts corresponding to viral master replication initiation protein (Rep) of BBTV	Resistance to BBTV	Shekhawat et al. (2012)
	AAB	miRNA of MusamiRNA156	Stunt and change in leaf anatomy	Ghag et al. (2015)

(continued)

Table 3.1 (continued)

Cultivar (genotype) Name		Genes introduced	Trait improved/ phenotype	Reference
	AAB	Aquaporins – membrane intrinsic protein gene (MusaPIP1;2)	Salt stress tolerance	Sreedharan et al. (2013)
	AAB	MusaWRKY71	Modified abiotic and biotic stress response, oxidative andsalinity tolerance	Shekhawat and Ganapathi (2013)
	AAB	MusaDAD1, MusaBAG1, MusaBI1	Resistance to Fusarium wilt, FOC race 1	Ghag et al. (2014b)
	AAB	Defensin (Sm-AMP-D1)	Resistance to Fusarium wilt, FOC race 1	Ghag et al. (2014a)
	AAB	Petunia floral defensin (PhDef1/PhDef2)	Resistance to Fusarium wilt, FOC race 1	Ghag et al. (2012)
	AAB	Intron hairpin RNA (ihpRNA)-mediated expression of small interfering RNAs (siRNAs) targeted against vital fungal genes (velvet and Fusarium transcription factor 1) factor 1 (Ftf1)	Resistance to Fusarium wilt, FOC race 1	Ghag et al. (2014c)
	AAB	MusaDHN-1	Abiotic stress, Drought and salinitytolerance	Shekhawat et al. (2011)
	AAB	MusaNAC68	Salinity and drought tolerance	Negi et al. (2016)
	AAB	Soybean β-1-3-endoglucanase	Resistance to Fusarium wilt, FOC race 1	Maziah et al. (2007)
	AAB	MusaSAP1	Drought and salinity tolerance	Sreedharan et al. (2012)
	AAB	MusaPIP2;6	Salinity tolerance	Sreedharan et al. (2015)
	AAB	MusabZIP53	Growth retardation, dwarf phenotype. Enhanced drought, salt and cold tolerance with stunted plants	Shekhawat and Ganapathi (2014)
'Virupakshi'	AAB	RNAi targeting the BBTV rep gene,	Resistance to BBTV	Elayabalan et al. (2013)
'Gonja manjaya'	AAB	Rice pattern recognition receptor (PRR), XA21	Resistance to bacterial wilt (Xanthomonas campestris pv. musacearum)	Tripathi et al. (2014)
	AAB	Maize cystatin	Nematode (Radopholus similis)	Roderick et al. (2012)
'Lady Finger'	AAB	Antiapoptosis animal genes: Bcl-xL, Ced-9, Bcl-2 3′ UTR	Resistance to Fusarium wilt, FOC race 1	Paul et al. (2011)

(continued)

Table 3.1 (continued)

Cultivar (genotype) Name		Genes introduced	Trait improved/ phenotype	Reference
'Pisang Nangka'	AAB	Rice thaumatin-like protein (tlps)	Resistance to Fusarium wilt, FOC race 4	Mahdavi et al. (2012)
'Sukali Ndiizi'	AAB[a]	Plant ferredoxin-like protein (Pfl p)	Resistance to bacterial wilt (*Xanthomonas campestris* pv. *musacearum*)	Namukwaya et al. (2012)
	ABB[b]	Carica papaya cystatin (CpCYS)	Resistance to nematode	Namuddu et al. (2013)
	ABB	Hrap	Banana Xanthomonas wilt (BXW)	Tripathi et al. (2010)
	ABB	Plant ferredoxin-like protein (Pfl p) gene	Banana Xanthomonas wilt (BXW)	Namukwaya et al. (2012)

[a]According to Promusa
[b]According to the reference

Gene Identification in Bananas and Plantains

Gene identification and function elucidation are needed in a genetic modification framework to improve different traits. Furthermore, banana genes could be used to generate cisgenic or intragenic bananas. Alternatively, indirect characterization could be performed by analyzing gene expression patterns by using different techniques including northern blot, RT-qPCR, and/or differential screening of cDNA libraries (Clendennen and May 1997; Liu et al. 2002; Van den Berg et al. 2004; Xu et al. 2007; Van den Berg et al. 2007; Caamal-Velázquez et al. 2007; Mbéguié-A-Mbéguié et al. 2008, 2009; Chen et al. 2011; Passos et al. 2012; Chen et al. 2016; Sánchez et al. 2016; Santos et al. 2016a; Chávez-Navarrete et al. 2019), or through transcriptomics using next-generation sequencing platforms (Wang et al. 2012; Li et al. 2012, 2013; Bai et al. 2013; Yang et al. 2015; Ravishankar et al. 2015; Backiyarani et al. 2015; Hu et al. 2015, 2017a; Zorrilla-Fontanesi et al. 2016; Wang et al. 2017; Sun et al. 2019; Tripathi et al. 2019a).

Promoter Analysis

Promoter sequences are used for constitutive and/or regulating expression of genes inserted by genetic transformation. A promoter could be defined as the DNA sequence located at the 5′ region of a coding region which is responsible of recruiting the transcription machinery to start transcription. An important characteristic of promoters is the presence of *cis*-acting elements which interact with transcription factors and are important for the activity characteristics of the promoters. Promoter

sequences could be characterized by in silico analysis to identify *cis*-acting elements responsible for the expression activity pattern. A review of in silico analysis of banana promoters is described by Santos et al. (2016a). Characterization of promoter activity could be performed by fusing the promoter sequences to a reporter gene. Therefore, the activity of the resulting reporter protein reflects the rate and pattern of transcription, which is directed by the promoter. In plants, three reporter genes are commonly used, including green fluorescent protein (*gfp*; GFP; Heim et al. 1995; Chiu et al. 1996), β-glucuronidase (*uid*A; *gus*, Jefferson et al. 1987), and firefly luciferase (*luc*, Ow et al. 1986). Furthermore, reporter genes could be used to standardize genetic transformation protocols (Sági et al. 1992, 1995a, b; May et al. 1995).

Promoters could be classified according to their type of activity in three classes (reviewed and adapted from Hernández-Garcia and Finer 2014): (i) constitutive promoters, which drive expression in all tissues and developmental stages; (ii) spatiotemporal promoters, which include tissue- or space-specific expression; and (iii) inducible promoters, which refers to specific expression after application of an external chemical signal or presence of a biotic or abiotic stress. Furthermore, synthetic promoters include the modification or combination of different promoters and/or defined regulatory elements, which could drive expression within the three classes described above. In banana, several promoters have been used to drive gene expression in genetically modified bananas and could be classified according to their source in viral (e.g., CaMV35S; Sági et al. 1995a, b), bacterial (e.g., Nos, May et al. 1995), plant (e.g., Ubi1 Sági et al. 1995a, b), and native (Act, Hermann et al. 2001). A detailed review of promoters used in banana transformation is described elsewhere (Santos et al. 2016b).

Table 3.2 Application of genome editing techniques in banana

Genome editing tool	Cultivar name (genotype)	Target gene	Phenotype	Reference
CRISPR/CAS9	´Boxi´ (AAA)	Phytoene desaturase (MaPDS)	Albino	Hu et al. (2017b)
CRISPR/CAS9	´Rasthali' (AAB)	Phytoene desaturase (RAS-PDS)	Albino	Kaur et al. (2018)
CRISPR/CAS9	'Williams' (AAA)	Phytoene desaturase (PDS)	Albino	Naim et al. (2018)
CRISPR/CAS9	'Gonja Manjaya' (AAB)	BSOLV ORFs	Resistance to BSV	Tripathi et al. (2019b)
CRISPR/CAS9	'Gros Michel' (AAA)	*MaGA20ox2*	Semi-dwarf	Shao et al. (2019)
CRISPR/CAS9	'Sukali Ndiizi' (AAB), 'Gonja Manjaya' (AAB)	Phytoene desaturase	Albino	Ntui et al. (2020).

New Breeding Techniques in Banana and Plantains

Different techniques have been applied for crop improvement. Genome editing is a promising tool for crop improvement and gene function elucidation. Main methodologies for genome editing include zinc finger domains (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/CAS9) (reviewed by Gaj et al. 2016). Furthermore, genome editing techniques have been applied for the improvement of tropical crops (reviewed by Rojas-Vásquez y Gatica-Arias 2019). Recently, genome editing has been accomplished in banana for different purposes (Table 3.2), increasing the toolbox of breeding techniques in banana and plantains.

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Chapter 4
The Stress as Inducer of Heritable Changes in Micropropagated Banana – The Hypothesis of Cytokinin Accumulation

Carlos Noceda and Douglas A. Steinmacher

Abstract Vegetative plant propagation may lead to heritable phenotypic changes, a phenomenon known as somaclonal variation. The underlying molecular causes relay in the breakdown of a preexisting chimerism, or in the induction by stress of rather controlled chromatin remodeling at epigenomic and genomic levels, as well as in less extent mutations, generally considered to occur randomly. The controlled epigenetic alterations may facilitate subsequent concrete genome reorganizations and, both together, the occurrence of other DNA sequence mutations in genomic regions constitutively more labile or that become more exposed. Thus, chromatin remodeling, as a plastic response of plant to manage stress, is a controlled effect that may facilitate specific genome changes probably not so random. This should result in more frequent variant phenotypes (somaclones), such as the dwarf types of banana and plantain generated after several cycles of micropropagation. Several studies pointed out that the banana (epi)genome might became unstable during increased multiplication cycles, resulting in higher somaclone indexes. The most applied plant growth regulator for banana micropropagation is the cytokinin 6-benzylaminopurine, which promotes the development of new shoots, another possible defense response of plant to the specific stress generated by the whole set of

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artificial culture conditions. The contents of such molecule and its metabolism in the plant along subcultures affect both organogenesis and somaclonal variation by inducing chromatin changes that affect developmental processes and phenotype, respectively. In the present chapter, we express our opinion about these facts on the bases of reviewed literature and our own experience.

Keywords Somaclonal variation · Stress · Epigenomic · Mutations

Introduction

Banana and plantain (*Musa* spp.) span important cultivars that contribute to food supply along the world, especially in tropical and subtropical areas. The production of *Musa* commercial crops is limited by two important factors: (1) scarce fertility and slow vegetative propagation rate in field and (2) several diseases and pests, mainly including virus, fungi, and nematodes. Biotechnologies are helping to overcome these problems by (1) enhancing vegetative propagation (micropropagation by organogenesis or somatic embryogenesis), especially of interesting genotypes, and (2) improving plant quality, for which optimized micropropagation methods are also essential.

Induction of mutations through several methodologies may provide improved genotypes, including the variation generated in vegetative propagation, especially in micropropagation. Nevertheless, these last types of changes are normally undesirable, since they lead to variant phenotypes –somaclonal variants – which usually decrease fruit production, generating important commercial losses.

Banana Somaclonal Variation

Somaclonal variation can be defined, in a wide sense, as the occurrence of molecular changes during plant vegetative propagation. Frequently, those changes are phenotypically manifested. The heritability, mitotic, and/or meiotic of the change depends on the type of molecular alteration, which can be of two main types:

1. Genomic, i.e., in the DNA nucleotide sequence. These changes may involve from a single nucleotide to wide chromosomic rearrangements or increments and consequently are highly heritable. These changes can be produced randomly, with a trend to happen in more labile regions of the genome, or associated with certain regulation levels and, consequently, in more specific DNA regions. These last types of alterations, such as those due to the activity of genetic transposable elements, are obviously more reversible.
2. Epigenomic, which normally involves chromatin alterations without directly affecting the deoxynucleotide sequence, but frequently altering gene transcrip-

tion or transposable elements activity, and in this last case ultimatelly affecting DNA sequence (Gao et al. 2007, Gaut and Ross-Ibarra 2008). Transposable elements are usually activated by their hypomethylation (Kaeppler et al. 2000). The epigenomic changes are usually regulated by subcellular machinery and are more reversible, especially after meiosis, than genomic changes randomly acquired. Epigenetic changes occur along both aging (e.g., of the in vivo or in vitro cultures) and development (e.g., multiplication through mitosis or de-differentiation and re-differentiation in an adventitious context, causing cell reprograming among different cell cycles).

There are two main causes of such molecular alterations:

1. Breakup of a preexisting chimerism, which is a different composition of the genome and/or epigenome in different cells of mother plants, thus leading to a different proportion and distribution of those distinct cells in the regenerants.
2. Induction by the stress caused by the environment surrounding the cells leading to the regenerants. Stress may affect genome and/or epigenome in two ways: (a) cell division acceleration may induce DNA replication mistakes or error in the epigenetic status maintenance, which tends to affect the more labile chromatin regions, and (b) the regulated plasticity of the (epi)genome involves the presence of specific chromatin regions prepared by evolution to be strategically modified as a response to stresses. In this last case, the phenotypic consequence under controlled conditions may be different to that which would be expected under natural stressing environment, and the degree of reversibility of the modification could also vary due to strong artificial and repetitive stress characterizing tissue culture.

The appearance of more frequent concrete phenotypic variants –also called somaclones when vegetative propagation is performed–, such as dwarf types of banana and plantain, suggests the existence of chromatin regions more susceptible to change. Thus, dwarfism is the most common undesired somaclonal variation in *Musa*, and it has been reported to represent approximately 80% of the total variants (Israeli et al. 1996). The bananas from the Cavendish group (AAA) are shown to be more prone to express this abnormality (Bairu et al. 2008).

In several banana and plantain cultivars, Oh et al. (2007) found six genomic regions more susceptible to be genetically changed by organogenic micropropagation. One of the variable regions codified expansin 1, an enzyme involved in cell expansion. This may explain the frequency of dwarf plants appearing among variants. Another labile region resulted highly variable, but also reversible. This may be due to a chimerism breakup, but also to epigenetic mechanisms. In fact, the change in this fragment and its reversibility can be explained on the basis of extra-genomic, epigenetic repair mechanisms, such as those involving RNA intervention. Furthermore, such region also has epigenetic variability (DNA methylation). Dwarf individuals appear hypermethylated, compared to the normal phenotype, in certain DNA cytosines, correlating changes in sequence. In preliminary results, we detected higher hypermethylation in certain sequence contexts for banana dwarf regenerants

(in preparation). In fact, epigenetic changes such as DNA methylation modifications could be the leading factor of somaclonal variation in many cases (Wang and Wang 2012). The frequency of concrete changes supports the idea of a post-adaptive base emerging under stresses.

The starting material for propagation (named explant in tissue culture) can be more or less susceptible to those stresses, depending on its age or position in the plant. The stress can also be caused by handling and/or culture conditions. The regulation by the cell of epigenetic alterations may facilitate subsequent concrete genome reorganizations and, considered together, the occurrence of other DNA sequence mutations in those genomic regions, becoming constitutively more labile or more exposed. Thus, chromatin remodeling, as a plastic response of plant to manage stress, is a cell-controlled effect that may facilitate specific genome changes, but probably not so random. Several studies pointed out that the banana (epi)genome might became unstable during increased multiplication cycles, resulting in higher somaclone indexes.

Benzylaminopurine as a Hit for Somaclonal Variation

Among culture conditions, the more important factor in causing stress is exposure to exogenous plant growth regulators (PGRs). Prolonged exposure to PGRs may lead to genetic and epigenetic changes (Vázquez and Linacero 2010). Different PGRs affect genomic chromatin remodeling (references in Miguel and Marum 2011) including DNA methylation (Lo Schiavo et al. 1989).

Different PGRs are used to induce mitosis in plant tissues. A class of PGRs which induces mitosis is cytokinins, which naturally occur in plants as follows:

1. Free bases (active forms)
2. Bases conjugated with the following molecules:
   (a) Nucleosides (ribosides): less active forms (Yamada et al. 2001; Lomin et al. 2015).
   (b) Glycosides (O- and N-glycosides): intermediates of detoxification pathways or ireversible deactivators of free forms (Blagoeva et al. 2004; Sakakibara 2006; Bairu et al. 2011). Nevertheless, O-glucosidated cytokinins may have some activity at physiological levels (McGaw and Hobgan 1985) or can be reversibly deglycosylated by β-glucosidase action (Brzobohatý et al. 1994; Falk and Rask 1995; Kristoffersen et al. 2000), and therefore they might act as inactive storage forms of this type of PGRs.
   (c) Nucleotides (van Staden and Crouch 1996): inactive forms of storage. These conjugated forms of cytokinins conform pools that can be rapidly activated when needed and thus contribute to control the levels of active cytokinins (Kiran et al. 2012).

Interestingly, inactive storage forms are much more abundant compared to the free bases (Kiba et al. 2013; Miyawaki et al. 2006; Svačinová et al. 2012; Takei et al. 2004). This fact indicates that the concentration of active cytokinins is tightly controlled to prevent unregulated signaling. This regulation is achieved by coordination of the enzymes involved in biosynthesis, modification (e.g., conjugation) and degradation (e.g., oxidation) of cytokinins. The occurrence, distribution and variation of individual cytokinins depend on plant species, tissue and developmental stage (Sakakibara 2006).

Cytokinins are also the main PGRs applied in banana micropropagation. Specifically, 6-benzylaminopurine (BAP), also named bencyladenine (BA), as well as its derivatives, are active and readily available substances which stimulate growth and metabolism in plants. BA is a naturally occurring cytokinin in very low amounts and is also chemically synthesized for commercial purposes (van Staden and Crouch 1996). BA and its derivatives are commonly used in plant biotechnology (Doležal et al. 2007) including micropropagation (George et al. 2008). In different cultivars of bananas, BA has been shown to induce shoot tip multiplication, and to stimulate growth of axillary and adventitious buds as well as foliar development of shoot tip cultures (Jafari et al. 2011).

BA is a very stable molecule but it is rapidly metabolized by plant cells. In fact, it is known that cytokinin oxidases exert very low activity against BA as compared to other cytokinins (Brzobohatý et al. 1994). On the other hand, BA might be conjugated with other compounds.

Studies in carrot and *Arabidopsis* have shown that exogenous high concentrations of BA are able to induce programmed cell death (PCD) in plant cultured cells by accelerating leaf senescence (Carimi et al. 2004), whereas physiological concentrations result into a delay in leaf senescence (Wang et al. 2019). PCD is defined as the genetically determined suicide of individual cells that occurs during normal development but also as a response to stressful conditions, i.e., it may be also exogenously induced (Kunikowska et al. 2013). This observation corroborates that stressful conditions induced by high concentrations of BA accelerate defense mechanisms and/or results in abnormal development.

Cytokinins act together with other PGRs to increase the rate of cell division and preferentially in genetically abnormal cells (Bayliss 1980). Random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) genetic markers did not detect somaclonal variation in banana from group AAB when tissues were exposed to high levels (up to 10 mg L^{-1}) of exogenous cytokinins (BA or kinetin) (Venkatachalam et al. 2007). Nevertheless, BA, especially when used at higher concentrations (such as 30 μM), has been shown to be a source of phenotypic and/or genotypic (even at chromosomic level) somaclonal variation in several species (references in Bairu et al. 2011), including banana (*Musa acuminata* or ploidy derivatives) (Giménez et al. 2001; Martin et al. 2006; Bairu et al. 2006). Not only high levels of BA but also those of the cytokinin topolin affected DNA sequence (Aremu et al. 2013). BA was found to show more undesired responses than its derivatives, the topolins, resulting in a higher abnormality index in, for example, both cv. Grand Naine and cv. Williams (Bairu et al. 2008). In these studies, there was a significant

increase in phenotype variation rate between the third and seventh multiplication cycles.

What is more, independently of the BA concentration applied, increased multiplication cycles results in a higher variation rate in banana (Bairu et al. 2006). Also, the number of subcultures with cytokinins in this crop was shown to affect not only phenotype but DNA sequence (Reuveni and Israeli 1990; Rodrigues et al. 1998; Muhammad and Othman 2005; Martin et al. 2006; Bairu et al. 2006; Ray et al. 2006; Sheidai et al. 2008).

Such increases in phenotype and genome variation might occur by the accumulation of conjugated BA over time, resulting in endogenous concentrations that are in some way stressful for plant development without necessarily generating a higher multiplication rate in the shoot proliferation phase of micropropagation. In fact, results with banana have shown that uptake of BA was linear throughout the culture period in *Musa* (Blakesley 1991; Blakesley and Constantine 1992) and that accumulation in tissues of BA-glucosides was 2.88-fold higher than free BA (Blakesley and Constantine 1992). In micropropagated *Spathiphyllum floribundum*, the main derivative of BA is BA-glucoside, which accumulates at the plant base and might in turn release the BA free form later along time resulting in different problems during greenhouse acclimatization, such as heterogeneity in growth and inhibition of rooting (Werbrouck et al. 1995). The balance between catabolism and storing, for example, by means of reversible conjugation, plays a role in the mean-term effect of PGRs. However, we still lack concrete data to confirm that the accumulation of BA-glucosides or BA released from their derivatives generates a major stress culminating in the somaclonal variation in *Musa.*

Apart from effects on genomes, although there is not a direct study in *Musa,* there are reports that support the hypothesis of an effect of certain DNA hypomethylation changes in cellular active cytokinin levels (Masuta et al. 1995; Tanaka et al. 1997; Li et al. 2008), which can be related to the catabolism of such PGRs (Li et al. 2013). Conversely, a possible effect of cytokinins in DNA methylation increases could explain the hypermethylation that we observed in dwarf somaclones of banana (in preparation). In fact, it has been postulated that transient dwarfism can be derived directly from epigenetic modifications, which may be due to a carryover effect of PGRs (Nwauzoma and Jaja 2013).

Somaclonal Variation and Other Molecules with Cytokinin Activity Different from Benzylaminopurine

BA is currently by far the most applied cytokinin in commercial banana micropropagation. This is mainly due to costs, effectivity and availability. However, studies using different cytokinins or their derivatives are continually being conducted in order to get suitable commercial application information. Thidiazuron, a molecule with strong cytokinin activity, did not strongly affect genetic or phenotypic stability,

nor did meta-topolin or its riboside in *Musa* spp. (Bairu et al. 2008; Noceda et al. 2012; Bhalang et al. 2018). Nevertheless, thidiazuron led to aberrant in vitro shoots in plantain (Roels et al. 2005) and low success rate was obtained with this molecule, so that only few attempts were made with it.

Another cytokinin types are the topolins. They, and especially meta-topolin, have been employed for culture initiation, protocol optimization, and counteracting various in vitro-induced physiological disorders in many species (Aremu et al. 2017). In Cavendish bananas, meta-topolin riboside was found to produce a significantly larger number of shoots compared to BA and free meta-topolin (Bairu et al. 2008), but all tested cytokinins showed similar index values for somaclonal variation (Bairu et al. 2008).

Novel topolin derivatives are being synthesized and evaluated on plant growth. Szüčová et al. (2009) reported an array based on synthetic hydroxy- and methoxy-BA N9-derivatives. Tetrahydropyran-2-yl and tetrahydrofuran-2-yl groups can protect their N-9-conjugated cytokinin, and some of their derivatives have shown to lead to a gradual conversion to the free active base and increased transport rates in *Arabidopsis* vasculature (Podlešáková et al. 2012). These phenomena resulted in a generally positive effect on plant vigor without the rooting inhibition usually observed when conventional cytokinins are exogenously applied, which was also observed with a N9-tetrahydropyranyl derivative applied in *Ulmus* micropropagation (reference in Podlešáková et al. 2012).

Specifically in banana cv. Williams, among different cytokinins tested, another tetrahydropyranyl derivative synthesized as described by Szüčová et al. (2009) showed several advantages over other cytokinins including BA, such as higher multiplication and rooting rates (Aremu et al. 2012a, b). Also, high concentrations of the molecule showed the lowest somaclonal variation on the basis of ISSR molecular markers when compared with meta-topolin and BA (Aremu et al. 2013).

Conclusion

Banana micropropagation is a very important technique for this crop, especially to diminish several diseases affecting its productivity. However, a tight control of the culture conditions and multiplication cycles is needed. Cytokinin accumulation may interact with chromatin at genetic and epigenetic levels. Thus, for practical purposes, it is important to note that the concentration of BA or its derivatives in culture medium, as well as time (i.e., duration of each multiplication cycle and number of multiplication cycles) and form (i.e., use of liquid or semisolid culture medium) of exposure to such PGR, should be taken into account during the micropropagation program in order to produce high quality plants with low occurrence of somaclonal variation. In addition, different types of cytokinins less prone to accumulation along subcultures and/or glycosylation as tetrahydropyran-2-yl-N9-derivatives are expected to reach commercial production scale allowing their commercial use.

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Chapter 5
Somatic Embryogenesis for Clonal Propagation and Associated Molecular Studies in Cacao (*Theobroma cacao L.*)

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Abstract Cacao (*Theobroma cacao* L.) cultivation faces several acute problems that reduce its productivity and competitiveness. Production inefficiency of seedlings for replanting remains an issue. Tissue culture is expected to provide pragmatic solutions. Establishment of a reliable, reproducible, and efficient in vitro plant regeneration system is a vital prerequisite for biotechnological application of crop improvement programs. Numerous types of explants and protocols for micropropagation have been studied and used for cacao, and somatic embryogenesis is the main system used to clonally propagate cacao. Although effective somatic embryo production is now possible, the limited frequency of somatic embryos' conversion to ex vitro seedlings still prevents the large-scale clonal propagation of cacao. Most papers in the literature have focused on testing the influence of different types and combinations of plant growth regulators with the aim of improving regeneration and the multiplication stage of some cultivars. Genotype is one of the most influential factors on the in vitro response of cacao. Despite this, no successful universal protocol has yet been developed for multiple genotypes, limiting the usefulness of current protocols to commercial biotechnology labs. This review summarizes current knowledge of how far in vitro plant regeneration systems in cacao have developed. This could help researchers and scientists to improve protocols to a wider range of genotypes as well as collecting, conserving, and breeding cacao to increase productivity.

Keywords *Theobroma cacao L.* · Somatic embryogenesis · Cell and tissue culture · Molecular mechanisms

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Introduction

Cacao, designated as *Theobroma cacao* by Carolus Linnaeus the eighteenth century botanist, is an important Neotropical perennial crop, on which the thriving global chocolate industry is based. Among the 26 species of the genus, *T. cacao* is the only plant cultivated on a large scale to produce chocolate. *Theobroma cacao* diverged from its most recent common ancestor 9.9 million years ago (Ma) (7.7–12.9 [95%HPD]), and the genera *Theobroma* occurred from 12.7 Ma (11.6–14.9 [95%HPD]) and thus coincided with Andean uplift from the mid-Miocene (Richardson et al. 2015). Although the term "cocoa" is generally used for the plant and its products, until the cacao seeds, commonly known as "beans", are harvested, fermented, and dried, then the product is known as cocoa. Beans are shelled and roasted and then ground to form a paste known as "liquor". Some cocoa liquor is pressed to extract the fat, known as cocoa butter. Cocoa liquor and butter are usually combined with sugar, milk, and other ingredients to form chocolate. In addition to its use as food, cocoa butter is also used in very small quantities in pharmaceutical and cosmetic products. "Cacao" was the Aztec word for chocolate, and *Theobroma* means "Food of the Gods," in keeping with the Aztecs' regard for the drink they made from cacao seeds. According to Aztec mythology, the cacao tree was brought to earth by the god, Quetzalcoatl, who planted it in Southern Mexico and in the Yucatan peninsula (Badrie et al. 2013).

In recent years, cacao bean production has been steadily declining while demand in local and international markets is growing. Aging trees, increasing exposure to biotic and abiotic stress, insufficient quality of young plants, and lack of recent elite plant material are several causal factors. Thus, the renewal of aging plantations and enhancing the quality of cacao by planting elite plant material are required to improve productivity. The cacao crop is grown with an approximate planting density of 1100 trees per hectare, and it has been estimated that with a replanting rate of 10%, there is an annual requirement for one billion units. Therefore, an efficient propagation method for cacao is essential to accelerate breeding programs and to avoid production shortages in the future.

In general, the use of sexual seeds, rooted cuttings, grafting (top and side), budding, and marcotting are the propagation methods mostly used. Traditionally, cocoa propagation has been done via seeds, because it is the simplest and most economical way for farmers. Given that *T. cocoa* reproduces naturally by cross pollination, sowing materials from seeds that generally exhibit a highly heterogeneous genetic background means that the agronomic yield of crops is highly variable. A hybrid seed is produced by pollination, in a controlled manner, of the flowers from selected clones. Hybrids produced in this manner have shown great precocity in fruiting and vigorous plant development, so their use in plantations is advised, given that their degree of variability is within acceptance limits. However, it is an expensive method, considering the need of intensive trained laborers.

Asexual or vegetative propagation plays an important role in faithfully reproducing the desirable characteristics of a selected tree or group of trees. Using rooted

cuttings is an easy technique, with a high multiplication rate, and free from graft incompatibility and root stock suckering and has low production cost, but this method requires large-scale projects, high investments in facilities, equipment, clonal gardens, and suppliers. Thus, intensive labor is required, with strict sanitation needed in the clonal garden before harvesting the scion sticks (with four to five buds per graft); during and after the rooting process, the lack of the taproots may arrest plant growth and yield during the dry season, especially in shallow soils, plus risk of disease infection by soil-borne pathogens in susceptible clones. Cloning propagation through grafting is quick-starting and an easy and simple technique and has low to medium production costs, and clones may be quickly propagated due to fast growth rate. This process and the techniques required can be performed using many different types of rootstocks (seedlings, stems, old tree trunks, high branches). However, it has constraints, including those mentioned above, and the bushy growth pattern is more pronounced, which is considered an undesirable feature.

Finally, marcotting or air layering is a vegetative propagation method that was developed to promote the formation of roots on attached branches of many species (Hartmann et al. 2002). This method has high production cost and slow rate of multiplication and is inappropriate for large-scale projects (Sena et al. 2015). In general, the vegetative propagation systems have low rate of propagation and undesirable morphological features observed in some propagules, which often lack normal dimorphic nature and display bush-like growth with a fibrous root system. Therefore, maintenance of such material is a more labor-intensive process and requires skilled workers (Traore et al. 2003).

There are a considerable number of improved genotypes, but one of the major limitations to exploiting this germplasm is the lack of methods of massive cloning of selected plants that are efficient from both the economic and the agronomic point of view. In this sense, modern techniques of propagation and massive multiplication as somatic embryogenesis can improve the production of plant material. As with many other woody species, somatic embryogenesis has been found to be a powerful tool for mass clonal propagation of cacao. In the last 40 years, cacao research in vegetative propagation through somatic embryogenesis has gained much prominence. More recently, somatic embryogenesis has been investigated under laboratory and field conditions as a feasible in vitro clonal propagation method for commercial production of cacao plants.

The main advantages of tissue culture methods include the possibility of rapidly generating asexually propagated, uniform plants with highly valued genetic traits. Additionally, for cacao, somatic embryogenesis offers a system for the clonal production of orthotropic plants with normal dimorphic architecture and taproot formation. The production and testing of disease-free materials and germplasm conservation via cryopreservation are other important potential contributions of plant tissue culture to the improvement, preservation, and distribution of cacao germplasm. In *T. cacao*, two techniques of in vitro micropropagation have been reported as direct and indirect somatic embryogenesis. Indirect somatic embryogenesis requires the additional step of cell dedifferentiation, unlike direct somatic embryogenesis, which does not require this step. This review aims to provide a

comprehensive summary of the advances to date in tissue culture and the associated biotechnological approaches applied to cacao, a historically recalcitrant species. Through a critical analysis of past notable achievements, we hope to assist researchers to refine approaches for improving the research of *T. cacao*.

Somatic Embryogenesis in Cacao: History

Somatic embryogenesis (SE) was described by Williams and Maheswaran (1986) as "the process via which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes." SE is a common process present in nature that occurs during both embryonic and post-embryonic plant development (Smertenko and Bozhkov 2014). In vitro SE can also be induced in vegetative explants or cells following treatment with plant growth regulators (PGR) or stresses such as osmotic shock, dehydration, water stress, ions of heavy metals, changes in pH of the culture medium, cold treatments and thermal shock, hypoxia, antibiotics, ultraviolet radiation, and chemical or mechanical treatments. Some studies have addressed correspondences and differences between zygotic and somatic embryogenesis and suggest that the patterning and specification events are quite similar except for a lack of the suspensor and dormancy in in vitro cultured somatic embryos (Hand et al. 2016). There are two different ways of inducing SE, direct somatic embryogenesis (DSE) and indirect somatic embryogenesis (ISE). In DSE, somatic embryos can be directly induced from the explant under certain conditions without any intermediate callus stage. Conversely, ISE occurs via an intermediate callus stage and has been observed in most species. Distinguishing between DSE and ISE can be difficult as both processes have been observed to occur simultaneously in the same tissue culture conditions. In contrast to primary somatic embryogenesis (PSE) induced from explant cells, secondary somatic embryogenesis (SSE) is the phenomenon whereby new somatic embryos are induced through existing somatic embryos (Guan et al. 2016).

ISE is a multistep regeneration process beginning with a proembryogenic mass (PEM), followed by somatic embryo formation, maturation, and conversion. A key point in ISE is the production of PEM, which consists of proliferating embryogenic cells at an intermediate state between callus and somatic embryo and a relatively disorganized structure. Auxin is required for the proliferation of PEMs but inhibits the development of PEMs into somatic embryos (Yang and Zhang 2010). Therefore, the most important step in vegetative cells that undergo SE must be to first gain the "embryogenic" state.

Studies on SE in cacao have been performed for over 40 years (Fig. 5.1). In the late 1980s and 1990s, considerable efforts were made in tissue culture to produce cocoa plants by SE (Table 5.1). The initiation of somatic embryo in cacao by using zygotic embryo explant was presented for the first time by E.B. Esan at the V International Cocoa Research Conference at Ibadan, held in Nigeria in September 1975, and the procedures were published in 1977 (Esan 1977). The phenomenon

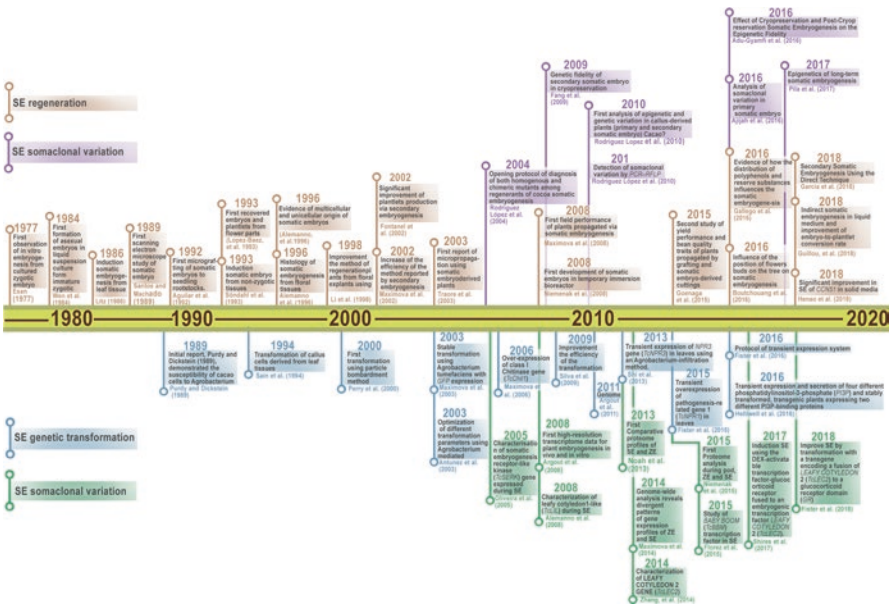

Fig. 5.1 Chronology of research in Theobroma cacao somatic embryogenesis (SE) and biotechnological interventions

was reported independently by Pence et al. (1979) and subsequently repeated by other researchers using immature zygotic embryos when inducing somatic embryos (Pence et al. 1979, Wang and Janick 1984). The compounds 2,4 dichlorophenoxyacetic acid (2,4 D) and 1-naphthaleneacetic acid (NAA) were found to be the most effective natural and synthetic auxins, respectively. Coconut water was added; nevertheless, plantlet regeneration could not be consistently obtained (Pence et al. 1980). Since then, somatic embryo induction on mother plant-derived tissues of several cacao genotypes was achieved in several laboratories in the world.

Kononowicz et al. (1984) demonstrated the dependence of the frequency of SE on concentrations of 2,4 D in the medium when cotyledons of cocoa are used as explants. The capacity to regenerate via SE may also be determined by the developmental stages of the zygotic embryo (Esan 1985). Subsequent studies on SE from culture of immature zygotic embryos reported direct and indirect pathways for SE differentiation (Adu-Ampomah et al. 1988; Duhem et al. 1989), while Wen et al. (1984) used liquid and semisolid medium for the somatic embryo induction. However, while a step forward, these methods did not produce clonal material that replicated the genotype of the source tree, and thus, genetic gain was lost. Afterward, research energies were directed toward the development of tissue culture systems from somatic tissues including leaves (Litz 1986) and nucellus (Chatelet et al. 1992; Figueira and Janick 1993; Esan 1992).

The first report on successful production of somatic embryos from nonsexual tissues and the conversion of these embryos into plantlets were provided by

Table 5.1 A list of granted patents, in chronological order, on *T. cacao* somatic embryogenesis (SE), prior to October 2019

Publication Number	Publication Year	Title	Inventors	Applicant(s)
US 4301619 A	1981	Plant tissue produced by nonagricultural proliferation of cacao embryos	Janick, Jules; Pence, Valerie	Purdue Research Foundation
US 4291498 A	1981	Method for production of mature asexual cacao embryos and product thereof	Janick, Jules; Hasegawa, Paul; Pence, Valerie	Purdue Research Foundation
US 4545147 A	1985	Asexual embryogenesis of callus from *Theobroma cacao* L.	Janick, Jules; Kononowicz, Halina	Purdue Research Foundation
US 5312801 A	1994	Somatic embryogenesis and plant regeneration of cacao	Sondahl, Maro; Chen, Zhenghua; Sereduk, Thomas; Bellato, Claudia; Liu, Si-Jiu; Bragin, Alvina	DNA Plant Technology Corporation; Hershey Foods Corporation
US 6150587 A	2000	Method and tissue culture media for inducing somatic embryogenesis, Agrobacterium-mediated transformation, and efficient regeneration of cacao plants	Guiltinan, Mark; Li, Zhijian; Traore, Abdoulaye; Maximova, Siela.	Penn State Research Foundation
US8921087 B2	2014	Cocoa somatic embryogenesis	Florin, Bruno Jean-Marie; Masseret, Bernard; Vachet, Caroline Denise Monique	Nestec SA
AU2014353082 B2	2017	Production of plants using somatic embryogenesis	García Rojas, Claudia Yanet; Días, Cristiano Villela; Marelli, Jean-Philippe	Mars, Incorporated

Lopez-Baez et al. (1993), Sondahl et al. (1993), and then Alemanno et al. (1996a, b, 1997), all using floral explants, including petals and staminodes. The advances made in the system of propagation through SE were patented first by Janick and Pence (1981) (Table 5.1). The patent included three fundamental steps: (1) proliferation of embryos by asexual embryogenesis through in vitro culture using a defined media, (2) the growth of the embryos in vitro in a media that will prevent premature germination, and (3) harvest of the in vitro grown cotyledonary tissue. Later, the same inventors patented a method for growth, development, and maturation of cocoa embryos from the precursor stage (Janick et al. 1981), and 4 years later, in 1985 the process of SE from callus was patented again (Janick and

Kononowicz 1985). In 1994, based on the work carried out in 1993, when nonsexual tissues such as the nucella were used as a source of explants, the process was again patented by Sondahl et al. (1994).

Speaking of efficiency, these propagation systems, while partially successful in producing somatic embryos, were still of limited usefulness, being applicable to only a few genotypes and suffering from low conversion rates. Subsequently, Li et al. (1998) reported a more efficient SE method capable of propagating a wide variety of cacao genotypes. Unlike the previous reports, primary somatic embryos were produced from floral explants at high frequencies using thidiazuron (TDZ) and 2,4 D. Li et al. (1998) achieved better somatic responses from many cacao genotypes using DKW complex salt (Driver and Kuniyuki 1984) than Lopez-Baez et al. (1993) did using Murashige and Skoog complex salt with the same explants (petals and staminodes). DKW complex salt provides a significantly higher concentration of calcium, sulfur, and magnesium compared to MS complex salt (Murashige and Skoog 1962).

Maximova et al. (2002) realized modifications of SCG medium reported by Li et al. (1998) included substitution of 6-furfurylaminopurine (Kin) with 6-benzyladenine (BA) and exclusion of the coconut water. The development of a secondary embryogenesis system utilizing primary somatic embryo cotyledon explants was achieved, which resulted in up to a 30-fold increase in somatic embryo production compared to PSE. These modifications made in the culture media were also patented by Guiltinan et al. (2000). The novel culture media of the invention included primary callus growth medium, secondary callus growth medium, embryo development medium, primary embryo conversion medium, secondary embryo conversion medium, and plant regeneration medium. In addition, the patent included the improvement of the cocoa processing tissues with *Agrobacterium* vectors and regenerating transgenic plants. The invention further relates to transgenic cocoa somatic embryos and plants obtained according to the methods of the invention. At the same time, Fontanel et al. (2002) achieved, from the work done by Lopez-Baez, the induction of primary and secondary embryogenesis. The main modifications were DKW macro-micronutrients in all mediums (induction, expression, maturation) without amino acids, the absence of PGRs in the expression medium, and without AIA and AIB in the maturation medium. All modifications allowed successful multiplication of embryogenic callus with a significant production of in vitro cocoa plants (500/plants/month/employee). From the culture media reported by Maximova et al. (2002) and Fontanel et al. (2002), numerous modifications have been made in each of the stages of differentiation and development of somatic embryos.

The progress in cacao SE has enabled the production of large numbers of aseptically grown cacao plants from flower staminodes and petal cultures. Traore et al. (2003) continued using apical and nodal stem explants from primary somatic embryo-derived plants for production of axillary shoots. Additionally, the authors established a successful protocol for rooting of micro-cuttings in vitro for improving protocol propagation by SE. From the studies, it has been found that the embryogenic response is genotype dependent, and the protocols are not applicable to all

genotypes (Tan & Furtek 2003). Cocoa remains a generally recalcitrant species, and the result has been unsatisfactory for commercial production of elite material. Recalcitrance is defined as the inability of plant cells, tissues, and organs to response readily to tissue culture. One of the factors often considered as a component of in vitro recalcitrance is a high phenolic content and oxidation of these compounds. In this sense, Alemanno et al. (2003) studied by histochemical means the composition and location of phenolic compounds in cocoa flowers and their evolution throughout the SE process. Later, Quainoo et al. (2008) found that SE was effective in eliminating the cocoa swollen shoot virus (CSSV) from infected cocoa trees producing disease-free clonal stock materials. The CSSV was drastically reduced from primary to secondary embryogenesis. In the same year, for a scaling-up step Niemenak et al. (2008) improved the efficiency of in vitro multiplication of cacao somatic embryos in liquid media. One-minute immersion every 6 h per day increased the production of somatic embryos approximately 13-fold after 3 months of culture. This culture system also increased the conversion of somatic embryos into torpedo-shaped forms. Likewise, Minyaka et al. (2008), with modification in $MgSO_4$ (24 mM) and K_2SO_4 (71.568 mM) concentrations in the culture media, managed to get direct somatic embryos on staminodes and petals.

The first research about field performance of plant propagated via SE was realized by Maximova et al. (2008). At Union Vale Estate, Saint Lucia, nine genotypes were planted in a field. At 4.5 years, counted from planting in the field, there were no major differences in all growth parameters (stem diameter, stem height, length of the longest jorquette branch, number of jorquette branches, first flowering and fruiting), among the propagation methods evaluated (SSE, micropropagation, and orthotropic rooted cutting), with exception of the orthotropic rooted cuttings. Trees grown from seeds were slightly taller than trees propagated by the other methods. Trees propagated as orthotropic rooted cuttings exhibited smaller average stem diameters, shorter stem heights to the jorquette, and shorter jorquette branches. The authors concluded that somatic embryo-derived plants demonstrated normal phenotypes in field conditions and have growth parameters like plants propagated by traditional methods. With the objective of enhancing the conversion of somatic embryos of cocoa into plantlets, Quainoo and Dwomo (2012) evaluated the effect of different modification in the concentration of Abscisic acid (ABA) in their embryo development (ED) medium. It was found that 0,0026 mg L^{-1} ABA was more effective in converting primary and secondary somatic embryos into plantlets, and the effects of ABA treatments on conversion of secondary somatic embryos into plantlets were superior to use of primary embryos.

In 2014, the process of propagation via SE was patented again, but this time the applicant was Nestec S. A and as inventors Florin, Bruno Jean-Marie; Masseret, Bernard; and Vachet, Caroline Denise Monique (Florin et al. 2014). This time, the indirect process by SE using explant material was patented which is subjected to (a) primary embryogenesis in the dark in a solid culture medium causing induction and expression to produce primary embryos; (b) secondary embryogenesis in which the primary embryos are treated in the dark in a solid or liquid culture medium to produce and multiply embryogenic callus, followed by treatment of the embryogenic

callus in the dark in a suitable liquid culture medium causing expression of the embryogenic callus to produce further new embryos; (c) pre-germination of the secondary embryos in a Petri dish on a solid medium, or in a bioreactor in a liquid medium, into pre-germinated secondary embryos at the cotyledonary stage; (d) ex vitro germination of the pre-germinated secondary embryos at the cotyledonary stage by sowing directly on a culture substrate in the greenhouse to produce the plantlets; and (e) development of the plantlets.

A second study of yield performance and bean quality traits of plants propagated by grafting and somatic embryo-derived cuttings was reported by Goenaga et al. (2015). In this study, 12 cacao clones were propagated by grafting and orthotropic rooted cuttings of somatic embryo derived at Corozal, Puerto Rico, and evaluated for 6 years of production under intensive management. It was found that propagation treatments had a significant effect on dry bean yield and pod index but not on number of pods produced. Average yield across varieties for both propagation treatments was 2087.9 $kg.ha^{-1}$ per year of dry beans. Dry bean yield of varieties propagated by grafting was 7% higher (2166.7 $kg.ha^{-1}$ per year) than those propagated by orthotropic rooted cuttings of somatic embryo-derived plants (2009.2 $kg.ha^{-1}$ per year). With few exceptions, flavor characteristics were not significantly affected by propagation treatments. The authors concluded that although there were significant differences between plant propagation treatments for some of the variables measured in this study, the use of SE was a viable propagation system for cacao. Succeeding with the aim of improving SE throughout a physiological approach, Boutchouang et al. (2016) evaluated the influence of the position of flower buds used as explants. Results obtained showed that callogenesis is induced on all explants independently of their origin, with an 80% average frequency. The frequency of embryogenesis was twofold higher in staminode-derived calluses from secondary plagiotropic fan branch and primary plagiotropic fan branch with respect to orthotropic main stem.

In order to understand the causes of lack of regeneration in some genotypes, Gallego et al. (2016) studied histologically the capacity of the somatic embryos to store different compounds. The study showed that, in somatic embryos of the regenerating variety, polyphenols were localized mainly in the periphery of the embryo (epidermal cells) and proteins were the main storage substance in the embryo expression medium, while the nonregenerating variety had a high presence of polysaccharides with random distribution of polyphenols at the end of the embryo induction step. In the same year, Garcia et al. (2016) reported modifications in the carbohydrate source and in PGRs for CCN51 and CCN10 protocol. The authors found that it is possible to achieve a high production of plants by SE, although the efficiency is highly genotype-dependent, and it is therefore necessary to optimize hormone balance and hormone type, as well as the explant type for each genotype. Also, through the use of SSE, it is possible to increase SE production at least tenfold, though the observed response variation between genotypes may reflect differences in endogenous and exogenously supplied hormones.

Subsequently, Bustamia and Werbrouck (2017) evaluated the induction and development of somatic embryos from staminode and petal explants of the ‘Sulawesi

2' clone using the protocol developed by Guiltinan and Maximova (2010) and the modified protocol of Fontanel et al. (2002). It was found that the IND medium from Fontanel et al. (2002) was able to induce a direct somatic embryogenic response on petal explants and ISE on staminode explants of 'Sulawesi 2'. Kouassi et al. (2017a, b) found that the auxins 2,4,5 trichlorophenoxyacetic acid (2,4,5 T) and 2,4 D in combination with KIN have a broad-spectrum action on SE induction. It was also demonstrated, for the first time ever, that it is possible to use 4-amino-3,5,6-trichloropicolinic acid (Picloram) and 2,4 D to induce callus and SE, respectively, in the elite cocoa genotypes. Also, the callus browning oxidation of the different genotypes was reduced, by two to three times, when the culture media were supplemented with various concentrations of $AgNO_3$. The addition of PVP (300 $mg.L^{-1}$) in the induction medium improved the rate of somatic embryos of the genotypes. The improvement of responses to SE in reducing the rate of browning of cultures will allow a mass production of the high-yield cocoa genotypes.

Most recently, Mars Inc. patented the ES production (AU 2014/353082 B2) in April of 2017 (García et al. 2017). This patent on the production of cacao plants claims micropropagation via direct SE. This method uses explants such as staminodes and petal base tissues for induction of primary embryos in a medium supplemented with benzylaminopurine (BAP). Subsequently, the epicotyl segments removed from primary embryos are placed in an induction medium containing BAP to induce direct secondary embryos, followed by further embryo development in a medium containing gibberellic acid. All the cultures are maintained in the light (photoperiod 16: 8 (light: dark)) at a temperature of 23–29 °C for a sufficient period to obtain embryos.

Clonal Propagation via Somatic Embryogenesis

SE in cacao is a tissue culture-based system starting from floral parts in which genetically identical embryos are formed. These embryos have the advantage of growing with the normal orthotropic-plagiotropic morphology of a seed-grown cacao plant, unlike those made from cuttings or grafting of plagiotropic shoots. This growth form may have important advantages over the plagiotropic plants in terms of normal tap root structure/function and lower pruning costs due to natural jourquette formation. SE has five events (induction, expression, maturation, and conversion into plants and development). The first event in SE is the induction phase, where cells acquire differentiated somatic embryogenic capacity by DSE or ISE. DSE occurs when minimal cell division precedes embryo formation, while in ISE, high amounts of callus proliferate before embryo formation. SE is only possible if the cells are competent and receive an appropriate inducing stimulus. Two types of inductive conditions have been recognized to permit differentiated cells to become undifferentiated competent cells: internal and/or external phytohormone or plant growth regulator (PGR) levels in tissue and stress factors, as osmotic shock substances exhibit different concentration changes (sucrose, polyethylene glycol, and

abscisic acid, among others), dehydration culture medium, water stress, ions of heavy metals, changes in pH of the culture medium, cold treatments and thermal shock, hypoxia, antibiotics, ultraviolet radiation, and chemical or mechanical treatments.

Among the first works of SE that contributed to the advancement of the propagation protocol and the techniques used to monitor the embryogenic development, it is important to highlight the study by Santos and Machado (1989). These authors cultured immature zygotic embryo with Zeatin (1 mg L^{-1}) and obtained somatic embryo over cotyledon surface-like small groups. With a scanning electron microscope, they could observe the developmental stages of embryos such as globular, early heart-shaped, torpedo stage and adult embryos (Table 5.2). In the search for options to increase efficiency in propagation by SE, outstanding research work was carried out by Aguilar et al. (1992) on micrografting techniques. In this study, the application of micrografting techniques allowed for the regeneration of complete plants in 10 months. The optimum age of the rootstock was found to be 3 weeks, a point at which secondary roots were beginning to develop. The best response of the micrograft occurred when the cotyledons were excised from the somatic embryos. However, the study was performed only for the genotype UF-613 and as rootstock IMC67 but had problems to achieve the conversion of somatic embryos in plants. Some subsequent studies were done using floral explants; however, unsatisfactory growth of somatic embryos forced the research community to search for different culture conditions.

Sondahl et al. (1993) studied the way to develop a method for producing somatic embryos from non-sexual explants like petals and nucellus tissue, their research team aimed for a regeneration rate of 4,3% for petals utilizing 2,21 mg.L^{-1} 2,4 D, 0,22 mg.L^{-1} KIN in the induction medium (IM) and 2% for nucellus utilizing 5 g. L^{-1} PVP, 40, 5% coconut water (CW), 60 mg.L^{-1} MES, 0,66 mg.L^{-1} 2,4 D. In the same year, Lopez-Baez et al. (1993) used all the tissues of the button flower (sepals, petals, staminodes y ovaries). The highest embryogenesis rate was obtained from explants cultured in 1 mg.L^{-1} 2,4 D and 0,25 mg.L^{-1} KIN for 21 days. In the maturation medium (MM) with 0,001 mg.L^{-1} BA after 28–35 days, they aimed for a ratio germination of 74%, conversion in plantlets of 66%, and an ex vitro transfer survival of 68%. However, the data collection for each of the seven genotypes was not done in the study and therefore did not represent a significant advance to the process. Figueira and Janick (1993) described an improved protocol for induction of nucellar embryony and for conversion of nucellar somatic embryos into seedlings. Furthermore, their results suggested that an elevated CO_2 concentration (20.000 mg. L^{-1}) benefits embryo conversion, and the preculture of somatic embryos in liquid medium is beneficial to somatic embryo development. A total of five culture media were formulated of development of the embryos: induction medium, expression medium, maintenance medium, maturation medium, and germination/conversion medium (Table 5.2). All media had combinations of different growth regulators, and all used the basal MS medium except for the germination medium used by WPM (Lloyd and McCown 1980).

Table 5.2 Somatic embryogenesis in *T. cacao* protocols: genotype, type of explant, basal media, different components and PGRs, culture conditions, and references

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
ICSLxSIAL244	Zygotic embryo	MS + 0,75 or 1 mg.L^{-1} Zeatin +2 g.L^{-1} Gelrite®	I: 16:8 light: dark (40,5 µmol m^{-2} s^{-1}) (30 days). 3000 lux T: 28 °C	Santos and Machado (1989)
UF-613, IMC67 (rootstocks)	Zygotic embryo (cotyledons from seeds)	*Induction medium* MS + 3 mg.L^{-1} BA +1 mg. L^{-1} NAA + 30 g.L^{-1} sucrose +0,8 g.L^{-1} Difco® Bacto Agar	I: Dark (90 days) T: 27 ± 2 °C	Aguilar et al. (1992)
		Expression medium MS + 500 mg.L^{-1} HC + 50 g.L^{-1} Sucrose	I: 16:8 light: dark (30 days). 1700 lux T: 27 °C	
	Young Petals, nucellus	*Young Petals Callus induction medium* MS + 2,21 mg.L^{-1} 2,4 D + 0,215 mg.L^{-1} KIN +30 g.L^{-1} sucrose +0,8 g. L^{-1} Difco® Bacto Agar	I: Dark (28 days)	Sondahl et al. (1993)
		Regeneration medium ½MS + 1126 mg.L^{-1} BA +0,2% AC + 30 g.L^{-1} sucrose +0,8 g.L^{-1} Difco® Bacto Agar	I: Dark (60–120 days) Periodic subcultures	
		Nucellus explants Callus induction medium Kao's medium +5 g.L^{-1} PVP 40 + 5% CW + 600 mg.L^{-1} MES + 0,66 mg.L^{-1} 2,4 D + 30 g.L^{-1} sucrose +0,8 g.L^{-1} Difco® Bacto Agar	I: Dark (28 days)	
EET48, EET64, EET94, EET228, CC260	Peduncle, sepals, petals, staminodes, stamens, ovary	*MI: Induction basal medium* MS + 0,4 mg.L^{-1} L-leucine +0,4 mg.L^{-1} L-lysine +0,2 mg.L^{-1} L-tryptophan +0,4 mg.L^{-1} L-arginine +1 mg.L^{-1} 2,4-D + 0,25 mg.L^{-1} KIN +3 mg.L-1 glycine +50 ml/L CW + v40 g.L^{-1} sucrose +2 g.L^{-1} Gelrite®	I: Dark (21 days) T: 26–27 °C	Lopez-Baez et al. (1993)

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
		EM: Expression medium MS + 0,2 mg.L^{-1} L-leucine +0,2 mg.L^{-1} L-lysine +0,1 mg.L^{-1} L-tryptophan +0,2 mg.L^{-1} L-arginine +1 mg.L^{-1} glycine +100 ml/L CW + 40 g.L^{-1} sucrose +2 g.L^{-1} Gelrite®	I: Dark (42–56 days) T: 26–27 °C	
		MM: Maturation medium ½MS +0,4 mg. L^{-1} L-leucine +0,4 mg. L^{-1} L-lysine +0,2 mg. L^{-1} L-tryptophan +0,4 mg. L^{-1} L-arginine +0,05 mg. L^{-1} AIA + 0,05 mg.L^{-1} AIB + 0,02 mg.L^{-1} GA_3 + 0,5 mg.L^{-1} adenine sulfate +40 g.L^{-1} maltose +2 g.L^{-1} Gelrite®	I: 12:12 light: dark (7 µE m^{-2} s^{-1}) (28–35 days) T: 26–27 °C	
		GM: Germination medium ½MS + 0,4 mg. L^{-1} L-leucine +0,4 mg. L^{-1} L-lysine +0,2 mg. L^{-1} L-tryptophan +0,4 mg. L^{-1} L-arginine +0,01 mg.L-1 NAA + 0,02 mg L-1 GA_3 + 0,2 mg.L^{-1} 2iP (isopentenyl adenine) + 1 mg.L^{-1} ABA +0,5 mg.L^{-1} adenine sulfate +1 g.L^{-1} AC (activated charcoal) + 80 g. L^{-1} maltose ó 40 g.L^{-1} Glucose +3 g.L^{-1} Gelrite®	I: 12:12 light: dark (60 µE m^{-2} s^{-1}) (28–35 days) T: 30–31 °C light/25–26 °C dark	
		CM: Conversion medium ½MS + 0,4 mg. L^{-1} L-leucine +0,4 mg. L^{-1} L-lysine +0,2 mg. L^{-1} L-typtophan +0,4 mg. L^{-1} L-arginine +0.15 g. L^{-1} AC + 5 g.L^{-1} glucose +3 g.L^{-1} Gelrite®	I: 12:12 light: dark (60 µE m^{-2} s^{-1}) (42–56 days) T: 30–31 °C light/25–26 °C dark	
		Ex vitro adaptation Vermiculite/sand/Perlite (2:2:1), solution nutritive ¼ MS	I: 12:12 sunlight/ dark (28–42 days) H: 90–95% T: 30–31 °C light/25–26 °C dark	

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
BC46, SCA12 (Scavina 12)	Zygotic embryo and nucellus	*Induction medium* ½MS + 0,10 mg.L^{-1} thiamine HCl + 0,49 mg. L^{-1} pyridoxine HCL + 0,11 g.L^{-1} Myo-inositol +0,5 mg.L^{-1} nicotinic acid +2 mg.L^{-1} glycine +40 g.L^{-1} sucrose +10% CW + 0,88 mg.L^{-1} 2,4-D + 0,1 mg.L^{-1} 2iP + 0,5 g.L^{-1} malt extract +10.000 (0,2%) PVP + 0,5 g.L^{-1} HC	I: Dark (60 days) A: 100 rpm T: 26 °C	Figueira and Janick (1993)
		Development medium ½MS + 0,10 mg.L^{-1} thiamine HCl + 0,49 mg. L^{-1} pyridoxine HCL + 0,11 g.L^{-1} Myo-inositol +0,5 mg.L^{-1} nicotinic acid +2 mg.L^{-1} glycine +40 g.L^{-1} sucrose +10% CW + 0,5 mg.L^{-1} 2iP + 0,1 g.L^{-1} malt extract +10.000 (0,2%) PVP. pH = 5,3. Semi-solid medium	I: Light (60 days)	
		Maintenance medium ½MS + 0,10 mg.L^{-1} thiamine HCl + 0,49 mg. L^{-1} pyridoxine HCL + 0,11 g.L^{-1} Myo-inositol +0,5 mg.L^{-1} nicotinic acid +2 mg.L^{-1} glycine +1000 mg.L^{-1} CH + 30 g.L^{-1} sucrose +8 g.L^{-1} Agar Sigma	I: Light (60 days)	
		Maturation medium: ½MS + 42,63 mg.L^{-1} sorbitol +10 g.L^{-1} sucrose	- 15 days in orbital shaker (100 rpm)	
		Conversion medium: WPM + 22,86 mg.L^{-1} fructose	I: 16:8 light:dark (60 µmol m^{-2} s^{-1}) G: CO2 (20.000 ppm) HR: 50–70% 31 °C/24 °C (day/night) VHO cool white fluorescent	

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
Catongo, Laranga, Marina, Sca 6-1, Sca 6-2, Sca 6xICS 1 #1, Sca 6xICS 1 #3, TSH 1112, RB48, EET 400, Pentagonia F1, ICS 1, ICS 16, ICS 39, ICS 67, Pound 7, Tomate Ceplac, UF 613	Staminodes	*PCG: Primary callus growth* DKW basal salts +250 mg. L^{-1} glutamine +200 $mg.L^{-1}$ Myo-inositol +2 $mg.L^{-1}$ thiamine-HCL + 1 $mg.L^{-1}$ nicotinic acid +2 $mg.L^{-1}$ glycine +20 $g.L^{-1}$ glucose +1,98 $mg.L^{-1}$ 2,4-D + 0,005 $mg.L^{-1}$ TDZ + 2 $g.L^{-1}$ Phytagel	I: Dark (14 days) T: 25 °C	Li et al. (1998)
		SCG: Secondary callus growth WPM basal salts low (Lloyd and McCown, 1980, Sigma M-6774) + Gamborg's vitamin solution +20 $g.L^{-1}$ glucose +2 $mg.L^{-1}$ 2,4-D + 0,30 $mg.L^{-1}$ KIN +5% CW + 2,2 $g.L^{-1}$ Phytagel	I: Dark (14 days) T: 25 °C	
		ED: Embryo development DKW + 100 $mg.L^{-1}$ Myo-inositol +2 $mg.L^{-1}$ thiamin-HCl + 1 $mg.L^{-1}$ nicotinic acid +2 $mg.L^{-1}$ glycine +30 $g.L^{-1}$ sucrose +1 $g.L^{-1}$ glucose +2,0 $g.L^{-1}$ Phytagel	I: Dark (60 days) T: 25 °C	
		PR: Plant regeneration medium $\frac{1}{5}$ DKW + 100 $mg.L^{-1}$ Myo-inositol +2 $mg.L^{-1}$ thiamin-HCl + 1 $mg.L^{-1}$ nicotinic acid +2 $mg.L^{-1}$ glycine +10 $g.L^{-1}$ glucose +5 $g.L^{-1}$ sucrose +0,2 $g.L^{-1}$ KNO_3 + 1,7 $g.L^{-1}$ Phytagel	I: 16:8 light:dark (50 µmol m^{-2} s^{-1}) (14 days) T: 25 °C	

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
UVE 1, UVE 5, UVE 9, UVE 22, EEG 29, TSH 565, GU 143, GF 23, IFC 5, KER 1, IFC 705, NA 32, NA 79, Sca 6	Staminodes	*PCG: Primary callus growth* (Li et al. 1998) DKW basal salts +250 mg. L^{-1} glutamine +200 mg.L^{-1} Myo-inositol +2 mg.L^{-1} thiamine-HCL + 1 mg.L^{-1} nicotinic acid +2 mg.L^{-1} glycine +20 g.L^{-1} glucose +1,98 mg.L^{-1} 2,4-D + 0,005 mg.L^{-1} TDZ + 2 g.L^{-1} Phytagel	I: Dark (14 days) T: 25 °C	Maximova et al. (2002)
		SCG Secondary callus growth (Li et al. 1998 modified) WPM basal salts low (Lloyd and McCown, 1980, Sigma M-6774) + Gamborg's vitamin solution +20 g.L^{-1} glucose +0,53 mg.L^{-1} 2,4-D + 0,315 mg.L^{-1} BA +2,2 g.L^{-1} Phytagel	I: Dark (14 days) T: 25 °C	
		ED: Embryo development (Li et al. 1998) DKW + 100 mg.L^{-1} Myo-inositol +2 mg.L^{-1} thiamin-HCl + 1 mg.L^{-1} nicotinic acid +2 mg.L^{-1} glycine +30 g.L^{-1} sucrose +1 g.L^{-1} glucose +2,0 g.L^{-1} Phytagel	I: Dark (60 days) T: 25 °C	
18 genotypes	–	*INDI: Induction* DKW + DKW vitamins +1 mg.L^{-1} 2,4-D + 0,25 mg.L^{-1} KIN +30 g.L^{-1} glucose +3 g.L^{-1} Gelrite®	I: Dark (32 days) T: 25 °C	Fontanel et al. (2002)
		INDexp: Expression DKW + DKW vitamins +30 g.L^{-1} glucose +3 g.L^{-1} Gelrite®	I: Dark (32 days) T: 25 °C	
		CM2: Callus multiplication MS + DKW vitamins +1 mg.L^{-1} 2,4,5 T + 0,25 mg.L^{-1} Adenine +30 g.L^{-1} glucose +3 g.L^{-1} Gelrite®	I: Dark (63 to 112 days) T: 25 °C	

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
		EM2: Expression MS + DKW vitamins +40 $g.L^{-1}$ sucrose +3 $g.L^{-1}$ Gelrite®	I: Dark (14 × 21 days) T: 25 °C	
		MM6: Maturation medium ½MS macronutrients + DKW micronutrients + DKW vitamins +0,01 mg. L^{-1} NAA + 0,02 $mg.L^{-1}$ GA_3 + 1 $g.L^{-1}$AC + 40 g. L^{-1} glucose +3 $g.L^{-1}$ Gelrite®	I: 12:12 light:dark (60 µmol m^{-2} s^{-1}) (28–42 days) T: 25–30 °C	
		ENR6: In vitro development ½MS macronutrients + DKW micronutrients + DKW vitamins +10 $g.L^{-1}$ glucose +5,0 $g.L^{-1}$ sucrose +2 $g.L^{-1}$ Gelrite®.	I: 12:12 light:dark (60 µmol m^{-2} s^{-1}) (28–56 days) T: 25 °C	
Sca6	Staminodes and petals	*PCG: Primary callus growth* DKW basal salts +250 mg. L^{-1} glutamine +200 $mg.L^{-1}$ Myo-inositoL +20 $g.L^{-1}$ glucose +2 $mg.L^{-1}$ 2,4-D + 5 $µg.L^{-1}$ TDZ. liquid medium	I: Dark (14 days) T: 25 °C	Niemenak et al., (2008)
		SCG: Secondary callus growth WPM medium and vitamins +20 $g.L^{-1}$ glucose +2 $mg.L^{-1}$ 2,4-D + 50 µg. L^{-1} BAP. Liquid medium	I: Dark (14 days) T: 25 °C	
		ED: Embryo development DKW salts + DKW vitamins (100 $mg.L^{-1}$ Myo-inositol +2 $mg.L^{-1}$ thiamin-HCl + 1 $mg.L^{-1}$ nicotinic acid +2 $mg.L^{-1}$ glycine) + 30 $g.L^{-1}$ sucrose +1 $g.L^{-1}$ glucose. Liquid medium	I: Dark (90 days) T: 28 °C 1-minute immersion every 6 h per day Media change every 30 days	
		SCG: Secondary callus growth medium (Maximova et al. 2002)	I: Dark (14 days) T: 25 °C	

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
		ED: Embryo development (Maximova et al. 2002)	I: Dark (90 days) with subculture every 14 days. T: 25 °C	
Sca6, IMC67, C151-61, SNK12, ICS40, POR, IMC67, PA121, SNK64 and SNK10.	Staminodes and petals	*The protocol used in this study was adapted from that of* Maximova et al. (2005) *PCG:* DKW + 250 mg.L^{-1} glutamine +100 mg.L^{-1} Myo-inositol +1 ml.L^{-1} DKW vitamin stock (100 mg.ml^{-1} Myo-inositol +2 mg.ml^{-1} thiamine-HCl + 1 mg.ml^{-1} nicotinic acid +2 mg.ml^{-1} glycine) + 20 g.L^{-1} glucose +3, 978 mg.L^{-1} 2,4-D + mg.L^{-1} 0,0099 TDZ	I: Dark (14 days) T: 25 °C	Minyaka et al. (2008)
		SCG: Secondary callus growth DKW + 0,5 ml.L^{-1} DKW vitamins +20 g.L^{-1} glucose +2 mg.L^{-1} 2,4-D + 0,25 mg.L^{-1} KIN +2 g.L^{-1} Phytagel	I: Dark (14 days) T: 25 °C	
		ED: Embryo development DKW + 1 ml.L^{-1} DKW vitamins +30 g.L^{-1} sucrose +1 g.L^{-1} glucose +2 g.L^{-1} Phytagel	I: Dark (21 days) T: 25 °C	
AMAZ 3-2, AMAZ 10-1, COCA 3370-5, GU 183 H.	Staminodes	*PCG* (Li et al. 1998) *SCG* (Maximova et al. 2002)	–	Quainoo and Dwomo (2012)

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
MCCS 14-056, POUND 7, TSH 1188, TSH 565, VB 1151, CCN 51, UF 613, PS 1319, Sca6, CCN 10	Staminodes and petals	*PCG* (Li et al. 1998 modified) DKW + vitamins DKW + 2 mg.L^{-1} 2,4-D + 5 µg.L^{-1} TDZ + 250 mg. L^{-1} L-glutamine +200 mg. L^{-1} Myo-inositol +20 g.L^{-1} glucose +2 g.L^{-1} Phytagel The DKW vitamin stock solution yielded final concentrations of 1 mg.L^{-1} nicotinic acid +2 mg.L^{-1} glycine +0.1 g.L^{-1} Myo-inositol +2 mg.L^{-1} thiamine–HCl	I: Dark (14 days) T: 27 °C	Garcia et al. (2016)
		SCG (Li et al. 1998 modified) WPM + Gamborg's vitamin solution +2 mg.L^{-1} 2,4-D + 50 µg.L-^{1} BAP + 20 g.L^{-1} Glucose +2 g.L^{-1} Phytagel	I: Dark (14 days) T: 27 °C	
		ED4: Embryos development-4 medium (It is a modification from ED medium used in Li et al. 1998 protocol) DKW + DKW vitamins +40 g.L^{-1} sucrose +2 g.L^{-1} Phytagel	I: Dark (56–70 days) T: 27 °C ± 2 °C	
		ED3: Embryos development-3 medium (It is a modification from ED medium used in Li et al. 1998 protocol) DKW + DKW vitamins +30 g.L^{-1} sucrose +2 g.L^{-1} Phytagel *Transfer to the ED3 medium every 14 days for 3 months*	I: Dark (56–70 days) T: 27 °C ± 2 °C	
		SCG: Secondary somatic embryogenesis (Maximova et al. 2002) 300 µg.L^{-1} KIN or 50 µg. L^{-1} BAP (depending on genotype)	I: Dark (14 days) T: 27 °C	

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
		ED4 medium DKW + DKW vitamins +40 $g.L^{-1}$ sucrose +2 $g.L^{-1}$ Phytagel	I: Dark (14 days) T: 27 °C	
		ED3 medium DKW + DKW vitamins +30 $g.L^{-1}$ sucrose +2 $g.L^{-1}$ Phytagel	I: Dark (84 days until emerge and reach their maturation) T: 27 °C ± 2 °C	
		EDL: Embryo development in light (PEC Primary Embryo Conversion) DKW salts +1X vitamins DKW + 20 $g.L^{-1}$ glucose +0.3 $g.L^{-1}$ KNO_3 + 45.65 mg. L^{-1} L-lysine +32.80 mg. L^{-1} L-leucine +51.05 mg. L^{-1} L-tryptophan +43.55 $mg.L^{-1}$ L-arginine +18.76 $mg.L^{-1}$ glycine +2 $g.L^{-1}$ Phytagel	I: 16:8 light/dark (84 days) (50–190 µmol m^{-2} s^{-1}) T: 27 °C ± 2 °C	
		EDL modified: Embryo development in light ½ DKW basal salts +1X DKW vitamins +0.3 $g.L^{-1}$ KNO_3 + 3.0 $mg.L^{-1}$ IBA (indole-3-butyric acid) + 1.8 $g.L^{-1}$ Phytagel	I: 16:8 light/dark (120 days) (50–190 µmol m^{-2} s^{-1}) T: 27 °C ± 2 °C	
SYS12, SYS13, SYS16, SYS24, SYS4, CCN51, TSH565, EET8, ICS1, ICS39, ICS60, ICS95	Staminodes and petals	*INDI* (Fontanel et al. 2002)	I: Dark (30 days) T: 27 °C	Henao et al. (2018)
		INDexp (Fontanel et al. 2002)	I: Dark (35 days) T: 27 °C	
		CM2 (Fontanel et al. 2002)	I: Dark (30 days) T: 27 °C	
		MM6 (Fontanel et al. 2002)	I: Dark (30 days) T: 27 °C	

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
		In vitro development (Fontanel et al. 2002) ½MS macronutrients + DKW micronutrients + DKW vitamins +0,4 mg. L^{-1} L-Leucine +0,4 mg. L^{-1} L-lysine +0,2 mg. L^{-1} L-tryptophan +0,4 mg. L^{-1} L-arginine +0,01 mg. L^{-1} NAA + 0,02 mg.L^{-1} GA_3 + 1 g.L^{-1} AC + 40 g. L^{-1} Glucose +3 g.L^{-1} Phytagel	I: 12:12 light/dark (60 days) (32 µmol m^{-2} s^{-1}) T: 28 °C ± 2 °C	
EET95, EET96, EET103, Sca6	Staminodes	*PCG:* (Li et al. 1998 modified) DKW macronutrients + DKW micro +200 mg.L^{-1} Myo-inositol +1 mg.L^{-1} nicotinic acid +2 mg.L^{-1} thiamine +2 mg.L^{-1} glycine +250 mg.L^{-1} glutamine +2 mg.L^{-1} 2, 4-D + 0,005 mg.L^{-1} TDZ + 50 mg.L^{-1} IP + 20 g.L^{-1} glucose +3 g. L^{-1} Gelrite®	I: Dark (14 days) T: 25 °C	Guillou et al. (2018)
		SCG: WPM macro y micronutrients +100 mg. L^{-1} Myo-inositol +1 mg. L^{-1} nicotinic acid +10 mg. L^{-1} thiamine +1 mg.L^{-1} pyridosine-HCL + 2 mg. L^{-1} glycine +2 mg.L^{-1} 2,4-D + 0,25 mg.L^{-1} KIN +50 ml.L^{-1} WC + 20 g.L^{-1} glucose +3 g.L^{-1} Gelrite®	I: Dark (14 days) T: 25 °C	

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
		CC2: Expression MS macronutrients + DKW micronutrients +100 mg.L^{-1} Myo-inositol +1 mg.L^{-1} 1 nicotinic acid +2 mg.L^{-1} thiamine +2,0 mg.L^{-1} glycine +0,4 mg.L^{-1} L-lysine +0,4 mg.L^{-1} L-leucine +0,4 mg.L^{-1} L-arginine +0,2 mg.L^{-1} L-tryptophan +0,025 mg.L^{-1} adenine-H_2SO_4 + 30 g.L^{-1} glucose +3 g.L^{-1} Gelrite®	I: Dark (84 days) T: 25 °C	
		CC21: Multiplication MS macronutrients + DKW micronutrients +100 mg.L^{-1} Myo-inositol +1 mg.L^{-1} nicotinic acid +2 mg.L^{-1} thiamine +2,0 mg.L^{-1} glycine +0,4 mg.L^{-1} L-lysine +0,4 mg.L^{-1} L-leucine +0,4 mg.L^{-1} L-arginine +0,2 mg.L^{-1} L- tryptophan +1 mg.L^{-1} 2,4,5 T + 0,25 mg.L^{-1} adenine-H_2SO_4 + 30 g.L^{-1} glucose +3 g.L^{-1} Gelrite®	I: Dark (42 days) T: 25 °C	
		G80: Maturation ½MS macronutrients + MS micronutrients +100 mg. L^{-1} Myo-inositol +0,7 mg. L^{-1} thiamine +0,5 mg.L^{-1} nicotinic acid +0,5 mg.L^{-1} pyridoxine-HCL + 2 mg. L^{-1} glycine +0,4 mg. L^{-1} L-lysine +0,4 mg. L^{-1} L-leucine +0,4 mg. L^{-1} L-arginine +0,2 mg. L^{-1} L-tryptophan +0,010 mg.L^{-1} NAA + 0,20 mg.L^{-1} isopentenyl adenine +1,0 mg.L^{-1} ABA +0,020 mg.L^{-1} GA_3 + 1 g. L^{-1} AC + 40 g.L^{-1} gucose +3 g.L^{-1} Gelrite®	I: Dark (42 days) T: 25 °C	

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
		ENR8: Germination MS macronutrients + DKW micronutrients +100 mg.L^{-1} Myo-inositol +1 mg.L^{-1} nicotinic acid +2 mg.L^{-1} thiamine +2 mg. L^{-1} glycine +0,4 mg. L^{-1} L-lysine +0,4 mg. L^{-1} L-leucine +0,4 mg. L^{-1} L-arginine +0,2 mg. L^{-1} L-tryptophan +30 g.L^{-1} glucose +3 g.L^{-1} Gelrite®	I: 12:12 dark/light (60 days) (65 µmol m^{-2} s^{-1}) (light red/white/ medium blue) T: 25 °C	
Different genotypes	Staminodes and petals	*Indirect Primary Somatic Embryogenesis* *PCG (Primary callus growth)* DKW sales+ DKW vitamins +20 g.L^{-1} glucose +250 mg.L^{-1} glutamine +200 mg.L^{-1} Myo-inositol +2,0 mg.L^{-1} 2,4 D + 5 ug.L^{-1} TDZ + 2,0 g.L^{-1} Phytagel	I: Dark (14 days) T: 25 ± 2 °C	Garcia et al. (2018)
		SCG (Secondary callus growth) McCown's (WPM) + 1 ml Gamborg's vitamin solution +20 g.L^{-1} glucose +2 mg.L^{-1} 2,4-D solution +0,05 mg.L^{-1} BA solution or 0.3 mg.L^{-1} Kinetin solution (depending of genotype) + 2,2 g.L^{-1} Phytagel	I: Dark (14 days) T: Between 25 and 30 °C	
		ED4: Embryo development DKW sales+ DKW vitamins +40 g.L^{-1} sucrose +2,0 g.L^{-1}	I: Dark (14 days) T: Between 25 and 30 °C	
		ED3: Embryo development DKW sales+ DKW vitamins +30 g.L^{-1} sucrose +2,0 g.L^{-1} Phytagel	I: Dark (14 days) T: Between 25 and 30 °C	

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
Different genotypes	Staminodes and petals	*Secondary somatic embryogenesis process using the indirect technique SCG (Secondary callus growth)* 2,3 g McCown's (WPM) + 1 mL Gamborg's vitamin solution +20 $g.L^{-1}$ glucose +2 $mg.L^{-1}$ 2,4-D solution +0,05 $mg.L^{-1}$ BA solution or 0.3 $mg.L^{-1}$ KIN +2,2 $g.L^{-1}$ Phytagel	I: Dark (14 days) T: Between 25 and 30 °C. Or liquid medium in the light I: light/dark 16:8 (14 days). In a shaker set at 100 rpm. T: Between 25 and 30 °C	Garcia et al. (2018)
		ED4 (Embryo development) DKW sales+ DKW vitamins +40 $g.\ L^{-1}$ sucrose +2 $g.L^{-1}$ Phytagel	I: Dark (14 days) T: Between 25 and 30 °C.	
		ED3: Embryo development DKW sales+ DKW vitamins +30 $g.\ L^{-1}$ sucrose +2 $g.L^{-1}$ Phytagel	I: Dark (14 days) T: Between 25 and 30 °C.	
		EDL (Embryo germination and conversion) DKW sales+ DKW vitamins+ (1X) Amino acids +20 $g.L^{-1}$ glucose +0,3 $g.L^{-1}$ KNO_3 + 1,8 g. L^{-1} Phytagel	I: Light/dark 16:8 (90 days) T: 25 ± 2 °C	
		EDL (Embryo germination and conversion) DKW sales+ DKW vitamins+ (1X) amino acids +20 $g.L^{-1}$ glucose +0.3 $g.L^{-1}$ KNO_3 + 1,8 g. L^{-1} Phytagel	I: Light/dark 16:8 (20 days) T: 25 ± 2 °C	
		PR: Plant Regeneration (used when the root is missing) ½ DKW sales+ ½ DKW vitamins+ (1X) amino acids +4,5 $mg.L^{-1}$ IBA + 10 $g.L^{-1}$ glucose +5 $g.L^{-1}$ sucrose +0,3 g. L^{-1} KNO_3 + 2 $g.L^{-1}$ Phytagel	I: Light/dark 16:8 (20 days) T: 25 ± 2 °C	

(continued)

Table 5.2 (continued)

Genotype	Type of explant	Basal media, different components, and PGRs	Culture conditions	References
		EDL (Embryo germination and conversion) DKW sales+ DKW vitamins+ (1X) Amino acids +20 g.L^{-1} glucose +0,3 g.L^{-1} KNO_3 + 1,8 g. L^{-1} Phytagel	I: Light/dark 16:8 (20 days) T: 25 ± 2 °C	
		Secondary Somatic Embryogenesis using the Direct Technique *IM: Induction medium (Direct somatic embryogenesis)* ¼ MS macronutrients + ½ MS micronutrients +42,5 mg.L^{-1} KH_2PO_4 + 21,5 mg.L^{-1} Fe-EDTA +1 mg.L^{-1} pyridoxine HCL + 1 mg. L^{-1} nicotinic acid +10 mg. L^{-1} thiamine +1 mg.L^{-1} BA +100 mg.L^{-1} Myo-inositol +40 g.L^{-1} sucrose +2,0 g. L^{-1} Phytagel	I: 16:8 light/dark (35 days) (80– 190 µmol m^{-2} s^{-1}) T: 27 °C	
		EM: expression medium (Direct somatic embryogenesis) MS + 1 mg.L^{-1} calcium pantothenate +21,5 mg.L^{-1} Fe-EDTA +1 mg.L^{-1} pyridoxine +1 mg.L^{-1} nicotinic acid +1 mg.L^{-1} thiamine +0,1 mg.L^{-1} biotin +0,6 mg.L^{-1} GA_3 + 100 mg.L^{-1} Myo-inositol +0,3 g.L^{-1} KNO3 + 40 g.L^{-1} sucrose + (1X) Amino acids +2,2 g. L^{-1} Phytagel	I: 16:8 light/dark (21 days) (80– 190 µmol m^{-2} s^{-1}) T: 27 °C	
		EDL (Embryo germination and conversion) DKW sales+ DKW vitamins+ (1X) Amino acids +20 g.L^{-1} glucose +0,3 g.L^{-1} KNO_3 + 1,8 g. L^{-1} Phytagel	I: Light/dark 16:8 (20 days) T: 25 ± 2 °C	

I Illumination, *T* Temperature, *H* Humidity, *A* Agitation, *G* Gas exchange

Li et al. (1998) published one of the most influential works in SE in *T. cacao*. In that research, the embryogenic response was achieved in 19 genotypes using the staminodes as the explant. Rapidly growing calli were induced by culturing staminode explants on a DKW salt-based primary callus growth (PCG) medium supplemented with 20 $g.L^{-1}$ glucose, 1,99 $mg.L^{-1}$ 2,4 D, and 0,001 $mg.L^{-1}$ TDZ. Calli were subcultured onto a WPM salt-based secondary callus growth medium supplemented with 20 $g.L^{-1}$ glucose, 1,99 $mg.L^{-1}$ 2,4 D, and 0,30 $mg.L^{-1}$ KIN. Somatic embryos were formed from embryogenic calli following transfer to a hormone-free DKW salt-based embryo development medium containing only sucrose. Many plants were regenerated from somatic embryos and established in soil in a greenhouse. Plants showed morphological and growth characteristics similar to those of seed-derived plants. From Li et al.'s study (1998), Maximova et al. (2002) reported the development of a secondary embryogenesis system utilizing primary somatic embryo cotyledon explants, which resulted in up to a 30-fold increase in somatic embryo production compared to PSE. The increase in the frequency of secondary embryogenesis was due to modifications of secondary callus growth medium (SCG) (Li et al. 1998) containing 2 $mg.L^{-1}$ 2,4 D and 0,30 $mg.L^{-1}$ BA. The modifications of SCG medium included substitution of kinetin with BA and exclusion of the coconut water. They also found that while primary embryos arise from clusters of cells forming embryonic nodules, secondary embryos arise predominantly from the division of single cells, in a pathway reminiscent of zygotic embryogenesis (ZE).

Concomitantly, Maximova et al. (2002) and Fontanel et al. (2002) developed on their own, a new protocol based on previously published works but with different substances in the PGRs. In the callus induction of primary embryogenesis, the main differences are as follows: in the medium of Fontanel et al. (2002), it does not have glutamine but tryptophan 1 $mg.L^{-1}$; the concentration of my-inositol is 100 $mg.L^{-1}$, whereas Li et al. (1998) used 200 $mg.L^{-1}$; Fontanel et al. (2002) have a higher concentration of glucose (30 $g.L^{-1}$) than Li et al. (1998) (20 $g.L^{-1}$); and the concentration of 2,4 D is less at 1.mg L^{-1} compared to 1.99 $mg.L^{-1}$ forf Li et al. (1998). Li et al. (1998) used 0.001 $mg.L^{-1}$ TDZ, but Fontanel et al. (2002) used 0.25 $mg.L^{-1}$ KIN. In the embryo expression medium of Fontanel et al. (2002), the basal salts and vitamins are of the DKW formulation, while in the medium of Li et al. (1998), the basal salts are WPM and the vitamins are Gamborg's. In Fontanel et al. (2002) medium, the glucose concentration is greater than 30 $g.L^{-1}$, and the medium is free of growth regulators, while in Li et al. (1998) it is 20 $g.L^{-1}$, 1.99 $mg.L^{-1}$ 2,4 D, and 0.30 $mg.L^{-1}$ KIN of growth regulators and 5% coconut water.

When inducing a secondary embryogenesis, the medium for Fontanel et al. (2002)s case has MS macronutrients, DKW micronutrients, and DKW vitamins, while Maximova et al. (2002) used WPM salts and Gamborg's vitamins like embryo expression medium. As a growth regulator, Fontanel et al. (2002) used 1 $mg.L^{-1}$ 2,4,5 T, while Maximova et al. (2002) used a combination of 0,53 $mg.L^{-1}$ 2,4 D and 0.3 $mg.L^{-1}$ BAP. For the expression of secondary embryos in Fontanel et al. (2002) s case, the medium has the macronutrients MS and DKW micronutrients and DKW vitamins without regulators, while Maximova et al. (2002) do not report this stage. Fontanel et al. (2002) have a medium for maturation of the embryos with half of the

basal salts MS, DKW micronutrients, DKW vitamins, 0.01 mg.L^{-1} NAA, 0.02 mg. L^{-1} gibberellic acid (GA_3), 1 g.L^{-1} activated carbon, and 40 g.L^{-1} glucose, while Maximova et al. (2002) do not report a specific means for maturation. However, Maximova et al. (2002) have an embryo development medium with DKW salts, 100 mg.L^{-1} myo-inositol, 2 mg.L^{-1} thiamine HCl, 1 mg.L^{-1} nicotinic acid, 2.mg L^{-1} glycine, 30 g.L^{-1} sucrose, and 1 g.L^{-1} glucose. The maturation medium in Fontanel et al. (2002)s case has half of MS basal salts, MS macronutrients, DKW micronutrients, and DKW vitamins plus increases to 50 g.L^{-1} sucrose and 10 g.L^{-1} glucose.

Minyaka et al. (2008) evaluated the effects of $MgSO_4$ and K_2SO_4 concentrations for somatic embryo differentiation using three genotypes (Sca6, IMC67, and C15161), using the protocol of Maximova et al. (2002). The effect of these two salts in culture media appears to be most efficient at the embryo development stage. At this stage, in studying recalcitrance of cacao, high concentrations of 2888,7 mg.L^{-1} $MgSO_4$ and 12471,3 mg.L^{-1} K_2SO_4 in the culture media induced direct somatic embryos on staminodes and petals of the Sca6 and IMC67 genotypes. The positive effect of $MgSO_4$ and K_2SO_4 on the acquisition of embryogenesis competence was further tested on seven cacao genotypes reported as non-embryogenic: SNK12, ICS40, POR, IMC67, PA121, SNK64, and SNK10. All these genotypes were able to produce somatic embryos depending on the $MgSO_4$ concentration. Thus, this result showed that the recalcitrance of cacao to somatic embryo differentiation can be overcome by screening for the suitable $MgSO_4$ or K_2SO_4 concentration. Studies of the influence of different K+/Mg2+ ratios (at normal sulfate concentration) on somatic embryo differentiation revealed that sulfate supply was the main factor promoting responsive explants and the proportion of embryos. Another relevant study was carried out by Quainoo and Dwomo (2012). They evaluated the effects of abscisic acid (ABA) on embryo development (ED) of primary and secondary somatic embryos in the different developmental stages (globular, heart, and torpedo) in order to overcome the low rate of conversion into plantlets, utilizing both Li et al. (1998)s and Maximova et al. (2002)s protocol. Genotype COCA 3370-5 was more efficient than genotype AMAZ 3-2 in terms of embryogenesis and response to ABA treatments. The most effective treatments for embryo conversion were 0,0026 mg.L^{-1} ABA at 2 weeks. The effects of ABA treatments on conversion of secondary somatic embryos into plantlets were superior to primary embryos.

More recently, Garcia et al. (2016) evaluated the embryogenic response in 10 genotypes (Table 5.2). The PCG and SCG media were used as reported by Li et al. (1998), but the ED medium was modified; the modifications were mainly in the carbohydrate source. In the experiment used sucrose 40 g.L^{-1} and 30 g.L^{-1} of glucose in ED4 and ED3 medium respectively, instead of 1 g.L^{-1} of glucose and sucrose 30 g.L^{-1} used in ED in the original medium. For CCN51 and CCN10, SE production was highest using a high concentration of glucose in the induction medium (80 g. L^{-1}). The other components are the same as used in the original protocol. SSE protocols using primary embryo explants proved to be more productive than PSE protocols, using floral explants. This difference could be attributed to the fact that, within a given explant, cotyledonary cells have more competition than the presumably smaller populations of competent cells in petals or staminodes. Alternatively, a

higher proportion of brown callus in the PSE-derived cultures might explain higher SE generation in SSE protocols, possibly due to larger populations of competent cells. The observed genotypic differences in SE productivity response likely reflect genetic variation in concentration/type of endogenously produced compounds, for example, polyamines, ethylene, phenolic compounds, auxin, etc., or to synergy of endogenously expressed PGRs with medium-supplied PGRs. Additional variation in donor flower bud size and/or the physiological stage might also underlie the observed performance variation (Teixeira et al. 2002). Henao et al. (2018) evaluated the protocols reported by Li et al. (1998) and Fontanel et al. (2002) in 12 genotypes, 5 Colombian genotypes, and 7 universal genotypes. The complete development of seedlings was completed in the MM6 medium in some genotypes, which is opposite to the results obtained by Garcia et al. (2016) who describe this development in ED medium. A substantial change by Henao et al. (2018) was included in the Fontanel et al. (2002)s protocol for the maturation and development of the embryos', only the MM6 medium, changes that allowed the optimization of resources and time in about 2 months. One of the main differences between Li et al. (1998)s and Fontanel et al. (2002)s maturation medium is that in MM6 the macronutrients are MS, while for ED are DKW; in addition, the MM6 medium is supplemented with activated carbon, amino acids, NAA, and GA_3.

One possible explanation for these results is a higher macronutrient concentration, especially nitrogen (KNO_3 and NH_4NO_3) in the MS medium compared to the DKW. In the MM6 medium with NAA combined with GA_3, an efficient germination was achieved, opposite to the findings in the medium without regulators (ED). This synergistic effect of auxin and gibberellin on the promotion of embryo conversion into seedlings has also been reported in other species (Paramageetham et al. 2004; Junaid et al. 2007). The latest publications on the subject have been made by Guillou et al. (2018) and Garcia et al. (2018). Guillou et al. (2018) report embryogenic cell lines in liquid medium starting from high frequency somatic embryogenesis (HFSE) callus. The growth kinetics of the cultures during the multiplication and the expression steps conducted in 250 mL Erlenmeyer flasks were described for three genotypes selected for their agronomical traits (EET95, EET96, and EET103). The multiplication of the embryogenic calluses in a medium containing 2,4,5 T at 1 $mg.L^{-1}$, initiated with an inoculation density of 20 $g.L^{-1}$ of callus, was achieved. As has been reported in other previous works, they found that the expression of the callus embryogenic capacity was conducted in an auxin-free medium. 1 g of callus inoculum produced 1000 to 1500 embryos within 5–7 weeks. In improving the plantlet regenerative capacities in liquid medium, supplementing the expression medium with myo-inositol as an osmotic agent at a concentration of 50 $g.L^{-1}$ increased the embryo-to-plantlet conversion rate from 13–16% to 40–48%.

Finally, Garcia et al. (2018) developed new and useful strategies to produce plants in liquid or solid media that improved the propagation process. DSE methodology does not require the production of the embryogenic callus; therefore, it does not require the use of 2,4 D and provides a way of obtaining a high number of normal embryos in a short time compared with the ISE procedures. To obtain direct secondary somatic embryos, the epicotyls from primary somatic embryos were

used. These explants were placed into a flask with 25 mL of liquid medium (IM) for 5 weeks (Table 5.2). The main components of liquid induction media were MS macro- and micronutrients, 42,5 mg.L^{-1} KH_2PO_4, 21,5 mg.L^{-1} Fe-EDTA, 100 mg.L^{-1} myo-inositol, 10 mg.L^{-1} thiamine, 1 mg.L^{-1} nicotinic acid, HCL 1 mg.L^{-1} pyridoxine, 1 mg.L^{-1} BA, and 40 g.L^{-1} sucrose. After this period, the direct somatic embryos in the globular stage were transferred into a flask with 50 mL of liquid expression medium (EM). The EM medium is formed from DKW vitamins, DKW amino acids, 1 mg.L^{-1} calcium pantothenate, 21,5 mg.L^{-1} Fe-EDTA, 0,1 mg.L^{-1} biotin, 0,6 mg.L^{-1} GA_3, 0,3 mg.L^{-1} KNO_3, and 40 g.L^{-1} sucrose. After a 21-day culture period in EM, the cultures are transferred into embryo development in light medium (EDL) but in the dark, with transfers every 14 days for embryo maturation. Using temporary immersion system bioreactors, the embryos mature, and the medium was changed to EDL liquid medium (2 mL of EDL medium per mature embryo) with temporary immersion 6–8 times a day with 1–2 min per immersion. The liquid medium was changed every 20–30 days (1–2 times) until the embryos have a radicle and 2–4 leaves. They are then transferred to a greenhouse for acclimatization and incubated at 25–30 °C with a 16:8 h light/dark photoperiod. The authors emphasized that primary SE is not widely used to produce cacao plants due to its high dependency on genotype. Additionally, SE is influenced by PGR type and balance as well as sugar concentration.

Molecular Mechanism of Somatic Embryogenesis

Propagation of elite varieties has been achieved through SE, but low efficiencies and genotype dependence are still a significant limitation for commercial scale propagation. SE is not completely understood; particularly, how do competent somatic cells form somatic embryos and the lack of knowledge of the mechanisms that govern this dramatic reprogramming of somatic cells represent the greatest limitation to the rational improvement of this method in propagating many important species. Understanding the underlying mechanism is important because SE is a more accessible model to understand ZE, which occurs when embedded in the maternal tissue. SE is also relevant to agriculture, as genetic engineering generally requires the regeneration of transformed cells into plants.

Recent advances in high-throughput sequencing systems and “omics” resources have facilitated generation of high-resolution transcriptome data for plant embryogenesis, both in vivo and in vitro, and thereby provide novel insights into the molecular basis of embryogenesis (Argout et al. 2008). Noah et al. (2013) and Niemenak et al. (2015) reported proteome profiles of cacao SE and their equivalent ZE at various developmental stages analyzed through 2D PAGE and nano-LC-MC. That work identified proteins that represent an array of functional categories, including seed storage compounds, stress response, photosynthesis, and translation factors (TF). TFs are compounds that occur in different proportions, either in both types of embryos or in one, and that are essential in proper development. In addition, gene

expression profiles of ZE and SE have been generated using whole-genome microarray (Maximova et al. 2014). That study reported that many genes including those encoding for transcription factors and genes related to flavonoid and lipid biosynthesis were differentially expressed between the two embryo developmental processes. The relatively higher expression of ethylene- and flavonoid-related genes during SE suggests that the developing tissues may be experiencing high levels of stress during SE maturation caused by the in vitro environment. The expression of genes involved in the synthesis of auxin, polyunsaturated fatty acids, and secondary metabolites was higher in SEs relative to ZEs despite lack of lipid and metabolite accumulation. These differences in gene transcript levels associated with critical processes during seed development are consistent with the fact that somatic embryos do not fully develop the large storage cotyledons found in zygotic embryos. Such results thus provide an insight into cacao SE at a molecular level, and the information has been used by researchers in developing and characterizing novel molecular markers for SE.

There are few studies in *T. cacao* (cocoa) about transcription factor-enhanced SE. De Oliveira et al. (2005) found that the *somatic embryogenesis receptor kinase (SERK)* gene in cacao (*TcSERK*) is highly expressed in initial embryogenic calli, repetitive embryogenic sectors, and somatic and zygotic mature embryos, suggesting that this gene plays a role during cacao embryo development and the maintenance of repetitive status. Alemanno et al. (2008) characterized *leafy cotyledon1-like (L1L)* in cocoa. *TcL1L*, a homologue of *AtL1L*, is expressed very early during cocoa ZE and SE and especially into the protoderm and epidermis. *TcL1L* was mainly expressed in young and immature somatic embryos, at the same location as that observed in zygotic embryos. The cells of this special cell layer are precisely the place of origin of secondary somatic embryos; increased expression of this gene has been detected in early stages of cacao ZE and SE.

Zhang et al. (2014) characterized the ortholog of the *A. thaliana leafy cotyledon2 gene* (*AtLEC2*) in *T. cacao* (*TcLEC2*). *TcLEC2* encodes a B3 domain transcription factor preferentially expressed during early and late zygotic embryo development. The expression of *TcLEC2* is higher in dedifferentiated cells competent for SE (embryogenic calli), compared to non-embryogenic calli. Ectopic overexpression of *TcLEC2* in cacao leaves was able to induce the expression of seed transcription factor genes, such as *TcAGL15, TcABI3*, and *TcLEC1*. The constitutive overexpression of *TcLEC2* in stably transformed cells resulted in greatly enhanced SE as early as 4 weeks compared to 6 to 7 without *TcLEC2* overexpression, implying that the enhanced activity of *TcLEC2* is enough to promote the efficiency of SE in cacao. Florez et al. (2015) studied *Baby Boom (BBM)* transcription factor. The ortholog of the *A. thaliana BBM* gene (*AtBBM*) was characterized in *T. cacao* (*TcBBM*). *TcBBM* expression was observed throughout embryo development and was expressed at higher levels during SE as compared to ZE. *TcBBM* overexpression in *A. thaliana* and *T. cacao* led to phenotypes associated with SE that did not require exogenous hormones. While transient ectopic expression of *TcBBM* provided only moderate enhancements in embryogenic potential, constitutive overexpression dramatically increased SE proliferation but also appeared to inhibit subsequent development.

Shires et al. (2017) reported an inducible SE system by exploiting a dexamethasone activatable embryogenic transcription factor to promote somatic embryo formation from juvenile leaves. This fourfold enhancement in embryo production rate utilized a glucocorticoid receptor fused to an embryogenic transcription factor *leafy cotyledon2 (TcLEC2)*. These activatable chimeric transcription factors contribute to the elucidation of the regulatory cascade associated with SE of plants, which could in the near future improve the propagation and regeneration of transgenic plants.

Finally, Fister et al. (2018) developed a more efficient embryogenesis system, in which tissue was transformed with a transgene encoding a fusion of *leafy cotyledon2 (TcLEC2)* to a glucocorticoid receptor domain (GR) to control nuclear localization of the protein. Upon application of the glucocorticoid dexamethasone (dex), downstream targets of *LEC2* involved in seed development were upregulated, and somatic embryos (SEs) were successfully regenerated from *TcLEC2-GR* transgenic flower and leaf tissue in large numbers. Additionally, exposure of *TcLEC2-GR* floral explants to dex increased the number of SEs compared to floral explants from control. They have demonstrated that regulating *TcLEC2* activity offers a powerful new strategy for optimizing SE pipelines for cacao.

Genetic Improvement

Genetic transformation is a biotechnological tool that has contributed to performing basic studies on the structure and function of genes. It has also been widely used to evaluate the potential of the introduction of genes from other organisms to improve characteristics in crops. The sequencing of the complete genome for many economically important crops such as cocoa has contributed significantly to their respective genetic improvement (Argout et al. 2011). In cacao, an initial report by Purdy and Dickstein (1989) demonstrated the susceptibility of cacao cells to *Agrobacteria,* a commonly used bacterium capable of introducing DNA into plant cells. Later, Sain et al. (1994) described transforming cacao cells. Although transformed callus cells derived from leaf tissues were obtained, no plant regeneration was recorded from those transformed cells. This was due to the lack of an efficient protocol to recover plants from leaf tissue-derived calli.

Later, Perry et al. (2000) and Santos et al. (2002) described the transformation using the particle bombardment method to demonstrate that reporter genes could be introduced into cacao cells and visualized. However, none of these efforts resulted in the regeneration of transgenic cacao plants. Subsequently, using *Agrobacterium tumefaciens*-based transformation of cultured somatic embryos, a transformation system for cacao capable of producing whole plants was established by Maximova et al. (2003). Using the green fluorescent protein marker gene to identify transgenic somatic embryos, the authors recovered a series of transgenic plants that were grown to maturity. The growth and development of the plants was shown to be the same as the control group. Transgene insertion, gene expression, and stability were all shown to be similar as in other transgenic plants of different species. Details of

some of the experiments in which various parameters were optimized were also published separately (Antunez et al. 2003).

The regeneration system of somatic embryos with *A. tumefaciens* cocultivation to obtain transgenic plants reported by Maximova et al. (2003) has been successfully used to produce transgenic cacao plants overexpressing cacao *class I Chitinase gene (TcChil1)* (Maximova et al. 2006). These transgenic plants showed enhanced fungal pathogen resistance against *Colletotrichum gloeosporioides*. Subsequently, the Silva et al. (2009) study was conducted to improve the efficiency of the transformation method. Shi et al. (2013) reported the characterization of a putative *NPR3 gene (TcNPR3). TcNPR3* was transiently expressed in cacao leaves using an *Agrobacterium*-infiltration method, and they reported that *TcNPR3* knockdown leaf tissues were dramatically more resistant to infection with *Phytophthora capsici* in a leaf bioassay, showing smaller lesion sizes and reduced pathogen replication. Fister et al. (2015) described transient overexpression of *TcNPR1*, a major transcriptional regulator of the SA-dependent plant immune system that it can increase pathogen tolerance to *Phytophthora tropicalis* in cacao leaves.

Most recently, an optimized method for transient transformation for several genotypes through *Agrobacterium* infiltration has been published by Fister et al. (2016). Likewise, Helliwell et al. (2016) reported transient expression, and secretion of four different phosphatidylinositol-3-phosphate (PI3P)-binding proteins in detached leaves greatly reduced infection by two oomycete pathogens, *P. tropicalis* and *Phytophthora palmivora*, which cause black pod disease. Also, stably transformed, transgenic plants expressing two different PI3P-binding proteins showed substantially enhanced resistance to both *P. tropicalis* and *P. palmivora*, as well as to the fungal pathogen *Colletotrichum theobromicola.*

Other Important Biotechnological Aspects

Somaclonal Variation

SE propagation protocols offer a greater capacity for the rapid propagation of elite trees but carry the risk of producing mutant regenerants in such numbers that economic viability of the approach is compromised (Plader et al. 1998). Somaclonal variation has been defined by Wang and Wang (2012) as genetic and phenotypic variation among clonally propagated plants of a single donor genotype and includes genomic large-scale deletions and gross changes in chromosome structure/number, directed and undirected point mutations, and epigenetic changes (histone acetylation, DNA methylation, chromatin remodeling, etc.). Detailed characterization of mutation profiles that arise during culture should improve our understanding of processes influencing mutation and allow the selection of protocols yielding the fewest/least severe changes.

The first study on somaclonal variation of *T. cacao* was performed by Rodriguez et al. (2004). The authors presented a simple protocol that enabled the provisional diagnosis of both homogenous and chimeric mutants among large regenerant populations. The assay exploits consistent differential amplification of alternate simple sequence repeat (SSR) alleles at heterozygous loci. Calibration of the relative amplification of alleles from two genotypes (LCTEEN162/S-1010 and SIAL 93) and the synthetic chimeras created from them revealed a strong linear relationship between "peak heights" representing alternate alleles following capillary electrophoresis. The assay predicts chimeric composition to a reasonable level of confidence (±5%) so long as the infrequent allele exceeds 15% of the template. The system was applied to 233 regenerants of cocoa SE and identified 72 (31%) putative chimeric mutants for slippage mutation or allele loss across two loci.

To evaluate genetic fidelity during the SE process, Fang et al. (2009) used microsatellites as molecular markers. Ten cocoa simple sequence repeats (SSR) for screening a population of primary somatic embryos and secondary somatic embryos were used. They found in the primary somatic embryos 38 1% of polymorphic profiles, while in secondary embryos, the frequency was 23.3%. Also, secondary somatic embryos cryopreserved through encapsulation-dehydration revealed no polymorphism. Likewise, Rodriguez et al. (2010a) characterized new mutations among a clonal population of plants regenerated via a SE used previously for cocoa cryopreservation, by cleaved amplified polymorphic sequence (CAPS). Furthermore, they found 26.3% of polymorphisms, results like previous reports of de novo microsatellite length mutations. In the same year, the same authors used 15 single sequence repeat (SSR) markers to study genetic change and they used 386 methylation-sensitive amplified polymorphism (MSAP) markers to study epigenetic change. The plant regenerant exhibited 35% polymorphism. Genetic variation initially accumulated with culture age but subsequently declined. Epigenetic profiles diverged between leaf and staminode samples from source trees, leaves of late regenerants exhibited significantly less genetic and epigenetic divergence from source leaves than those exposed to short periods of callus growth Rodríguez et al. (2010b).

Ajijah et al. (2016) using 19 SSR markers found 97–100% level of similarity among regenerated plantlets. In the same year, Adu-Gyamfi et al. (2016) evaluated the effect of in vitro conservation, cryopreservation, and post-cryopreservation generation of somatic embryos, on the appearance of epigenetic somaclonal variation using methylation-sensitive amplified polymorphisms (MSAP). Somatic embryos accumulated epigenetic changes, but these were less extensive than in those regenerated after storage in liquid nitrogen. Furthermore, the passage of cryopreserved embryos through another embryogenic stage led to further increases in variation. Interestingly, this detected variability appears to be in some measure reversible. The outcome of this study indicates that the cryopreservation-induced phenotypic variability could be, at least partially, due to DNA methylation changes.

Finally, Pila et al. (2017) explored the relationship between DNA methylation, the long-term secondary SE, the embryogenic potential, and global DNA methylation levels in young (12 months old), aged (36 months old), and extra (39 months

old) SE subjected to different 5-Azacytidine (5-azaC) treatments. Global DNA methylation levels increased in aged somatic embryos with long-term in vitro culture, but 5-azaC-supplemented treatments resulted in unaltered levels. They found that the DNA methylation pattern during SE was not affected by 5-azaC, and DNA methylation increased during SE expression. The outcome of this study suggested that the long-term SE in cacao induced the decline on embryogenic potential, which can be reversible through 5-azaC supplementation.

Conclusion

There is great demand for high-quality plantlets of cacao to ensure long-term sustainability of cocoa production. Therefore, to be commercially viable in cacao, SE has to express the embryogenic pattern that donor material with a high rate of competent cells is present. Any variation in endogenous PGR's levels would likely impact embryogenic capacity and further compound underlying genotype-to-genotype variation response. The relationship between phenology and SE response has been studied and suggests that both flowering and fruiting levels can seasonally impact SE. Commercial cacao propagation is possible, but more research into its physiology, molecular biology, and genetics is needed to understand all these processes. Thus, it will be important to adjust this protocol for new clones where there is no preliminary information available. SSE is more efficient than primary SE. Implementation of modern molecular tools in cacao biotechnology research will undoubtedly be an integral part of this process.

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Chapter 6
Use of Biotechnology in Forestry Breeding Programs for Natural Resources and Biodiversity Conservation: Creating Super Trees for the Future

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Abstract Owing to the increasing human population and the increasing global demand for wood, its consumption is exceeding the natural rate of regeneration in many areas worldwide. Despite only 3% of the world's forested land is plantation forest, plantations are highly productive; and with further improvement in genetic composition of planting stock as well as applying biotechnology, additional productivity increases can be obtained. For this reason, it is necessary to enrich traditional breeding programs with biotechnological tools able to increase the quantity and quality of the forestry plants produced. FAO's definition of forest biotechnology encompasses different techniques for cloning forest trees. Forestry companies are currently considering clonal propagation as a good source of forestry plants. Clonal propagation can be achieved by various means: grafting, rooting of cuttings, coppicing, or in vitro propagation. Several methods of clonal propagation are being practiced with conifers. Along this chapter, a summary of some of the different approaches to improve *Pinus* spp. clonal propagation will be described, particularly those made in our laboratory.

Keywords Forestry biotechnology · Breeding programs · Natural resources · Biodiversity conservation

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Introduction

The increase in the human global population leads to an increase on the global demand for wood consumption. At the current rate of consumption, forests in many areas are exceeding their natural regeneration capacities (Fenning and Gershenzon 2002). For this reason, it is necessary to enrich traditional breeding programs with biotechnological tools able to increase the quantity and quality of forestry plants produced (Bonga 2015). Using in vitro technologies, organogenesis is generally restricted to the young seedling as the explant source. For this reason, initially, organogenesis techniques in *Pinus* species in order to produce clonal plants from selected seeds were developed. Then, in order to reproduce exactly the genotype of the donor plant, adult trees were used after applying various rejuvenating pretreatments, e.g., pruning, and spraying with cytokinins, using vegetative buds of different *Pinus* species or fewer needle primordia of 3- and 7-year-old trees. Main problems associated with this technique are low in vitro rooting, small acclimatization percentage, poor growth, etc. For all these reasons, in 2007 we concentrated all our efforts in the development of somatic embryogenesis systems. Somatic embryogenesis is a fascinating developmental pathway through which plants can be regenerated from bipolar structures derived from a single or a few somatic cells and that was first described more than 50 years ago in carrots by Reinert (1958) and Steward (1958). *Pinus* spp. somatic embryogenesis presents different problems, however. During the last few years, we have focused in overcoming some of the problems: the competence window problem, the low initiation frequencies, the low rates of maturation, poor germination rates, low regeneration capacity in conserved cell lines, etc. Moreover, we developed combined systems to increase the efficiency of SE in embryogenic cell lines with recalcitrance to be cryopreserved and procedures in different conifer species including hybrids. In parallel, one of our main research areas of interest was the study of the physiological mechanism controlling the tolerance to drought conditions in *Pinus* species. During the last few years and taking into account all the knowledge generated, as well as the fact that it has been found that using different temperatures during the process of embryo formation produced clonal somatic plants with different phenology, our challenge was being able to modulate the drought tolerance in *Pinus* spp.; Different stressful environmental conditions have been applied along the different stages of somatic embryogenesis (initiation, proliferation, and maturation) in order to obtain clonal plants with tolerance to different degrees of water stress. Preliminary results have showed that somatic plants coming from EMs initiated at lower temperatures showed higher water use efficiency than controls. At the same time, amino acid and sugar analyses and the ultrastructure at cellular level were studied in order to know the structural changes adapt after extreme temperatures (30, 40, 50, and 60 °C) as well as the metabolites involved in the different SE response.

Organogenesis

Seed Organogenesis

Plant propagation through tissue culture may be accomplished by employing callus, organ, cell, and protoplast cultures. Although tissue explants from tree species are generally difficult to grow and differentiate in vitro, the first types of cultures have been experimentally employed with varying degrees of success for micropropagation of a number of tree species (Ahuja 1988). Although initially callus cultures were employed for plantlet regeneration, now mostly organ cultures are employed for clonaI propagation. In this sense, organogenesis is generally restricted to the young seedling as the explant source (Bonga 2017), and these kinds of techniques have been studied for several conifers in the last 30 years (von Arnold and Hawes 1989; Nugent et al. 2001; Tang and Newton 2005).

In 1957, Skoog and Miller (1957) proposed that a balance between auxin and cytokinin determines the morphogenic competence of an explant in in vitro culture. Manipulation of the composition and ratio of these plant growth regulators inside the tissue is often the primary empirical approach to the optimization of in vitro culture. During this process of plant growth regulator optimization, abnormal or unusual organ development is often observed and attributed to plant growth regulator imbalance (Ramage and Williams 2004).

In conifers, seeds usually are the initial explant to induce organogenesis; seed organogenesis involves a four step process: (1) induction and development of adventitious buds on embryonic explants, (2) elongation of shoot buds, (3) multiplication of shoots, and (4) rooting of the shoots and their transfer to ex vitro conditions (Thorpe et al. 1991). In *Pinus radiata*, Aitken-Christie et al. (1988) developed an organogenesis protocol to generate large numbers of meristematic nodules from zygotic embryos. In the aforementioned protocols, shoot induction was achieved in radiata pine with 22 μM benzyladenine (BA) for 3 weeks. Stange et al. (1999) studied the effect of different BA and thidiazuron (T) concentrations to test their effect on the number and quality of the shoots obtained. Currently, BA is the most widely used cytokinin in plant micropropagation due to its effectiveness and affordability, but it has been reported to provoke hyperhydricity in some species (Bairu et al. 2007). Hyperhydricity is a critical factor as hyperhydric explants present several morphological and physiological disorders (Hazarika 2006). These explants are difficult to root, are more susceptible to infections, and present low survival rates when transferred to the greenhouse (von Arnold and Eriksson 1984).

Naturally occurring cytokinins are adenine derivatives found in higher plants (Strnad 1997), and they can be classified by the configuration of their N6-side chain as isoprenoid (zeatin) or aromatic cytokinins (BA and m-T) (Mok and Mok 2001). Meta-topolin (m-T) has been studied as an alternative phytohormone for micropropagation in *Pinus pinaster* (De Diego et al. 2008) and *Pinus pinea* (Moncaleán

et al. 2005). Later on, in our laboratory, Montalbán et al. (2011) tested different aromatic cytokinins in order to obtain improvement in seed organogenesis process, but they found the incubation period more important than the type and concentration.

The initial explant type is another important factor. Although in several protocols in *Pinus* spp. isolated cotyledons have been used (Alonso et al. 2006; Álvarez et al. 2009), other authors (Lambardi et al. 1993; De Diego et al. 2011) pointed out that organogenesis using isolated cotyledons generally presented lower survival percentages than using whole zygotic embryos, and the whole plant regeneration was delayed probably due to damages in the cotyledons when they are isolated. For instance, in *Pinus pinaster* (De Diego et al. 2011) and *P. radiata* (Montalbán et al. 2012), mature embryos are the best explants for fast and better regeneration.

Once the induction and elongation of shoots have been achieved, rooting is attained through the application of exogenous auxins to the culture medium. In radiata pine, a combination of a naturally occurring auxin (indole-3-butyric acid (IBA)) with a synthetic auxin (1-naphtalene acetic acid (NAA)) has been used for root induction in past years (Hargreaves et al. 2005).

Bud Organogenesis

In order to reproduce exactly the genotype of the donor plant, attempts to achieve in vitro regeneration from adult trees were carried out by Hugues-Jarlet and Nitsch (1988) using female floral buds. However, these authors did not obtain complete plant regeneration. Micropropagation protocols by micrografting or by microcutting partially solved the problems for cloning adult trees (Dumas and Monteuuis 1995). Nevertheless, these studies revealed that young or rejuvenated material was necessary to obtain regenerated plants, because the rooting from mature material showed problems by developing adventitious roots. Several approaches have been made to achieve this goal and different rejuvenating pretreatments, e.g., pruning and spraying with cytokinins (Monteuuis et al. 2011), and have been tested. In our laboratory, vegetative buds of different *Pinus* species have been used; in this sense, we achieved clonal plants from *P. pinaster* (De Diego et al. 2008), *P. sylvestris* (De Diego et al. 2010), *P. pinea* (Cortizo et al. 2009), *P*. sylvestris (De Diego et al. 2010), and *P. radiata* (Montalbán et al. 2013) (Fig. 6.1a, b). In the abovementioned species, rooted and acclimatized clonal plants were obtained, but after several months, depending on the species, plants showed adult trees characteristics. After reaching this extraordinary goal, we identified the problems associated with this technique: low in vitro rooting, low acclimatization percentage, poor growth, etc. For all these reasons, this technique can be used to reproduce an elite tree as well as to study aging process, but it is quite difficult to scale it up as a vegetative propagation method. For this reason, in 2007 we moved all our efforts to the development of somatic embryogenesis systems.

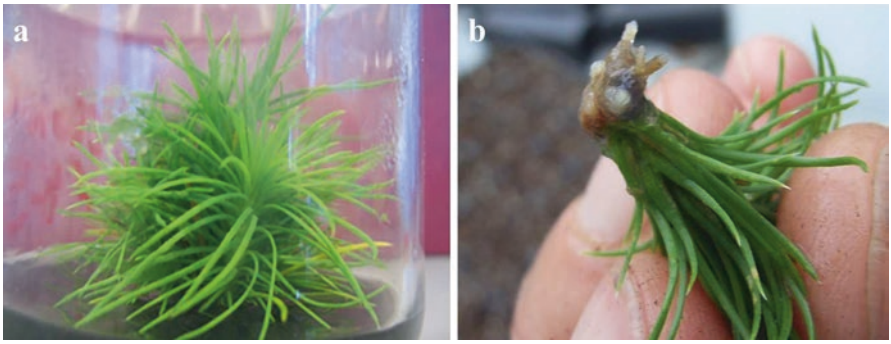

Fig. 6.1 *Pinus radiata* shoots coming from organogenesis from adult buds (**a**) and isolated reinvigorated rooted shoot after rooting stage (**b**)

Somatic Embryogenesis: Main Bottlenecks

Somatic embryogenesis (SE) is a fascinating developmental pathway through which plants can be regenerated from bipolar structures derived from a single or a few somatic cells that were first described more than 50 years ago in carrot by Reinert (1958) and Steward (1958). In forestry, somatic embryogenesis has been the most important development for plant tissue culture, not only for mass propagation but also for enabling the implementation of biotechnological tools that can be used to increase the productivity and wood quality of the plantation forestry. The development of SE in forest trees started in 1985, and nowadays many studies are focused on the optimization of conifer SE system to the development of multi-varietal forestry (Park 2002). However, these advances for many *Pinus* spp. are not sufficiently refined to be implemented commercially. In our laboratory, several efforts have been made in order to overcome the main bottlenecks of somatic embryogenesis process in radiata pine. In this sense, we focused in overcoming some of the problems:

1. *Narrow competence window*: Green cones are usually stored at 4 °C for 1 (Salajova and Salaj 2005) to 4 weeks (Yildirim et al. 2006) while processing them; as a consequence, a high amount of human resources is needed to introduce the amount of initial explants demanded for initiation stage in a short period of time. For this reason, it is necessary to develop systems able to increase the efficiency of the SE process. In our laboratory, a storage method for green cones was developed, and we were able to report that it is possible to maintain this kind of plant material of *P. radiata* for over a month, and an increment of initiation rates can be obtained. Moreover, cold treatment does not affect proliferation rates or maturation percentages (Montalbán et al. 2015).
2. *Low initiation frequencies*: Initiation of SE (Fig. 6.2a) is influenced by the developmental stage of immature embryos, the genotype of the parent trees, and the formulation of the tissue culture medium (Klimaszewska et al. 2001; Pullmann

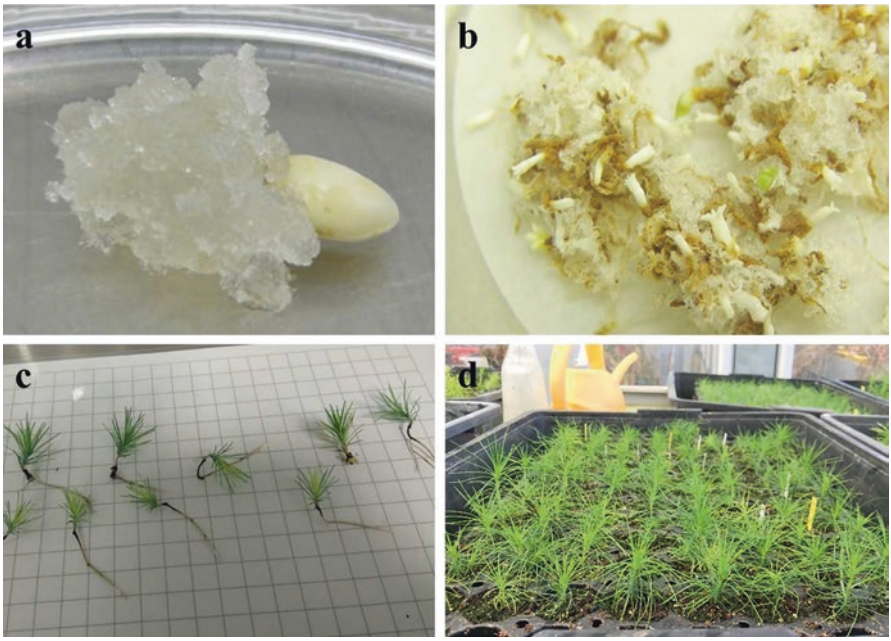

Fig. 6.2 *Pinus radiata* embryogenic tissue able to transfiere to proliferation stage (**a**); *Pinus radiata* somatic embryos after 16 weeks in maturation media (**b**); *Pinus radiata* germinated embryos (**c**) able to be acclimatized to ex vitro conditions in the greenhouse (**d**)

et al. 2004; Park et al. 2006). In this sense, for many economically important pine species, the initiation frequency of SE is insufficient for commercial application. Improvement in SE initiation is important for developing varietal lines as well as managing genetic diversity (Park et al. 2006). The nitrogen source has been crucial for initiation and proliferation stage as well as to determine the quality and quantity of somatic embryos obtained months later (Montalbán et al. 2011).

3. *The low rates of maturation*: In conifer SE, the development of mature somatic embryos (Fig. 6.2b) into plantlets occurs without the benefit of the megagametophyte, which is a major organ for storage of both lipids and proteins. For this reason, the composition of the culture medium takes on special importance in this phase, as it must be a substitute for the megagametophyte in order to supply adequate amounts of nitrogen and carbon (Ling and Leung 2002). However, there were no studies in the *Pinus* genus where the effect of different media on germination was tested, although some authors studied the influence of pre-germination treatments (Jones and van Staden 2001), light quality (Merkle et al. 2006), or explant's position on the media (Aronen et al. 2009; Percy et al. 2000). Based on our experiments, we recommend the following protocol: 100 mg of embryonal mass per 70 mm diameter filter paper disc, no subcultures during the entire maturation period, no pre-germination treatment needed due to the high

quality of the somatic embryos obtained, and high conversion of somatic embryos into plants, resulting in a significant saving of cost and labor (Montalbán et al. 2010).

4. *Poor germination rates*: Culture containers may cause anatomical and physiological changes that have negative effects on rooting and ex vitro acclimatization of somatic plantlets. The control of these factors could contribute to the improvement of somatic embryogenesis systems in conifers, especially in pines. Recent studies showed that supplying auxins to the culture medium did not improve the number of rooted explants (Fig. 6.2c). Furthermore, our results demonstrated that container type had a strong effect on rooting and acclimatization percentages. In this regar, explants cultured in Ecoboxes® showed a significant increment in survival after the acclimatization stage (Fig. 6.2d) (Montalbán and Moncaleán 2019).
5. *Low regeneration capacity in conserved cell lines*: The commercial deployment of SE in conifer species can only be accomplished when this technology is combined with cryopreservation of embryogenic cell lines (ECLs) (Park 2002). Cryopreservation is the key element of conifer SE programs making long-term storage of embryogenic tissue (ET) possible, arresting biochemical and most physical processes (Panis and Lambardi 2006), while lengthy field testing of cell lines is being carried out (Park et al. 2016). There are studies where storage at −80 °C has been tested for periods ranging from days to months to preserve pine somatic embryos (Percy et al. 2000), coconut pollen grain (Machado et al. 2014), testicular cells of sturgeon (Golpour et al. 2016), or human serum and plasma (Zander et al. 2014). Taking into account the abovementioned reports, we carried out an experiment to assess whether it was possible to store radiata pine ECLs over a year in an ultra-low temperature freezer (−80 °C) without loss of regeneration capacity (Fig. 6.3). Our results demonstrated that ECLs from radiata pine could be preserved at −80 °C for over a year and that recovered tissue proliferates, maturates, germinates, and acclimatizes even better than non-stored ECLs

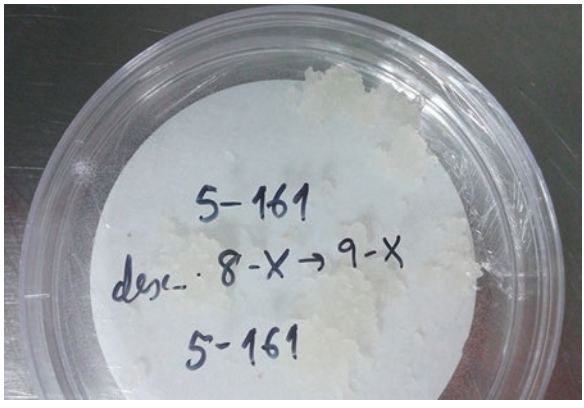

Fig. 6.3 Embryogenic tissue growing from an embryogenic line stored for a year at −80 °C

(Montalbán and Moncaleán 2017). Further research will determine if this storage system is feasible for longer periods.

Organogenesis and Somatic Embryogenesis Combined Method

As was mentioned above, propagation via SE is an effective method in propagating elite plants, but some genotypes show low embryo quality or germination frequencies making large-scale production of the genotypes too expensive and therefore eliminates these genotypes from production. A novel approach to overcome these kinds of problems associated with SE has been carried out in our laboratory. It consists on the development of a combined SE and organogenesis protocol. For this purpose, the effect of different initial explants, BA concentrations and induction periods, and culture conditions was assayed in somatic embryos of *Pinus radiata*. Once the radiata pine somatic embryos are obtained, up to 19 rootable shoots can be obtained from a single embryo, these shoots present rooting percentages around 60%. Considering that we can obtain more than 150 embryos per 100 mg of ET (Montalbán et al. 2010), theoretically by using the described method, more than 1700 rooted shoots can be produced (Montalbán et al. 2011). Moreover, these shoots before rooting can be propagated and continuously used as a source for plant regeneration. Furthermore, this approach facilitates the application of genetic manipulation procedures; and over time, numerous plants, representing clones, can be regenerated from a single shoot. Thus, in vitro micropropagation via induction of SE and organogenesis can be very useful for plant genetic resource management. Another useful approach to increase the viability of somatic embryos for the organogenesis process is to store them at 4 °C. Briefly, once somatic embryos are obtained, the cold storage procedure can be initiated. The embryos are poured onto a filter paper disc (Whatman® no.2) in a Petri dish containing 1/2 LP supplemented with 3% sucrose medium without plant growth regulators. Then, the Petri dishes are stored at 4 °C in the dark. After 1 year, isolated embryos can be used in two different ways: growth reactivation and germination (80% success) or shoot induction to follow the organogenic process described previously.

Drought Tolerance Induction

In parallel, one of our main research areas of interest was the study of the physiological mechanism controlling the tolerance to drought conditions in *Pinus* species (De Diego et al. 2012, 2013a, b, 2015). During the last few years and taking into account all the knowledge generated, as well as the fact that it has been found that different temperatures applied during the process of embryo formation produced

clonal somatic plants with different phenology (Kvaalen and Johnsen 2008), our challenge is being able to modulate the drought tolerance in *Pinus* spp. Different stressful environmental conditions have been applied along the different stages of somatic embryogenesis: initiation (García-Mendiguren et al. 2016; Pereira et al. 2016), proliferation (Pereira et al. 2017), and maturation (Moncaleán et al. 2018), in order to obtain clonal plants with different degrees of water stress tolerance. Preliminary results have showed that somatic plants coming from EMs initiated at lower temperatures showed higher water use efficiency than control ones (Montalbán et al. 2016). When the stress was applied at the maturation stage, the highest temperature reached (28 °C) led to a significantly lower water availability than the other temperatures tested, and ECLs matured in culture medium with the lowest water availability (10 $g.L^{-1}$) produced a significantly higher number of somatic embryos (Se). When the concentration of cytokinins was analyzed, it was observed a similar trend for CK concentrations in Se cultured at 18 and 23 °C when the agar concentration increased from 8 to 9 $g.L^{-1}$, except iPR, DHZR, and cZOG. When considering the results by CK type or CK-metabolite, this trend was also observed in all cases (Moncaleán et al. 2018). When the relation between IAA and active CKs (bases, ribosides) was evaluated, we could observe low values in Se developed at standard temperature (23 °C) independently of the gellan concentration in the culture media (Moncaleán et al. 2018). The highest amount of free bases, the active forms of cytokinins (Klemš et al. 2011), was found in Se developed under 18 and 23 °C and low gellan gum concentration, treatments showing the highest water availability. So, our results are in agreement with other authors describing that a decrease in water availability could be related to the rapidly utilization of CK bases and tightly regulated by mechanisms that prevent their accumulation at high levels (Moncaleán et al. 2005; Stirk et al. 2005). Our results suggest that none of the phytohormones found acts alone in the acquisition of embryogenic maturation capacity; it seems that the dynamic and complementary actions of the auxin and cytokinin pathways regulate several developmental processes, and their ability to crosstalk makes them ideal candidates for mediating stress adaptation responses, in agreement with Bielach et al. (2017) as well as initiating various signal transduction pathways (Kohli et al. 2013). In summary, biotechnology will be a useful tool to increase the productivity and the quality of forestry plants in the near future. Funding this research was funded by MINECO (Spanish Government) Project (AGL2016-76143-C4-3R), CYTED (P117RT0522) and DECO (Basque Government).

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Chapter 7
Stress Modulation in *Pinus* spp. Somatic Embryogenesis as Model for Climate Change Mitigation: Stress Is Not Always a Problem

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Abstract Climate change is leading to higher temperatures and lower precipitation; this fact can have a negative impact on plant performance and survival. Latest findings have revealed that the conditions in which zygotic embryogenesis takes place have an impact on the adaptive capacity of the resulting plants. Somatic embryogenesis provides us a potent biotechnological tool to manipulate the physical and chemical conditions (water availability) along the process and to study their effect in the final success of the process in terms of quantity and quality of somatic plants produced. The development of somatic cells to somatic plantlets comprises three stages: induction of embryonal masses, maturation of embryogenic tissues, and conversion into somatic plants. Our experience in somatic embryogenesis in *Pinus* spp. enables us to explore the possibility to modulate the quality in terms of abiotic stress tolerance of somatic plants modifying environmental conditions during the initial stages of the process. Our results have shown that the modification of environmental conditions affected not only the success of the process in some species of pines but also the water use efficiency of the somatic plants after several months in ex vitro conditions. In the chapter, we will show the different responses obtained in all the stages of the somatic embryogenesis process as well as the

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response obtained after drought periods in plants growing in the greenhouse under ex vitro conditions.

Keywords Stress modulation · Somatic embryogenesis · Climate change · Mitigation · *Pinus* spp

Introduction

As a result of being sessile organisms that are continuously exposed to a huge amount of external stimuli and changing environments, plants have evolved and managed to acquire different mechanisms to rapidly detect and respond to a great variety of stress factors, both biotic and abiotic (Xia et al. 2015). Among abiotic stresses, drought and high temperatures are among the most common environmental constraints for plants. In fact, due to climate change, extreme heat waves and long drought periods are becoming more and more frequent (Duliè et al. 2013), which cause a serious threat not only for natural ecosystems but also for all type of plantations and cultivars (Allen et al. 2010). These conditions are already challenging the survival and productivity of forests (Manzanera et al. 2017). Conifers, by far the largest and most diverse gymnosperm group that grow in a wide range of climate zones, are of great importance both ecologically and economically since they make up most of the biomass in many biogeographic zones of the Northern Hemisphere and provide wood and non-wood products (von Arnold et al. 2018). Nonetheless, the current situation driven by climate change foretells big difficulties to satisfy the future demand of forest products and services (Fenning et al. 2008).

At the molecular level, heat and drought are known to affect a great number of physiological traits. They trigger inhibition of photosynthesis, senescence, water, and osmotic imbalances and production of reactive oxygen species and modify the permeability and fluidity of cell membranes, among others (Larkindale and Knight 2002; Sangwan et al. 2002; Vacca et al. 2004; Feller and Vaseva 2014). However, the great adaptability and phenotypic plasticity of plants offer the opportunity to combine traditional breeding techniques with modern biotechnological tools to obtain plants pre-adapted to different environmental conditions (Conrath et al. 2015). This pre-adaptation strategy is mainly ruled by epigenetic variations, which endow plants with some kind of "memory" that results in modified gene expression patterns (Davies et al. 2011). In this context, somatic embryogenesis (SE) could be a useful tool to modulate future plant tolerance and therefore their behavior in ex vitro conditions. SE is a biotechnological tool in which embryos arise from somatic cells and commonly includes a set of stages such as induction, multiplication (proliferation), maturation, and conversion into plantlets (Heringer et al. 2018). Variation of the culture conditions during initial steps of the SE process is known to have

long-lasting effects in plants, even altering some developmental traits years later (Kvaalen and Johnsen 2008). Consequently, SE apart from being one of the most effective techniques to obtain large amounts of cloned material in conifers opens the door to a promising research field that could help to unravel the molecular mechanisms by which plants face stress.

To this purpose, in the last few years our research team has been applying different culture and environmental conditions during the different steps of SE, in order to assess if those changes in conditions can determine the success of the SE process itself in terms of plant production and plant quality, but also the behavior of somatic embryo-derived plants ex vitro. In parallel, numerous studies have been carried out to evaluate the effect of those conditions at morphological levels as well as to analyze different metabolites, aiming to detect early stress markers involved in the acquisition of tolerance. To enable a broader understanding of these mechanisms, two *Pinus* species from contrasting habitats have been employed in all experiments: *P. radiata* and *P. halepensis*.

Stress Modulation on Initial Stage of Somatic Embryogenesis

The Outcome of Different Water Availabilities and Temperatures at Initial Stages

As referred above, two *Pinus* species have been used in our experiments: *P. radiata* and *P. halepensis*. *R*adiata pine is a conifer native from California and México. It has a great economic relevance being suitable for a great range of uses due to its rapid and versatile growth and the desirable quality of its pulp and timber. It is the most widely planted pine in the world (Espinel et al. 1995; Dungey et al. 2009). *P. halepensis* Mill., commonly named Aleppo pine, is a species native to the Mediterranean region, widespread from Spain to Algeria (Botella et al. 2010). It is important for environmental conservation (Montalbán et al. 2013), due to its great ecological plasticity, since it can thrive under semi-arid climatic conditions in a wide variety of soils and can be used in large afforestations (Maestre and Cortina 2004).

With prior protocols established for SE in both species, two separate experiments *per* species that investigated the stress influence along the process by applying different environmental conditions, at induction and proliferation steps, were performed as indicated in Fig. 7.1.

In the initial set of experiments, different gellan gum concentrations (2, 3, and 4 gL^{-1}), to increase or reduce water availability, were added to the induction media, and the explants were then stored at three different temperatures (18, 23, and 28 °C). Immature megagametophytes from four seed families in *P. radiata* (García-Mendiguren et al. 2016a) and five in *P. halepensis* (Pereira et al. 2016) were used as explants. Different environmental conditions were applied only at the induction

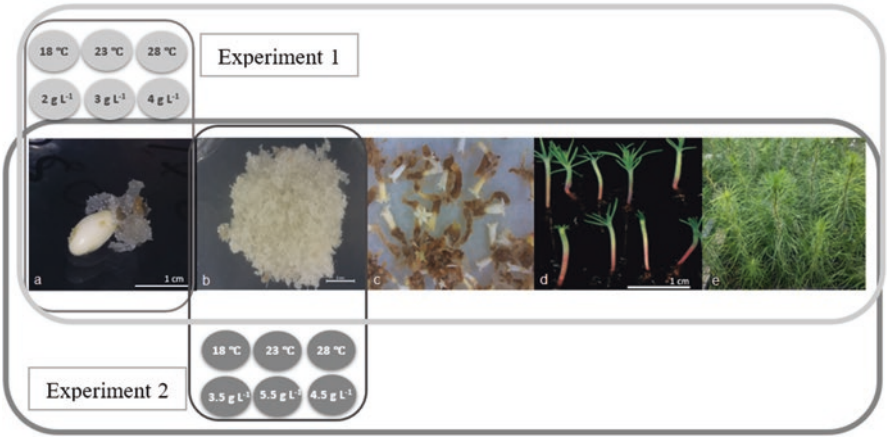

Fig. 7.1 Scheme of the experiments carried out in order to test the stress influence along the process by applying different environmental conditions, at induction and proliferation steps of somatic embryogenesis in *Pinus* spp. (**a**) Embryogenic mass extrusion from immature megagametophyte of *P. halepensis*. (**b**) Established embryogenic mass in proliferation of *P. halepensis*. (**c**) Somatic embryos obtained from embryogenic masses of *P. radiata*. (**d**) In vitro germinated somatic plantlets of *P. halepensis* and (**e**) acclimatized somatic plants of *P. radiata* growing in the greenhouse. Each experiment comprised nine different treatments and was individually developed for each species

stage, and then the cultures were maintained at standard conditions on the subsequent stages of the process. In both species, initiation rates of embryonal masses (EMs) were significantly different among seed families, temperatures, and gellan gum concentrations (García-Mendiguren et al. 2016a; Pereira et al. 2016). As expected, both showed significant differences on initiation rates between seed families. Since the effect of the mother plant cannot be equally reproduced unless the same families are used for a future experiment, a higher number of family seeds can reduce the variability of the results (Montalbán et al. 2013, 2015). Regarding the water availability, it is known that water availability in the culture media plays an important role in the phases of EM initiation and proliferation (Choudhury et al. 2008; García-Mendiguren et al. 2016a), and both species showed higher initiation rates in explants cultured with the lowest water availability (4 gL^{-1}). These results agree with reports in *P. monticola* (Percy et al. 2000) and *P. pinea* (Carneros et al. 2009) and are in contrast to some authors who have shown that when gellan gum increases, the initiation of embryogenesis is negatively affected (Harry and Thorpe 1991; Becwar et al. 1995; Pullman and Skryabina 2007). Nonetheless, the highest initiation rate of EMs in *P. halepensis* was obtained in cultures maintained at 23 °C (control temperature), while the lowest temperature, 18 °C, led to better initiation rates on *P. radiata*. Bonga et al. (2010) suggested that changes in temperature regimes might improve initiation and proliferation since temperature stress may promote cellular reprogramming. We relate this difference to the optimal temperature at which these species grow in the field, which is usually lower in the case of

radiata pine. Concerning the subsequent phases of SE in Aleppo pine, the initiation temperature only had a significant effect at proliferation stage, with initiation temperature 18 °C leading to lower rates. All embryogenic cell lines (ECLs) were able to produce somatic embryos (*se*), and there were no differences on the number of *se* produced or at the germination rates. García-Mendiguren et al. (2016a) showed that EMs of radiata initiated at 28 °C exhibited higher proliferation percentages and produced the highest number of *se*. A second experiment was developed with the same purpose, to evaluate the effect of different environmental conditions along the SE process, for both species at the proliferation stage. In this case, different gellan gum concentrations (3.5, 4.5, and 5.5 gL^{-1}) were added to the proliferation media, and again, the explants were stored at three different temperatures (18, 23, and 28 °C). Standard conditions were applied at all other stages of the process.

The results presented by García-Mendiguren et al. (2016a) on radiata pine showed that more water availability, 3.5 gL^{-1} gellan gum, led to lower proliferation rates. EMs incubated at the highest temperature, 28 °C, also showed lower proliferation rates. Nonetheless, no significant effects on maturation rates or the number of SE produced were observed. Pereira et al. (2017) gave results for Aleppo pine, reporting opposite findings in *P. radiata*, showed that the interaction between temperature and water availability significantly affected the number of *se* produced per gram, and germinations rates, in agreement with results obtained by Kvaalen and Johnsen (2008) in *Picea abies*. A significantly higher number of *se* were obtained when the cultures were proliferated at 28 °C with 3.5 gL^{-1} gellan gum, whereas the highest germination rate was achieved from EMs proliferated at 28 °C in a culture media supplemented with 5.5 gL^{-1} gellan gum. As a conclusion, it can be stated that regarding *P. radiata* the initiation stage is crucial to all further steps, from initiation to maturation of *se*, having long-term effects on embryogenesis. On the other hand, with *P. halepensis*, manipulation of environmental conditions during the initiation stage only influenced the success of initiation and proliferation, while during the proliferation of embryonal masses, it caused an effect only several months later, in the production of somatic plants at the germination stage.

Protein Profiles of Somatic Embryos Derived from Different Water Availabilities and Temperatures at Initials Stages

SE can occur in tissue and cell cultures of a wide range of species, from herbaceous to woody plants. Nonetheless, the mechanisms of initiation and *se* development are not fully understood, so in recent years SE has been studied using discovery proteomics, in order to get deeper insight into the process (Klubicová et al. 2017). Proteomics is a very useful tool for studying stress tolerance, allowing the identification and quantification of stress-tolerance-associated proteins (Pinheiro et al. 2014), with the possibility to use protein markers for improving selection. The utilization of the global transcript expression or protein profiling (Lippert et al. 2005;

Zhao et al. 2015) has been used as an approach to generate such markers and for a better understanding of SE regulation. In this sense, a 2D proteomic analysis was carried out, aiming to determine if the abovementioned environmental conditions during the induction phase of SE in *P. radiata* resulted in differences in the protein profiles of *se* (García-Mendiguren et al. 2016b). Protein patterns in *se* from ECLs from treatments producing high percentages of initiation (18/23 °C, 4 gL^{-1}) and low percentages of initiation (28 °C, 2/3 gL^{-1}) were evaluated and compared. In *se* from treatments that led to low percentages of initiation, a prominent group of proteins involved in defense responses, such as the osmotically inducible protein OsmC, chaperone protein, and vicilins, was identified. Chaperones are known as heat shock proteins (HSPs), whose biological role is to assist folding of unfolded or misfolded proteins under stress conditions (Efeoğlu 2009), and are also known for their roles in the maturation, as they act as oxidative stress regulators (Marsoni et al. 2008). Proteins associated with other functional groups, such as the response to ROS, important signaling molecules reported to act in plant responses to biotic and abiotic stresses (Delledone et al. 2001), and in the development of SE of conifers (Zhang et al. 2010), or proteins related to gene expression, were also found in lower proportions. The presence of proteins involved in stress and metabolic responses indicates that changing environmental conditions seem to influence all the SE process.

Effect of Extreme Temperature Stress on the Induction Phase

Following on from the studies on the effects of different gelling agents and culture temperatures in the success of the SE process, further experiments were carried out in radiata pine with extreme temperature stress, in order to examine if those stresses can establish an epigenetic mark in the embryogenic cells, leading to plants with different ability to grow ex vitro under unfavorable conditions. For these studies, high temperatures (30, 40, 50, and 60 °C) for different incubation periods during initiation (Castander-Olarieta et al. 2019) were applied. Simultaneously, the success of the SE process was evaluated, and different morphological and molecular analyses were performed.

In line with previous studies (García-Mendiguren et al. 2016a; Pereira et al. 2016), the application of high temperatures for long exposure time periods during initiation had detrimental effects during the first steps of the SE process (initiation and proliferation), which reduced considerably the genetic diversity that could be induced by this technique. Nonetheless, the same treatments provoked an increase in the production of *se*, which agrees with the results reported by Fehér (2015). These authors postulated that stress acts like a selective pressure during initial phases of SE, improving the efficiency of the forthcoming steps. It is worth highlighting that the *se* obtained presented morphologies that usually led to low germination and rooting rates (Pullman et al. 2003). On the other hand, our results suggest that high temperatures for short periods did not provoke a decrease in initiation or

proliferation rates, but also promoted the production of *se*. Moreover, in this case, the embryos obtained were of better quality, bigger, and more elongated, suggesting that mild stresses enhance not only the production but also the quality of the embryos formed. For a better understanding of the effects of thermal stress during SE, an exhaustive micromorphological and ultrastructural analysis of the EMs was carried out. This study confirmed the presence of three cell types (embryogenic cells, tube-like cells, and suspensor cells) and three organizational structures (PEMI, PEMII, and PEMIII) in EMs, as already observed for other conifer species by Filonova et al. (2000) and Steiner et al. (2016). As a general trend, the application of high temperatures resulted in the loss of polarity and cellular disorganization of embryogenic structures (Fig. 7.2a, b, c, d), coupled with an increase in the number of vacuoles, plastolysome-like structures, whorls by cytoplasmic membranes, and starch grains around the nuclei (Fig. 7.3), all of them symptoms of programmed cell death (Smertenko and Bozhkov 2014).

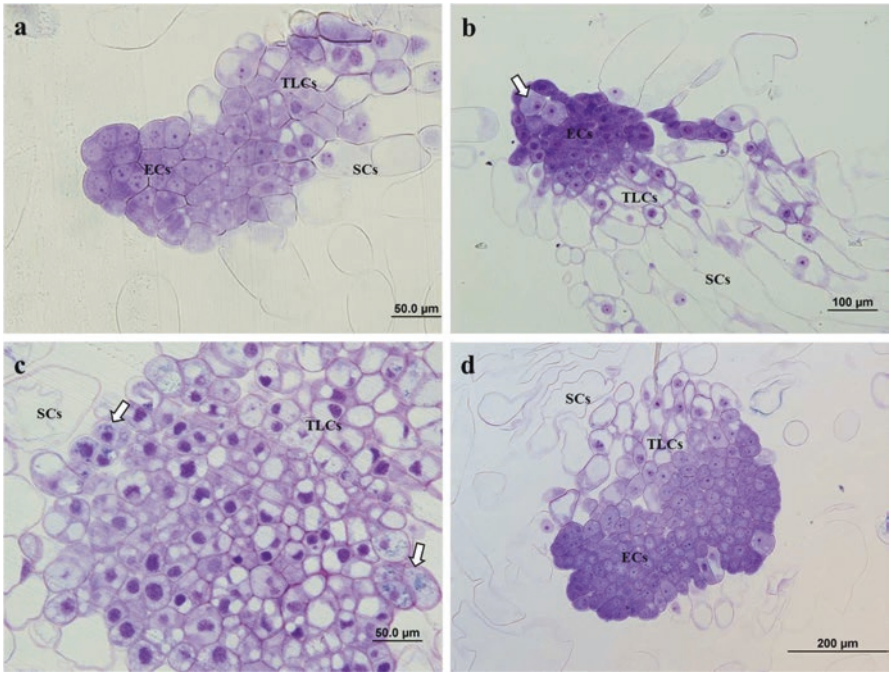

Fig. 7.2 Light microscopy and histochemical analyses of proembryogenic masses of *Pinus radiata* induced at different temperatures. The pictures show different cell types: embryogenic cells (ECs), tube-like cells (TLCs), and suspensor cells (SCs). (**a**) PEMIII from 23 °C presenting a clear polarization and well-organized structure. (**b**) PEMIII from 40 °C starting to lose polarization with enlarged TLCs arising in the middle of embryonal areas (arrow). (**c**) Embryonal area from 50 °C showing cells with advanced symptoms of programmed cell death (high vacuolation, compacted heterochromatin) and lost polarization. Small phenolic grains are also visible (arrows). (**d**) PEMIII from 60 °C forming a big cluster of compacted and well-organized ECs

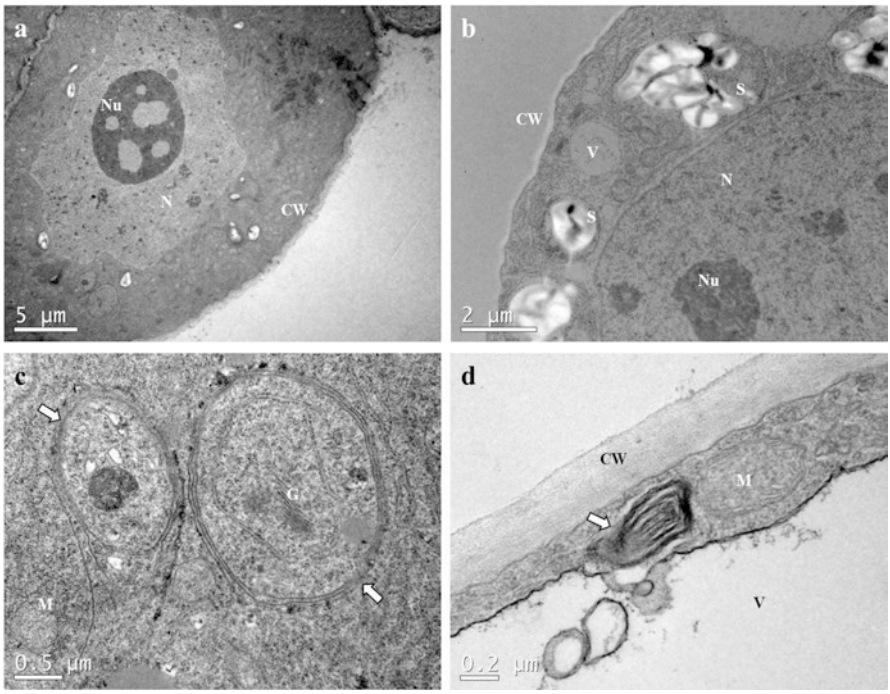

Fig. 7.3 Transmission electron microscopy images of ECs and TLCs from proembryogenic masses initiated at different temperatures. Cells present a great variety of organelles: nuclei (N), nucleoli (Nu), cell wall (CW), vacuole (V), mitochondria (M), Golgi bodies (G), and plastids with starch grains (S). (**a**) ECs from 23 °C with a dense cytoplasm and just a few S. (**b**) Details of ECs from 40 °C showing symptoms of programmed cell death such as a great number of V and S surrounding the N. Plastolysome-like structures engulfing different organelles (**c**) and whorls of cytoplasmatic membranes (**d**) from ECs and TLCs from 50 °C treatment, respectively (arrows)

Activation of programmed cell death by temperature was also reported by DaMatta and Cochicho Ramalho (2006). This phenomenon is known to increase the formation of suspensor cells (Abrahamsson et al. 2012), which together with the accumulation of starch grains around the nuclei are the principal causes of loss of embryogenic competence and formation of abnormal se (Morel et al. 2014; Merino et al. 2018). This was also observed with the application of high temperatures for long exposure times in *P. radiata* (Castander-Olarieta et al. 2019).

It is noticeable nevertheless that the same studies revealed an enhancement in the embryo formation when pulse-like treatments were applied. These conditions favored the presence of bigger and more developed embryogenic areas (Fig. 7.2d), which may explain the increase in embryo production and embryo quality previously observed.

Among the multiple fine-tuning strategies developed by plants against stress, readjustment of biochemical pathways and accumulation of elevated amounts of metabolites are two important examples. Heat and drought have overlapping roles

(Jia et al. 2017) and are known to activate similar molecular mechanisms driven by osmotic imbalances (Shinozaki et al. 2003; Gimeno et al. 2009). In forest species, these events lead to an enhanced synthesis and accumulation of soluble carbohydrates and amino acids (Patakas et al. 2002).

Sugars are described as taking an active part in numerous stress-mediated signal transduction pathways, and their activity as osmolytes, membrane stabilizers, and oxygen radical scavengers is well documented (Moradi et al. 2017; Woodrow et al. 2017). However, Castander-Olarieta et al. (2019) did not find pronounced differences in the carbohydrate profile of EMs exposed to different temperatures. However, the same authors detected significant differences in the amounts of certain amino acids, some of which are well known as playing important roles in plant stress tolerance acquisition. They are involved in osmotic adjustment, pH regulation, detoxification of reactive oxygen species, and synthesis of secondary metabolites (Corcuera et al. 2012; De Diego et al. 2015).

In opposition to numerous studies that highlight the importance of proline as one of the most active amino acids in stress responses (Wang et al. 2019). Castander-Olarieta et al. (2019) showed a clear participation of branched-chain amino acids in response to high temperatures. Isoleucine was the best example, whose levels increased with higher temperatures, suggesting its contribution to the synthesis of stress-induced proteins and to the regulation of gene expression (Joshi et al. 2010). Tyrosine also accumulated in all heat-shock treatments. This amino acid is a precursor of a wide range of secondary metabolites, among which phenolic compounds are of great importance because of their antioxidant role (Eliášová et al. 2017). Interestingly, light microscopy analysis carried out by the same authors revealed the presence of these compounds in the vacuoles of EMs subjected to the highest temperatures (Fig. 7.2c).

Future Perspectives

After having tested the feasibility of modulating the productivity and the quality of *se* by modifying different culture conditions during initial steps of SE in two pine species from contrasting habitats, our research team is also paying special attention to the effect of extreme temperatures (40, 50, and 60 °C) for different incubation periods, during the initiation phase, in SE of *P. halepensis*, for which information in this regard is still scarce. In that way we would be able to obtain more information about the different behaviors of this pine species and the mechanisms they employ to face stress.

Likewise, we consider it is of great interest to unravel the role that phytohormones are playing in these developmental and stress-response processes. Plant hormones, apart from modulating numerous physiological and developmental responses (Wani et al. 2016) and being essential during in vitro organogenesis and SE (Moncaleán et al. 2003, 2005, 2018; Montalbán et al. 2011), are active compounds during stress responses (De Diego et al. 2012, 2015).

Following the studies previously described (García-Mendiguren et al. 2016b), a deep study of the proteomic response of EMs and *se* to extreme temperatures will be performed. Proteomics is a very useful tool for studying stress response. The identification and quantification of proteins associated with stress-tolerance response accompanied with the possibility to use protein markers for improving selection can help the understanding of SE regulation, the mechanisms involved in the process, and the possible tolerance of the plants obtained (Klubicová et al. 2017).

Furthermore, we are also testing the drought resilience of somatic plants generated from EMs cultured under high temperatures, in order to assess if all the changes observed in previous studies at in vitro stages persist until reaching plant level and alter their capacity to face stress. This could prove a great advance toward the production of plants pre-adapted to unfavorable conditions. Finally, we are examining the expression patterns of stress-related genes and their methylation state on EMs, *se*, in vitro somatic plants and plants subjected to stress in the greenhouse.

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Part II
Current Topics in Biodiversity, Conservation, and Environmental Impact

Current Topics in Biodiversity, Conservation, and Environmental Impact will focus on innovative ideas for the enrichment of biodiversity, conservation biotechnology, biodiversity for biocontrol, and environmental biotechnology. Environmental biotechnology will cover the use of biotechnology to aid the recovery of water and land contaminations focusing on wastewater biosorption and soil bioremediation of dangerous elements such as heavy metals.

Chapter 8
A Protective Role for Accumulated Dry Matter Reserves in Seeds During Desiccation: Implications for Conservation

Hector Pérez, Lisa M. Hill, and Christina Walters

Abstract We live in an unprecedented time of plant biodiversity loss. Current extinction rates are three orders of magnitude faster than extinction rates measured over geologic time. Similarly, 30% of plants are threatened with extinction. This information is startling when one considers that humans depend on plants for life. Fortunately, several systems exist to conserve plant genetic diversity. Seed storage within genebanks represents the most widely utilized system for plant conservation. One advantage of genebanking is that seed viability can be maintained for decades to centuries. Seeds must tolerate extensive post-harvest drying (5–10% moisture content) and cold (−18 °C) to maintain shelf-life for these periods. However, many important tropical seeds cannot tolerate drying to these levels thus precluding genebank storage. But what separates desiccation-tolerant from sensitive seeds? Previous hypotheses related to protective roles for certain sugars and proteins or the formation of intracellular glasses are insufficient. For instance, desiccation-tolerant and desiccation-sensitive seeds accumulate the same types and levels of protective molecules. Likewise, desiccation-sensitive seeds form intracellular glasses if dried sufficiently. Alternatively, using seeds of a tropical palm as a model, our work identifies a critical minimum level of cellular dry matter accumulation for appropriate desiccation tolerance. Our model suggests that cells must acquire >35% dry matter reserves to avoid lethal desiccation stress prior to genebanking. This level of dry matter accumulation may serve as a reference point for future breeding efforts or manipulation of the seed developmental program to enhance desiccation tolerance.

Keywords Conservation · Dry matter · Seed desiccation · Genebank

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Introduction

Humans are living in an unprecedented time of wild plant biodiversity loss. For instance, Pimm et al. (2014) estimate that 30% of plant species are threatened with extinction. The same authors maintain that current extinction rates, measured as extinctions per million species years, are three orders of magnitude faster than extinction rates measured over geological time. These two pieces of information combined with the fact that humans depend on plants for survival are unsettling. Moreover, we are not only losing plants in danger of extinction. We are losing crop wild relatives that contain important genetic information. We are losing undiscovered species that form networks that provide crucial ecological services. We are also losing many plants that may serve as sources of medicines, foods, fiber, and aesthetic reasons. At the same time, we are breeding crops with new traits that should be preserved. Many systems exist for conservation of plant genetic diversity each with benefits and limitations (Frankel et al. 1995). For example, a country may decide to designate vast areas as reserves for in situ conservation. In this scheme, plants can be conserved in their original community. Alternatively, constraints may exist regarding the purpose, management, and financing of such reserves. Ex situ conservation represents another set of methods for preserving of plant genetic diversity. Field genebanks have the advantage of continual evaluation of important traits and direct utilization of plant materials in breeding programs. However, field genebanks are expensive to operate and generally cannot cover all the genetic diversity represented in a species. Plants in field genebanks are also exposed to pests, pathogens, and natural disasters. Tissue culture genebanks represent a good method to conserve species that propagate asexually and/or do not readily produce seeds. However, this method is costly and requires labor with technical skills. Contamination and somaclonal variation in culture also represent other risks. Seed genebanks are advantageous because large quantities of seeds can be stored in relatively small area. Such stores may encompass most of the genetic diversity of a species. Management of seeds in storage is typically less labor intensive than other methods, and seeds are generally stored in an environment free of pests and pathogens. Yet, multiplication of accessions is essential prior to a critical drop in viability, and seed genebanks may require electricity or other critical infrastructure (FAO 2014). Nonetheless, seed genebanks represent the most globally utilized method of ex situ conservation.

Seed Desiccation Tolerance: An Essential Functional Trait for Seed Genebanking

The essential prerequisite to realize advantages associated with seed genebanking is that seeds must tolerate considerable levels of post-harvest desiccation (Walters 1998). International genebank standards suggest drying seeds in equilibrium with

15% relative humidity prior to storage (FAO 2014). This translates to a tissue water potential of about −260 MPa. Recall that most plant tissues succumb when water potential falls below about −2 MPa. Fortunately, predictions estimate that about 75–80% of plant species produce seeds that tolerate the levels of desiccation necessary for storage under genebank conditions (Dickie and Pritchard 2002). Conversely, many important tropical species produce seeds that do not tolerate the extreme levels of drying necessary for genebank storage. However, what separates desiccation sensitive from tolerant seeds? Previous thinking centered on the protective role of accumulated proteins, such as LEAs, sugars, and the formation of intracellular glasses that maintain cellular integrity during drying. Yet, these hypotheses evolved given that desiccation-sensitive and desiccation-tolerant seeds possess the same types, levels, and distribution of important macromolecules. Intracellular glasses also form within cells of sensitive seeds if dried sufficiently (Berjak and Pammenter, 2008; Bewley et al. 2012; Kermode and Finch-Savage 2002; Vertucci and Farrant 1995; Walters 1998; Walters et al. 2005, 2010). If this is the case, then why does seed desiccation sensitivity exist?

Building a Dry Matter Accumulation-Based Model to Address Seed Desiccation Sensitivity

We took a seed developmental approach to investigate the question of seed desiccation sensitivity. Specifically, we examined the accumulation of dry matter reserves, seed water relations, and changes in cellular volume associated with desiccation stress. We sought to determine how dry matter accumulation and changes in seed-water relations throughout seed development influence changes in cellular volume. This resulted in a model of permissible cellular contraction during drying (Pérez et al. 2012). Previous work from Merryman (1974) and Steponkus (1995) was the basis for our model. Those authors advanced a minimum critical cellular volume theory. For example, cellular volume in a hydrated cell begins to change at the onset of desiccation. Cells begin to shrink as these volume changes occur. Desiccation imposes various mechanical stresses on membranes and organelles as cellular volume decreases. Essentially, cells cannot recuperate from mechanical stresses imposed by drying when cellular volume decreased to 50–65%. Cellular damage manifests itself upon rehydration as water enters cells and volume began to increase.

Another important component of our model centers on differences in seed developmental programs between seeds expressing desiccation sensitivity or tolerance. Desiccation-tolerant seeds exhibit developmental patterns during histo-differentiation that is characterized by large increases in fresh mass and water content. Here the mother plant maintains vascular connections to developing seeds in order to maintain tissues in a hydrated state necessary for cell growth and division. Seeds begin accumulating dry matter during the latter stages of histo-differentiation. Subsequently, large increases in dry matter denote the reserve accumulation phase.

Water content also decreases during this phase as accumulated dry matter reserves begin displacing cellular water. An important aspect of this phase is that desiccation-tolerant seeds exhibit the phenomena of physiological maturity. In other words, dry matter accumulation reaches a maximum as cellular volume becomes packed with proteins, lipids, and carbohydrates. Maturation drying represents the final phase of seed development. Here, seed water content drops considerably as vascular connections between seeds and the mother plant sever. Relative humidity gradients between seeds and the surrounding environment may drive continued drying.

Desiccation-sensitive seeds exhibit patterns like tolerant seeds during the early stages of development. However, desiccation-sensitive seeds continue accumulating dry matter and do not appear to reach a state of physiological maturity prior to shedding from the parent plant. Also, desiccation-sensitive seeds typically do no present large decreases in water content during the final stage of development. Therefore, two important points emerge. First, all cells display a minimum critical cellular volume. Second, the accumulation of dry matter reserves varies between desiccation-tolerant and desiccation-sensitive seeds. We utilized seeds of *Pritchardia remota* (Arecaceae) to develop our model of lethal reductions in cellular volume. *Pritchardia* represents a group of tropical palms occurring throughout Hawaii and the south Pacific region. Our study focused on the accumulation of dry matter reserves and water relations in developing embryos. Likewise, we employed transmission electron microscopy (TEM) to visualize cellular architecture and how this changed during embryo development. We worked with embryos for several reasons. First, seeds are massive compared to embryos and are composed principally of endosperm (Fig. 8.1). Seeds require 2–3 months to complete germination, while embryos germinate in tissue culture within 1–2 weeks (Pérez et al. 2008; Pérez et al. 2012). Moreover, development of the endosperm obscures developmental changes in embryos. For instance, compare the *y*-axis scale in Fig. 8.2a, b.

We calculated changes in embryo water potential throughout development and noted that embryo water potential reached −25 MPa at seed shedding (Fig. 8.3a). This water potential is below the lethal limit for many desiccation-sensitive seeds (Walters et al., 2005). We also dried embryos over a stream of N_2 and determined a critical water potential of −49 MPa. Embryos did not survive lower levels of drying as indicated by in vitro embryo germination assays (Pérez et al. 2012). We then examined how embryo water content changed with changes in water potential during development (Fig. 8.3b). We used this information along with cell measurements collected from TEM images to calculate changes in cellular volume.

Embryos are in the early stages of development at 100 days after anthesis (DAA). Embryonic cells are highly vacuolated and filled mostly with water. A lack of dry matter reserves also characterizes these cells (Fig. 8.4a). Alternatively, embryos are physiologically mature by 340 DAA. This signifies that embryos reached maximum accumulation of dry matter. Vacuoles are still evident but appear filled with some level of dry matter. Likewise, the cytosol also displays accumulation of dry matter (Fig. 8.4b, c). About 30% of the cell volume was occupied by dry matter reserves (Pérez et al. 2012).

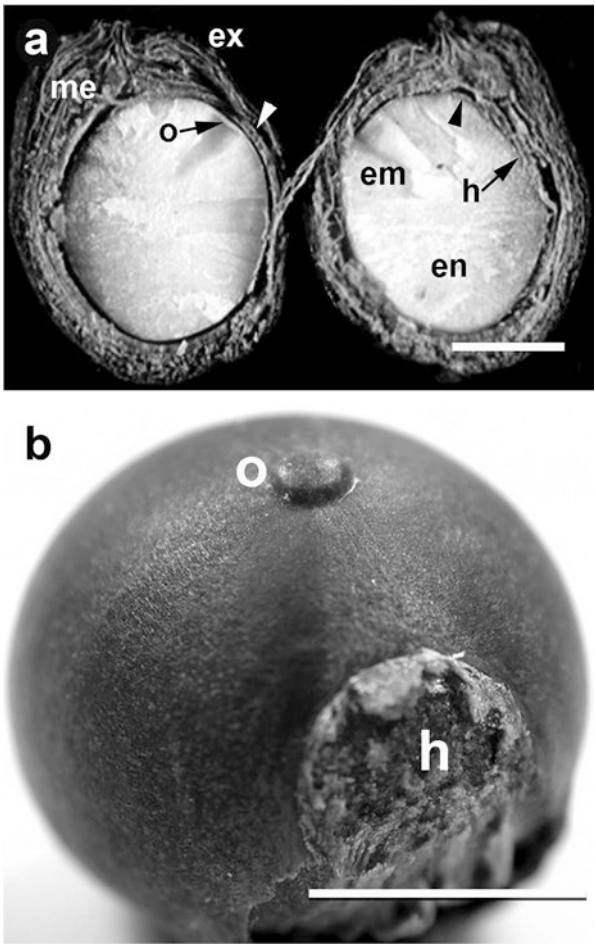

Fig. 8.1 (**a**) Cross section of *Pritchardia remota* fruit and seed. The pericarp consists of the fleshy exocarp (ex), fibrous mesocarp (me), and thick endocarp (white arrowhead). The seed is composed primarily of endosperm (en) and a relatively thin testa (black arrowhead). The reduced embryo (em) sits beneath the operculum (o) and adjacent to the hilum (h). (**b**) Close of the seed showing the operculum and hilar scar. Scale bars in (**a**) and (**b**) 1 and 2 cm, respectively. (Adapted from Pérez et al. (2008))

Small changes in cellular volume were evident at either stage of development when we applied low levels of desiccation stress (−0.5 MPa). However, cellular volume in young embryos reduced by about 65–80% following drying stress at −5 or −15 MPa. Cells enter the range of lethal cell volume reduction and did not germinate in vitro (Fig. 8.5). In contrast, cellular volume reductions do not enter the lethal range following desiccation stress at −5 or −15 MPa for mature embryos due to some buffering via accumulated dry matter. However, desiccation stress to −49 MPa induces lethal reductions in cellular volume (Fig. 8.5) as confirmed via in vitro germination assays (Pérez et al. 2012).

This is important when considering storage of germplasm in accordance with international genebank standards. These standards call for drying seeds in equilibrium with 15% relative humidity. This relative humidity value translates to about −260 MPa. *Pritchardia* embryos are capable of tolerating some levels of desiccation

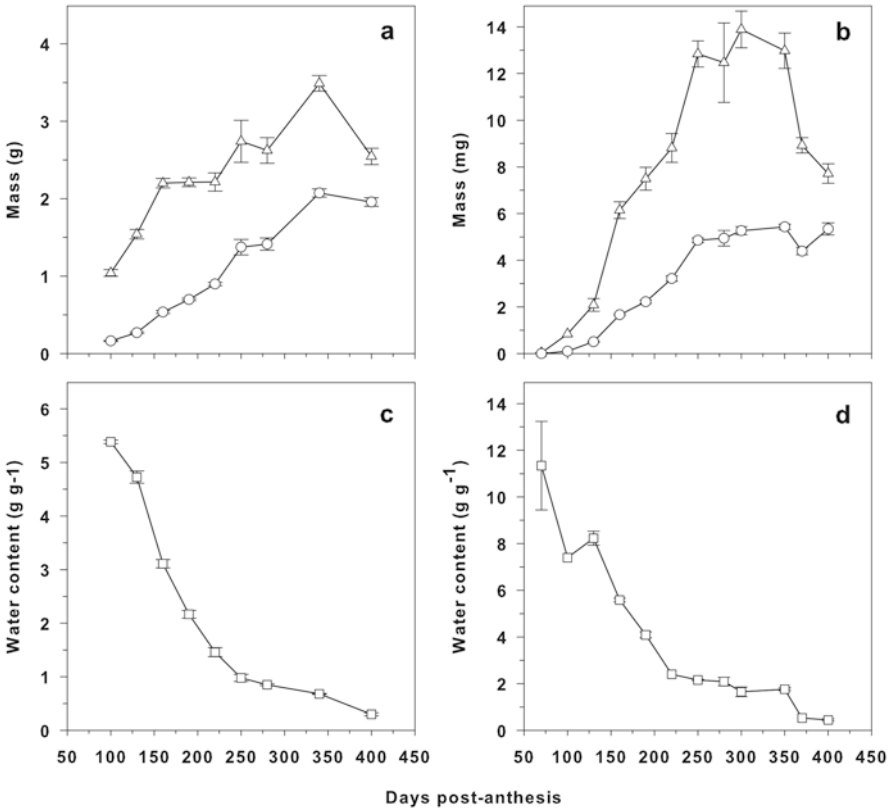

Fig. 8.2 Changes in accumulation of dry matter reserves and water content for *Pritchardia remota* seeds (**a**, **c**) and embryos (**b**, **d**) during development. Fresh mass, dry mass, and water content denoted by triangles, circles, and squares, respectively. Error bars denote standard error of the mean. (Adapted from Pérez et al. (2012))

stress but are sensitive to the level of drying necessary for long-term storage within genebanks.

Conclusions

Our measurements suggest that cell volume within developing seeds and/or embryos must consist of at least 35% dry matter reserves to avoid lethal reduction in cellular volume. Cellular volume will decrease to some extent when cells achieve this level of dry matter reserves. The key difference is that volume reductions will not be catastrophic to membranes and organelles. Accumulating more than 35% is advantageous to mitigate high levels of mechanical stress imposed by extreme drying. We propose that many species producing desiccation-sensitive seeds, like many tropical

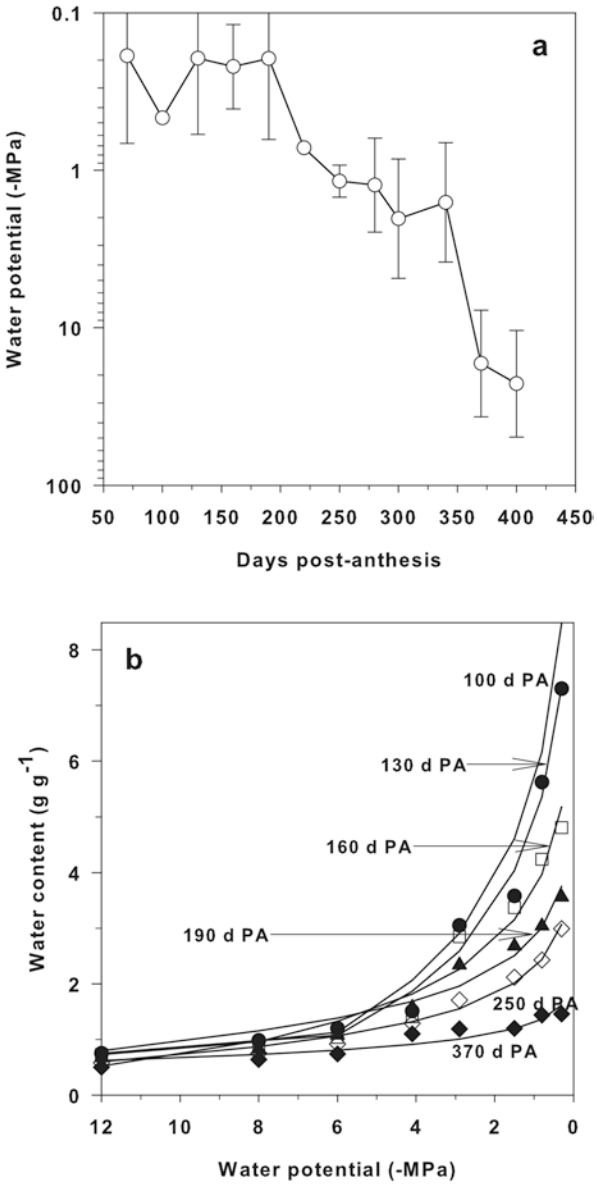

Fig. 8.3 *Pritchardia remota* embryo water relations during development. (**a**) Changes in water potential throughout the developmental program. (**b**) Pressure-volume curves calculated from water content-water potential relationships of embryos measured using polyethylene glycol solutions of various water potentials. Curves were calculated from regression (r2 range 0.92–0.98) of water content with ln (water potential) for water potentials ≤ -8 MPa. (Adapted from Pérez et al. (2012))

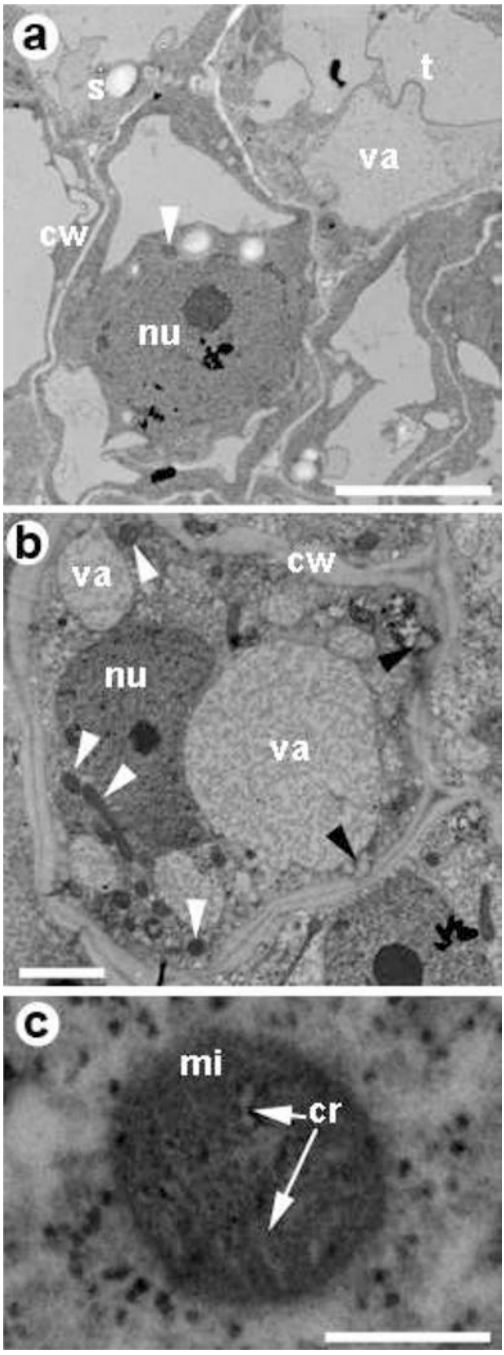

Fig. 8.4 Ultrastructural changes for *Pritchardia remota* embryos at 100 (**a**) and 400 (**b**, **c**) days postanthesis. Detail of mitochondria in (**c**) showing de-differentiation. Scale bars in (**a**), (**b**), and (**c**) are 5 µm, 2 µm, and 200 nm, respectively. Black arrowhead, lipid droplet; cr cristae, cw cell wall, mi and white arrowheads, mitochondria, nu nucleus, s starch granule, t tonoplast, va vacuole. (From Pérez et al. (Pérez et al. 2012). Cambridge University Press ©, reprinted with permission)

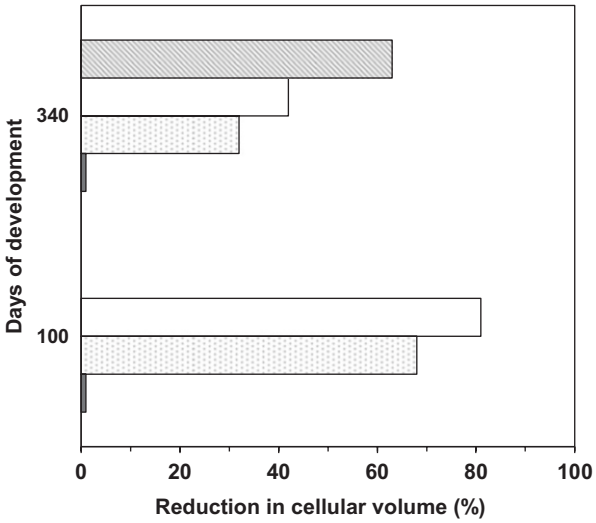

Fig. 8.5 Modeled reductions in cellular volume for developing *Pritchardia remota* embryos following various levels of desiccation stress induced by exposure to drying in equilibrium with water potentials of −0.5 (black bar), −5 (dotted bar), −15 (white bar), or −49 (diagonal crosshatch bar) MPa. Gray vertical bar represents the range of lethal reductions in cellular volume proposed by Meryman (1974) and Steponkus et al. (1995)

species, do not accumulate sufficient dry matter reserves prior to shedding from the parent plant. Perhaps the 35% value may serve as a reference point for future breeding efforts or research on the manipulation of seed development programs to improve seed desiccation tolerance. Clearly, working with more tropical species will strengthen our model.

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Chapter 9
Biodiversity in Ecuador and Its Immense Potential for Agricultural Pest Control

Carmen Isabel Castillo Carrillo

Abstract Biological control in Ecuador was studied and applied before 1937; unfortunately extensive and detailed reports are non-existent or not easily found. These valuable details are not commonly known in our country. We have examined published information about biological control of agricultural pests in universities and research centres to find contributions that could be applied directly or to serve as models for other cases. This chapter offers a deep compilation of the biodiversity identified as natural enemies of agricultural pests from the first reports found until the last few years in Ecuador. Researchers of Instituto Nacional de Investigaciones Agropecuarias (INIAP) together with undergraduate to postgraduate students and academics from several higher agrarian education branches have scanned information from cropped and non-cropped areas to find natural enemies of agricultural pests. Great findings are available, but usually in local publications. This is the opportunity to highlight these discoveries and offer the technology for enterprises, entrepreneurs and progressive farmers who will dare to generate and use natural enemies for pest control in a healthier and environmentally low-impact agriculture.

Keywords Biodiversity · Ecuador · Agricultural pest control · Environmental agriculture

Introduction

Applied biocontrol in agriculture has a history of more than 80 years in Ecuador. Classical and inundative biological controls have been applied to several staple and cash crops and in fruit and ornamental trees covering more than 300,000 hectares in

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the country (Castillo Carrillo et al. 2020 in press). Several research programmes and surveys have been done over time, but some of them are poorly known. The purpose of this chapter is to offer a compilation of the biodiversity used in inundative and conservationist biocontrol of agricultural pests in Ecuador, as well as allowing the results of research and surveys to serve as sources of information to develop more programmes of applied effective biocontrol. The chapter will be divided into nine groups to address the findings: recommendations and protocols that have been developed about parasitoids, predators, entomopathogenic nematodes, bacteriophages, entomopathogenic viruses, entomopathogenic fungi, nematode-parasitic fungi, antagonist and plant-growth-promoter fungi, and beneficial pseudomonads.

Parasitoids

Parasitoids were used to control pests in extensive acreage in the coastal regions in Ecuador in the 1980s, mainly in maize, soya bean, cotton, banana and sugar cane. The parasitoid belonging to the *Trichogramma* genus (Hymenoptera: Trichogrammatidae) was used against the sugarcane borer *Diatraea saccharalis* Fab. (Lepidoptera: Pyralidae) and the fall armyworm *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). The initiator of this massive use over a period of 10 years was the company Biológicos Ecuatorianos S. A. (BIOESA), which annually produced 3 billion parasitoids. Sadly, the company went out of business because the demand for parasitoids went down (Castillo Carrillo et al. 2020 in press). A good attempt at biocontrol of the coffee berry borer *Hypothenemus hampei* (Coleopteran: Scolytidae) was the use of the imported parasitoids *Prorops nasuta* Waterston (Hymenoptera: Bethylidae) and *Cephalonomia stephanoderis* (Hymenoptera: Bethylidae) from Africa (Klein-Koch 1988). After multiplication and releases for 3 years, these species reached parasitism rates of 23% and 83%, respectively (Mendoza et al. 1994). In other case, millions of *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) were introduced in Ecuador in 1978 from the USA, to control the cottony-cushion scale *Icerya purchasi* Maskell (Hempitera: Margarodidae) in fruit trees and ornamental plants (Molineros 1984). The last two cases are the main examples of classical biocontrol used in Ecuador in earlier times.

Nowadays, one of the main extensive crops in Ecuador is sugarcane. In 2017, more than 100,000 hectares were planted mainly in the coastal region, and biological pest control is used in an approximately one-third of the total acreage. *Billaea claripalpis* (Wulp) (Diptera: Tachinidae), a parasitic tachinid, is grown and used against the sugarcane borer *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae). Centro de Investigación de la Caña de Azúcar del Ecuador (CINCAE; Sugar Cane Research Centre of Ecuador) has been a leader in this biocontrol system and has inventoried more than 26 species that are natural enemies of pests in this crop (Mendoza 2018).

Potato is a staple crop in the Ecuadorian highlands. More than 30,000 hectares of potato have been planted and rely on chemical sprays to control pests. Nevertheless,

the search of natural enemies for pest control is constant. The parasitoid *Copidosoma koehleri* Blanchard (*Hymenoptera: Encyrtidae*) was found parasitizing eggs of the potato tuber moth *Phthorimaea operculella* (Zeller) (*Lepidoptera: Gelechiidae*). *Apanteles* sp. (*Hymenoptera: Braconidae*) was found to have parasitized larvae of the Andean potato tuber moth *Symmetrischema tangolias* (Gyer.) (*Lepidoptera: Gelechiidae*) (Báez and Gallegos 2013). More natural enemies of potato pests are reported in later groupings.

Banana, another cash crop grown on the coast, has an important scale insect pest *Dysmicoccus texensis* Ferris (*Hemiptera: Pseudococcidae*), which feeds by sucking plant sap and transmitting the Banana streak viruses (BSV), causing economic losses. The parasitoid *Hambletonia pseudococcina* Compere (Hymenoptera: Encyrtidae) was studied for mass-rearing to control *D. texensis*. The scale insect was best reared in pumpkin and the best diet to increase the longevity of the parasitoid was using 1 mL of water plus 4 mL of honey and 0.01 mg of pollen. Field releases of 200 parasitoids succeeded in controlling the scale with more than an 80% parasitism. After 3 months from the releases, there was a level of 44% of parasitism still present in the field (Cuzco 2014; INIAP-EELS 2014).

In rice, *Tibraca limbativentris* Stal (Hemiptera: Pentatomidae) is an important pest in Ecuador. In 1992, the egg parasitoid *Telenomus* sp. (Hymenoptera: Scelionidae) was found controlling the pest in a range of 44–70% in eight localities in Guayas and Los Rios provinces. When the eggs parasited by *Telenomus* become black, after 8–12 days the parasitoid emerges. A female wasp can lay 35–40 eggs per hour (Mata 1992).

In 1999, there was a study to determine a mass-rearing system for the parasitoid *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae), using eggs of *Sitotroga cerealella* Olivier (Lepidoptera: Gelechiidae). *T. pretiosum* emerged in a range of 70–92% when reared in a temperature range of 14 °C to 26 °C at 90% of relative humidity in 11–12 days. This research determined a storage period of 25 days of the parasited eggs at 4 °C before starting to reduce parasitoid survival (Moreno 1999).

In 2016, in San Cristobal island in the Galapagos, Instituto Nacional de Investigaciones Agropecuarias (INIAP; National Institute of Agriculture Research) studied the presence of *Ageniaspis citricola* Logvinovskaya (Hymenoptera: Encyrtidae) as a parasitoid of the citrus leaf miner *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae), with a parasitism rate from 56% to 60%. *A. citricola* was the main natural enemy of the leaf miner because others such as Coccinelidae (Coleoptera), Dolichopodidae (Diptera), Chrysopidae (Neuroptera), Vespidae (Hymenoptera) and Anthocoridae (Hemiptera) were found in very low quantities (INIAP-EELS 2016).

Fruit flies are the most important pests in fruit production in the coastal region of Ecuador. The main species are *Ceratitis capitata* (Wiedemann), *Stenoma cecropia* Wiedemann, *A. obliqua* Macquart, and *A. striata* Schiner (all Diptera: Tephritidae). To control these species, the farmers assign between 10% and 15% of the total production budget. INIAP conducted research to assess the capacity of parasitism of *Diachasmimorpha longicaudata* (Ashmead) (HYMENOPTERA: Braconidae) in the

following fruit fly species: *D. longicaudata* parasited 65% of *C. capitata* larvae, 72% of *A. fraterculus*, 80% of *A. serpentina*, 74% of *A. striata*, and 15% of *A. obliqua*. The highest parasitism was in *A. serpentina* with 80%. The survival means for *D. longicaudata* reared in *C. capitata* were 19 days for females and 15 days for males, in *A. fraterculus* they were 15 days (female) and 14 days (male), in *A. serpentina* were 31 days (female) and 14 days (male), in *A. striata* were 16 days (female) and 12 days (male), and in *A. obliqua* 3 days for female (no males emerged) (Sánchez 2007).

Oil palm (*Elaeis guineensis* Jacquin) is planted in more than 300,000 hectares in Ecuador. One of the major pests in this crop is the leaf eater *Stenoma cecropia* Meyrick (Lepidoptera: Stenomidae). La Asociación Nacional de Cultivadores de Palma Aceitera (ANCUPA; National Association of Palm Growers) made an inventory of the natural enemies of this pest. Larval parasitoids *Elachertus* sp. and *Horismenus* sp. (Hymenoptera: Eulophidae) were found attacking early larval stages (1–4 instar), the parasitoid *Aprostocetus* sp. (Hymenoptera: Eulophidae) parasiting late instars (5–8) and *Brachymeria* sp. (Hymenoptera: Chalcididae) parasiting pupa stages (Quishpe 2009). Other natural enemies detected by this study will be described in some of the next groupings.

Naranjilla (*Solarium quitoense*) is an Andean fruit cultivated in the warmer edges of the eastern and western highlands. The fruit borer (*Neoleucinodes elegantalis* (Guenée) (Lepidoptera: Crambidae)) is the most significant insect pest in naranjilla, causing economic losses of around 90% of the production. In an extensive survey performed in Pichincha, Tungurahua, Pastaza, and Napo provinces, larval parasitoids such as *Meteorus* sp. (Hymenoptera: Braconidae) and *Lymeon* sp. (Hymenoptera: Ichneumonidae) were found. *Copidosoma* sp. (Hymenoptera: Encyrtidae) was the most relevant, found in some localities in a parasitism percentage around 1.36% (Sosa 2009).

Studies on *Aphidius colemani* Viereck (Hymenoptera: Aphidiidae) were performed to establish a rearing protocol. At laboratory conditions (19 °C, 46–60% RH) and using *Brevicoryne brassicae* (L.) (Hemiptera: Aphididae) as host, *A. colemani* has a life circle of 13.5 days with a 72% of emergence, but at warmer conditions (25 °C, 60–70% RH) the cycle was completed in 10.5 days with 65% emergence. In 1 year, it is possible to have between 27 and 34 generations. When fed with honey and water, the female adults survived for 17.3 days and the males for 38.3 days. *A. colemani* parasited few aphids of *Aphis medicaginis*, and none of *A. cytisorum*. *B. brassicae* was reared on cabbage and broccoli in the field to provide food for the aphids. In white-painted plastic bottles, some leaves with aphids are placed and parasitoids added in a ratio of 8:1, getting a 97% of parasitoid emergence (Reyes 2009).

To control of the corn fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae), releases of *Telenomus remus* Nixon (Hymenoptera: Scelionidae) were studied in field experiments. At INIAP's research station Litoral Sur, a temporary rearing platform was established for both host and parasitoid. Wasps were released in a corn trial every 7, 15, and 30 days in batches of 41 individuals per plot. Releases every week were the most effective in controlling *S. frugiperda* showing less plant damage (Castillo 2013).

Predators

Podisus connexivus Bergroth (Hemiptera: Pentatomidae) was studied in order to control eight species of lepidopteran larvae (noctuids) of economic importance in agriculture: *Peridroma saucia* Hübner, *Copitarsia turbata* H., *C. consueta* (Walker), *Agrotis deprivata* W., *A. ipsilon* (Hufnagel), *Dargida graminivora*, *S. frugiperda* and *Helicoverpa zea* (Boddie), with *P. connexivus* showing a feeding preference on *A. deprivata*. In general, the pentatomid preferred the sixth-instar noctuid larvae. Also, it was mentioned the predator showed cannibalism when mass reared, possessed a longevity of 100 days, and an oviposition rate of 290 eggs. The adult stage was the most aggressive predator (Pérez 1992).

Three plant hosts for *Orius florentiae* (Herring) (Hemiptera: Anthocoridae), a predator of thrips, were assessed for laboratory rearing. Adults of the anthocorid were collected in Chimborazo province and placed (7 couples) in plastic bottles containing water and flowered stems of potato, lupinus (*Lupinus mutabilis*), and cassia (*Cassia canescens*). Cassia was the best host for *O. florentiae* with a total of 486 laid eggs (in 11 days), with a mean of 51 egg hatched, and 50 adults developed (Ortega 2010).

Orius sp. was identified as predator of the banana rust thrips, *Chaetanaphothrips signipennis* (Bagnall) (Thysanoptera: Thripidae) in Los Rios province. In laboratory studies, the predation capacity of thrips by the anthocorid stages was as follows: *Orius* at the III development stage had percentages of predation from 31% to 43% on thrips in the I, II, and III stages, and *Orius* at the IV development stage had percentages from 18% to 48% on same three first stages. *Orius* of the V development stage had predations from 53% to 67%. Adults of *Orius* were the predators of the pre-pupa in a 79%, pupa in an 80%, and the adult thrips at 91% (INIAP-EELS 2015).

In order to assess its potential as a predator, the life cycle of *Cybocephalus nipponicus* Endrody-Yöunga (Coleoptera: Cybocephalidae) reared and fed with the banana scale *Aspidiotus destructor* Signoret (Hemiptera: Diaspididae) was studied in laboratory conditions. The beetle was fed with different development stages of *A. destructor*. The mean time to complete the life cycle of *C. nipponicus* was 27 days in total, 5 days as egg, 8 days as larva and 14 days as pupa. *C. nipponicus* adults live around 40 days. *C. nipponicus* ate a mean of 10 nymphs of the first stage, 15 of the second stage, 24 immature scales, 26 mature scales, and 44 eggs of *A destructor*, for 43 days. Groups of 5 predator larvae ate 21 female scales and 12 male pupae of the scale, in an 8-day period. Confined multiplication of the predator beetle reached 153% (Arias 2006).

For oil palm, two predators of *S. cecropia* were identified as natural enemies of this defoliator. The ant *Brachymyrmex* sp. and a species of the Reduviidae family were found eating larvae of the fifth to eighth instars (Quishpe 2009).

Laboratory and field trials were performed to study the predatory action of *Coenosia attenuata* Stein (Diptera: Muscidae) over *Liriomyza* spp. (Diptera: Agromyzidae) on *Gypsophila paniculata* and *Trialeurodes vaporariorum* Westwood

(Hemiptera: Aleyrodidae) on tomato (*S. lycopersicum*). Releases of a mean of 2.1 *C. attenuata* per plant showed an 80% of control efficiency for *Liriomyza* spp., and a 41.4% for *T. vaporariorum* (Urbano et al. 2018). Further research gave a protocol for mass-rearing of *C. attenuata* in a substrate made with 50% of coffee fibre plus 50% of a mix (60% peat + 40% zeolite). This substrate obtained the right capacity for water content and porosity. Adults and larvae of *Drosophila melanogaster* and *Liriomyza* spp. were added as food for the predator. Plastic containers held the mix where *C. attenuata* laid eggs and larvae fed on it (Albán et al. 2018). Details of *D. melanogaster* and *Liriomyza* spp. rearing are in Albán et al. (2018).

Further research for *C. attenuata* rearing tested mixes using oil palm fibre (with and without manure), earthworm humus (with and without manure), and only manure. Temperature conditions in the greenhouse were 19–30 °C and the RH of the substrates were around 70%. All substrates were suitable for *C. attenuata* rearing. Best results for egg hatching were obtained by the substrates using oil palm fibre and manure (90% hatching). The life cycle was completed in 29 days (Anrango et al. 2018).

Entomopathogenic Nematodes

In 2005, soil sampling for entomopathogenic nematodes took place in potato systems (fields and storage places) and in natural vegetation in the provinces of Carchi, Cotopaxi, Chimborazo and Tungurahua. Fifteen isolates of the genus *Steinernema* and 13 of the genus *Heterorhabditis* were identified (Argotti et al. 2011), and 8 isolates were efficient in controlling *Premnotrypes vorax* (Hustache) (Coleoptera: Curculionidae) in greenhouse and field trials (Chacón 2011).

Among the pests causing low yields (in oil palm), the root borer *Sagalassa valida* Walker (Lepidoptera: Glyphipterigidae) is one of the most detrimental and common pests in the northern region of the coast for oil palm. The National Association of Oil Palm Growers (ANCUPA) reports a survey of natural enemies of the root borer in Esmeraldas province. Eighteen isolates of entomopathogenic fungi and 19 isolates of bacteria were found, but the most abundant was the entomopathogenic nematode population of Heterorhabditidae. Even though the nematode species was not identified, trials in the laboratory determined a population of 7000 infective juveniles are necessary to reach a mortality rate of 86% of the root borer larvae, with a mean lethal time of 9 days (Cofre 2010). Next step was to study the mass-rearing of the entomopathogenic nematodes. Three methodologies to multiply nematodes were studied. Two artificial media based in potato–yeast–sweet potato, pig liver and dog food were tested together with multiplication in *Galleria mellonella*, the bee wax moth. The best multiplication system was the use of *G. mellonella* at a time of 14 days, when it had the highest population of infective juveniles. A field trial was performed to assess the best doses per 3-year-old palm in the field (a low dose with 750,000 infective juveniles, a medium with 1.5 million, and a high with two million) to control the root borer *S. valida*. All the doses were compared with a control using

Endosulfan® (10 ml/palm in three applications). The dose of 1.5 million nematodes gave similar control levels as the chemical treatment in the field (Chica 2010).

Ten entomopathogenic nematodes were molecularly analysed in 2016. Nematodes were extracted from infected larvae of three main potato pests *Phyllophaga* sp. (Coleoptera: Scarabaeidae), *P. vorax* and *Tecia solanivora* Povolny (Lepidoptera: Gelechiidae) from three provinces Carchi, Chimborazo and Cotopaxi in a previous survey. Collected nematodes were preserved and renovated according to Lacey 1997. ITS (800–1000 bp) and 28S (500–800 bp) regions were amplified by PCR with TW81/AB28 primers (Reid et al. 1997) and D2A/D3B primers (Nadler et al. 1999). Sequencing was performed and all the isolates corresponded to the species of *Steinernema feltiae* (INIAP-EESC-DNPV 2016).

Bacteriophages

Bacteriophages are viruses that replicate within bacteria and are responsible for a bacterial mortality rate of 10–80% (Weinbauer 2004). Resent research has been developed to study the effect of viruses to control the bacterial disease 'black spot' caused by *Pseudomonas syringae* pv. in tomato (*S. lycopersicum*) in Ecuador. The bacteriophages were identified using a transmission electron microscopy, as particles with the characteristic morphology of Myoviridae family of the Caudovirales order. Phages were isolated from agricultural soil samples. In the laboratory, trials showed lysis of bacteria causing the decrease of the proliferation of the plant pathogen. The phage cocktail was analysed by spot test and double-layer agar (Lara 2015). More detailed procedures are found in Lara's research. Another effective isolation of beneficial viruses can be found in López 2015.

Another research programme isolated bacteriophage (spot test) from soil of naranjilla (*S. quitoense*) plantations in Napo province and tested them against *Pectobacterium carotovorum* subsp. *brasiliense* (bacteriosis). Infection by the bacteria was reduced to a range of 66–74% (Flores et al. 2018).

Entomopathogenic Viruses

For this review, only one report was found about the use of entomopathogenic virus as a biocontrol agent of the Andean potato moths *T. solanivora* and *P. operculella* in potato tubers for consumption and for seed potatoes in the highlands. The McKnight Foundation, the Catholic University of Ecuador, Institut de Recherche pour le Développement (IRD) of France and INIAP worked from 2006 to 2012 on the development of a commercial biopesticide based on the JLZ9F (Baculovirus) virus in combinations with use of *B. thuringiensis kurstaki* (Bacu-Turin) to control the moths (Suquillo et al. 2012). Sadly, no agreement has been made with producers to scale up the microbial control agent (Castillo Carrillo et al. 2020 in press).

Entomopathogenic Fungi

This group of natural enemies has been studied extensively for the biological control of agricultural pests than any other group in Ecuador. The results are promising, and some Ecuadorian companies are mass producing and commercializing the bioproducts (see some examples in Castillo Carrillo et al. 2020). Furthermore, extensive plantations of several crops are applying entomopathogenic fungi against pests. Small farmers also are using biocontrol as part of their own responsibility for a cleaner agriculture. Some examples are present in the next lines. As it was mentioned above, potato is a stable crop which provides food security to low-income farmers in the highlands. In surveys to find entomopathogenic fungi for future alternative tools to control important pests as the Andean weevil *P. vorax* in potato (Barriga 2003; Guapi et al. 2013; Landázuri 2003) and the herbivore *Macrodactylus pulchripes* Blancharé in maize (Ayala 2006; Rueda 2005), more than 30 isolates of *Beauveria bassiana*, 20 of *Metarhizium* sp. and 4 of other species were obtained (Gallegos et al. 2011). Their growth rate, sporulation and pathogenicity were determined in the laboratory and in the field. Also, formulations were developed for isolates that significantly reduced both pest species (Ayala 2006; Barriga 2003; Guapi et al. 2013; Landázuri 2003; Rueda 2005). In another survey performed in Pichincha province, isolates of native fungi were obtained from soil samples to study the potential as biocontrol agent of *P. vorax* and *Phyllophaga* sp. in potato and pastures. Seventeen isolates had more than 80% of spore viability, and trials in the laboratory and field showed that the *Metarhizium* isolates caused 70% of larval mortality (in a concentration of 2.00E6 CFU/soil gr). The *Beauveria* isolates found in this survey were not effective in controlling larvae of the two pest species (INIAP-DNPV 2013). Molecular characterization of some the mentioned isolates was performed to show the genetic diversity of the entomopathogenic fungi (Arahana et al. 2013).

A bioformulation base on clay and *B. bassiana* was tested in two localities (Pusniag and Huaconas La Merced) in Chimborazo province. The entomopathogens were isolated from the Andean potato weevil *P. vorax* found in same province. Pusniag has a level of 59–73% relative humidity and a mean temperature of 10 °C. The soil had 1.8% of organic matter, and the experiment was performed during the dry season (from June to January). The original population of the weevil was high, 29 adults per square metre. Meanwhile, the second locality, Huaconas, has 61–71% of relative humidity, a mean temperature of 11 °C. The soil had 9.8% of organic matter and during the development of the potato plants, the plot had irrigation. The original population was 17 adults per square metre. In Pusniag, in summary, at the end of the experiment the percentage of non-infected tubers was 30%; meanwhile, in Huaconas, the percentage was 87% of clean tubers. In Pusniag, using the *B. bassiana* directly on rice was better than the formulation with clay, with the opposite in Huaconas. There, the bioformulation had better control of the weevil due to its better sporulation than the rice because it just formed mycelia. In Huaconas, the biocontrol was higher due to the better soil

moisture. In both localities, during the harvest, it was observed that *Beauveria* infected larvae near the tubers. The cost of the bioformulation was $61/kg, and the amount of the formulation applied was 768 kg/ha. Both need improvement. Nevertheless, when farmers start to apply biological control with entomopathogens, the benefits are cumulative through time (Guapi et al. 2013). In early 2000, INIAP with an IPM-CRSP project studied biocontrol of the banana black weevil *Cosmopolites sordidus* (Germar) on the coast of Ecuador, by using isolates of *B. bassiana* (Solís et al. 2001).

In 2005, on San Cristobal island in the Galapagos, a survey of 20 soil samples in the agricultural area of the island was done to find entomopathogenic fungi to be used in biological control of pests. Infected insects were also collected, and fungi were isolated from them as well. Twenty-eight isolates were purified. Genera such as *Beauveria*, *Metarhizium*, *Paecilomyces*, *Aspergillus*, *Fusarium*, *Mortierella* among other were identified. Another objective of this research was to study some of the isolates as effective entomopathogenic fungi for two important pests in banana in the island, *Cosmopolites sordidus* (Germar) (Coleoptera: Dryophthoridae) and *Metamasius hemipterus* (Olivier) (Coleoptera: Curculionidae). Three *Beauveria* isolates (from soil and one from infected insects), and one isolate of *Metarhizium* (from an infected insect) were tested against the insect pests to know the mortality percentage, lethal time and lethal dose among other parameters. One of the best entomopathogens was an isolate of *Beauveria* from soil-attacking *M. hemipterus* because it induced total mortality after 9 days, the first dead insects appeared after 2.3 days and the lethal time mean was 3.2 days. A lethal dose mean of 2.26×10^8 spores/ml was obtained for *M. hemipterus* (Valverde 2005).

Next, some examples of application of inundative biological control using entomopathogenic fungi. An oil palm plantation located in Orellana province in the Ecuadorian rainforest applied a native isolate of *Paecilomyces tenuipes* (Peck) Samson on 500 hectares of this crop against *Lincus* sp. (Hemiptera: Pentatomidae), which is a vector of a pathogen causing wilting. Another trial made by this company tested *Trichoderma* sp. on 5 hectares to control of *Demostipa* sp. (Palmar del Río, 2017). Other research performed by an enterprise in Esmeraldas province used a mixture of *B. bassiana*, *Metarhizium* sp. and *B. thuringiensis* to control *S. cecropia* and *Opsiphanes cassina* Felder (Lepidoptera: Nymphalidae) on 2000 hectares of oil palm (Castillo Carrillo et al. 2020 in press). In a study performed by ANCUPA, the best natural enemy found for the herbivore *S. cecropia* on oil palm was the entopathogenic fungus *Cordyceps spegazinii*. This study revealed a total mortality of larvae with a dose of 3.57×10^8 spores/ml in 15 days. In the first 2 days of treatment, dead larvae were found, meanwhile the LT50 was reached at 4.48 days. The average for lethal dose was 1.421×10^8 spore/ml. Lower concentrations also led to total mortality, but it took more time compared with the control (water), which registered no mortality (Quishpe 2009). *Alurnus humeralis* Rosenberg (Coleoptera: Chrysomelidae) is controlled using *Metarhizium* sp. on 200 hectares of palm in two plantations in Esmeraldas and St. Domingo de los Tsáchilas provinces. In other extensive pineapple plantations in the coast region, pests such as *Metamasius* sp.

(a serious pest) and sometimes *Rhynchophorus palmarum* (L.) (Coleoptera: Curculionidae) damage the fruit. A mixture of *B. bassiana* and *Metarhizium* sp. is used for the control of these pests (Castillo Carrillo et al. 2020 in press).

Rice, in Ecuador, is planted on more than 360,000 hectares, mainly in the coastal region (ESPAC 2017). A cocktail of *B. bassiana*, *Metarhizium* sp. and *Lecanicillium* sp. is used to reduce the pest attack of *Hydrellia* sp. (Diptera: Ephydridae), *S. frugiperda*, *Diatraea* sp., *Tagosodes orizicolus* (Muir) (Homoptera: Delphacidae) (Castillo Carrillo et al. 2020 in press). For *T. orizicolus* that causes direct damage and is a vector of a virus, a trial to test some entomopathogenic fungi including *Isaria fumosorosea*, *Isaria* sp., *L. lecanii*, *Metarhizium* sp. y *P. lilacinum* was performed. The results showed control in less than the 50% of the pest population after 72 hours but after 120 hours the nymph mortality reached a 73%, using *Metarhizium* sp., 60% using *Isaria* sp., 40% for *L. lecanii* and *P. lilacinum*, and 26% using *I. fumosorosea* (INIAP-EELS 2017).

Nematode-Parasitic Fungi

The plant pathogenic nematode *Radopholus similis* causes losses around 80% in banana plantations in Ecuador. Ecuadorian isolates of *Purpureocillium lilacinum*, a fungus reported as a natural enemy of the nematode *R. similis*, have been studied as positive population killer of nematodes in the field (INIAP-EELS 2004). In 2017, INIAP studied bioformulations of *P. lilacinum* to obtain longer survival and a consequent better use of wettable powders and granules. Also, quality control of the formulations was assessed. The procedures used for production are discussed below.

Spore production: To increase the level of the spores, sterile rice (121 °C for 15 minutes) with a 25% of humidity in plastic bags was used as the substrate, by inoculating 10 ml of broth (2.59×10^9 conidia/ml) per 100 g of rice. The rice was incubated for 7 days at 25 °C. For formulations as wettable powder and emulsionable concentrate, spores were harvested from the rice using a sieve and dried at 26 °C. The proportions in the formula were 42% of transport material, 42% of a dispersant agent, 6% of a binder material and 10% of dry spores. For dispersible granules and covered granules, spores were washed using 40 ml of water (plus Triton at 0.001%) for 100 g of rice. Dispersible granules formulation: *P. lilacium* broth was used in a proportion of 35.18%, 4.66% of gums and 60.16 of dispersant. A manual extruder was used to make the granules. Granules were dried at 15 °C for 5 days previous to its packaging.

Covered granules formulation: The dough was made using 4% of gums, 4% of liquid and 92% of *P. lilacium* broth. The mix of the dough and the granules (support, e.g., zeolite, clay) was 1:3. When the granules were covered, two additional layers of dry powder were added in a proportion of 1:1. After drying the covered granules at room temperature, they were packed and sealed. For the emulsionable concentrate formula, the liquid transporter was mineral oil at 92.55%, 5% of a dispersant agent

and 2.45% of dry spores. The oil, the dispersant and the spores were mixed at 150 rpm for 30 minutes and then 15 minutes at 200 rpm. The mix was stored in plastic containers at 6 °C. Spore content of all formulations was constant for at least 2 months, and spore viability was stable. Also, purity was preserved in the formulations throughout the processes. The advantages of entomopathogenic fungi formulations are based on their stability at storage and transport temperatures and the optimization of the beneficial fungi (Viera et al. 2018, INIAP-EESC-DNPV 2018, see Bermeo 2017 for formulation procedures).

The fungus *Pleurotus ostreatus* was studied as a nematicide as well as its nematostatic activity against the potato cyst nematode *Globodera pallida* in the laboratory. Direct exposure with the hyphae and a broth were assessed. The mycelium was isolated in agar–water and then the broth filtered and ready to use. To 2 ml of the broth at four different treatments (0%, 50%, 75% and 100%), 25 nematodes (juvenile II) contained in 1 ml of water were placed in six well plates. Observations of the nematode motility were performed after 1, 2 and 3 days. The nematicide effect of the hyphae had a positive correlation between exposure time and mortality of *G. pallida*. At 3 days the mortality was over the 80% level. The nematostatic effect of the hyphae correlated negatively, as when time increased, fewer static nematodes were found. The best treatment was at 1 day of exposure with 34% of static nematodes. After this time, the hyphae started to colonize. The nematostatic action of the filtered broth resulted in 65% stasis when its concentration was 100% for 8 hours, and the nematicide effect of the broth was a 42% of *G. pallida* mortality when it was a 100% concentrate for 1 day of exposure. Both, the hyphae and the broth presented nematostatic and lethal activity against *G. pallida* in laboratory trials. Scale up trials in the field are the next step (Arteaga et al. 2018).

Antagonist and Growth-Promoter Fungi

Trichoderma due to its efficient broad-spectrum inhibitor against pathogens is recognized as a biocontrol fungus. *T. asperellum* secretes compounds such as chitinases, glucanases, and proteases, as well as xylanases that degrade the cell walls of fungi and contribute to mycoparasitism. *T. asperellum* produces primary metabolites that are precursors of antimicrobial compounds; it also produces a variety of antimicrobial secondary metabolites and peptides. Furthermore, it induces plant resistance and enhances plant immunity against pathogens (Wu et al. 2017). As a growth promoter, *T. asperellum* was studied together with the nematode–parasitic–fungus *P. lilacinum* in an organic banana farm on the coast of Ecuador. The trial had 33 plots with 80–90 banana plants per plot. Quality and quantity of roots and population of the plant pathogenic nematodes *Radopholus similis* and *Helicotylenchus multicinctus* were evaluated. In general, *P. lilacium* increased the root quantity by 25% and *T. asperellum* by 19%. There was an initial evaluation of the nematode population before the treatments. Treatments were applied every 15 days and evaluations every

month. The reduction of the *R. similis* populations by the use of the combination of *T. asperellum* plus *P. lilacium* went from 25% to 60% of reduction. Only using *T. asperellum*, the reduction went from 30% to 41% and for *P. lilacium* from 15% to 52%; meanwhile the chemical treatments went from 22% to 56%. The reduction of the *H. multicinctus* populations by the use of the combination of *T. asperellum* plus *P. lilacium* went from 36% to 38%. Only using *T. asperellum*, the reduction was around 14% and for *P. lilacium* from 21% to 31%; meanwhile the chemical treatments went from 13% to 24% (INIAP-EELS 2016, 2017).

For the *T. asperellum* isolates, INIAP and AgResearch from New Zealand developed and assessed formulations. Four prototypes of bioformulations (wettable powder, emulsifiable concentrate, and covered and dispersible granules) were developed using conidia of *T. asperellum*. The prototypes showed appropriate viability and stability after 6 months of storage at 4 °C compared with the control stored at 30 °C, where the effect of temperature reduced the conidia germination by 100%. Briefly, proportions for the formulations are presented in the next section. Mass production of *T. asperellum* and conidia extraction are detailed in Perdomo (2018). The formulation of wettable powder was based on bentonite and powder (1:1) at 86.91%, casaba starch at 7.1% and dry conidia at 5.99%. The formula for the emulsifiable concentrate was based on the use of paraffin oil (86.67%), a Triton x-100 solution (5%) and pure conidia (8.33%). To make a coated granule formulation, a biomatrix has to be prepared in advance using Triton x-100 water solution (11.44%), canola oil (0.52%), Xanthan gum (0.52%), and pure conidia (4.49%). To prepare the granules, zeolite (77.81%), mix of bentonite and powder 1:1 (4.17%), powder (1.04%), and the biomatrix (16.98%) are used. Details of the preparation can be found in Perdomo (2018). One more formulation was tested in this research, dispersible granules. Their formula was based on the use of Gellan gum (0.83%), mix of bentonite–powder 1:1 (52.96%), casaba starch (3.3%), Triton x-100 at a 0.1% water solution (35.80%) and pure conidia (7.1%). In general, formulation brought stability and better preservation of *T. asperellum* after 6 months of storage at 6 °C. The best formulation was the wettable powder stored at 4 °C for 6 months presenting 85% viability of conidia and colony concentration of 1×10^8 CFU/g (Perdomo 2018).

In cacao, *Moniliophthora roreri* (Cif and Par) (causing the disease frosty pot rot) and *Crinipellis perniciosa* (Stahel) Aime and Phillips-Mora are two important pathogens attacking the plants. Two native isolates of *Trichoderma*, *T. koningiopsis* and *T. stromaticum* Samuels & Pardo-Schulth were tested in the field against the pathogens. Lowest damage and highest yield increase were obtained when treated with *T. koningiopsis* (Solís and Suarez 2006). The company Estuardo Quirola Lojas controls frosty pod rot with products based on *T. harzianum*, *T. viride* and *T. pseudokoningii* Rifai in more than 200 hectares with the possibility of expanding the area in a short time (Castillo Carrillo et al. 2020 in press). Several cases of applied biocontrol have been used in the last years in Ecuador to control pathogens as shown in the following discussion. As an antagonist fungus, and as a cultural practice, *Trichoderma* sp. is used to control *Phytophthora palmivora* infestations attacking

the rest of the foliage cut from pineapple plants after harvest. *Trichoderma* in combination with *Matarhizium* is used massively in oil palm cultivations to control pests and diseases in the coastal region. There is a report of more than 350 hectares of rice treated with a mix of *Trichoderma* sp., *B. subtilis* (Ehrenberg) and other bacteria that has total control of the rice blast *Pyricularia oryzae* (Cavara). Around 80 hectares of roses use *Trichoderma* spp. to reduce infections of soil-borne pathogens and nematodes (Castillo Carrillo et al. 2020 in press). Also, *T. harziaum* Rifai has been used on more than 60 hectares of broccoli for 14 years. Pathogens such as *Plasmodiophora brassicae* Woronin have been already eradicated from the soil, and other pathogens as *Sclerotinia* sp. and *Rhizoctonia* sp. have adequate control (90% efficacy), *Alternaria* sp. and *Botrytis* sp. (50% efficacy) and damping off disease (85% efficacy) (Moreno 2018).

Avocado is becoming a crop of high economic importance in Ecuador for local consumers and for export. Usually, cultivated varieties grow on grafted rootstocks. The growth of rootstocks in the nursery is a critical stage, therefore the study of use of beneficial microorganisms for improving plant nutrition and growth is necessary. In a research collaboration by INIAP and AgResearch from New Zealand, the effect of inoculation of *T. harzianum* or *Glomus iranicum* var. *tenuihypharum* on avocado rootstock seedlings was evaluated. The use of *T. harzianum* significantly increased the absorption of nitrogen and magnesium by the roots, also the aerial parts increased the absorption of nitrogen, calcium, magnesium, manganese and copper. *G. iranicum* significantly increased the absorption of calcium and iron by the root. Neither of the two microorganisms influenced the absorption of sulphur but produced an increase in absorption of several macro and micronutrients (Sotomayor et al. 2019).

Blackberry (*Rubus glaucus*, Benth) in Ecuador is mainly planted in the centre of the country, in valleys located in the highlands. INIAP and AgResearch also studied the effect of *T. asperellum* (1.53 × 109 CFU/g) in blackberry's yields. The inoculation of *T. asperellum* increased the crop yield by 17% (Viera et al. 2019).

Organic banana farms together with INIAP were testing the stability and survival of a *T. asperellum* formulation made as a covered grain compared with the fungus grown in rice and tested in the field. The two treatments were applied in two different ways, one in a hole next to the plant and the other on the soil surface. The granules applied in a hole resulted in better survival and multiplication of the microorganism in the field (INIAP-EELS 2018).

Beneficial Pseudomonads

In 2014, INIAP tested several isolates of *Pseudomonas* spp. to assess their effect over the development of the soil-borne fungus *Gaeumannomyces graminis* which is a plant pathogen on rice. No effects on inhibition or control were found probably because the trials were performed in vitro over some specific artificial media. Literature mentions that these beneficial pseudomonads colonize the root system

Table 9.1 Summary of the natural enemies (nine groups) used against agricultural pest in Ecuador presented in this chapter

Natural enemy	Agricultural pest
Parasitoids	
Trichogramma	*Diatraea saccharalis, Spodoptera frugiperda*
Billaea claripalpis	*D. saccharalis*
Copidosoma koehleri	*Phthorimaea operculella*
Apanteles sp.	*Symmetrischema tangolias*
Hambletonia pseudococcina	*Dysmicoccus texensis*
Telenomus sp.	*Tibraca limbativentris*
Trichogramma pretiosum	*Sitotroga cerealella*
Ageniaspis citricola	*Phyllocnistis citrella*
Diachasmimorpha longicaudata	*Ceratitis capitata, Anastrepha fraterculus, A. obliqua, A. striata, and A. serpentina*
Elchertus sp., *Horismenus* sp., *Aprostocetus* sp., and *Brachymeria* sp.	*Stenoma cecropia*
Meteorus sp., *Lymeon* sp., and *Copidosoma* sp.	*Neoleucinodes elegantalis*
Aphidius colemani	*Brevicoryne brassicae*
Telenomus remus	*Spodoptera frugiperda*
Predators	
Podisus connexivus	*Peridroma saucia, Copitarsia tubata, C. consueta, Agrotis deprivata, A. ípsilon, Dargida graminivora, S. frugiperda, and Helicoverpa zea*
Orius florentiae	Thrips (Thysanoptera)
Orius sp.	*Chaetanaphothrips signipennis*
Cybocephalus nipponicus	*Aspidiotus destructor*
Brachymyrmex sp.	*S. cecropia*
Coenosia attenuata	*Liriomyza* spp., and *Trialeurodes vaporariorum*
Entomopathogenic nematodes	
Steinernema feltiae, genus *Heterorhabditis*	*Premnotrypes vorax, Sagalassa valida, Phyllophaga* sp., and *Tecia solanivora*
Bacteriophages	
Family Myoviridae	*Pseudomonas syringae* pv. Tomato
Bacteriophages not identified	*Pectobacterium carotovorum subsp. brasiliense*
Entomopathogenic viruses	
Baculovirus JLZ9F	*T. Solanivora*
Entomopathogenic fungi	
Beauveria bassiana, Metarhizium sp.	*P. vorax*, *Macrodactylus pulchripes, Phyllophaga* sp. *Cosmopolites sordidus, Metamasius hemipterus, Stenoma cecropia, Rhynchophorus palmarum, and Opsiphanes cassina*
Paecilomyces tenuipes	*Lincus* sp.
Trichoderma sp.	*Demostipa* sp.
Cordyceps spegazinii	*S. cecropia*

(continued)

Table 9.1 (continued)

Natural enemy	Agricultural pest
Metarhizium sp.	*Alurnus humeralis*
B. bassiana, Metarhizium sp., and *Lecanicillium* sp.	*Hydrellia* sp., *S. frugiperda, Diatraea* sp., and *Tagosodes orizicolus*
Isaria fumosorosea, Isaria sp., *L. lecanii, Metarhizium* sp., and *P. lilacinum*	*T. orizicolus*
Nematode-parasitic fungi	
Purpureocillium lilacinum	*Radopholus similis*
Pleurotus ostreatus	*Globodera pallida*
Antagonist and growth-promoter fungi	
T. asperellum	*Radopholus similis, and Helicotylenchus multicinctus*
T. koningiopsis, and T. stromaticum	*Moniliophthora roreri, and Crinipellis perniciosa*
Trichoderma spp., and *Matarhizium*	*Phytophthora palmivora*
Trichoderma harzianum	*Plasmodiophora brassicae, Sclerotinia* sp., *Rhizoctonia* sp., *Alternaria* sp., and *Botrytis* sp.
Trichoderma sp. and *Bacillus subtilis*	*Pyricularia oryzae*
Trichoderma spp.	*Botrytis* sp. *Oidium* sp., and *Peronospora* sp.
T. harzianum, Glomus iranicum var. *tenuihypharum*	Avocado pathogens
T. asperellum	Blackberry and banana pathogens
Beneficial pseudomonads	
Pseudomonas spp.	*Gaeumannomyces graminis*

and protect it against pathogens, thereby improving plant growth and yield. Therefore, it is suggested to perform trials doing soil inoculations when plants are growing to see if artificial infections with *G. graminis* take place or not, due to the effect of the presence of pseudomonads in the rice root system (INIAP EELS 2014).

Conclusions

This review has summarized most of the more relevant research about applied biocontrol in Ecuador. Nevertheless, there are important research operations not cited here that deserve attention, but unfortunately space and time are limited. Though Ecuador possesses a high diversity of agricultural pest species, natural enemies are present in significant numbers and ecological richness as well. In this chapter, more than 40 natural enemies used against approximately 70 agricultural pests are presented (Table 9.1). Thus, valuable experience in biocontrol is now available, including techniques that could be applied directly or serve as the basis for more research. These are opportunities to identify and use successful methods for pest control which can lead us into a healthier and environmental low-impact agriculture.

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Chapter 10
Native Biodiversity: A Strategic Resource to Accelerate Bioeconomy Development in Latin America and the Caribbean

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Abstract Latin America and the Caribbean (LAC) comprise one of the regions with the largest endowments of natural capital in the world due to its great diversity and specific endemism. For this region, natural resources are usually the basis of its economy. However, the rapid socio-economic growth of LAC has generated pressure on natural resources and a continued loss of biodiversity. This region faces new challenges that will allow it to adapt to progress in a sustainable way. Thus, it is essential to integrate biodiversity and traditional and scientific–technological knowledge into production processes. The bioeconomy emerges as an alternative to promote the socio-economic and environmental development of LAC, which each country must face with a territorial vision. The bioeconomy must be based on a strategy with three fundamental pillars: conservation, knowledge and sustainable use of resources. The bioeconomy generates added value and promotes long-term growth in the region, through the production of food and medicines, among others obtained from local biodiversity. In this regard, the possibility of carrying out sustainable development in different territories is extended. Thus, it is necessary to contribute to greater knowledge of native flora and promote its conservation and sustainable use through scientific research and technology transfer.

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Introduction

This chapter invites us to reflect on the scope of biodiversity of the Latin America and the Caribbean (LAC) region in the face of the new challenges of sustainable development and to identify new opportunities linked to the bioeconomy. We encourage you to think about how this new paradigm emerges as a socio-economic model that recognizes the fundamental role of science in the integration of traditional and scientific–technological knowledge. This chapter is arranged in four different sections. At first, the concept of biodiversity is approached, and we are asked how its loss affects LAC countries from an environmental, social and economic point of view. The second and third sections present a synthesis of the concepts of bioprospecting, biocommerce and bioeconomy, and how they relate to the new global challenges of balancing the promotion of human development with the preservation and restoration of natural capital. To conclude, the fourth section focuses on the current opportunities and challenges to face in the development of the bioeconomy in vulnerable LAC territories.

Biodiversity: Concept and Its Importance for Latin America and the Caribbean (LAC)

Historically, the human being uses and interacts with biological diversity on a daily basis in different ways. Nevertheless, only a very small percentage of biodiversity is used directly by people. However, people rely indirectly on a much larger amount of biodiversity without realizing it. Anyhow, the concept and all its implications are not fully understood since they do not contemplate all its aspects and dimensions. The Convention of Biological Diversity (CBD 1992) indicates that understanding the full value of biodiversity is not simple. In addition to the gaps remaining in the understanding of biodiversity and ecosystem functioning, each individual stakeholder may have different values for the same attribute of biodiversity. The CBD has defined biodiversity as ‘the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems’.

In accordance with the National Biodiversity Index, LAC has eight megadiverse countries, which are located on Amazon basin and in Mesoamerica (Bolivia, Brazil,

Colombia, Ecuador, Perú, Venezuela, Costa Rica and Mexico). This is based on estimates of country richness and endemism in four terrestrial vertebrate classes and vascular plants, according to the country size (Global Biodiversity Outlook 2001). Also, LAC has a great variety of unique and still little-known ecosystems, such as desert and semidesert regions (Patagonian steppe); wet and dry pampas (south of Brazil, Uruguay and Argentina); the Pantanal of Brazil, Bolivia and Paraguay; and marine ecosystems and their continental platform (Rice et al. 2018). LAC includes 28% of terrestrial and freshwater world ecoregions with a highly distinctive or irreplaceable species composition. The region presents 20% of the key biodiversity areas identified worldwide. It also has some of the most extensive wilderness areas on the planet, such as the Pacific Northwest, the Amazon and Patagonia. In the Americas, there are a total of more than 122,000 species, among which about 29% are seed plants and provide 33% of the plants that are used by humans (Joly 2018). In addition, LAC is one of the main sustainable biomass producers and has other elements that would allow greater and better use of the region's resources and biological knowledge. This could include scientific and technological capabilities, development of industrial bioenergy infrastructure and commercial relations with the rest of the world.

This biodiversity provides goods and environmental services related to human well-being, called 'ecosystem services'. Ecosystem services are the different resources and processes that the environment provides, and they must be accompanied, exploited and protected. Accordingly, due to its high biodiversity, LAC has a bigger contribution over people's quality of life than the global average. However, most countries in this region are using nature at a rate that exceeds its capacity of resilience. This inappropriate use of resources affects the quality of human life, and it can have adverse consequences in economic, social and ecological aspects.

Contributions of Native Biodiversity to People and Quality of Life

Human well-being is the result of many factors directly or indirectly linked to biodiversity and its ecosystem services, for example, the human being has cultivated throughout history 7000 species of plants for food. Besides, biodiversity provided among others, fibre, fuels, genetic resources, biochemicals, natural remedies, medicines and water. So, the populations have benefited over the last century from the conversion of natural ecosystems to human-dominated ecosystems and the exploitation of biodiversity. However, the intensive and unsustainable food production caused biodiversity loss, ecosystems changes and a reduction of the contributions of nature to the quality of people's life.

In respect of pharmacological products, mankind historically has used medicines originated from biodiversity. Microorganisms, flora and fauna provide extensive knowledge which carry important benefits for biological, health and

pharmacological sciences. This is one of the most sophisticated sources of new biocompounds and products with high economic value, such as bioactive ingredients (digestive, cardiovascular, analgesic, antitumor, immunological, among others) and food supplements (Valli et al. 2018). In this context, new approaches like bioprospecting play a strategic role because they allow the generation of technological developments based on genetic resources.

Bioprospecting can be defined as the systematic search and development of new sources of chemical compounds, genes, microorganisms, macro-organisms, and other valuable products from nature (Timmermans 2001). This provides the necessary knowledge to find new products and characterize the properties of native flora in order to give them additional value, which generates a direct benefit for people (Boeri et al. 2018). There are different points of view for the discovery of bioactive compounds from plants, such as ethnobotany, which is based in popular uses of medicinal and food plants. This allows the scientific validation of traditional knowledge, and it is of great importance for pharmaceutical and cosmetic industries. Access to the knowledge and practices related to the use and harnessing of plants promotes conservation of this cultural and local heritage (Boeri et al. 2017). Generally, the regions of greatest biological diversity on the planet are inhabited by native peoples, who have associated traditional knowledge. Recently, bioprospecting has broadened its reach, including exploration and research on the understanding that native peoples possess in relation to the diversity of genetic resources and their management techniques.

On the other hand, bioprospecting is an effective tool in the search for economically valuable active compounds to promote the commercialization of biodiversity. In this sense, understanding the importance of biodiversity for LAC in particular allows the promotion of biocommerce initiatives, which seeks to obtain sustainable profit from the benefits of biodiversity. Biocommerce is understood to include activities of collection, production, transformation and commercialization of goods and services derived from native biodiversity, under the criteria of environmental, social and economic sustainability (UNCTAD n.d.). This term was adopted during the Convention of Biological Diversity (CBD) and agreed by the Andean Development Corporation (CAF), the Community of Andean Nations (CAN) and the United Nations Conference on Trade and Development (UNCTAD). Within biocommerce, three sectors were identified and prioritized: natural ingredients and products for the pharmaceutical and cosmetic industry, natural ingredients and products for the food industry and sustainable tourism (UNCTAD 2017).

Biocommerce has tried to adapt this concept to the business fields related to biodiversity products and the various types of actors that make up the production chain. In general, biocommerce integrates rural, small and medium-sized producers, for whom research and development are not usually central elements. Most of these new markets respond to the continuous increase in consumer demand for products and services that incorporate criteria and principles of social and biological sustainability. Consumers often prefer natural products of sustainable origin that are more environmentally friendly and contribute to social equity. Countries in the LAC region are dependent on natural resources to provide the basis of much of their

economies. To integrate biodiversity into economic and social development strategies, it is necessary to understand those aspects of it that sustain poverty reduction and other sector-specific development activities. This knowledge can incorporate biodiversity-related objectives in the decision-making process of productive sectors and government policy.

Biodiversity as the Main Input for Bioeconomy

As mentioned above, biodiversity and ecosystems provide a variety of contributions for human well-being. Therefore, biodiversity is the basis of the bioeconomy, defined as 'the knowledge-based intensive use of biological resources, processes, technologies and principles, for the sustainable provision of goods and services across all sectors of the economy' (IICA 2019). The new bioeconomy paradigm, which contributes to fulfilling Sustainable Development Goals (SGDs), should be based on three pillars: conservation, knowledge and sustainable use. Globally, there is a general consensus in which the bioeconomy is presented as a model to align social and economic development with the care of the environment, decarbonization and mitigation of the effects of climate change, in accordance with the SDGs. The importance of these relationships is clearly established by analysing the possible contributions of the bioeconomy to the fulfilment of at least 11 of the 17 SDGs, summarized in the 2030 Agenda.

Knowledge, in turn, is a new value which is also the basis of bioeconomy. We could say that it is the application of the knowledge of the economy to living beings. It is jointly supported by technologies, not only on biotechnology, but also on the epistemic convergence of economics, biology and ecology. Will the economy be biologizing or is it changing the way we see the world? This change was much needed. It is rapidly evolving towards a broad vision for sustainable development that is about leveraging new knowledge and technologies that converge and empower each other to offer new and previously unthinkable options as possible in the near future. It is also about a total change in the role of biological resources in the structuring of economies and the pursuit of social welfare (Hodson de Jaramillo et al. 2019). For LAC countries, these trends represent a new and powerful opportunity. The region is not only a major producer of sustainable biomass, it also has important developments in its scientific–technological capabilities, as well as in its industrial infrastructure and in the development of bioenergy, where it has become one of the main players in international markets. In LAC, the concept of bioeconomy has been consolidating and is promoted as an alternative model for sustainable development and green growth.

The extraordinary biodiversity richness of this region of the world and its great capacity for biomass production offer enormous potential for the production and processing of products and represent a strategic point to promote diversification of agricultural and agro-industrial production. But it is necessary to pursue actions to reduce emissions and to mitigate and adapt to climate change along value chains

(Rodríguez et al. 2017). While the region is very heterogeneous, it generally has a good underutilized potential of high-level trained and infrastructure resources. As the discourse of bioeconomy was born in Europe, more specifically in Germany, it is necessary to develop and discuss a bioeconomy model for the region, where biodiversity is the differential factor.

In this sense, the development of regional policies that promote and drive bioeconomy is essential. Not all countries have explicit bioeconomy policies or strategies, although some, such as Argentina, Brazil, Colombia and Ecuador, have expressed an interest in formulating them. Argentina, Colombia, Paraguay and Uruguay have initiated activities in the knowledge-based bioeconomy (bioprospecting, agricultural technologies), in the form of international cooperation agencies, such as the Economic Commission for Latin America and the Caribbean of the Nations (ECLAC) and IICA, which have been promoting articulation and cooperation for the development of the bioeconomy in policy aspects and alliances between the private sector and research (Germany Bioeconmy Council BÖR, 2018). In the case of Central America, the potential of the bioeconomy is considered to be directly linked to agricultural production and research, development and innovation, and some groups have been working on traditional native products underutilized but with interesting potential. It is considered that the capacity to supply natural resources in these countries, including water and biodiversity, as well as the resources to provide ecosystem services, should be maintained and promoted, all under the activities of adaptation and mitigation of climate change, the effects of which have been openly manifested in recent years, especially in the Caribbean (Vega and Madrigal 2017).

There has been very interesting progress in some of the countries in the LAC region. For example, in Argentina, a national process has been proposed for the development of a regional bioeconomy framework strategy linked to the concept of smart territories (Rodríguez 2018). In Argentina bioeconomy began in 2013, and a push to develop this national bioeconomy policy has started from a listing of the proper needs (http://www.bioeconomia.mincyt.gob.ar/). In 2017 the Argentine government published a document entitled ‘Argentine Bioeconomy’, which presents the action plan for the development of bioeconomy in the country, and, subsequently, a programme that highlights the need to implement strategies in policies and defined actions. For its part, the Grain Stock Exchange has emphasized the importance of the energy and food sectors, recommending the development of biotechnology policies to promote their application in food production, in the pharmaceutical sector and in the protection of the environment (Hodson de Jaramillo 2018). The Brazilian government has also promoted bioeconomic developments for more than five decades with policies, laws and strategies. The main focus has been the sustainable use of biodiversity, biotechnology and the production of biofuels (Germany Bioeconomy Council – BÖR 2018).

According to Lewandowski (2018), the proper advancement of bioeconomy requires meeting the following five assumptions: (i) natural conditions, which emanate from the richness of biodiversity, soils, and agroclimatic conditions that enhance biomass production, bioenergy and bioproducts in bulk; (ii) labour

resources and trained human resources; (iii) knowledge resources to foster innovation with the corresponding investments in research, development and innovation; (iv) financial resources for investment in all value chains, especially venture capital; and (v) support infrastructure in terms of transport, information and communications (Hodson de Jaramillo 2018). Successful adoption of the bioeconomy requires social discourse on the ways in which society can reconcile economic growth and sustainability, for which it is necessary to adapt and adjust all time initiatives and agendas, as well as the necessity for cooperation (Schütte 2018). At the recent World Summit on Bioeconomy (April 2018), topics of global relevance for political and research agendas were defined, mainly focused on the relationship of the bioeconomy to climate change and the impact on health, digitization and convergent technologies ('bio', 'nano', 'info'); communication and public trust in transformative sciences and technologies; interdisciplinary education and training at all levels; biodiversity as a resource and basis for bioeconomy, marine and ocean bioeconomy; innovative sources of funding; and bioeconomy in cities. Recommendations included the establishment of international coordination and knowledge-sharing mechanisms with participants in this meeting, and with United Nations organizations through development of biodiversity and innovation forums, especially the Paris Agreement on Climate Change (GBS 2018).

A key consideration is how to link the increase the flow of results in science and technology to their social application for the common good, so that they are a factor of well-being for the population and, moreover, a central element that contributes to the sustainability of the planet (Hodson de Jaramillo 2018). The work that is being carried out in some countries of the region, such as Argentina, Brazil, Ecuador, among others, on the knowledge and bioprospection of native biodiversity are fundamental to facilitate this transfer of knowledge to society. Rodríguez et al. (2019) emphasize that harnessing the potential offered by the bioeconomy requires an adequate understanding of the basis of available biological resources, related scientific and technological capabilities and market capacity and consumer acceptance of new bioeconomy products. Rodríguez et al. (2019) also said that valorisation is not only understood in monetary terms (mainly due to desirable qualities of the product and its valuable benefits), it includes the intrinsic values of biodiversity and ecological functions. In the agriculture, forestry and fisheries sectors, as well as in the production of food, biochemicals, biomaterials and bioenergy, bioeconomy policies and programmes should generally recognize and assess the functionality and quality of biological resources rather than as a quantitative 'biomass'. In this context, the studies and deep understanding of biodiversity create new possibilities for its sustainable uses. However, it should not only focus on the use and potential of biodiversity, but also on the aspects of species reproduction, propagation and domestication of plant resources, which allow a country and/or geographic areas to set conservation strategies (Boeri et al. 2018). It is important to consider that beyond promoting the use of plant genetic resources in a sustainable way, the possibility of pursuing development in different territories is extended. Thus, it is necessary to produce greater knowledge of the native flora and favour its conservation and sustainable use through scientific research and technology transfer. Scientific knowledge is a

fundamental tool for generating added value to the natural resources of a region, increasing benefits and promoting sustainable use and conservation of native ecosystems. For this, the economic and social value of biodiversity in each territory needs to be evaluated and better understood.

Bioeconomy Development in Patagonia, a Vulnerable Territory from LAC

In the extra-Andean Patagonia, overgrazing, deforestation and unsustainable agriculture have combined with natural erosion to carry ecosystems to critical points of deterioration and consequent biodiversity loss (Ravelo et al. 2011). Almost 80% of its entire surface shows signs of deterioration and desertification, and 58% of it was classified as irreversible (Mazzonia and Vázquez 2009; Ravelo et al. 2011). This situation affects the ability to produce goods and services. Probably, the current greatest threat to Patagonian ecosystems is the lack of knowledge about their native flora and the impacts generated by environmental degradation. In this region, the native flora has abundant endemic genera and species, and the International Union for Conservation of Nature (IUCN) has described it as one of the centres of high plant diversity (Villamil 1999; Soriano et al. 1995). The identity of Patagonia includes its biodiversity as integral part, a foundation of its character and its essence. In a world of growing demands and pressure for the use of natural resources, the challenge is to link knowledge and conservation of biodiversity with its potential uses and sustainable management.

Arid and semi-arid environments, such as those in the Patagonian region, are a potential source of new phytopharmaceuticals. There plants species are exposed to several stress conditions that affect their physical, chemical and biological process and induce metabolic transformations to ensure their survival. In these hostile environments, natural selection favours the presence of species with high levels of defence systems to protect tissues (Coley et al. 1985). The synthesis of secondary metabolites can be a plant defence strategy (Vilela et al. 2011). Associated with this, there is a rich and diverse range of natural compounds with peculiar chemical structures that are one of the most sophisticated sources of molecular models for medicinal chemistry and the production of new drugs. In this context, bioprospecting is essential to find and characterize potential sources of food, medicine, aromatic and medicinal plants. The use of ingredients and raw materials obtained from native species has increasing interest in several industrial sectors and an economic potential with growth rates between 8% and 10% (Aramendis et al. 2018).

On the basis of the above, it is important to indicate that of the 1211 new low-molecular weight prototypes introduced in the world market as pharmaceutical products, 60% derive from natural products, imitate them or are designed from them (Newman and Cragg 2016).

The North Patagonian region offers numerous native and naturalized species, which constitute an immense source of vegetable products used as aromatic, medicinal, dyestuffs, sweeteners, etc. and represent an economic potential for the region (Boeri et al. 2017a; Boeri et al. 2017b; Boeri et al. 2018; Piñuel et al. 2019a; Piñuel et al. 2019b). Therefore, the development of bioprospecting is an economically viable activity for the sustainable management of ecosystems and possible development of regional markets.

In recent decades, Patagonian berries have attracted substantial attention and have been the subject of extensive bioprospecting research due to their antioxidant properties and their potential in promoting health (Fuentes et al. 2019). Blueberries among other fruits have gained popularity and significant attention. They have been denominated 'superfruits' as a term to promote the health benefits of these fruits due to its high levels of antioxidants, fibres, vitamins, minerals and other nutrients that improve health (Chang et al. 2019). In this sense, recently our work group has been focused in the bioprospecting of *B. microphylla* fruits (popularly known as calafate). This is a native species from Argentinian Patagonia that produces small edible fruits of great importance for the regional economy. Traditionally, they have been and are used in food and medicinal preparations. These fruits have a high concentration of phenolic compounds, mainly anthocyanins, which, along with their high antioxidant activity, increase the value and quality of calafate berries. The proteins present in this species seeds were evaluated, and their gastric and duodenal digests presented antioxidant activity. These contributions on characterization of the components of Calafate fruit can increase consumers' interest and can be used as a functional ingredient or simply as an ingredient in foods supplements. Finally, this study on a native species from Patagonia is an example of how knowledge on regional natural resources and bioprospecting allow incorporate added value, generate products with local identity and diversify activities related to regional industries.

Conclusions

Considering that LAC have 8 of the 17 most megadiverse countries in the world, wide genetic resources, productive landscapes diversity and biomass production capacity, bioeconomy is an opportunity for this region. What makes this new economic model different is the incorporation of knowledge for the definition of new alternatives and productive paths, fostering the production and the intensive use of knowledge related to biological resources, processes and principles, and the sustainable supply of goods and services in all economic sectors, thus migrating from the unsustainable oil-based economy to the biomass economy (Hodson de Jaramillo 2018). In order to be successful, the adoption of this model requires decisive political determination as well as strong commitment from all the stakeholders. It should be note that some sectors associate bioeconomy with neoliberalism. There are authors who see bioeconomy as the 'neoliberalization' of nature, that is, the prevalence of market values and as a way to benefit the interest of large companies

interested in commercializing innovation and new technologies and not small producers (Lewandowski 2018). So, the discussion of a Latin American bioeconomy should include ethical, environmental, economic, social and legal aspects, such as intellectual property rights and the implications of patents on cost and cost accessibility of technology in developing countries, access biodiversity resources legally and share the benefits of developments equitably (CBD and Nagoya Protocol), as well as public awareness and acceptance of developments (Hodson de Jaramillo et al. 2019). In this context, bioprospecting makes it possible to transform this biodiversity into high-value products to markets related to biocommerce and diversify the productive matrix of a region in an environmentally friendly way, according to the Sustainable Development Goals. In this sense, in the LAC region, biocommerce shows significant successful experiences, which include the placement of native products in the international food markets, cosmetic industry, pharmaceuticals, timber species, among others. Always considering its three pillars, economic, environmental and social sustainability, including culture and facilitating access to all actors and fair distribution of benefits. To conclude, LAC have also the necessary scientific and technological capacities for the development of these sectors and the need to find new alternatives for a more sustainable and inclusive development.

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Part III
Biodiscovery Applications and Added-Value Bioproducts

Biodiscovery Applications and Added-Value Bioproducts will review the novel techniques for the discovery, characterization, and industrial applications of bioproducts. This part will describe advantages of using plants and microorganisms for the discovery and development of new molecules and materials. The part will also provide examples of the applications of biomass and by-products from the food, forestry, and agroindustry for multiple purposes. In this part, smart approaches will be addressed to add value to different bioproducts or their production processes.

Chapter 11
Low-Cost Crop Waste Biosorbent Technology for Removing Toxics and Pollutants from Wastewater

Mayra Vera, Christian Cruzat, and María Eulalia Vanegas

Abstract Wastewater pollution is an urgent environmental concern. The negative effects of pollution on both humans and the environment have become a subject of intense discussion. Several techniques have been developed for the removal of pollutants from water. Most of these techniques adopt a combination of physical, chemical, and biological processes, which include photo-oxidation, chemical coagulation, sedimentation, filtration, disinfection, and adsorption. Nevertheless, adsorption is one of the most efficient methods for removal of pollutants from water, offering many economical, technological, and ecological advantages. The search for new low-cost technologies to remove contaminants from aqueous solution will be well embraced. Low-cost adsorbents can be employed to remove recalcitrant compounds from aqueous solution inexpensively and can be effective for removing both organic and inorganic contaminants. Biosorption technology using different biomasses (agro-industrial waste or organic waste that are considered composting material) is a promising alternative for the treatment of effluents that contain pharmaceuticals and heavy metals. These processes have certain advantages from both economic and environmental points of view, such as availability, abundance, the renewable nature of the adsorbent material, their low cost, and ease of operation of the treatment plant. Consequently, it is important to find low-cost alternative materials instead of traditional. Among the different agro-industrial wastes, sugarcane bagasse, cocoa shell, peanut husk, among others, are attractive materials for removing toxic heavy metals and pharmaceutical products from wastewater. To date, several researchers have reported on removal of different pollutants by using sorbents.

Keywords Wastewater · Water pollution · Biosorbent · Toxic removal · Agro-industrial waste uses · Biomasses

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Introduction

Contamination of the environment by heavy metals has become a major concern in recent years. Various industries produce and discharge wastewater containing heavy metals into the environment, posing a serious environmental threat to human health and the ecosystem. Untreated wastewater from industries and homes eventually end up in rivers and other aquatic systems that are a source of livelihood for humans. Most of these rivers are used as source of drinking water by rural dwellers without any form of treatment, thus increasing the chances of suffering ill health. Contamination and redistribution of toxic metals, metalloids, radionuclides in the environment as well as introduction of a plethora of organic pollutants necessitates ever-increasing standards of pollutant detection and treatment. The deleterious effects of organic and inorganic pollutants on ecosystems and on human health are well known, and huge expenditure is devoted to industrial treatment methods to prevent or limit discharges. Apart from physical and chemical methods of treatment, biological methods have been in place for many years such as standard sewage and water purification treatments as well as auxiliary reed bed and wetlands approaches.

Heavy metals are known to persist in the environment and become a risk for aquatic organisms. Biosorption uses the ability of dead/inactive biomass to remove heavy metals from aqueous solutions. The major advantages of the biosorption technology are its effectiveness in reducing the concentration of heavy metal ions to very low levels and the use of inexpensive biosorbent materials. The utilization of agro-wastes as adsorbent is currently receiving wide attention because of their abundant availability and low cost. In recent years, applying biotechnology in controlling and removing heavy metal pollution has received much attention, and gradually became a hot topic in the field of metal pollution control because of its potential application. An alternative process is (bio)sorption, which can be defined as the removal of metal or metalloid species, compounds, and particulates from solution by biological materials. The idea of using locally available, low-cost agricultural plant materials for the research make sorption the preferred method.

Biosorption

Biosorption can be defined as a physico-chemical process used for removing substances from solution by biological material through spontaneous redistribution; traditional definitions were generally related to removal of heavy metals by microbial material (Gadd 2008). However, due to recent research, new applications have extended the definition, and nowadays the term biosorption has been extended to remove other compounds, such as dyes, pharmaceutical products, drugs, fertilizers, and pesticides, using living or dead biomass including all kinds of microbial, plants, and derived products.

Biosorption process therefore involves a liquid phase (aqueous solution) containing the sorbate (inorganic or organic pollutants) and biosorbent (substrates of biological origin).

There are a lot of biological materials that have a high potential for removing pollutants under environmental applications, including living biomass (fungi, algae, other microbial cultures) (Kumar et al. 2016), industrial solid wastes (sewage sludge, activated sludge, fermentation) (Norton et al. 2004; Hammaini et al. 2003), agricultural solid waste (fibres, leaves, fruit peels, rice straw, sugarcane bagasse) (Ali et al. 2012; Nahar et al. 2018), and natural residues. However, the constant challenge is employing biomass that is cheap, abundant, renewable, efficient, and environment-friendly.

The cost and value (valorization) are the major principles of sustainable development in a strategy for using local available agriculturally based biosorbents. This overall concept evaluates the technical feasibility for biosorption processes that can give added value. Agricultural wastes have high percentages of cellulose, hemicellulose, and lignin that contain a variety of functional groups (amine, carbonyl, hydroxyl) responsible for adsorption. Many researchers used cheap biosorbents such as nut shells, fruit peels, bagasse waste, risk husk, corncobs, olive oil factory waste (Abdolali et al. 2014; Malkoc et al. 2006; Peñafiel et al. 2019b), among others. The modification with pretreatments can modify the surface, increasing binding sites (de Oliveira et al. 2019). Therefore, by-product or agro-industrial waste are promising materials for being readily available and low-cost biomaterials, and these materials can be disposed of without expensive regeneration, reducing total treatment cost.

Mechanism of Biosorption

Due to the high variety of functional groups, biosorption is a complex process affected by several mechanisms that can operate simultaneously. The activity of the functional group can appear as a possibility for ion exchange and for formation of a donor–acceptor complex, as well as the ability to form chelate poly-ligand spatial complexes (Nikiforova and Kozlov 2016). There are many factors affecting biosorption processes, such as chemical structure, molecular size, charge, and available surface area.

Physical adsorption is a surface phenomenon with no specific interaction, and can involve weak Van der Waal forces, hydrogen bonds, polarity and steric interactions, dipole-induced dipole interaction, $\pi - \pi$ or electrostatic interaction (Ali et al. 2012); therefore, it is a reversible process and dependent on pressure as well as temperature. The functional groups have non-selective affinity for the metal ion species and bind to a wide range of metals and have been evaluated as monocomponents; but in most cases, when there are more than one type of metal ion, the removal of one may be influenced by the presence of other (Sud et al. 2008). In a

multicomponent system, there exist a competition for the adsorption sites on the surface, although the metals have generally the same charge, and the smallest size ion has a best adsorption favourable with highest adsorption capacity. For example, when metal ions such as Cd(II) and Pb(II) are studied in a bimetal solution in batch mode using banana peel, the mobility of the lighter ion (CdII) is more than the heavier ion (PbII) and makes adsorption favourable (Muhamad et al. 2010; Anwar et al. 2010).

Ion-exchange, where the metal ions are binding by replacing the initially occupied charged ion on biosorbent, is a reversible mechanism. Witek-Krowiak and Reddy (2013) reported the removal of Cr(III) and Cu(II) from aqueous solutions using soya bean meal, and the biosorption mechanism was due to ion-exchange, chelation and further precipitation of metal ions on the surface of biomass.

Another mechanism is the precipitation of insoluble metal species as metal oxides and hydroxides on biomass surfaces. Many cases are reported where the sorption is enhanced with pH value, but that is not a real adsorption process improvement; it is an alternate sorption mechanism, where the bulk solubility limit is reached, and the metal ion is removed from aqueous solution by the bulk precipitation. A surface precipitation model includes an initial, rapid, adsorption mechanism followed by a slower surface precipitation covering the adsorbed layer (Schneider et al. 2001). Therefore, it is important to consider the metal ion speciation as a function of pH in order to evaluate the precipitation contribution in biosorption process.

Complexation involves a ligand centre in the biomass (atoms with lone pair electrons to donate) like divalent oxygen (hard acids), trivalent nitrogen atoms or sulphur atoms (soft acids), and can form monodentate or multidentate ligands for binding metal species. The concept of hard/soft ligands predicts the bonds formed, ionic or covalent respectively (Gadd 2008). This mechanism can inhibit desorption.

Chelation can be defined as a binding of a metal ion with an organic molecule (ligand) to form a ring structure. Amine groups play an important role in metal ion binding. The chelation takes place through dative bonds with the lone pair of electrons within nitrogen present in the $–NH_2$ groups of biomasses. Panda et al. (2007) reported that the binding of Ni(II) on husks of *Lathyrus sativus* was probably chelation through dative bond formation.

Finally, when other pollutants are present in synthetic and real aqueous solution (other metal ions and organic materials), metal biosorption capacity can change through synergism, antagonism and non-interaction. Calculating the adsorption rates can illustrate the effects in multicomponent conditions, and the presence of co-solutes could enhance the intermolecular forces between pollutants.

Parameters Affecting Biosorption

Different factors affect the biosorption rate which include temperature, pH value of solution, nature of biosorbents, surface area-to-volume ratio, concentration of biomass, initial metal ion concentration and metal affinity to biosorbent.

Under batch conditions, a biosorbent is mixed with a metal-containing solution within a simple reactor and operated until thermodynamic equilibrium is reached. In a continuous-flow system (packed bed), synthetic wastewater is continuously fed to a biosorbent column with an upward or downward stream.

Factors Influencing Batch Biosorption

Studies in adsorption by batch experiments (discontinuous) provide a measure of the efficiency of adsorption in removing specific elements and the maximum adsorption capacity. In addition, they are useful in determining optimum operating conditions (solution pH, particle size, contact time, solute concentration, temperature, agitation rate, etc.) as well as the mechanisms involved in the process. Of these, the pH usually plays a major role in biosorption and seems to affect the solution chemistry of metal ions or another pollutant (metal, dyes, pharmaceutical compounds) and the activity of the functional groups of the biomass.

Temperature For efficient removal of metal ions from environment samples, the optimum temperature needed to be investigated. It is generally assumed that biosorption is carried out between 20 and 35 °C. High temperatures above 45 °C may result in adsorbent damage which in turn affects metal uptake process (Abbas et al. 2014; White et al. 1997; Ahalya et al. 2003; Goyal et al. 2003). As temperature increases, it usually enhances biosorptive removal of adsorptive pollutants by increasing surface activity and kinetic energy of the adsorbate but may damage physical structure of biosorbent (Park et al. 2010).

pH The pH of the solution is the most important parameter which affects the surface charge of biosorbent, the degree of ionization, and speciation of the adsorbate – all of which affect the sorption of the sorbent material, solubility of metal ions and number of binding sites on the biomass. The kinetics of sorption strongly depends on the initial pH of the solution since at lower pH, the biosorption of metals is affected (Greene and Darnall 1990; Deng and Wang 2012). General range of pH for metal uptake is between 2.5 and 6. Above this limit, metal uptake ability of biosorbent gets compromised (Abbas et al. 2014).

It has been shown that the affinity of cationic species for the functional groups present on the cellular surface is strongly dependent on the pH of the solution. The biosorptive capacity may be low at low pH values, and increases with pH until reaching an optimum level. However, at higher pH (8 and upwards), metals begin to precipitate due to formation of M(OH). At low pH values, cell wall ligands are closely associated with hydronium ions and restrict the biosorption of M^{n+} as a result of competition between H_3O^+ and M^{n+} with bacterial biosorbent cell wall ligands. As the pH increases, more ligands, such as carboxyl, phosphate, imidazole, and amino groups, would be exposed and carry negative charges which attract M^{n+} and biosorb it onto the cell surface (Joo et al. 2010).

Effect of agitation speed As agitation speed increases, it enhances biosorptive removal rate of adsorptive pollutant by minimizing its mass transfer resistance, but may damage the physical structure of a biosorbent (Park et al. 2010).

Effect of biosorbent size If biosorbent size decreases, it is favourable for batch process due to higher surface area of the biosorbent, but not for column process due to its low mechanical strength and clogging of the column (Park et al. 2010).

Concentration of biomass The concentration of biomass is directly proportional to the metal uptake (Abbas et al. 2014; Gadd and White 1985; Modak and Natarajan 1995). Metal uptake depends on biding sites. More biomass concentration or more metal ions may restrict the access of metal ions to binding sites (Nuhoglu and Malkoc 2005). The dosage of a biosorbent strongly influences the extent of biosorption. In many instances, lower biosorbent dosages yield higher uptakes. An increase in the biomass concentration generally increase the amount of solute biosorbed, due to the increased surface area of the biosorbent, which in turn increases the number of binding sites. Conversely, the quantity of biosorbed solute per unit weight of biosorbent decrease with increasing biosorbent dosage, which may be due to the complex interaction of several factors. An important factor at high sorbent dosages is that the available solute is insufficient to completely cover the available exchangeable sites on the biosorbent, usually resulting in low solute uptake. Also, as suggested by the interference between binding sites due to increased biosorbent dosages cannot be overruled, as this will result in a low specific uptake (Bilal et al. 2018).

Initial metal ion concentration The initial concentration provides an important driving force to overcome all mass transfer resistance of metal between the aqueous and solid phases (Zouboulis et al. 1997). Increasing amount of metal adsorbed by the biomass can be dependent upon initial concentration of metals. Optimum percentage of metal removal can be taken at low initial metal concentration. Thus, at a given concentration of biomass, the metal uptake increases with increase in initial concentration (Abbas et al. 2014).

Effect of contact time It is necessary to identify the step that governs the removal rate of sorption process using different models kinetic.

Effect of initial concentration of sorbate In general, the isotherm studies for solid–liquid systems are carried out by changing the amount of sorbate in the solution. The performance of the sorbents is usually gauged by its uptake, which can be calculated by fitting the Langmuir isotherm model to the actual experimental data.

Taking account that biosorption is an eco-friendly and cheap method of removing metals from the environment, research conducted during last five decades has provided vast amounts of information about different types of biosorbents and their mechanism of metal uptake, with examples given in the following sections.

Fly ash was used as a low-cost sorbent for the removal of Cd(II), Pb(II), and Cr(VI) ions from aqueous solutions. The influence of pH, initial concentration of metal ions, equilibrium contact time, and temperature were studied using batch sorption experiments. The equilibrium for Cd(II), Pb(II), and Cr(VI) ions was attained with 90-, 100-, and 120-min contact times, respectively. The optimum pH values for Cd(II), Pb(II), and Cr(VI) removal were found to be 8.5, 6.5, and 2, respectively, with the sorption kinetics fitting a first-order kinetic model (Khan et al. 2009).

Dinesh and Kunwar (2002) investigated the use of low-cost activated carbon derived from bagasse, an agricultural waste material, as a replacement for the current expensive methods of removing heavy metals from wastewater. With a view to find a suitable application of the material, activated carbon has been derived, characterized and utilized for the removal of cadmium and zinc. The uptake of cadmium was found to be slightly greater than that of zinc and the sorption capacity increased with increase in temperature. The adsorption studies were carried out both in single- and multi-component systems. Studies were conducted to delineate the effect of temperature, initial adsorbate concentration, particle size of the adsorbent and solid-to-liquid ratio. It was concluded that the adsorption occurs through a film diffusion mechanism at low as well as at higher concentrations.

The biosorption characteristics of Cr^{6+} from aqueous solution using sugarcane bagasse was investigated. Experimental parameters affecting the biosorption process, such as pH, contact time, initial metal concentration, and temperature were studied. The Cr^{6+} biosorption onto sugarcane bagasse was favoured by pH, temperature 25 °C, and with 10 $g.L^{-1}$ biosorbent dosage (Alomá et al. 2013).

A biosorption experiment of the heavy metals (Fe, Cr, Cu, Mn, and Zn) was conducted without controlling for any experimental parameters (e.g., pH, temperature, or other compounds present in the effluent samples) by using four agricultural wastes or byproducts, namely rice husk, sawdust, lemon peel, and eggshell in which 20 g of each biosorbent was added to 1 L of effluent samples and stored for 7 days. The biosorption capacity of each biosorbent is ranked as follows: eggshell, sawdust, rice husk, and lemon peel. Furthermore, the biosorption affinity of each metal ion was found in the following order: Cu and Cr (both had similar biosorption affinity), Zn, Fe, and Mn (Nahar et al. 2018).

In this study, two agricultural residues, cotton stalks and apricot seeds, were used to adsorb copper and lead in solutions. Sorption capacities of agricultural wastes were significantly affected by solution pH, adsorbent mass and adsorbent particle size. The adsorption efficiency of two agricultural waste was in the order cotton stalk > apricot seed and the agricultural wastes adsorbed metal ions in the order of Pb > Cu. This study has indicated that cotton stalk and apricot seed could be employed as low-cost alternatives in wastewater treatment for the removal of heavy metals (Kahraman et al. 2008).

Pagnanelli et al. (2002) have carried out a preliminary study on the use of olive mill residues as heavy metal–sorbent material. The results revealed that copper was maximally adsorbed in the range of 5.0–13.5 $mg.g^{-1}$ under different operating conditions.

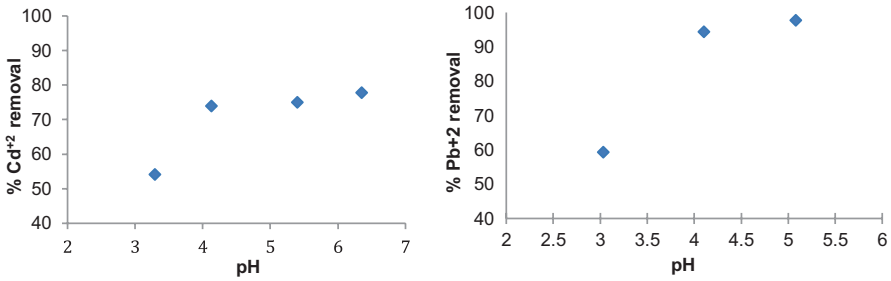

Fig. 11.1 Effect of pH on removal of cadmium and lead

Vera et al. (2016) investigated the use of sugarcane bagasse as a biosorbent in removing lead and cadmium from a mining wastewater sample. The parameters affecting the biosorption process, such as pH, contact time, concentration of the metal species and kinetic biosorption was studied. According to Fig. 11.1, best percent cadmium removal was 77.81% at a working pH equal to 6, and 99.76% for lead at pH of 5, which is reached after 10 min. It was noted for both lead and cadmium that as the metal concentration increases the amount of metal adsorbed per gram of adsorbent. The experimental data of biosorption of lead and cadmium favourably reproduce Elovich models and pseudo-second order, with correlation coefficients (R^2) for lead 1 and cadmium from 0.9986.

Characterization Techniques

Among the different types of heavy metal bio-absorbents, we have a wide range of analyses that can characterize these compounds and observe their encapsulation power, and their physico-chemical relationship with samples to be absorbed with respect to the bio-absorbents, but there are some techniques that stand out for their ease and depth in characterizing of bio-absorbent and analyte. These efforts are to detect physical and chemical changes in the structure of compounds and thus better understand the phenomena they utilize (Hamza et al. 2019). Among these techniques, we can include scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XDR), thermogravimetric analysis (TGA), and the different adsorption isotherm studies.

Normally it is necessary to analyse which is the group or chemical component in lignocellulosic residues (which in many cases are similar but the quantity varies) and is this the real cause of metal retention. Functional groups such as hydroxyl, carboxyl, sulfhydryl, sulphonate, etc. have been proposed to be responsible for metal sorption binding by different biosorbents, and their importance for uptake depends on factors such as, quantity of sites, accessibility, chemical state, or affinity between metal and site. Analysing the structure of lignocellulosic compounds, cellulose provides 30–50% of the structure as units composed of β-D-glucopyranose

sugars, with approximately 65% of cellulose highly oriented, without leaving access to water or other solvents. On the other hand, hemicellulose and lignin provide between 20–30% and 15–25%, respectively of the remaining biomass. Therefore, it is through these latter groupings that are supposed to interact with the heavy metals. There are also small amounts of water, ash, cyclic hydrocarbons, and organic and inorganic materials present in lignocellulosic sources (Chabannes et al. 2018).

Fourier transform infrared spectroscopy (FTIR) is a valuable technique to identify functional groups present on the surface of the adsorbents before and after the adsorption process. This technique can determine the presence and interaction analysis of certain chemical groups in all samples, such as amino radicals (NH−), carboxyl (−COOH), and hydroxyl (OH−). When FTIR spectra show a strong peak at 3445 cm^{-1}, it represents the –OH stretching of phenol group of cellulose and lignin, and if a peak appears at 2927 cm^{-1}, this indicates the presence of a $–CH_2$ aliphatic compound. The appearance of peaks at 1735 cm^{-1} and 1633 cm^{-1} indicates the presence of C–O stretching of an aldehyde group and C–C stretching of a phenol group, respectively. Similarly, the peaks between at 1508 cm^{-1} and 1372 cm^{-1} in the spectrum of a bio-absorbent can be due to C=C of aromatic ring. After adsorption, the shifting of –OH and the slight decrease in intensity indicate the involvement of OH in the adsorption phenomena. Scanning electron microscopy (SEM) is performed in order to obtain information about the surface morphologies of un-activated, activated and activated with metals-loaded adsorbent, at different magnifications, and to enhance the electron conductivity of the samples, it can be done by coating with gold particles. The particle size(s) and Polydispersity Index of adsorbent before accumulation of metal were determined using zeta-sizer (Zetasizer). X-ray diffraction (XRD) spectra are performed to determine changes in the structure of bioadsorbents that can help determine the existence of interactions between heavy metals and biosorbents. Thus, because of the amorphous nature of biosorbent compounds it yields complex spectra, with no defined peaks in the absence of metals. On the contrary, when we have absorption of heavy metals, defined peaks appear, characteristic of the crystalline structures of heavy metals inserted in a mixture of complex peaks of an amorphous structure (Noli et al. 2019).

Also and complementary to the above methods, thermogravimetric analysis (TGA) is used for the characterization of thermal degradation of adsorbent before and after metal accumulation. In addition, it is used to measure the material's thermal stability and the rate of change of weight of a sample as a function of time and temperature in a controlled atmosphere. It is primarily used to determine the thermal and/or oxidative stability of materials as well as their compositional properties. The weight of a sample is recorded as a function of temperature under air or inert atmosphere, such as helium or argon. To slow down the oxidation, the measurement is performed under a lean oxygen atmosphere (1–5% O_2 in N_2 or He). To obtain information about the elemental compositions of the biosorbents, the carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) contents of each sample are measured by using a CHNS Analyzer. The percentage of oxygen is calculated by subtracting the C, H, N, and S contents from 100%. Subsequently, the adsorption studies are also focused on determining the effect of the presence of one adsorbate on the

adsorption of a second adsorbate. The adsorption isotherms are usually modelled by using the Langmuir and Freundlich isotherm models (Wang et al. 2019). A summary of different characterization techniques using agro-waste materials is shown in Table 11.1.

Modelling

Parallel to the extensive experimental activity carried out during the last decades on heavy metal biosorption, several mathematical models, most in the form of empirical correlations, have been developed to elucidate and represent the heavy metal adsorption on biomass binding sites (Volesky and Schiewer 2000). Mathematical modelling represents a useful tool to describe the complex mechanisms characterizing the biosorbent and the solute interactions and assists in the optimization and design of biosorption processes. The biosorption models are classified into two main categories based on the mode of operation (batch or continuous) used to conduct the process. Although most industrial applications prefer a continuous mode of operation, batch experiments have to be used to evaluate the required fundamental information, such as biosorbent efficiency, optimum experimental conditions, biosorption rate and possibility of biomass regeneration.

Batch System

Equilibrium Study

Biosorption process generally started with batch mode, this experiment compares the biosorption capacity (q_e, mg.g^{-1}) and percentage of removal (%) versus the residual concentration of the pollutant in the solution at equilibrium, Eqs. (11.1) and (11.2):

$$q_e = \frac{(C_0 - C_e)V}{m} \tag{11.1}$$

$$\text{Removal}(\%) = \frac{(C_0 - C_e)100}{C_0} \tag{11.2}$$

Where C_0, C_e are the concentration of pollutant in the initial solution and at equilibrium in the aqueous phase after adsorption (mg.L^{-1}), V is the volume of the solution (L), and m is the mass of the biosorbent (g).

The main operating factors are evaluated in order to optimize the experimental conditions (solution pH value, biosorbent dose, initial sorbate concentration,

Table 11.1 Different characterization techniques results done by various researchers using agro-waste materials

Biosorbent	Technique	Analysis	Reference
Coffee residue	Adsorption isotherms	The BET surface area was determined by means of the standard BET equation applied in the relative pressure range from 0.06 to 0.3 m^2/g	Boudrahem et al. (2011)
	Thermogravimetric analysis (TGA)	Raw coffee residue exhibits only one prominent wave of weight loss [between 200 and 400 °C] with a maximum centred at 270 °C, followed by a slow weight loss with decreasing rate. A considerably different pattern is exhibited by the H_3PO_4-treated lignocellulosic material. Through this temperature range of 400–900 °C, two DTG peaks appear with maxima at 525 °C and 700 °C. Thus, impregnation with H_3PO_4 brings about considerable effects on the course of pyrolysis of coffee residue: (i) it shifts degradation to considerably higher temperatures, (ii) it enhances the early dehydration stage combined with an evolution of low-boiling volatiles, (iii) it strongly retards the evolution of volatiles where up to 600 °C only around 50% weight is lost, and (v) it promotes the carbon yield. These observations suggesting mechanism, involving dissolution of some chemical components with bond cleavage, followed by recombination at other sites forming new polymeric structures that are thermally more resistant	
	Infrared spectra of the Fourier transform (FTIR)	FTIR spectra present for the activated carbon impregnated with H_3PO_4 (50% optimal chemical ratio). They show the following bands: 4000 cm^{-1} and 3500 cm^{-1} correspond to the –OH groups of the phenol function; 2906 cm^{-1} and 2847 cm^{-1} correspond to stretching C–H groups of aliphatic, olefinic, and aromatic structures; 1556 cm^{-1} is attributed to stretching C=O of the carbonyl groups in quinine as well as γ-pyrone structure; and 1424 cm^{-1} corresponds to stretching C–O or O–H deformation in carboxylic acids	
Palm shell	Adsorption isotherms	The BET surface area was found to be 0.2979 m^2/g	Sreelatha et al. (2010)
	X-ray diffraction (XDR)	The X-ray diffraction pattern shows a peak centred around 21° corresponding to a 002 reflection of disordered packing of micrographites. The peak is broad and suggests an amorphous structure. The peak at 21° corresponds to an interlayer distance of 0.423 nm which suggested a disordered carbonaceous interlayer.	

(continued)

Table 11.1 continued

Biosorbent	Technique	Analysis	Reference
	Infrared spectra of the Fourier transform (FTIR)	Possible interactions of pre-treated with sulphuric acid (APSP) and dye was studied by comparing the features of the spectra obtained from IR spectroscopy studies of dye, dye-loaded, and dye-unloaded APSP. APSP showed a broad frequency at ~3419.42 cm^{-1}, which can be assigned to N–H/–OH stretching. After the adsorption of the dye molecule the peak is shifted to 3424.65, 3383.76, and 3174.57 cm^{-1} for AO-II, DSB, and AV-7 dyes, respectively. The peak at ~1625.29 cm^{-1} was assigned to the N–H bond of amine or characteristics of the elongation of the aromatic –C=C- bonds which also have been shifted to 1617.62, 1608.24, and 1609 cm^{-1} for AO-II, DSB, and AV-7 dyes, respectively. The peak at 1378.58, which corresponds to C–N stretching, is missing after the adsorption of dye molecules. The peak at 1200 cm^{-1} is associated with the C–O stretching of the aromatic ring which is also shifted to a lower frequency region	
	Scanning electron microscope (SEM)	The morphology of APSP was studied using a scanning electron microscope. The surface is magnified 1000 times which shows that the adsorbent has an irregular, rough, and porous surface with identifiable micropores and mesopores	
Corncob and peanut shell precursors	Adsorption isotherms	The BET surface area was found to be 862 m^2/g and 654 m^2/g for corncobs-based or peanut shells–based activated biocarbons, respectively	Wiśniewska and Nowicki (2019)
	Viscosity	Polyacrylic acid (PAA) with the weight average molecular weight equal to 2000 Da	
	Zeta potential (ZP)	The electrophoretic mobility of the solid particles (in the absence of adsorbates) takes negative values, and it is not possible to determine the isoelectric point (IEP) value. In the case of both activated carbons, the addition of Pb(II) cations causes a noticeable decrease in the absolute values of electro-kinetic potential, which probably results from the accumulation of their positive charges at the stiff and mobile parts boundary	
	Scanning electron microscope (SEM)	The small, bright fragments, which can be observed in both SEM images, may be a consequence of the mineral matter presence in the porous structure of the studied samples. The mean pore size of both activated carbons equal to approx. 2.5 nm determines the penetration of PAA macromolecules into their interiors. This is confirmed by the SEM images. In such a case the separation of these aggregates containing activated carbon and binding hazardous adsorbates is facilitated	

Chitosan (CH) and thiobarbituric acid (TBA) to form chitosan/thiobarbituric acid (CT)	Ultraviolet-visible (UV-Vis) spectroscopy analysis	UV analysis. The electronic absorption spectra of chitosan/thiobarbituric acid exhibited an absorption maximum at 226 nm, which can be assigned to the $\pi \rightarrow \pi^*$ transition, whereas the absorption bands at 282 and 363 nm can be assigned to $n \rightarrow \pi^*$ intra-ligand transitions	Bhatt et al. (2018)
	Infrared spectra of the Fourier transform (FTIR) analysis	FTIR analysis showed that intense broadbands in the 3300–3500 cm^{-1} region can be attributed to N−H and the O−H stretching vibrations in chitosan/thiobarbituric acid (CT). The absence of peak at around 3600 cm^{-1} can be attributed to the keto-tautomer of thiobarbituric acid, evidencing the formation of CT. Furthermore, there were either slight shifts in characteristic peaks of CT or change in intensities after adsorption of mercury species. The amide III band shifted from 1319 cm^{-1} in CT to ~1304, 1302, and 1301 cm^{-1} in CT–Hg^0, CT–Hg_2^+, and CT–CH_3Hg^+, respectively	
	Scanning electron microscopy–energy-dispersive X-ray (SEM–EDX) analysis	The EDX spectra showed the presence of sulphur in CT, indicating the incorporation of 2-thiobarbituric acid into chitosan. Furthermore, the presence of mercury peaks after adsorption of inorganic, methyl, and elemental mercury confirmed the adsorption of all of the three mercury species onto CT	
	Nuclear magnetic resonance (NMR) analysis	The NMR spectrum of CT and proposed structure of CT, respectively. The peaks at 2.05–2.07 ppm were attributed to the three methyl protons of N-acetyl glucosamine, and those at 3.18 ppm, to the 2 protons (H_2) of glucosamine. The overlapping signals from 3.7 to 3.9 ppm were attributed to protons connected to the nonanomeric C3−C6 carbons in the glucopyranose ring of chitosan chain, and the signal at 5.029 ppm was attributed to the anomeric proton and to the proton connected to C5 of the pyrimidine ring of thiobarbituric acid	
	Differential scanning calorimetry (DSC) analysis	Differential Scanning Calorimetry (DSC) analysis curves showed two exothermic peaks at 279 °C and 302 °C in CH and 285 °C and 301 °C in CT were attributed to the thermal decomposition of amino residues. Furthermore, no exothermic peak was observed at around 400 °C, thereby indicating that the N-acetyl groups were not present on the polymer matrix and only amino residues were observed, which are less thermally stable than the N-acetyl.	

(continued)

Table 11.1 continued

Biosorbent	Technique	Analysis	Reference
	Thermogravimetric analysis (TGA) analysis	Thermogravimetric analysis (TGA). Both CH and CT exhibited similar thermal behaviour. The weight loss in the 40–100 °C region was due to the loss of adsorbed water or that absorbed in the inner polymeric network. The weight loss that occurred at ~200–300 °C was slightly less in CT as compared to that in CH, which could be due to the supramolecular cross-linking of thiobarbituric acid in the polymer structure, leading to slower thermal degradation of the biopolymer. The weight loss observed at 300–400 °C can be ascribed to degradation of polysaccharide units and breakdown of the glucopyranose ring. The weight loss in the 400–500 °C range could be due to emission of volatile products, such as CO and CO_2, and the formation of the carbon residue.	
	X-ray diffraction (XRD)	X-ray diffraction (XRD) analysis of CH, CT, and mercury-loaded CT samples. The XRD pattern of CT showed a broad peak at around $2\theta = 20°$, indexed as the 101 plane of crystal form I, which is present due to the amorphous state of chitosan. The higher peak intensity for CT at 19.93° could be attributed to the intermolecular hydrogen bonding patterns and crystalline structure. Furthermore, the position of the peak at 19.93° for CT shifted to 21.95°, 19.98°, and 20.27° on interaction with Hg^0, Hg^{2+}, and CH_3Hg^+, respectively. Furthermore, the binding of the sulphur species with Hg^0, Hg^{2+}, and CH_3Hg^+, respectively, was also supported with the generation of new peaks at 46.55, 41.21, and 42.73°, respectively. The crystalline index was calculated to be 50.07% using crystalline index (%) = $[(I_{100} - I_{am})/I_{110}]x100$, where I_{100} denotes the maximum intensity at ~20° and I_{am} is the intensity of amorphous diffraction at 16°, whereas the crystallite size was found to be 1.24 nm for the peak at 19.939° using Scherrer's expression	
	X-ray photoelectron spectroscopy (XPS)	X-ray photoelectron spectroscopy (XPS) analysis. In CT, the binding energy at 164.6 eV corresponded to organically bound sulphur. The typical S $2p_{3/2}$ binding energy for unbound thiols was between 163 and 164 eV, suggesting that the thiol group of thiobarbituric acid is free after layering with chitosan. After the adsorption of Hg(II), the appearance of peak at 161.7 eV suggested binding of thiol group to mercury, whereas the peak at 163.2 eV corresponded to the free thiol group. Similar signals were observed after adsorption of elemental mercury and methyl mercury	

Orange peel	Infrared spectra of the Fourier transform (FTIR)	The orange peel FTIR spectrum showed the peak of the hydroxyl group (–OH) ranges from 3424 to 3450 cm^{-1}, the C=O bond of non-ionic carboxylic acids in the range 1735–1750 cm^{-1}, the symmetric and asymmetric bonding of C=O vibration of ionic carboxylate group in the range of 1617–1637 cm^{-1}, and the symmetrical vibration of ionic carboxylic group in the range of 1300–1500 cm^{-1}. The peak that appears in the 3440 cm^{-1} shows the presence of free hydrogen or the O–H bond in alcohols, phenols, and carboxylic acids present in pectin, cellulose, and lignin. This peak has changed 8 units in the orange peel after absorption, which indicates effect of this group on the absorption of thorium. Due to the FTIR spectrum of orange peel, the effect of hydroxyl and carboxylic groupings, that are abundantly present in this adsorbent, is known in the biosorption of thorium. The absorption of thorium on orange peel is most likely due to the electrostatic attraction between these functional groups and the metal cation. The carboxylic group loses proton at high pH ($–COO^-$) and has a negative superficial potential, which increases the metal cation absorption capacity	Gh et al. (2018)
	X-ray fluorescence (XRF)	The results show that the percentage of calcium and potassium ions decreased significantly after adsorption, and on the other hand, with the adsorption of thorium, the percentage of thorium in adsorbent after absorption increased (93% by weight). So, calcium and potassium ions are replaced by thorium ions during the adsorption process. By measuring the concentrations of calcium and potassium ions in samples taken from the column outlet solution can also study the same mechanism of the process	
Tangerine peel	Infrared spectra of the Fourier transform (FTIR)	As can be seen for untreated tangerine peel, a broad absorption peak around 3400 cm^{-1} indicates the existence O–H stretching vibrations occur within a broad range of frequencies, indicating the presence of ‘free’ hydroxyl groups and bonded O–H bands of carboxylic acids. After-chemical treatment resulted in shifting of this peak towards higher value of wave numbers (around 3430 cm^{-1}), which corresponds to polymorphic form of cellulose. Also, for untreated peel, peak of medium intensity in the area of carbonyl group, around 1740 cm^{-1}, can be attributed to acetylene and uronic ester groups of pectin, lignin, and hemicelluloses or to ester bonding of carboxyl groups from ferulic and coumaric acid of lignin and hemicelluloses	Memić et al. (2018)

(continued)

Table 11.1 continued

Biosorbent	Technique	Analysis	Reference
Wheat straw	Infrared spectra of the Fourier transform (FTIR)	Ground, dried wheat straw (*Triticum aestivum*) was subjected to FTIR analysis for its characterization in terms of functional groups (adsorption sites). The characteristic functional group present in wheat straw was C=O (1650.8 cm^{-1})	Ali et al. (2011)
Sugarcane bagasse	Ultraviolet-visible (UV-Vis) spectroscopy analysis	The absorbance calculated of lignin solution at 280 nm. Furthermore, furfuraldehyde and hydroxymethylfurfural in the acid solution were quantified by HPLC. The conversion factor for furfuraldehyde and hydroxymethylfurfural into cellulose and hemicellulose are 1.37 and 1.29, respectively	Rocha et al. (2015)
	Determining the amount of carbohydrates by HPLC	The amounts of cellobiose, glucose, xylose, arabinose and acetic acid present in the acid hydrolysate of lignocellulosic materials were quantified by HPLC. The concentrations of glucose and cellobiose were converted into cellulose, concentrations of xylose and arabinose were converted to hemicellulose, and concentration of acetic acid was converted to acetyl groups. The masses were divided by the dry weight of the original material and multiplied by the hydrolysis factor: 0.90 and 0.95 for glucose and cellobiose to cellulose, respectively; 0.88 for xylose and arabinose to hemicelluloses and 0.72 for the conversion of acetic acid to acetyl group	
	Elemental analysis	The content of carbon, hydrogen, nitrogen and sulphur was determined in an element analyzer. The oxygen content was determined by the difference. As average, the bagasse samples contained 42.2% of cellulose, 27.6% of hemicelluloses, 21.6% of lignin, 5.63% of extractives and 2.84% of ashes. In general, the composition of these values is also within the ranges state, which are 40–50% for cellulose, 25–30% for hemicelluloses, and 20–25% of lignin. (Lignin is thought to be linked mainly to side-chain sugars present in the hemicelluloses, as arabinose and galactose)	

Carica papaya	Infrared spectra of the Fourier transform (FTIR) biosorbent	The FTIR spectrum of raw biomass showed several distinct and sharp absorptions at 3409 cm^{-1} (indicative of primary amide, $–CONH_2$ group), 2925 cm^{-1} (indicative of –CH stretching), 2359 cm^{-1} (indicative of = $N–H_2^+$ group), 1742 cm^{-1} (indicative of five ring lactone), 1627 cm-1 (indicative of amide I band of amide bond in N-acetyl glucosamine polymer or of the protein peptide bond), 1439 cm^{-1} (indicative of the bending of CH_2CH_3), and the band at 1036 cm^{-1} (indicative of sulphur compounds, S=O). The FTIR spectra of papaya wood exposed to Hg(II) ions indicated no shifts or change in any of the characteristic absorbance bands present in raw biomass with the exception of a peak shift at 3434, 2362, and 1645 cm^{-1}. The spectra exhibited absorptions at approximately 3434 cm^{-1}, suggesting the occurrence of secondary amide, –CONH–. The results implied mainly involvement of amide groups in sorption of Hg(II) ions. Although slight changes on the other absorption frequencies were observed	Basha et al. (2008)
	Scanning electron microscopic (SEM)	Electron micrographs of the raw and Hg(II) sorbed biomass of *C. papaya* clearly distinguish two cases. The surface of raw biomass is rough and irregular with a large area for metal–surface interaction which formed a cage like structure after Hg(II) sorption. The exact reason for this formation is not known at this moment; however, the chemical interaction between functional groups of the biomass and mercury ion may be responsible for the formation of cage-like structures. Energy-dispersive X-ray analysis (EDX) provides elemental information through analysis of X-ray emissions caused by a high-energy electron beam. The spectra recorded in spot profile mode indicate the presence of C, N, O, Al, Na, K, and S. These signals are due to X-ray emissions from the fibre and proteins present on the cell wall of the biomass. Additional signals of Hg are noted, indicating the binding of metal ions on the biomass. Moreover, for the sample after biosorption, was observed on the EDX spectrum that the peaks of K disappeared. This observation could suggest that the mercury species has replaced K^+ on the cell wall of *C. papaya*, thereby signifying an ion-exchange mechanism as one of the mechanisms of Hg(II) biosorption for this biosorbent	
	Atomic absorption spectroscopy (AAS)	The pH for all experiments was 5.0 except for effect of pH where the pH was varied from 3.0 to 8.0. This is irrespective of the pH PZC (6.25) to avoid metal ion precipitation	Gilbert et al. (2011)

(continued)

Table 11.1 continued

Biosorbent	Technique	Analysis	Reference
Tamarindus indica seed powder (TSP)	Infrared spectra of the Fourier transform (FTIR)	*Tamarindus indica* seed powder (TSP) mainly contains polysaccharide with fats, tannins, proteins and amino acids in minimum proportion. The FTIR spectra of TSP shows the presence of several functional groups, indicating the complex nature of TSP. The broad and strong band at 3450 cm^{-1} suggests the presence of OH and NH2 groups. The peak at 2904 cm^{-1} can be attributed to CH stretching vibrations while the peak appearing at 1652 cm^{-1} arises from CO stretching in amide groups. The peak at 1465 cm^{-1} represents CH3. The peak around 1160 cm^{-1} indicates CN stretching vibration. The peak at 1060 cm^{-1} corresponds to CO stretching vibration of alcohols and carboxylic acids. After Cu(II) biosorption, the band at 3450 cm^{-1} corresponding to OH and NH groups shifts to the lower frequency (3390 cm^{-1}). Thus, it can reasonably be concluded that OH and NH_2 groups may be the main binding sites for Cu(II) onto TSP. Also, the peaks at 1652 cm^{-1} and 1060 cm^{-1} shift to 1615 cm^{-1} and 1030 cm^{-1} respectively. This shift in peaks suggests that CO and CO groups participate in the Cu(II) binding process. Hence, FTIR spectral analysis reveal that functional groups like NH_2, OH, CO, and CO present on TSP surface are involved in Cu(II) biosorption	Chowdhury and Saha (2011)
	Scanning electron microscopic (SEM)	SEM is one of the most useful tools for studying the surface morphology of a biosorbent. The SEM images of native TSP and Cu(II)-loaded TSP. SEM image of TSP before metal biosorption shows a rough, uneven and heterogeneous surface with porous structure. The rough surface can help increase the surface area available for biosorption of Cu(II). However, the porous textural structure is not observed on the surface of Cu(II)-loaded TSP. The surface morphological change can be linked to precipitation/complexation of Cu(II) on the biosorbent surface	
	Adsorption isotherms	The BET surface area, total pore volume and average pore diameter of the biosorbent before metal biosorption was found to be 29.46 m^2/g, 0.0189 cm^3/g, and 67 Å, respectively.	
	X-ray diffraction (XRD)	The XRD pattern of TSP and Cu(II) loaded TSP. The XRD pattern of TSP does not show any characteristic peaks indicating amorphous nature of the biosorbent. The amorphous nature suggests that metal ions could easily penetrate into the surface of the biosorbent as was observed in the case of gum kondagogu. The XRD pattern of metal-bound TSP shows distinct and complex peaks, indicating the deposition of Cu(II) on the surface of TSP	

Citrus maxima peel (CM), passion fruit shell (PF), and sugarcane bagasse (SB)	Scanning electron microscopic (SEM)	The biosorbents used in this study uptake metal ions through complexation or ion exchange processes, which might be influenced by the surface properties of the test biosorbents. SEM micrographs with valleys and peaks exhibiting the roughness of CM and PF and the absence of protrusions for SB. In the SEM images, the biosorbents exhibited limited pore volume and are not porous. The biosorbents uptake metal ions through the functional groups on their surfaces. The pore volume and specific surface area are less crucial than the BET surface area, ZP, and pH values of the biosorbents. The specific surface area of the biosorbent was lower than 2 m^2/g. The results demonstrated that the pore volume and surface area are not critical in this study	Chao et al. (2014)
	Infrared spectra of the Fourier transform (FTIR) biosorbent	FTIR spectroscopy was used to determine the functional groups before and after adsorption present at the biosorbent surface. The spectra are similar before and after adsorbing Cu^{2+}. The broad absorbance at 3200–3600 cm^{-1} indicates the presence of OH or COOH groups. A weak absorbance at 1690–1760 cm^{-1} suggests the presence of the C–O group. The absorbance band at 1200–1220 cm^{-1} might result from the CO and OH groups in COOH, and the absorbance at 1050–1300 cm^{-1} might result from the C–O group on the surface. Thus, the FTIR spectra indicate that the biosorbent surfaces are rich in polymeric hydroxyl groups, CHN and COO groups, and OH groups of polysaccharides. Agricultural by-products usually are composed of lignin and cellulose and other functional groups of lignin (i.e. alcohols, aldehydes, ketones, carboxylic, phenolic and ether groups), which all have the ability to some extent to bind heavy metals by donation of an electron pair from these groups to form complexes with metal ions in aqueous solution	

(continued)

Table 11.1 continued

Biosorbent	Technique	Analysis	Reference
	Zeta potential (ZP)	The biosorbent zeta potentials (ZP) for various pH values determine the ion exchange potential. A negative ZP indicates a negative surface charge on the adsorbent surface, and vice versa. Significantly, the table shows negative ZP values for the entire pH range tested. Thus, metal ions may adsorb to the biosorbent surface by an ion-exchange mechanism. The results demonstrated that ion exchange is one of the adsorption mechanisms for metal ions in the test biosorbents. The cation-exchange capacities (CEC) values of the biosorbents arise from the functional groups present on their surfaces. The CEC values of the CM, SB, and PF are 47.3, 11.8, and 26.9 (meq/100 g), respectively. The dissociation of hydrogen ions or other cations from a functional group under specific pH conditions generates an exchangeable cation. Functional groups such as carboxyl, amino, and hydroxyl on the surface of the biosorbents provide complexation sites for metal ions. Groups containing oxygen or nitrogen atoms provide nonbonding electrons for coordination with the divalent metal's ions. Thus, the surface functional groups are the dominant factors in determining adsorption capacity. According the main element composition of the biosorbents, which predominantly contain carbon and oxygen, and no nitrogen, consequently amino groups are not present in substantial quantities, whereas carboxyl and hydroxyl groups are the potentially dominant groups	
Cashew nut shells	Thermogravimetric analyzer coupled with a Fourier transform infrared spectrometer (TG-FTIR)	The volatile pyrolysis product species identified in this study are (i) carbon monoxide (CO); (ii) carbon dioxide (CO_2); (iii) tar (by difference using gravimetric data); (iv) water (H_2O); (v) methane (CH_4); (vi) ethylene (C_2H_4); (vii) formaldehyde (CH_2O); (viii) formic acid (HCOOH); (ix) carbon sulphide (COS); (x) acetic acid (H_3CCOOH); (xi) methanol (CH_3OH); (xii) ammonia (NH_3); (xiii) hydrogen cyanide (HCN); (xiv) isocyanic acid (HCNO); (xv) acetone (H_3COCH_3); (xvi) phenol (C_6H_5OH); and (xvii) acetaldehyde (H_3CCHO). Tar evolution patterns and yields were determined by difference, using the sum of gases quantified by FTIR and the balance curve obtained thermogravimetrically	Tsamba et al. (2007)

Olive cake	Elemental analysis CHNS (this technique consists of the de-termination of the percentage of C, H, N and S)	The elemental analysis of the biosorbent. The sample was mainly composed of carbon (51.57%) and oxygen (35.33%). If the results are compared with the ones obtained in previous studies of the untreated olive cake, it was observed that the carbon content was concentrated after the washing, whereas the oxygen content was reduced. The nitrogen, hydrogen and sulphur contents remained almost constant after the treatment. The concentration of carbon could be due to the elimination of inorganic compounds as it showed the reduction of ash that occurred after the treatment. The reduction of oxygen could be due to the elimination of water caused by the drying carried out. The elemental analysis showed similar results to the ones obtained by other authors for hydrolysed live cake. On the other hand, the proximate analysis. In this case, as aforementioned, ash and moisture contents were reduced with respect to untreated olive cake. The volatiles were the major compounds present in the sample (70.08%)	Fernández-González et al. (2018)
	Differential scanning calorimetry (DSC) analysis	The first peak of DTG curve appeared at temperatures around 100 °C, corresponding to the moisture content and the external moisture bounded by the surface tension. The second peak appeared at a temperature around 280 °C, corresponding to the decomposition of hemicellulose. Another peak was observed around 380 °C, which could be attributed to cellulose. Those two peaks appeared for the hydrolysed olive cake and not for the untreated olive cake. In the untreated one, it appeared as one broad peak whereas in the washed sample it appeared as two peaks which have slightly shifted to the right with respect to the untreated sample. It could be because leaching promoted the thermal stability of the material. The degradation of lignin occurred in a broad range of temperatures, appearing as a baseline of the DTG curve and not as a well-differenced peak	
Olive stone and sugar cane bagasse	Elemental analysis CHNS (this technique consists of the de termination of the percentage of C, H, N and S)	Characterization of a biosorbent is an important analysis for understanding the behaviour or the mechanism of cadmium removal on the surface of biosorbent. These in order to quantify the amount of C, H, N, S and O by to elemental analysis and the moisture content of olive stone and bagasse by-products. Elemental analysis results showed that olive stone and bagasse have a similar elemental composition, being composed mainly of carbon (51.78% and 48.57% for olive stone and bagasse, respectively) and oxygen (41.77% and 45.58% for olive stone and bagasse, respectively). The H concentration was 6.33% and 5.41% for olive stone and bagasse, respectively. There is no sulphur on both olive stone and bagasse. The moisture content was lower than 5% for both biosorbents	Moubarik and Grimi (2015)

(continued)

Table 11.1 continued

Biosorbent	Technique	Analysis	Reference
	Infrared spectra of the Fourier transform (FTIR)	FTIR analysis is primarily used to identify the chemical groups present in the biosorbents. FTIR spectra before Cd^{2+}biosorption. Direct information on the presence of surface functional groups can be obtained from FTIR studies. FTIR results revealed very similar spectra for olive stone and bagasse, which confirmed that both have similar functional groups. In order to illustrate the distinct attributions, the corresponding assignments identified for the olive stone and the bagasse. The FTIR analysis shows the absence of nitrogen and sulphur groups in the olive stone and the bagasse structure, whereas we note the presence of different oxygen groups, mainly carbonyl, alcohol and phenol groups, ethers and esters. Hence, the good sorption properties of the olive stone and bagasse towards Cd^{2+} ions can be attributed to the presence of these functional groups	
	Adsorption isotherms	The results show that the olive stone pores are mesopores while bagasse pores are macropores. The average pore diameter of olive stone and bagasse was found to be 12.7 nm and 57.2 nm, respectively. The specific surface area of olive stone and bagasse is 0.378 m^2/g and 0.487 m^2/g, which was supported by the data reported in other researches. The strong interaction between adsorbate molecules and pore walls further controls the filling of micropores during adsorption process	
	Zeta potential (ZP)	Zeta potential is the manifestation of surface charge density of the adsorbent. Surface charge density has a significant effect on Cd^{2+} metal ions adsorption on olive stone and bagasse. It obtained the variation of zeta potential of olive stone and bagasse as a function of pH and the pH at the point of zero charge (pH_{pzc} of olive stone is 4.2 and pH_{pzc} of bagasse is 5.7), similar results have been reported by other researchers. The pH_{pzc} value is the point at which surface functional groups do not contribute to the pH of the solution. Above this pH value, the surface charge becomes negative and the adsorbent will take up the cations with higher affinity	

	Scanning electron microscopy (SEM)	Olive stones and bagasse by-products used in this study were analysed by SEM in order to examine their morphology. The SEM image of olive stones and bagasse before cadmium adsorption show that pores of different sizes and different shapes could be observed. As shown in these images, the prepared olive stones and bagasse have irregular structure, which can favour the biosorption of Cd^{2+} ions on different parts of biosorbent. These images confirm the previous results obtained by the analysis of the porosity of olive stones and bagasse	
Soya bean	Infrared spectra of the Fourier transform (FTIR)	The FTIR spectra of soya bean as an adsorbent of Pb and As show before and after biosorption of heavy metal respectively. A broad band between 3201 and 3518 cm^{-1} in both adsorbents is indicative of the presence of free and hydrogen-bonded OH groups on adsorbent surface. This stretching is due to both the silanol groups (Si–OH) and adsorbed water (peak at 3400 cm^{-1}) at the adsorbent surface. The stretching of OH groups bound to methyl groups shows a signal between 2852 and 2924 cm^{-1}. Similarly, peaks at 1743 and 1317 cm^{-1} are indicative of carboxyl group, while those at 1539.20 and 1458.18 cm^{-1} are indicative of $–CH_2$ and $–CH_3$ groups. The peak at 1251, 1035, 896, and 850 cm^{-1} is due to $–SO_3$ stretching, C–O stretching, C=O stretching, and aromatic –CH stretching. The presence of polar groups on the surface of adsorbent is likely to give considerable cation-exchange capacity to the adsorbents. The peaks at 783 cm^{-1} indicate the presence of Si–H	Gaur et al. (2018)
	Field emission gun scanning electron microscopes (FEG-SEM)	The SEM micrograph of un-activated, activated and activated metals (Pb and As)-loaded adsorbent. As mentioned previously, the activated adsorbent contains exposed cavities and pores which favour the adsorption of metals. From it could be inferred that surface texture of the soya-based adsorbent changes drastically after the adsorption of the heavy metals. The surface of the soya-based biosorbent also appears uneven and irregular with cavities which facilitate interaction of heavy metals (Pb and As) with the biosorbent surface, leading to proper metals adsorption. An alternation in rough surface was examined after the use of Pb and As in the adsorption studies	

(continued)

Table 11.1 continued

Biosorbent	Technique	Analysis	Reference
	Dynamic light scattering (DLS) Zeta meters	The instrument Zetasizer uses the phenomenon of dynamic light scattering to reveal the particle size of the sample through the measurement of Polydispersity Index. The dynamic light-scattering measurement shows polymer particles around 0.1 μm with a polydispersity of 7. Particles in suspension/solution undergo Brownian motion which is induced by the bombardment of the solvent particles that moves due to their thermal energy. When the laser light hits the particle surface, the smaller particles move faster as the rate of intensity of light is directly proportional to the size of particle. Also, the movement of the particle depends on the motion of solvent molecule	
	Thermo-gravimetric analysis (TGA)	Thermal stability of soya waste–based bioadsorbent is directly dependent on decomposition temperature of its various oxides and functional groups. The surface groups present on carbons moiety and those formed as a result of interaction with oxidizing gases or solutions are generally quite stable even under vacuum at temperatures below 150 C, irrespective of the temperature at which they are formed. However, when the carbons are heated at higher temperatures, the surface groups decompose, producing CO (150–600 C), CO_2 (350–1000 C), water vapour and free hydrogen (500–1000 C). The principal experimental variables which could affect the thermal degradation characteristics in air and nitrogen flow in a TG experiment are the pressure, purge gas flow rate and the weight of sample. In the present study, the operating pressure was kept slightly positive; the purge gas flow rate was maintained at 200 C	
Core–shell structured biosorbent derived from chemical-mechanical pulp (CMP) and carboxymethylated cellulose fiber (CMF)	Infrared spectra of the Fourier transform (FTIR)	The differences of functional groups between original CMP and the as-prepared CMF. Obviously, the spectra showed that the broad bands appeared at around 3250–3500 cm^{-1} was consistent with the hydroxyl groups; meanwhile, the signal at roughly 2920 cm^{-1} may be due to the C–H stretching vibration. The broad peak at 1064 cm^{-1} can be attributed to the stretching vibration of C–O–C. Comparing the spectrum of CMP with that of CMF, the peaks at 1600 cm^{-1} and 1400 cm^{-1} were related to the C=O stretching and bending vibration, respectively. The results confirmed that –COOH was successfully introduced into CMF	Wang et al. (2019)

	Scanning electron microscopes (SEM) and laser scanning confocal microscope (LSM)	In the process of carboxymethylation, lignin would wrap on the surface of the CMF like a 'shell' and prevent the swelling of CMF. Figure 11.3c shows the different fluorescence intensity of the cross-section of CMFs after dyed with anionic fluorescent brightener. We found that the surface of the as-prepared CMF showed a strong fluorescence (white) while the internal exhibited a weak fluorescence (dark), which was mainly due to the core–shell structure of CMF and the different electrostatic repulsion between the lignin–cellulose constitution and fluorescent brightener. Compared with the lignin-based shell of CMF, he cellulose-based core with more negative charges had a stronger electrostatic repulsion and a less adsorption capacity toward fluorescent brightener, thus leading to the weak fluorescence in the internal.	
	X-ray diffraction (XRD)	XRD shows the characteristic peaks of cellulose I present in the CMP samples were 14.8° and 22.5°, respectively. For CMF, a new peak appeared at 21°, which was a typical peak for cellulose II, indicating that high sodium hydroxide concentration could change the cellulose crystal form during the process of carboxymethylation.	
Banana peels waste	Infrared spectra of the Fourier transform (FTIR) biosorbent	FTIR spectra of banana peels particles with a particle size of <250 μm (before and after the pre-treatment stage) were analysed and it shows number of peaks that indicate a complex nature of these particles. It is apparent from this figure that the following peaks 1735–1559 cm^{-1}, 1531.5–1409 cm^{-1}, and 1413.6–1024 cm^{-1} were shifted due to the pretreatment stage. The peaks 1735–1559 cm^{-1}, 1531.5–1409 cm^{-1}, and 1413.6–1024 cm^{-1} were assigned as C=O stretch, C=C stretch, and C=N stretch, respectively. However, the peak of 2326–2048.9 cm^{-1} that was attributed to the OH stretch was not shifted.	Al-Mohammedawi et al. (2019)
	Scanning electron microscopes (SEM)	SEM showing the surface morphology of banana peels particles before and after the pretreatment stage show that microporous structure and rough texture were observed for this sample, which can enhance the performance of banana peels particles as biosorbents. Similar observations were also previously reported when using banana peels as treatment agents. After pre-treatment stage, it was observed that the porous structure of banana peels particles was increased, and the binding sites were occupied. This can be attributed to the adsorption of ammonium, in addition to the possible transfer of carbon compounds from banana peels particles into brewery wastewater.	

temperature). Different isotherm model equations are used to study the biosorption equilibrium, of which the Langmuir and Freundlich are probably the most widely used with a high rate of success.

Langmuir isotherm. Biosorption takes place at specific homogeneous sites on the surface of the biosorbent forming monolayer coverage and the molecule adsorption capacity on one site is independent of its surroundings Eq. (11.3):

$$q_e = \frac{q_m K_L C_e}{1 + K_L C_e} \tag{11.3}$$

where q_m is the maximum binding capacity ($mg.g^{-1}$), and K_L is the Langmuir constant that represents affinity between the sorbent and sorbate through to the free energy of biosorption ($L.mg^{-1}$).

Freundlich, is empirical model, and assumes a multilayer biosorption on a heterogeneous surface with no uniform distribution of heat of adsorption Eq. (11.4):

$$q_e = K_F C_e^{1/n} \tag{11.4}$$

where K_F is a constant relating the biosorption capacity ($mg.g^{-1}$)($L.mg^{-1}$)^n and $1/n$ is a dimensionless empirical parameter relating the biosorption intensity, higher the $1/n$ value, more favourable is the adsorption, which varies with the heterogeneity of the material and when $n = 1$, all sorption centres are equivalent. The model is widely used to describe the adsorption from diluted solution but yields no maximum value of adsorption at saturation.

Sips model takes account three parameters from Freundlich and Langmuir models, and assumes that each adsorption site interacts with a single molecule or ion of the adsorbate surface Eq. (11.5):

$$q_e = \frac{q_m \left(K_s C_e\right)^{1/n}}{1 + \left(K_s C_e\right)^{1/n}} \tag{11.5}$$

Where K_s is sips binding constant ($L\ mg^{-1}$)^n, and n is the heterogeneity factor; n close to or equal to 1 takes place in biosorbents with homogeneous active sites whereas a value close to 0 takes places in biosorbents with heterogeneous active sites. The n_s parameter obtained with the Ni(II) and Cr(III) adsorption data in simple systems was 0.51 and 0.65 respectively, these values between 0 and 1 describe the degree of homogeneity/heterogeneity of the adsorption sites due a greater concentration of superficial groups with the same capacity of adsorption in terms of energy according Guarín-Romero et al. (2019).

BET (Brunauer, Emmett and Teller) isotherm equilibrium model is a multilayer model, and it assumes that a Langmuir isotherm applies to each layer and that there is equal energy of adsorption for each layer except for the first layer Eq. (11.6):

$$q_e = \frac{q_s C_{BET} C_e}{\left(C_s - C_e\right)\left[1 + \left(C_{BET} - 1\right)\left(\frac{C_e}{C_f}\right)\right]} \tag{11.6}$$

Where q_s is the theorical saturation capacity using the Langmuir isotherm (mg g^{-1}), C_{BET} the BET constant which indicates energy of surface interaction ($L.mg^{-1}$). In Table 11.2, the main results of different types of natural agro-industrial wastes and by-products for heavy metal removal are summarized.

In most cases, Langmuir isotherms were able to simulate the experimental data better than Sips and Freundlich models with a high correlation coefficient and is often used to determine the maximum sorption capacity of biosorbent.

The majority of authors consider the low sorption capacity of byproducts and agro-industrial waste to be a main problem for their application but is necessary to note that those sorbents work most effectively in treatment of very dilute solutions, enabling very low concentration of pollutants to be achieved.

Table 11.2 Adsorption isotherms models data obtained on biosorption of different ion metals and other pollutants on agro-waste biosorption

Biosorbent	Pollutant	Operation conditions			Results			References
		pH	T (°C)	C_0 (mg. L^{-1})	q_{exp} (m. g^{-1})	Isotherm model	q_{cal} (m.g^{-1})	
Banana peels	Cd (II) Pb (II)	3 5	25	0.05		Langmuir	q_m: 2.18 5.71	Anwar et al. (2010)
Soybean meal	Cr (III) Cu(II)	5	20	100	15.6 14	Sips	q_m: 23.5 39.6	Witek-krowiak (2013)
Olive oil	Cr (VI)	2	60	200	16.49	Langmuir	q_m:18.69	Malkoc (2006)
Aloe vera waste	U(VI) Cd(II)	4 5			201.2 70.4			Noli et al. (2019)
Pretreated orange peel	Cr(III) Fe(III)	3		10 30		Langmuir	q_m: 9.43 18.19	Lugo-Lugo et al. (2012)
Corncobs	Sulfamethoxazole	6	20	20	0.56	BET	q_s: 0.50	Peñafiel et al. (2019a)
Grape pomance	Aflatoxin B1 Zearalenone Ochratoxin A Fumonisin B	7	37	1 1 2 2	4.9 1.12 0.64 0.12	Sips Langmuir Freundlich Langmuir	q_m: 4.7 2.7 2.5 1.6	Avantaggiato et al. (2013)

Kinetics Study

The adsorption rate between sorbate and biosorbent in equilibrium is evaluated with kinetic models and rate limiting steps. The pseudo-first and pseudo-second order are the most widely used kinetic models to evaluate the biosorption of heavy metals and emerging compounds on agro-waste.

Pseudo-first order. Adsorption process occurs through first-order chemical reactions, where the rate constant is independent of concentration Eq. (11.7):

$$\frac{dq_t}{dt} = k_1\left(q_e - q_t\right) \tag{11.7}$$

Where k_1 is rate constant of pseudo-first order model (min^{-1}) and q_t is amount adsorbed at time t ($mg.g^{-1}$). Generally, the calculated q_e differs significantly of experimental q_e although high correlation coefficient is obtained.

Pseudo-second order. Also considered a chemical reaction but second order. Involving valence forces through sharing or exchange of electrons between biomass and metal ions Eq. (11.8):

$$\frac{dq_t}{dt} = k_2\left(q_e - q_t\right)^2 \tag{11.8}$$

Where k_2 is rate constant of pseudo-second order model ($g.mg^{-1}.min^{-1}$).

Intraparticle diffusion model presented by Weber and Morris is a model derived from Fick's second law of diffusion Eq. (11.9):

$$q_t = k_p t^{1/2} + C \tag{11.9}$$

Where K_p is the intraparticle diffusion rate constant ($mg.g^{-1}.min^{-1/2}$) and C is a constant for any experiment (mg/g) and has been reported by several researchers to involve two forms, the first one to get a straight line that passes through the origin when adsorption capacity at any time (q_t) plotted against the square root of time ($t^{1/2}$), and the second one, when two or three steps are involved in the whole process, in the plot q_t versus $t^{1/2}$, has multilinearity, the first step considers an external instantaneous surface adsorption, the second step is controlled by intraparticle diffusion therefore is a gradual adsorption, and the third step is the final equilibrium step (Wu et al. 2009). Wu et al. plotted the curves with various initial adsorption factors (R_i) and considered four zones of the initial adsorption according to R_i value; from 0 to 1 were classified, zone 1 for weakly initial adsorption, zone 2 for intermediately initial adsorption, zone 3 for strongly initial adsorption and zone 4 pro completely initial adsorption. For example, the kinetics for phenol compounds on active carbons belonged to zone 3. Therefore, the shape of adsorption isotherm is so important and is associated with the formation of monomolecular or multimolecular layer adsorption via both strong and weak adsorbate–adsorbent interactions (Abdolali et al. 2014)

Continuous Systems

Most research using biosorbents for metal ions are based on batch kinetic and batch equilibrium studies. However, in the practical operation of full-scale sorption processes, continuous-flow fixed-bed columns are often chosen. In such systems, the concentration profiles in the liquid and sorbent phases change in both space and time. From the perspective of process modelling, the dynamic behaviour of a fixed-bed column is described in terms of the effluent concentration–time profile, that is, the breakthrough curve. As a result, the development and application of predictive and simulative mathematical models for the design of continuous biosorption processes represent an important area in environmental engineering. The determination of model parameters and the verification of model validity can be obtained by well-designed, laboratory-scale experiments. With potentially enormous environmental applications in detoxification of metal-bearing industrial effluents, biosorption processes are considered as not only technically feasible but also economically very attractive.

Mathematical Backgrounds

From a practical point of view, biosorption processes to great scales are carried out in a continuous way (Park et al. 2010). In these systems, the concentration in the liquid phase and in the solid phase varies both in space and in time, due to which, the design and optimization of fixed-bed columns is especially difficult if not approached through a quantitative model. In this sense, the dynamic behaviour of fixed-bed columns is described in terms of 'exit concentration–time', that is to say, what is known as breakthrough. A typical breakthrough curve represents the relationship of the entry concentrations to the exit concentrations in time or the circulated volume. When the fluid passes through the column, an area of mass-transfer is defined that varies from 0% of the initial concentration (corresponding to the sorbent without solute) to 100% of the initial concentration (corresponding to the total saturation).

The point where the metal concentration in the effluent reaches a certain value, generally related with the permitted limit for that metal by regulations and environmental norms, is called the breakthrough point, and corresponds to the breakthrough time (t_b) that allows determining the volume of treated effluent. From a practical point of view, the saturation time (t_s) is set when the metal concentration in the effluent has a value between 90% and 95% of the initial concentration of that metal in solution.

To describe the breakthrough curve, numerous models have been proposed that can be from semi-empiric simple models of a few parameters that exist and easy to solve mathematically, to rigorous models that consider axial dispersion in the flow direction, resistance to the film diffusion, diffusion intra matter, which can include

diffusion in the surface and in the pores that generally require complicated numeric solutions (Chu 2003). These models are not only used to analyse and interpret experimental data, they also are used to predict the response of the systems when the operation conditions are changed (Joo et al. 2010). The performance of packed-bed column is described according to the concept of the breakthrough curve that is the plot of time versus effluent concentration:

Effluent volume is calculated from Eq. (11.10):

$$V_{\text{eff}} = Qt_{\text{t}} \tag{11.10}$$

where t_{t} represents the total time (min) and Q is the flow that circulates through the column (mL. min^{-1}).

The area under the breakthrough curve obtained by integrating the adsorbed concentration versus time plot can be used to find the total adsorbed metal quantity. Total adsorbed metal quantity in the column (q_{t}) for a given feed concentration and flow rate is calculated from Eq. (11.11):

$$q_{\text{t}} = \frac{Q}{1000} \int_{t=t_{\text{t}}}^{t=0} C_{\text{R}}\, dt \tag{11.11}$$

where C_{R} is the concentration of retained metal (mg.L^{-1}).

Total amount of metal sent to column is calculated from Eq. (11.12):

$$m_{\text{t}} = \frac{C_0 Q t_{\text{t}}}{1000} \tag{11.12}$$

Total removal percent of metal (column performance) with respect to flow volume can also be calculated from the ratio of total quantity of metal adsorbed to the total amount of metal passed through the column from Eq. (11.13):

$$\text{Removal } (\%) = \frac{q_{\text{total}}}{m_{\text{total}}} \tag{11.13}$$

The breakthrough curve, typically S-shaped, represents a valuable tool for evaluating the biosorbent efficiency. Indeed, the amount of solute removed at saturation can be easily evaluated by calculating the area above the breakthrough curve, whose slope provides information about the column service time. The main features of the breakthrough curve are the breakthrough and saturation/exhaustion points which theoretically correspond to an abrupt rise (infection point) in the effluent concentration plot and the complete column saturation, respectively. The time elapsed until attaining the breakthrough point directly affects the service time of the column. In laboratory experiments, packed bed columns are usually operated until the saturation point is attained. Conversely, in industrial applications, the column is usually regenerated when the effluent metal concentration exceeds a breakthrough/service point, which is prefixed depending on the metal toxicity. When the breakthrough point is attained, the effluent concentration can slowly rise to the saturation point

(flattened breakthrough curve). However, it is preferable to have a steep slope which corresponds to a shorter mass transfer zone (Vijayaraghavan and Yun 2008). The shape of the breakthrough curve is affected by many parameters, such as flow rate, inlet metal concentration, pH, bed height and bed particle size (Kumar et al. 2016).

Most of the models for continuous sorption systems have been developed to predict the breakthrough curves. Some examples are reported in Table 11.3. The Adams–Bohart model is usually applied to the initial part of the breakthrough curve and is obtained by combining two kinetic equations, the first describing the solute

Table 11.3 Column adsorption models (Patel 2018; Ahmed and Hameed 2018; Acheampong et al. 2013; Vijayaraghavan and Prabu 2006)

Breakthrough curve model	Equation	Remarks	Nomenclature
Thomas model	$\frac{C_0}{C} = 1 + \exp\left(\frac{k_{TH}}{F}\left(Q_0 M - C_0 V_{eff}\right)\right)$	One of the most widely used methods to describe column biosorption, which assumes Langmuir kinetic of sorption–desorption and second-order reversible reaction kinetic	k_{TH} is the Thomas model rate constant ($mg^{-1}\ g^{-1}$), Q_0 is the maximum solid-phase concentration of the solute (mg g^{-1}), V_{eff} is the volume of metal solution (L)
Adams–Bohart	$\frac{C}{C_0} = \exp\left(k_{AB} C_0 - k_{AB} N_0 Z / v\right)$	Rate of adsorption depends upon concentration of the sorbing species and residual capacity of adsorption	k_{AB} is the kinetic parameter ($L.mg^{-1}.min^{-1}$) N_0 is the saturation concentration (mg. L^{-1}), Z is the bed length (cm) and v is the superficial velocity ($cm.min^{-1}$)
Yoon–Nelson model	$\frac{C}{C_0} = \frac{\exp\left(k_{YN} t - \tau k_{YN}\right)}{1 + \exp\left(k_{YN} t - \tau k_{YN}\right)}$	Assumes that the rate of decrease in the probability of adsorption for each sorbate molecule is proportional to the probability of sorbate sorption and the probability of sorbate breakthrough on the sorbent	k_{YN} is the Yoon–Nelson model rate constant (min^{-1}), τ is the time required for 50% adsorbate breakthrough (min)
Modified dose–response model	$\frac{C}{C_0} = 1 - \frac{1}{\left(V_{eff} / b_{MDR}\right)^{a_{MDR}}}$		a_{MDR} and b_{MDR} are the modified dose–response model constants

(continued)

Table 11.3 (continued)

Breakthrough curve model	Equation	Remarks	Nomenclature
Bed depth service time (BDST)	$t = \frac{N_0}{C_0 v} Z - \frac{1}{k_{BDST} C_0} \ln\left(\frac{C_0}{C_t} - 1\right)$	Based on the Bohart and Adams quasi-chemical rate law, the rate of the sorption process is directly proportional to the fraction of sorption capacity still remaining on the media. This model is provided by the relationship between bed depth and service time	k_{BDST} is the rate constant ($L.mg^{-1}.h^{-1}$), v is the linear velocity ($cm.h^{-1}$)
Clark model	$\frac{C}{C_0} = \left(\frac{a}{\left(1 + A_c e^{-rt}\right)}\right)^{\frac{1}{n-1}}$	Column adsorption is mass-transfer concept with combination of Freundlich isotherm and behaviour of flow in column is of piston type.	n is the Freundlich parameter and A and r are the Clark Constants. The introduction of parameter a to take into account that the curve is not perfectly symmetrical at the point $C = 0.5C_0$

transfer from the liquid phase, the second governing the sorption accumulation on the biosorbent A similar equation has been obtained by Wolborska (1989), who also takes the solute axial diffusion into account. The Thomas model has been used in the linear form to quantify the maximum adsorption capacity of the adsorbent bed. In the Clark model, the breakthrough curve is obtained by adopting the Freundlich equation. The model introduced by Yoon and Nelson (1984) is much simpler as it does not require specific information about the adsorbate/adsorbent system.

Vera et al. (2019) investigated the use of lead(II) and cadmium(II) biosorption with sugarcane bagasse and cocoa shell in fixed-bed columns. The hydrodynamic study was performed by varying the diameter and height of the column, as well as the diameter and mass of the biosorbent, to determine the best hydrodynamic conditions, which resulted in a flow through the column of 2.12 $mL.min^{-1}$. The experimental data were adjusted to several models that describe the rupture curve for one and two component systems.

As we see in Fig. 11.2a, b, for single-component systems the area under the curve is greater for Cd^{+2} than for Pb^{+2}, deducing that the greater absorption corresponds to Pb^{+2}, which it is due to a greater affinity to the bagasse active sites for Pb^{+2} more than Cd^{+2}. Table 11.4 shows the parameters obtained from the analysis of the experimental data presented in the rupture curves, for single-components and two-components with the sugar cane bagasse.

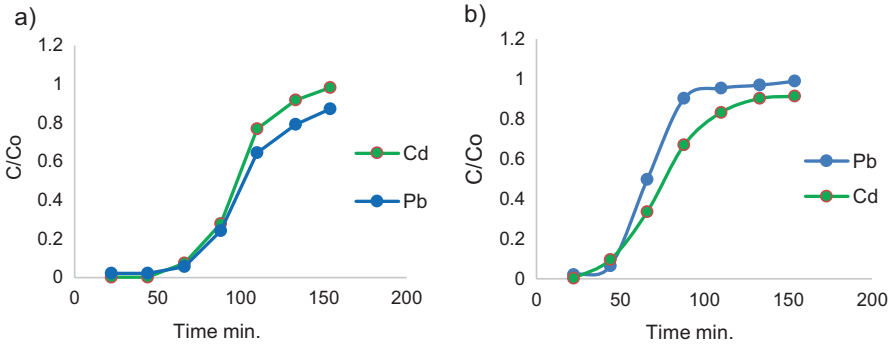

Fig. 11.2 (**a**) Rupture curve for adsorption of Pb^{+2} and Cd^{+2}, in a column of fixed bed filled with sugar cane bagasse in single-component system. (**b**) Rupture curve for adsorption of Pb^{+2} and Cd^{+2} in a column of fixed bed filled with sugar cane bagasse in two-component system

Table 11.4 Rupture curves parameters for adsorption of Pb^{+2} and Cd^{+2} in columns of fixed bed using sugar cane bagasse

		Single-component system		Two-component system	
Parameters		Pb(II)	Cd(II)	Pb(II)	Cd(II)
Rupture curves	tr (min)	74	70	47	43
	ts (min)	168	128	88	129
	Vr (mL)	118.4	112	76.61	70.09
	q_b (mg.g^{-1})	0.154	0.146	0.1	0.091
	SNU (cm)	15.67	5.14	13.05	18.67
	M_{total} (mg)	2.29	2.29	2.33	2.33
	q_t (mg)	2.08	2.07	2.08	2.16
	% adsorption	91	90	89.3	92

According to data obtained by the rupture curves, in the single-component and two-component system, the amount of metal that passes through the column (Mtotal) and the maximum adsorption capacity (q_t), they are equivalent to Pb^{+2} and Cd^{+2}. In the single-component system, the parameters which varied in lead and cadmium, they are the times of rupture and saturation (tr y ts), the volume treated at rupture point (Vr), the adsorption capacity (q_b), the not used fixed lying (SNU), being reported higher values for Pb^{+2} than for Cd^{+2}.

Regarding removal, the bagasse showed an adsorption percentage, practically equal for lead and cadmium. However, in the two-component system, data shows that lead and cadmium interaction in solution caused a significant decrease in all values of the variant parameters from single component systems, though some parameters were superior for cadmium and others for lead, as in case of rupture time, in lead (47 min) it was slightly superior than cadmium (43 min), so the volume treated for lead (76.6 mL) is superior than cadmium (70.09 mL), moreover for saturation time, the higher value registered was for cadmium, (129 min) compared to

lead (88 min), that means after the rupture, the competition of the active sites by metals of concern, favoured Cd^{+2}, this one which had the highest affinity for lead. The adsorption capacity proved to be equal for both metals, at the no used fixed bed, cadmium reported the highest value with 18.63 cm. About removal, the bagasse showed a higher adsorption percentage for cadmium with a removal of 92% instead of lead which showed 89%. In conclusion, in the single-component system, lead has greater affinity to sugar cane bagasse active sites than cadmium, however, when these two metals are in interaction, there is a greater affinity by cadmium, the affinity order being Cd > Pb.

As shown in the Fig. 11.2a, Dose–Response model is the best-adjusted model to the rupture curve of experimental adsorption of Pb^{+2}, with a R^2 factor correlation of 0.97. The Thomas model is the least adjusted to rupture curve, though its R^2 is more than 0.9. The Yoon–Nelson model adjusts acceptably to experimental data with a R^2 equal to 0.99; however the values of the time required to retain 50% of initial metal, τ, they are very similar to those obtained experimentally in sugar cane bagasse, which coincides with the findings of several researchers studying different biosorbent–metal systems in fixed bed column. For the adsorption of cadmium (Fig. 11.2b), the Yoon–Nelson model is the best curve that fits to the experimental data with a R^2 registered of 0.99; the values of τ, experimentally and calculated by the model are equal. The Dose–Response model has an allowable adjustment with cadmium experimental rupture curve, with a R^2 of 0.9. The least adjusted model is Thomas', though its R^2 is equal to 0.97 the generated curve, underestimates the experimental values.

For two-component system, Yoon–Nelson, Thomas, and Dose–Response models were separately applied, in order to appreciate the adjustments of rupture curves modelled with experiments. Figure 11.3a compares experimental rupture curves of lead and cadmium with those adjustment curves obtained from the Yoon–Nelson model. The Yoon–Nelson model does not adjust properly to experimental rupture

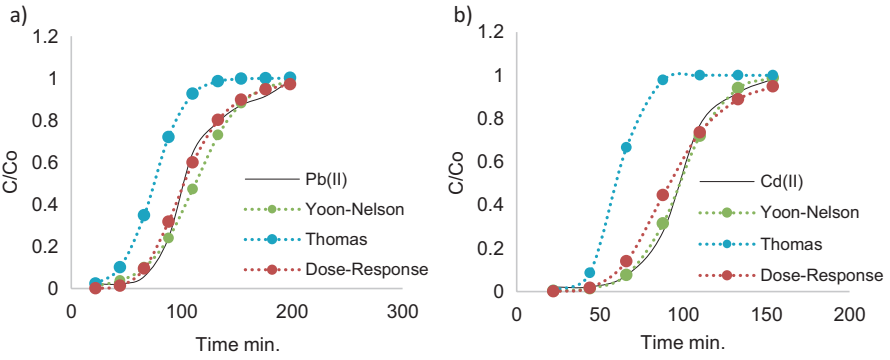

Fig. 11.3 (**a**) Comparison between Pb^{+2} experimental rupture curves and those obtained by applied models based on time in column filled with sugar cane bagasse. (**b**) Comparison of the experimental rupture curves of Cd^{+2} with those obtained by applied models based on time in column filled with sugar cane bagasse

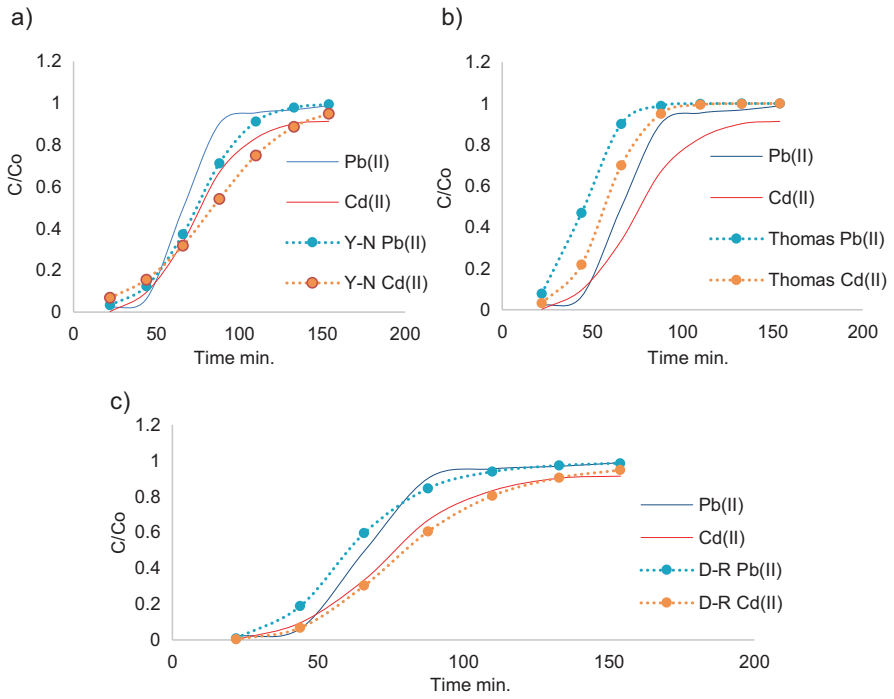

Fig. 11.4 (**a**) Comparison of the experimental rupture curves of Pb^{+2} y Cd^{+2} with those obtained by Yoon–Nelson model in column filled with sugar cane bagasse in T–C system. (**b**) Comparison of the experimental rupture curves of Pb^{+2} y Cd^{+2} with those obtained by Thomas in column filled with sugar cane bagasse in T–C system. (**c**) Comparison of the experimental rupture curves of Pb^{+2} y Cd^{+2} with those obtained by Dose–Response model in column filled with sugar cane bagasse in T–C system

curves of lead and cadmium in a two-component system; the modelled curves with Yoon–Nelson showed a R^2 of 0.94 for lead and 0.93 for cadmium. Figure 11.3b compares the experimental rupture curves of lead and cadmium with those adjusted curves obtained from Thomas model (based on time). The curves obtained by the Thomas model does not adjust to any studied metals (Fig. 11.4).

From all models based on time, the Dose–Response model is the best adjusted to experimental data for both lead and cadmium. With R^2 values equal to 0.96 for lead and 0.99 for cadmium. Regarding the adsorption capacities, the experimentally obtained values for sugar cane bagasse in a two-component system, corresponds to 0.1 $mg.g^{-1}$ for lead and 0.09 $mg.g^{-1}$ for cadmium, similar values were obtained from Thomas model, 0.094 $mg.g^{-1}$ for lead and 0.11 $mg.g^{-1}$ for cadmium. Using the parameters found with this model, it is possible to obtain an expression that reproduces the columns behaviour in other experimental conditions, with no need for further experiments. The biosorption of Pb^{+2} and Cd^{+2} with cocoa shell in fixed bed was also investigated. The experimental data were fitted to several models describing the breakdown curve for single component and two component systems. The

removal percentages of lead and cadmium in single-component systems are 91% and 90%, respectively. In bicomponent systems with Pb–Cd the percentage of lead removal was 88% and cadmium was 90%. The Dose–Response model in two-component and single-component systems was the one that best reproduced the experimental rupture curves throughout the measured range (Vera et al. 2018).

Rodriguez (2015) investigated the removal Cr^{6+} and Ni^{2+} by sugarcane bagasse in two up-flow fixed-bed columns in series have been presented. The experimental data were adjusted for several kinetic models that described the breakthrough curve obtained for a single column and for two columns in series. The Dose–Response model is the one that better adjusts the experimental data for the studied metals with a high correlation coefficient.

Perspectives

Over the last years, biosorption has received considerable attention from academic researchers, becoming one of the most promising and cost-effective alternative technologies for heavy metal removal and recovery from industrial wastewaters. However, despite the high number of scientific studies on biosorption, several technical and scientific aspects still need to be clarified for the commercialization and the spread of this technology at industrial scales. Based on these considerations, future research may be focused on the characterization and identification of new materials to be used as biosorbents with higher cost-effectiveness and biosorption efficiency, enhancement of selective metal biorecovery through biosorption in multi-metal systems, and development of analytical tools based on deterministic mathematical models able to describe multi-sorbate systems.

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Chapter 12
The Status of Plant and Plant–Microbe Interactions Related to Medicinal Agents

David J. Newman

Abstract For millennia, medicinal plants have been the source of medicines initially for everyone, but since approximately the middle of the twentieth century, in the West, their use has been decreasing significantly as drugs that though frequently came directly from plants and/or microbes were substituted for direct usage of plant extracts or mixtures. Currently one can estimate that over 70% of the world's population still have access only to medicinal plants. Ancient systems that compiled data on how to collect and use such 'mixtures' are still used extensively, with examples being traditional Chinese Materia Medica (or TCM), the Ayurvedic system in India, Arabic herbals, and Korean and Japanese equivalents, with similar systems used in the Americas among indigenous peoples. In the West, beginning with the isolation and purification of morphine in the early 1800s in France, and continuing with the subsequent isolation and identification of significant other compounds, probably culminating in the synthesis of acetyl salicylic acid (aspirin) in Germany in the late 1800s, the drugs in use, though in a significant number of cases based upon initially plant-associated compounds, are now made in chemical laboratories, even though the original 'lead' came from nature, initially from plants.

Keywords Plant–microbe interactions · Medicinal agents · Discovery · Ancient systems · Plant compounds

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Current Status

Though the data presented below (Figs. 12.1 and 12.2), about the numbers of small molecules and their sources, are from information published in 2016 (Newman and Cragg 2016) covering drugs approved worldwide from 1981 to 2014 for all diseases except cancer, and then from approximately 1950 to 2014 for antitumour agents, a review in preparation that will extend the data series through the end of 2018 shows almost the same ratios of sources as the 2016 review for small molecules. The number of biologicals has dramatically increased in nominal terms, but this is due to the

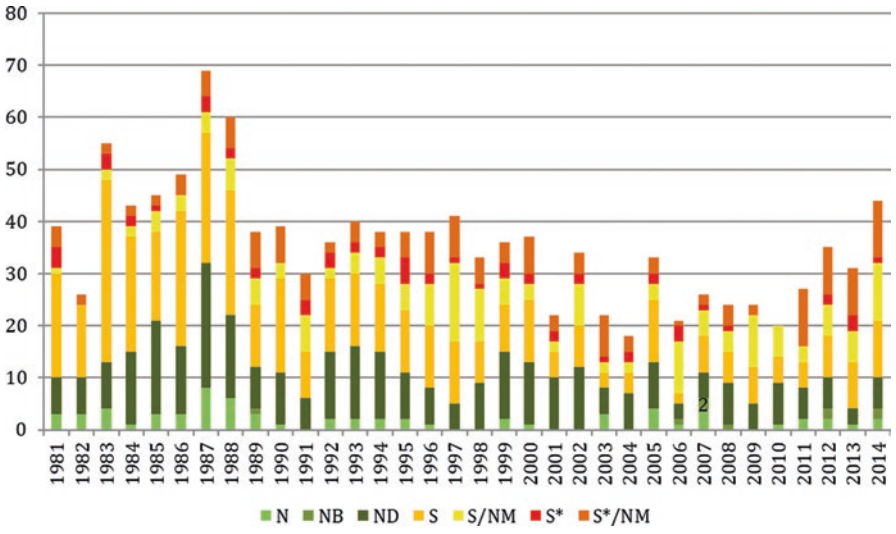

Fig. 12.1 Source of drugs from 1981 to 2014 inclusive. (Coding from Newman and Cragg 2016)

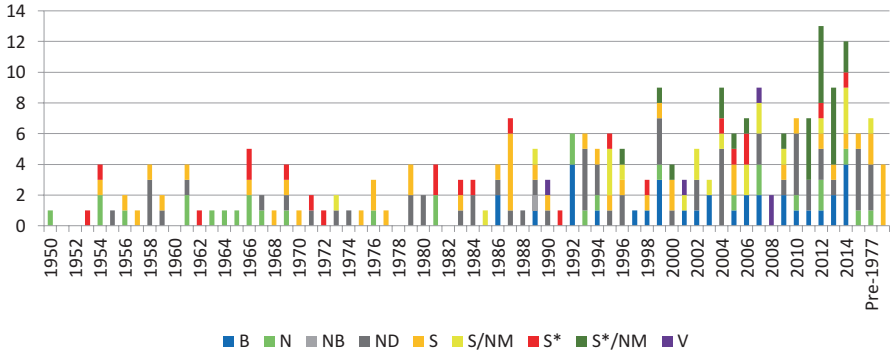

Fig. 12.2 Antitumour agents from 1950 to 2014

approval of a large number of what are known as ‘biosimilars’. These are biologics whose patents have expired and now are being made and sold by many pharmaceutical companies worldwide. In general terms they can be considered to be generic biologicals, and thus are not counted in any subsequent listings.

From a plant-source perspective, there are four natural products that are used directly as drugs (initially in the West but now in use worldwide) or as the ‘raw material’ from which to make semisynthetic variations. The reason for modifications by chemical means usually has to do with pharmacological and/or toxicological problems with the original material, even though it went into clinical use initially. These may be discussed individually with comments as to how they have developed.

Taxol

This compound, taxol (Fig. 12.3(1)), is isolated from the American yew tree, *Taxus brevifolia*, with the tree being part of a random collection made by the US Department of Agriculture in the 1960s, as part of a programme with the National Cancer Institute looking for potential antitumour agents. The story has been told many times and contrary to reports from Chinese and Indian sources that *Taxus* species were used for treatment of cancer, none have survived inspection. Taxol was shown to have a unique (at the time) mechanism of action, in that it literally ‘froze’ the tubulin system in cells. Note that it did not matter if the cell was a normal one or a cancerous variant. The levels of taxol in the yew tree was such that approximately six 150 mm diameter trees were required for the isolation of one treatment dose, and as the compound was found in the lower bark, the tree was destroyed. National Cancer Institute (NCI) funded significant numbers of synthetic attempts at total synthesis, but when a French group demonstrated that a precursor, 10-desacetylbaccatin III (Fig. 12.3(2)) could be converted into a derivative of taxol (taxotere) (Fig. 12.3(3); approved in France), and that the needles of *Taxus baccata* contained that precursor in significant quantities, a method was rapidly found and taxol was finally approved in the USA in 1992 for the treatment of ovarian cancer. The story has been told many times (Suffness and Wall 1995) but the full history of the many variations on the method of delivery has never been fully told. Subsequently, a method using callus cultures became the standard way of producing taxol for pharmaceutical use and also for modification. Currently there are three approved drugs based on the skeleton, taxol (Fig. 12.3(1)), taxotere (Fig. 12.3(3)) and cabazitaxel (Fig. 12.3(4)), with others in various phases of clinical trials.

Since this original work, many reports have surfaced, beginning in 1993, of endophytic fungi that can produce taxol in low quantities. To date, although two microbes have been shown to definitively produce taxol when fermented, including having their complete genomes sequenced and all intermediate enzymes in the pathway identified, microbes have not replaced plant cell tissue culture. There is one

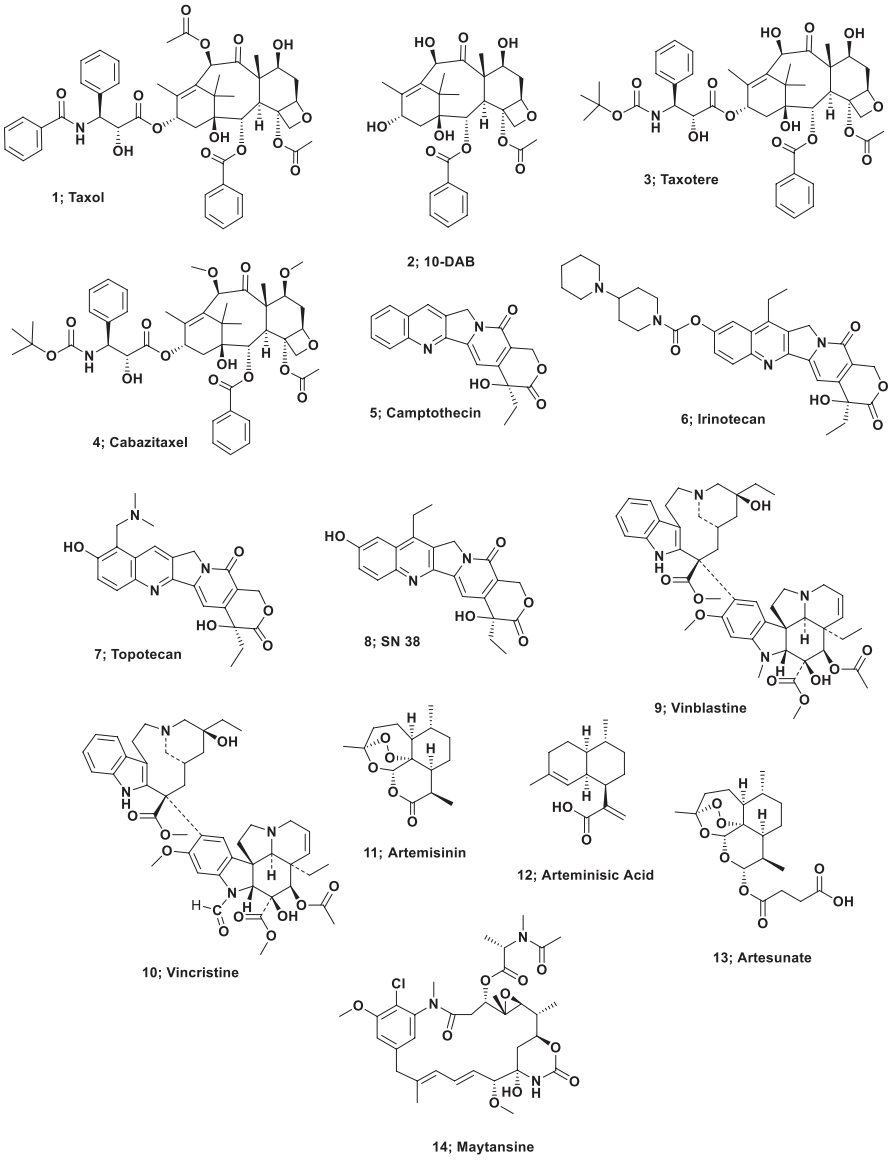

Fig. 12.3 Compound structures

aspect that has never been published, and that is whether or not the callus culture is axenic without any microbe within the plant cell matrix. This would be a simple operation to perform, but no evidence either way has been reported. A general discussion of such an involvement was provided in a review by Newman (2018).

Camptothecin

Camptothecin was also isolated from an NCI collection by the same chemistry group (Wall and Wani) who previously identified taxol (Fig. 12.3(5)). The source tree, genus *Camptotheca*, was actually collected from a herbarium in California, though the tree is a native of the Himalaya. There are many claims that these plants were used for cancer treatment under the Ayurvedic system, but when one realizes that cancer is very difficult to diagnose in the absence of a laboratory, and that camptothecin itself is highly toxic due to breakdown of the internal lactone, then one needs to be careful in assessing such claims. The pure natural product was pulled from clinical trials by NCI in the 1970s due to the toxicity of the breakdown product. Subsequently two companies produced a synthetic variant. The one in Japan synthesized irinotecan (Fig. 12.3(6)), while the other in the USA synthesized topotecan (Fig. 12.3(7)), both of which entered use against cancer in the 1990s. Subsequently, the breakdown product of irinotecan, SN38 (Fig. 12.3(8)) is now in use with a chemical modification as a 'warhead' on an antibody–drug conjugate and a number of these are currently undergoing clinical trials at the moment.

As is the case with taxol, there have been a significant number of reports that invoke the presence of an endophytic microbe (usually a fungus) isolated from a tree that 'produces' camptothecin, that gives camptothecin on fermentation, but that similarly to the taxol-producing fungus, loses its ability to produce on repeated fermentation. However, very recently, an Indian group demonstrated production by an endophytic bacterium that contained a plasmid. If the bacterium was 'cured of the plasmid', then camptothecin production ceased, and on replacement of the plasmid, production reoccurred. Whether the plasmid is producing the material or an inducer is not yet known (Newman 2018).

Vinca Alkaloids

The two compounds vincristine (Fig. 12.3(9)) and vinblastine (Fig. 12.3(10)) were first isolated from the flowering plant *Catharanthus roseus*, also known as the rosy periwinkle, in the late 1950s as a result of an indigenous medicinal use that was in fact incorrect. The plant had been used in the Philippines during World War II as a treatment for diabetes. Following World War II, scientists at Lilly, the company that was the major producer of insulin in the USA, tested the plant extracts and pure materials isolated from it in diabetic and regular mice. Instead of acting as an insulin mimic, they realized that the leukocyte (white cell) count in the mice very significantly decreased, so the latter thought was, could this also work in patients with leukaemia (a blood-based cancer where the white cell levels rise to levels well above the normal figure)? This was a disease that at times occurred in young children and had a very high mortality rate in such patients in the 1950s. Suffice to say that these

two plant-sourced molecules effectively reduced the mortality to single figures. The complete biosynthetic sequence of these compounds has been identified though it took many years (Caputi et al. 2018).

Currently over 500 modifications of the basic skeletons have been synthesized, but to date only three variations have been approved since the initial approvals of the vinca alkaloids in the early to mid-1960s. These are listed without structures but have the date of first launch in parentheses, vindesine (1979), vinorelbine (1989) and vinflunine (2010). Currently the production for use is from a specific plantation in the USA. There have been significant comments on what appeared to be a 'removal' of indigenous knowledge, but as mentioned earlier the usage was incorrect, and the information was roughly 60 years before the Convention on Biodiversity, with absolutely no correlation between diabetes and leukaemia. To date there have been two published reports of microbial production of these agents (Guo et al. 1998; Yang et al. 2004).

Artemisinin

This is a true use of traditional Chinese medicine (TCM) information and dates back to the Vietnam War, when Dr. Tu YuYu was tasked by the Chinese leaders to search for a potential antimalarial that could be used by North Vietnam soldiers, as they could not obtain quinine or synthetic antimalarials (Fig. 12.3(11)). She finally went back into records of TCM over 2000 years old and realized that a warm decoction rather than the use of boiling water (which was customary in TCM extractions), enabled her to isolate and identify artemisinin from the sweet wormwood (*Artemisinin annua*). When the structure was finalized, the reason for the chemical instability became obvious, that is, the bridging dioxy group. This work led to the award of half of the 2015 Nobel Prize for Physiology or Medicine to her.

From a plant-derived biotechnology perspective, this compound could be almost considered the 'poster child', as the plant source is slow-growing and the whole plant is destroyed in isolating the compound. The US Department of Energy gave a grant to Dr. Jay Keasling (and a company developed from the work), which enabled the Keasling group to take the gene clusters from the plant that produced the precursor, artemisinic acid (Fig. 12.3(12)) and express them in brewer's yeast (Ro et al. 2006). The original idea was to produce the final compound, but a relatively simple chemical process (even using flow reaction chemistry) enabled the production of significant levels of artemisinin that could then be converted into the desired drug form, one of which is artesunate (Fig. 12.3(13)). The World Health Organization wants to reduce the overall cost to US$1 per dose, and the French pharmaceutical house Sanofi is working with the Keasling process in order to reach this goal.

Plant–Microbe Interactions

Where an Ecuadorian investigation of endogenous medicinal plants could be a significant contribution would be in the study of endophytic and/or epiphytic microbes (bacteria and fungi) that occur in/on the medicinal plants themselves, provided that there is some idea as to what chemical compounds 'produced' by the plant might be the medicinal agent or agents.

Such an effort would require coordination, probably including contiguous countries scientists with relevant skills in order to isolate and genetically characterize potential 'producers'. That this is not a farfetched idea can be seen from the work in Germany by the group at Dortmund who demonstrated that without question the anti-cancer candidate maytansine (Fig. 12.3(14)) was not the product of the plant from which it was first isolated, but was produced by a consortium of microbes in the plant's rhizosphere and then taken up by the plant as a protective agent. This is different than the cases involving taxol and probably camptothecin as there the potential-producing microbes were within the plant itself as endophytes (Kusari et al. 2014). A relatively recent discussion as to the role of microbes in producing agents found in higher organisms was published by Newman in 2018.

That microbes associated with a plant, even if not known to produce medicinal agents, may well have significant value, can be seen by the recent publication from the Piel group, where they demonstrated that just the leaf of *Arabidopsis thaliana* which can be thought of as the '*Drosophila*' of the plant world, has over 200 microbes that can be visualized from just one leaf, and when DNA sequenced, these organism contained very significant numbers of previously unknown biosynthetic gene clusters (BGCs) that had the potential to produce novel compounds (Helfrich et al. 2018). Equally, if any could be grown successfully, then the techniques very recently published by the Seyedsayamdost group at Princeton University in the USA (Xu et al. 2019) could also be used to elucidate the potential for further work.

What has to be recognized is that the information from indigenous peoples and also from major compendia of medicinal plant use is that there are specific requirements as to when the plants are collected from the aspects of altitude, growth status, weather conditions and specific site(s). Although these requirements were made centuries ago before the discovery of microbes, when one realizes that the plants exist in an 'ocean of endophytic and epiphytic microbes' with multiple chemical signals being exchanged across genomic kingdoms, then the requirements as to 'how, where and when' make sense. There have been reports over the years of a lack of reproducibility of activities from the nominally same plant but collected at a different location or time/season. If looked at from the aspect of microbial–plant signalling, then these observations make sense, and need to be considered in any such research programme.

Potential for Use of Plant-Associated Waste

There have been a variety of publications from World Health Organization (WHO), various universities in the USA and the reference that is attached from Indian authors (Paritosh et al. 2017), that can be adapted very easily in small to large agricultural organizations in Ecuador, that have the potential to produce usable gases and also to produce nutrients that can be reused with animal husbandry. Some of the presentations at the recent Banana/Computational Intelligence Methods for Bioinformatics and Biostatistics (CIBB) symposia in Ecuador touched on these, but perhaps more thought needs to be applied within the geographic areas.

A Caveat

What has to be realized by the granting authorities in Ecuador, and other countries in South America in particular, is that no one country has the capabilities to perform a lot of this work, but a transnational consortium, even based upon the Andean Pact nations, could certainly investigate the potential of the areas medicinal plants and look for the actual producer(s) and work with them. Scientific relationships with other countries would also be necessary, as the scientific equipment necessary is not available in most areas of the world. A successful variation on this would be the International Cooperative Biodiversity Groups (ICBG) program that is just completing between the School of Pharmaceutical Sciences in Sao Paulo state in Brazil and the group at the Harvard Medical School in the USA, studying the microbes involved with ants in the Brazilian Amazon. This program was the winner of an open competition and was jointly funded by São Paulo Research Foundation (FAPESP) in Sao Paulo state and the National Institutes of Health (NIH) in the USA.

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Chapter 13
Bringing New Function to Packaging Materials by Agricultural By-Products

Benjamin Le Delliou, Olivier Vitrac, and Sandra Domenek

Abstract The development of functional additives for packaging polymers starting from by-products of the agriculture and food industry is a very active research field. Different examples are discussed concerning the formulation of polylactides (PLA) with the aim to increase ductility and barrier properties or developing active packaging using by-products of the vegetable oil industry and lignocellulosic materials. Moreover, the potential of the production of polyhydroxyalkanoates from agricultural by-products is shown and recent results concerning their blending with auxiliary polymers with the aim to obtain packaging materials are also available. Notwithstanding the renewable origin of such novel technological adjuvants for packaging materials, their safety must be ensured, and they need to comply with existing regulations. The fundamental principles and means for risk management are summed up. When performance and safety are designed together, there is a high potential for agriculture and food industry to contribute to the development of new packaging systems, safer for consumers and environment.

Keywords Polylactide · Polyhydroxyalkanoate · Packaging · Barrier properties · Mechanical properties · Food Contact Materials · Regulation · Nanocellulose · Vegetable oil industry · Lignin

Introduction

Due to the superior properties of thermoplastics, the packaging sector is the largest user of polymer materials. Polymers enable the production of lightweight materials, easily processed into packages with specific design and mechanical properties.

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The large use of those materials causes, however, several environmental issues, mostly linked to the risk of persistent pollution in the environment and the use of fossil resources. The development of biodegradable and biobased polymers is one of the answers to these questions. Combined with the use of renewable resources, it allows one to step towards a circular economy by bringing new value to agricultural production. In this perspective, agriculture and food industry can become suppliers of polymer materials and not only users of these materials.

Academic research brings the necessary expertise for screening potential (by) products and improving the functionality of these polymer materials. Indeed, new biodegradable and biobased resins sometimes lack the technical properties for successful replacement of existing materials. This is a challenge to rise to and to invent novel strategies for building the desired function by the creation of original assemblies and morphologies. Furthermore, constraints such as availability, ecological impact and security of materials need to be respected.

Within this framework, examples will be discussed based on the experience of our laboratory concerning the development of novel packaging materials based on agricultural by-products by taking into account functional and security aspects. The main focus will be on biobased polyesters, mainly polylactides (PLA), which account for the highest volume in packaging applications, and polyhydroxyalkanoates (PHAs), which are actually an emerging field.

Formulation of Polylactides

Introduction on PLA Synthesis, Properties, and Degradation

The basic constitutional unit of PLA (Fig. 13.1) is lactic acid. Lactic acid (2-hydroxy propionic acid) is an α-hydroxy acid with an asymmetric carbon atom and exists either as L(+) or D(−) stereoisomers. The L− isomer is produced in humans and other mammals, whereas both the D- and L-enantiomers are produced in bacterial systems. The large majority of lactic acids produced today are obtained by bacterial fermentation of simple sugars in the L-conformation. The abbreviation of the polymer ‘PLA’ stands for either for poly(lactic acid) or for poly(lactide), depending on the polymerization route. Poly(lactic acid) is obtained by direct polycondensation of lactic acid. Because of the difficulty in reaching high molecular weights, this synthesis route is scarcely used. The majority of PLA is polylactide obtained by

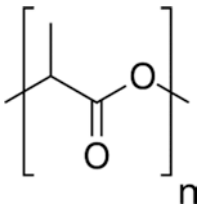

Fig. 13.1 Chemical structure of PLA

ring-opening polymerization (ROP) of lactides (Ducruet and Domenek 2015), a synthesis pathway first reported by Wallace Carothers in 1932 (Carothers et al. 1932).

Three forms of lactide can be obtained by high-temperature distillation: L,L-lactide, D,D-lactide, and D,L-lactide (meso-lactide). The type of lactide and the proper management of the ROP allows producing macromolecules with controlled stereochemistry (Stanford and Dove 2010). The stereo control of the macromolecular chain induces large changes in the thermal stability and crystallization behaviour of PLA. PLA with random distribution of enantiomers and containing more than 93% of L-lactic acid is semi-crystalline, while PLA containing L-lactic acid between 50% and 93% is amorphous. D-lactic acid, in high proportion, prevents PLA crystallization (Auras et al. 2004). In contrast, enantiopure poly-L-lactic acid (PLLA) and poly-D-lactic acid (PDLA) can form a stereo complex due to stereoselective interactions (mostly van der Waals forces) locking the chains into a novel material with largely changed physical properties, for instance increased crystallization and melting temperature (Stanford and Dove 2010).

PLA is a permitted Food Contact Material (FCM) by European and North American Legislation. It is generally formulated with some technological adjuvants, but at moderate levels with antioxidants and lubrication agents identified (Gratia et al. 2015; Lalanne et al. 2010; Salazar et al. 2017). A summary of the major properties of PLA is presented in Table 13.1. PLA is efficient in maintaining food quality because of its high resistance to fatty food and barrier to aroma compounds (Salazar et al. 2014). It is however noteworthy that PLA has high affinity for aromatic molecules, which at very low concentrations can cause plasticizing and induced crystallization (Salazar et al. 2012).

PLA is a glassy polymer at room temperature. The melting temperature is dependent on the D/L ratio, being situated in a range between 140 °C and 180 °C. The stereo complex melting temperature is shifted to much higher temperatures (Table 13.1). PLA presents brittle fracture behaviour with necking and stress

Table 13.1 Main properties of polylactides (PLA)

			Reference
Density	1.24	g/cm^3	(Auras et al. 2004)
Glass transition temperature Tg	55–60	°C	(Ducruet and Domenek 2015)
Melting temperature	140–180	°C	(Saeidlou et al. 2012)
Stereo complex.	260	°C	(Stanford and Dove 2010)
Thermal decomposition temperature	360	°C	(Carrasco et al. 2010; Kopinke et al. 1996)
Elongation at break	5–10	%	(Domenek et al. 2018)
Young modulus	3500	MPa	(Domenek et al. 2018)
Yield strength	40–70	MPa	(Domenek et al. 2018)
Oxygen permeability 23 °C, 0% RH	2–3 10^{-18}	m^3.m/(m^2.s.Pa)	(Sonchaeng et al. 2018)
Water vapour permeability 23 °C	$1{\cdot}10^{-14}$–$1{\cdot}10^{-13}$	Kg. m/(m^2.s.Pa)	(Sonchaeng et al. 2018)

whitening. The main deformation mechanism is shear banding and shear-band-nucleated crazing. In the ductile deformation region after the necking, the preferred plastic deformation mechanism of PLA is then standard crazing (Domenek et al. 2018). Oxygen barrier properties of PLA are intermediate between high volume polymers such as poly(ethylene) (PE) and poly(ethylene terephthalate) (PET), while water vapour permeability is 10 to 1000 times higher compared to PET and PE.

The degradation of PLA can be abiotic, that is uniquely relying on chemical processes, or biotic, that is involving metabolic processes of microorganisms. Abiotic thermal degradation can occur during processing because aliphatic polyesters such as PLA have limited thermostability. The degradation processes can start at temperatures as low as 215 °C (Kopinke et al. 1996), but the fastest degradation kinetics are observed around 360 °C (Carrasco et al. 2010; Gupta and Deshmukh 1982; Kopinke et al. 1996). The mechanisms of the thermal degradation of PLA are rather complex, involving thermal hydrolysis by trace amounts of water, zipper-like depolymerization, inter- and intra-molecular transesterification, and random oxidative main chain scission (Södergård and Stolt 2002). Those reactions can be accelerated by the presence of impurities or residual catalysts. Thermal hydrolysis and intramolecular transesterifications are the preponderant degradation routes of PLA during processing, as has been reported by several authors (Taubner and Shishoo 2001; von Oepen and Michaeli 1992; Wang et al. 2008). Careful drying, minimizing process temperature and residence time are important process parameters for PLA extrusion. In particular, Salazar et al. (2017) showed that the main degradation products of PLA formed during extrusion were lactides, alcohols, and aldehydes. They quantified several odorous compounds with low perception thresholds, such as 2-methyl 2-propanol or acetaldehyde. The acetaldehyde concentration was however between 3.6 and 18 times lower compared to values obtained during PET production. Those compounds disappeared during storage of PLA under ambient conditions and were not detectable any more after 2 months (Salazar et al. 2017). In practice, this means that PLA does not lead to perceivable alteration of organoleptic properties of foodstuff. A successful experiment testing the sensorial properties of chocolate packed in PLA films was reported by Ruellan et al. (2016).

Hydrolysis kinetics of PLA in the mild temperature conditions ($T < 40$ °C) of an agricultural environment are of high importance for the design of products using mulch films. Upon immersion, water molecules can penetrate only the amorphous phase, and crystallites are an excluded volume. Amorphous domains are therefore degraded first, and PLA materials thus undergo bulk erosion starting from the surface (Hakkarainen et al. 1996). In the presence of water and a catalyst, ester hydrolysis occurs, bringing about a decrease of molecular weight. The accumulation of oligomers with acid ends or lactic acid can have an autocatalytic effect on the ester hydrolysis. It can be shown that the degradation of bulk pieces in humid environments proceeds faster than pieces immersed in water. In the latter case, acidic degradation products are leached out from the sample and washed away, which decreases the autocatalytic degradation process (Gerometta et al. 2019).

Biodegradability of polymers is a multifunctional property referring to end-of-life options of materials in different biological environments (industrial or home

composting, anaerobic digestion, etc.). According to the ISO EN 13423 it refers to a process wherein materials are degraded under the action of microorganisms into carbon dioxide, methane (only in anaerobic conditions), water, inorganic compounds, and new biomass. The kinetics of this degradation are dependent on the specific biodegradation conditions (temperature, water activity, presence of microorganisms and their nature, oxygenation, etc.) (Ruggero et al. 2019). Biodegradation can therefore involve initial chemical processes such as dissolution, hydrolysis and enzyme-catalysed degradation, but also oxidation and photolysis. In the case of PLA, an abiotic (catalysed) hydrolysis step seems to be always involved leading to the formation of oligomers, that are able to be assimilated by microorganisms, which will metabolize them to CO_2 and water under aerobic conditions (Auras et al. 2004; Pitt et al. 1981). Biodegradation of PLA has been studied in soil, in sea water and in compost. The biodegradation kinetics vary as a function of the PLA characteristics (molecular weight, stereochemistry, crystallinity degree) and the environment. For example, in soil activation time can be very long, bringing about long degradation times of PLA if abandoned in natural environments. A field test in Greece showed complete disintegration of PLA only after 1 year, while it was accomplished in 60 days in industrial composting conditions involving temperatures higher than T_g (Haider et al. 2019). In simulated composting conditions, such as liquid media, PLA mineralization by microorganisms can be completed after only 60 days (Ruellan et al. 2016).

Toughening PLA Using Agricultural By-Products and Their Derivatives

As shown in Table 13.1, PLA is a brittle polymer with relatively low toughness. This limits its applications in both rigid and flexible packaging. Improving toughness using biobased and biodegradable substances is therefore a very active research field. Agricultural by-products can be a source of technical adjuvants, such as plasticizers, fibres, and polymers for the formulation of blends. The influence of plasticizers on the mechanical properties of PLA has been extensively studied and recently reviewed (Ruellan et al. 2015a). Plasticizers essentially increase the free volume inside polymer matrixes and decrease the interchain interactions (Ruellan et al. 2015a), which eases the slip of one macromolecule with respect to another, decreasing the cooperativity volume (Araujo et al. 2019). The macroscopic consequence of these mechanisms is decrease in the glass transition temperature and increased elongation at breaks. Numerous monomeric and oligomeric plasticizers were tested, the most prominent of which were polyethylene glycol (PEG) with variable molecular masses and citrate derivatives, such as acetyl tributyl citrate (ATBC) (Ruellan et al. 2015a). The transition from brittle to ductile behaviour of formulated polymers is generally obtained when the plasticizer concentration is large enough to decrease the glass transition temperature near the mechanical testing temperature. To give an example, ductile PLA was obtained with ATBC concentrations higher than 15 wt% (Courgneau et al. 2011; Courgneau et al. 2012).

An alternative possibility to increase toughness of polymers is the use of impact modifiers. Most impact modifiers are materials softer than the polymer matrix (e.g. rubbers). The role of the softer phase is to absorb the mechanical energy of an impact and to stop fracture propagation in case of formation of crazes or breaks. This latter effect can also be obtained by the inclusion of plasticizers at concentrations higher than the miscibility limit. In that case, the plasticizer phase separates and forms tiny droplets able to stop the fracture propagation (Ruellan et al. 2015c). For preserving biodegradability and biobased carbon content in plasticized PLA, biobased and biodegradable molecules were extensively screened as plasticizers (Ruellan et al. 2015a).

To give some examples, epoxidized soybean oil (Al-Mulla et al. 2010; Ali et al. 2009; Xiong et al. 2012; Xu and Qu 2009) or epoxidized palm oil (Al-Mulla et al. 2010; Silverajah et al. 2012a, 2012b) were tested with some success. Very interesting properties could be obtained using deodorizer distillates, which are by-products of the vegetable oil industry. They can be easily introduced in the PLA matrix by twin-screw extrusion. It was shown that deodorizer distillates from the palm oil refinery improved the elongation at break of PLA up to 180%, while the T_g (45 °C) remained higher than room temperature and the yield strength at 24 MPa (Ruellan et al. 2015b, 2016). This result is reported in Fig. 13.2. The deodorizer distillates of

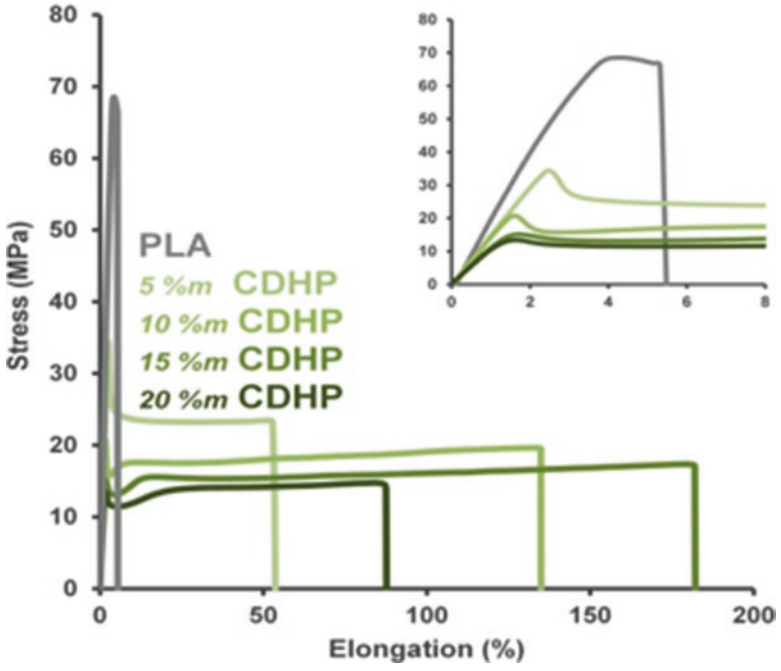

Fig. 13.2 Tensile properties of polylactides (PLA) (grey line) and PLA formulated with palm oil deodorizer distillate (PODD -CDHP) at different concentrations (5, 10, 15, 20 wt%) (green lines). The insert zooms on the properties of the neat PLA. (Adapted from Ruellan et al. 2015b)

the palm oil refinery contain different types of free fatty acids which crystallize at temperatures below and above room temperatures. The mixture of liquid and crystalline inclusions in PLA induced extensive crazing with numerous microcracks, the formation of which slowed down fracture propagation and yielded a large delay of specimen fracture (Ruellan et al. 2015b). The properties were stable during aging for 6 months (Ruellan et al. 2016). Overall migration tests at laboratory scale showed compliance with European legislation limits. The materials were fully biodegradable (Ruellan et al. 2016). Preliminary Life Cycle Analysis showed an advantage of using such by-products over petrochemical plasticizers. Those results show that the use of agricultural by-products compatible with current polymer processing technologies can mediate large property gains of PLA.

Increasing Barrier Properties of PLA with Agricultural By-Products

Gas barrier properties of polymers are an important use property for many applications, in particular food packaging. The sorption/diffusion model (Graham 1864) rules the permeability (P) of gas and vapours in polymers, establishing that P is the product of two fundamental contributions, the diffusion coefficient (D) and the solubility coefficient (S), that is, $P = D \cdot S$. The permeability is therefore influenced by different intrinsic factors of a given polymer, such as the free volume fraction (FFV) of the polymer, the glass transition temperature (Tg), the cohesive energy density (CED), the molecular orientation, and the copolymerization level. Furthermore, the inclusion of impermeable structures in the polymer matrix increases the tortuosity of the diffusive pathway, which macroscopically decreases D. The excluded volume of the impermeable structures also decreases the available volume for penetrant sorption, which macroscopically decreases S.

The increase of the tortuous pathway of diffusion inside PLA was largely investigated by Sonchaeng et al. (2018). Two different approaches were used: (i) increasing the polymer crystallinity, since the crystallites are impermeable to small molecules (Colomines et al. 2010; Guinault et al. 2012; Nassar et al. 2017); and (ii) adding nanofillers. Although most studies concerned mineral nanoparticles (Sonchaeng et al. 2018), some results were obtained employing agricultural products from lignocellulosic biomass (Espino-Pérez et al. 2018; Espino-Perez et al. 2016; Fabra et al. 2016; Sanchez-Garcia and Lagaron 2010).

The crystallization of PLA does not improve the gas barrier properties in all cases; in some configurations even adverse effects can be obtained. This counterintuitive result is caused by the formation of densified amorphous phases in the vicinity of PLA crystallites, which constitutes an accelerated pathway for diffusion (Guinault et al. 2012; Nassar et al. 2017). Densified phases are formed preferentially upon crystallization of PLA from the glassy state at low temperatures, conditions which favour coupling between amorphous and crystalline phase of the

polymer (Nassar et al. 2017; Righetti and Tombari 2011). Thus, the annealing of PLA with regards to improved barrier properties needs to be carried out at high temperatures (higher than 110 °C) ideally on nucleated materials (Nassar et al. 2017).

The second technology for increasing barrier properties is the use of nanofillers. In particular, bionanocomposites of PLA were developed using cellulose nanostructures. Nanocelluloses can be obtained from different sources of lignocellulosic biomass. Although most of the nanocelluloses produced today come from the paper industry, many reports exist showing the production of nanocelluloses from by-products of the food and agriculture industry. For example, they can be successfully obtained from barley husks and agave bagasse (Espino et al. 2014) or banana residues (Alzate-Arbelaez et al. 2019). Cellulose microfibrils (MFC) have nanosized diameters (2–20 nm depending on the origin) and a fibre length in the micrometre range. The crystalline parts of MFC can be isolated by several mechanical and chemical treatments, affording cellulose nanocrystals (CNC) or nano whiskers. Dimensions of CNC range from 200 nm up to 1–2 μm in length and about 8–50 nm in diameter (Habibi 2014).

Nanocelluloses have very high oxygen barrier properties in the dry state. The oxygen permeability ($P(O_2)$) can be in the order or one to two magnitudes lower than the one of the widely used polymer polyethylene vinyl alcohol (Fukuzumi et al. 2009). Sanchez-Garcia and Lagaron (2010) and Sanchez-Garcia et al. (2008) compared the effect of the same content of MFC and CNC on $P(O_2)$. The addition of 5 wt.% of MFC had no effect on gas barrier properties while 5 wt.% of CNC led to a decrease in oxygen permeability. Espino-Perez et al. (2013) reported similar results. The main difficulty in using CNC or MFC for barrier applications is their swelling in environments with a water activity higher than 0.6. The swelling substantially decreases the barrier properties (Fukuzumi et al. 2013). Surface grafting techniques were developed to decrease this water sensitivity of CNC with some success. A sample result is reported in Fig. 13.3, which displays the water sorption isotherm of native CNC and surface-grafted CNC. In the range of low water activities, both sorption isotherms were essentially superposed. The native CNC showed an upswing of the water mass uptake at activities >0.6 due to granule swelling and water condensation. The surface grafting of CNC produced a shift of the upswing towards higher water activity and less mass uptake (Espino-Perez et al. 2016).

Swelling phenomena give rise to non-Fickian diffusion, a phenomenon with is often overlooked. PLA and CNC films and their composites are subject to polymer relaxation-driven non-Fickian water diffusion (Davis et al. 2013; Espino-Pérez et al. 2018). In the case of films thinner than 80 μm, relaxation phenomena can be observed at experimental times longer than 2 hours and the relaxation-driven component can be preponderant (Fig. 13.4) (Almeida et al. 2020; Espino-Pérez et al. 2018). Recently, a mathematical model was developed which was able to separate Fickian transport kinetics and polymer relaxation-driven transport. With the help of this model, characteristic relaxation times can be obtained and used for the optimization of the transport properties of nanocomposites in presence of swelling penetrants (Almeida et al. 2020).

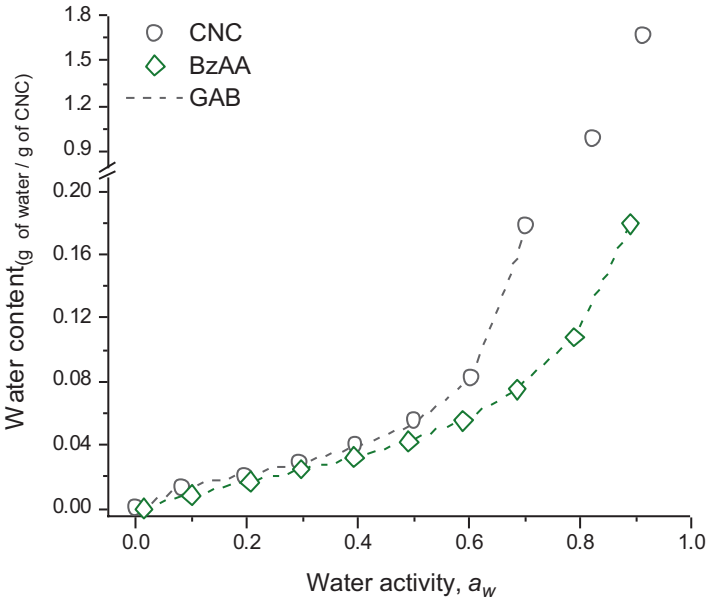

Fig. 13.3 Water sorption isotherm of cellulose nanocrystals (CNC) powders (black circles) and powders of CNC-bearing surface grafts of benzyl acetic acid (BzAA, green diamonds). (Adapted from Espino-Perez et al. 2016)

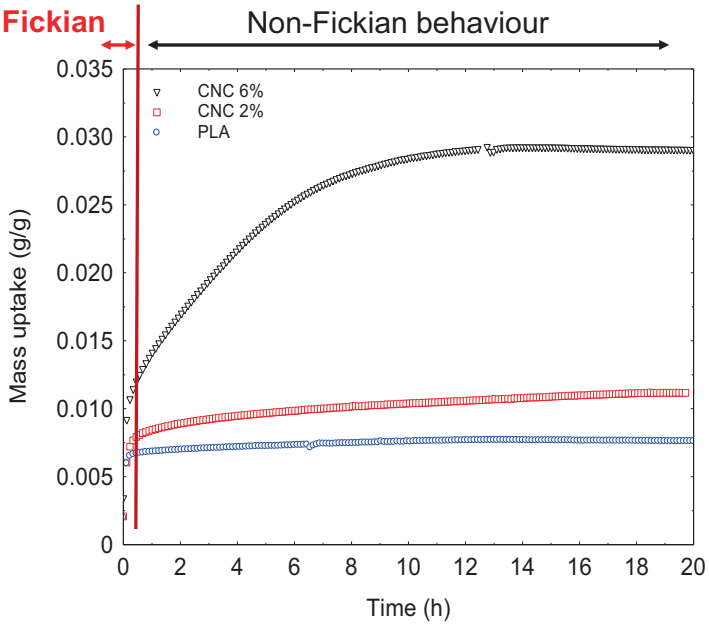

Fig. 13.4 Non-Fickian water vapour mass uptake of polylactides (PLA) and cellulose nanocrystals (CNC) nanocomposites at different CNC concentrations (2 wt% and 6 wt%). (Adapted from Espino-Pérez et al. 2018)

In conclusion, high gas barrier properties and in particular, high oxygen barrier properties are needed in food packaging for the protection of oxidation sensitive goods. Biobased materials such as nanocelluloses have very interesting potential in this respect, but they need to be shielded from water vapor. This challenge is yet to be met.

Lignin as Source for Antioxidants and Active Packaging

An alternative or supplementary means to protect oxidation sensitive foodstuff during shelf-life is the use of active packaging. This technology consists of the inclusion of antioxidant molecules in the packaging material. Generally, active packaging films rely on migration of the antioxidant to the foodstuff. However, some evidence exists in the literature that it is possible to develop materials with antioxidant surface activity (Crouvisier-Urion et al. 2016; Domenek et al. 2013; López de Dicastillo et al. 2012). In that context, lignin, an abundant and low-priced by-product of biorefinery processes based on lignocellulosic biomass (bioethanol, paper making), is an interesting source of raw materials. Lignin is a polyphenolic copolymer constituted of three subunits (coumaryl, coniferyl and syringyl alcohols), arranged in a hyper-branched structure and containing reactive free phenolic hydroxyl groups. Furthermore, lignins contain often additional free phenolic acids (e.g. ferulic acid). Phenolics are well-known antioxidants due the ease of proton abstraction and the stabilization of free radicals in the quinone resonance structure.

The antioxidant activity of lignin is already largely documented (Naseem et al. 2016). A drawback of lignin in the field of active packaging is that lignin cannot form films by itself. It needs to be included in a polymer matrix, which raises compatibility issues between lignin and the carrier polymer. PLA was investigated as a carrier polymer (Domenek et al. 2013; Yang et al. 2015b, 2016a). An interesting result concerned the extrusion processing of lignin–PLA blends. The thermal treatment leads to lignin degradation and liberation of free phenolic acids. This enhanced the antioxidant activity of the PLA/lignin films, which shows that such materials can be potentially developed using existing polymer processing technology (Domenek et al. 2013). In response to aggregation issues of lignin in the carrier polymer, fabrication methods of lignin nanoparticles were developed and different types of compatibilizers between lignin and PLA were experimented (Yang et al. 2015a, 2016a).

A very interesting result was obtained combining CNC as dispersants and lignin nanoparticles in PLA. The resulting materials had enhanced strength and antioxidant and antimicrobial properties (Yang et al. 2016a). Among alternative carrier materials for lignin, the use of chitosan is very promising. Chitosan is a derivative of chitin, which is an abundant by-product of the fishing industry (crustacea shells) (Crouvisier-Urion et al. 2016, 2017; Yang et al. 2016b). Chitosan and lignin are incompatible polymers since chitosan is much more polar than lignin. Therefore, aggregates are formed upon dispersion (Baumberger et al. 1998; Núñez-Flores et al.

2013), the size of which can however be decreased using lignin nanoparticles or high-shear or high-pressure processing methods (Crouvisier-Urion et al. 2017; Yang et al. 2016b). Figure 13.5a–c shows the impact of high-pressure homogenization on the decrease of the size of lignin aggregates in chitosan and its impact on the antioxidant activity of the blends. The homogenization treatment did not impact the overall radical scavenging activity of the films, as can be observed by the superposition of the RSA kinetics (Fig. 13.5d–e) but changed the importance of the contribution of dissolved lignin residues. High-shear and high-pressure treated lignin included more small molecules likely to be extracted into the contacting medium, where they develop antioxidant activity. Lignin can thus have antioxidant activity on the film surface and via the extraction of active compounds in the foodstuff (Crouvisier-Urion et al. 2017). Both mechanisms can be used for developing active packaging, but the safety of the materials needs to be very carefully instructed.

Antioxidants are furthermore important technological adjuvants of plastics and rubbers, because the addition of antioxidant phenolics is the most convenient and effective way to keep polymers, such as polyolefins and polyesters, from oxidative damages during melt processing, aging and weathering. For this purpose, the

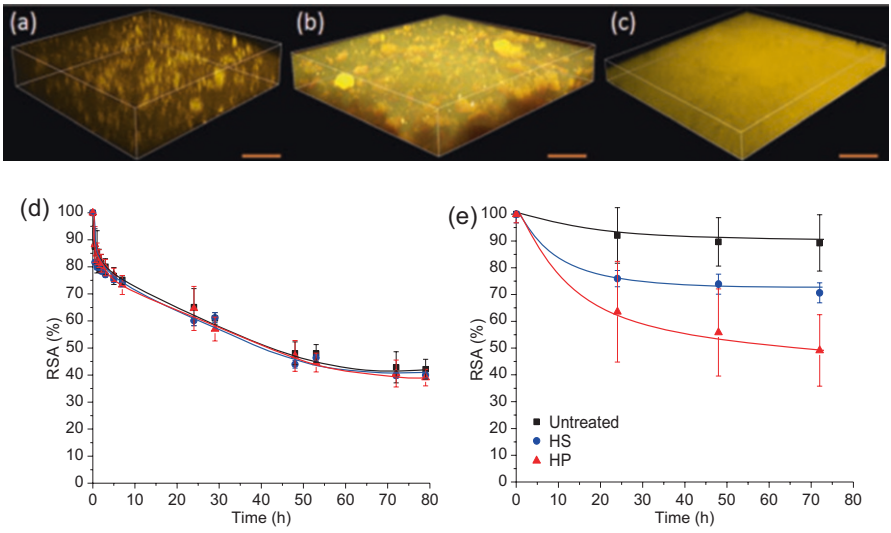

Fig. 13.5 Effect of high-shear (HS) and high-pressure (HP) treatment on lignin aggregates and antioxidant activity of chitosan films. (**a–c**) 3D reconstruction from two photon fluorescence microscopy observations performed at 750 nm excitation with 563–588 filter selection for chitosan–lignin film for the (**a**) untreated, (**b**) HS-treated and (**c**) HP-treated lignin. The scale bar represents 40 μm. (**d–e**) Reduction of the radical scavenging activity (RSA, %) determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and representing the kinetics of the decrease of the concentration of free radicals (DPPH) in ethanol–water solution; (d) overall kinetics of films submersed in an ethanol–water solution containing the free radical DPPH, including untreated, HP-treated, and HS-treated lignin; (**e**) analysis of the radical scavenging activity of lignin residues released from these films ($n = 6$). The lines are to guide the eye. (Adapted from Crouvisier-Urion et al. 2017)

employment of natural antioxidants, such as lignin-derived *p*-hydroxycinnamic acids (*p*-coumaric acid, ferulic acid and sinapic acid) is often hindered by their low thermal stability. Thermal stabilization can be obtained by derivatization. To that aim, a novel synthesis method following the principles of green chemistry using a *Candida antarctica* lipase B was developed. This enzyme can catalyse transesterification reactions of *p*-hydroxycinnamic acids with biobased diols (1,2-ethylene glycol, 1,3-propanediol, 1,4-butanediol, glycerol and isosorbide) in organic solvents or directly in the reactants without solvent under mild conditions (Pion et al. 2014; Reano et al. 2016b). The macrobisphenols obtained showed thermal stability at temperatures higher than 280 °C (Pion et al. 2013). The analysis of the structure–activity relationships defined the optimal structure with regards to antiradical and antioxidant activity (Reano et al. 2015). The highest antiradical activities were obtained with saturated esters and sinapic moieties. The saturated ferulic acid–based bisphenols had slightly lower activity, but ferulic acid is more available than sinapic acid (Reano et al. 2015). Ferulic acid–based compounds were tested in PP and poly(butylene succinate) (PBS) against the commercial benchmark Irganox® 1010 (tetrakis-(methylene-(3,5-di-*t*-butyl-*p*-hydroxycinnamate)) methane, CAS 6683-19-8), a widely used petrochemical antioxidant.

Figure 13.6 sums up the results of the oxidation induction time (OIT) of mixtures of PBS and the bisphenols at different concentrations. The highest OIT was obtained with tris-*O*-dihydroferuloyl glycerol (GTF), and all biobased molecules were more efficient than Irganox® 1010 (Reano et al. 2016a). In PP, the results were not favourable for the biobased molecules, because of their low solubility in the very apolar polymer matrix. This problem can be solved by adapting the polarity of the molecules. For that, a new class of bisphenols based on glycerol derivatives was designed using fatty acids from vegetable oils. They showed high antiradical power and thermal stability, which makes them potent antioxidant additives for polyolefins (Hollande et al. 2018). The endocrine disrupting activity of the bisphenols was tested in vitro, and no estrogenic activity was observed (Hollande et al. 2018). To sum up, lignins are a very promising raw material for high value-added substances. They can either be used with only minor preparation or serve as a starting material for chemicals synthesis in the aim to produce novel antioxidants or active packaging materials.

Blending of Polyhydroxyalkanoates (PHAs)

In the last decades, numerous studies addressed the development of PHAs, and some applications of PHAs exist today in the biomedical field, taking advantage of the biocompatibility of PHAs. PHAs feature high biodegradability in natural environments, even in seawater (Dilkes-Hoffman et al. 2019). They have some promising properties for larger-scale applications in the field of plastics, mainly linked to

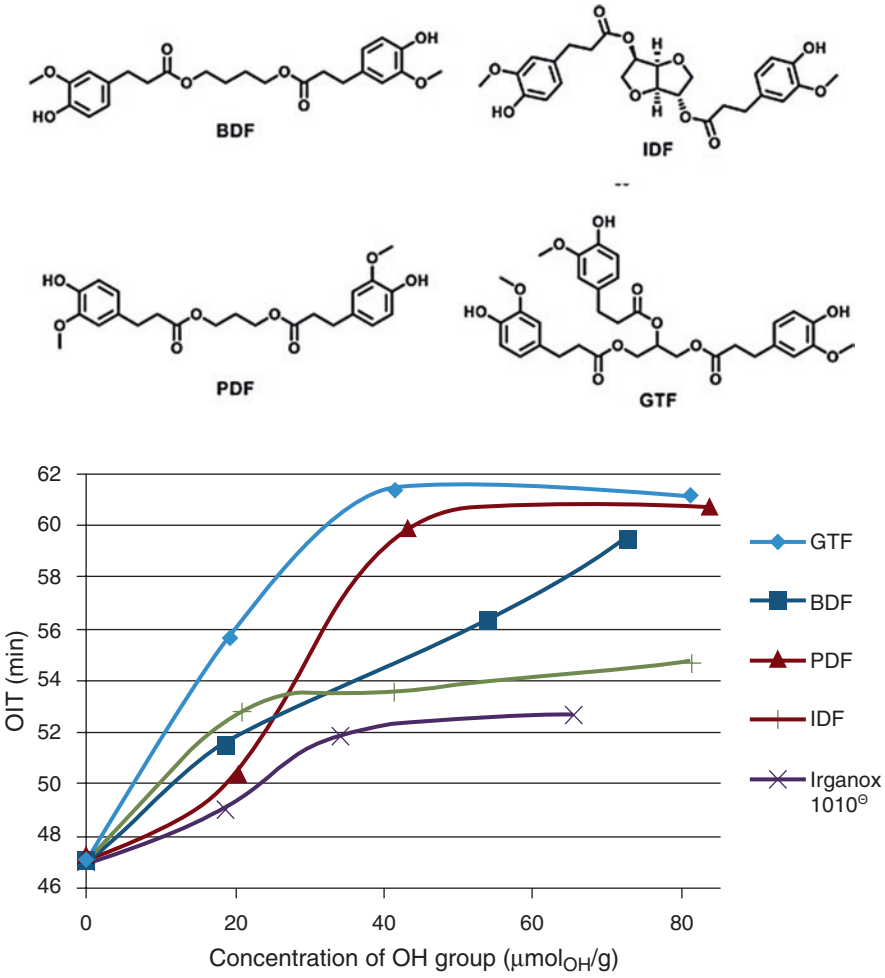

Fig. 13.6 Antioxidant properties of ferulic acid–based antioxidants (upper part) tested by the Oxidation Induction Time (OIT) (thermogravimetric test) in poly(butylene succinate) recorded as a function of concentration of free phenolic hydroxyls. Irganox® 1010 is the petrochemical benchmark. (Adapted from Reano et al. 2016a)

their high crystallinity, which are high barrier properties to gases compared to other bioplastics and high heat-deflection temperature (HDT) (Laycock et al. 2014). Today, the major hurdles for materials applications are a lack of mechanical properties and low thermal stability during plastics processing. The latter point can be partly solved by the use of chain extenders, which allow crosslinking of PHA chains during the processing, and therefore maintain molecular weight, or blending strategies such as blending PHAs with other biodegradable polymers.

Introduction to PHAs

Polyhydroxyalkanoates (PHAs), discovered in the 1920s by Maurice Lemoigne, are fully biodegradable thermoplastic polyesters produced by microorganisms as carbon and energy storage compounds in the presence of excess carbon and limited macronutrients, such as oxygen, nitrogen or phosphorus. Depending on the length/number of carbons of the monomer unit, they can be classified as short-chain-length or medium-chain-length PHAs. The most prominent group is short-chain-length PHAs, mainly represented by poly(3-hydroxybutyrate) P(3HB) and the copolymer P(3HB-co-3 HV) (Fig.13.7) (Laycock et al. 2014; Pakalapati et al. 2018).

The general fabrication process consists of a two-step fermentation, where the first step consists of microbial growth until high cell densities are obtained, and the second step entails macronutrient limitation to trigger PHA production and intracellular accumulation. The downstream processing consists of breaking down the microbial cells to liberate the PHA granules and their purification by solvent or surfactant-based extraction techniques. This process is relatively cost-intensive as compared to standard polymer synthesis. Apart from the costs linked to downstream processing and purification, other variation in costs that affect the overall pricing are the type(s) of fermentation, pure cultures needing sterile conditions or mixed cultures in non-sterile environments, and the choice of fermentation substrates (pure sugars, by-products, wastes) (Laycock et al. 2014; Pakalapati et al. 2018).

The pure culture process under sterile conditions of P(3HB) was first patented in 1959 by Grace and Company (Baptist and Ziegler 1965). This process is still in use and the few commercially available PHAs grades are synthesized via pure culture fermentation using in most cases *Cupriavidus necator.* PHA production based on mixed cultures offers a viable solution with decreased processing costs, because microbial consortia are stable without aseptic conditions (Laycock et al. 2014). Synthesis of PHAs in mixed cultures was first observed in 1974 in wastewater treatment plants designed for biological phosphorus removal (Wallen and Rohwedder 1974). A supplementary advantage is that mixed cultures are able to adapt to various complex feedstocks, opening the door for cost reductions by using agricultural by-products.

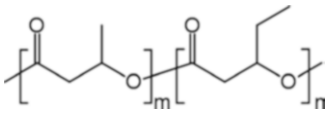

Fig. 13.7 Chemical structure of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)

Use of Agricultural By-Products of PHA Production

Production of PHA requires the use of a carbon source as the fermentation substrate. However, the cost of the carbon source greatly contributes to the overall cost of PHA production and can be as high as 40% of total operating cost (Leong et al. 2017; Posada et al. 2011). Using carbon sources coming from waste or by-products of agriculture and food industry is an appealing solution for cost reduction. Furthermore, revalorization of wastes is a possibility to step towards a more circular economy. The change of the carbon source can have a large impact on the productivity of the microbial culture. A trade-off needs to be reached between cost of fermentation substrates and PHA productivity. This topic has consequently received large industrial and academic interest. For example, Kumar et al. (2016) used four types of food industry wastes including hydrolysates of pea shells, apple pomace, potato peels and onion peels. They were tested for PHA production individually or mixed with pea shells in different ratios. The authors found that all hydrolysates were suitable for the growth and PHA production by *B. cereus EGU43* and *B. thuringiensis EGU45*. Biowaste combinations achieved high PHA yields, with the combination of potato peels (PP) and pea shells (PS) achieving the highest PHB yield, but dependent on the PS:PP ratio as PS:PP 1:2 and 2:1 produced 20 and 405 mg/L of PHB, respectively. Addition of glucose was effective in enhancing the PHB yield but varied upon PS:PP ratio from 485 to 335 mg/L at 1:2 + 1% glucose supplementation and 2:1 + 1%G, respectively. In another example, wastewater from different fruits and vegetables processing plants were used at carbon sources for PHBV with very high HV content (up to 35 mol%) (Elain et al. 2016; Lemechko et al. 2019).

Vegetable oils represent another inexpensive carbon source to synthesis PHAs at lower cost than carbon sources of high purity. Their potential as feedstock is linked to their chemical composition, which includes esters of glycerine and a high content of monocarboxylic acids. Olive oil, corn oil and palm oil are reported to be effective in producing the homopolymer poly(3-hydroxybutyrate) using *Cupriavidus necator* H16 (Fukui and Doi 1998). Virgin and waste sesame oil could be successfully used as carbon source for PHBV production by *C. necator* H16 (Taniguchi et al. 2003). Similarly, waste frying oil could be used to synthesis PHB with homopolymer concentrations similar to PHBV from glucose (Verlinden et al. 2011).

Another promising way to obtain PHAs at lower cost comes from microalgae. Microalgae are part of a heterogeneous group of microorganisms, photosynthetic, eukaryotic or prokaryotic, and Gram negative (Olaizola 2003). Their photosynthetic behaviour offers the ability to use light as source of energy and inorganic nutrients for growth and to synthesize valuable compounds. Species, culture conditions and extraction methods still need to be defined to take full advantage of microalgae as viable source to produce PHAs (Costa et al. 2019).

Physical–Chemical Properties of PHAs

PHAs are rubbery materials at room temperature (glass transition temperature, Tg, of PHB is typically 5 °C), wherefore they are often compared in the literature to polyolefins. Even if the tensile strength is comparable to the yield stress of polypropylene (PP) or polyethylene (PE) (elongation of break typically 400 for PP and 600–700 for LDPE), the elongation before a break is largely inferior (Table 13.2). Typically, PHAs attain less than 5% of maximum elongation, which makes them extremely brittle materials. Also, their impact strength is low, with fractures propagating readily through the material. The reason is most likely the occurrence of very large spherulites with poor coupling between crystalline and amorphous phases. The use of nucleating agents for decreasing spherulite sizes was shown to be a successful means for slightly increasing their mechanical properties (Laycock et al. 2014). Moreover, physical aging kinetics is fast, bringing about rapid embrittlement of the polymers. The embrittlement is also favoured by cold crystallization of PHA because that crystallizing temperature is near room temperature (Gérard 2013). The most commonly used PHBV, contains 3 mol% HV and shows a Young Modulus of about 2.9 GPa, a tensile strength of 38 MPa and very poor elongation at break of about 3%. The thermo-mechanical properties of PHBV can be tuned by increasing the percentage of HV in PHBV copolymers (Anjum et al. 2016), but even at high 3 HV ratios the polymer remains relatively brittle (Table 13.2). PHBVs have also a narrow processing window and degrade rapidly upon melting, they have a low shear viscosity and melt elastic strength, which negatively impacts their processability in blow extrusion (Anjum et al. 2016; Cunha et al. 2016). This strongly limits their industrial application, because most flexible packaging and thin films are obtained by blow extrusion.

Table 13.2 Thermo-mechanical properties of different Poly(3-hydroxybutyrate-co-3-hydroxyvalerate), PHBV copolymers

Polymer	3 HV content (mol%)	Young Modulus GPa	Tensile strength MPa	Elongation at break %	T_m (°C)	T_g (°C)	Reference
P(3HB)	0	3.5–4	40	3–8	173–180	5–8	(Anjum et al. 2016)
P3(HB-*co*-3 HV)	3	2.6	32	3.5	167	N/A	(Corre et al. 2012)
	9	1.9	37	N/A	162	N/A	(Anjum et al. 2016)
	15	0.5	11	9	142	−5	(Lemechko et al. 2019)
	35	0.04	1.2	13	150	−12	

Formulation and Blending of PHAs with Polyesters and PLA

A widely used strategy to improve the thermomechanical properties of brittle polymers consists in blending them with a more ductile material, thus combining polymer properties in a synergetic way. Moreover, this route can help reducing material costs, since blending a high-cost material with a low-cost polymer will decrease the total cost. Most polymers are immiscible, and this leads to phase separation. Depending on blend ratio, different morphologies can be developed. When one polymer is in the majority, this will lead to a nodular structure where droplets of the minor polymer are dispersed throughout the continuous matrix. When both polymer ratios are close, a co-continuous phase morphology will develop starting from a system-specific threshold ratio. Immiscible blends exhibit two T_g, corresponding to the properties of each polymer. Only one T_g will be observed for miscible polymers. In partially miscible blends a shift and/or broadening of the T_g of both or only one component can be observed approaching an individual T_g. The use of a ductile material is expected to improve the toughness of the overall material. Miscible blends often shown an enhancement in modulus and strength over unblended constituents due to specific interactions between both polymers. A positive synergistic effect on mechanical properties of immiscible blends can be obtained, but surface compatibility must be ensured to avoid poor tensile and impact strength (Robeson 2007). PHBV and PBS obtained by melt-blending showed, although immiscible, significantly enhanced moduli compared to that of the neat PBS and increased elongation at break compared to neat PHBV (Phua et al. 2015).

In particular, the blending of PHAs with poly(lactide), PLA, has received interest, because of the already existing commercial applications of PLA. Blends were tested for the biomedical field (He et al. 2014; Shabna et al. 2014), for mulch films (Dharmalingam et al. 2015), for textiles (Szuman et al. 2016), and for technical applications such as packaging (Boufarguine et al. 2013; Martelli et al. 2012; Tri et al. 2013; Zembouai et al. 2014). The interest of using immiscible blends between PLA and PHB or PHBV is that the PHA can accelerate crystallization kinetics of PLA (Tri et al. 2013) and increase the elongation an break typically up to 40% (Boufarguine et al. 2013; Gerard et al. 2014; Modi et al. 2013) and up to 60% with optimized morphologies (Boufarguine et al. 2013). The ternary blend of PLA, PHBV and PBS afforded a good stiffness–toughness balance by improving the toughness and flexibility of PLA (Zhang et al. 2012). The failure mode changed from brittle fracture of the neat PLA to ductile behaviour. In that study there was partial miscibility between PHBV and PLA but limited compatibility of both polymers with PBSA. The authors suggested that the optimum performances were obtained for a composition PHBV/PLA/PBSA of 60/30/10 (Zhang et al. 2012). Blends and multilayers were also used to increase the barrier properties of PLA. The dispersion of 10% of PHBV lamellas in PLA induces a decrease of gas permeability, which is slightly more pronounced than seen with only one thick layer of PHBV in PLA. Indeed the helium permeability decreased from 120×10^{-18} (m^3.m)/

(m^2.s.Pa) to 77.6 and 74.3 × 10^{-18} (m^3.m)/(m^2.s.Pa) with 3 and 129 layers, respectively (Boufarguine et al. 2013).

To overcome the negative effects induced by immiscible polymer blends, compatibilization is usually used to improve the mechanical properties due to enhanced interfacial adhesion, which helps stress transfer across the interface. Moreover, the morphology will tend towards finer structures with a large decrease in nodule size by preventing coalescence between particles as illustrated in Fig. 13.8. Immiscible polymer blends can be compatibilized by in situ/reactive compatibilization by a third component which contains a reactive functional group (i.e., anhydride, epoxy, etc.) which will react with blend constituents (Utracki 2002). PHBV/PBS (80/20) compatibilized via in situ reactive extrusion using dicumyl peroxide (DCP) as radical initiator at a DCP content of 0.5 wt%, dramatically improved the mechanical properties compared to non-compatibilized blends (Ma et al. 2012), and the morphology was much finer. The strain-stress behaviour changed from brittle to ductile with an elongation at break increasing from 8% to 400%. This improvement was attributed to the formation of a PHBV-*g*-PBS copolymer, which was assumed to be located at the interface between the polymers and thus act as compatibilizer (Ma et al. 2012). DCP was also used for the compatibilization of PLA/PHBV (Dong et al. 2013). DCP improved the melt strength of the blends through partial crosslinking which was further confirmed by improved melt viscosity. The blends had enhanced impact strength and impact toughness from about 13 to 20 kJ.m^{-2} after addition of 0.5 wt% DCP in PHB/PDLLA (70/30) blend (Dong et al. 2013).

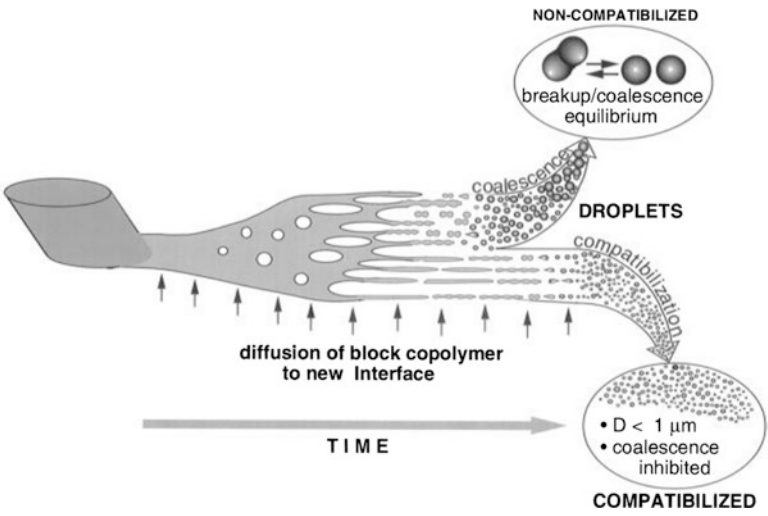

Fig. 13.8 Schematic of morphology development during melt blending. As pellets or powder of the minor phase soften, layers peel off. These stretch out into sheets which break up into fibres and then droplets. (Reprinted with permission from Macosko et al. 1996. Copyright (2020) American Chemical Society)

Safety of Formulated Biobased and Biodegradable Food Contact Materials

General Principles and Binding Regulations

US Legislation The biological origin of the polymer or its ability to be degraded by microorganisms does not preclude the obligation of testing the suitability of the proposed material for food contact. According to section 201(s) of the FD&C Act (FEDERAL FOOD, DRUG, AND COSMETIC ACT, last revision 24 June 2019), possible contaminants originating from food contact materials (food contact substances are defined specifically in section 401(s) of the FD&C Act) are called indirect additives and correspond to any substance, which may reasonably become a component or otherwise affects the characteristics of any food. Substances that are generally recognized by experts, qualified by scientific training and experience, to be safe under the conditions of its intended use are exempted. In this respect, incorporating food components into the packaging material as filler, plasticizer, adhesive, or antioxidant could be exempted from any restriction and be preferable to the use of alternative synthetic substances. However, because a pesticide chemical residue in or on a raw agricultural commodity or processed food requires a specific approval, the safety of agricultural by-products and possibly of by-products originating from food production should always be established. Scientific procedures include the application of scientific data (analytical, calculations, simulations, or other scientific studies), information, and methods, whether published or unpublished, as well as the methods to evaluate the toxicology and the chronic exposure to consumers. In short, if it is impossible in the present state of scientific knowledge to establish with complete certainty the absolute harmlessness of the use of any by-product, a safety assessment should be carried out. In determining safety, the probable ingestion of the substances originating from the considered by-products must be considered, as well as its cumulative effect of the substance in the diet, considering any chemically or pharmacologically related substance or substances in such diet.

EU Legislation The EU framework is built on very similar foundations (safety requirements for the manufacturing, processing, and distribution of food contact materials), but with different conditions of application. General safety requirements laid down by Regulation (EC) No 1935/2004) apply to any of the seventeen groups of materials identified by the EU Commission, and for which specific measures exist already or could be applied in the future (see Annex I of Regulation 1935/2004/EC). As a general rule, all food contact materials and articles should be manufactured in compliance with good manufacturing practice (further defined in Regulation (EC) No 2023/2006), so that under normal and foreseeable conditions of use they do not transfer their constituents to food in quantities that could endanger human health, bring about an unacceptable change in the composition of the food, or a deterioration of its organoleptic characteristics. Such rules do not exist for today for materials incorporating by-products originating from agriculture or food production. They are aimed at strengthening the self-assessment and responsibility of the manufacturers, processors, and distributors of food contact materials and components.

The concept of specific measures is essential in the EU legal system as it encourages the harmonization at EU level towards the highest standard among all member states. The adoption of such specific measures lies with the European Commission, which may adopt such measures but is not obliged to do so. In most cases, specific measures are adopted via the 'regulatory procedure with scrutiny' in which the European Parliament plays a scrutiny role. Without being exhaustive, the specific measures include the list of authorized substances (positive list), purity standards and special conditions of use for positively listed substances, specific and/or overall limits on the migration of certain constituents into the food, basic rules for checking compliance with the harmonized rules, authorization procedure for substances not yet in the positive list. In short, what is not accepted should not be used without asking for an authorization at the European Food Safety Authority (EFSA). As a general rule, EFSA has 6 months to issue its opinion as to whether, under the intended conditions of use of the material or article in which it is used, the substance complies with the general safety requirements of the framework Regulation 1935/2004/EC. If the opinion is favourable, EFSA will publish an opinion including the designation of the substance and its specifications, recommendations or restrictions of use, an assessment of the analytical method, and/or calculation method used for risk assessment. From 2004 to 2015, EFSA has evaluated 348 substances (Karamfilova and Sacher 2016).

Specific Measures and Specific Migration Limits in the EU The level of harmonization at the EU level is far from being complete. Specific measures exist so far only for plastics and recycled plastics, ceramics, regenerated cellulose film, and active and intelligent packaging. For other groups of materials such as rubbers, silicones, textiles, paper and board, printing inks, adhesives, coatings and varnishes, waxes, and wood, such measures may exist at a national level. The principles of safety assessment for plastic materials used alone or in multilayers are summarized in Fig. 13.9. For accepting a new substance, the level of the studies depends on the expected level of migration, that is to say on the level of mass transfer to the food in contact. Once the substance is accepted, the level of migration must be lower than the overall migration limit (60 $mg \cdot kg^{-1}$ or 10 $mg \cdot dm^{-2}$) and the eventual specific migration limit (SML). The value of the SML is inferred from toxicological data and a conservative chronic exposure scenario for an adult of 60 kg ingesting 1 kg of food packed in 6 dm^2. SML can be verified by migration testing or by migration modelling (Zhu et al. 2019b).

Overall Migration Limits (OML) in the EU OML provide a global assessment of the inertia of food contact materials by assessing the total amount of non-volatile substances transferrable to the food. OML testing is achieved by weighing the packaging before and after packaging. The methodology is not sufficiently accurate to be related to specific migration limits, and the standard conditions (OM1. OM7) are not related to time and temperature conditions used to determined SML. The overall migration limit for plastics is 60 $mg \cdot kg^{-1}$ for children or infants and can be replaced by the limit of 10 $mg \cdot dm^{-2}$ in other cases (Table 13.3).

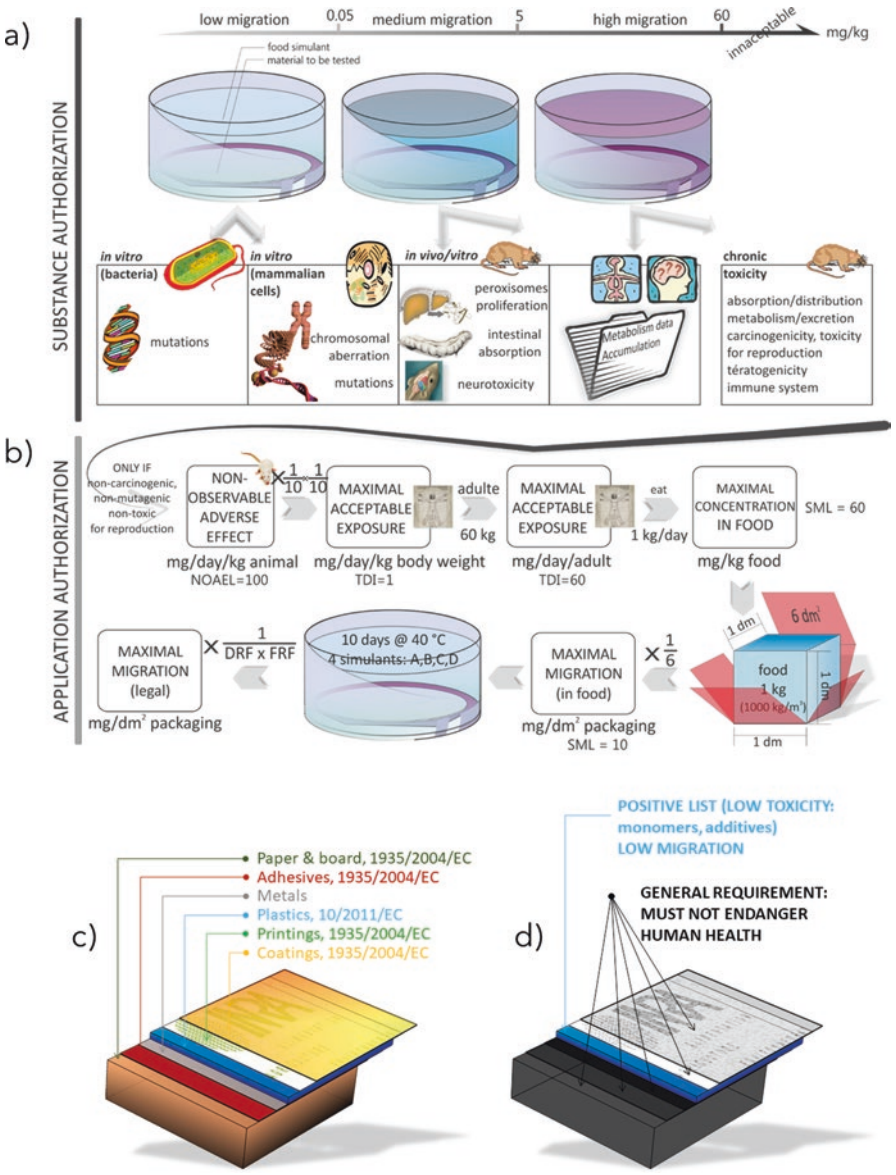

Fig. 13.9 Principles of safety assessment applied in EU (**a**) for the authorization of new substances, (**b**) for the verification of specific migration limits, (**c**–**d**) principles in multi-materials (here a laminate intended to be in contact with food). DFR Simulant D Reduction Factor, *FRF* Fat Reduction Factor, NOEL Non-observable Adverse Effects Level, TDI Tolerable Daily Intake

Table 13.3 Test conditions for evaluating overall migration limits after the European Regulation (EC) No 10/2011

Test code	Food contact conditions	Test conditions
OM1	Any food contact at frozen and refrigerated conditions	10 days at 20 °C
OM2	Any long-term storage at room temperature or below, including heating up to 70 °C for up to 2 h, or heating up to 100 °C for up to 15 min	10 days at 40 °C
OM3	Any contact conditions that include heating up to 70 °C for up to 2 h, or up to 100 °C for up to 15 min, which are not followed by long-term room or refrigerated temperature storage	2 h at 70 °C
OM4	High-temperature applications for all food simulants at temperature up to 100 °C	1 h at 100 °C
OM5	High-temperature applications up to 121 °C	2 h at 100 °C or at reflux or alternatively 1 h at 121 °C
OM6	Any food contact conditions with food simulants A, B, or C, at temperatures exceeding 40 °C	4 h at 100 °C or at reflux
OM7	High-temperature applications with fatty foods exceeding the conditions of OM5	2 h at 175 °C
OM8	High-temperature applications without storage as alternative to OM1, OM3, OM4, OM5, and OM6	Food simulant E for 2 h at 175 °C and food simulant D2 for 2 h at 100 °C
OM9	High-temperature applications with long-term storage as alternative to OM1, OM3, OM4, OM5, and OM6	Food simulant E for 2 h at 175 °C and food simulant D2 for 10 days at 40 °C

Food simulants: A (10% ethanol), simulant B (3% acetic acid), simulant C (20% ethanol), simulant D1 (50% ethanol), and simulant D2 (vegetable oil)

Sensorial Evaluation

The risk of deterioration of organoleptic characteristics needs to be verified, in particular, for bio-sourced and biodegradable components of the food packaging. The risk of aging, hydrolysis, and interactions with food are particularly high at all stages of its processing, storage, and final use. No specific guidance has been proposed to perform sensorial tests, but it is recommended to perform the evaluation on the food in worst-case conditions rather than on food simulants. The tests should be carried out in a way to detect off-flavours, bad taste or odours. Triangular tests are the most used by the industry (two samples are identical) as they enable the evaluator to identify easily false positive.

What Is Acceptable According to EU Rules for Thermoplastics

Though it was initially constructed for synthetic substances, Regulation (EC) No 10/2011 for thermoplastics evolved progressively to integrate bio-sourced additives, monomers and more recently polymers obtained by fermentation. Criteria of

purity prevail and currently, the concept of GRAS (generically recognized as safe substances) does not hold. For plastics obtained by microbial fermentation recovered with or without subsequent solvent extraction, it is stated that 'potential health risk may occur from the migration of non- or incompletely reacted starting substances, intermediates or by-products of the fermentation process (EC No 10/2011). In this case, the final product should be risk assessed and authorized before its use in the manufacture of plastic materials and articles. Among the 885 positively listed initial substances, 314 additives or monomers can be obtained by fermentation, but only one polymer (3-hydroxybutanoic acid-3-hydroxypentanoic acid, copolymer, i.e. PHBV) has been authorized. Current authorization requires this polymer to be produced by the controlled fermentation of *C. necator* (formerly *Alcaligenes eutrophus)* (strain H16 NCIMB 10442) with mixtures of glucose and propanoic acid as carbon sources.

In the general case and without prior authorization, by-products and bioproducts could be used in finished products under the same conditions, which have been accepted in general terms for recycled material. Materials or articles could incorporate non-listed substances, when they are present behind a plastic functional barrier, preventing them to migrate in detectable amounts. The limit of detection is set to 0.01 $mg \cdot kg^{-1}$ and it excludes any mutagenic, genotoxic or reprotoxic substance as well as aggregates or molecular systems intermediate between dispersed solutes and nanoparticles. It is finally emphasized that other specific measures are not yet adopted at EU level and the complete requirements for multi-material and/or multilayer materials and articles should be verified on a case basis for each member state, based on their own national provisions.

The Design of Materials Incorporating By-Products, Bioproducts or Polymers Obtained by Fermentation

The evaluation by EFSA, FDA and other bodies of biobased and biodegradable materials used alone or as components shows the various risks (summarized) in Table 13.4. The burden to find a compromise between many characteristics including risk assessment, food shelf-life, environmental impact, processing and retailing conditions can be found by using rapid engineering methods of packaging design. Failure mode effects and critical analysis (Nguyen et al. 2013) have been successfully applied to packaging design along with rapid environmental prototyping methods (Zhu et al. 2019a). These methods are particularly suitable to tackle the risk of cross-contamination in presence of complex supply chains (Nguyen et al. 2019; Nguyen et al. 2017). The software and databases parts of open-source projects (Vitrac 2015; Vitrac and Nguyen 2018) and a European initiative for massive online courses (Vitrac 2019).

Table 13.4 Critical risk associated to the use of by-products, bioproducts or polymers obtained by fermentation. Summarized from EFSA and FDA evaluation documents

Issue	Possible corrective action
Lack of control on the purity of raw materials: Presence of residues	Specific control plan, purification, avoiding uncontrolled sources in fermentation, adding a functional barrier
Extensive of chemical solvents	Solvent substitution, extraction at solid state, solvent drying
Strong sensitivity to humidity (e.g. the properties in humid conditions differ from those in dry ones)	Restriction to dry food, avoiding frozen and chilled conditions, avoiding cooking and heating conditions
Rapid hydrolysis or oxidation during storage	Controlling storage time and temperature before use; food products with short shelf-life; dry food
Strong interactions with the fat content of food	Restriction to dry food, non-processed food, overpackaging
Lack of barrier properties to adhesive and ink components	Avoiding printing and laminates
Plasticized formula with high risk of migration	Avoiding the use of plasticizers, high temperature

Conclusion

The examples discussed show that numerous studies were already carried out in the field of valorisation of agricultural by-products for increasing functionality of packaging materials, and that those approaches can have equal or higher performance than currently used technologies.

The development of functional additives for packaging polymers, or packaging polymers themselves starting from by-products of the agriculture and food industry, hold high potential for the transition of the manufacturing industry towards increased use of renewable resources and the possibility for agriculture and the food industry to develop novel value-added substances. Agricultural and food industries today are users of packaging materials and by use of agricultural by-products in the packaging materials, the agriculture industries could thus become suppliers of those products. This would close the circle of fabrication, utilization, recycling and re-fertilization of soils by biodegradation for the production of new biomass used to prepare new of packaging materials. This would be clearly a step towards a circular economy.

However, many technological hurdles need yet to be overcome to develop the academic findings into industrially viable products. These include standardization and quality management of raw materials which are today wastes. Scale-up of productions processes, and very importantly ensuring safety of the raw materials and packaging. In particular, the safety and legislative requirements need to be considered in the early steps of development, thus designing a novel generation of packaging safer for consumers and environment.

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Chapter 14
Use of Banana Crop Wastes to Develop Products in the Aquaculture and Plastic Sectors

Rubén Paz, Mario Monzón, Gisela Vega, Noelia Díaz, and David Pestana

Abstract The banana plant is a species that bears fruit only once in its life cycle. After the harvesting, the pseudostem is left on the plantation without generating a relevant nutritional value for the soil. Despite the high content of antioxidants of this subproduct, currently it is occasionally used for cattle feeding. This work is focused on the valorisation of this waste through the extraction of the natural fibre for its use in the development of 100% biodegradable materials (cover bags for banana crop and bags for fish feeds) and as a natural additive for high production volume in the plastics industry. For this purpose, the necessary technology for the mechanical extraction and processing of the banana fibre has been developed. In addition, during the extraction process another important subproduct is generated: the pulp. This by-product has the peculiarity of having low fibre content and, consequently, improved properties for its application as an additive in easy-to-digest fish feeds. To take advantage of this property, the approach consists of the use of this subproduct of the extraction process as a natural source of antioxidants for fish feeds in aquaculture, thus being an alternative to the synthetic antioxidants currently used. This approach fosters the circular economy through the increased added value of the banana crop, ensuring the use of wastes in other industries.

Keywords Banana crop · Use of wastes · Banana fibre · Banana pulp · Circular economy

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Introduction

Banana cultivation and aquaculture are key activities for the economy of the Canary Islands (Spain). In the case of banana growing, around 50% of the European production comes from this region. Once the bananas are harvested, organic wastes (mainly banana pseudostems) are usually left in the plantation. These wastes have the disadvantage of releasing gaseous emissions and do not provide significant nutritional value for the soil.

Worldwide, agriculture activity causes 10.1% of the total greenhouse gas emissions. Nitrous oxide (N_2O) is the main greenhouse gas coming from agricultural soil emissions. This gas is derived from nitrogen mineral fertilizers, manure spreading and nitrogen from crop waste. Regarding carbon dioxide emissions (CO_2), agriculture activities do not emit significant amounts and, additionally, provide the massive stockpile of carbon, thus reducing the amount of CO_2 in the atmosphere. On the other hand, the aquaculture sector represents another basic activity for the Canary Islands. In this context, it is important to find alternative raw materials for fish feed diets and formulations, without competing with human food and bearing in mind their sustainability (Ramírez et al. 2019).

Another problem nowadays is the massive use of plastics, mainly coming from non-renewable petrochemical sources. Despite the dependency on global oil reserves and price increases, the consumption of plastic is still increasing, a reason why the substitution of petrochemical sources by renewable resources for plastic manufacturing is completely necessary. The total EU consumption of plastics is close to 50 Mt. (Ortega et al. 2010). From this consumption, 38% is related to packaging, resulting in 16 Mt/year of waste due to the single-use nature of these products. This problem also occurs in the agriculture sector, where plastics are usually left on the farms and do not degrade. To mitigate this effect, several types of bioplastics such as polyhydroxyalkanoates (PHAs), polylactides (PLA) and starch plastics are being used in packaging applications, which is also a challenge for the agricultural sector.

Banana crops by-products have several applications. Indigenous people from Indo-Malaysian Regions and Southeast Asia use them as food or wrapping materials for traditional meals. Another application is the usage as animal feed, but an additional processing step is required to reduce the water content. Moreover, banana by-products have bioactive constituents that can be used as nutraceutical products or even as natural preservatives for foods. Regarding non-food applications, banana by-products also have high potential as raw materials for industrial applications as they are independent from the agro-food-based market and do not require extra planting areas. For example, natural fibres from banana sub-products can be utilized for the production of paper. In the case of banana pseudostems and leaves, they have also potential for ethanol production or even to be used as substrate for the production of non-food cellulose, cellulolytic enzymes, organic acids, heavy metal absorbers or fertilizers (Padam et al. 2014). On the other hand, Begnini et al. (2019) created a biodegradable film based on cassava starch reinforced with nanocellulose (obtained from banana fibre) and glycerol.

Several authors have also investigated the banana fibre properties as reinforcing material for composites. Liu et al. (2009) analysed the properties of banana-fibre-filled composites based on HDPE/Nylon-6 (80/20, w/w) blends with different compatibilizer concentrations and made by injection moulding. Biswal et al. (2012) studied the thermal stability and flammability of banana-fibre-reinforced polypropylene (PP) nanocomposites made by compression moulding with nanoclays. Moreover, banana fibre has been also used as reinforcement in polyethylene (PE) rotomoulded parts, demonstrating capabilities to enhance the mechanical properties (flexural/tensile modulus and flexural strength), but significantly reducing the impact strength (Ortega et al. 2013). Other authors have also proved the feasibility of using banana fibre (mixed with 50% of wool fibre) to produce continuous yarn (Ortega et al. 2016) and the subsequent woven production to be used as technical textile for composite materials made by compression moulding. However, the presence of wool fibre in the yarn, which facilitates the yarn production, leads to reduced mechanical properties of the composite reducing the real potential of banana fibre (Monzón et al. 2019). Despite the high potential of banana fibre and its composites, most of the research and applications made so far were at the laboratory scale, mainly because of the lack of cost-effective processes to obtain the banana fibre. Therefore, an automated banana fibre extraction process would increase its application to a larger extent (Venkateshwaran and Elayaperumal 2010).

Life BAQUA Project

The Life BAQUA project aims to implement a circular economy through the use of banana crop waste in other sectors, thus reducing the carbon footprint and employing a more sustainable and eco-friendly use of resources. As commented above, after the harvesting of the bananas, the pseudostem is left on the soil. Life BAQUA project uses these pseudostems to extract the natural fibre present in it so that it can be used as a natural additive for industrial applications, such as reinforcement for biobased and biodegradable plastics, and also to substitute for synthetic additives present in polymeric plastic parts in high volume production sectors.

On the other hand, the project also seizes the 'potential advantages' in the banana pulp resulting from the fibre extraction process. This by-product has antioxidant properties and low fibre content, reasons why it is proposed to be used as a natural additive for fish feeds in aquaculture.

In summary, Life Baqua project is structured around the following four technical objectives:

- Optimization of waste management of banana production through an innovative extraction process that turns a waste into a resource.
- Environmental benefits derived from the improved agro-wastes management, which is aligned with the European roadmap 2050 towards a low carbon economy.

- Development of a circular economy strategy between sectors involved in the project: natural fibre as an additive for biobased/biodegradable plastics and/or as substitute for synthetic additives; and pseudostem pulp as natural antioxidants in fish feed.
- Improvement of the nutritional quality in fish feed.

Automated Extraction Process of Banana Fibre

The following sections describe the different steps and subsystems developed to automate the process and make it feasible.

Extraction Subsystem

The extraction system applies three main processes: tuxying, scraping/brushing and cutting. The first module extracts the part of the leaf in which the fibre concentration is very low (tuxying), to prepare a thinner sheet that will be scraped and brushed in the second module to remove impurities. After the extraction, the fibre is cut on the top, which is not processed as it is the holding zone of the leaf. The following paragraphs explain these steps.

Once the pseudostem arrives from the farm, the leaves are manually separated and flattered in a roller machine, thus facilitating the feeding for the next step. The leaves are then introduced (vertically and one by one) in the conveyor system of the automated extraction fibre machine (Fig. 14.1).

This conveyor system transports the leaves along the different steps of the process. In module 1, the leaves are scraped (from the top to the bottom) by blades on both sides. The softer side (the internal zone, which is thicker and contains very few fibres compared with the external side) is removed. Once the leaf is moved out of module 1 by the conveyor system, a rope system reshapes it into a round shape to easily enter module 2. There, the leaf is scraped again by blades in a rotary system. Moreover, some of the blades contain brushes to improve the cleaning and quality of the extracted fibre.

Once the fibre leaves module 2, it is necessary to separate it from the rest of the leaf. A cutting system with a saw is used for this purpose. Finally, the fibre must be dried to facilitate the subsequent chopping process. For this reason, it is dried outdoors for 2–3 days, thus taking advantage of the favourable climate conditions of the Canary Islands, with the consequent energy savings. Regarding the pulp obtained during the fibre extraction process, it can be delivered as it is obtained in the extraction machine, but then it has to be processed to be added to fish feed (Díaz et al. 2017, 2018).

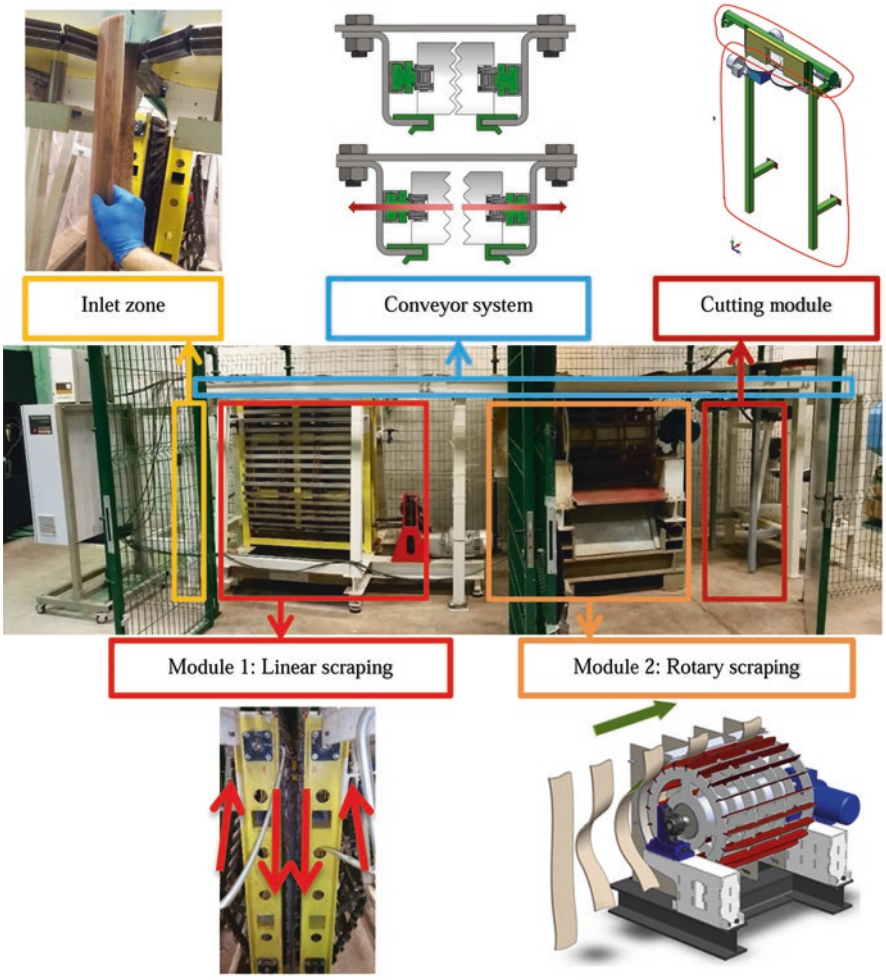

Fig. 14.1 Extraction fibre subsystem

Chopping Subsystem

For the production of short fibres, a chopping machine was designed and manufactured. It consists of rollers and a conveyor belt to guide the fibres to the blade where they are cut. The length of the fibre can be regulated by the conveyor belt speed control and/or the cutting tool speed control, producing the nominal length of 2 mm. The fibre is fed into the machine through a hopper (Fig. 14.2) that guides the fibre along the conveyor belt. This conveyor belt pushes the fibre towards the fibre addressing zone, where a group of vertical and horizontal rollers continue to transport the fibre to the cutting zone. Both the conveyor belt and vertical/horizontal

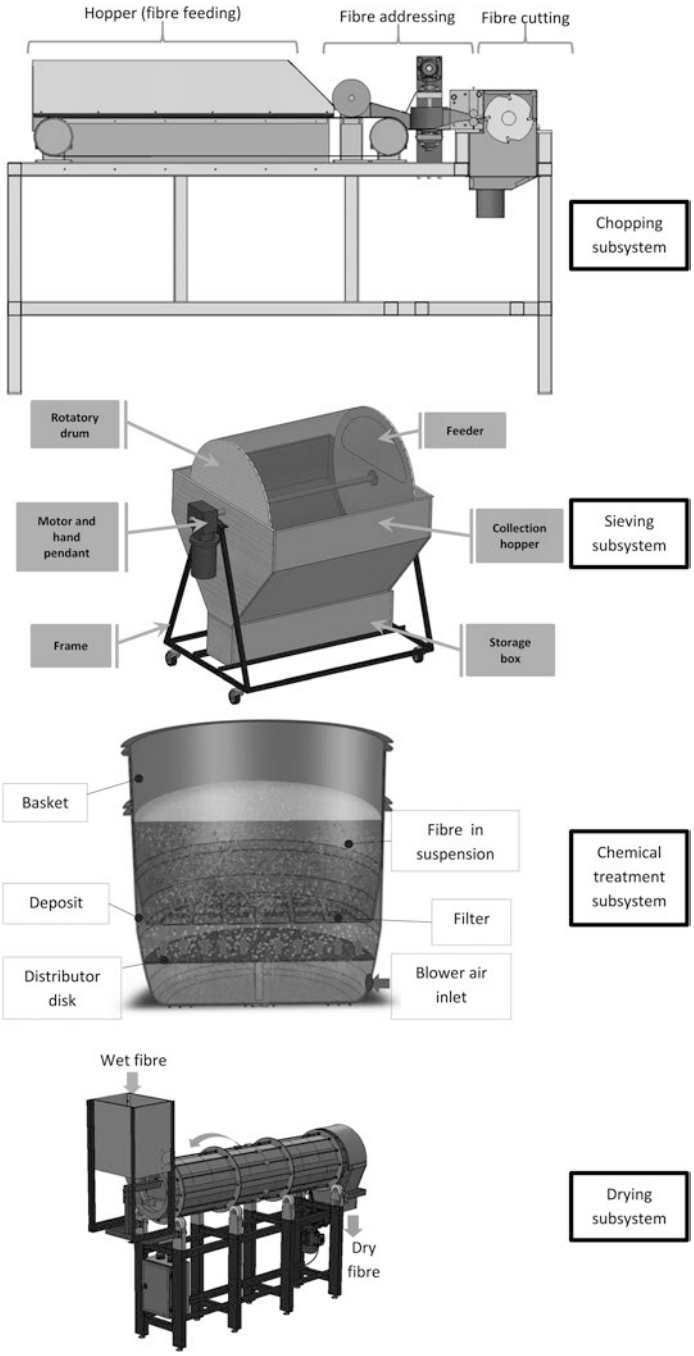

Fig. 14.2 Design of the chopping, sieving, chemical treatment reactor and drying subsystems

rollers are connected through a chain and are also properly sized to achieve the same linear speed (constant feeding speed). On the other hand, the horizontal rollers located at the end of the fibre addressing system include a spring system that allows the automated adjustment of the separation between the rollers, thus guaranteeing a good holding of the fibre regardless of the amount or thickness of it. Once the fibre is cut by the cutting tool, a vacuum system sucks it into a bag.

Sieving Subsystem

When the end of a bunch of fibre is released from the horizontal rollers of the chopping machine, the cutting blade can drag it, thus obtaining larger fibres than desired (longer than 2 mm). This can be a problem for the subsequent applications of the fibre, especially for the compounding process. To mitigate this, a sieving subsystem was developed (Fig. 14.2). It consists of a big rotatory drum with a mesh all around its perimeter. The fibre is placed inside this mesh and the drum rotates, thus sieving the fibre according to the mesh size. A galvanized steel hopper guides the sieved fibre to the box situated on the bottom. The fibre is then collected from this box at the end of the process. From the experimental tests, it was observed that the shortest fibre tends to be on the bottom on the box, while the longest is always on the top. For this reason, a later blending process was included in the pilot plant design (Fig. 14.3). On the other hand, the waste fibre retained in the drum (long or dirty fibre) can be used for other applications.

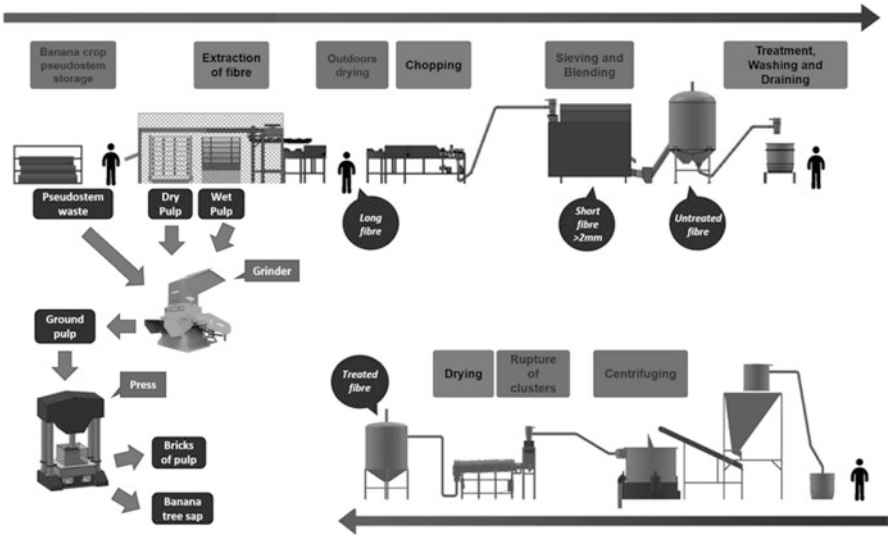

Fig. 14.3 Flowchart of the integration in a pilot plant

Chemical Treatment Subsystem

A chemical treatment in the fibre can provide several advantages: the improvement of the compatibility between the plastic matrix and natural fibre, thermal stability during the processing time, enhanced mechanical properties of the resulting composite, and enlarged contact surface between plastic and fibre. The use of NaOH as a chemical agent for the reduction of hemicelluloses and lignins in fibre is well known and usually applied in fibres such as sisal or abaca. In the case of banana fibre, it is also known that the treatments with 1 N NaOH improves its thermal stability, facilitating its use as a part of composite materials in combination with plastics such as polyethylene (PE) or polypropylene (PP).

For these reasons, a reactor was developed with a nominal production of 5 kg of dried treated fibre per day, thus ensuring the nominal production required for the pilot plant. One of the key parts of the reactor is the solid–liquid (fibre-treatment solution) separation system for the recovery of the treated fibre. To facilitate this separation, the reactor was divided into two bodies: the external deposit and the internal basket (Fig. 14.2).

The internal part consists in a basket (removable body) with a filtering mesh in the bottom, thin enough to prohibit the loss of fibre through its pores. This basket has a bottom rigid enough to support the weight of the soaked fibre during the draining process. On the other hand, the fixed body of the reactor (deposit) includes an air inlet and a distributor disk that guarantees a good distribution of the compressed air before reaching the fibre. This air provides the proper agitation to the fibre during the process, thus improving the efficiency of the treatment.

The complete treatment subsystem is composed of the previously described reactor and two tanks (one for the treatment solution and the other one for a water rinsing), all of them connected by pipes and pumps. The fibre is placed inside the basket and immersed in the liquid phase, which is pumped from the corresponding tank. The blower air is introduced to shake the fibre.

Once the treatment is done, the basket is removed from the residual fibres, so that the fibres are deposited on the filter, while the treatment solution drains through and remains in the container. Afterwards, the liquid phase is pumped back to its tank and the washing operation starts. The procedure is similar: the basket (with the fibre inside) is placed in the container, the water is pumped, and the blow air produces the agitation.

Drying Subsystem

After the chemical treatment, a drying process is needed to remove the moisture from the chopped and treated fibre to achieve the required conditions for its storage and subsequent compounding process. A drying machine was developed with the goal of reducing the relative water content of the banana fibre to below 15%. The machine consists of a drum slightly inclined towards the outlet to ensure a continu-

ous feeding. The drum has an internal helix that pushes the chopped fibre along the drum, while several infrared (IR) emitters allocated inside the drum (in the top) heat the fibre. Additionally, the drum has several ridges that drag the material during the rotation and cause a cascade of material, thus facilitating the drying process as the particles of fibre change their orientation and position. During the tests, it was observed that the fibre was too wet after the chemical treatment. Therefore, a centrifugation step was considered necessary to reduce the moisture before the fibre is fed into the drying subsystem. This idea was implemented in the design of the pilot plant (Fig. 14.3). On the other hand, it was also observed that the wet fibre tended to agglomerate, forming clusters that remain together during the drying process and that are undesired for the compounding extrusion process. For this reason, a vibration system was added in the inlet of wet fibre of the drying machine to break those clusters before the fibre gets into the machine. Figure 14.2 shows the redesigned machine. With this stage, the whole process is completed (fibre extraction, outdoor drying, chopping, chemical treatment and drying). The fibre is then packed and delivered for its use.

Integration of the Process in a Pilot Plant

All the previous steps were integrated in a pilot plant (Fig. 14.3). This flowchart shows all the required machines and stages, also including the steps needed to process the pulp. In this regard, both the pseudostem waste and the pulp obtained from the extraction fibre machine are ground and pressed to remove the water content. The bricks obtained are then suitable for delivery and prepared to be used as additive for fish feed.

This pilot plant allows the production of different types of fibre depending on the processes applied. The following list summarizes the types obtained, from less to more processed fibre:

- Untreated long fibre: The first output is the long fibre obtained after the extraction fibre process and outdoor drying.
- Untreated short fibre (larger than 2 mm): Once the fibre is chopped and sieved, the waste from this last process can be used in several applications. This fibre is longer than 2 mm and has a lower quality than the other outputs as the sieving process also removes the dirty fibre.
- Untreated short fibre (shorter than 2 mm): This is the fibre obtained from the sieving–blending machine. Therefore, the quality is higher than the previous one.
- Treated short fibre: This is the previous fibre after being treated and dried. It is the result of the complete process.

Depending on the application, a different type of fibre can be used. In the case of the plastics sector, the short fibre configuration is the most suitable to facilitate the compounding extrusion process. Regarding the chemical treatment, it can vary according to the specific application and polymeric matrix used.

Applications in the Plastic Sector

The short fibre obtained from the previous process has been applied to the production of reinforced injected parts with conventional plastic and bags with a biodegradable matrix.

Reinforced Injected Parts

Plastics parts made of conventional polymers such as high-density polyethylene (HDPE), acrylonitrile butadiene styrene (ABS) and polystyrene (PS) can be reinforced with treated fibre to improve the mechanical strength up to eight times, with a maximum fibre inclusion of 20%. In the case of banana fibre, the tests showed that the higher the percentage of banana fibre, the higher the mechanical strength and stiffness (elastic modulus), but the composite is more brittle. On the other hand, the use of natural fibre to reinforce plastic parts allows the reduction of synthetic products and makes possible the substitution of high environmental impact products by others with a lower impact and cost. This idea was applied in several products, such as in the production of mounting sleeves of ventilation systems. To make this possible, the fibre is firstly mixed with HDPE in different proportions to produce pellets by compounding extrusion. Once the material is extruded, a cutting system is used to obtain pellets. The resulting pellets are then used as raw material for the injection moulding process. These parts were tested to measure the stiffness. The tests consisted in deforming the circular shape until achieving an internal diameter of 106 mm. The force applied is measured with a load cell. The results showed that the force needed to deform the part was higher as the amount of fibre was increased.

Biofilm

Another application of the fibre is the production of biofilm through a film-blowing extrusion line. In this case, the fibre is blended with different biodegradable and biobased matrix polymers in a compounding extrusion machine. The pellets obtained are then introduced in the extruder of a film-blowing line to produce bags. These bags can be applied in the agriculture sector to speed up the ripening process of banana bunches (banana sleeves), or as a container for fish feed. These bags were then tested in a banana plantation to analyse their behaviour in terms of status of the bananas and bags after the harvesting. A conventional bag was also used as control group. The matrix polymers used were Danimer, Mater-bi, Bio-PBS and PLA. From the experiments, it could be concluded that all the bags had a good appearance and integrity, except the PLA bag, which was too stiff and was broken due to the environmental conditions. Regarding the banana status, all the bunches of bananas

tested were in good conditions. After several tests, it was concluded that Mater-bi matrix was the best option for the production of banana sleeves because the growing of the fruit was faster, and no scratches were found on the bananas.

Fish Feed for Aquaculture

The effect of the introduction of different banana crops by-products on fish diets was also analysed. The by-products tested were the pulp obtained from the fibre extraction process and the banana flowers. Both of them were used in different percentages in fish feed production for tilapia and seabass species. From the tests carried out it was observed that the level of antioxidants in banana pulp was not significant to be used as an additive with that characteristic. However, it was found that banana flowers had high levels of polyphenols, thus having antioxidant properties. On the other hand, the subsequent tests demonstrated that banana pulp could be used as a substitute of scratch or wheat flour for fish feed production, with the advantage of not competing with human food. In this regard, it was observed that the inclusion of 6% of banana pulp did not cause liver or intestine damage on seabass and tilapia species. As a result of this study, it was concluded that banana crops by-products are promising raw materials for sustainable aquaculture diets. An inclusion of 6% of banana pulp and 3% of banana flowers demonstrated to be appropriate for tilapia and seabass species. The banana pulp reduces the amount of scratch/wheat flour used and the banana flower provides high levels of polyphenols. This results in more sustainable and higher quality diets.

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Chapter 15
Use of Agroindustrial Biomass for Biofuel and Enzyme Discovery and Production

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Abstract Biomass is all biologically produced matter and means a biological reservoir of energy. The list of biomass wastes is large, for example, agriculture, horticulture, forest residues, food processing, and municipal solid wastes, among others. Biomass is widely available; its use reduces the amount of wastes and the biofuels obtained from it are considered renewable energy. Crop cultivation activities (e.g., rice, banana, maize, and sugarcane) produce large quantities of agricultural wastes. These (lignocellulosic) materials are composed of cellulose, hemicellulose, and lignin, and cellulose is the most abundant organic polymer on Earth, but the conversion of lignocellulose into reducing sugars is more difficult than the conversion of starch. Microorganisms play an important role in the global carbon cycle, and microbial decomposers have been isolated from raw materials

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as reported in many publications. These microbes secrete, among other enzymes, ligninases, hemicellulases, and cellulases, which hydrolyze the polymers present in the agroindustrial wastes. The search for microbes with good performance on lignocellulosic materials is expanding to other niches and currently many extremophiles are under characterization. Microbial enzymes are produced by different approaches: solid state fermentation, liquid submerged fermentation, or by genetic engineering of the lignocellulolytic microorganism or by heterologous expression in model microorganisms. Biological treatment of lignocellulose improves the efficiency of hydrolysis, and compared to other pretreatments, it does not require handling chemical substances. Other challenges to overcome in biofuel production are the presence of inhibitors, mainly furan derivatives, which are generated during physical and chemical pretreatment of lignocellulosic biomass. Research is now focused on developing methods for detoxification and identification or the development of much more tolerant fermentative microbes. Current efforts are paying attention to the consolidation of processes for biofuel conversion in order to make them more efficient and economically feasible. This chapter reviews the state of the art for valorization of biomass to produce enzymes and biofuels.

Keywords Valorization of biomass · Biofuels · Agroindustrial · Enzymes

Introduction

The imminent decline of the world's oil production, its high market prices, and environmental impacts have made the production of biofuels reach unprecedented volumes over the last 10 years. An increase in energy demand is due to rapid industrialization and high population growth. This is why there have been intense debates discussing the impacts of biofuels. Biofuels are one of the most promising alternatives (Alzagameem et al. 2018). The environmental impacts of programs that encourage biofuel production, farmland and requirements, and the impacts on food production are also discussed. International organizations and political leaders have concluded that the use of biofuels is inevitable, but international and national regulations should pay attention to the use of land and the environmental impacts caused by biofuel production (Koonin 2006).

It is also important to consider the different existing options for raw materials and different technological routes to produce biofuels as well as aspects of the cost of production and the relationship of their economic viability with international oil prices. Fossil fuels are used to meet the growing energy requirements of the industrialized world. A problem with this is that the world demand for oil has grown faster than its production. However, oil, which boosted the economic growth of the last century, is about to reach its peak. Besides, the CO_2 emitted when burning fossil fuels is an important cause of climate change. Limited oil reserves and environmental concern have increased the need to seek alternative sources of energy (Alzagameem et al. 2018).

The industry produces large volumes of biomass waste, which increases its disposal and pollution problems, representing a loss of biomass and important nutrients that can be recovered and recycled. The use of biomass, in particular for the production of biofuels, is becoming more interesting and necessary. Biofuels (biodiesel, bioethanol) are direct and immediate substitutes for liquid fuels used in transportation and can be easily integrated into logistics systems in operation. Methane, a gaseous fuel, can also be produced from waste biomass and can be used directly as an energy source or can be passed through turbines to generate electricity (Kazemi Shariat Panahi et al. 2019).

Lignocellulosic biomass provides a unique low-cost resource for the sustainable production of liquid fuels on a scale sufficient to have a substantial impact on demand.

Lignocellulosic Biomass: Chemistry

Evolution has selected lignocellulose to be the key element of plant structure and consequently to be resistant to biotic and abiotic stresses. These characteristics represent a bottleneck in the industrial processing of biomass. Recalcitrance to saccharification is a major limitation for the conversion of lignocellulosic biomass.

The knowledge of the chemistry of biomass is key for the development of energy-efficient biorefinery processes. In general, the chemistry of lignocellulosic biomass is extremely complex, involving extensive ranges of chemical compounds. Cellulose, hemicellulose, lignin, proteins, and fats are the primary chemical compounds present in the biomass together with lesser amounts of other chemicals such as vitamins, dyes, and flavors (Maity 2015).

Cellulose is the main building material out of which plants are made, and it is the most abundant naturally occurring biopolymer. Various natural fibers such as cotton and higher plants have cellulose as their main constituent. It is a linear homopolymer composed of D-glucopyranose units linked by β-1,4-glycosidic bonds (Fig. 15.1). It mainly contains carbon (44.44%), hydrogen (6.17%), and oxygen (49.39%). These carbohydrate polymers consist of tens to hundreds to several thousand monosaccharide units (Lavanya et al. 2011).

Hemicellulose is a group of heterogeneous polysaccharides, which are formed through biosynthetic routes different from that of cellulose. Like cellulose, most hemicellulose functions as supporting material in the cell wall. It contains pentoses (xylose and arabinose), hexoses (glucose, galactose, and mannose), and sugar acids (D-glucuronic and D-galacturonic acids). The content and chemical structure of hemicelluloses can vary within different biomass types and even in the same plant. Hardwood hemicelluloses are xylans whereas softwood consists of glucomannans. Mannose is the most important hemicellulosic monomer followed by xylose, glucose, galactose, and arabinose (Saha 2003).

Lignin is a racemic, heteropolymer consisting of three hydroxycinnamyl alcohol monomers, differing in their degree of methoxylation: p-coumaryl, coniferyl, and

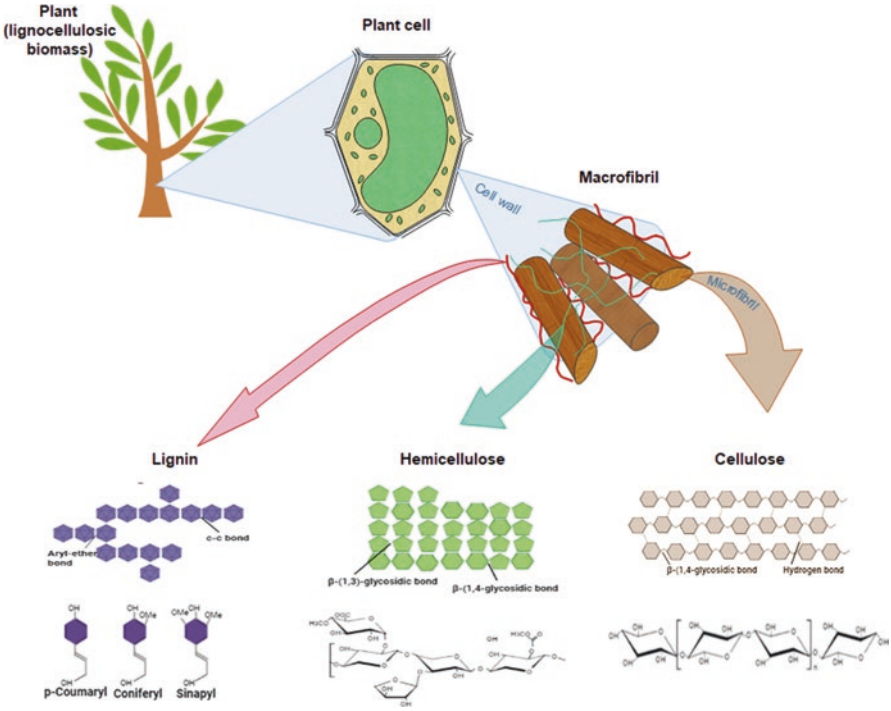

Fig. 15.1 Schematic structure of lignocellulose

sinapyl alcohols. The composition of lignin is dependent on the source of biomass; in general, hardwood, softwood, and annual plants (grasses) contain similar monomers although the relative content can vary significantly (Rößiger et al. 2018).

The structure and the quantity of cellulose, hemicellulose, and lignin vary according to species, tissues, and the maturity of the plant cell wall (Table 15.1) (Martínez et al. 2005; Monlau et al. 2012; Park et al. 2016).

The breakdown of lignocelluloses is a necessary step in biofuel production processes. Mechanical pretreatments improve digestibility and conversion of saccharides; for example, studies have shown that particle sizes must be reduced to 0.5–2 mm to decrease heat and mass transfer limitations and to reach a well-accepted level of digestibility. Currently, mechanical size reduction steps are not cost effective, but these are combined with moderate chemical or physicochemical cotreatments that reduce energy consumption, maintain cellulose polymer structures, and increase enzymatic accessibility (Adapa et al. 2010; Vyas et al. 2018). Pretreatments change the lignocellulosic matrix and make the holocelluloses more accessible to enzymes or chemical hydrolysis to release the reducing sugars (da Silva et al. 2016;

Table 15.1 Chemical composition of lignocellulosic biomasses

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood	35–50	24–40	18–25
Softwood	45–50	25–35	25–35
Corn cob	36	23	17
Wheat straw	30	50	15
Corn fiber	14.28	16.8	8.4
Wood	80–95	0	0
Barley straw	40–44	28–30	20–22
Bagasse	50	20	30
Nutshell	25–30	25–30	30–40
Paper	15–20	80–85	0
Newsprint	40–55	25–40	18–30
Fast growth pasture	45	31.4	12
Hard waste of cattle	1.6–4.7	1.4–3.3	2.7–5.7
Chemical paper pulp waste	60–70	10–20	5–10
Sunflower stalk	31	15.6	20.2
Pine	43.3	21.5	28.3
Eucalyptus	64.1	18.4	21.5
Spruce	45.5	22.9	27.9

Monlau et al. 2012). An effective pretreatment process has the following main objectives (da Silva et al. 2016; Kumari and Singh 2018):

(a) Release of sugar.
(b) Avoid loss or degradation of released sugars.
(c) Limit the production of inhibitory products.
(d) Reduce energy demands.
(e) Minimize the cost of biofuel production.

The conversion of lignocellulosic materials in biofuels is dependent not only on their biochemical composition but also on the organization of their constituents and the interaction between them. Biological, chemical, and physicochemical conversions of lignocellulosic materials are affected by a number of factors such as the crystallinity of the cellulose, the structure of hemicelluloses, structural surface area and pore volume, lignin composition, content, and cross-linking (Monlau et al. 2012; Taherzadeh and Karimi 2008), all of which depend on the species, tissues, plant part, and plant maturity. We will focus on ethanol and biogas (i.e., methane) production since they are the main biofuels produced so far from lignocellulosic biomasses.

Biofuels

Biogas

Biogas consists of around 50–60% methane (CH_4), 40–50% carbon dioxide (CO_2), and some minor constituents, such as hydrogen sulfide (H_2S) and water. The production of biogas from a variety of biological or lignocellulosic wastes is considered favorable because of its economic and environmental benefits (Kumari and Singh 2018; Hakawati et al. 2017).

Basic Concepts of Anaerobic Digestion

Anaerobic digestion is the collective process of the breakdown of biological material carried out by a group of microorganisms in the absence of oxygen (Bhatia et al. 2018). The general scheme of the conversion of organic substrates to biogas is indicated in Fig. 15.2 and consists of four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis.

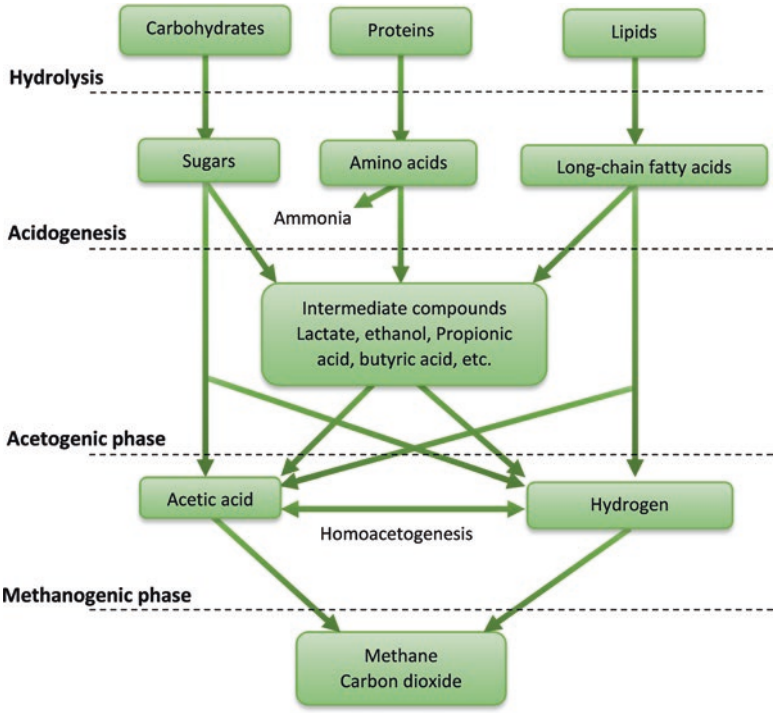

Fig. 15.2 Diagram of anaerobic digestion of complex biopolymers to methane

Hydrolysis

At this stage, biopolymers such as polysaccharides, proteins, nucleic acids, and fats are initially hydrolyzed, by extracellular enzymes secreted by hydrolytic microorganisms, into organic monomers, which can be utilized either as substrates by fermentative organisms (amino acids, sugars) or by anaerobic oxidizers (fatty acids). Depending on the structure of the cell wall, the extracellular enzymes have different locations. In Gram-positive bacteria, extracellular enzymes cross the membrane and may be temporarily restricted by the cell wall but eventually diffuse into the environment. Some enzymes, however, remain attached to the outer surface of the membrane and may be temporarily restricted (Demirel and Scherer 2008; Zhang et al. 2007).

Acidogenesis

In this stage, organic monomers are utilized as substrates by fermentative organisms (amino acids, sugars) or by anaerobic oxidizers (fatty acids). The carbon products from these reactions are intermediate compounds, such as propionate and butyrate, which may later be converted to acetate and hydrogen (Demirel and Scherer 2008).

Acetogenic Phase

During this stage the fermentative, syntrophic, and homoacetogenic bacteria are the main contributors to acetate production, and it is one of the most important intermediaries in anaerobic digestion. Fermentative and syntrophic acetogenic bacteria convert fatty acids into acetate, biogas (H_2 and CO_2), and other by-products while homoacetogenic bacteria can use H_2 and CO_2 to produce acetate (Table 15.2). The accumulation of H_2 dissolved in liquid or high partial pressure of H_2 can alter the flow of electrons in the biochemical pathways of microorganisms and inhibit fermentation and the acetogenic phase (Demirel and Scherer 2008; Nie et al. 2007).

Methanogenic Phase

Methanogens are strict anaerobic microorganisms that produce methane as part of their energy metabolism. The list of substrates for growth can be divided into three groups (Table 15.3). In the first group, the electron donor is H_2, formate ($HCOO^-$), or some alcohols, and the electron acceptor is CO_2, which is reduced to methane. The methanogenic microorganisms capable of using H_2 as an electron donor to

Table 15.2 Stoichiometry of acetogenic reactions

	Syntrophic acetogenic reactions
1	Propionate $C_2H_5COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$
2	Butyrate $C_3H_7COO^- + 2H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$
3	Propionate $C_2H_5COO^- + 2HCO_3^- \rightarrow CH_3COO^- + 3CHOO^- + H^+$
4	Butyrate $C_3H_7COO^- + 2HCO_3^- \rightarrow 2CH_3COO^- + 2CHOO^- + H^+$
5	Propionic acid $C_2H_5COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2$
6	Butyric acid $C_3H_7COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$
7	Butyric acid $2C_3H_7COOH + CO_2 + 2H_2O \rightarrow CH_4 + 4CH_3COOH$
8	Glucose $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$
	Homoacetogenesis
9	$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$

Reviewed in (Nie et al. 2007; Oude lferink et al. 1998; McCarty and Smith 1986)

reduce CO_2 are known as hydrogenotrophic (Demirel and Scherer 2008; Whitman et al. 2006). The formate molecules can also be an important substrate, although its concentration in methanogenic environments is low, because it is rapidly produced and consumed (Demirel and Scherer 2008). In the second group, the substrates are a variety of compounds containing methyl groups, which are reduced to methane (Ward et al. 2008; Whitman et al. 2006). Finally, in the third group, the synthesis of methane comes from an acetoclastic reaction, in which the methyl carbon of the acetate is reduced to methane and the carboxyl carbon is oxidized to CO_2; the ability to catabolize this substrate is limited to the *Methanosarcina* and *Methanosaeta* (*Methanothrix*) species (Whitman et al. 2006).

Conversion of acetate to methane is the limiting step in the production of biogas, because methanogenic microorganisms have a low growth rate, which results in a relatively low population size. Under normal conditions, about 70% of methane is formed by acetoclastic methanogens and the remaining 30% by hydrogenotrophs.

Factors Influencing Anaerobic Digestion

Temperature

It influences digestion of the substrate, the growth, and metabolism of microorganisms. Anaerobic digestion can be performed in three temperature ranges: psychrophilic (10–20 °C), mesophilic (25–45 °C), or thermophilic (45–65 °C). Thermophilic

Table 15.3 Stoichiometry of methanogenic reactions

	First group:
1	Hydrogen $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$
2	Formate $4CH_3COO^- \rightarrow CH_4 + 3CO_2 + 2H_2O$. This shows an acetate decarboxylation, not from formate. It should be $4HCOO^-$ *to* $CH_4 + 3CO_2 + 2H_20$.
3	2-propanol $4C_3H_7OH + CO_2 \rightarrow CH_4 + 4C_3H_6O + 2H_2O$
4	Ethanol $C_2H_5OH + CO_2 \rightarrow CH_4 + 2CH_3COO^-$
5	Methanol $CH_3OH + H_2 \rightarrow CH_4 + H_2O$
6	Methanol $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$
	Second group
7	Methylamine $4CH_3NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+$
8	Dimethylamine $2(CH_3)_2NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_4^+$
9	Trimethylamine $4(CH_3)_3N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_4^+$
10	Dimethyl sulfide $2(CH_3)_2S + 2H_2O \rightarrow 3CH_4 + CO_2 + 2H_2S$
	Third group
11	Acetate $CH_3COOH \rightarrow CH_4 + CO_2$

Reviewed in (Whitman et al. 2006; Demirel and Scherer 2008)

conditions have different benefits: increase the solubility of organic compounds, make faster biological and chemical reactions, and increase the death of pathogens (Appels et al. 2008; Sung and Liu 2003).

pH and Alkalinity

Different anaerobic microorganisms have different pH ranges for growth and metabolism, and methanogenic archaea are extremely sensitive to changes in pH; their optimum values are between 6.5 and 7.2. On other hand, fermentative bacteria are less sensitive and can function in a wider range between 4.0 and 8.5 (Appels et al. 2008). As mentioned above, the limiting group for biogas production is aceto-clastic methanogens, so it is necessary to keep pH neutral for successful anaerobic digestion (Moosbrugger et al. 1993).

Two factors associated with pH are the alkalinity of the system and the production of volatile fatty acids (VFAs). During the production of VFAs in anaerobic

digestion, the pH tends to decrease. This fall in pH can be prevented by methanogenic microorganisms that produce compounds with buffer capacity such as carbon dioxide, ammonium, and bicarbonate (Appels et al. 2008).

Toxicity

A wide variety of substances inhibit microbial growth or have negative effects on anaerobic digestion and decreased methane production and fatty acid accumulation. The degree of inhibition/toxicity is variable mainly due to different interactions among anaerobic microbes (e.g., antagonism or synergism). Some common inhibitors for anaerobic digestion are long-chain fatty acids (LCFAs), ammonia, nitrogen, and sulfate.

LCFAs are surface-active compounds that in aqueous systems behave like synthetic surfactants. The nonionized form of LCFAs bind to the microbial cell surface and are then absorbed into the cell. Subsequently, acyl-CoA synthetase activates LCFAs, which are then degraded with a sequential removal of two-carbon units (via beta-oxidation), with acetate as the product. However, a high concentration of LCFA is inhibitory. LCFAs show acute toxicity toward anaerobic consortia by adsorption onto the cell wall/membrane, interfering with the transport or protective functions of the cell wall (Salminen and Rintala 2002; Chen et al. 2008). On the other hand, various substances, including albumin, starch, bile acids, and cholesterol, may reduce the toxicity of LCFAs due to the formation of complexes or competitive adsorption on the cell wall. Bentonite and calcium are also substances that may prevent inhibition by LCFA since they promote calcium-LCFAs precipitation (Salminen and Rintala 2002).

Ammonium cations (NH_4^+) and free ammonia (NH_3) are the two predominant forms of inorganic nitrogen in anaerobic digestion; they are produced during the degradation of proteins and urea, and both are toxic. Free ammonia is more toxic because it passes through the cell membrane of microorganisms, causing proton imbalance and potassium deficiency (Appels et al. 2008; Chen et al. 2008).

Concentrations around to 200 $mg.L^{-1}$ of ammonia are beneficial for the anaerobic digestion process, because nitrogen is an essential nutrient for anaerobic microorganisms. The concentration of total ammoniacal nitrogen (TAN) causing 50% reduction in methane production is in the range of 1700–14,000 $mg.L^{-1}$. The difference between these concentrations can be attributed to the variety between substrates and inoculum, environmental conditions (temperature and pH), and periods of acclimatization (adaptation of methanogenic microorganisms to high concentrations of ammonia) (Chen et al. 2008).

Sulfate is commonly found in many wastewaters. Under anaerobic conditions, sulfate is used as an electron acceptor and is reduced to sulfide by sulfate reducing bacteria (SRB). Sulfate reduction inhibits methanogenesis at two stages of the process: at the first stage, by competition for organic or inorganic substrates, which decreases methane production, and second stage, by the toxicity of sulfide on several microbial groups (Appels et al. 2008; Chen et al. 2008).

SRB can metabolize a large number of substrates such as alcohols, organic acids, aromatic compounds, and long-chain fatty acids. Similarly, they compete with fermentative and acetogenic bacteria or methanogenic archaea for acetate, H_2, propionate, and butyrate. Normally, inhibition through competition does not occur in the hydrolysis because SRBs are not capable of degrading biopolymers; therefore, they rely on fermentative microorganisms to degrade these compounds and metabolize degradation products. On the other hand, acetogenic and methanogenic microorganisms are affected by the presence of SRB because they compete for the same fermentation products (Appels et al. 2008). In anaerobic systems, methanogens and syntrophic bacteria that degrade propionate are generally more sensitive to sulfide inhibition (Cirne et al. 2008).

Advantages and Disadvantages of Anaerobic Treatment

Anaerobic digestion has several advantages over other types of organic waste treatment (Claassen et al. 1999; Pipatmanomai et al. 2009) among which are:

- *High efficiency*. Good removal efficiencies are achieved in these systems, even at high organic loads and low temperatures.
- *Simplicity.* The construction and operation of reactors for anaerobic digestion are relatively simple.
- *Flexibility.* Anaerobic treatment can easily be applied on a small or large scale.
- *Less space requirement.* The spaces are smaller compared to conventional systems.
- *Low production of sludge.* The amount is low compared to aerobic treatment technologies due to the growth rates of methanogens. In addition, this sludge can be stored for a long time without a significant reduction of activity; so it can be used as inoculum to start-up new reactors.
- *Low energy consumption.* Unless the influent of the reactor needs to be heated, the energy consumption is minimal. The energy is recovered as biogas. Its physical and chemical properties are similar to natural gas and can be used for heating, lighting, production of electricity, or replacing of diesel or gasoline in transport vehicles (Fig. 15.3).

The main disadvantages of anaerobic digestion are:

- *Low removal of pathogens and nutrients.* The pathogens and nutrients are partially eliminated; a refining treatment is necessary.
- *Possible long start-up period*. When inoculum is not adequate, start-up in the anaerobic reactor may require a longer time than start-up in an aerobic one, mainly due to the low growth rate of methanogenic microorganisms.
- *Possible bad smell.* Hydrogen sulfide (H_2S) is produced during the anaerobic process, especially when there is a high concentration of sulfate in the influent.

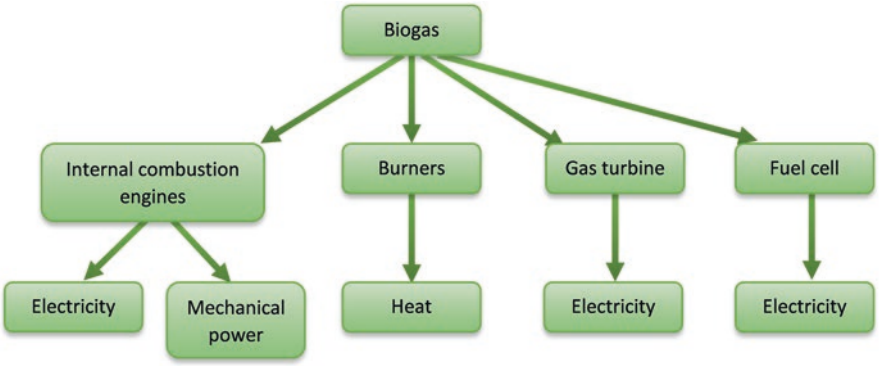

Fig. 15.3 Main uses of methane

Current Technologies for Biogas Production from Lignocelluloses

Numerous technical solutions exist for anaerobic digestion of lignocellulosic sources (Table 15.4). One of the most important parameters for considering in the selection of the best technique is total solid (TS) concentration. Anaerobic digestion can be classified as wet, semidry, and dry when TS of substrate are <10, 10–15, or >15%, respectively (Zhang et al. 2018). Wet anaerobic digestion (W-AD) is the most common technology used in facilities dedicated to continuous treatment of liquid inputs, or solid in a liquid mix. W-AD generates low level of sludge, is easy in operation and maintenance, and acceptable methane production yield per unit mass of organic material is fed to a digester (Chiumenti et al. 2018; Li et al. 2013). Thus, W-AD is widely applied to treat lignocellulosic sources such as livestock and poultry manure, food waste, and energy crops (Zhang et al. 2018). The most common reactor in W-AD is a vertical continuously stirred tank reactor (CSTR) that works in a single-stage digestion; however, many treatment plants have two-stage processes where the second stage digester can be a CSTR, up-flow anaerobic sludge blanket (UASB), fixed film, or other reactor types. In a two-stage system, the hydrolytic-acidogenic stage is separated from the methanogenic stage, overcoming the drawbacks of a single stage system. Two-stage systems can exhibit increased process stability, aiding in processing substrates with high organic load and increasing the methane yield (Li et al. 2020).

However, for high solid content wastes, such as agricultural crop straws, dry anaerobic digestion (D-AD) is a better choice (Zhang et al. 2018). Advantages of D-AD technology include lower pretreatment of feedstock, reduction of size in the case of organic waste, and removal of inert materials is not necessary (as needed in W-AD to prevent clogging of pumps, pipes, or over accumulation in the digesters). Additionally, in the case of solid inputs, W-AD normally requires dilution with water, liquid inputs, or digestate recirculation (Bhatia et al. 2018). D-AD processes

Table 15.4 Anaerobic digestion of different lignocellulosic sources

Substrate	Operating conditions	Biogas production	Methane concentration (%)	Methane yield	Conclusions	References
Corn Stover (CS) and chicken manure (CM)	W-AD in glass bottles CS is mixed with tap water and inoculum (sludge of municipal wastewater treatment plant) with total solids of 5.1–5.6%	0.1 L-CH_4. $L_{reactor}^{-1}$	14.4	0.3 mL.g^{-1} VS_{added}	Codigestion improved methane yield compared with using CS or CM alone under W-	Li et al. (2013)
	D-AD in glass bottles CS is mixed with tap water and inoculum (sludge of municipal wastewater treatment plant) with total solids of 20.1–22.4%	0.2 L-CH_4. $L_{reactor}^{-1}$	14.8	0.2 mL.g^{-1} VS_{added}	AD and D-AD conditions. D-AD registered higher methane production compared with W-AD	
	W-AD in glass bottles. CM is mixed with tap water and inoculum (sludge of municipal wastewater treatment plant) with total solids of 5.1–5.6%	0 L-CH_4. $L_{reactor}^{-1}$	0	0 mL.g^{-1} VS_{added}		
	D-AD in glass bottles CM is mixed with tap water and inoculum (sludge of municipal wastewater treatment plant) with total solids of 20.1–22.4%	0.8 L-CH_4. $L_{reactor}^{-1}$	29.4	0.9 mL.g^{-1} VS_{added}		
	Codigestion in W-AD in glass bottles Both f raw materials were mixed with tap water and inoculum (sludge of municipal wastewater treatment plant) with total solids of 5.1–5.6%, CS:CM ratio 1:3	4 L-CH_4. $L_{reactor}^{-1}$	61.4	21.2 mL.g^{-1} VS_{added}		

(continued)

Table 15.4 (continued)

Substrate	Operating conditions	Biogas production	Methane concentration (%)	Methane yield	Conclusions	References
	Codigestion in D-AD in glass bottles. Both raw materials were mixed with tap water and inoculum (sludge of municipal wastewater treatment plant) with total solids of 20.1–22.4%, CS:CM ratio 1:3	13 L-CH_4. $L_{reactor}^{-1}$	64.8	8.5 mL.g^{-1} VS_{added}		
Cow manure and agricultural products (corn silage, triticale, ryegrass, alfalfa, and straw)	Codigestion in a full-scale dry anaerobic digestion plant was monitored for 478 days. Temperature in the reactor was set to 38 °C. Total solids of 21.9%	5000–11,000 m^3 per day	53.8–63	44–220 $m^3.t^{-1}$	The dry fermentation can potentially achieve similar biogas to W-AD. Relevant aspects include retention time, loading rate, and recirculation of digestate and leachate	Chiumenti et al. (2018)
Kitchen waste (KW) and fruit-vegetable waste (FVW)	Codigestion by two-phase anaerobic digestion. The TS of KW and FVW was adjusted to 10% before using. Ratio KW/FVW was 1:1. The acidogenic phase reactor was made out of a 2.5 L glass bottle, and the methanogenic phase reactor was an UASB; both reactors were performed at 30–33 °C	325 mL per day	na	na	The two-phase AD system with 50%KW can not only dispose more KW than the two-phase AD system with 25% KW but also have the better stability in MPR. Therefore, the results suggested that the 50% KW was the best ratio in this two-phase AD system.	Yang et al. (2013)

Switchgrass (W-AD)	W-AD of eight biomass feedstock was compared with D-AD. Effluent from a mesophilic liquid anaerobic digester fed with municipal wastewater sludge and food waste was used as inoculum In D-AD, each lignocellulosic biomass was mixed with inoculum to obtain TS content of 18–19%. The mixed materials were loaded into 1-L glass reactor and incubated at 37 °C In W-AD, each feedstock was mixed with deionized water and inoculum to obtain a mixture of 5% TS. The mixtures were loaded in 2-L glass jars and incubated at 37 °C	1.6 $L.L_{work}^{-1}$	na	111 $L.kg^{-1}$ $VS_{feedstock}$	No significant difference in methane yield between W-AD and D-AD except for wastepaper and pine were found. However, the volumetric productivity was two- to seven-fold greater in D-AD system compared with W-AD system, except for paper. Methane yields from corn Stover, wheat straw, and switchgrass were 2–5 times higher than those from yard waste, maple, and pine biomass. Pine had very low biogas yield, indicating the need for pretreatment prior to D-AD	Brown et al. (2012)
Switchgrass (D-AD)		12.3 $L.L_{work}^{-1}$	na	116.9 $L.kg^{-1}$ $VS_{feedstock}$		
Corn Stover (W-AD)		1.8 $L.L_{work}^{-1}$	na	124 $L.kg^{-1}$ $VS_{feedstock}$		
Corn Stover (D-AD)		14 $L.L_{work}^{-1}$	na	131.8 $L.kg^{-1}$ $VS_{feedstock}$		
Wheat straw (W-AD)		2 $L.L_{work}^{-1}$	na	139.1 $L.kg^{-1}$ $VS_{feedstock}$		
Wheat straw (D-AD)		12 $L.L_{work}^{-1}$	na	123.9 $L.kg^{-1}$ $VS_{feedstock}$		
Yard waste (W-AD)		1 $L.L_{work}^{-1}$	na	59.7 $L.kg^{-1}$ $VS_{feedstock}$		
Yard waste (D-AD)		6.2 $L.L_{work}^{-1}$	na	49.3 $L.kg^{-1}$ $VS_{feedstock}$		
Leaves (W-AD)		1.1 $L.L_{work}^{-1}$	na	81 $L.kg^{-1}$ $VS_{feedstock}$		
Leaves (D-AD)		6.4 $L.L_{work}^{-1}$	na	75.3 $L.kg^{-1}$ $VS_{feedstock}$		
Wastepaper (W-AD)		3.8 $L.L_{work}^{-1}$	Na	312.4 $L.kg^{-1}$ $VS_{feedstock}$		
Wastepaper (D-AD)		3 $L.L_{work}^{-1}$	Na	15.3 $L.kg^{-1}$ $VS_{feedstock}$		
Maple (W-AD)		1 $L.L_{work}^{-1}$	Na	57.2 $L.kg^{-1}$ $VS_{feedstock}$		
Maple (D-AD)		6.4 $L.L_{work}^{-1}$	na	46.9 $L.kg^{-1}$ $VS_{feedstock}$		
Pine (W-AD)		1 $L.L_{work}^{-1}$	na	54.3 $L.kg^{-1}$ $VS_{feedstock}$		
Pine (D-AD)		3.2 $L.L_{work}^{-1}$	na	17 $L.kg^{-1}$ $VS_{feedstock}$		

(continued)

Table 15.4 (continued)

Substrate	Operating conditions	Biogas production	Methane concentration (%)	Methane yield	Conclusions	References
Sewage sludge and rice straw	Dry codigestion under mesophilic anaerobic conditions. The inoculum was acclimated at 35 °C for 2 months. Dry codigestion was carried out in a batch reactor made of glass. Total solids of 20%. C/N ratio 26:1	2.0 L per day L	36–59	518 mL.g^{-1} VS	The treatment duration was reduced by 3 times from average 75 days under mesophilic conditions to average 25 days under thermophilic conditions, indicating that thermophilic dry codigestion can significantly improve the utilization efficiency of reactors	Chu et al. (2015)
	Dry codigestion under thermophilic anaerobic conditions. The inoculum was acclimated at 55 °C for 2 months. Dry codigestion was carried out in a batch reactor made of glass. Total solids of 20%. C/N ratio 26:1	12.2 L per day L	41–60	602 mL.g^{-1} VS		
Corn straw	Two-stage anaerobic digestion with pretreatment (hydrolysis). The hydrolysate was obtained by combined two-step pretreatment with dilute hydrochloric acid and celluloses. The acidogenic stage was performed in a CSTR reactor at 38 °C and an UASB was performed as the methanogenic reactor at 38 °C	na	na	270 mL.g^{-1} COD_{added}	The hydrolysate from lignocellulosic biomass was an excellent source for the methane production. VFAs produced in the acidogenic stage were appropriate for the subsequent conversion into methane	Li et al. (2020)

Wheat straw	Liquid hot water (LHW) pretreatment to enhance the anaerobic digestion. The inoculum used was obtained in a mesophilic biogas plant fed with slaughter wastewater. LHW was conducted to 0.4–2.5 Mpa and the final temperature was in the range 150–224 °C over 30 min. The AD was performed in bottles at mesophilic conditions and TS content of 3.2%	145 mL CH_4 per day	na	200 mL.g^{-1} VS_{added}	The increasing intensity of LHW pretreatment gradually broke the highly crystallized structure of wheat straw. The optimum condition appeared at 175 °C and 30 min, increasing the methane production until 62.9%	Shang et al. (2019)
	The AD was performed in bottles at mesophilic conditions and TS content of 3.2%	35 mL CH_4 per day	na	120 mL.g^{-1} VS_{added}		
Sugarcane bagasse	The anaerobic digestion was performed in serum bottles.	na	na	170 mL.g^{-1} $VS_{substrate}$	The maximum accumulative methane yield was obtained from the thermophilic microaerobic pretreated sugarcane bagasse and followed by the alkali pretreated sugarcane bagasse, which were 29.3% and 11.8% higher than that of untreated sample, respectively. As for total methane yield, TMP was more efficient than AP	Fu et al. (2015)
	Thermophilic microaerobic pretreatment (TMP) on anaerobic digestion. Inoculum used was anaerobic sludge from a wastewater treatment plant. Microaerobic pretreatment was carried out in 300 ml serum bottles injected the oxygen load of 10 mg/$VS_{substrate}$. The anaerobic digestion was performed in serum bottles	na	na	225 mL.g^{-1} $VS_{substrate}$		
	Alkali pretreatment (AP) on anaerobic digestion. Inoculum used was anaerobic sludge from a wastewater treatment plant. During the alkali pretreatment, the NaOH dose was 2% of substrate (TS) and the loading rate was 65 g/L (TS of sugarcane bagasse loaded per liter effective volume of digester). The anaerobic digestion was performed in serum bottles	na	na	190 mL.g^{-1} $VS_{substrate}$		

na not available

can be found as batch or continuous systems, but the batch mode is generally preferred since it is easier to operate and resembles the in-vessel composting process, familiar to waste managers and farmers. Notwithstanding many advantages, D-AD also has some disadvantages, which include long degradation times and potential accumulation of toxic and inhibitory compounds (e.g., VFAs, ammonia, and heavy metals) due to the high TS content (Rocamora et al. 2019).

In addition to the TS concentration, it is important to consider that lignocellulosic materials are recalcitrant to microbial digestion due to the complex structure of cellulose-hemicellulose-lignin. Furthermore, lignocellulosic biomasses have high organic load and low nitrogen content, making the anaerobic digestion process more difficult; high lignin percentage; high C/N ratio; and contamination with pesticides affect the anaerobic digestion. Most of these problems are overcome by the addition of a cosubstrate, and this process is called anaerobic codigestion. Codigestion of two or more organic materials enhances the biogas yield up to 25–400% in comparison with monodigestion of feedstock (Kainthola et al. 2019; Li et al. 2020).

As the complex structure of lignocellulosic materials is the rate-limiting step of anaerobic fermentation, pretreatment to destroy interlinks between lignin, cellulose, and hemicellulose is essential to improve biodegradability; agriculture residues with no pretreatment result in low biogas yield. Pretreatment methods include physical, chemical, biological, or combined strategies, and selection depends on the characteristics and structure of the biomass. The best strategy is the one that allows increasing biodegradable substrates without loss of matter during the process (Martínez-Gutiérrez 2018; Li et al. 2020). In recent years, special attention has been paid to the addition of conductive materials during anaerobic treatment with the aim of promoting direct interspecies electron transfer (DIET). This strategy has been shown to increase methane yield and remove VFAs due to the synergistic activity of microorganisms during the anaerobic digestion (Paritosh and Vivekanand 2019).

Enhancement in Biogas Production Using a Conductive Material DIET

Direct interspecies electron transfer (DIET) was reported for first time in 2014 when pili between *Geobacter* and *Methanosaeta* bacteria in coculture was observed (Rotaru et al. 2014). Not all exoelectrogenic bacteria are able to generate pili; however, the addition of conductive materials increases the genera of bacteria that are able to exchange electrons (Xu et al. 2019). DIET using stainless steel as a conductive support had a kinetic advantage 108-fold than *interspecies hydrogen transfer* (IHT) to compete for the electron donor.

Electron transport can be done by shuttle molecules such as hydrogen or formate. Traditionally, IHT and interspecies formate transfer (IFT) have been intrinsically linked with interspecies electron transfer (IET). However, problems with H_2 partial pressure and formate concentration have been reported. Recently, many investigations have paid attention to DIET, which has become widely accepted.

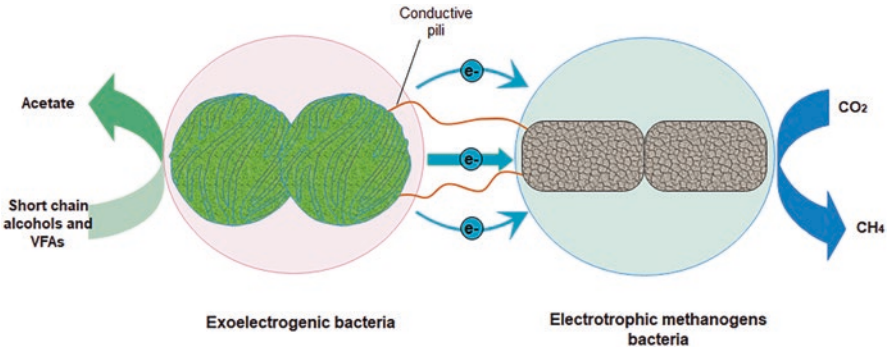

Fig. 15.4 Schematic representation of direct interspecies electron transfer (DIET) between exoelectrogenic and electrotrophic bacteria. This syntrophic association enables degradation of short chain volatile fatty acids (SCVFA) and alcohols in exoelectrogenic bacteria, and the pili is the biological bridge that allows the passage of electrons to electrotrophic methanogens, which use them to reduce carbon dioxide to methane

DIET is another form of IET. Exoelectrogenic bacteria and electrotrophic methanogens are the microbial partners in DIET (Fig. 15.4), and they establish syntrophic association to degrade short chain volatile fatty acids (SCVFA) and alcohols. Recently, granular-activated carbon (GAC) showed high performance in promoting DIET and improved methane production yields (Valero et al. 2018). GAC works as a support for microbial communities and as an electron conductor between exoelectrogenic bacteria and electrotrophic archaea. Compared with other conductive supports, GAC conductivity is significant higher (3600 $\mu S \cdot cm^{-1}$); meanwhile stainless steel conductivity is 667 $\mu S \cdot cm^{-1}$, magnetite 160 $\mu S \cdot cm^{-1}$, and biochar 5 $\mu S \cdot cm^{-1}$. GAC has large surface area, and it is a low-cost material, making it ideal to use as microbial support for further DIET-based biogas production (Valero et al. 2018).

Paritosh and Vivekanand (2019) investigated the effect of hardwood biochar on D-AD of wheat straw. Results showed that 10 g/L of hardwood biochar led to a two-fold increment in methane yield (223 $L.kg^{-1}$ VS) compared to the control (110 $L.kg^{-1}$ VS). Therefore, it is necessary to further investigate these new anaerobic digestion techniques for later application in the treatment of lignocellulosic residues to increase methane yields and biogas quality since carbon-based conducting materials for D-AD of agricultural stubble have not been explored.

Bioethanol

Since fossil oil is expected be exhausted within the next 40 or 50 years (Zabed et al. 2017; Zhao et al. 2018; Olguin-Maciel et al. 2019), introduction of bioethanol in the transport sector is considered a necessary strategy for reducing the dependence on fossil energy. Ethanol contains 35% oxygen, the reason why its combustion is ~15%

more efficient than gasoline, with lower emission of harmful gases. In addition, gasoline contains sulfur that contributes to sulfur oxide emissions to the atmosphere, causing acid rain, and is also known as a potential carcinogen. Ethanol contains negligible amount of sulfur, which is another advantage.

First- and Second-Generation Ethanol

The oil crisis of the 1970s caused countries with low oil reserves to turn to other sources of fuel for transport. Brazil was one of the first countries to propose the use of ethanol as an energy source creating the Proalcool program, and some years later, cars were able to use a blend of gasoline-ethanol. This was possible due to the important sugar-alcohol industry that developed in this country with an efficient technology. This industry was based on sugarcane as a primary crop, and the high sucrose productivity in varieties of this crop (up to 50% of culm dry biomass) made possible its exploitation at commercial scales (Moore 1995; de Souza et al. 2014). Some years later, the USA initiated its own program with the Energy Policy Act of 1978 that established a subsidy on bioethanol produced locally from corn grains or imported from other countries (Tyner 2008). Due to climate and land characteristics, corn is the most efficient cultivar in the USA for starch ethanol production. In 2018, ethanol production from corn starch in the USA attained 16.1 billion gallons (USDA 2019). Another crop with potential for ethanol production is sweet sorghum due to the fact that the process for ethanol production is similar to that of sugarcane. The processes to obtain ethanol from the feedstocks mentioned in this section are classified as first generation under the premise of energy security. Nowadays, sustainability criteria have gained more importance because they assess the real environmental, economic, and social impact of ethanol production.

Ethanol Sustainability

With the advent of climate change debates, the sustainability of renewable energy sources is being revised. Nowadays, energy security is being displaced as the main reason for the use of biofuels in the energy matrix of a country needing to lower GHG emissions. The use of ethanol made from biomass is considered carbon neutral because as the biomass grows, it absorbs CO_2 from the atmosphere, which will offset the CO_2 produced when the ethanol is burned (Bhatia et al. 2012; US EIA 2019). The use of 10% ethanol blends can reduce greenhouse gas emissions by 12–19% (Gronchi et al. 2019). In the last decade, the sustainability of ethanol production and its use as a fuel have been well documented (Goldemberg et al. 2008; Hattori and Morita 2010).

A net energy balance and a life cycle analysis are key procedures to assess the sustainability of a given process. In the case of fuel ethanol, these analyses showed that in order to improve the energy efficiency, the use of fossil fuel as an energy

source should be avoided. Instead, the combustion of residual biomass is advised (cogeneration). The higher impacts to the environment are due to crop cultivation. The negative effects of land use, inorganic fertilizers, and water requirements should be lowered. The use of residual biomass is one approach to lower impact on environment. Cellulosic feedstocks include grasses, fast-growing trees, sawdust, and stalks. Nonconventional starch sources can also be used. Finally, the use of organic fertilizers and pesticides is encouraged.

Ethanol recovery by distillation is highly energy demanding as a great volume of liquid has to be heated to separate ethanol from water in a mixture where water accounts for approximately 90% of total volume. Still, this water has to cool to be disposable. As mentioned above, one measure already implemented at large scale is the use of residual biomass as energy source. The use of other renewable sources, and in this case of solar energy, should be studied in order to lower the use of fossil fuel in the ethanol recovery step. Ethanol produced from lignocellulosic biomass is classified as second-generation ethanol. The challenges that need to be overcome to obtain the fermentable sugars necessary to ethanol production are discussed in the "Lignocellulosic Biomass and Chemistry of Biomass" section, and they are similar to those descriptions for the "Biogas Production Under Biogas" section.

In their work, Aguilar-Sánchez et al. (2018) analyzed different scenarios for ethanol production from sweet sorghum juice (first-generation ethanol) and stalks (second-generation ethanol). The best scenario in terms of energy efficiency is the direct fermentation of sweet sorghum juice (from the stalks), rich in fermentable sugars, and the use of stalk biomass for cogeneration by its combustion. Positive net energy ratios of 1.1 and 1.9 were attained when stalks were used for cogeneration using a steam turbine or a combined cycle process, respectively.

The alkaline-oxidative pretreatment of the stalks for lignocellulose breakdown resulted in the higher energy demand and the worst environmental performance step. This indicates that for lignocellulosic biomass, it is important to develop more environmentally friendly pretreatments, which can be attained by the use of biological pretreatments. The enzymatic breakdown of lignocellulose can be carried out at relatively low temperatures (less energy needed) and mild pH values (less toxicity of residues). The formation of inhibitors is also prevented due to near ambient temperatures applied during the pretreatment. Finally, the search of more efficient enzymes to deconstruct the lignocellulosic matrix is the subject of new studies all around the world.

One last topic in ethanol production is economic viability. As energy from fossil fuel origin is still extensively used, the production costs of ethanol depend on international oil prices. This is more obvious in the case of lignocellulosic ethanol due to the additional, high-energy demand, pretreatment process. In order to guarantee the use of ethanol (or any biofuel) in the energy matrix of a country, public policies have been implemented. The mandatory use of gasoline/ethanol blends is accompanied with a well-designed legal framework, which includes incentives to producers, and norms that ensure the quality of the blends. More than 64 countries have participated in various programs to use gasoline/bioethanol blends. Sugarcane and corn starch are the main ethanol feedstocks, but the increase in global demand pushes for the use of other biomasses (Zhao et al. 2018; Olguin-Maciel et al. 2019; Gronchi et al. 2019).

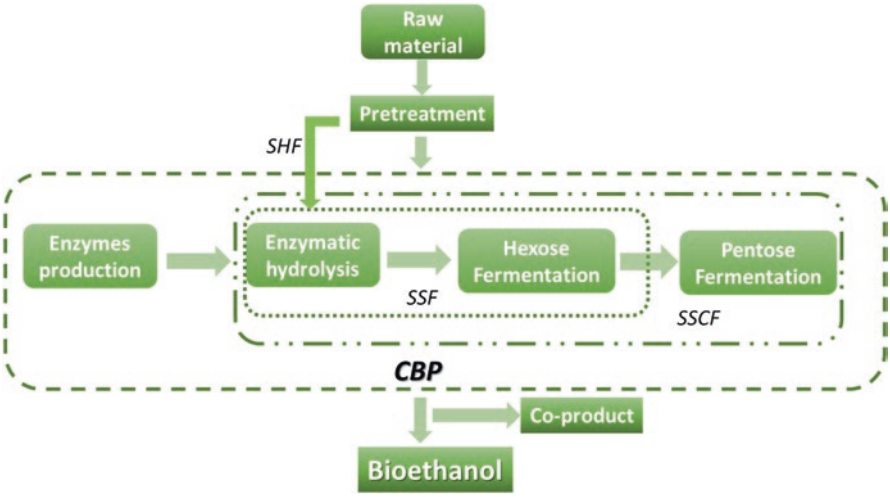

Fig. 15.5 General process for ethanol production from biomass. SHF, separate hydrolysis and fermentation; SSF, simultaneous saccharification and fermentation; SSCF, simultaneous saccharification and cofermentation; CBP, consolidated bioprocessing

It is important to mention that the chain value of ethanol production needs to be continuously improved. The search for cultivars with higher productivities is important in the case of energy crops already in use to lower production costs. To minimize adverse ambient impacts, the use of fossil energy should be replaced by renewable energy and in the case of lignocellulosic ethanol, it is necessary to develop new (chemical or biological) pretreatment procedures with low energy demand and low ambient impacts.

The work reported by Velázquez-Valadez et al. (2016) is an example of the efforts made toward the establishment of sustainable pretreatments. An alkaline-oxidative pretreatment of residual agave biomass was carried out with 82.6% lignin removal. It was followed by hydrolysis with an enzyme mixture where synergetic enzymes enhanced the saccharification; RS yield was 29% higher in comparison with the use of individual enzymes. The reaction medium from the pretreatment step was only neutralized and substituted by citrate buffer solution without washing the biomass, hence avoiding the use of water, in an effort to improve sustainability.

Strategies to Produce Bioethanol

Recent advancements in bioethanol production mainly focus on the improvement of technology to increase ethanol yield per unit of biomass and to reduce cost to make its production more efficient and economically viable. Figure 15.5 shows the general pro-

cess for bioethanol production. Different strategies for bioethanol production exist, depending on using the different stages independently, or in combination.

SHF (Separate Hydrolysis and Fermentation)

Separated hydrolysis and fermentation (SHF) is the conventional method for bioethanol production. In this configuration, the enzyme production, hydrolysis of biomass, and hexose and pentose fermentation are carried out in separate reactors. This allowed optimum conditions for hydrolysis and fermentation, but accumulation of sugars inhibit the enzymatic hydrolysis and reduce efficiency (Parisutham et al. 2014; Dahnum et al. 2015; Devarapalli and Atiyeh 2015).

SSF (Simultaneous Saccharification and Fermentation)

The disadvantages of SHF led to the development of the simultaneous saccharification and fermentation (SSF) process. In SSF, hydrolysis and fermentation are carried out simultaneously in a single reactor; enzyme and yeast are put together and sugars are immediately consumed, decreasing the inhibition of glucanases and β-glycosidases (Vohra et al. 2014; Dahnum et al. 2015; Devarapalli and Atiyeh 2015).

Sugars are much more inhibitory in the process than ethanol itself; thus, SSF can reach higher hydrolysis rates, yields, and ethanol concentrations than SHF. In addition, less equipment is required in SSF and it is easier in handling; moreover, the ethanol in the broth prevents the growth of undesired microorganisms (Fan 2014; Vohra et al. 2014; Dahnum et al. 2015; Devarapalli and Atiyeh 2015). The main challenge in SSF is to meet adequate conditions enabling hydrolytic activities and fermentation by microorganisms at the same time. Enzymatic hydrolysis is best at ~50 °C while most fermenting microbes need 28 °C–37 °C for ethanol production; likewise, optimal pH is different for enzymes and fermentative microorganisms (Vohra et al. 2014; Dahnum et al. 2015; Devarapalli and Atiyeh 2015).

Kluyveromyces marxianus has shown good performance in SSF. Many strains of this yeast grow well at 45–52 °C and can efficiently produce ethanol at temperatures between 38 °C and 45 °C. Moreover, *K. marxianus* offers additional benefits such as high growth rate and the ability to utilize a wide variety of sugar substrates (e.g., arabinose, galactose, mannose, xylose) at high temperatures (Bhatia et al. 2012; Blanch 2012; Vohra et al. 2014).

SSCF (Simultaneous Saccharification and Cofermentation)

In the simultaneous saccharification and cofermentation (SSCF) process, glucose and xylose released during the pretreatment and hydrolysis are cofermented in the same reactor. The use of a mixture of yeasts that assimilate both hexoses and pen-

toses has been proposed, but hexose-utilizing microorganisms grow faster, limiting the production of ethanol from pentoses. Although some microorganisms (e.g., *K. marxianus*) are capable of fermenting both types of sugars, efficient production of ethanol needs genetic modification of microorganisms already adapted to ethanolic fermentation (Bhatia et al. 2012; Vohra et al. 2014; Devarapalli and Atiyeh 2015).

CBP (Consolidated Bioprocessing)

Technologies for biofuel production tend toward consolidation; in recent years, the concept of "direct microbial conversion (DMC)" (Demain et al. 2005), currently known as "consolidated bioprocessing (CBP)", has emerged as an efficient method for saccharification and fermentation to produce ethanol and other organic acids (Sharma et al. 2018, 2019). CBP involves the integration of enzyme production, enzymatic hydrolysis, and fermentation, using a single type of microorganism in a single bioprocessing system. The integration of all these biological processes needed for ethanol production entails great potential to save capital and operational costs (Moreno et al. 2017; Kumar and Gupta 2018). Actually, considering investment capital for equipment, raw materials, yield of ethanol, and loss expenditures, estimation for CBP was \$0.04 gal^{-1} while for SSCF it was \$0.19 gal^{-1} (Parisutham et al. 2014; Vohra et al. 2014; Mbaneme-Smith and Chinn 2015).

The concept of CBP was proposed in 1996, and increasing evidence supports that CBP may be feasible. CBP research has focused on the development of new and even more effective CBP microorganisms, which has been a key challenge (Lynd et al. 2005; Okamoto et al. 2014).

Since the growth of the microorganism, hydrolysis, and fermentation are carried out simultaneously, and the greatest difficulty is finding the ideal conditions for all phases (Ali et al. 2016). To develop a microorganism suitable for efficient CBP, genetic engineering should render (1) fermentative capacity in a hydrolytic organism or (2) amylolytic or cellulolytic capacity to a fermentative organism. However, these strategies bring other difficulties since coexpression of multiple genes is not efficient, and some recombinant genes express poorly or with low activity. In addition, recombinant microorganisms show genetic instability, poor tolerance, and low productivity. Thus, the search for native organisms capable of performing a CBP is essential to increase the number of wild genes available for genetic manipulation and development of a good microorganism capable of performing CBP efficiently (Fan 2014; Ali et al. 2016).

Although CBP has many advantages in comparison with other processes, it has still many limitations: limited microbial growth, long fermentation periods, byproducts (e.g., organic acids), and meagre yields. Currently, almost all native microorganisms suitable for CBP of complex raw materials need nutrients or the addition of exogenous enzymes. Interestingly, Olguin-Maciel et al. (2019) reported that *Trametes hirsuta* Bm-2 strain is capable of producing ethanol from *Brosimum alicastrum* seed flour without pH adjustments and no exogenous enzyme or nutrient

addition. The amount of ethanol was low and needed optimization, but it was a promising result.

Strategies to Develop Microorganisms Suitable for CBP

The CBP strategy for bioethanol production requires an organism or organism consortium that is capable of synergistic and compatible production of hydrolytic enzymes and fermentation with a high yield of bioethanol. This organism can be developed via two strategies: the native and the recombinant strategy (Fan 2014; Ali et al. 2016). The microorganism should achieve rapid saccharification of biomass, use multiple simple sugars (e.g., glucose, cellobiose, xylose), and tolerate toxic compounds derived from pretreatment and fermentation (Fan 2014; Ali et al. 2016; Ábrego et al. 2017).

The native strategy of CBP makes use of microorganisms with a natural capability of deconstructing lignocellulosic biomass to fermentable sugars. The most studied CBP microbe is *C. cellulolyticum*. CBP microbial candidates are classified into three groups: fungi, free-enzyme bacteria, and cellulosome-forming bacteria (Olson et al. 2012; Zuroff et al. 2013; Nagarajan et al. 2019). Most of the work to identify CBP microorganisms has paid attention on anaerobic cellulolytic bacteria. However, native cellulolytic anaerobes have branched pathways that yield a wide variety of fermentation products in addition to ethanol. Thus, the major goal is to improve ethanol yield by deleting the branched pathways. Other goals include increasing CBP microorganisms' tolerance to ethanol and improving the use of hexose and pentose sugars. However, the CBP microorganisms identified so far are far short of expectations for efficient alcohol production (Olson et al. 2012; Zuroff et al. 2013; Fan 2014; Ábrego et al. 2017). One alternative strategy is the use of coculture of microbes or the use of microbial consortia, since it allows combining their specific metabolic capacities (Olson et al. 2012). A microbial consortium is a population composed of two or more strains, interacting with each another. Mixed strains achieve the tasks that are difficult or impossible for a single culture (Liu et al. 2018). A consortium can be a mixture of natural symbionts (natural consortia), mixtures of selected microbes (artificial consortia), or a mixture of native microbes with genetic engineering microorganisms (synthetic consortia) (Bernstein and Carlson 2012).

The recombinant strategy of CBP aims to endow fuel-producing microorganisms with hydrolytic activities. Therefore, prior to engineering microorganisms, it is important to select a host with the desired characteristics. The emphasis is on strains that can utilize low-cost substrates, have high product yield, competitive fitness, and are more robust to environmental stresses (Fan 2014; Ábrego et al. 2017). Once an eligible host is identified, then additional desirable characteristics that should be introduced by genetic engineering need to be defined – whether targeting particular enzymes, modifying enzymes by rational engineering, or changing the metabolic flux through metabolic engineering (Yan and Fong 2017) is necessary. The major challenges to overcome in recombinant strategies include regulating the expression of heterologous genes (to proper levels), adverse effects of the coexpression of mul-

tiple foreign genes on cell performance, improper protein folding, and no secretion of recombinant proteins (Fan 2014; Nagarajan et al. 2019).

Enzymes for Lignocellulose Biomass Deconstruction: Discovery and Production

The demand for novel enzymes with high process performance to replace traditional chemistry is increasing and predicts a market of more than 10 billion USD in 2024. Microbial enzymes are employed in almost all industries, that is, pharmaceutical and food industries, manufacturing of pulp and paper, soap and detergent, textile, and biofuels. Microorganisms represent the largest proportion of biomass on Earth, and environmental analysis of microbial communities currently estimates that only from 0.1% to 1% of these microorganisms are known because most of them are not culturable using traditional microbiological methods (Culligan et al. 2014). Therefore, microorganisms remain key sources for obtaining new enzymes.

Enzymes for Lignocellulosic Biomass Deconstruction

Microorganisms that degrade cellulose can convert it into soluble sugars either by acidic or enzymatic hydrolysis. There are three main types of cellulases: (1) endoglucanases act internally in the cellulose chain by cleaving the β-1,4 bonds to release nonreducing ends; (2) exoglucanases remove the cellobiose from the nonreducing end of the cellulose chain; and (3) β D-glucosidases breakdown cellobiose and small cello-oligosaccharides into glucose molecules (Dabhi et al. 2014).

Microorganisms or a suitable cocktail of enzymes known as hemicellulases can hydrolyze hemicellulose, but the complexity of hemicellulose typically requires the action of a broad set of enzymes such as endoxylanases, endomannanases, xylosidases, glucosidases, arabinosidases, galactosidases, mannosidases, and glucuronidases (López-Mondéjar et al. 2016). Hemicellulases are produced by many species of bacteria and fungi as well as several plants. Several hemicellulases, including xylanases and mannanases, have been identified in *Trichoderma reesei*, and most commercial hemicellulase preparations are produced by genetically modified *Trichoderma* or *Aspergillus* strains (Mussatto and Teixeira 2010).

The structure of lignin is mainly composed of phenolic and nonphenolic residues. Some fungi and bacteria secrete enzymes to metabolize lignin. These enzymes comprise lignin peroxidases, manganese peroxidases, and laccases. These glycoproteins contain heme groups that require hydrogen peroxide as an oxidant. Lignin peroxidase degrades lignin to nonphenolic units. Manganese peroxidase acts on lignin with phenolic and nonphenolic units through lipid peroxidation reactions. Manganese is oxidized, which in turn oxidizes the phenol rings to phenoxy radicals,

leading to the splitting of the compounds (Kantharaj et al. 2017; Madadi and Abbas 2017).

High levels of fermentable sugars from lignocellulosic biomass require the progressive, synergetic, noncompeting action of ligninases (ligninolytic enzymes as laccases), hemicellulases (e.g., xylanases, mannanases, and arabinases), and cellulases (i.e., endoglucanases, exoglucanases, cellobiohydrolases, and β-glucosidases).

Strategies to Produce Lignocellulolytic Enzymes

Screening of Lignocellulolytic Microorganisms

Since ancient times, microorganisms have been used to obtain products useful to mankind even if the people did not fully understand how, and one example of this is obtaining alcoholic beverages by Sumerians and Babylonians approximately 6000 years BC. With the development of methodologies, increasingly sophisticated instruments and, above all, the industrial revolution that led to the demand for food, drugs, and other products of commercial interest, enzymes were discovered.

Microorganisms can produce enzymes in a short time, in large quantities, and with the possibility of modifying enzyme production by genetic manipulations. In recent times, new strains, with high capacity to degrade xenobiotic compounds (derived from modern industrial processes), have been isolated from hostile environments such as extreme pH, salinity, temperature, or in the presence of toxic compounds (Anbu et al. 2016).

The demand for nonpolluting fuels, bioremediation of pollution, global warming, and the wide availability of plant material wastes (Dashtban and Qin 2009) has led to the search for free microorganisms, microbial consortia, or enzymes useful for the degradation of raw materials (Gonçalves de Siqueira et al. 2010; Kausar et al. 2010; Dabhi et al. 2014). Many fungi secrete several lignocellulolytic enzymes of synergistic action, contributing significantly in nature to the breakdown of lignocellulosic residues. The detection of activity in agar plates is the most common test for screening by using substrates that, when degraded, form halos that are easy to detect or develop color or fluorescence (Apolinar-Hernández et al. 2016; Berini et al. 2017; Canseco-Pérez et al. 2018). Screening microorganisms from sugarcane, bananas, and other agroindustrial wastes have enabled isolation of microorganisms capable of degrading complex carbohydrates (Cuervo-Fernandez et al. 2007; de Lima et al. 1999; Dabhi et al. 2014) as well as growing in the presence of industrial contaminants. For example, Medeiros et al. (2000) screened fungi for carbohydratases. They compared the cellulolytic activities of two strains of *Trichoderma harzianum* (T4 and T6), one *Acrophialophora nainiana*, and one *Humicola grisea* var. thermoidea cultivated in liquid medium with banana cluster stems as the carbon source. They found that *H. grisea* showed the greatest enzymatic activities of β-mannanase, β-xylanase, β-xylosidase, α-arabinofuranosidase, and cellulase.

Frequently, the screening of microorganisms is carried out on the main sources available in the country. In Nigeria, Arotupin (2007) reported that bacteria, fungi, and yeasts collected in cassava wastes, and selected using starchy and carboxymethyl cellulose media, show high cellulase and amylase activities. The best microbial cellulase producers were *Aeroccocus viridans*, *Bacillus* sp., *Corynebacterium manihot*, *Aspergillus niger*, *Articulospora inflata*, *Geotrichum candidum*, and *Candida utilis*.

Kausar et al. (2010) in Malaysia isolated 49 fungal strains from various natural and induced composts derived from rice straw. Four isolates were selected by high growth rate, biomass production, and lignocellulolytic activities. Finally, they identified a lignocellulolytic consortium of *A. niger* and *Trichoderma viride* with fast and efficient composting of rice straw and conversion into value-added products. Similarly, in Brazil, Gonçalves de Siqueira et al. (2010) screened cotton agroindustrial wastes and isolated strains of *Aspergillus oryzae*, *A. terreus*, *Emericella nidulans*, *Penicillium citrinum*, *Fusarium verticillioides*, *Fusarium proliferatum*, and *Paecilomycesum lilacinum*. Characterization in liquid medium with cotton residues as a carbon source showed that *A. terreus* was the best, with activities of xylanase, mannanase, pectinase, endoglucanase, FPase, and avicelase.

Forests have also been a point of attention from which to screen lignocellulolytic microorganisms because forest biomasses are rich in lignin and largely recalcitrant to hydrolysis. In the Czech Republic, López-Mondéjar et al. (2016) studied the cellulolytic potential of forest soil bacteria. They isolated the cellulase-hyperproducer strains *Mucilaginibacter* L294, *Pedobacter* O48, and *Luteibacter* L214. To obtain comprehensive information of these bacteria, they sequenced their genomes and then sequenced their proteomes (from fungal growth on plant biomass and on microcrystalline cellulose). They found that *Mucilaginibacter* and *Pedobacter* synthesize cellulases and hemicellulases while *Luteibacter* produces cellulose-binding transglycosylases when grown on forest wastes as the carbon source.

Not only are lignocellulosic materials used as sources of microorganisms but also any biomass that is abundant in the region. In India, Devi and Kumar (2012) performed microbial screening using the manure of several animals (cow, elephant, horse, camel) for strains with cellulolytic activity. For screening, they used solid media containing cellulose, carboxymethylcellulose, and xylan, identifying one strain of *Trichoderma* sp. and one *Aspergillus* sp. with the highest activities.

In the literature, there are a large number of reports like the above descriptions. In summary, the large diversity of fungal and bacterial species has shown potential for lignocellulosic deconstruction. Microorganisms from agroindustrial residues or manure have a high capacity for degradation of complex compounds such as hemicelluloses or lignin. It is not a rule, but microorganisms from a particular source are usually good for hydrolyzing that material. Then regional screening of lignocellulosic microorganisms is recommended. The importance of the screening is to define the best fungal and bacterial strains that help in the use of biomasses.

Production of Microbial Enzymes: Solid-State and Submerged Fermentations

Early alternatives for recovering microbial enzymes were solid-state fermentation (SSF) and submerged/liquid fermentation (SmF). SSF (Fig. 15.6a) is a process carried out in the absence or near absence of water, with microorganisms growing on

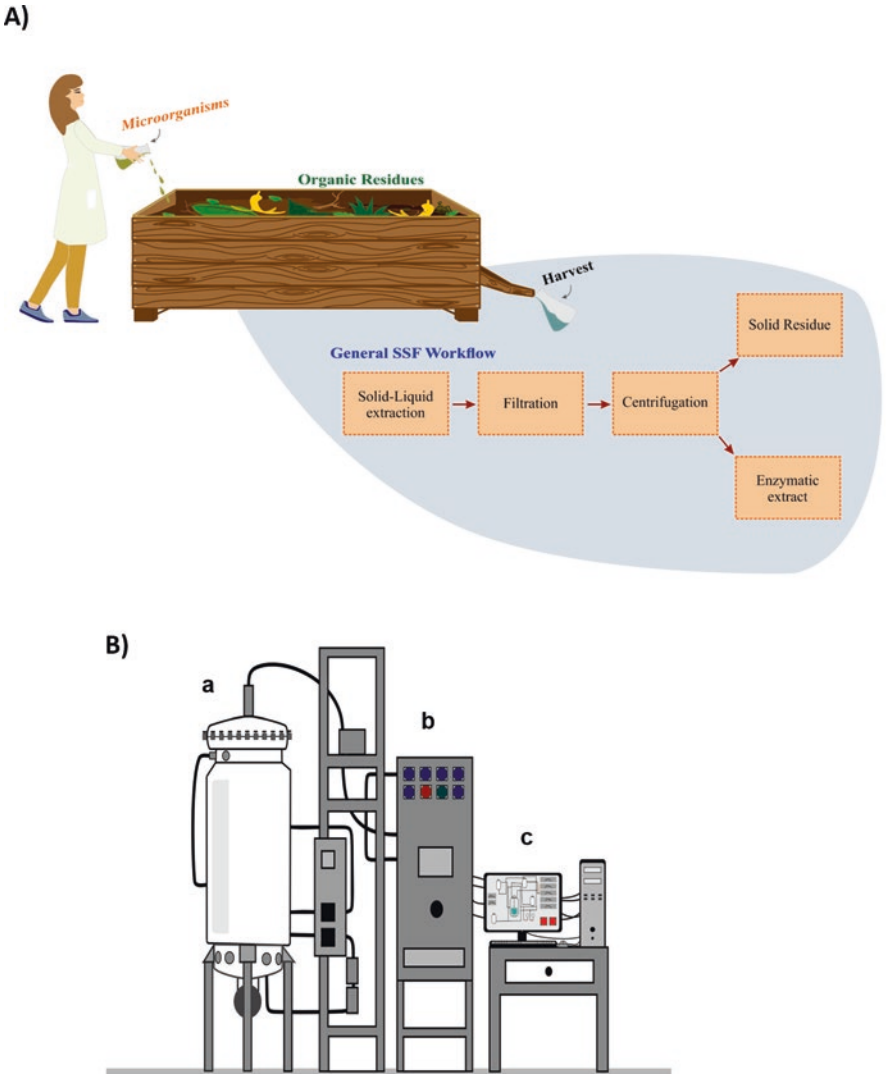

Fig. 15.6 Microbial production of enzymes under solid-state fermentation SSF (**a**) and submerged/liquid fermentation, SmF (**b**). In (**b**), the components of a liquid-phase bioreactor: (a) bioreactor; (b) pilot plant control panel; (c) SCADA pilot plant (Supervisory Control and Data Acquisition)

lignocelluloses or celluloses as nutrient sources. In this fermentation technique, the substrates are utilized very slowly, and microbes grow slower as well. The metabolism exhibited by microorganisms is different in SSF and SmF; enzymes and other products are commonly more concentrated in SSF than in SmF: eight-fold cellulase from *Bacillus* sp.; 50-fold esterase from *Aspergillus niger*; 16-fold cellulose from *Trichoderma viride*; 7.5-fold polygalacturonase from *A. niger* (Lizardi-Jiménez and Hernández-Martínez 2017). SSF is relatively a simple and cheap process that uses low-cost biomaterials with minimal or no pretreatment for bioconversion, similar to composting and ensiling. It has been exploited in Asian regions from ancient times, but attention is growing due to the increasing use of different types of organic wastes and the large list of added-value products that can be obtained (Yazid et al. 2017). Agricultural wastes such as bagasse, straw, stem, stalk, cobs, fruit peel, and husk have been raw materials for enzyme recovery. For example, Jatropha deoiled seed cake was used for the production of *Thermoascus aurantiacus'*s cellulases under SSF without any pretreatment. In this work, endo-β-1,4-glucanase (124.44 U/g) and β-glucosidase (28.86 U/g) were recovered, and their characterization showed maximum activity at 70 °C and pH 4. These cellulases were successful in the saccharification of sugarcane bagasse (Dave et al. 2012). The report from Dabhi et al. (2014) is another example of enzyme production by SSF. These authors used the bacterial consortia of *Cellulomonas cartae*, *P. fluorescence*, *P. putida*, and *Bacillus megaterium* to produce cellulolytic enzymes such as endo-1,4- βD- glucanase, exo-1,4- βD-glucanase, and β-glucosidase on banana residues. They found the greatest peaks of enzymatic activities between 20 and 25 days. These works support the potential of SSF in lignocellulose enzyme production.

However, more than 75% of the large-scale production of industrial enzymes is produced using SmF (Fig. 15.6b), because it better supports the utilization of genetically modified organisms and the control of multiple parameters that influence the production of enzymes is easier in SSF. Any slight deviation from the specified parameters will result in an undesirable product, and SSF has limited reproducibility of results (Lizardi-Jiménez and Hernández-Martínez 2017). But SmF faces challenges too including the use of water-based medium, maintenance of a homogenous environment with good distribution of nutrients and oxygen, and control of pH and temperature, and the capital investment required for SmF is about 78% more than that for SSF. Then, workers choose the mode of fermentation according to their purposes and applications.

There are different alternatives for the hydrolysis of lignocellulosic biomasses by SmF, for example, to grow the selected lignocellulolytic microbe on the raw material, or adding consortia of microorganisms, or cell-free extracts (a cocktail of enzymes). The selection of microorganisms is important to prevent the antagonistic action of microbes or enzymes. For example, Mena-Espino et al. (2011) cultured *Pleurotus ostreatus* and *Phanaerochaete chrysosporium* on banana waste as the only carbon source and used the crude cellulolytic-ligninolytic enzymatic extracts for the saccharification of banana stem. Both extracts were added, independently, on

the pretreated material, which contained 42.4 gL^{-1} of reducing sugars (RS). Surprisingly, although *P. ostreatus*'s cellulolytic activity was almost half (17,777.78 UL^{-1}) compared to *P. chrysosporium*'s (31,296.30 UL^{-1}), the latter extract produced a decrease in RS (to 2.27 gL^{-1}), and meanwhile the former extract produced an increment in RS to 63.65 gL^{-1}. The probable explanation is that *P. chrysosporium*'s extract contained 5.6-fold of manganese peroxidase and 38-fold lignin peroxidase activities in comparison with the other extract, and these enzymes require H_2O_2. *P. chrysosporium* secretes glucose oxidase that consumes glucose for H_2O_2 production. Although the *P. chrysosporium* strain secreted more cellulolytic activity, its glucose oxidase and peroxidases act antagonistically to the goal of recovery of RS in the saccharification.

Searching for Lignocellulolytic Enzymes in the Postgenomics Era

Genomics

Industrially important enzymes are derived from a small group of bacterial and fungal strains, primarily *Bacillus subtilis*, *Bacillus licheniformis*, *Candida antarctica*, *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma* sp. However, the catalogue of enzymes for industrial demands is still insufficient. Thus, the search for new biological sources of enzymes is still continuing. One effective strategy is to collect microbes from different environmental niches and screen their ability to hydrolyze selected substrates. The detection of activity by the development of halos or color on selected substrates in agar plates is the most common test. However, this procedure is a time-consuming task and takes a few years for the sampling of microorganisms for gene identification and enzyme characterization.

The costs of sequencing a microbial genome is decreasing rapidly: a high-quality draft now costs less than $100, and a finished genome sequence less than $500. This has enabled the rapid generation and release of genome sequences, including microorganisms from diverse environments. The Microbial Genome Database contains 6318 complete genomes, including 5861 bacteria, 254 archaea, and 203 eukaryotes (Uchiyama et al. 2019), meanwhile the 1000 Fungal Genomes Project (http://1000.fungalgenomes.org) reports more than 1000 genomes. Bioinformatics tools allow biotechnologists to make predictions on unknown gene function and find novel and better enzymes. The first data mining was carried out by homology-based approaches, using Blast scores, but inherent limitations of this strategy led to the development of nonhomology-based methods with more sensitive and precise algorithms (Luo et al. 2012; Uchiyama et al. 2019). In parallel, protein databases such as Pfam (https://pfam.xfam.org/), UniProt (https://www.uniprot.org/), RCSB-PDB (https://www.rcsb.org/), and BRENDA (https://www.brenda-enzymes.org/) are continuously growing. Likewise, web servers or programs for protein structure

modeling have been developed, for example, HHpred (https://toolkit.tuebingen.mpg.de/tools/hhpred), HMMSTR/ Rosetta (http://www.bioinfo.rpi.edu/~bystrc/hmmstr/server.php), Robetta (http://robetta.bakerlab.org/), SWISS-MODEL (https://swissmodel.expasy.org/), among others, and these tools help in the prediction of novel enzymes.

Survey of the genomes of acid-tolerant thermophilic lignocellulolytic fungi (*Myceliophthora thermophila, Thielavia terrestris, Thielavia heterothallica, Chaetomium thermophilum, Thermomyces lanuginosus, T. thermophilus, Rhizomucor miehei, Talaromyces cellulolyticus and Malbranchea cinnamomea*, *Acidothrix acidophila, Acidomyces acidophilus* and *Hortaea acidophila*) highlighted *Thielavia terrestris* as harboring the largest number (473) of carbohydrate-active enzymes among these fungi, a similar number to the well-known cellulase producer *Trichoderma reesei*. *T. terrestris* and other species of this genus exhibited faster growth on rice straw, secreted a wider range of enzymes, showed high enzymatic activity, thermostability, and stability under acidic conditions, consistent with predictions from genomic results (Thanh et al. 2019). In addition, comparison of the genomes of lignocellulose-degrading thermophilic fungi to related mesophiles reveals that thermal adaptation includes a reduction of the genome size and an increased frequency of the amino acids Ile, Val, Tyr, Trp, Arg, Glu, and Leu (IVYWREL) in proteins.

Fungal endoglucanases are the major source of enzymes for industrial applications. In the genome of *Aspergillus fumigatus*, 18 different genes encoding endoglucanases have been identified. One of them, belonging to the GH7 family and predicted with a molecular weight of 48.19 kDa and isoelectric point of 5.03, was heterologously expressed in *Kluyveromyces lactis*, resulting a thermostable enzyme (optimum at 60 °C, but 40% active at 80 °C) with catalytic activity in a pH range from 4.0 to 8.0, suitable for the paper industry and production of biofuels (Rungrattanakasin et al. 2018). Likewise, searching for laccases, the analysis of the whole genome of *Pseudomonas putida* retrieved eight different laccase-like gene sequences, one of them with the motif MTHHSED on the N terminus. The enzyme was expressed in *E. coli* Rosetta (DE3), and the recombinant protein exhibited stability over a broad temperature and pH range and good potential in the degradation of synthetic textile dyes (Mandic et al. 2019). These are only few examples, but the number of reports of genome-wide identification of enzymes is impressive, contributing to the rapid identification of new enzymes.

Metagenomics

In the last two decades, the development of culture-independent methods based on collecting biological material from environmental samples (metagenomics) has revealed the potential of unculturable microorganisms as sources of novel enzymes. The metagenomics approach involved the construction of DNA libraries, cloning the DNA in plasmids (for small inserts), fosmids and cosmids (~40 kb), and BAC

vector for large inserts (≥100 kb); fosmids and BAC inserts prevent the assembling of chimera genomes and enabled recovering of full-length genes and gene clusters, allowing the identification of the genetic context of genes (Berini et al. 2017).

The best strategy to search for enzymes in a genomic library is by functional screening. Functional screening allows discovery of new classes of enzymes that lack homologies to known sequences (Batista-García et al. 2016). The detection of activity in agar plates is still the most common test. A more sophisticated method for functional screening was described by Tan et al. (2014, 2016) who performed heterologous complementation to screen phytases from agricultural and garden soils. These authors found thermostable phytases with unusual sequences by growing the *E. coli* EPI300 clones on media with phytate as the sole phosphorous source. In other reports, heterologous complementation has been successfully used to find nucleases, RNAse H, and polyhydroxyalkanoate synthase (Ngara and Zhang 2018). However, for ease and lower cost, the earlier functional screening method is largely used to search lipases, esterases, proteases, and other hydrolases.

The other search is by sequence. Sequence-based screening is used to retrieve conserved catalytic domains by PCR reaction or hybridization. However, in the last decade, next-generation sequencing technologies became more efficient and cheaper and opened a new era of in silico screening. Direct sequencing and bioinformatics analysis of metagenomic DNA are enabling the discovery of new enzymes and bypassing the laborious steps of library construction. This strategy is rapidly triggering the finding of new enzymes, including largely divergent ones (Barriuso and Martínez 2015; Koutsandreas et al. 2019; Stewart et al. 2019; Zarafeta et al. 2016).

Berini et al. (2017) published a comprehensive review of searching by metagenomics of industrially relevant enzymes. DNA sources have been highly diverse and comprise among others soil, compost, insect gut, cattle rumen, hydrothermal springs, agriculture bagasse, and feces, and in much of these reports the DNA was cloned in libraries. The list of isolated enzymes includes endoglucanases, β-glucosidases, β-xylanases, α-rhamnosidases, amylases, chitinases, esterases, lipases, oxidoreductases, serine proteases, cysteine proteases, metalloproteases, phytases, etc.

The bulk of enzymes discovered by metagenomics to date are prokaryotic. The presence of large introns in eukaryotic genes makes the transcription and translation in heterologous hosts difficult and limits metagenomic analyses. In the last decade this was overcome through metatranscriptomics and metaproteomics (Bailly et al. 2007; Blank et al. 2018; Damon et al. 2012), and these technologies have been widely used in prokaryotes as well. But currently from these approaches, few industrially relevant biocatalysts were isolated: a cellulase (Takasaki et al. 2013) and a phosphatase (Kellner et al. 2011).

For biofuel production, metagenomics has mainly targeted the searching of enzymes for lignocellulose saccharification (Batista-García et al. 2016; Obeng et al. 2017), and some remarkable cellulases have been obtained. Some interesting results are now described. Garg et al. (2016) isolated one endoglucanase from a metagenome library constructed from soil (collected in the outer region of the Institute of Microbial Technology, Chandigarh, India). This enzyme is the most halotolerant

and halostable cellulase described so far, since it is able to maintain 100% activity after one-year incubation in 1 M NaCl, 90% activity in 2 M NaCl, 80% activity in 3 M LiCl, and 60% activity in 2 M KCl. The halotolerance arises due to the presence of acidic residues on the protein surface; in addition, the enzyme possesses four nonconserved cysteines, which are conserved, contributing to its thermal stability. In other reports three interesting metagenome-sourced endoglucanases were described by Yang et al. (2016); these enzymes are acidophilic (pH range 4.5–6.5), halotolerant (active in 5 M NaCl), and thermostable (60–70 °C). Lignocellulosic biomasses need to be pretreated prior to enzymatic hydrolysis, and salts widely used for lignocellulose pretreatment inhibit the activity of cellulases (specially the presence of chloride ions). Thus, these halotolerant and thermostable enzymes are very promising for lignocellulosic saccharification.

The last step in cellulose saccharification is mediated by β-glucosidases, which are prone to inhibition by glucose. Gomes-Pepe et al. (2016) described a β-glucosidase not inhibited by the product, and on the contrary, activity increased by 36.8% at 50 mM of glucose, and the enzyme was still 50% active at 350 mM of glucose. Another very attractive fact of this enzyme for the biofuel industry is its tolerance to ethanol, up to concentrations of 500 mM (the enzyme retains 86% of activity), while at 1 M ethanol it still retains 41% of residual activity. Thus, metagenome-based enzymes can contribute to overcome bottlenecks in lignocellulosic bioethanol production.

Esterases/lipases have been also extensively searched for using metagenomics (Borchert et al. 2017; López-López et al. 2014; Yan et al. 2017), and some of them show transesterification activity. Sahoo et al. (2017) reported the production of biodiesel using different waste vegetable oils with a thermostable lipase, resistant to many organic solvents (isopropanol, DMSO, methanol, DMF, ethanol, dichloromethane, acetone) isolated by metagenomics from Taptapani Hot Spring, Odisha, India. Likewise, Yan et al. (2017) discovered a lipase with transesterification activity from a hot spring field in Niujie, Eryuan, Yunnan province, China. The maximal biodiesel yield obtained was 350 mmol/L (at a molar ratio of methanol of 10.5:1) and 1% of water content. The enzyme still produces biodiesel at 5% of water content, but at 6% the competitive hydrolysis reaction is favored, and the amount of free fatty acids rapidly increased.

It is worth mentioning that some of the metagenome-sourced enzymes have been subjected to patents, such as a cellulase from bovine rumen (patent WO2014142529 A1) (Ko et al. 2013), lipase isolated from soil (patent CN103834626 A), and protease from compost (patent CN103409443 A) as well as a few carbohydratases suitable for biofuel production (Berini et al. 2017). Examples of commercial enzymes coming from metagenomic approaches are Pyrolase 160™-Diversa Corporation (San Diego, CA, USA), which is a β-mannanase from *Thermotoga maritime* cloned from DNA isolated from a deep sea hydrothermal vent (Parker et al. 2001); Phyzyme® XP-Danisco-Dupont (Marlborough, UK), which is a high thermostable phytase from *Escherichia coli* (Ravindran et al. 2006) used to improve digestibility of phytin-bound phosphorus, calcium, energy, and amino acids in monogastric animal diets; and Fuelzyme®—Verenium Corporation (San Diego, CA, USA), an

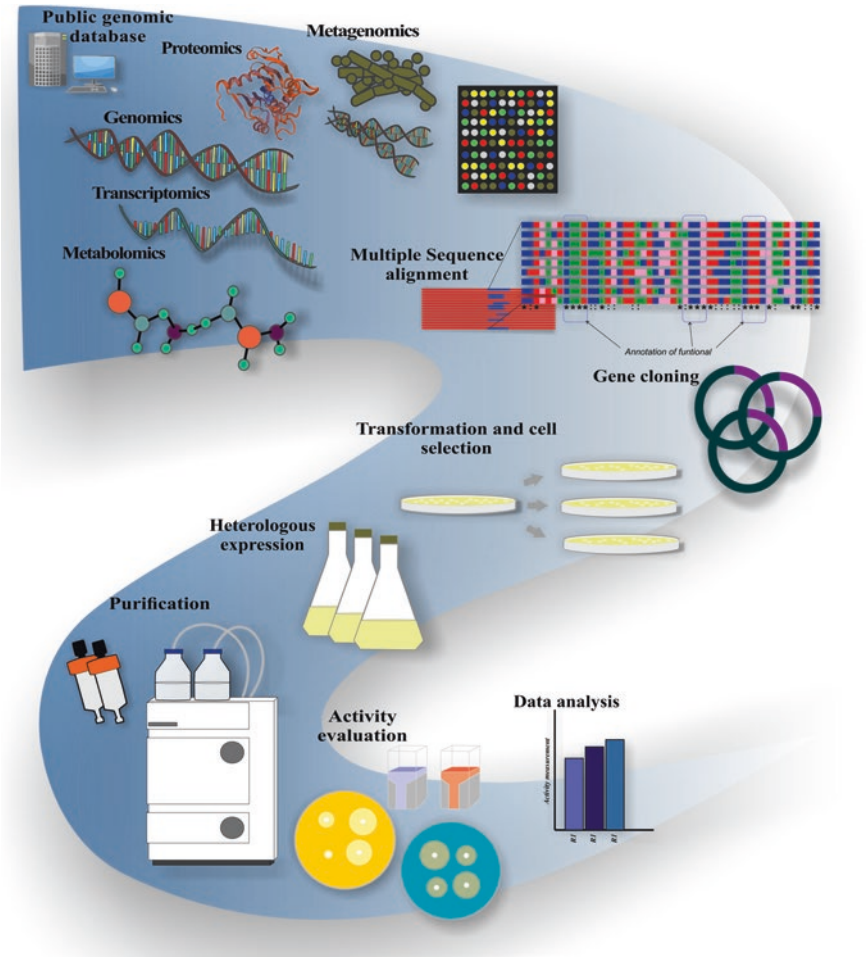

Fig. 15.7 Genomics and metagenomics strategies. Schematic workflow of modern strategies for discovering new enzymes

alpha-amylase produced by a *Thermococcus* sp. isolated from a deep sea hydrothermal vent (Dalmaso et al. 2015). This information highlights the relevance of metagenomics in the current discovery of novel enzymes with industrial applications. Figure 15.7 presents the pipeline of genomics and metagenomics approaches for enzyme discovery.

On the other hand, the knowledge of the constituents of the cellulolytic machinery, its structure, and organization has enabled mining metagenome data from cellulose-rich niches and has increased understanding of how free-living microbes interact in nature. Reconstruction of the microbial draft genomes predicts a mutualistic interaction between the dominant microbes and beneficiary microbes, suggesting they survive by depending on each other by acquisition and exchange of

metabolites. Concerning the cellulolytic process in the niche, dominant cellulolytic microbes as *Clostridium* act as helpers that produce endoglucanases, and other bacteria such as *Cloacibacterium*, *Exiguobacterium*, *Acetivibrio*, and *Tolumonas* cross-feed by oligosaccharide uptake (Cui et al. 2019).

Recombinant Expression of Enzymes

Heterologous expression of proteins existed in pregenomics times. However, in the postgenomics era it is without doubt a necessary tool (Luo et al. 2012). Strains with a history of being safe (nonpathogenic and nontoxigenic) are selected for the production of industrial enzymes. *Escherichia coli* is the favorite bacterial heterologous expression system for many proteins so far, but the expression of secreted lignocellulolytic enzymes has been inefficient in *E. coli*. Significant progress in improving results have been achieved by changing promoters and optimizing codon usage, but the translocation step across a double membrane system in this bacterium negatively affects the yield of secreted proteins. Better results have been rendered in Gram-positive bacteria *B. subtilis* and *Lactococcus lactis*. In the case of eukaryotic organisms, the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* have successfully expressed cellulases, hemicellulases, and pectinases. Alternative hosts for the heterologous expression of eukaryotic enzymes are the yeasts *Kluyveromyces lactis* and *Yarrowia lipolytica* (Benedetti et al. 2019).

From Genetic Engineering of Cellulases to Designer in Vitro and in Vivo Cellulosomes

Plant cell wall–degrading machinery of aerobic and anaerobic microorganisms differ considerably in organization. Cellulases and hemicellulases of anaerobes, particularly clostridia and rumen microorganisms, frequently assemble into cellulosomes, a large (molecular weight > 3 MDa) multienzyme complex. First described as large protuberances on the surface in the thermophilic, anaerobic bacterium, *Clostridium thermocellum* in the 1980s, it is now known that the cellulosome complex contains not only cellulases but also hemicellulases, pectinases, polysaccharide lyases, carbohydrate esterases, and glycoside hydrolases (Fontes and Gilbert 2010).

The cellulosomal machinery contains enzymes and noncatalytic modules (Fig. 15.8), the dockerins, and cohesin modules, which bind each other and form a large protein, the scaffoldin. The dockerin domain in the hydrolytic enzymes binds to cohesin modules in scaffolding, allowing cellulosome assembly. Scaffoldings also contain a cellulose-specific family 3 CBM (CBM3a) that targets the cellulosome to the plant cell wall and crystalline cellulose, a C-terminal divergent dockerin that targets the bacterial cell envelope, and an atypical cohesin that tethers the cellulosome to the surface of the bacterium (Bayer et al. 2008).

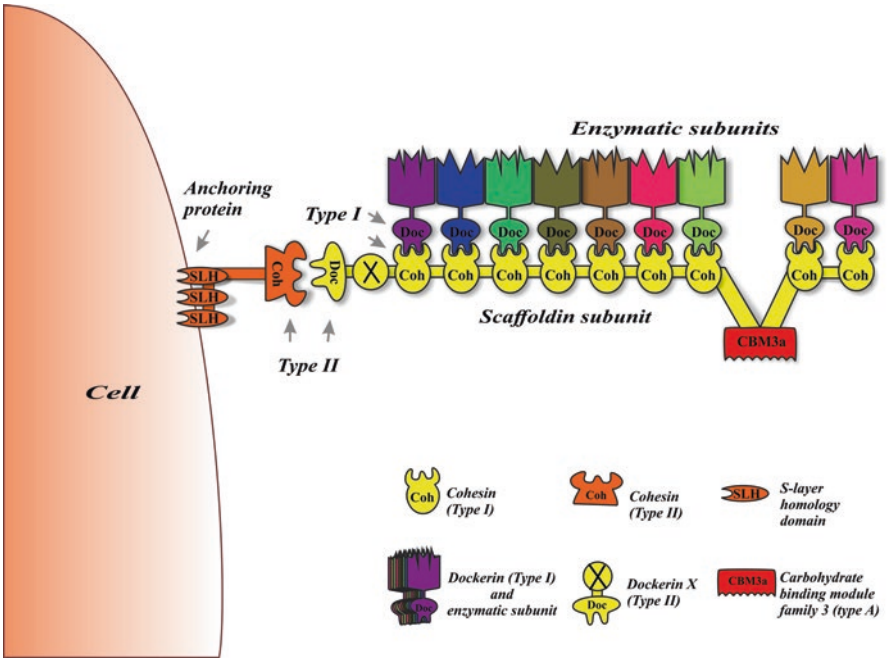

Fig. 15.8 Schematic representation of cellulosome composition and structure organization. Modular cellulases and hemicellulases produced by anaerobic microbes contain a dockerin, which associate strongly with cohesin in noncatalytic scaffolding. A carbohydrate-binding module (CBMs) brings the cellulosome to cell wall, and finally the cellulolytic complex associates with the cell surface through anchoring domain surface layer homology (SLH)

The knowledge on how the cellulosome assembles has allowed the design of minicellulosomes. The first in vitro construct bound endoglucanase E (EngE) from *C. thermocellum* to miniCipA, which was found to contain cohesin I and II domains, that interact with enzymes containing dockerin I and II. Synergism occurred when enzymes were associated with miniCipA. CipAs also contains CBM, which is critical for maximizing activity (Doi et al. 2003).

Pagès et al. (1997) showed that there is no cross-species interaction between cohesins and dockerins, but chimeric cellulosomes could be constructed by adding cellulosomal enzymes, dockerins, and cohesins from two bacterial species, *C. thermocellum* and *C. cellulolyticum*. In vitro construction of minicellulosomes showed that the formation of chimeric minicellulosomes contributed to higher activity on crystalline cellulose than free enzymes, that the CBD contributed to higher activity by bringing the enzymes closer to the substrate, and that the composition of the neighboring enzymes had a positive effect on activity. However, although artificial minicellulosomes show synergistic effects, the native cellulosomes still showed much higher activity than a combination of minicellulosomes (Doi et al. 2003). Moraïs et al. (2012) used the designer cellulosome approach and integrated six dockerin-bearing cellulases and xylanases from *Thermobifida fusca* into a chimeric scaffolding, engineered to bear a cellulose-binding module and cohesin modules.

The chimeric cellulosome was able to degrade untreated wheat straw (up to 1.6-fold compared to the wild-type free enzymes), and it was 42% as efficient as the natural cellulosomes of *Clostridium thermocellum*, which was important progress. By combining the cohesin-dockerin interactions and engineered proteins, new types of synthetic cellulosomes have been generated (Gunnoo et al. 2016). However, natural cellulosomes exhibited higher activity than synthetic complexes due to the greater flexibility of the native versus engineered scaffold, according to analyses by small-angle X-ray scattering (Chundawat et al. 2016).

First attempts to construct cellulosomes in vivo failed. In the first report, the cellulosome genes were introduced in *C. acetobutylicum*. The bacterium had the genes and produced an extracellular cellulosome complex of >665 kDa, confirmed by SDS-PAGE/Western blot analysis, but the bacterium could not grow on cellulosic materials. Also, the recombinant minicellulosome could not degrade Avicel or bacterial cellulose (Sabathé and Soucaille 2003). Efforts continued and then successful scaffoldings of minicellulosome components in vivo (e.g., in *E. coli*, *Bacillus subtilis*) and the MiniCbpA scaffolding from *C. cellulovoran* were successfully expressed in *B. subtilis*, *Saccharomyces cerevisiae*, and *Corynebacterium glutamicum* (Gunnoo et al. 2016).

Characterization of cellulosomal enzymes linked to native or engineered scaffolding proteins has revealed that enzyme complexation is critical to cellulose hydrolysis. Interaction between dockerin and cohesin and endoglucanase or xylanase increases their hydrolytic abilities on the substrates. Then, insoluble lignocellulosic materials can be converted to fermentable sugars by in vitro or in vivo cellulosome or minicellulosome systems. By combining the use of synthetic biology and metabolic engineering, the building blocks are used for engineering recombinant cellulosomes, and they are tethered to cell surfaces by using hydrophilic domains (HLD) or surface layer homology (SLH) domains as an anchoring module (Fontes and Gilbert 2010; Hyeon et al. 2016). For cell surface display on yeast, a glycosylphosphatidylinositol is used as the anchor and fused with the scaffolding protein at the C-terminus (Hyeon et al. 2016). Whole-cell biocatalysts that display lignocellulosic hydrolytic enzymes have shown continuous production of reducing sugars at high yields by direct degradation of lignocellulose hydrolysis, and the rapid consumption of glucose by nearby cells prevents inhibition of endoglucanase and β-glucosidase (Hyeon et al. 2016). Cell surface display of endoglucanase E (CelE) and β-glucosidase A (BglA) on *Corynebacterium glutamicum* increased by six-fold in the conversion of rice straw, 3.1-fold with miscanthus, and 3.3-fold with rape stem to RS, compared to secreted cellulases complexes, and the biosystem retained activity after reactions at 70 °C. Even more, β-glucosidase was able to retain activity at 80 °C (Kim et al. 2014).

Recently, Leis et al. (2018) generated a recombinant *E. coli* with 60 cellulosomal components from *C. thermocellum*, producing a highly thermostable complex with optimal activity around 60–65 °C and optimal pH of 5.8, consistent with optimal parameters of the native cellulosome. These authors reported for the first time comparable activity with the commercially available fungal enzyme cocktail (Cellic CTec2) on microcrystalline cellulose Avicel and on softwood pulp. Fungal enzyme

cocktails are commonly used, and their effectiveness had not previously been matched by bacterial systems.

Analogous structures to cellulosomes have been reported in anaerobic fungi, which are known to assemble plant biomass–degrading enzymes by sequence-divergent noncatalytic dockerin domains (NCDDs). Recently, genomes of the anaerobic fungi *Anaeromyces robustus*, *Neocallimastix californiae*, and *Piromyces finnis* were sequenced with single-molecule technology (PacBio) and cellulosomes validated with proteomics, revealing an average of 312 NCDD containing proteins per fungal strain and 95 large fungal scaffoldings that bind to NCDDs. Fungal dockerin and scaffolding domains are largely divergent from their bacterial counterparts, but several catalytic domains originated via horizontal gene transfer with gut bacteria. In addition, fungal cellulosomes include novel GH3, GH6, and GH45 enzymes. These findings suggest that the fungal cellulosome evolved independently but took some useful activities from bacterial neighbors, resulting in a chimeric structure (Haitjema et al. 2017). However, much remains to be elucidated from fungal cellulosomes.

For their part, biomass-degrading aerobic fungi secrete synergistic cocktails of individual enzymes with one or several catalytic domains per enzyme. Resch et al. (2013) compared cellulase cocktails expressed by the fungus *Hypocrea jecorina* and a cellulosome preparation secreted from the bacterium *C. thermocellum*. They found that free fungal enzymes were more active on pretreated biomass meanwhile cellulosomes were much more active on purified cellulose. Observation by transmission electron microscopy showed different mechanisms of cellulose deconstruction by free enzymes and cellulosomes. When both preparations were combined, dramatic synergistic deconstruction of cellulose was observed. Thus, combination of fungal and bacterial cellulolytic machineries will eventually overcome biomass recalcitrance. Research continues to make these promising strategies useful for saccharification and the utilization of lignocellulosic biomass for bioenergy (Kahn et al. 2019; Ren et al. 2019; Wang et al. 2019).

Concluding Remarks and Perspectives

From a long time ago, the large volume of lignocellulosic biomasses has been proposed as raw materials to produce biofuels, but the complex composition and structure of lignocelluloses have prevented their effective exploitation. Pretreatments of lignocellulose are necessary to improve digestibility and release RS, but most of them release or produce toxic metabolites that inhibit enzymatic saccharification and the growth of fermentative microorganisms. However, recent isolation of microbes that are able to grow in lignocellulosic hydrolysates and the discovery of thermostable and halotolerant enzymes are opening new opportunities for the use of lignocelluloses. Novel glucose-insensitive endoglucanases and β-glucosidases, which are not inhibited by sugars, are also largely promising. Protein modeling programs and mutagenesis are helping in the understanding of the molecular bases

responsible for all those tolerances, which allow us to search the huge postgenomic databases for microorganisms or enzymes with those desirable characteristics.

The thermophilic bacterium *C. thermocellum* is documented to be the most efficient system for cellulose hydrolysis so far. The current knowledge concerning the composition and organization of enzymes in cellosomes enables the search for cellulolytic clusters in assembled genomes and metagenomes, providing synthetic biology and genetic engineering with new and powerful biobricks.

Since the available list of promising genes for genetic engineering is large and growing constantly, the next challenges will be to achieve successful and efficient heterologous expression. Coordinated expression of multiple genes, correct protein folding and maturation, and efficient secretion of free enzymes or even more, and complete enzymatic machineries are some of the challenges to be overcome to obtain synergistic work of the saccharolytic and fermentative enzymes. The challenges are many but the pieces to assemble the puzzles are now beginning to be available, and surely powerful native microorganisms will be isolated and microbial cell factories will be created. No doubt, strong lignocellulolytic microorganisms will be screened from available regional biomass to optimize hydrolysis and fermentation of these materials. DIET has demonstrated its potential to enhance the production of biogas. Further research is necessary to establish the DIET-based production of methane from lignocelluloses. Likewise, research should focus on improving CBP for ethanol production. The final challenge will be to take the best results from the lab and take them to a large scale.

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Chapter 16
Temperature Profiles During the Fermentation of Cacao *'Nacional'* to Understand Chocolate Quality

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Abstract The quality of chocolate is affected by a multitude of factors including cacao cultivar, maturity, fermentation management, drying, roasting, grinding, conching and, the addition of ingredients. In the sequence of unit operations, perhaps the most complex is fermentation because it is affected by the variability of microbial populations, the variability in the amount of mucilage, and the variability in the concentration of fermentable sugars. Several fermentation studies have been published but very few detail the progress of the fermentation in multiple points in a 1-m^3 fermentation box. Both yeast and bacterial fermentations of cacao produce heat. Therefore, we monitored temperature profiles as indirect indicators of microbial activity. Measurement of fermentation temperature at the centre of fermentation boxes and only five other locations in a box have been previously reported. In this research study, we continuously monitored temperature profiles in 27 locations in a symmetric cubic matrix in a 1-m^3 box. Temperature right before the removal from one box to the next varied by 10–13 °C for a single batch and up to 16 °C for six batches. The maximal reported temperature was 54 °C. Highest temperatures occurred in the highest layer and lowest temperatures in the lowest layer. However, there was not a clear symmetry in the temperature profiles of the corners in each layer, suggesting non-uniform microbial growth or non-uniform heat losses. Alternation of top and bottom layers when changing from one box to the next

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reduced the heat non-uniformities. Cut tests at each stage of fermentation indicated the progress of the fermentation is very variable among batches and within a batch. Finally, analysis of headspace volatiles confirms the very large variability of fermentation conditions within and among batches.

Keywords Temperature profiles · Fermentation · Cacao '*Nacional*' · Chocolate quality

Introduction

The chocolate confectionary industry is valued at approximately $100 billion while the value of cocoa beans is only $10 billion (Schwan and Fleet 2015). About 95% of the cacao production in the world is from the bulk quality cultivar *'Forastero'*, and it is used by the largest chocolate manufacturers to produce chocolate bars and other confections that most consumers are familiar with (Fowler 2009). By virtue of its high yield and disease tolerance, as well as by the economies of scale, the prices of chocolate confections that use cacao *'Forastero'* are considerably lower than those that use fine flavour cultivars and are made by craft chocolatiers. However, cacao from fine flavour cultivars such as *'Criollo'* or '*Nacional*' are preferred and are the most commonly used by the craft growing craft chocolate industry. The U.S. craft chocolate industry was worth $100 million in sales in 2015 (Shanker 2017). Today there are approximately 500 craft chocolate makers in the United States. For example, while a 4.25-oz dark chocolate bar from Hershey is sold for less than $2.00, a craft dark single source chocolate bar ranges from $10.00 to $20.00. Ecuador is the fourth largest producer of cacao in the world and the largest producer of fine flavour cacao, in particular, the cultivar '*Nacional*'. Ecuador is also the second largest exporter of cacao to the United States. Cacao '*Nacional*' produces cocoa liquors with very rich and complex flavour profiles that have very desirable and distinctive floral notes. Indeed, the flavour perception of such liquors produce at first a subtle fresh citrus flavour followed by astringency, dry fruits (raisin, prune), climaxing with nutty (almonds, pecan) retro-nasal aroma and resolving with subtle floral aromas. However, the quality of each fermentation batch is variable and does not always result in what would be considered as 'ultra-premium'.

A large body of research on the manufacture and quality of chocolate exists and has recently been reviewed (Gutierrez 2017). Hundreds of papers have been published on the flavour of cacao and chocolate. Approximately 600 aroma-active compounds have been identified in cacao or chocolate. However, their mechanisms of formation during fermentation, drying, roasting and conching or their sensory characteristics as a result of their interactions have not been fully elucidated (Aprotosoaie et al. 2016). In contrast, much less research exists on cacao fermentation, in particular, a clear characterization of the effect of fermentation on chocolate quality. This gap of knowledge has been identified, but it is being filled mostly by European

researchers as evidenced by a recent review (De Vuyst and Weckx 2016; Romanens et al. 2018). Some efforts have been undertaken to survey the genetic diversity of *'Nacional'* cultivars (Solorzano et al. 2012; Loor et al. 2009; Boza et al. 2014). Associating individual *'Nacional'* cultivars to cocoa liquor quality is practically impossible because cacao is produced by a large number of small farms that ferment it under non-standardized and uncontrolled conditions. Indeed, because fresh cocoa beans are perishable, fermentation is done locally. In many cases, fermentation is done by small farmers in primitive ways, sometimes on piles on the ground, covered with banana leaves or plastic tarps. Some companies consolidating cacao from different farms can, in part, address this issue.

However, to reduce the cost of transportation and the concentration of waste, mature cacao pods are harvested, beans and mucilage are separated from the shell (Fig. 16.1) at the farm and transported in sacs to the fermentation site. The time of harvest and distance between the farm and the fermentation site is variable. In some cases, cacao starts fermenting in transit and, in some unfortunate instances, it moulds. Cacao fermentation is spontaneous; that is, produced by environmental microorganisms. It begins with an anaerobic yeast fermentation that converts sugars to ethanol, followed by aerobic fermentation mostly driven by acetic acid bacteria. Fermentation produces flavour precursors that are converted into the desired flavours upon drying and roasting. After beans are dried and roasted, they are ground until smooth cocoa liquor or cocoa mass is produced.

Expert tasters taste the liquor and rate the batch of fermented and dried beans. Historical private, unpublished data files of quality from companies that ferment cacao have allowed the identification of farms that supply the beans that produce the best cocoa liquor. In such places, better quality control can be achieved; however, the non-uniformity in the quality of the fermented beans is still staggering. The only occasional quantitative measurement done is determining temperature at 1–5 locations when the fermentation is done in wooden boxes by inserting a thermometer. The duration of each stage of fermentation is coarsely controlled. Cacao '*Nacional'* is fermented in wooden boxes and can produce cocoa liquors with complex and very desirable flavour profiles. Large farms also do the fermentation in wooden boxes

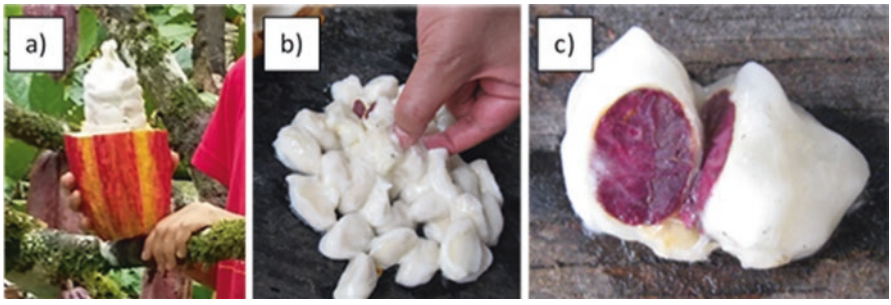


Fig. 16.1 Cacao fruit. (**a**) pod cut in half showing the intact beans on the top half, (**b**) cocoa beans separated from the shell and (**c**) cocoa bean cut in two, showing the white mucilage

and better control the duration and aeration of fermentation, typically by arranging sets of three boxes in a stair fashion (Fig. 16.2).

At the box in the top, the alcoholic fermentation takes place for one to two days, then the beans are shovelled to the second box, which allows mixing and aeration. In this second stage, acetic acid fermentation becomes dominant. After two additional days, the beans are shovelled to the last box where the acetic acid fermentation continues for about two more days. At the end of a fermentation batch, the adequacy of the fermentation is determined visually using a cut test. Depending on the colour and texture of 50 beans cut longitudinally, it is determined whether or not the batch was properly fermented. However, the flavour of two different batches with similar results in the cut test, and from the same cacao cultivars can be very different, indicating that the fermentation process plays a critical role in the quality of the final product. The objective of this study was to determine the variability of the fermentation of cacao *'Nacional'* through the detailed characterization of the temperature profiles, cut tests during 96-h fermentations and characterizing the metabolite profiles of the cocoa liquors.

Materials and Methods

From August to November of 2017, six batches of cacao *'Nacional'* were fermented at Compañía Exportadora de Cacao de Aroma y Orgánico (CECAO), Guayaquil, Ecuador. Wooden boxes were instrumented with a cubic mesh of 27 thermocouples type K, two temperature data loggers model RDXL12SD and one temperature data logger model RDXL4SD from Omega Engineering Inc. (Norwalk, CT, USA) that collected temperature data at 5-min intervals for the duration of the entire fermentation process. When cacao was transferred to the next box, the thermocouples were moved as well. Figure 16.3a is a schematic representation of the thermocouple mesh, and Fig. 16.3b is a picture of the fermentation box with the thermocouples.

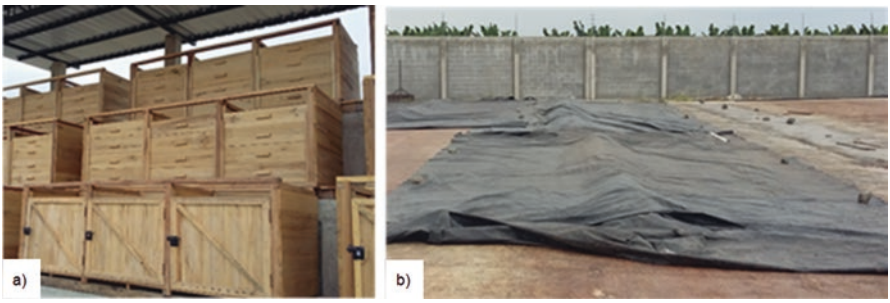


Fig. 16.2 (**a**) Fermentation boxes (1 m^3 each) and (**b**) fermentation of cacao CCN-51 in Lagarto, Kaoka, Ecuador

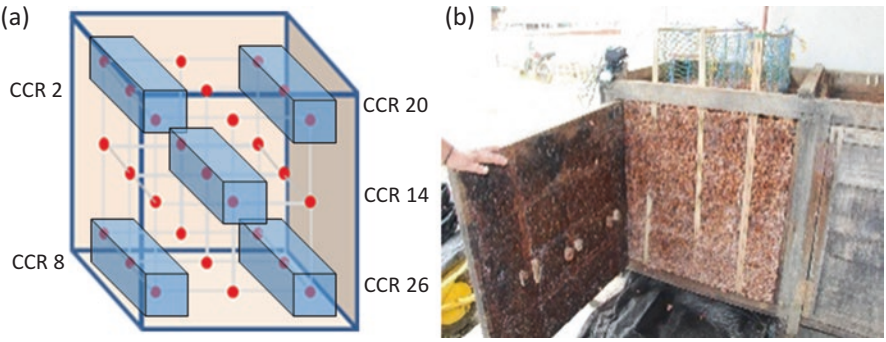
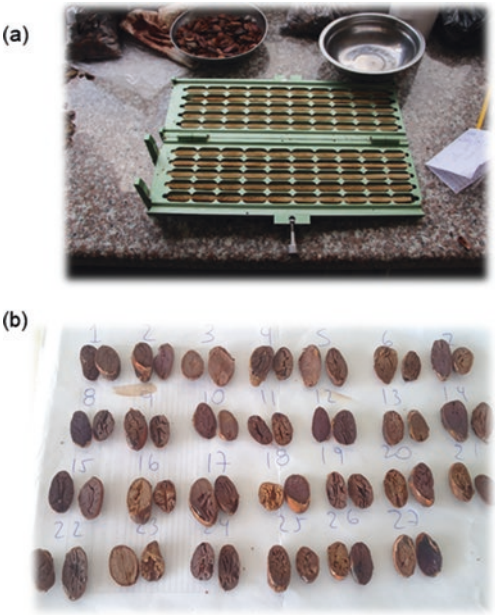


Fig. 16.3 (**a**) Schematic representation of the distribution of thermocouples in a cacao fermentation box; (**b**) photo of a fermentation box with beans and the thermocouples installed

Cacao was carefully transferred from box to box ensuring that the top layer of the first box became the bottom layer of the second box and that very little mixing occurred horizontally to ensure that the temperature represented the temperature history of specific groups of beans.

Beans from four corners and the centre of the fermenter were collected longitudinally as indicated by the blue clusters in Fig. 16.3a. Each cluster grouped samples around three thermocouples. Smaller samples were not practical as they are insufficient for grinding on a bench-scale melanger. Cocoa liquors were prepared after sun-drying fermented beans for one day, roasting and grinding in a melanger for 30 min. Cocoa liquors were poured into rectangular chocolate moulds and were kept refrigerated at 4 °C until analysed except during transportation from Ecuador to the United States. Aliquots of 10 g were placed in 50-mL vials sealed with a septum and melted in a water bath at 55 °C. Abundance of volatile components was analysed by solid phase micro-extraction (SPME) coupled to gas chromatography-mass spectrometry (GC-MS).

Among 15 of the most abundant compounds, 10 were identified using NIST or Wiley tenth edition compound databases and 5 were labelled as unknowns. Results were analysed using principal component analysis to identify metabolite profiles associated with location in the box. Beans from locations near each of the thermocouples were also collected to carry out cut tests during the process of moving the beans from one box to the next. Figure 16.4a shows the standard guillotine used to cut beans longitudinally. Figure 16.4b is a sample of cut beans from each of the 27 locations in a batch. Figure 16.4c shows an example of the visual characterization of the extent of fermentation based on duplicate cut beans from each location of the box where thermocouples were placed.



1	2	3	4	5
V – GF	GF – GF	GF – GF	GF – P	P - GM
6	7	8	9	10
GF – GM	GF – GM	GF – GM	GF – GM	GF – GM
11	12	13	14	15
V – GF	GM – GM	GF – V	P – V	GF – GM
16	17	18	19	20
GF – V	GF – GM	GF – GM	GF – GM	GM – GM
21	22	23	24	25
GF – V	V – V	GF – V	GM – V	GF – V
26	27			
V – V	V - GF			

Fig. 16.4 (**a**) Cocoa bean guillotine, (**b**) cut beans from different locations in the box, (**c**) visual assessment of the extent of fermentation for beans from each location

Results and Discussion

Temperature Profiles

Temperature is an indicator of microbial activity during fermentation. Indeed, both anaerobic alcoholic fermentation and aerobic acetic fermentation are accompanied by the generation of heat. To our knowledge, this is the most comprehensive study on the temperature profiles in a cacao fermentation box. As is well known, temperature increased as the fermentation progressed. Each batch reached different average temperatures at the end of each 48-h stage of the fermentation. For example, some batches reached 44 °C after 48 h of alcoholic fermentation while some only reached 36–37 °C. Similarly, some batches had locations where the maximal temperature reached approximately 54 °C while others only reached about 46 °C. In their compilation, Schwan et al. (2015) reported that the maximal temperature ranged from 43 °C to 53 °C with most studies reporting 47 °C–50 °C The lowest maximal temperature was reported for a fermentation carried out in a stainless steel tank (Pereira et al. 2012).

Presumably, heat losses to the surroundings caused the lower temperature. Figure 16.5 is an example of the considerable variability of temperature in one

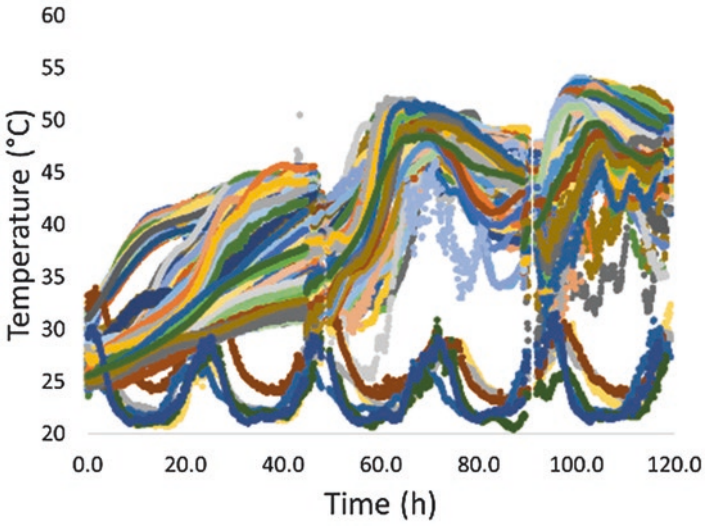


Fig. 16.5 Temperature fluctuations during a single batch of cacao *'Nacional'* fermentation at each of the location shown in Fig. 16.3a

fermentation batch. With the exception of the yellow curve at the bottom that recorded the ambient temperature fluctuations, all the other curves represent the temperature at each of the 27 locations in the fermentation box. Large variability occurred at the end of the alcoholic fermentation, right before transferring to the second box, and then at the very end of the fermentation.

The maximal temperature was 53.6 °C for the batch shown in Fig. 16.6 and the maximal standard deviation was 3.8 °C. These results suggest that depending on the location at which the beans were, their extent of fermentation was different. Although mixing during transfer mitigates the variability, and some 'cold' beans may 'catch up' with the fermentation after transfer, non-uniformities are unavoidable in the current setup. Indeed, the beans corresponding to the bottom of the fermentation box (e.g. grey line in Fig. 16.5) are the coldest at the end of the first 48 h. However, the temperature of these beans become among the highest when located at the top layer between 48 and 96 h. Vigorous fermentation continued up to approximately 100 h as indicated by the increase in temperature. Afterwards, microbial activity decreased to the point where heat losses are greater that the heat produced by aerobic bacteria. The duration of fermentation affects the degree of protein hydrolysis and thus the formation flavour precursors (Caligiani et al. 2016).

Replication of fermentation resulted in surprisingly large variability in temperature profiles as a function of fermentation time as shown in Fig. 16.6. Differences of up to 16 °C exist as a result of the non-uniformities within a batch (Fig. 16.5) as well as the batch to batch variability. The rate of heating varies a lot from batch to batch, which is probably due to differences in the level of inoculum, as well as the time of

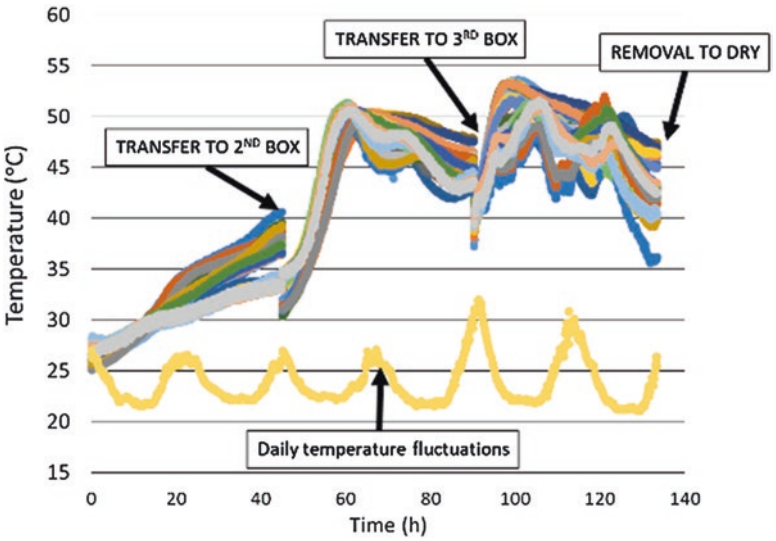


Fig. 16.6 Temperature fluctuations for three different batches of cacao '*Nacional*' fermentation at each of the location shown in Fig. 16.3a

the initial temperature. The initial temperature is dictated by the time of the day when the box is filled as well as any heating during transportation. During transportation in plastic or yute sacks, the beans heat up due to sun irradiation and begin to ferment in the sacks used to contain the beans.

Cut Tests

The cut test is the standard method to visually determine the adequacy of cacao fermentation. In each layer of the fermentation box, the number of well fermented beans increased as the fermentation progressed as shown in Fig. 16.7. However, the batch to batch variability was so large that it was difficult to ascertain significant differences. The large number of "well-fermented" beans after the first 48 h of fermentation does not indicate that these beans are truly well fermented as the aerobic acetic fermentation is not yet dominant. Rather, the cut test indicates the incomplete fermentation because of the presence of violet and slate beans. Although violet and slate beans were not found on the top layer, this might be due to the sampling size of the experiment. The number of violet and slate beans decreased with fermentation as expected. The variability shown in Fig. 16.7 as well as the variability in the cut tests done every 48 h during fermentation are clear indicators of the non-uniformity of the fermentation.

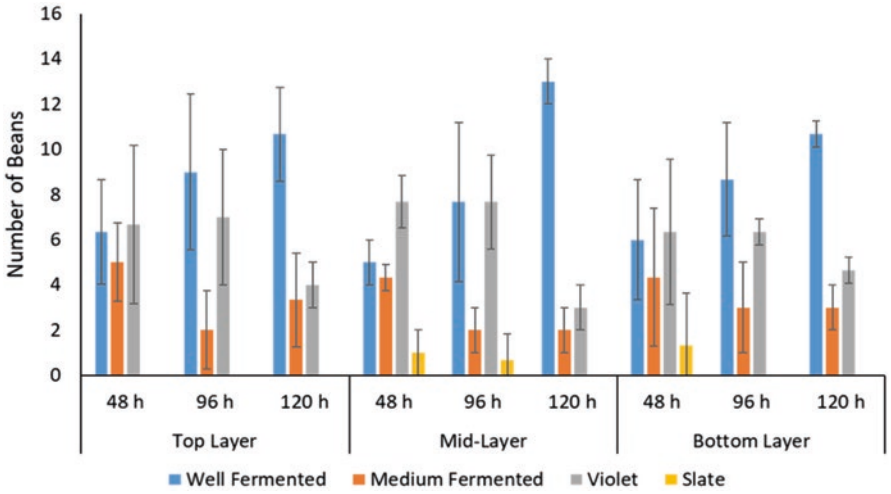


Fig. 16.7 Evolution of the fermentation progress in each layer of the fermentation as indicated by the cut tests. Bars represent the mean number of beans at the observed stage of fermentation. Error bars represent the standard deviation ($n = 3$)

Volatile Metabolites of Cocoa Liquors

To elucidate whether consistent fermentation occurred at extreme locations of the box (i.e. corners and centre, Fig. 16.3a), metabolite profiles from GC-MS analyses of volatile compounds were analysed using principal component analysis. Figure 16.8a plots the first two components that account for 50.5% of the variance in compound abundance. No clustering by location was identified. However, three individual samples were very different, each (CCR2, CCR20 and CCR26) corresponding to samples in a different corner from different batches.

Data dispersion around the mean for the abundance of volatile compounds ranged from 2.4% to 148% RSD with the median of 27.0% RSD. These observations confirm the non-uniformity of fermentations. Furthermore, the temperature profiles of the centre point of samples CCR2, CCR20 and CCR12 where near the extremes compared to the temperature profiles of the other centre points as indicated by contrasting Fig. 16.8b, c. Samples CCR2 and CCR20 had anomalous drops in temperature towards the last portion of the fermentation. Both correspond to the top layer of the box at the end of the fermentation that may be attributed to cooling by wind.

The concentrations of 1,3-butanediol, 2,3-butanediol and 2-butanone,4-(acetyloxy) were highly variable. The highest concentration of each of these and other compounds was at a different location for each batch, confirming the lack of reproducibility within a fermentation batch. We did not find any detectable linalool in any of the preliminary batches, which is consistent with the expert tasters who did not report any floral notes on these batches.

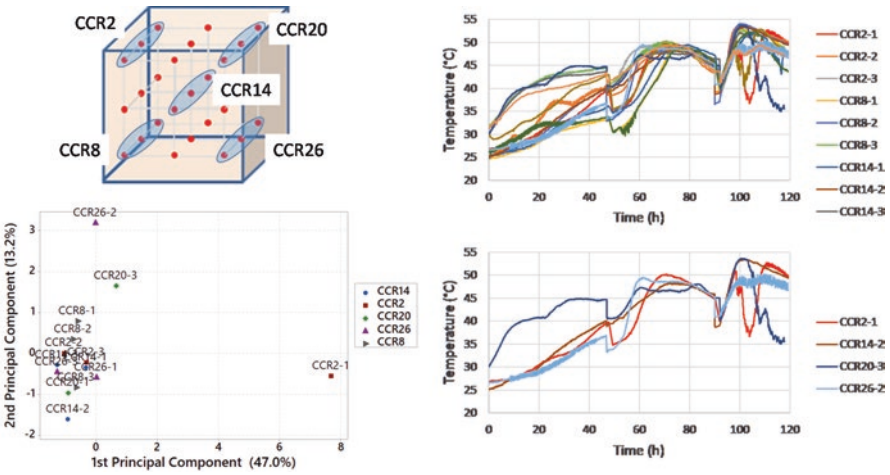


Fig. 16.8 (**a**) Score plot from the principal component analysis (PCA) of volatile compound from samples at the corners (CCR2, CCR 20, CCR8 and CCR 26) and centre (CCR 14) of the fermentation box; (**b**) temperature profiles for the samples experienced by the middle point of each location (centre point for each cluster highlighted in blue in Fig. 16.3a; (**c**) temperature profiles of samples with greatest differences in PCA

Conclusions

The very wide variance in temperature profiles, cut test results and metabolite abundance are the reasons for the absence of statistically significant differences. However, because the extent of fermentation at any point in time during the fermentation is not controlled and varies depending on the location in the fermentation box, that is, the independent variable is not controlled, the response of the dependent variables (i.e. temperature profiles, cut tests and metabolite profiles) also cannot be controlled. Therefore, it is necessary to reduce the sources of variability in the rate of fermentation. Shelling the cacao pods should be done at the same location where the beans are to be fermented. Fermentation should be started at the same time of the day (i.e. beans should be at the same temperature), and the level of inoculum should be similar. Moving the beans from one box to the next should be based on determination of the extent of the fermentation rather than on the basis of fermentation time.

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Chapter 17
Ethno–Phytopharmacology: Product Validation Process Based on Traditional Knowledge of Medicinal Plants

Javier Mussin and Gustavo Giusiano

Abstract Ethno-phytopharmacology studies the traditional use of plants for the prevention and cure of several diseases. It provides multidisciplinary research on components of medicinal plants, their identification and description, properties, modes of action and interactions with the human organism. Search for new bioactive drugs is another aim of these experimental investigations. Since the World Health Organization (WHO) supports and encourages the introduction of traditional medicine resources into health systems around the world, the use of medicinal plants has shown a marked increase. For this reason, interest in applying scientific methods to validate or refute the traditional use of these plants with the rigors of evidence-based medicine to assess safety, efficacy, and quality has become increasingly important. These three concepts govern the twenty-first century therapy inherent to any conventional drug and allow medicinal plants to aid in the development and advancement of modern medicine, serving as a starting point for the design of new, better, and healthier drugs. In this chapter, parameters to validate medicinal plant attributes such as selection and harvest, extraction and processing methods, analytical techniques to isolate and identify bioactive metabolites, biological activity screening, and other aspects are discussed.

Keywords Ethno-phytopharmacology · Human health · Traditional medicine · Medicinal plants · Drugs

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Introduction

Plants have been used as medicine throughout human history by different cultures. Through trial and error learning, the human being learned to differentiate plants with beneficial effects from those that were toxic or inactive as well as which combinations or processing methods had to be used to obtain the desired results. Their countless uses have been documented and transmitted through generations, including organized traditional medical systems (Ayurveda, Unani, Kampo, and traditional Chinese medicine, among others). On the other hand, herbalism, folklore, and shamanism are widely practiced in Africa and South America. These are based on a "confidential information learning system" that is only passed to the next generation by a shaman, healer, or herbalist; therefore, scientific validation and information on the plants are less documented than in other systems (Fabricant and Farnsworth 2001).

Plant-based prepared medicines contain a variety of chemical compounds, many of which contribute or are responsible for their medicinal benefits. The compound to which the activity is attributed is named as the active compound, and its presence and concentration depend on a series of factors, among them, the plant's species, harvesting time, the type of soil, the part of the plant utilized, the processing, etc. The diverse composition, occasionally unknown, of products based on medicinal plants, makes it difficult to ensure the quality of the different production batches without adequate controls. In many countries, these products are launched onto the market without these controls and without the necessary safety and efficacy studies. The fact that a herbal product that has demonstrated to be safe and efficient during the validation process, having a defined and constant composition, is one of the most important prior qualifications to produce a quality medicament (Kunle et al. 2012).

According to the World Health Organization (WHO), approximately 80% of the world population, especially inhabitants of developing countries, use medicinal plants or products derived from them as part of their health care due to their lack of access to laboratory-derived drugs (WHO, IUCN, WWF 1993; WHO 2013). The "WHO Traditional Medicine Strategy 2002–2005" program aimed to keep natural medical practices as possible therapeutic alternatives. The "WHO Traditional Medicine Strategy 2014–2023" program reappraises and sets out the course for traditional and complementary medicine usage in the next decade (WHO 2002, 2013).

In this chapter, we discuss the concept and origin of ethno-phytopharmacology, and the basic parameters to validate the attributes of medicinal plants are described. These parameters include, information recompilation, selection, collection and processing of the plant material, extraction methods, evaluation of the biological activity with special emphasis on antimicrobial activity, isolation processes for, and identification of, active compounds, and, finally, safety and efficacy studies.

Ethno-Phytopharmacology

The encouragement in the research of bioactive compounds based on plants started at the beginning of the twenty-first century when Friedrich Sertürner managed to isolate the analgesic and the agent of sleep from the opium poppy, which he called *morphium* (morphine) in reference to the Greek god of dreams, Morpheus (Atanasov et al. 2015). Since then, medicinal plants played a key role in the development and advancement of modern medicine, acting as a starting point for the development of new drugs.

In the last 100 years, a great number of bioactive compounds based on medicinal plants have become essential for modern medicine, examples being digoxin from *Digitalis* spp., vincristine and vinblastine from *Catharanthus roseus*, morphine and codeine from *Papaver somniferum*, and atropine from *Atropa belladona*. Additionally, it is estimated that 60% of antitumor and antiinfectious drugs that are already on the market or into the trial phase are derived from nature (Newman and Cragg 2020a). Recognized examples of antiparasitic agents (malaria effectively) are quinine from *Cinchona* spp. and artemisinin isolated from *Artemisia spp*. In 2015, the Nobel Prize for Physiology or Medicine was awarded in part to Dr. Youyou Tu in the ethnomedicine field for her contribution to drug therapy based on artemisinin (isolated from *Artemisia annua*), which reduced malaria impact and mortality rates, saving millions of lives globally (Andersson et al. 2016).

Between 1981 and 2019, 1394 new chemical entities belonging to the small molecules group were approved, 65% were derived or inspired by nature. Most of these compounds come from microbial sources or as a result of the interaction of microorganisms with plants (Newman and Cragg 2020a). However, the potential use of plants as a source for new drugs is still not fully explored. It is estimated that only around 6% of the approximately 250,000–500,000 plant species have been well studied in terms of biological activity and 15% have been studied phytochemically. It has been determined that 80% of medicinal plants' isolated bioactive compounds have an ethnomedical use identical or related to the instructions for which the plant has been prescribed (Fabricant and Farnsworth 2001).

The word ethno-phytopharmacology comes from the following Greek terms:

- *Ethnos* = race, culture
- *Phyton* = plant
- *Pharmakon* = drug, medicine
- *Logos* = discourse, explanation

Ethno-phytopharmacology comprises a multidisciplinary approach including studies about the traditional use of plants for the diagnosis, prevention, and treatment of diseases and also research on the medicinal plants' components, their identification and description, biological properties, mode of action, and interaction with the human body.

Such studies allow the scientific validation of medicinal plants' attributes that once rested solely on their habitual and popular use, but also they provide new bioactive drugs with the rigors of medicine based on evidence, providing safety, efficacy, and quality to the patient. These three concepts rule the current therapeutic use in the twenty-first century of any conventional drug. Ethno-phytopharmacology allows medicinal plants to intervene in the development and advancement of modern medicine, acting as a starting point for the development of new drugs.

Validation of Medicinal Plants

According to the Pan American Health Organization (PAHO) (Arias 1999), a medicinal plant is any wild or cultivated plant used for a medical purpose. A herbal medicine is a manufactured product that contains one or more therapeutically active components exclusively extracted from plants (aerial or non-aerial parts, juices, resins, oils, etc.) either raw or processed. However, these plant-based products must undergo a scientific validation process in terms of efficacy, safety, and quality. The belief that all plant-based products are a good treatment just because they are obtained from nature ought to be discarded. In many cases, these products are entirely ineffective and, occasionally, even toxic. At the same time, we should not ignore the possibility that many medicinal plants could be simple mediators acting as placebos in a cultural context and do not contain pharmacologically active molecules for the disease indicated by ethnomedical information (Gertsch 2009). Figure 17.1 shows the validation process stages for products based on medicinal plants; all of them are of great importance.

Selection of Plant Material

There are many approaches to select the vegetal species for the pharmacological study: the ethno-pharmacological approach, the random approach, the chemosystematic approach, among others. Throughout millennia, human beings were improving their knowledge about medicinal plants by trial and error experience and transferring that knowledge. Therefore, the ethno-pharmacological approach is still the most efficient one for the discovery of new molecules. This approach is based on the selection of the plant species according to its traditional medical use (Atanasov et al. 2015). It is estimated that 74% of bioactive compounds derived from plants were discovered after checking the ethno-pharmacological approach (Ncube et al. 2008).

Information from the ethnic group about how they use the plant is extremely important. The part of the plant that they use (flowers, leaves, roots, etc.) and how they process it can give hints to the best method for the active compound extraction. The formulation and posology used will provide information about pharmacological

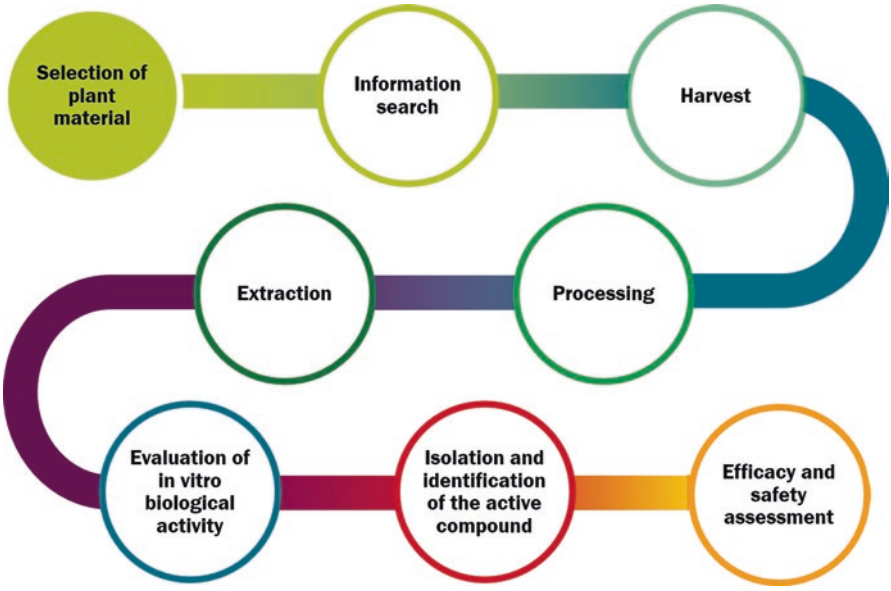

Fig. 17.1 Validation process stages of products based on medicinal plants

activity, oral versus non-oral intake, and the doses to be tested. However, certain considerations have to be taken into account when the ethno-pharmacological approach is chosen to select the plant material; for example, each ethnic group has its own health and disease concepts, just as in different health systems. The signs and symptoms must be interpreted, translated, and related to medical concepts, allowing a study focused on a particular biological activity (Rates 2001).

Information from organized traditional medical systems (Ayurveda, Unani, Kampo, and Chinese traditional medicine), herbalism, folklore, and shamanism can be obtained from a number of sources, including books, articles about plants, review articles, footnotes made by the botanist on herbaria vouchers, fieldworks, and online database like NAPRALERT and USDA –Duke (Fabricant and Farnsworth 2001; Cos et al. 2006).

Information Search

Once the plant has been selected based on its ethnomedical knowledge, it is necessary to gather the greatest possible amount of scientific endorsement information. Aspects to consider in order to plan the collection of the plant to be utilized (Fabricant and Farnsworth 2001; WHO 2003) include:

- Learning about its organography, morphology, and physiology. Allows determination of the variations that a plant can undergo during the different seasons and

stages of life. The plant's age and the moment of harvest could affect the chemical composition and influence the presence or absence of the active compound and, in consequence, its medicinal properties. Learning the plant's morphology allows identification of the correct part that must be used
- Knowing the botanical geography, meaning the place where the plant grows and its abundance, as well as the place where it will be harvested
- Learning the legal aspects. If the plant is in danger of extinction or its cultivation is forbidden, obtaining the permits necessary to collect and study them

A global preliminary study about the plant is important because another aspect that determines chemical composition are its endophytic organisms, such as fungi and bacteria. Occasionally, compounds present in the collected plant material may be metabolites produced by endophytic organisms or induced products by the plant as a result of the interaction with the said organism (Giusiano et al. 2010; Atanasov et al. 2015). In fact, a good number of those thought to be "plant-derived" natural products have been shown to be metabolites produced as a result of interaction with endophytic and epiphytic microorganisms (Newman and Cragg 2020b).

Harvest

In most cases, the plants are directly collected from their natural habitat, so accurate identification is paramount. It is important that the botanist is capable of identifying the species and also prepare the material for the preservation of the herbarium so as to secure reference material ("voucher sample"). For unambiguous identification, it may be necessary to use a combination of methods, such as genetic and chemical analyses, in addition to the morphological and anatomic characterizations. On the other hand, the plants' habitat and the legal permits, in particular that of protected species, must be respected when collecting from nature.

The WHO (2003) and the European Medicines Agency (EMA) (2006) developed guidelines on good agricultural and collection practices for medicinal plants in order to promote sustainable collection techniques and reduce environmental problems. In addition, the Convention on Biological Diversity (WHO 1992) and the Nagoya Protocol (Convention on Biological Diversity 2011) on access and benefit sharing must be respected.

The plant's chemical composition depends not only on the identity of the species and the harvesting time, but also on the composition of the soil, the topography, geology, vegetation, altitude, weather and environmental conditions, daylight hours, among other variables; therefore, all this information must be gathered, and also obtain photographic records and specify the coordinates of the harvest site.

The harvest area must be far from pollution and agricultural crops since the presence of heavy metals and agrochemical compounds may affect the interpretation of the plant's properties. Harvesting should be avoided near to drainage ditches, roadsides, mine tailings, garbage dumps, and industrial facilities that could produce

toxic emissions. Similarly, harvesting medicinal plants in and around active pastures, including riverbanks downstream from pastures, should be avoided in order to avoid microbial contamination from animal waste (WHO 2003; EMA 2006; Atanasov et al. 2015).

The harvest plan must allude to the species, to the part of the plant (root, leave, fruit, etc.), and the amounts to be collected. Ecofriendly and nondestructive harvest systems, which will vary considerably from one species to another, must be applied. For instance, in the collection of trees and bushes roots, the main roots must not be cut off or unearthed and cutting off the central root must be avoided; only lateral roots ought to be located and collected (WHO 2003).

Based on the information compiled prior to collection, to secure the best quality and quantity of components with possible biological activity, the plant material must be collected during the right season or period, even the time of the day (as mentioned earlier under information). The components and/or concentrations may vary during the growth cycle (young or adult plant, or plant age) and even during the day. Additionally, it should be kept in mind that each part of the plant has its most appropriate harvest time when, in general, the active compound is in its highest concentration. For example, leaves are collected when photosynthesis is most active, meaning when they are green, mostly before or during the blooming phase, in dry weather (no rain), and in the morning when dew has evaporated (WHO 2003).

The harvested plant material should not come into direct contact with the soil and must be placed in baskets, mesh bags, or other clean and airy containers, without vegetal remnants from previous harvest activities. If the underground parts of the plant are the ones used (like the roots), the dirt residue must be removed (WHO 2003).

The use of plants under controlled conditions of cultivation ought to be considered over those harvested from nature. This allows the production of a homogeneous material, largely guaranteeing the chemical homogeneity and reducing the disadvantages associated with an uncontrolled environment (Rates 2001; WHO 2003). After harvest, transformation processes and compound degradation may occur making it a necessary requirement that the plant material goes to the processing stage as soon as possible.

Processing

The processes previous to extraction allow the removal of impurities and the avoidance of alterations on the plant material, so as to secure a high-quality crude material. Postharvesting alterations may be classified into external and internal alterations. External alterations are made by humidity, sunlight, heat, and the presence of strange material, such as dirt, insects, parasites, microorganisms, etc. Internal alterations may be caused by enzymatic reactions, autoxidation reactions, and reactions between components of the plant. These alterations can be reduced by following the harvest and processing stages step by step.

Plant material processing can be divided into five stages:

- Classification
  Every strange or contaminated material must be discarded and if it doesn't comply with the organoleptic characteristics sought. A thorough material check has to be made, preventing it from being contaminated by insects, parasites, etc., either with fragments of other plants, or other parts of the itself plant that are not wanted, or decaying materials. An organoleptic evaluation must be made, including appearance, damage, size, color and odor (WHO 2003).
- Washing and sanitizing
  To get rid of debris that may be present on the surface and ensure the microbiological quality of the plant material, crude material must be subjected to a washing and sanitizing process after harvest. The washing must be done with plenty of potable water in order to remove soil debris, dust, spores, etc., that may affect the quality of the final product.
  Sanitizing may be carried on through chemical or physical methods. The choice of the optimal method will depend on the type of material to be sanitized, on its volumes, and on its possible costs. Nevertheless, the chemical method by immersion in aqueous sodium hypochlorite solution (NaOCI) is still the most used. The solution concentration and the immersion duration time depend on the plant material. In general, the plant material is sanitized using an aqueous solution of NaOCI 0.5–2% over 5–10 minutes. Afterward, several rinses are performed with sterile purified water, followed by straining or centrifugation of the material to remove any remnants of NaOCI and water (Fuentes-Fiallo et al. 2000).
- Drying
  The aim of the drying or dehydrating process is to reduce the plant water content with the aim of preventing alteration of the active compound. It also allows its storage and conservation for a longer period. There exist a great number of drying techniques, and the choice will depend on the part of the plant, the material amount, and the advantages and disadvantages of each technique. Indigenous peoples generally use a natural outdoor drying process until it reaches a steady weight. They perform this process in a ventilated place and, depending on the part of the plant, this process is carried out in the sun or shade (Fuentes-Fiallo et al. 2000; WHO 2003).
  Occasionally, herbal medicines are made out of recently harvested fresh plants as infusions, decoctions, or crushing the plant in a mortar or stone so as to apply it on skin or ingest it. In these cases, when traditional use warrants it, the drying process is omitted and can go straight to the extraction stage. To avoid alterations of unstable bioactive compounds that may be present, the time that goes by between the harvest and the extraction process must be the least possible. On the other hand, the fresh plant can undergo a stabilization process through freezing, lyophilization, use of alcohol vapors, etc. Stabilization causes the irreversible denaturation of the plant enzymes, allowing the maintenance of its relatively immutable chemical composition (Rates 2001).

- Storage
  Storage is not recommended when the active compound and stability of the plant material are unknown since the material can be exposed to alterations. But if storage is required, its conditions must be very well detailed. In general terms, the plant material must be stored labelled, protected from sunlight and contamination sources, in a cool and dry location, with humidity and temperature controls (WHO 2003).
- Grinding and sifting
  Occasionally, the plant material undergoes a grinding/milling and sifting process in order to improve the extraction of the compounds. When grinding/milling, smaller particles are obtained, increasing the contact surface and enhancing the extraction of the compounds; at the same time, sifting produces uniform sized particles. Generally, sieve sizes vary between 5 and 0.2 mm (Hilbay et al. 2016). However, if the particles are too fine, lumps/clumps can be formed and make the extraction difficult.
  It is recommended to perform this process just before extraction. In contact with air, the plant material not ground/milled slowly loses its volatile compounds. Nevertheless, a higher reduction of these compounds is observed when it is ground/milled (Muñoz 1996).

Extraction

Several extraction techniques are available. Nevertheless, when the chemical nature of the involved components with biological activity is known (once more the information search is crucial), the extraction methods must lead to the acquisition of these components with the highest yield and purity possible. Traditional healers mainly use water to prepare the medicines, but it has been found that the majority of the active components that have been identified from plants are poorly soluble in water, while extracts from organic solvents have shown a greater biological activity (Ncube et al. 2008; Das et al. 2010). However, if the active compound is unknown, a generalized method cannot be described.

When the chemical composition is unknown, the extraction method can be based on the information about how the plant is used in popular medicine, or the investigator can perform several extractions with solvents of increasing polarity to make sure to extract most of the compounds. The extraction with solvents of increasing polarity is the most useful method since it allows one to separate the components of a complex mixture and see which is the fraction or fractions that show activity. On the other hand, plant essential oils, which are a complex mixture of volatile components, mainly terpenes and terpenoids are also worth recovering and to do this step, the most utilized technique is still steam distillation.

The extraction process is a very critical stage because the active components can be lost if the technique is not appropriate. Moreover, an inadequate extraction method can cause the decomposition of the natural product (Rates 2001; Ncube et al. 2008; Das et al. 2010; Nazzaro et al. 2017). Therefore, when the active compound is unknown, the plant material has to be separated into two parts: one part for obtaining the essential oils and the other for extraction with solvents of increasing polarity. It is important to determine the amount of plant material needed to obtain a sufficient amount of extract and/or essential oils in order to perform all subsequent tests. Successful extraction of active components will depend on the solvents used at this stage; thus, the solvent properties should include low toxicity, ease of evaporation, fast absorption, preservative action, and not cause the extract to complex or dissociate (Das et al. 2010).

The extracts (hexene, methanol, aqueous, etc.) can be obtained by maceration at room temperature for 12–24 hours with periodic stirring. The longer the solvent and plant material are in contact, the greater the extraction of the plant compounds until a chemical equilibrium is reached. Similarly, stirring the mixture and increasing the solvent-plant material ratio improves the extraction of the compounds. The ratio of solvent and dry plant material that is generally used is 10:1 (v/w). Depending on the plant, the part used, and the information gathered previously; the extraction time, the pH, the temperature, the size of the particle, and the relation between the amount of solvent and plant material can be modified (Das et al. 2010). Once the extract has been obtained, it must be filtered and then the solvent removed by concentration under vacuum and lyophilized until a dry residue is obtained. Extraction and evaporation should be performed at low temperatures so as not to alter thermolabile components. For aqueous extracts, only lyophilization is generally used (Brusotti et al. 2014). The recommended procedure for extracting compounds from medicinal plants is outlined in Fig. 17.2.

Evaluation of In Vitro Biological Activity: Emphasis on Antimicrobial Activity

Using the ethno-pharmacological approach as a guiding thread, the evaluation of the biological activity of plant-based products is necessary in order to validate the traditional use and to search for the most active extracts. Discovering active compounds from plants requires a multidisciplinary approach in which success depends on a well-chosen set of in vitro and in vivo tests.

The extracts obtained are assayed using biological tests selected based on the alleged bioactivity. For each biological property to be studied, the available in vitro assays should be used first since the in vitro bioassays are faster and require smaller amounts of samples (Cos et al. 2006; Brusotti et al. 2014). The choice should optimally combine simplicity with good sensitivity and reproducibility. Internationally accepted and standardized methods should be chosen.

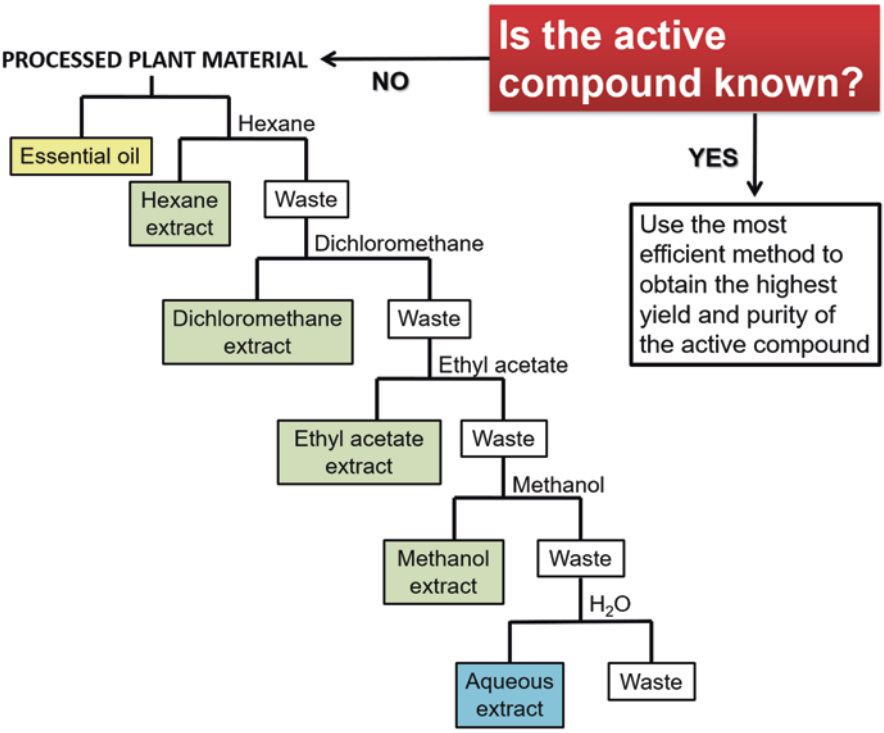

Fig. 17.2 Stages of the extraction process from medicinal plants

Due to a great diversity of available assays for each biological property (antibacterial, antifungal, antiviral, antiparasitic, antineoplastic, antioxidant, etc.), this discussion will only focus on in vitro assays directed toward antibacterial and antifungal activities that meet these requirements. Other available in vitro antimicrobial activity methodologies are described in the review published by Balouiri et al. (2016).

Currently, there is no consensus on which is the most appropriate methodology to determine the antifungal and antibacterial activity of plant extracts, since they are complex mixtures of compounds. However, the Clinical & Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standardized reference methods to evaluate in vitro antibacterial and antifungal activities for pure compounds. Laboratory procedures and conditions set forth in these documents can be adapted and used for plant extracts to perform comparable and reproducible tests and results.

CLSI and EUCAST broth microdilution methods allow a quantitative evaluation of the in vitro inhibitory activities of a component against bacteria and fungi. The minimum inhibitory concentration (MIC) is obtained, defined as the lowest concentration of the compound or drug to inhibit the growth of microorganism, expressed in $\mu g.mL^{-1}$ or $mg.L^{-1}$. The main differences between the CLSI and the EUCAST methods lie in the inoculum size and the reading method, visual in the case of CLSI

and spectrophotometric in EUCAST. To evaluate the biological activity of plant-based products, we recommend the CLSI document because the spectrophotometric reading could be affected by colored extracts or turbidity as a consequence of the chemical and hydrophobic nature of some components present in the extracts (Balouiri et al. 2016).

The broth microdilution method requires the preparation of two-fold serial dilutions of the "antimicrobial" to be tested (e.g., 64, 32, 16, 8, 4, 2, and 1 $\mu g.mL^{-1}$) in a liquid culture medium dispensed in a 96-well microplate. Subsequently, the plates are inoculated with a standardized suspension of the microorganism to be tested. Once the incubation period is done, the plate is examined, and the MIC determined (Cantón et al. 2007; CLSI 2020). Following the steps set out in the CLSI documents allows the tests to be reproducible (inter- and intralaboratory), and the results to be comparable with other antimicrobial agents. Nevertheless, some modifications to the standardized protocol are necessary in order to test natural products. Those modifications must be the least possible alterations to the protocol. In the following section we give some considerations that have to be taken into account using these techniques:

- For fastidious microorganisms with strict nutritional requirements, it is necessary to make some modifications, such as those proposed by Rojas et al. (2014) to study the susceptibility of *Malassezia* spp. Likewise, some authors have proposed modifications to improve the MIC reading based on the use of resazurin or tetrazolium salts (Balouiri et al. 2016); however, as it was previously said, the established protocol should be followed as strictly as possible.
- To define the in vitro activity of an antimicrobial agent against a microorganism, reference methods include categories for clinical use, such as being susceptible, susceptible-dose dependent, resistant, or nonsusceptible. These categories allow one to infer whether a treatment at a defined dose of antimicrobial agent can stop the infection caused by a specific microorganism. Plant extracts and pure components isolated from plants are not discussed in the reference methods, therefore, can only be used to obtain MIC values and usually cannot make categorical interpretations (CLSI 2020).
- In the reference documents, the MIC reading endpoint can be MIC-0, MIC-1, and MIC-2. These are defined as the lowest concentration of an antimicrobial agent capable of inhibiting 100%, ≥80% and ≥50% growth, respectively, as compared with the antimicrobial agent-free growth-control well. The MIC reading endpoint varies according to the drug and the microorganism to be tested; for example, for itraconazole, MIC-0 is used for filamentous fungi and MIC-2 for *Candida* spp. The selection of the MIC reading endpoint is based on the distribution of MIC, pharmacokinetic and pharmacodynamic parameters, animal models, treatment, and clinical evaluation (Espinel-Ingroff et al. 2012). Therefore, as there is no consensus for plant extracts, it is necessary to determine the three reading endpoints.
- Dimethyl sulfoxide (DMSO) is recommended for use as a solvent for plant extracts and isolated compounds because of its advantages from other solvents.

However, it is important to take into account that DMSO is toxic for many microorganisms; therefore, to avoid inaccurate results, the final concentration in each well should be ≤1% (CLSI 2020).

- The test microorganisms will be determined after considering the traditional ethnomedical use of the plant. Sometimes it is necessary test the extract or the isolated compound activity against different genera and species of microorganisms (e.g., Gram-positive and Gram-negative bacteria, yeast, filamentous hyaline and dematiaceous fungi, etc.). To obtain conclusive statistical values, the test should be carried out against a considerable number of strains of the same species. An antimicrobial agent can show different MIC against different microorganisms even against isolates of the same species.
- Reference strains and positive controls have to be included in order for the assay to be considered a reproducible and comparable method. The positive controls can be, for example, antimicrobial drugs in clinical use (penicillin, fluconazole, etc.). For a correct interpretation of the microplate, it is recommended to use the distribution showed in Fig. 17.3.

One key point in the evaluation of the in vitro activity of medicinal plant-based products is to establish the concentrations at which an extract is considered active for a particular biological activity and for a specific assay method. Often, different opinions are found on what is the significant concentration of the biological activity of an extract. Some authors use excessively high concentrations of plant extracts to identify a biological property. Paracelsus' well-known quote reads "*dosis sola facit venenum*" (it is the dose which makes a thing poison). The problem is that high concentrations result in a markedly increased incidence of false positives in vitro tests. This lack of criteria causes loss of time and money. It should be understood

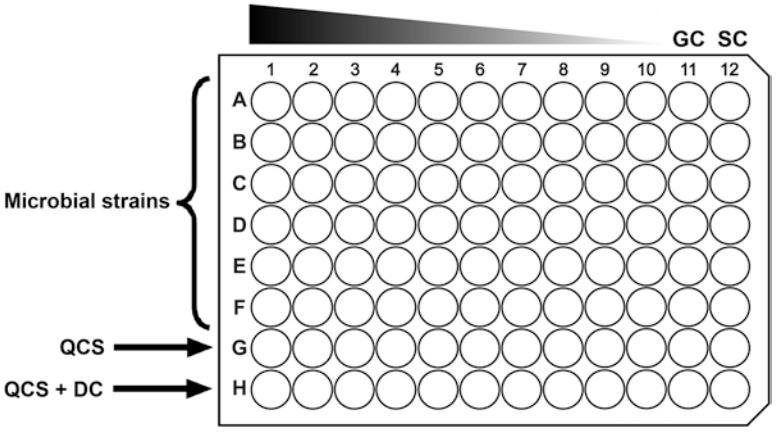

Fig. 17.3 Schematic distribution of the antimicrobial agent, microorganisms, and controls in the 96-well microplate to determine MIC. GC growth control, SC sterility control, DC drug control, QCS quality control strain

that the concentration determines the meaning. Unfortunately, there are no standards; therefore, the biological activity criteria should be well supported.

The in vitro evaluation method for the biological activity must be as sensitive as possible to allow the detection of weakly active compounds or those in low proportions in the extract; at the same time false positives have to be recognized and eliminated (Gertsch 2009). Using the broth microdilution method, we can establish the criteria to determine the antibacterial and antifungal activity, having in mind the following points:

1. Some authors consider that an extract is active with a value of MIC ≤1000 μg.mL^{-1} and ≤50 μg.mL^{-1} or ≤100 μg.mL^{-1} for a pure compound (Salvat et al. 2001; Holetz et al. 2002; Pessini et al. 2003; Svetaz et al. 2004; Malheiros et al. 2005; Sanches et al. 2005; Tanaka et al. 2006; Kuete 2010; Alvino Leite et al. 2015). Other authors establish more stringent values: MIC ≤100 μg.mL^{-1} for an extract and MIC ≤25 μM for a pure compound (Cos et al. 2006; Brusotti et al. 2014). Nevertheless, it is important to know that extracts are a complex mixture of compounds present in different proportions and that, in few occasions, the active compound is found in a proportion higher than 20%. On the other hand, according to CLSI, certain drugs categorized as susceptible against specific microorganisms present higher MIC values as it is the case of sulphonamides against *Salmonella* spp. and *Staphylococcus* spp. with a value of MIC ≤256 μg.mL^{-1} (CLSI 2020). Hence, by using too strict a criterion, the opportunity to detect potential active compounds could be missed.
2. At MIC values ≥2000 μg.mL^{-1}, the incidence of false positives increases significantly, that is, observation of growth inhibition as a result of using high and toxic concentrations. Moreover, technical difficulties may arise for the reading of the MIC due to turbidity or low solubility of the extract (Cos et al. 2006).
3. CLSI documents test MIC values that are exponential values of the number 2 raised to a positive or negative integer, that is, 2^x (CLSI 2018).
4. Some authors make a classification of the active extracts (Holetz et al. 2002; Pessini et al. 2003; Tanaka et al. 2006; Kuete 2010; Alvino Leite et al. 2015).
5. The application of a less rigorous endpoint, such as MIC-2, represents a better endpoint reading of the in vitro activity of some compounds (CLSI 2017a). On the other hand, CIM-2 and CIM-1 are associated with fungistatic/bacteriostatic drugs while CIM-0 is usually associated with drugs of biocidal action (CLSI 2017b, 2018). Therefore, use of CIM-2 would allow us to detect potential compounds with antimicrobial activity that would not be detected with CIM-0.

Based on the previous points, using the broth microdilution method, the antibacterial/antifungal activity of an extract can defined as follows.

The extract is considered:

- Inactive: MIC-2 >1024 μg.mL^{-1}
- Low/weak activity: MIC-2 ≥512–1024 μg.mL^{-1}
- Moderate activity: MIC-2 ≥128–<512 μg.mL^{-1}
- Strong/good/significant activity: MIC-2 <128 μg.mL^{-1}

However, the activity of the extract will be determined by the potency of the active compound and its proportion in the extract. Moreover, synergistic or antagonistic interactions among compounds present in the extract may occur.

A pure compound is considered:

- Inactive: MIC-2 >256 μg.mL^{-1}
- Low/weak activity: MIC-2 ≥128–256 μg.mL^{-1}
- Moderate activity: MIC-2 ≥16–<128 μg.mL^{-1}
- Strong/good/significant activity: MIC-2 <16 μg.mL^{-1}

For a better characterization of the activity, another parameter to evaluate is the minimum bactericidal concentration (MBC) or the minimum fungicidal concentration (MFC). MBC and MFC are defined as the concentration of antimicrobial agent needed to kill 99.9% of microorganisms compared to the initial inoculum. These parameters are determined from the microdilution plate, after the MIC reading, by subculturing the contents of the wells in which no growth was observed. After incubation, the number of colonies forming units per milliliter is calculated (UFC.mL^{-1}) and the MBC or MFC is determined. In certain cases, MBC and MFC have proved to be better predictors of the clinical response. Currently, only the standardized reference method for determining the MBC has been developed (CLSI 1999); however, methods have been proposed to determine the MFC from modifications of the standardized method for bacteria (Espinel-Ingroff et al. 2002; Pfaller et al. 2004).

To estimate if an extract or a pure compound has bactericidal, bacteriostatic, fungicidal, or fungistatic action, the MBC/MIC or MFC/MIC ratios can be used (Hazen 1998; Pfaller et al. 2004; Meletiadis et al. 2007).

Ratio ≤4 is considered bactericide/fungicide

Ratio >4 is considered bacteriostatic/fungistatic

Nevertheless, the most recommended in vitro method to determine the type of action is the time-kill assay. The most appropriate methodology to perform this test is described in the CLSI M26-A document and provides information whether the antimicrobial effect is time dependent or concentration dependent. Modifications to this method have been proposed for use against fungi. This method also allows to determine if a combination of two compounds has a synergistic, antagonistic, or no interaction effect against a specific microorganism (Pfaller et al. 2004). Another method widely accepted to determine the interaction between two compounds is the checkerboard assay (Meletiadis et al. 2002; Odds 2003).

Before conducting the broth microdilution assay, in cases where one has several extracts or isolated compounds, it is advisable to perform a screening test to determine the most promising sample(s). This procedure "pre-test" will save time and money.

As a screening method, the agar dilution test described in CLSI M07 document (CLSI 2018) with modifications can be performed, which has shown to have a high correlation with the broth microdilution method. The "antimicrobial agent" to assay is incorporated into the nutrient agar medium in a concentration to obtain a qualitative evaluation (active or inactive), 1024 μg.mL^{-1} for extracts, and 256 μg.mL^{-1} for pure compounds. The inoculum is applied onto the agar surface using a replicator.

After incubation, at least a 50% reduction in the growth of the tested microorganisms in comparison to the control must be observed. The main advantage of this test lies in the fact that it allows a qualitative evaluation of an extract against many microorganisms on the same plate. Modifications such as growth medium and incubation time may be necessary to perform a proper screening prior to performing the broth microdilution method.

Bioautography is another method that is used. It basically consists of a separation of the compounds present in the extract using thin-layer chromatography (TLC) and then detecting the fractions that produce microbial inhibition by overlaying on a microbial test plate. Even when bioautography separates and identifies the active fractions, it is necessary to determine the MIC using the broth microdilution method. Variations of this methodology are extensively described by Dewanjee et al. (2015).

Isolation and Identification of the Active Compound

Once the in vitro biological activity of extracts has been checked, the next step is to isolate and to identify the active compounds. In general, the integration of different separation methods is necessary. Otto Sticher (2008) reviewed aspects and practical applications of the main separation techniques.

Currently, bioassay-guided fractionation is one of the most modern techniques for identifying bioactive natural products. This approach involves the repetitive fractionation of the extracts and the evaluation of their biological activity until the isolation of the pure compounds with the selected biological activity. Brusotti et al. (2014) described different strategies of the bioassay-guided fractionation for the discovery of drugs from plant extracts.

The next step is to elucidate the structure of the active compound. Different techniques are applied, including HPLC- UV-DAD, HPLC-MS, GC-MS, HPLC-SPE-NMR, UHPLC-DAD-TOF-MS, among others. The selection of the techniques will depend on the chemical nature of the compounds and the equipment available. Combinations of sensitive analytic techniques (HPLC, GC) with spectroscopy methods (MS, NMR) allow faster identification of known components. HLPC and GC are widely used to create profiles and natural product fingerprints for quantitative analysis and the quality control. The HPLC connected to simple detectors can record chromatographic traces in order to create profiles or quantification (Brusotti et al. 2014).

In recent years, the application of modern analytical techniques in conjunction with metabolomics and network pharmacology would help to identify and discover new active compounds has been postulated. Natural product researchers begin to utilize collaborative computerized networks to identify relationships among metabolite data. This enabled to establish the world's largest data warehouse for natural products. This repository, named Global Natural Products Social Molecular Networking (GNPS), is a web-based mass spectrometry ecosystem that aims to be an open-access knowledge base and share raw, processed, or identified tandem mass

(MS/MS) spectrometry data. GNPS aids in identification and data from initial acquisition/analysis to post publication can be freely accessed at https://gnps.ucsd.edu (Newman 2020).

The fast identification of plant extract components is an important step in the medicinal plants' validation process, allowing utilization of financial resources and effort only on the most promising compounds. However, attention should also be paid to known molecules that may have the biological property under study, but this activity was never reported.

The objective of isolation and identification of the active compound from the medicinal plant is based on being able to attribute a biological activity to one or more specific compounds present in the plant. When the active compound is isolated, we make sure to eliminate compounds that may have a toxic or antagonistic effect as well as to corroborate any existence of a synergistic effect between the components of a mixture. Furthermore, it allows an investigator to obtain standardized products based on medicinal plants. The standardization of plant-based products is the process that establishes a set of standards or inherent characteristics, constant parameters, definitive qualitative and quantitative values, which guarantee quality, efficacy, safety, and reproducibility. This involves the adjustment of the product's preparation to give a defined compound amount with known biological activity, obtaining high quality products, and subjected to rigorous quality controls throughout the manufacturing process (Kunle et al. 2012).

Efficacy and Safety Assessment

To evaluate the safety profile and the active compound efficiency, a preclinical and a clinical phase must be designed. In general terms, the preclinical phase (set of in vitro tests and animal models) allows initial evaluation of the adverse effects. The aim is to reduce and anticipate the risk for humans. The clinical phase (clinical trials to human tests) seeks to prove the therapeutic efficacy. FDA and other drug-regulating organizations encourage researchers to contact them in every phase of the test. One of the most frequent causes of the interruption of the new drug development is the appearance of toxic effects while the lack of efficacy contributes in a smaller proportion.

The step toward animal testing depends on the bioactivity already demonstrated and the additional information on the animal with which the tests will be made. In the initial phases, in vitro tests take priority over studies in animal models. In vitro cytotoxicity evaluation assays against human cell lines represent a very important criterion in the validation of the safety of new drugs and should always be included in parallel with the in vitro biological activity evaluation tests. On the other hand, tests on lower animals should be prioritized over higher animals, for example: first tests are performed on *Caenorhabditis elegans*, *Danio rerio* (zebrafish), *Drosophila melanogaster*, or *Galleria mellonella*; then on rodents (rats, mice, hamsters, guinea pigs, etc.) and, finally, on mammals such as rabbits, monkeys, etc. These decisions

Table 17.1 Characteristics of clinical trial phases

	Phase 0 "Exploratory"	Phase I	Phase II	Phase III	Phase IV
Description	First-in-man early trial to determine if drug engages its expected target	Initial safety evaluations, determine safe dosage range, identify common side effects, study toxicity profile of the drug	Begin to explore efficacy while maintaining safety	Final confirmation of safety and efficacy	Any trials conducted after FDA approval of the drug
Number of subjects	10–15 healthy volunteers	20–80 healthy volunteers	100–300 volunteers with the targeted medical condition	1000–3000 subjects with the targeted medical condition	Number of subjects depends on trial endpoints
Dose	Single, low dose (<1% of dose calculated to produce a clinical effect)	Single dose Single ascending dose Multiple ascending dose	Multiple dose trials, often conducted against placebo	Multiple dose trials, ascending doses	Variable
Endpoint	Not expected to show clinical effect or significant adverse effects. Helps to choose between competing chemical analogs for further study	Escalation of dose ends when unacceptable side effects occur; the previous dose is considered the maximum tolerated dose	Explores clinical effects against the targeted condition and reveals the less-common side effects	Confirms clinical efficacy of the drug against the targeted condition and evaluates safety and side effects	Confirms clinical efficacy and safety and explores other possible drug uses; may be required as a condition of drug approval
Timing	Can be conducted with prior approval while final IND review is pending	Together with Phase 0 trials, first clinical trials conducted in an IND process	Conducted after report to FDA of results of Phase I trials	Conducted after report to FDA of results of Phase II trials	Conducted after release of the drug by the FDA for marketing

Adapted from Van Norman (2016)
FDA U.S. Food and Drug Administration, *IND* investigational new drug

are generally based on scientific, economic, and ethical reasons (Atanasov et al. 2015). Nonmammal animal models have the advantage that they require less working time, cost, and space. Moreover, they provide a better statistical value because the usual number of experimental animals is 300 individuals for *C. elegans* versus 30–50 in mice, increasing the reliability of the results.

Rats and mice tests are crucial for the evaluation and validation of the biological activity of natural products and isolated compounds. These animal models have

short reproduction cycles and human-like physiology. They provide a deeper pharmacological picture since they allow assessment of the efficacy and the pharmacokinetic, metabolic, and toxicological phenomena. In addition, the use of genetically modified rodents allows discovery of new pharmacological targets and to elucidate mechanisms of actions.

Nonrodent mammal species such as rabbits, pigs, and monkeys also are widely used in the preclinical phase. These animal models present limitations, not only a higher price, but also more pronounced ethical considerations. However, the FDA and EMA regulatory guidelines require safety tests on at least two mammalian species including a nonrodent before authorization of human trials (Atanasov et al. 2015). Due to considerations on animal welfare, high cost, and labor intensity, the number of tests in animal models is generally kept to a minimum.

During the verification of the compound efficacy in animal models, several parameters should be considered, such as the route of administration, the dose to be applied, the experimental reading, and the use of a positive control, if available. Besides, it should be borne in mind that reactions to treatment in animals cannot be extrapolated directly to humans in all cases since there are differences in the response between species. In addition, there are certain reactions that are difficult to determine in animals, such as headache and depression. Therefore, the step toward conducting human trials always constitutes a risk and in order to qualify for a clinical trial application there must be an adequate balance between benefit and risk.

Once the safety and efficacy of the compound in animals has been demonstrated, the next step is clinical trials. Clinical trials are divided into phase 0, I, II, and III trials. Postapproval surveillance trials are generally called phase IV trials. Characteristics of the different clinical trials phases are summarized in Table 17.1.

Concluding Remarks

The validation process of products based on traditional knowledge of medicinal plants is long, expensive, and faces many challenges. Most extracts and compounds obtained from medicinal plants are only evaluated in in vitro tests and do not follow the subsequent stages necessary to ensure their efficacy, safety, and quality. It is estimated that of 5000–10,000 compounds, only one reaches the market and the process takes approximately 15 years (Fig. 17.4). However, as the WHO Traditional Medicine Strategy 2014–2023 program emphasizes, this is the process necessary to validate or refute, with scientific language, the ethno-pharmacological properties of a medicinal plant and certify it. In addition, it processes favor the discovery of new chemical entities and the development of new drugs. Although natural products are not patentable, the use of a given natural product or a new synthetic compound using the information obtained from that natural product or the method of producing that substance can be patented (Tallmadge 2018).

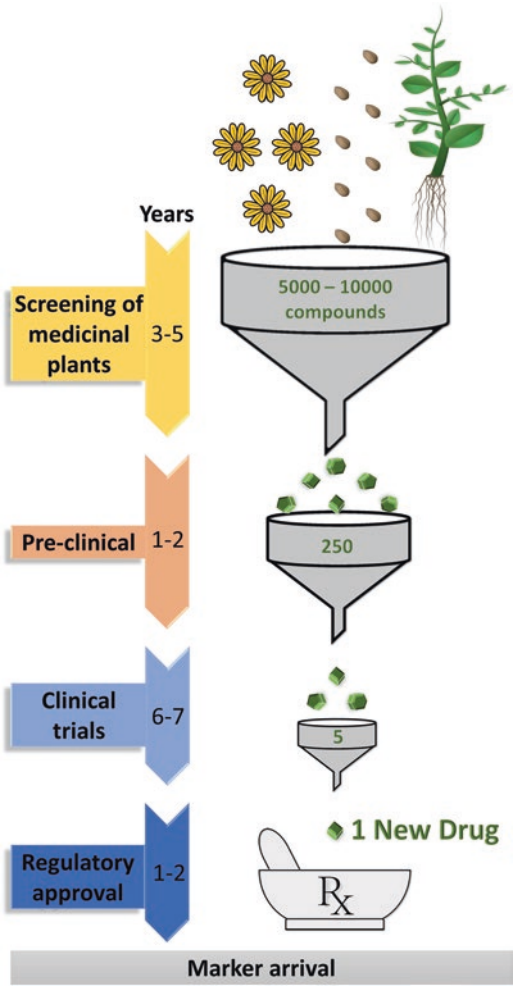

Fig. 17.4 Timeline of the discovery and development of new drugs from medicinal plants. (Adapted from Matthews et al. (2016))

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Part IV
Advances in Crop Protection and Biocontrol

New alternatives for crop protection are always a necessity for the agroindustry. With the rise in resistance to conventional control in many established pathogens and with more emergent diseases, it is imperative to use environmentally friendly and effective control. This part will present the recent information about several crop diseases in Latin American settings and novel alternatives for the control and biocontrol of established and emergent plant disease.

Chapter 18
Biological Control as a Key Tool for the Management of Invasive Species in Latin America and the Caribbean

Yelitza Colmenárez, Carlos Vásquez, Elisangela Gomes Fidelis, and Natalia Corniani

Abstract The attack of pests and diseases represents one of the main limitations for agricultural production in the Neotropical region. The intensification of trade between Latin American countries, the Caribbean, and other regions, among other factors, has resulted in the introduction of a large number of invasive pests in the Neotropical region, affecting crop production and causing large significant losses. Despite efforts to prevent their entry to Latin America and the Caribbean, through the establishment of quarantine systems in the different countries and the implementation of tactics to reinforce phytosanitary surveillance, each year new pests are reported to be introduced in areas where they were not present. This when added to the effects of climate change, represents a challenge for plant protection since it favours the displacement of pests to new areas due to the increase of temperature and changes in the climatic conditions, facilitating the establishment of some introduced species. This chapter presents the use of biological control agents through the implementation of programmes adapted to local conditions as a key strategy for the sustainable management of pests currently present in and potential pests to the region. Initiatives are also presented to strengthen the quarantine system and phytosanitary surveillance in a joint effort with institutions and government agencies of the countries where CABI implements the Plantwise and Action on Invasives programmes, and sustainable production projects with the objective of reinforcing food security in Latin American and the Caribbean countries.

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Keywords Biological control · Invasive species · Climate change · Neotropic · Plant protection

Introduction

Importance of Preventing the Entry of Invasive Species

Natural environments are continuously submitted to severe transformations, including movement of species beyond the limits of their native geographic ranges into areas in which they do not naturally occur, where they can inflict substantial changes (Gaston 2009). Thus, considering changes inflicted by alien species on the properties of an ecosystem an increasing number of studies that consider the environmental impacts have been published (Blackburn et al. 2014). However, according to Ricciardi et al. (2013) predictive understanding of the ecological impacts of invasive species has developed slowly, owing largely to an apparent lack of clearly defined hypotheses and of a broad theoretical framework. In this regard, confusion about terminology used for the designation of non-indigenous species, which alternatively have been called 'exotic', 'introduced', 'invasive', and 'naturalised', is particularly acute, leading to confusion about ecological concepts (Colautti and MacIsaac 2004).

After an alien species invasion, strategies addressing preservation or restoration of healthy ecosystems should be developed which include an initial survey of native and alien species (and their impacts) to define a base for comparison as the programme progresses (Wittenberg and Cock 2001).

Klapwijk et al. (2016) divided strategies against invasive species into three categories: prevention and interception, early detection and surveillance, and reporting and management.

1. *Prevention and interception*: Both are considered the first and most cost-effective options. According to Wittenberg and Cock (2001), exclusion methods should be based on pathways rather than on individual species since the former will provide the most efficient way to concentrate efforts at sites where pests are most likely to enter and thus intercept several potential invaders linked to a single pathway. Prevention of invasions includes interception based on regulations enforced with inspections and fees, treatment of material suspected to be contaminated with non-indigenous species, and prohibition of particular commodities in accordance with international regulations (Wittenberg and Cock 2001; Klapwijk et al. 2016).
2. *Early detection and surveillance*: During the early stages of invasions the invasive species are generally rare, making it difficult to detect them. Checking at

potential entry points (ports, airports, etc.) and in sensitive areas is mandatory since monitoring would improve the capacity to respond quickly to pest invasions, although it requires extensive resourcing and enforcement from the central authorities (Klapwijk et al. 2016).

3. *Reporting and management:* Eradication and/or prevention of spread of the detected invasive species is a crucial step carried out by the National Plant Protection Organisations (NPPO) based on pest risk analyses. However, this is hampered due to variable inspection protocols across different countries, even being visual inspections, which are less effective, and on the other hand, once biosecurity is breached, responses by each country may also be different, including not reporting or delaying reporting of incursions even of high-risk organisms (Brasier 2008). In fact, official reports of invasive organisms' presence are often made several years after detection, allowing invasive species to spread before eradication measures can be taken (Landeras et al. 2005).

Although eradication is often costly, it could be efficient, mainly when referred to invasive species with low level populations, low reproductive rates, and no dormant life stages (such as vertebrates, especially mammals) (Clout and Veitch 2002). For success to be achieved, eradication programs must meet a set of conditions including proper planning, a commitment to complete, putting the entire population of the target species at risk, removing them faster than they reproduce, and preventing reinvasion, and support from local people is desirable. Table 18.1 shows a description of the invasive species reported in Latin America and the Caribbean region.

Importance of Biological Control for Invasive Species Management

Many of the most important insect and mite pests, nematodes, and plant pathogens as well as the majority of the most invasive weed species are exotic, and these invasive species cause severe damage to agricultural, forest, and urban ecosystems costing billions of dollars annually and threatening the integrity of natural environments and the viability of endangered species (Perrings et al. 2002; Foy and Forney 1985).

Classical biological control constitutes a cost-effective and sustainable management strategy that potentially can mitigate costs and impacts of biological invasions on biodiversity. Classical biological control can be used to manage populations of a wide variety of invasive species (invasive plants, invertebrates, plant pathogens, and some vertebrates) that negatively impact biodiversity and ecosystem services (IUCN 2018).

Literature reports show various examples of successful cases in which biological control has contributed in the control of invasive species. Thus, populations of the rubber vine (*Cryptostegia grandiflora*), an asclepiadaceous species native to South West Madagascar that became invasive in Queensland (Australia) was effectively controlled by the Madagascan rust fungus, *Maravalia cryptostegiae*, which reduced

Table 18.1 List of invasiveinsectspecies in Latin America and the Caribbean region

Species	Comments about the species	Distribution
Acromyrmex octospinosus (Hymenoptera: Formicidae)[a]	Leaf-cutting ants *Acromyrmex octospinosus* are regarded as serious pests of crops. In the wild, they are a threat to many species of native plants that are vulnerable to defoliation.	Guadeloupe
Aedes albopictus (Diptera: Culicidae)[a]	The Asian tiger mosquito is spread via the international tire trade (due to the rainwater retained in the tires when stored outside). The tiger mosquito is associated with the transmission of many human diseases, including the viruses dengue, West Nile, and Japanese encephalitis.	Argentina, Barbados, Bolivia, Brazil, Cayman Islands, Chile, Colombia, Costa Rica, Cuba, Dominican Republic, Guatemala, Honduras, Mexico, Nicaragua, Panamá, Paraguay, Trinidad and Tobago, Venezuela
Anoplolepis gracilipes (Hymenoptera: Formicidae)[a]	*Anoplolepis gracilipes* (so-called because of its frenetic movements) has invaded native ecosystems and caused environmental damage from Hawaii to the Seychelles and Zanzibar. On Christmas Island in the Indian Ocean, it has formed multi-queen supercolonies. It is also decimating the red land crab (*Gecarcoidea natalis*) populations. Crazy ants also prey on, or interfere in, the reproduction of a variety of arthropods, reptiles, birds, and mammals on the forest floor and canopy. Their ability to farm and protect sap-sucking scale insects, which damage the forest canopy on Christmas Island, is one of their more surprising attributes. Although less than 5% of the rainforest on Christmas Island has been invaded so far, scientists are concerned that endangered birds such as the Abbott's booby (Sula abbotti), which nests nowhere else in the world, could eventually be driven to extinction through habitat alteration and direct attack by the ants.	Bolivia, Chile, Mexico, Panama

(continued)

Table 18.1 (continued)

Species	Comments about the species	Distribution
Anthonomus grandis (Coleoptera: Curculionidae)[a]	*Anthonomus grandis* is a brown to greyish-brown beetle native of Mexico to Central America and invasive in the United States. A. grandis feeds and develops only in cotton and closely related tropical (malvaceous) plants. In temperate zones *A. grandis* spends the winter in an adult reproductive dormancy where it subsists without food until it returns to cotton in the early spring. In sub-tropical and tropical areas adults are periodically active during warm periods of the non-cotton production seasons and will feed and reproduce whenever suitable hosts are available. *A. grandis* has caused serious losses to the cotton industry in the United States. Recent eradication programs and management strategies have reduced *A. grandis* populations dramatically and have prompted a rebound in the cotton market within the United States.	Argentina, Brazil, Colombia, Paraguay, Venezuela
Apis mellifera *scutellata* (Hymenoptera: Apidae)[a]	Warwick Kerr brought *Apis mellifera scutellata* from Africa to South America in 1957 to help revive the failing Brazilian beekeeping industry, which was using various European subspecies of *Apis mellifera* L., unsuitable for the South American environment. The queens and workers of several colonies were accidentally released, and these aggressive bees hybridized with local colonies. *A. m. scutellata* has been gradually spreading ever since, causing economic, social, and ecological problems due to the more aggressive behaviour shown by these hybrid bees.	Puerto Rico
Bemisia tabaci (Hemiptera: Aleyrodidae)[a]	This whitefly species has been reported from all continents except Antarctica. Over 900 host plants have been recorded for B. tabaci, and it reportedly transmits 111 virus species. It is believed that *B. tabaci* has spread throughout the world through the transport of plant products that were infested with whiteflies. Once established, *B. tabaci* quickly spreads and through its feeding habits and the transmission of diseases, it causes destruction of different crops around the world. *B. tabaci* is believed to be a species complex, with a number of recognized biotypes and two described extant cryptic species.	Brazil, Costa Rica, Curaçao, Dominican Republic, Jamaica, Mexico, Puerto Rico, Saint Kitts and Nevis, Saint Lucia

(continued)

Table 18.1 (continued)

Species	Comments about the species	Distribution
Ceratitis capitata (Diptera: Tephritidae)[a]	*C. capitate* is considered a major tephritid fruit fly pest of economic importance attacking more than 300 different hosts, primarily temperate and subtropical fruits. The medfly as it is commonly called has invaded many countries and caused major economic losses for fruit farmers. *C. capitate* shows the ability to tolerate cooler climates better than most other species of fruit flies. It lays its eggs under the skin of fruit, usually around already broken skin. Due to this reproduction habit, *C. capitate* thrives in agricultural areas where fruit is left out and becomes damaged. It spreads to new locations via exports and the local sale of fruit that contains eggs.	Argentina, Brazil, Colombia, Ecuador, Guatemala, Honduras, Panama, Nicaragua, Paraguay, Peru, Uruguay, Venezuela
Diaphorina citri (Hemiptera: Psyllidae)[b,c]	*Diaphorina citri* (Hemiptera: Psyllidae) is native to Southeast Asia and is considered one of the more important pests in Citrus and other rutaceous plants. Also, D. citri vectors cause a devastating disease called 'huanglongbing' or citrus greening disease transmitted by the bacterium *Candidatus Liberibacter asiaticus* that reduces fruit yield and quality. *D. citri* has been introduced into other regions, including Latin American and the Caribbean regions.	Antigua and Barbuda, Bahamas, Barbados, Belize, Brazil, Cayman Islands, Colombia, Costa Rica, Cuba, Dominica, Dominican Republic, Guadeloupe, Haiti, Honduras, Jamaica, Paraguay, Puerto Rico, United States Virgin Islands, Uruguay, Venezuela
Harmonia axyridis (Coleoptera: Coccinellidae)[a]	The harlequin ladybird is native to Asia and has been used extensively around the world for biological control of various aphid species. While it is a popular control agent, it has also brought with it several negative effects. Its establishment appears to decrease the diversity of native Coccinellidae. *Harmonia axyridis* can also quickly become a human nuisance when it seeks shelter during the winter months and takes up residency in the walls and insulation of houses and other structures. Surprisingly, *Harmonia axyridis* has also attained status as a pest of fruit production, particularly in the vineyards of the midwestern United States.	Argentina, Brazil, Venezuela

(continued)

Table 18.1 (continued)

Species	Comments about the species	Distribution
Homalodisca vitripennis (Hemiptera: Cicadellidae)[a]	The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar), is a xylem-feeding leafhopper native to southeastern United States and regions of northern Mexico. This insect is an important vector of the xylem-limited bacterium, *Xylella fastidiosa*. This pathogenic microorganism is responsible for many economically important diseases including phony peach disease, numerous leaf scold and scorch diseases, variegated citrus chlorosis, and Pierce's disease of grapes. This insect has been accidently introduced to California, Arizona, and several South Pacific islands where it threatens the grape and citrus industries. They lay their eggs inconspicuously below the epidermis of plant leaves which has allowed them to spread to new locations through the nursery trade at an alarming rate.	Chile, Honduras
Linepithema humile (Hymenoptera: Formicidae)[a]	The Argentine ant invades sub-tropical and temperate regions and is established on six continents. Introduced populations exhibit a different genetic and social makeup that confers a higher level of invasiveness (due to an increase in co-operation between workers in the colony). This allows the formation of fast-growing, high-density colonies, placing huge pressures on native ecosystems. For example, *Linepithema humile* is the greatest threat to the survival of various endemic Hawaiian arthropods and displaces native ant species around the world (some of which may be important seed dispersers or plant pollinators), resulting in a decrease in ant biodiversity and the disruption of native ecosystems.	Chile, Cuba, Peru, Mexico

(continued)

Table 18.1 (continued)

Species	Comments about the species	Distribution
Maconellicoccus hirsutus (Hemiptera: Pseudococcidae)[a]	The pink hibiscus mealybug is a polyphagous pest of a wide range of ornamental and agricultural plant species. Native to tropical and subtropical Asia and Africa, M. hirsutus forms colonies covered by a white waxy, elastic ovisac material. Feeding causes plant deformation and lowered aesthetics which can result in heavy economic losses. The overall potential annual cost of control and damages to the U.S. economy from *M. hirsutus* has been estimated to be around US$ 700 million, with the global estimate being around US$ 5 billion. While chemical and physical control methods are generally ineffective, effective biological control of *M. hirsutus* has been achieved in several countries.	Barbados, British Virgin Islands, Cayman Islands, Dominica, Guadeloupe, Grenada, Mexico, Montserrat, Puerto Rico, Saint Kitts and Nevis, Saint Lucia, Saint Martin, Trinidad and Tobago, Turks and Caicos Islands, Venezuela
Monomorium floricola (Hymenoptera: Formicidae)[a]	The primarily arboreal flower ant (*Monomorium floricola*) is one of the world's most broadly distributed tramp ants. Most occurrence records of M. floricola are in tropical and sub-tropical regions from latitudes above 30°; populations in latitudes above 35° are found in heated buildings or inside greenhouses. *M. floricola* has been identified as a significant arboreal predator of insect eggs; in Guam it is recognized as one of three most important ant species attacking eggs of native butterflies resulting in their reduced populations.	Antigua and Barbuda, Aruba, Bahamas, Barbados, Bolivia, Brazil, British Virgin Islands, Cayman Islands, Colombia, Costa Rica, Cuba, Curaçao, Dominica, Dominican Republic, Ecuador, Grenada, Guadeloupe, Guatemala, Haiti, Honduras, Jamaica, Martinique, Mexico, Montserrat, Nicaragua, Panama, Paraguay, Puerto Rico, Saint Kitts and Nevis, Saint Lucia, Saint Martin, Saint Vincent and the Grenadines, Trinidad and Tobago, Venezuela

(continued)

Table 18.1 (continued)

Species	Comments about the species	Distribution
Paracoccus marginatus (Hemiptera: Pseudococcidae)[d,e]	This mealybug species is native to Mexico and Central America, and currently it widely distributed in the Caribbean Island, United States, and other regions worldwide. P. marginatus is polyphagous that can damage a large number of tropical and subtropical fruits, vegetables, and ornamental plants and is a potential threat to commercial papaya plantations.	Antigua and Barbuda, Bahamas, Barbados, Belize, British Virgin Island, Cayman Island, Costa Rica, Cuba, Dominican Republic, Grenada, Guadeloupe, Guatemala, Haiti, Martinique, Mexico, Monserrat, Netherlands Antilles, Puerto Rico, Saint Kitts and Nevis, Saint Lucia, Saint Martin, U.S. Virgin Island, United States (Florida and Hawaii)
Paratrechina longicornis (Hymenoptera: Formicidae)[a]	The crazy ant is a tramp ant, which, by definition, is an ant that is widely dispersed through commerce and other human-assisted avenues. It is extremely easy to identify by observing its rapid and erratic movements. *Paratrechina longicornis* is highly adaptable to various environments and can be a major pest. It occurs in large numbers in homes or outdoors and is capable of displacing other ants and possibly other invertebrates. *Paratrechina longicornis* forages over long distances away from its nest, making the nest hard to find and the ants difficult to control.	Anguilla, Antigua and Barbuda, Argentina, Aruba, Bahamas, Barbados, Brazil, British Virgin Islands, Cayman Islands, Chile, Colombia, Costa Rica, Cuba, Dominica, Dominican Republic, Ecuador, Grenada, Guadeloupe, Guatemala, Haiti, Honduras, Jamaica, Martinique, Mexico, Montserrat, Nicaragua, Panama, Paraguay, Peru, Puerto Rico, Saint Kitts and Nevis, Saint Lucia, Saint Martin, Saint Vincent and the Grenadines, Trinidad and Tobago, Turks and Caicos, Venezuela

(continued)

Table 18.1 (continued)

Species	Comments about the species	Distribution
Pheidole megacephala (Hymenoptera: Formicidae)[a]	*Pheidole megacephala* is one of the world's worst invasive ant species. Believed to be native to southern Africa, it is now found throughout the temperate and tropical zones of the world. It is a serious threat to biodiversity through the displacement of native invertebrate fauna and is a pest of agriculture as it harvests seeds and harbours phytophagous insects that reduce crop productivity. *Pheidole megacephala* is also known to chew on irrigation and telephone cabling as well as electrical wires.	Brazil, Chile, Cuba, Ecuador, Mexico, Puerto Rico
Philornis downsi (Diptera: Muscidae)[a]	Adult flies feed on fruit, but larvae are semi-hematophagous (blood and tissue-feeding) parasites of birds. *P. downsi* larvae were first discovered in finch nests on Santa Cruz Island in 1997, although retrospective examination of insect collections show that the fly was present in the Galapagos Islands as early as 1964. Since then the parasite has spread to 12 of the 13 main Galapagos Islands and its larvae have been found in 64–100% of Darwin's finch nests. The blood sucking larvae cause mortality in up to 76% of nestlings. For this high impact, it is given the highest risk ranking amongst introduced insects and amongst diseases/parasites.	Ecuador
Raoiella indica (Acari: Tenuipalpidae)[f]	R. indica feeds on abaxial surface of arecaceous and musaseous plants showing yellowish on both surfaces of the leaflets. Both eggs and active stages of this mite are dark red in colour with black markings. *R. indica* is considered as an important pest of coconut in the Caribbean region and South America and potentially can cause damage in banana and plantain.	Barbados, Brazil, Colombia, Cuba, Dominica, Dominican Republic, Grenada, Guadeloupe, Guatemala, Haiti, Jamaica, Martinique, Puerto Rico, Saint Lucia, Saint Vincent and the Grenadines, Trinidad and Tobago, United States Virgin Islands, Venezuela

(continued)

Table 18.1 (continued)

Species	Comments about the species	Distribution
Rhynchophorus ferrugineus (Coleoptera: Curculionidae)[a]	The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Dryophthoridae), is widely distributed in southern Asia and attacks various palm species such as Phoenix sylvestris, Cocos nucifera, and *Metroxylon sago*. Recently it was accidentally introduced into the Caribbean where a monitoring program was established to determine the population level and distribution of infestations on Aruba and Curacao using commercially available pheromone traps.	Aruba, Curaçao, Netherlands Antilles
Schizotetranychus hindustanicus (Acari: Tetranychidae)[g]	The Hindu mite or citrus nest-webbing mite was originally described from citrus from southern India. In the early 2000, it was reported in northwestern Venezuela, Colombia, and Brazil. Feeding symptoms appear on the upper leaf surface along the main rib, later extending to the entire leaf blade; fruits become uniformly silvered and hard under severe infestation levels.	Brazil, Colombia, Venezuela
Scyphophorus acupunctatus (Coleoptera: Dryophthoridae)[a]	This species is becoming a major pest of native Agavaceae and Dracaenaceae species worldwide. Native to Mexico, it has decimated populations of agave crops, particularly those species used in the tequila and henequen industries. The importation of ornamental agave plants worldwide has facilitated *S. acupunctatus*' establishment in many parts of the world, particularly in Central America and the Caribbean, in Africa, Asia, and South America. On its host species, it causes rot and sometimes mortality due to its larvae boring holes which then facilitates the micro-organism entering the host. Due to the species being found generally inside the host species, typical insecticides have proven ineffective. However, research on the species' pheromones has shown that these could be a potential management tool, attracting individual adults away from hosts to collection sites.	British Virgin Islands, Cayman Islands, Costa Rica, Cuba, Dominican Republic, Haiti, Guatemala, Honduras, Jamaica, Nicaragua

(continued)

Table 18.1 (continued)

Species	Comments about the species	Distribution
Solenopsis geminate (Hymenoptera: Formicidae)[a]	This ant has spread almost worldwide by human commerce. It usually invades open areas but can easily colonise human infrastructure and agricultural systems, such as coffee and sugarcane plantations in hot climates. Its greatest known threats are its painful sting and the economic losses due to crop damage caused by its tending of honeydew-producing insects. *Solenopsis geminate* is known to reduce populations of native butterfly eggs and larvae. It has the potential to displace native ant populations but is susceptible to competitive pressures from other ant species.	Antigua and Barbuda, Argentina, Bahamas, Barbados, British Virgin Islands, Ecuador, Trinidad and Tobago, Turks and Caicos Islands
Solenopsis invicta (Hymenoptera: Formicidae)[a]	*Solenopsis invicta* is an aggressive generalist forager ant that occurs in high densities and can thus dominate most potential food sources. They breed and spread rapidly and, if disturbed, can relocate quickly so as to ensure survival of the colony. Their stinging ability allows them to subdue prey and repel even larger vertebrate competitors from resources.	Bahamas, British Virgin Islands, Cayman Islands, Paraguay, Puerto Rico, Trinidad and Tobago, Turks and Caicos Islands
Solenopsis richteri (Hymenoptera: Formicidae)[a]	It is commonly known as the black imported fire ant and is native to South America. It builds large mounds that can reach 46 cm in height. *Solenopsis richteri* damages crops, impedes recreational activities, and can undermine roads and asphalt. It is also very dangerous to those who experience anaphylaxis from the venom of its bite. Eradication of *Solenopsis richteri* is not an option. It can be controlled but this is an ongoing process.	Paraguay
Steneotarsonemus spinki (Acari: Tarsonemidae)[h]	*Steneotarsonemus spinki* Smiley is distributed geographically in Asia, the Caribbean region, North, Central, and part of South America, and it is considered the most important rice pest worldwide. Other than rice, more than 70 species of plants have been reported as host including wild rice, Argentine grass, etc.	Costa Rica, Cuba, Haiti, Nicaragua, Panama, Puerto Rico, Colombia, Venezuela

(continued)

Table 18.1 (continued)

Species	Comments about the species	Distribution
Tapinoma melanocephalum (Hymenoptera: Formicidae)[a]	It is known as a tramp ant as its spread around the globe has been assisted by human activities. It is highly flexible in the habitats it occupies, providing there is some form of disturbance allowing it to establish ahead of more dominant ant species, and it nests readily outdoors or indoors. *Tapinoma melanocephalum* is a household pest, disturbs greenhouse environments, and can transport pathogenic microbes in hospitals.	Anguilla, Antigua and Barbuda, Aruba, Bahamas, Barbados, Brazil, Cayman Islands, Colombia, Costa Rica, Cuba, Dominica, Dominican Republic, Ecuador, Guadeloupe, Haiti, Honduras, Jamaica, Martinique, Mexico, Nicaragua, Panama, Paraguay, Peru, Puerto Rico, Saint Kitts and Nevis, Saint Lucia, Saint Vincent and the Grenadines, Trinidad and Tobago, Venezuela
Tecia solanivora[i] (Lepidoptera: Gelechiidae)	*Tecia solanivora* is part of the complex of gelechid moths attacking potato (Solanum tuberosum L.). It is native to Central America and is considered in several countries of this area and in the Andean countries (Venezuela, Colombia and Ecuador) as one of the main pests of potato crops.	Costa Rica, Colombia, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama, Venezuela
Trogoderma granarium (Coleoptera: Dermestidae)[a]	*Trogoderma granarium* is considered a pest of considerable impact to stored foodstuffs. It maintains its presence in food storage in very low numbers and can survive long periods of time in an inactive state.	Mexico, Venezuela
Vespula germanica (Hymenoptera: Vespidae)[a]	It is commonly known as the German or European wasp and is a social wasp species. In introduced regions, where it is often more successful than in its native range, it efficiently exploits important food resources, such as nectar and insects, that native fauna may depend on. *V. germanica* displays many characteristics that make the species a successful invader, and a new colony can be established from a single inseminated female.	Argentina, Chile

(continued)

Table 18.1 (continued)

Species	Comments about the species	Distribution
Xylosandrus compactus (Coleoptera: Scolytidae)[a]	Originally from Asia, *Xylosandrus compactus* has spread to various coffee-growing areas throughout the world where it causes damage not only to agricultural crops but also to native forest trees. Beetles (Coleoptera) in the family Scolytidae, to which *Xylosandrus compactus* belongs, are among the most damaging insects worldwide. Because most scolytids breed under bark or inside wood, it has long been recognized that scolytids can easily be moved through international trade.	Brazil, Cuba

[a]ISSG (2009)
[b]Invasive Species Compendium (2020a)
[c]Quereshi et al. (2009)
[d]Mani et al. (2012)
[e]Invasive Species Compendium (2020d)
[f]Invasive Species Compendium (2020b)
[g]Invasive Species Compendium (2020c)
[h]Plantwise Knowledge Bank (2020)
[i]EPPO Global Database (2020)

the weed by up to 90% in some areas, allowing for more cattle production and reducing the costs of weed control, and also, experience gained in Queensland has benefitted other places where the rubber vine had colonized (https://www.cabi.org/projects/our-impact/biocontrol-of-invasive-species/).

Cassava mealybug (*Phenacoccus manihoti* Matile-Ferrero) was accidentally introduced in Africa causing damage to a staple crop that is particularly important in times of drought, leading to famine (Herren and Neuenschwander 1991). In the 1970s the cassava mealybug population erupted and spread rapidly, mainly due to it not having any natural enemies. Surveys carried out in Bolivia and Brazil found a native parasitic wasp which was introduced to Africa and within a decade had reduced the mealybug population by 95%.

Although the Asian citrus psyllid, *Diaphorina citri*, is native to Asia where is considered an important pest in citrus plantations, it is currently known to occur throughout the southern part of the United States, Caribbean islands, Central and South America, but invasion routes remain undetermined (Guidolin et al. 2014). The Asian citrus psyllid is currently the major threat to the citrus industry as it is the vector of *Candidatus Liberibacter*, the causal agent of huanglongbing disease (HLB) or 'greening' that seriously affects large numbers of citrus cultivars in several countries (Tsai et al. 2002). Efforts to control the Asian citrus psyllid have been made. Thus, *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae), a *D. citri* ectoparasitoid native to India, has been released in Réunion Island and Florida (United States) causing a significant reduction in 'greening' severity (Quereshi et al. 2009). Moreover, this parasitoid has been found in Brazil and Puerto Rico where releases have not been documented (Pluke et al. 2008).

The red palm mite, *Raoiella indica* Hirst is a notorious example of invasion. It was introduced in the Caribbean in 2004, in Martinique (Flechtmann and Etienne 2004), and rapidly moved to other islands (Rodrigues et al. 2007). Approximately, 5 years later it reached almost all Caribbean islands, Florida (United States), Mexico, Venezuela, Colombia, and the north of Brazil (Roraima State) (Carrillo et al. 2011; Kane et al. 2012; Návia et al. 2011; Vásquez et al. 2008). This tenuipalpid mite is of Oriental origin and it can cause severe damage to Arecaceae, especially coconut (*Cocos nucifera* L.), but also to Musaceae and other plant families (Flechtmann and Etienne 2004; Flechtmann and Etienne 2005; Etienne and Flechtmann 2006).

After its introduction into the region, some efforts have been made to search for natural enemies controlling the RPM. Thus, some studies searching for effective natural enemies have been carried out, resulting in the phytoseiid *Amblyseius largoensis* (Muma) as the most frequent predator associated with the pest on coconut in Americas (de Moraes et al. 2012; Gondim Jr. et al. 2012; Vásquez and de Moraes 2013).

As part of the efforts searching for native and exotic natural enemies of RPM, Brazilian researchers conducted surveys in Réunion Island and Thailand to identify potential biological control agents for this pest. In a study which compared the biology of a population of *A. largoensis* found in Réunion Island (Indian Ocean) with a population from Roraima State (northern Brazil), it was observed that the oviposition period, prey consumption, and net reproductive rate were significantly higher for the Réunion Island population, suggesting that further investigation could determine whether that population should be released in South America to control the pest (de Moraes et al. 2012; Domingos et al. 2013; Morais et al. 2016). Another population of *A. largoensis* and *Amblyseius cinctus* Corpuz-Raros & Rimando associated with the RPM on coconut palms in Thailand was also introduced in Brazil, and *A. largoensis* was efficient in controlling RPM. (Domingos et al. 2013). On the other hand, Colmenárez et al. (2014) carried out a survey to determine population trends and entomopathogenic fungi associated with the RPM in Trinidad, Antigua, St. Kitts and Nevis, and Dominica reporting 27 fungal isolates of which 15 isolates belonged to the genera *Cladosporium*, three to *Simplicillium* spp., and one to *Penicillium*, showing that *Simplicillium* and *Penicillium* isolates found in association with the RPM populations are of high potential for further use in pest management programs.

The papaya mealybug, *Paracoccus marginatus* Williams & Granara de Willink, is a poly-phagous pest, considered an important invasive species, was introduced in the Caribbean Islands and Florida (United States) in 1994–2002 and since then, it was rapidly reported as being present in different countries in Latin America and the Caribbean (CABI 2019). As part of the biological control, generalist predators such as *Cryptolaemus montrouzieri* Mulsant, lady beetles, lacewings, and hover flies have been reported as attacking the papaya mealybug (Walker et al. 2003). Encyrtid endoparasitoid wasps such as *Acerophagus papayae* (Noyes and Schauff), *Anagyrus loecki* (Noyes and Menezes), *Anagyrus californicus* Compere, and *Pseudaphycus* sp. were also reported as specific parasitoids of *P. marginatus* (Meyerdirk and

Kauffman 2001). From this group, *Acerophagus papayae* Noyes and Schauff has been highlighted as one of the most effective biocontrol agents of *P. marginatus* (Nisha and Kennedy 2016; Colmenárez et al. 2017). In the Caribbean, the introduction of some parasitoid species, such as *A. papayae*, reduced the pest population of *P. marginatus* from 82% to 97% (Meyerdirck and DeChi 2003).

Prediction Models and Climate Change Influence for the Establishment of New Invasive Species

Climate change is altering temperature, precipitation, the frequency of extreme weather events, atmospheric composition (mainly CO_2 concentration) and land cover, which are the key factors affecting species survival and, consequently, inducing stress of ecosystems (Simberloff 2000; Dukes and Mooney 1999). On the other hand, biological invasions are an important factor affecting biodiversity, being associated with nearly 60% of species extinctions (Bellard et al. 2018).

Predictions of the impact of climate change on biodiversity have frequently been based on an 'envelope' modelling approach which combines environmental variables and current distributions of species in order to predict distributions of species under future climate scenarios (Araújo et al. 2006; Thuiller et al. 2005). As invasion processes are a biological process, climate change is also expected to alter it (Bellard et al. 2018). Because climate change has a potential effect on fundamental biological processes, it will interact with other existing stressors influencing the distribution, spread, abundance, and impact of invasive species (Gritti et al. 2006). Although it is difficult to predict the effects of climate change on ecological systems, invasive species are likely to respond in ways that should be qualitatively predictable, and some of these responses will be distinct from those of native counterparts (Hellmann et al. 2008).

Several previous studies have stated that invasive species could be favoured by climate change (Thuiller et al. 2011; Vilà et al. 2007; Dukes and Mooney 1999); however, these studies have provided contradictory evidence, and no consensus has been reached (Bellard et al. 2018). Thus, more discussions about the distinctive consequences of climate change for invasive species are needed that evaluate key hypotheses to develop general theories and adaptive management about invasive species and climate change (Hellmann et al. 2008). In this regard, Harrington et al. (1999) have postulated two approaches for studying the impacts of climate warming on trophic interactions: to examine relationships between long-term or spatially extensive biological datasets and abiotic data, usually meteorological, available over a similar scale and to model interactions on the basis of experimentation that includes novel conditions expected in the future. Based upon the 'invasion pathway', these later authors identified five possible consequences of climate change, some of them being unique to invasive species because of traits and qualities

associated with invasion, and in other cases sharing qualitative responses with native species, but the mechanisms or the outcomes are distinct (Hellmann et al. 2008).

During recent decades, the number of new invasive alien species discovered or reported per annum (rates of invasive alien species) for a recipient region has been increasing all over the world (Huang et al. 2011). This fact has been attributed to increasing international trade (Westphal et al. 2008) but rarely linked to climate changes that can directly or indirectly influence the successful establishment of an introduced alien species in new regions (Walther et al. 2002).

Although human activities could promote species movement, their subsequent establishment and dispersion at the new environment is strongly associated with altered site conditions due to climate change (Walther et al. 2002). Using basic research on ecological and physiological processes that are sensitive to climatic variables such as temperature and precipitation, Walther et al. (2002) stated that warmer temperatures at the end of the twentieth century have affected the phenology of organisms, the range and distribution of species, and the composition and dynamics of communities. Bellard et al. (2018) demonstrated the role of climate change as a main factor for the future distribution of invasive alien species, and they found that climate change will more frequently contribute to a decrease in species range size than an increase in the overall area occupied for the plants and vertebrates studied while the ranges of invertebrates and pathogens are more likely to increase following climate change.

Barbet-Massin et al. (2018) assessed the predictive accuracy of species distribution models (SDM) in predicting the expansion of the Asian hornet (*Vespa velutina nigrithorax*), a species native to China that is invading Europe at a very fast rate. These authors compared occurrence data from the last stage of invasion (independent validation points) to the climate suitability distribution predicted from models calibrated with data from the early stage of invasion, and they observed that SDM could adequately predict the spread of *V. v. nigrithorax*, which appears to be partially climatically driven. Based on climate projections from general circulation models and statistical models, Capinha et al. (2013) evaluated future distributions for the threatened European crayfish fauna in response to climate change, watershed boundaries, and the spread of invasive crayfish, which transmit the crayfish plague, a lethal disease for native European crayfish. They observed that the number of suitable areas decreased for native crayfish; meanwhile the overlap with invasive crayfish plague-transmitting species was predicted to increase.

In regard to impacts of climate change on natural enemies of pest species, Thomson et al. (2010) summarized the following effects:

(a) Alteration of the fitness of natural enemies in response to changes in host/prey quality and size induced by temperature and CO_2 effects on plants
(b) Decrease of the susceptibility of herbivores to predation or parasitism by altering life cycles of herbivores in response to plant phenological changes

(c) Decrease of the effectiveness of natural enemies to exert biocontrol if pest is introduced into regions outside the range of distribution of their natural enemies although a new community of enemies might then provide some level of control
(d) Alteration of the abundance and activity of natural enemies through adaptive management strategies adopted by farmers to cope with climate change, since these strategies may lead to a mismatch between pests and enemies in space and time, decreasing their effectiveness for biocontrol

As the global climate change will provoke the potential breakdown of current biological control agents and consequently promote pest outbreaks, suitable approaches are needed to improve biological control (Thurman et al. 2017). According to van Lenteren (2012), increases of future pest damage could be counteracted by augmentative releases to maintain high densities of biological control agents even in suboptimal conditions. However, biological control agents performing well under specific environmental conditions will probably perform less efficiently when these conditions vary and thus we would expect that biological control agents suited for future climate scenarios will differ from those relied upon today (Thurman et al. 2017; Collier and van Steenwyk 2004). Consequently, efforts should be increased to identify biological control agents better adapted to the novel environmental conditions and be able to optimize control for pests under future climate scenarios (Thurman et al. 2017).

Because of the diverse and often indirect effects of climate change on natural enemies, predictions will be difficult unless there is a good understanding of the way environmental effects impact on tri-trophic interactions (Thomson et al. 2010). Probably parasitoids are significantly more affected by climate-induced perturbations, which will be modulated by direct effects on the organisms involved (effects on physiology and metabolism). The responses of those organisms and subsequent tri-trophic interactions and understanding what these effects might be is of critical importance (Furlong and Zalucki 2017).

Biological Control as a Key Tool for the Management of Invasive Species in Latin America and the Caribbean

Latin America and the Caribbean are the regions with the greatest biological diversity on the planet, and they host several of the world's megadiverse countries such as Brazil, Colombia, Ecuador, Mexico, Peru, and Venezuela (UNEP 2010; UNEP-WCMC 2016). South America harbours about 40% of the Earth's biodiversity, mainly in the Amazon rainforest which is the world's most biodiverse habitat (UNEP 2010, 2012), and high levels of endemism are observed in the region as 50% of the plant life of the Caribbean is unique, and this biodiversity also represents a source of abundant genetic resources for Latin America and the Caribbean region (UNEP 2010).

On the other hand, since LAC exhibits good climatic conditions it always has maintained a strong comparative advantage in agricultural production; thus LAC exported about 16% of global food and agriculture between 2012 and 2014. However intensive international trade increases the likelihood of pest species being introduced to this region, and thus challenges to crop production is higher insomuch as population is increasing rapidly (Fig. 18.1). The need to contribute to end hunger, achieve food security, and improve nutrition are key steps to sustainable development (UN 2019).

Impact of the Action on Invasive and Plantwise Programmes

Problems with invasive weeds, insects, plant diseases, and animals are increasing rapidly worldwide, consequently resulting in economic, social, and environmental impacts threatening the economic growth mainly of the world's most vulnerable people. In consequence, several international organizations, including CABI, are developing and implementing solutions for invasive species around the world based upon primary research to support global actions on the Action on Invasive Programme, helping to protect livelihoods and the environment.

In this regard, CABI's global Action on Invasive Programme is focused on an environmentally sustainable, regional, and cross-sectoral approach to managing

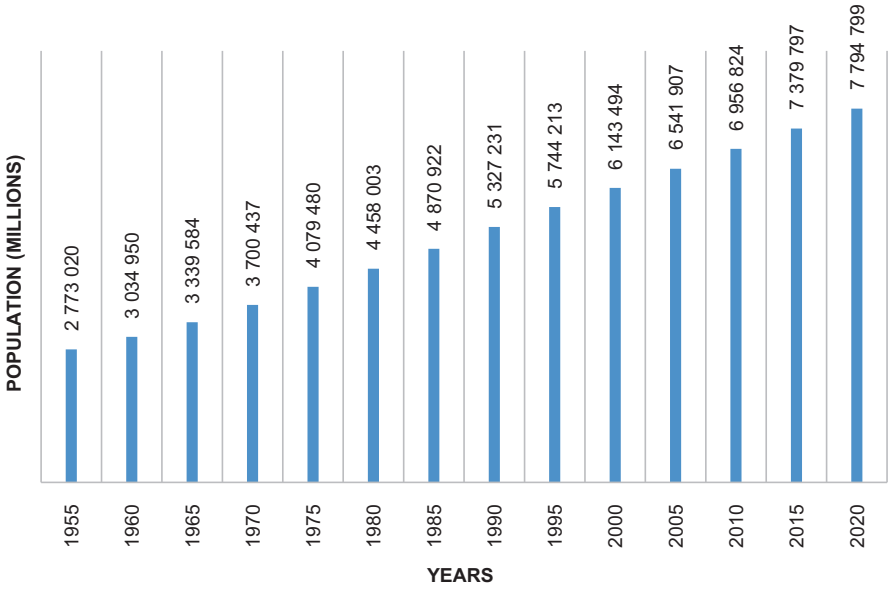

Fig. 18.1 World population increase. Figure developed by authors using World Population Prospects 2019 (UN 2019)

invasive species, which is based on a systems-based approach to managing biological invasions across sectors in three stages:

(a) Prevention: developing and implementing biosecurity policies to prevent the arrival and spread of invasive species and raising awareness of potential threats at a local level
(b) Early detection and rapid response: building capacity to develop and implement surveillance and emergency action plans for detecting and eradicating new invasions
(c) Control: scaling up existing invasive species management solutions, embedding control options in policy, and making sure that those living in rural communities have access to best practice and locally adapted solutions and are actively engaged in their implementation

CABI's global Action on Invasive Programme operates concomitantly with the Plantwise programme, which aims to help farmers to reduce their crop losses, working closely with national agricultural advisory services and establishing a global plant clinic network, where trained plant doctors are able to advise farmers to find practical solutions to crop management. Plant clinics work just like clinics for human health: farmers visit with samples of their crops, and plant doctors diagnose the problem and make science-based recommendations on ways to manage it. The Plantwise programme has been endorsed by member countries in 2011 as they recognized that CABI is well placed due to its network of centres in Africa (Kenya, Ghana), Asia (China, India, Malaysia, Pakistan), Europe (Switzerland, UK), and the Americas (Barbados, Bolivia, Brazil, Costa Rica, Grenada, Jamaica, Trinidad & Tobago, and Peru). Currently Plantwise has established a sustainable network of over 3700 plant clinics in 34 countries around the world (www.plantwise.org).

According to Colmenárez et al. (2019), the Plantwise approach is based on three inter-linked components:

1. An ever-growing network of locally run plant clinics, where farmers can find advice to manage and prevent crop problems. Trained agricultural advisory staff learn methods to identify any problem on any crop brought to the clinics, with the support of a national and international network of diagnostic laboratories, and provide appropriate recommendations guided by national and international best practice standards.
2. Improved information flows between everyone whose work supports farmers (e.g. extension, research, input suppliers, and regulators). Collaboration within national plant health systems enables these actors to be more effective in their work to improve plant health with concrete benefits for farmers.
3. The Plantwise knowledge bank, a database with online and offline resources for pest diagnostic and advisory services, provides both locally relevant, comprehensive plant health information for everyone and a platform for collaboration and information sharing between plant health stakeholders.

The plant clinic network is reinforced by the Plantwise Knowledge Bank, a gateway to practical online and offline plant health information, including diagnostic

resources, best-practice pest management advice, and plant clinic data analysis for targeted crop protection. Together, these two unique resources are part of the Plantwise approach to strengthen national plant health systems. The stronger the national plant health system, the better equipped the country will be to help farmers provide a safe and sustainable food supply and improve their livelihoods.

The problem of invasive species is not a recent issue, but climate change, trade, and tourism are all exacerbating the situation and increasing the need for effective responses at local, national, and regional levels. Thus, it is imperative that the sustainable development goals (SDGs) include a goal to 'introduce measures to prevent the introduction and significantly reduce the impact of invasive species on land and water ecosystems and control or eradicate the priority species'.

Action on Invasives is designed to enable countries and regions to adopt this approach through four interrelated work packages:

(a) Stakeholder engagement: fostering the right partnerships
(b) Providing best practice solutions for invasive species
(c) Community action: bringing information and action to scale
(d) Knowledge and data: creating and using knowledge

While the aim of Action on Invasives is to strengthen overall capacity to tackle invasive species, many of the activities focus on priority species as case studies. The first focus species are fall armyworm (*Spodoptera frugiperda*) (FAW), *Tuta absoluta*, and parthenium weed (*Parthenium hysterophorus*). Similarly, part of the national capacity involves regional and international collaboration; so Action on Invasives is working through selected countries as foci from which activities can be regionalized. The first countries for implementation are Ghana, Kenya, Pakistan, and Zambia.

As described by Colmenárez et al. (2016), the Plantwise theory of change refers to the following linkages that need to be strengthened (Fig. 18.2).

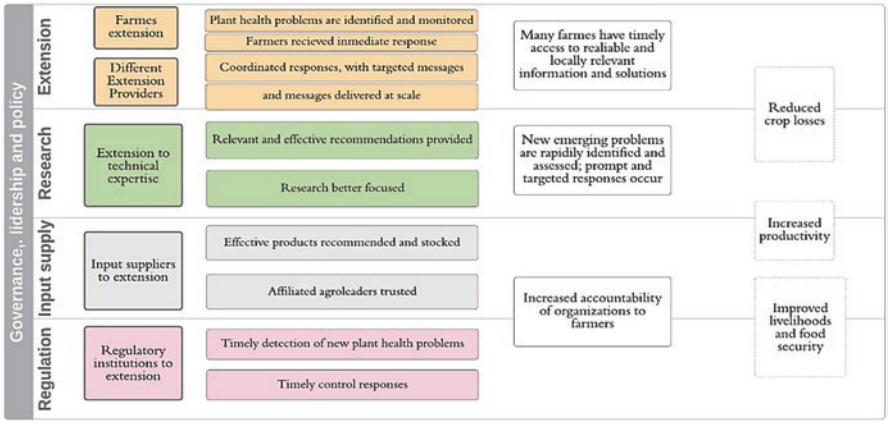

Fig. 18.2 Plantwise theory of change. (From Colmenárez et al. 2016)

Linkage between farmers and extension services in plant clinics: extension staff trained to diagnose plant disease or pest problems (plant doctor) place plant clinics so that farmers can bring any crop problem to the clinic.

Link different extension providers through clinics and the Knowledge Bank: regular meetings are made between plant doctors and plant clinic implementing organizations in order to share information on plant health problems and then this information is used in extension activities at different levels (local, district, or national).

Linkage between extension staff and technical expertise: there are networks of diagnostic laboratories associated with plant clinics for support in case of unknown problems. In these networks, researchers diagnose any new and emerging diseases and share their knowledge and expertise with plant doctors and then farmers. The Knowledge Bank supports extension staff with information about pests and plant health problems and records those encountered by farmers in their region.

Link extension and input suppliers: Plant clinics aim to work with trusted agro-input dealers to ensure that the products recommended by plant doctors are locally available and to promote codes of practice to help ensure ethical trading.

Extension staff should interact with government institutions: in cases where new pests are difficult to be identified at plant clinics or at outbreaks of the pest, government institutions (Ministry of Agriculture, NPPOs, etc.) are immediately informed in order to improve national pest lists as well as enabling early alerts to be issued and rapid response measures. The Plantwise Knowledge Bank provides a mechanism to capture data and enables those working in the national plant health and regulatory bodies to analyse the data as part of any pest risk analysis. The Knowledge Bank will allow countries to manage data in ways that will help them spot local problems before they flare up and become acute problems at the national level.

Some Case Studies of the Participation of CABI on the Management of Invasive Species Globally

Fall armyworm (*Spodoptera frugiperda*), a major maize pest in the Americas, has been found in Africa and Asia, and it has spread rapidly and is considered a serious invasive insect pest (FAO 2017). In the absence of any control method, the fall armyworm (FAW) has the potential to cause maize yield losses ranging from 8.3 to 20.6 million tons/year in just 12 maize-producing African countries, which can represent economic losses estimated at US$2.48–6.19 billion (Day et al. 2017). As part of integrated pest management practices, biological control plays an important role (FAO 2017; Day et al. 2017). FAO highlighted the importance of disseminating the management practices of FAW in order to ensure a proper management of the pest at field level. In this process, the CABI Plant Health Clinics, established as part of the Plantwise programme, have been considered as an important mechanism for

facilitating dissemination of the FAW management options to a wider number of smallholder farmers (FAO 2017).

Since its creation in 2009, Plantwise has expanded in several countries to the stage where it has directly reached about 1,900,000 farmers as well as indirectly through farmer-to-farmer exchange and other spill-over effects. The plant clinics are the entry point, where farmers bring to the plant doctors the queries about the problems they have with their crops. More and more plant clinic data are being stored in the knowledge bank and used as the basis for decision-making by plant health stakeholders and to provide critical information such as pest distribution maps, an example of online diagnostic tool and crop management support (CABI 2017). Plant clinic data is also being used in different ways, including the selection of research topics, determination of real problems at the field level, pest

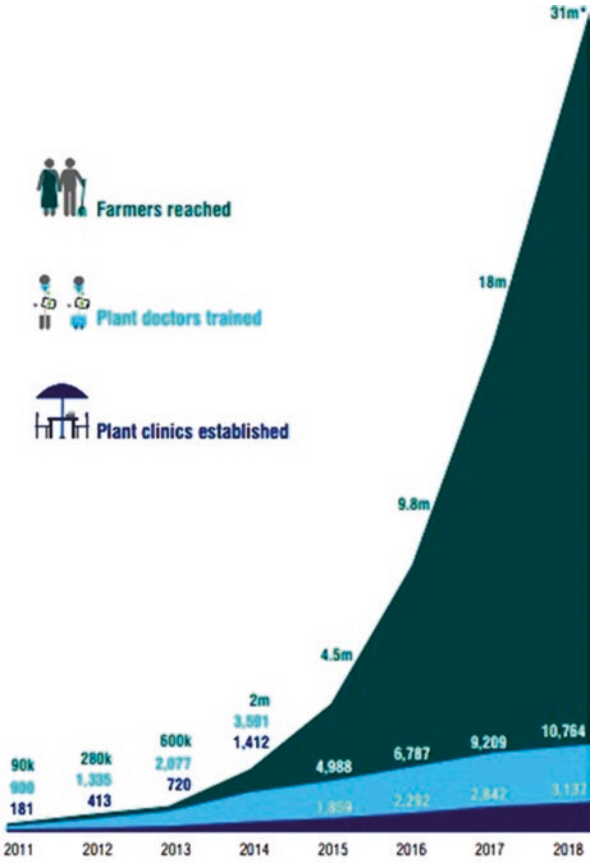

Fig. 18.3 Plantwise impact and progress (Plantwise 2018). Determined through estimations of primary reach (farmers reached directly through Plantwise activities) and secondary reach (farmers reached indirectly, e.g. as a result of plant doctors operating outside of Plantwise and farmers receiving advice from peers who visited plant clinics). Diagram not to scale

surveillance, reviewing the invasive species management practices, and distribution in the country. Thus, Plantwise will continue scaling up and reached 31 million female and male farmers in 2018 through the implementation of the Plantwise approach in a total of 34 countries (Fig. 18.3).

The FAW management strategies in Africa are focussed on identifying sustainable management practices to control the pest. Some advice and recommendations are directly available from the Americas, where both maize and FAW are native (FAO 2017). The FAO's broad framework for collaboration called South-South cooperation has also been highlighted as an important mechanism to transfer sustainable technology from the Americas to Africa for the control of FAW. As part of the dissemination process, the fall armyworm problem is frequently raised at Plantwise plant clinics, and brochures have been developed to provide information on the current extent of the fall armyworm invasion in Africa, known prevention, detection and control measures, short-term and long-term impacts of fall armyworm in Africa, and the invasion's potential impact on trade. The approaches include carrying out farmer perception surveys of fall armyworm impacts on maize, modelling the environmental suitability of Africa for fall armyworm, and carrying out national and continental economic analyses. Plant clinics provide the opportunity for developing smallholder capacity for managing the FAW in a sustainable manner (FAO 2017). Success of the Plantwise Program has primary relied on the support from farmers, governments, advisory services, NGOs, other plant health stakeholders and program donors (CABI 2018). The increasing number of partnerships has led to significant success for the program and consequently for low income farmers in countries where the program is being executed. CABI will maintain its close engagement with national and local partners in order to ensure a shared vision and commitment towards reaching sustainability of the Plantwise approach.

The establishment of a national plant clinics network conducted by trained extension officers as plant doctors allows the access of sustainable methods of control such as biological control by farmers. During the plant clinic sessions, famers can understand the technology of application of the biocontrol agents recommended, ensuring the adoption and the correct use of the bioproducts (Colmenárez et al. 2019).

In addition, other moth species, including *Tuta absoluta* have invaded several African countries causing economic impact on crops. However, pest resistance has developed due to heavy pesticide use to manage moth populations, thus supporting the need to find alternative biological based approaches that are economical and safer for farmers and consumers as well as for the environment (Mansour et al. 2018). Plant clinics have been an important tool in management of *T. absoluta* since PMDGs have been developed for Ethiopia, Kenya, Malawi, Tanzania, Uganda, and Zambia by providing recommendations that should contribute to strategies and/or criteria for controlling this pest, including use of pesticides Class II, III, and U (Rwomushana et al. 2019).

Sustainable management of invasive organisms must be based on an ecological approach and making biological solutions available to farmers, requiring regulators and input providers work together. The FAW action plan in Ghana was focused on four key elements: collaboration, awareness, surveillance, and research, and a

management process that identified challenges such as the engagement of input dealers on recommended insecticides, the engagement of the media through training and press briefings/releases, improving two-way communication between national and local stakeholders, and identifying and harmonizing the activities of new collaborators. In Kenya, the programme has facilitated introduction of products based on a naturally occurring virus to control FAW and to produce a pheromone to disrupt FAW mating. Also, discussions have also been held with Koppert Biological Systems on facilitating access to biological control agents for *T. absoluta* (CABI 2017, 2018).

Parthenium hysterophorus: *Parthenium*weed is invasive in many countries around the world, including South Asia, where an eco-climatic model suggests that many uninvaded areas are a good climatic match for this noxious weed (McConnachie et al. 2010). The weed causes several problems including disruption of the ecology of grasslands and invades woodlands through aggressive competition and allelopathy. By inhibiting the growth of other plants, it poses serious health hazards to livestock and can cause severe allergenic reactions in people, and it has been reported to reduce crop yields from 40% to 97%. In terms of pasture production, this noxious weed has been found to reduce livestock carrying capacities by as much as 90% (McConnachie et al. 2010). On the other hand, in 2018, Action on Invasives supported the development of national action plans for *Parthenium* in Pakistan by focussing on two biological control lines: improving the efficacy of the beetle *Zygogramma bicolorata* (already present in Pakistan) and releasing more than 1000 individuals at two sites to increase its overall range in Pakistan, as well as importing *Listronotus setosipennis* from South Africa, although testing and training are also in progress. In fact, a course on invasion biology and classical biological control of weeds was held in Pakistan in order to reinforce weed biocontrol knowledge, particularly on *Parthenium* (CABI 2018).

Final Considerations

Biological control has been proven to be an efficient and sustainable method of control. Within integrated pest management programmes, biocontrol can suppress populations of currently present and introduced pests.

The commercialization and distribution of natural enemies is a determining factor for the use of biological control agents at the field level. However, it is important that farmers have access to a proper advisory service; in this way, the establishment of a network of plant clinics, organizing practical sessions to clarify questions and visualizing the recommended sustainable practices, is critical. It is important to involve farmers in the discussions about the recommended practices, including the technology of application for biocontrol agents to ensure a high adoption level and the correct use at the field level; in addition, they can help in the process of early detection of new introduced species, reinforcing the surveillance system in the country.

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Chapter 19
Current Research on Andean Fruit Crop Diseases

Erika Benítez, William Viera, Patricia Garrido, and Francisco Flores

Abstract Several fruit crops that are native to the Andes hold significant potential for growing their market share. International demand for these commodities is often unmet as phytosanitary issues hinder their production and exportation. In this chapter we describe the main diseases affecting the most relevant Andean fruits grown in Ecuador, including tree tomato (*Solanum betaceum* Cav.), naranjilla (*Solanum quitoense* Lam.), babaco (*Vasconcellea* × *heilbornii*), blackberry (*Rubus glaucus* Benth.), and cherimoya (*Annona cherimola* Mill.), and examine current research aiming to improve integrated management of such diseases. Even though symptoms associated with viral, fungal, and bacterial diseases are common in most Andean fruit crops in Ecuador, their etiology is often unknown. In recent years, several causal agents of prevalent Andean fruit diseases have been described and characterized, but some remain elusive, constituting a threat for productivity. Black foot of blackberry caused by *Ilyonectria* and *Dactylonectria* species, and babaco mosaic virus infection are examples of plant diseases that have only been described in Ecuador. Although research on Andean fruit diseases is still lacking, tools for adequate treatment, timely diagnosis, and propagation of healthy plant material are now available or under development. Such efforts will likely result in increased fruit

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production and a better phytosanitary landscape that will aid in opening new markets for Andean fruit exportation.

Keywords Andean fruit · Crop diseases · Diagnostics · Ecuador · *Solanum betaceum* Cav. · *Solanum quitoense* Lam. · *Vasconcellea* × *heilbornii* · *Rubus glaucus* Benth. · *Annona cherimola* Mill

Introduction

Fruit crops that are indigenous to the Andes have been important for local consumption for centuries but, despite their great potential for exportation, the international market outreach of these products is negligible. Productivity of Andean fruit crops in Ecuador is low and could be significantly improved by implementing plant breeding (Viera et al. 2019a) and integrated pest management (IPM) programs. The successful execution of an IPM program depends on how much is known about the pests and diseases affecting the crop. In this book chapter we will discuss current research on the etiology of emerging diseases from Andean blackberry (*Rubus glaucus* Benth.), naranjilla (*Solanum quitoense* Lam.), babaco (*Vasconcellea* × *heilbornii*), cherimoya (*Annona cherimola* Mill.), and tree tomato (*Solanum betaceum* Cav.), some of the most important Andean fruit crops in Ecuador.

Andean Blackberry (*Rubus glaucus* Benth)

Blackberry belongs to the genus *Rubus* which is ubiquitous around the world. The predominant cultivated species in Ecuador is *Rubus glaucus*, commonly known as mora de Castilla or Andean blackberry. The fruit is endemic to the Northern Andean region (Marulanda et al. 2012) and is grown commercially at altitudes between 2200 and 3000 meters above sea level, from Mexico to Ecuador. The main production areas of blackberry in Ecuador are located in the inter-Andean valley (Iza et al. 2020), in the provinces of Tungurahua, Bolívar, Cotopaxi Chimborazo, Imbabura, Pichincha, and Carchi. Tungurahua generates 34% of the total production followed by Cotopaxi and Bolivar with 30% and 17%, respectively (MAGAP 2017).

Ecuadorian exports can be maintained year round as the fruit is continuously harvested. Yields peak from September to November while losses of up to 10% are common during the rainy season, from December to April. The international market demand for Andean blackberry is increasing and United States is the main consumer, receiving 69% of Ecuadorian exports. Both international and domestic demands for Andean blackberry are often unmet. In Ecuador, losses of this crop are

mainly attributed to inadequate management (33%), pests or diseases (28.2%), and drought or frost (28%) (MAGAP 2017). The main diseases affecting Andean blackberry in Ecuador are gray mold, powdery mildew, and black foot (Villares et al. 2016). There are also reports of gray mold, caused by the fungus *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*) (Acosta et al. 2020). This pathogen can attack crops at any stage of their development and can infect different aerial parts of the plant, affecting yield and quality of the crop (Villares et al. 2016). In Andean blackberry, the main damage occurs in the fruits where signs of the fungus appear as a gray cottony mycelium with profuse sporulation that causes rot and mummification. Powdery mildew is caused by *Oidium* sp. (Aldás & Tarquino 2013), which covers the underside of infected leaves with mycelium and conidia. Severe infections can affect plant development and significantly reduce productivity (Espín 2010).

Fungal wilt observed in blackberry plantations from Ecuador was initially associated with *Fusarium,Verticillium*, and *Cylindrocarpon* (Martínez 2014). However, it was later found that the symptoms were caused by fungi from *Dactylonectria* and *Ilyonectria,* genera that have *Cylindrocarpon*-like anamorphs, thus recognizing the disease as black foot of Andean blackberry (Sánchez et al. 2019). Black foot disease, first described in 1961 (Maluta and Larignon 1991), is a serious disease that affects the production of several crops (Alaniz 2008; Agustí-Brisach and Armengol 2013). Black foot disease in *Rubus glaucus* is characterized by wilting, rot of root and crown, and small leaves with chlorosis and curly tips (Sánchez et al. 2019). Symptoms of black foot disease were first observed in 2010 with an incidence of 13.3% in the provinces of Bolivar and Tungurahua (Fig. 19.1).

In 2017, Iturralde standardized an inoculation methodology of *Ilyonectria* sp. in Andean blackberry plants using 1×10^6 conidia/ml solution in plants with wounded roots. As a result, wilt and necrosis of the neck and roots were observed. In this study, *Dactylonectria torresensis* was identified as one of the causal agents of black foot disease in Andean blackberry (Iturralde 2017).

Pathogenicity tests were repeated for *D. torresensis* along with species of *Ilyonectria* associated with black foot, *I. vredenhoekensis*, *I. robusta,* and *I. venezuelensis,* determining their infective capacity by inoculating 35 healthy Andean blackberry plants for each fungal species. Crown necrosis and wilting of the basal leaves were evaluated 4 months after inoculation. While mock inoculated plants

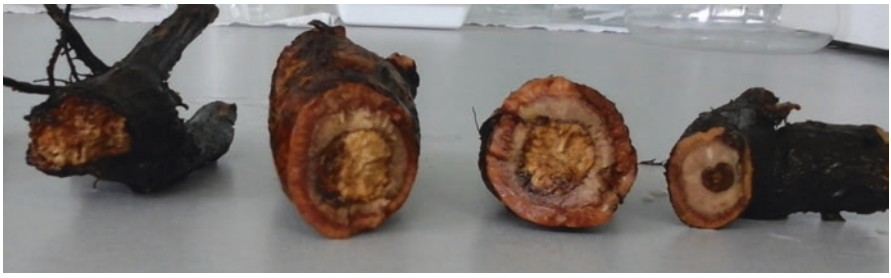

Fig. 19.1 Crown and roots of *Rubus glaucus* showing symptoms of black foot

were symptomless, all inoculated plants showed necrosis of the crown and wilting of the basal leaves with no significant differences among all the species tested. Fungi were identified morphologically and molecularly using the histone gene 3 and a fragment of the translation elongation factor 1 alpha. Maximum likelihood and Bayesian multilocus analyses revealed the close relationship between the species that cause black foot (Sánchez et al. 2019). All sequences are available in GenBank, and isolates used for pathogenicity tests are stored in the Universidad de las Fuerzas Armadas culture collection. Breeding programs can access these isolates to test new Andean blackberry lines for tolerance to black foot using the standardized method for inoculation.

Since 2012 as part of the Central-Sierra Fruticulture Program, INIAP developed a trial on Andean blackberry crops from Tisaleo, Tungurahua Province, where the soil was periodically treated for 5 years with *Trichoderma* spp. (Viera et al. 2019b; Viera et al. 2020), a potential agent for biological control of black foot (Martínez et al. 2019). To determine whether there are differences between the fungal communities of soil untreated and treated with *Trichoderma* spp., Benítez (2019) determined the taxonomic profiles of soil samples from the Tisaleo trial. Eight samples from the rhizosphere of Andean blackberry were collected, four from soil treated with *Trichoderma* and four from soil that was not treated. Total DNA was extracted, and high-throughput sequencing of the internal transcribed spacer was performed. After bioinformatic processing of the sequences, an average of 125,255 sequences per sample were obtained, which were grouped into 289 different operational taxonomic units. Additionally, each OUT was assigned to a different trophic group (i.e., pathotroph, saprotroph, or symbiotroph). No differences were found regarding trophic groups. Relative abundance of *Dactylonectria,Ilyonectria* and *Trichoderma* were also calculated. No significant differences were found for the abundance of *Ilyonectria* or *Trichoderma* while relative abundance of *Dactylonectria* was higher for treated soils (Fig. 19.2). The results were counterintuitive as higher *Trichoderma* and lower *Dactylonectria* and *Ilyonectria* concentrations were expected in treated soils.

Naranjilla (*Solanum quitoense*)

Naranjilla is a native fruit from Ecuador, grown mainly in the Amazon region and in the Andean zone: Chimborazo, Cotopaxi, Imbabura, Pichincha, and Tungurahua provinces (INIAP 2018). It has great export potential to international markets throughout the year. Its cultivation is of economic importance due to its profitability and market acceptance (Silva et al. 2016).

Few diseases have been described for this crop, but vascular wilt, caused by *Fusarium oxysporum,* is probably the most relevant. Vascular wilt of naranjilla is characterized by flaccidity and chlorosis that advances from the crown moving up through the stem, resulting in the abscission of leaves and flowers causing losses of up to 80% of the yield (Ochoa et al. 2001). *Cladosporium* sp., *Glomerella cingulata*,

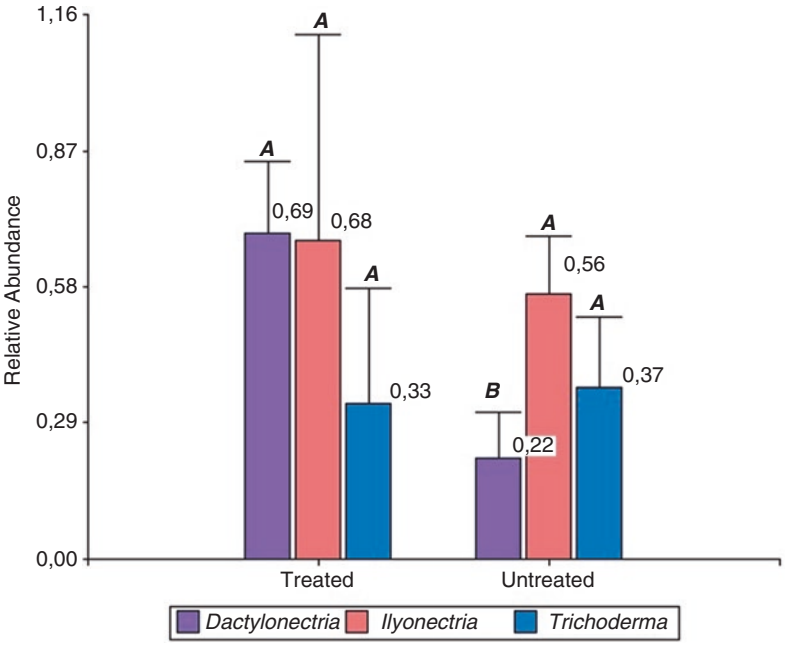

Fig. 19.2 Relative abundance of *Ilyonectria, Dactylonectria* and *Trichoderma* in soils that were treated and not treated with *Trichoderma* spp.

Verticillium albo-atrum, *Armillaria*, *Phoma* sp., *Alternaria* sp., *Sclerotinia sclerotiorum*, and *Colletotrichum* sp. have also been described as fungal pathogens of naranjilla (Rojas et al. 2010; Tamayo et al. 2001). The oomycete *Phytophthora infestans* can cause late blight in naranjilla, a disease that can be very aggressive under warm-humid conditions where it can cause total loss of the crop (Ochoa et al. 2010). Bacterial pathogens of naranjilla include *Ralstonia solanacearum, Clavibacter michiganensis,* and *Erwinia chrysantemi* which cause wilt and rot of fruits and stems (Bolaños et al. 2017; Tamayo et al. 2001).

Symptoms of viral infection (i.e., blistering, mosaic, leaf deformation, and chlorosis) have been observed for many years; causal agents were first described in Colombia (Tamayo et al. 2001) and more recently in Ecuador (Green et al. 2018; Ramos et al. 2019). In 2019, Ramos, collected leaf samples of naranjilla plants with symptoms of virosis (Fig. 19.3) from the experimental farm of the National Institute of Agricultural Research (INIAP) in the province of Pichincha. Total RNA was extracted from the leaves and sequenced using HTS technology. A bioinformatic pipeline was designed for virus discovery. Trimming and filtering of low-quality reads were performed with Trimmomatic_0.38 (http://www.usadellab.org/cms/?page=trimmomatic) and duplicated reads were removed with the dedupe script from BBMap_38.56 (http://sourceforge.net/projects/bbmap/).

Since the genome of *S. quitoense* was not available, deduplicated reads were mapped to the *S. tuberosum* genome using Bowtie2 – 2.3.3.1 (Langmead and

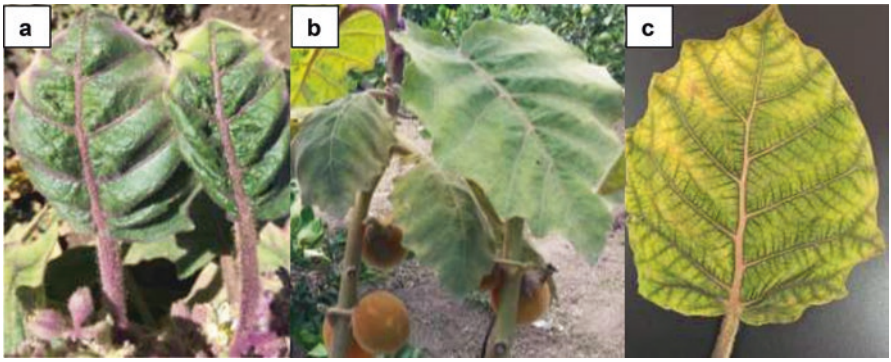

Fig. 19.3 Leaves of *Solanum quitoense* showing symptoms of virus infection. (**a**) Deformation, chlorosis, and blistering; (**b**) deformation and mosaic; (**c**) intervein yellowing

Table 19.1 Virus found in *Solanum quitoense*

Genus	Species	No. of contigs	Average Kmer coverage	% Identity	Accession number
Ilarvirus	*Potato yellowing virus*	29	6885	98.73	MH937420
Polerovirus	*Tobacco virus 2*	1	636	95.55	KY038943
Polerovirus	*Potato leafroll virus*	24	388	96.72	JQ420902
Torradovirus	*Tomato torrado virus*	1	16	96.17	MH587229
Solendovirus	*Tobacco vein clearing virus*	10	2	81.70	AF190123
Cavemovirus	*Cassava vein mosaic virus*	1	1	78.24	U59751

Salzberg 2012) and mapped reads were removed with SAMtools_1.7 (Li et al. 2009). The best assembly was achieved with Spades – 3.13.0 (Bankevich et al. 2012). Assembled contigs were compared with a database consisting of all viral sequences stored in Genbank using BLAST. Six different viruses were found in Naranjilla with potato yellowing virus having the highest average Kmer coverage (Table 19.1) (Ramos et al. 2019). In addition, other viruses such as Cucumber mosaic virus (CMV), Potato yellow vein virus (PYVV) and Alstroemeria necrotic streak virus (ANSV) have been reported by Gallo et al. (2018). Most commercial varieties of naranjilla reproduce asexually and can easily accumulate viruses; thus information on the virome of naranjilla will aid in diagnosing diseased plants and establishing a stock of disease-free plants for reproduction.

Babaco (Vasconcellea heilbornii)

Babaco is a natural interspecific hybrid between *Vasconcellea stipulata* Badillo (toronche) and *V. pubescens* (chamburo) A. DC. Kuntze native to southern Ecuador (Janick and Paull 2008). Babaco fruit contains high amounts of potassium, vitamin C, vitamin B2, B3, and vitamin A; therefore, it is considered of high nutritional value. It has attractive characteristics for industrialization, since babaco is a seedless fruit with thin skin which makes its processing relatively simple. It is mostly produced in Ecuador and New Zealand but attempts to grow it commercially have also been made in United States, Italy, Spain, France, Switzerland, Canada, and the Netherlands (Janick and Paull 2008; Scheldeman et al. 2011). In most countries where babaco is planted, commercialization has been difficult due to the large fruit size, lack of promotion, unfamiliar taste, or cost of crop maintenance and market. In Ecuador, babaco production is hindered by several plant pathogens.

In Ecuador, babaco is cultivated in Atuntaqui, Imbabura province; Tumbaco and Perucho, Pichincha pronvince; Gualaceo and Santa Isabel, Azuay province; Vilcabamba and Catamayo, Loja province; and Patate and Baños, Tungurahua province, the latter being the province with the greatest extension of this crop, representing 57% of the national production, estimated between 150 and 200 hectares cultivated throughout Ecuador (Espinoza 2016). Babaco viruses could be causing significant economic losses and reduction in productivity. In babaco plantations along the Ecuadorian highlands (Imbabura, Pichincha, Tungurahua, Azuay, and Loja provinces), characteristic symptoms of viral infection such as mosaic chlorosis, blistering, and deformation of the leaves have been observed for decades. Previously, these symptoms were associated with papaya mosaic virus (PapMV) (Robles Carrión et al. 2016) due to their serological and morphological characterization in viral isolates of tobacco leaves. Nevertheless, the causative agent was molecularly characterized by Álvarez-Quinto et al. (2017), where they described it as a new species of the genus *Potexvirus* and suggested the name babaco mosaic virus (BabMV) due to the host and the symptoms it produces. Five other viruses, including an Umbra-like virus, a Cheravirus, a Nepovirus, and a Deltapartitivirus that were not previously described, have been recently found in babaco (Cornejo-Franco et al. 2020) Given the asexual reproduction of the babaco by sprouts, stakes, or grafts, babaco virosis is spreading rapidly in the regions where it is cultivated.

Moncayo (2018) designed primers for early diagnostics and performed a phylogeographic analysis of the pathogen that would determine the most likely center of origin of the virus. Leaf samples of babaco plants with symptoms of virosis (Fig. 19.4) were collected in from Imbabura, Pichincha, Santo Domingo, Tungurahua, Azuay, and Loja provinces. The presence of a potexvirus was detected by reverse transcriptase polymerase chain reaction (RT-PCR) using generic primers. As the sequence of the complete genome of BabMV had not been described, a multiple sequence alignment of the whole genome of eight of the species closest to BabMV (senna mosaic virus, papaya mosaic virus, alternanthera mosaic virus,foxtrail mosaic virus, bamboo mosaic virus, clover yellow mosaic virus,

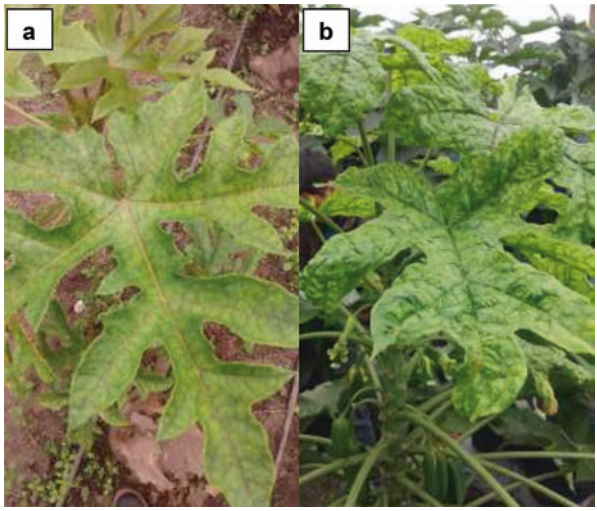

Fig. 19.4 Babaco leaf with symptoms of (**a**) mosaic and (**b**) blistering

Table 19.2 Primers for the amplification of part of 60% of the genome of babaco mosaic virus

Name		Sequence	Length	M T°	Product size
AMV1	F	CCCACTTTCGATGCAAACAC	20	55 °C	500–600 bp
	R	CCIGIICCIGCIACIGCATG	20	62 °C	
AMV2	F	CACCARCAGGCIARRGAYGA	20	55 °C	500–600 bp
	R	CTCAAACTGCAGCATCGCCCC	21	62 °C	
AMV3	F	TAGTGAGCCGCTTGTTGTCC	20	57 °C	700–800 bp
	R	CGTCVCKGTASSWRCCTCCG	20	61 °C	
AMV4	F	AACADRCTIGGRGTBTACTC	20	56 °C	500–600 bp
	R	CAATTCGCCTTGGGGTTTGAG	21	57 °C	
AMV5	F	GCTATAGGTATAGGATTAGC	20	46 °C	500–600 bp
	R	CCRTCRAAGAAATCRAAVGC	20	52 °C	
AMV6	F	AAYGARCTBAGRMTIGAYTGG	21	48 °C	500–600 bp
	R	AAATAGGGTGTACTTTCCAG	20	50 °C	

allium virus X, tamus red mosaic virus) was used to design six pairs of degenerate primers for the amplification and sequencing of 60% of BabMV genome (Table 19.2). These primers could be used to amplify and sequence a large portion of the genome of other potexviruses. Alternanthera mosaic virus (AltMV) and papaya mosaic virus (PapMV) with a 74% similarity at the amino acid level of the coat protein (CP) but with a 97% similarity with the virus BabMV.

The proposed diagnostic test consisted of an RT-PCR with primers designed from a conserved region of the CP protein based on the multiple sequence alignment of 20 BabMV viral isolates (BMVdF-TCAGAGTCATCHAACAYTGGAA/BMVdR-TGADGATTCACCAGARATC). The test was positive for all samples of babaco leaves with symptomatic virosis regardless of geographic region and was able to detect up to 8 ng/μL of viral RNA without cross-reacting with closely related potexviruses.

The phylogeographic analysis of BabMV was performed with the complete coat protein (CP) gene of 27 viral samples from the provinces of Imbabura, Pichincha, Santo Domingo, Tungurahua, Azuay, and Loja. It was found that the CP of the virus mutates at a substitution rate of 4.8×10^{-3} substitutions/site/year. The analysis estimated the origin of the virus between 1790 and 1960 and suggested that the most likely center of origin of BabMV is the province of Cañar. Nevertheless, a larger sample size is needed for a more accurate estimation.

Cherimoya (*Annona cherimola Mill)*

Scheldeman et al. (2002) mentioned that cherimoya was native to the southern part of Ecuador in the province of Loja and nothern of Peru; however, Larranaga et al. (2017) stated by genetic analysis the this fruit crop has a Central American origin. According to Guerro (2012), cherimoya is a crop of low economic and industrial relevance in Ecuador; however, due to its high yield per tree, large fruit size, and high level of brix degrees, cherimoya could become a commercial crop of exportation.

Diseases in cherimoya plants can negatively affect the marketability of the fruit. Root rot of cherimoya, caused by *Dactylonectria macrodydima,* causes symptoms of necrosis in the roots and crown from the epidermis to the vasculature, which results in reduced vigor, shortened internodes, and the development of secondary roots (Auger et al. 2015). Other potentially important diseases of cherimoya include anthracnose caused by *Colletotrichum gloeosporioides*, armillaria root rot caused by *Armillaria mellea*, cylindrocladium spot caused by *Cylindrocladium colhounii*, root rot caused by *Phytophthora* spp., pink disease caused by *Erythricium salmonicolor*, pseudocercospora spot caused by *Pseudocercospora* sp., and bacterial wilt caused by *Ralstonia solanacearum* (Ploetz 2003; Mayers and Hutton 1987).

In Ecuador, during the rainy season of 2016 (March–April, September–October), brown spot symptoms were observed on leaves and fruit of cherimoya in the orchards of Pichincha province. Leaves showed necrotic black-to-brown lesions bordered with chlorotic tissue around the central vein and the apex; fruit damage was limited to the epidermis. To identify the causal agent, Guevara et al. (2019) plated sterilized leaf tissue on potato dextrose agar (PDA) media supplemented with chloramphenicol. After 6 days of incubation at 25 °C, gray to brown colonies grew which were morphologically congruent in shape and color with *Alternaria alternata*. DNA from three isolates was extracted, and the elongation factor-1α gene (EF-27 1α) was amplified and sequenced. This result confirmed the presence of *Alternaria alternata*. In addition, Koch's postulates were confirmed by inoculation of the isolates in healthy cherimoya plants, leaves, and fruits (Fig. 19.5). This was the first report of *Alternaria alternata* causing brown spot disease in cherimoya in Ecuador.

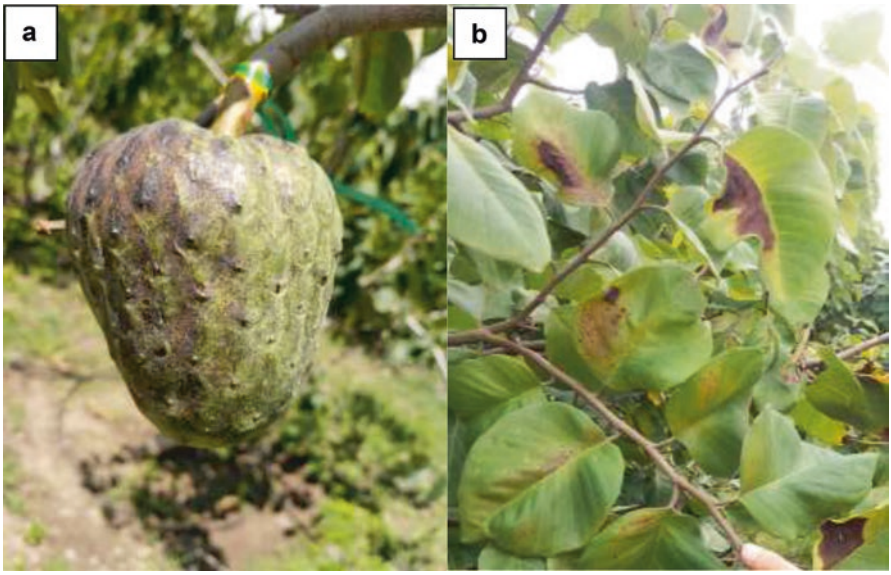

Fig. 19.5 Brown spot disease on *Annona cherimola* (**a**) fruit and (**b**) leaves

Tree Tomato (*Solanum betaceum* Cav)

Tree tomato is a native plant from the subtropical forests of the Andean region of South America (Acosta-Quezada et al. 2012; Bohs 1989). It is a fruit with a lot of potential for its organoleptic characteristics and for having a high nutritional value (Morón 2000). In Ecuador, this fruit is cultivated by small and medium farmers in around 4320 ha (SIPA-SIGAP 2017). It grows in regions between 1500 and 2800 m of altitude in dry temperate and subwarm humid climates (Albornoz 1992; León et al. 2004).

Tree tomato cultivars are susceptible to anthracnose (*Colletotrichum acutatum* and *C. tamarilloi*), blight (*Phytophthora infestans*) stem black spot (*F. solani*), vascular wilt (*F. oxysporum*), oidium (*Oidium* sp.), alternariosis (*Alternaria* sp.), nematodes (*Meloidogyne incognita*), and the virotic tree tomato complex (Tamayo 2001; León et al. 2004; Feican et al. 2016; Caicedo et al. 2017; Espinoza et al. 2017). The management of these diseases is based on the use of pesticides that in most cases is irrational and not always efficient; so sometimes the farmer leaves the crop before the plant produces its potential. The use of pesticides demands investment expenses, has side effects on the environment, can affect the health of the farmer, and there may be residues of the pesticides in the fruit.

Anthracnose is one of the main phytosanitary problems because of its high incidence (Viera et al. 2016; Caicedo et al. 2017). This disease occurs at any age of the plant. In the fruits, the initial spot infection is black, sunken, and circular with defined edges. This advances rapidly and covers the fruit, which produces mummification (Falconi et al. 2013). Losses caused by this disease range from 50% to 100% (Fig. 19.6) (Saldarriaga-Cardona et al. 2008).

Fig. 19.6 Fruit damage caused by *Colletotrichum acutatum* in *Solanum betaceum*

Fig. 19.7 Virus symptomatology observed in *Solanum betaceum* orchards

Another big problem in this fruit crop is the incidence of virosis. Symptoms such as foliar blistering, yellowing, chlorosis, leaf deformation, and spots with tan or oily appearance can be associated with the presence of virus in tree tomato orchards (Fig. 19.7). However, these symptoms cannot be related to a specific virus agent without other diagnostic methods like RT-PCR or transmission electron microscopy. The symptoms observed in tree tomato plantations are caused by a viral complex that includes different taxonomic types of virus such as potyvirus, polerovirus,

and tobamovirus (Sivaprasad et al. 2015; Sivaprasad et al. 2016; Espinoza et al. 2017). A complex of three viruses (PLRV, PVY, and ToMV) or at least two of them (PLRV and PVY) has been detected when samples have been analyzed. Other viruses such as tomato spotted wilt virus (TSWV), potato virus V (PVV), and Peru tomato mosaic virus (PTV) (Yeturu et al. 2016; Insuasti et al. 2016) have been also reported in plantations in Ecuadorian orchards.

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Chapter 20
Biotechnological Tools for the Development of Foc TR4-Resistant or -Tolerant *Musa* spp. Cultivars

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Abstract Bananas and plantains (*Musa* spp.) are one of the commercially important tropical fruit crops in the world. *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (Foc), is one of the most damaging diseases affecting *Musa* worldwide. The banana export industry based on the 'Gros Michel' (*Musa* AAA) cultivar in Latin America and the commercial production of the 'Manzano' (*Musa* AAB) cultivar in Cuba were decimated during the Foc race 1 outbreak in the twentieth century. Currently, the Foc tropical race 4 (TR4) has caused serious damage to the export standards for the Cavendish subgroup in Southeast Asia and continues to spread among banana-producing countries. Around 80% of banana cultivars produced worldwide are susceptible to this race, which represents an imminent threat for producing countries of Latin America and the Caribbean where the pathogen has not yet been detected. Biotechnological tools offer the opportunity to obtain resistant or tolerant cultivars to Foc through the combination of somatic embryogenesis and breeding techniques. In vitro mutagenesis using gamma radiations with a ^{60}Co source alongside *Agrobacterium tumefaciens*–mediated genetic transformation with anti-fungal genes will provide mutant and transgenic lines for early screening under various conditions. Tissue culture facilitates the rapid cloning of variants selected from greenhouse and field conditions. In addition, the use of antagonistic bacteria and fungi for the management of the pathogen can increase the resistance level of the new cultivars. In particular, the selection and study of the mechanisms of action of native microorganisms with antagonistic properties against Foc, such as *Bacillus* and *Trichoderma*, may contribute to the management of the disease.

Keywords Banana · Biocontrol · In vitro mutagenesis · Genetic transformation

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Introduction

Banana and plantain (*Musa* spp.) serve as important sources of staple food and fruit around the world and collectively are considered the world's leading fruit crop, with a production value reaching over 100 million tons per annum (FAO 2019). As a staple food, banana is an important export commodity in Africa and Asia, ensuring food security for millions of people (Aurore et al. 2009). One of the major constraints in the global production of banana is the *Fusarium* wilt disease, which is caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) (Ploetz 2006).

Foc gains entry into the plant host via its roots. Once inside, it colonises the rhizome and travels up the pseudostem where it blocks the water-conducting xylem vessels, thus preventing the transport of water and nutrients to the aerial parts of the plant. External symptoms of *Fusarium* wilt start with the yellowing and wilting of the older leaves and progress to the younger leaves until the plant dies. Internally, the plants show brown discoloration and necrosis of xylem vessels in the rhizomes and stems. The disease incidence varies depending on the cultivar, the environment and the level of inoculum but can extend to total crop loss in heavily infested fields (Moore et al. 2001).

Foc is a soil-borne pathogen that produces chlamydospores enabling its survival in the soil in the absence of the host. It is also known to survive on weed hosts in a non-pathogenic manner (Hennessy et al. 2005). Once the soil is infested with Foc, it generally becomes unsuitable for replanting for many years thereafter (Stover 1990). Furthermore, unlike the black Sigatoka leaf spot caused by *Pseudocercospora fijiensis*, the other major fungal pathogen affecting the banana industry worldwide, *Fusarium* wilt cannot be controlled by fungicides (Ploetz 2006). Outbreaks of *Fusarium* wilt decimated the banana industry primarily based on the cultivar 'Gros Michel' (AAA) in Central America in the 1950s. The pathogen was subsequently named Foc race 1, and the outbreak forced the industry to shift production to Foc race 1–resistant cultivars of the Cavendish (AAA) subgroup (Stover 1990).

Foc tropical race 4 (TR4) was recognised for the first time in 1990 in Taiwan. It caused serious damage to the current standards for export in crops of the Cavendish subgroup in several Southeast Asia countries (Butler 2013). Subsequently, a succession of TR4 incursions in banana-growing regions in Asia, the Middle East, the Indian subcontinent, Africa and even Europe was reported (Zheng et al. 2018). The first report of the occurrence of TR4 in Latin America was in samples originating from the department of Guajira in the northeast of Colombia, which is one of the leading global bananas producing countries in the region (García-Bastidas et al. 2019). TR4 has a wide range of hosts. More than 80% of the banana cultivars that are produced globally are susceptible to infection, including the banana subgroup (*Musa* AAB), important AAA and ABB cooking bananas, and various types of AA, AB, AAA as well as dessert bananas type AAB (Ploetz 2009). Cavendish now accounts for >40% of world banana production with export markets amounting to 15% of the total production (FAO 2019).

The creation of genetic variability and improvement of banana through conventional breeding is hampered for several reasons. Most of the cultivated bananas originated from crosses between *Musa acuminata* subspecies or *M. balbisiana* and *Musa acuminata*. The new cultivars, mostly diploids and triploids, exhibit parthenocarpic fruits and have reduced male and female fertilities. These characteristics, along with very low clonal propagation rates, caused a reduced genetic pool from which to search for desirable traits while low fertility impeded sexual hybridisation (Lopez et al. 2017). In addition, the presence of integrated sequences of the banana streak virus in the *M. balbisiana* genome limits the use of these genotypes as donors for traits as disease resistance and abiotic stress tolerance. Therefore, traditional breeding in banana encounters significant limitations, which could be overcome through the use of biotechnological tools.

The development of plant cell tissue and organ culture, combined with mutation breeding using in vitro mutagenic techniques in the last 20 years, has made it possible to transfer part of the conventional breeding programs from the field to laboratory conditions.

The induction of genetic variations by different methods and techniques is a basic tool used for years for plant improvement by the development of new cultivars with abiotic stress tolerance, resistance to pests and diseases and the improvement of quality and agricultural performance (Arene et al. 2007; Xu et al. 2012). This has been assessed on bananas by different authors (Alves and Lima 2000; Jain 2005, 2010; Lopez et al. 2017; Abdulhafiz et al. 2018; Ali et al. 2020). According to Xu et al. (2012) these techniques enable an increase in genetic variability as well as the rapid multiplication of the obtained mutants. The combined use of in vitro mutagenesis and tissue culture can therefore play an important role as alternate options in banana breeding. Tissue culture has an important role in developing new banana cultivars. Through this technology, a large number of improved mutant varieties have been released for commercial cultivation in many crops, demonstrating the economic value of the mutation breeding technology (Kharkwal and Shu 2009; Jain and Suprasanna 2011).

Somatic embryogenesis has contributed greatly to plant regeneration of bananas (Afza et al. 1996; Strosse et al. 2003) as well as protoplasts isolation (Assani et al. 2001, 2002, 2006; Xiao et al. 2007). Plants have been regenerated from banana somatic embryos derived from explants such as meristems, rhizome tissues, leaf bases, immature zygotic embryos and young male flowers of diploid and triploid banana. Clonal propagation of bananas has been accomplished by culturing meristems, rhizomes, inflorescences and others (Strosse et al. 2003).

Somatic embryogenesis technology has been applied in several crops for both mass propagation and genetic improvement. Among its advantages is the unicellular origin of somatic embryos which minimises or eliminates formation of chimeric regenerated plants. Also, embryogenic cell suspensions are ideal plant materials for the induction of mutations due to the production of mutated somatic embryos. The process can be automated, since the somatic embryos can be grown in a bioreactor for large-scale production and subsequent in vitro selection. Finally, the

embryogenic cells can be stored for long periods of time by cryopreservation which facilitates preservation of the mutants (Suprasanna et al. 2012).

Gamma irradiation has been reported to be the main physical mutagen for mutation induction in *Musa* although chemical mutagenesis has also been used in several *Musa* accessions (Jain 2010; Lamo et al. 2017). Banana mutant lines with improved agronomic traits such as height reduction, larger fruit size and early flowering have been identified (Jain 2010; Lamo et al. 2017). Moreover, banana mutant lines with resistance to *Fusarium oxysporum* f. sp. *cubense* (Hwang and Ko 1987; Bhagwat and Duncan 1988; Jain 2010) have been identified and further evaluated for confirmation of the resistance trait.

Genetic transformation of elite cultivars is one of the most promising strategies for the development of resistant varieties against *Fusarium*. This technology makes use of a gene pool which broadens every day as more genome sequences become available and new genes are being structurally and functionally characterised. Additionally, exciting new breeding techniques like CRISPR/Cas9 with its myriad of possibilities are diluting the boundaries between transgenic and non-transgenic organisms.

There are few options for *Fusarium* wilt management. Several factors complicate the development of effective long-term measures, especially the perennial characteristic of the crop and the polycyclic nature of the causative agent (Ploetz 2015). However, measures to alleviate and prevent the disease are implemented in all affected regions. The reduction of the use of chemical fungicides in agriculture is a necessity to reduce production costs and to avoid negative repercussions on the environment and human health (Pérez-Vicente 2016). Considering the urgency of finding new alternatives for the effective management of *Fusarium* wilt, the use of antagonistic microorganisms turns out to be an economically and ecologically sustainable strategy (Gang et al. 2013; Shafi et al. 2017).

The concept of biological control was proposed by Baker and Cook (1974) in their book *Biological Control of Plant Pathogens*. Later, Baker et al. (1984) defined it as 'the set of mechanisms to take advantage of the metabolic, biochemical, mechanical and/or physical reactions of the specific native microorganisms of an environment, capable of articulating or individually inhibiting a pathogen without altering the ecological balance'. These mechanisms include resistance induction, anti-biosis, competition and direct interaction with the pathogen either by parasitism or by predation (El Arbi et al. 2016).

Microbial biological control agents (MBCAs) applied in agriculture present several advantages when compared to chemical pesticides: (1) they are safer, (2) they mitigate environmental damage and bring less risk to human health, (3) they are effective in small quantities, (4) are more rapidly degraded than chemicals and (5) can be used in the conventional or integral management of diseases (Berg 2009). On the other hand, it is stated that the inhibitory action of MBCAs does not occur through the independent action of a single mechanism but that the suppression of diseases in plants is achieved by their joint action (Poritsanos 2005; Hernández-Rodríguez et al. 2010). Many authors even believe that the combination of several

microbial species with biocontroller effects could be more efficient than the use of individual species (Poritsanos 2005; Liu et al. 2017).

Over the years, several strategies and methodologies have been developed to identify the potential of MBCAs in vitro and/or in vivo. Generally, these agents have been described as fungi and bacteria with characteristics that allow them to control soil-borne diseases through direct antagonism or through defence stimulation reactions in plants, or both at the same time (Lecomte et al. 2016).

Related to the above, several investigations have demonstrated the antagonistic effect of MBCAs against *Fusarium* spp. (Alabouvette et al. 2012; Lecomte et al. 2016). For the control of *F. oxysporum*, the scientific literature indicates that fungi have been evaluated more than bacteria (68% versus 32%). Special attention has been given to species of the genus *Trichoderma* (53% of fungi), recognised for their potential as MBCA (Gajera et al. 2013). Other representative microorganisms of this group are non-pathogenic *Fusarium* strains (23%) and *Penicillium* strains (10%) (Lecomte et al. 2016). These authors indicated that most of the products marketed for the biological control of *F. oxysporum* in ornamental plants are composed of *Trichoderma* strains.

Likewise, Pérez-Vicente et al. (2009) showed the significant anti-fungal effect of a strain of *Trichoderma* (*T. harzianum* A24) against Foc. These authors showed that the application every 3 months of this strain in healthy plants from tissue culture decreased the incidence of Foc R2 in banana plantations. The procedure allowed production for more than 5 years in soils where the disease had previously destroyed plantations of the 'Donkey CEMSA' (ABB) cultivar. However, the use of *Trichoderma* even today only guarantees the reduction of the population and not the eradication of the pathogen in the soil (Pérez-Vicente 2016). In this chapter, we report different strategies for the development of Foc-RT4-resistant or -tolerant *Musa* spp. cultivars with special regard to biotechnological tools. Thus, we report a comprehensive literature review about in vitro mutagenesis using gamma radiations alongside *Agrobacterium tumefaciens*–mediated genetic transformation with anti-fungal genes, and how the use of antagonistic bacteria and fungi for the management of the pathogen can increase the resistance level of the new cultivars.

In Vitro Mutagenesis

Genetic variation is the starting point of any breeding programme. Genetic variation may already be present in nature, may be obtained after several years of selection or may be produced through hybridisation. In vitro mutagenesis refers to mutations induced by treating explants or in vitro cultures with a mutagen, followed by mutant screening and characterisation. This process involves a sequence of essential steps: choice of proper target plant material (explants or cultures), choice of a mutagen and determination of a suitable dose, post-treatment management and a series of subcultures, and regeneration of plants for mutant selection (Abdulhafiz et al. 2018).

The in vitro techniques have created new opportunities for the improvement of vegetative propagated plants (García 2000). In 1959 Melchers and Bergmann reported in vitro selection in cell suspension cultures. Since then, several in vitro selected variants have been investigated for their value in plant improvement (Maliga 1984; Dugdale et al. 2000). Tissue culture also allows for the handling of large populations for mutagenic treatment and the cloning of selected variants. It also offers the possibility of rapid execution of the propagation cycle of sub-culture aimed at separating mutated from non-mutated sectors (Ahloowalia 1998). Induced mutation in vitro has several advantages including the convenience in treatment of large number of propagules due to miniature size, the effective use of chemical mutagens and a lower requirement for mutagen doses. Novak et al. (1997) reported that tissue culture techniques were being developed for the induction of heritable variations useful in *Musa* breeding. In vitro mutagenesis may also contribute to conventional breeding programs by extending the genetic base available for recombination. Host-plant resistance, developed either by conventional breeding or by application of biotechnology, is thought to be the most economic and sustainable means of managing pests and diseases (Lorenzen et al. 2009; Pathak et al. 2016).

Mutagenic agents, such as radiation and certain chemicals, can be used to induce mutations at a higher frequency, generating genetic variation from which desired mutants may be selected (Roux 2004). The types of irradiation potentially available for mutagenesis include ultraviolet radiation (UV light) and ionising radiation (using X-rays, gamma-rays, alpha and beta particles, protons and neutrons). The effect of UV light on DNA (deoxyribonucleic acid) creates pyrimidine dimers that act by blocking the DNA transcription and replication with only a small portion mutated (Lestari 2012).

Gamma rays are the most commonly used mutagenic agent in in vitro mutagenesis experiments (Roux et al. 2004). They possess a high degree of accuracy, sufficient reproducibility and deep penetrating power into the biological materials, thus increasing variations in their physical and chemical compositions (Yamaguchi et al. 2003). Gamma irradiation results in small deletions (1–10 bp) while neutrons cause 300 bp to 12 kbp deletions and chemical mutagens result in point mutations mainly G/C-to-A/T transitions (Morita et al. 2009). Genetic improvement with the use of induced mutations based on mutagenic agents produce changes similar to natural mutations but in a relatively shorter time and in greater quantity (Donini and Sonnino 1998).

Irradiation using X-rays on plants for mutation has received much attention in recent years due to changes in plant cell structure and metabolism. These include dilation of thylakoid membranes, alteration of photosynthesis, modulation of the anti-oxidant system and accumulation of phenolic compounds. X-rays are ionising radiation and interact with the atoms or molecules of the medium to produce free radicals in the cells. These radicals can damage or modify important plant cell components as well as the morphology, anatomy, biochemistry and plant physiology depending on the doses of irradiation. It has been generally accepted that reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl ($HO^{\cdot}$) radicals are produced by the radiolysis of water. Among these

ROS, H_2O_2 is a normal metabolite in cells under optimum plant growth conditions and is not particularly cytotoxic, but when its concentrations are increased by environmental stresses and ionising radiation, it can lead to cell lethality. To cope with the damage caused by the ROS, the cells possess a complete and integrated endogenous enzymatic defence system. Indeed, peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) represent the endogenous enzymatic defences of the plant cell that become active during the cellular lesion. In fact, it has been reported that the activities of these sweeping enzymes are generally increased in various plant species after treatment with ionising radiation. A wide range of physiological modifications have been described by many researchers on plants exposed to X-rays. The commonly observed abnormalities are improvement or inhibition of germination, growth of seedlings and other biological responses (Mbaye et al. 2017).

The most critical part of induced mutagenesis is the selection of an effective mutagen and determination of the LD_{50} value, given that it varies with the mutagen and the genotype. Higher concentrations may lead to tissue damage or an increase in mortality. Likewise, the duration of the treatment should be enough to permit hydration and infusion of mutagen into the explants (Saraswathi et al. 2016). These authors treated shoot tips and in vitro grown proliferating buds of banana cv. 'Rasthali' (Silk, AAB) with various concentrations and durations of chemical mutagens: EMS (ethyl methane sulfonate), NaN_3 (sodium azide) and DES (diethyl sulphate) and concluded that the LD_{50} is a pre-requisite for mutation breeding, and it seemed to vary with the mutagen efficiency, genotype and explant type.

Use of tissue culture combined with mutagenic agents in *Musa* spp. allowed genetic variability to be increased and improved certain agronomic characters such as fructification, yield and resistance to pathogens (Ho et al. 1994). Three induction systems have been developed from mutations for the genetic improvement of *Musa* spp. The first was based on irradiation of the suckers in vivo before the extraction and cultivation of in vitro buds, but the authors only reported a low number of mutants (De Guzman et al. 1982). The second was supported by induction techniques of in vitro bud mutations; recovery of the mutants to avoid or reduce the emergence of chimeras and micropropagation of the desirable mutants (García et al. 2002) gave better results. In the third induction system Roux (2004) referred to the use of cell suspensions of embryogenic banana as an explant for in vitro mutagenic treatment in cultivars 'Williams' (AAA) and 'Three Hand Planty' (AAB). However, the author found that the radiation dose to be used varied with the genetic constitution of each cultivar, so it was necessary to conduct radio sensitivity studies when using other genotypes. On the other hand, Kulkarni et al. (2004) in 'Grande naine' (AAA) conducted a study of gamma radiation sensitivity only at the level of establishment of the cell suspension culture.

In the Institute of Plant Biotechnology (IBP) in Cuba, a program for the improvement to *Fusarium* wilt in the cultivars 'Gross Michel' (*Musa* AAA) and 'Manzano' (*Musa* AAB) was developed. The combination of physical mutations (gamma rays) applied on adventitious buds in vitro with artificial inoculation using a highly pathogenic strain of *Foc* and natural infection in the field was used. The agronomic characters of potentially resistant or tolerant *Fusarium* wilt mutants and the natural

infection by the disease were evaluated during a 5-year period under field conditions. In the final phase of the study, three mutants were selected that showed levels of resistance or tolerance to the disease as well as adequate agronomic characters and yield and then extended to tests in other areas of the country to verify if they maintained these improved characters (Bermúdez-Caraballoso et al. 2002). Recently one of these mutants was evaluated against Foc TR4 in the Fruit Tree Research Institute (FTRI), Academy of Agricultural Sciences (GAAS) of Guangdong Province, People's Republic of China. The Gros Michel (GMIBP-542) mutant was evaluated under field conditions and compared with several cultivars with different levels of resistance to the pathogenic agent. The planting framework was in lines of 10 plants on a soil heavily infected with Foc TR4 where clone evaluation trials of the Guangdong banana improvement programs are conducted. The trials were continued for 12 months. During the trial the number of infected plants was evaluated, and the infection percentages were calculated. As a result, the evaluated mutant presented the highest infection frequency values under field conditions (100%), and it can be considered as very susceptible to Foc TR4.

Using physical mutagens (gamma rays), Roux (2004) reported different banana mutants with improved morphological characters and resistance to diseases, such as bunch size and cylindrical shape (Klue Hom Thong KU1), plant height (dwarfism) (SH-3436-L9), and increased tolerance to *Fusarium oxysporum* f. sp. *cubense* (Mutiara and Novaria) and to the toxin of *Mycosphaerella fijiensis* (GN35-I to GN35-VIII).

More recently, Bermúdez-Caraballoso et al. (2016) in the Institute of Plant Biotechnology (IBP) developed a methodology to irradiate, with ^{60}Co source gamma rays, embryogenic cell suspensions of banana cv. 'Grande naine' (*Musa* AAA) until their conversion to plants. Different doses of radiation (0, 30, 40, 50, 60, 70 and 80 Gy) were applied to embryogenic cell suspensions in the multiplication phase and the embryos were later formed, matured and germinated. To determine the ex vitro response of the population of plants obtained these were transferred to greenhouse conditions. The results showed that with somatic embryos formed, no differences were observed between the effect of the different doses of radiation applied and the control. However, the radiation dose affected the percentage of somatic embryo formation and germination. Plants with phenotypic variations were regenerated with 40 Gy. Results from the greenhouse showed that as radiation doses increased up to 50 Gy, the frequency of variations increased. With higher doses of radiation, the survival of the plants was affected. Actually, the population of plants obtained were used for a screening to Foc under greenhouse and field conditions.

Smith et al. (2006) obtained an extra-dwarf Cavendish cultivar with resistance to sub-tropical race 4 *Fusarium*wilt. Similarly, Mishra et al. (2007), using the same strategies, obtained three banana cultivars belonging to the AAA and AAB genomic groups (Basari, Chakkarakela and Rasthali) in the greenhouse, which exhibited morphological variations such as thick, shiny, dark-green leaves, ovate leaves and dwarf plants with a rosette of leaves. Similarly, Ganapathi et al. (2008) using the banana cultivar Giant Cavendish (*Musa* spp. AAA group) selected mutants for their improved agronomic traits, including reduced height and early flowering.

In the banana hybrid FHIA-21 one mutant with height reduction and with promising agronomic and post-harvest characteristics and resistance to black Sigatoka was obtained. The research in this tetraploid hybrid indicated that physical mutagenesis using a ^{60}Co source was able to generate a large amount of variability in the population (Bermúdez-Caraballoso et al. 2010). These results demonstrated that the use of this strategy is valid for the genetic improvement of *Musa*.

Recently, Damasco et al. (2019) using gamma irradiation coupled with in vitro technology developed BBTV resistance in banana cv. 'Lakatan'. Ten resistant lines were selected after several generations of evaluation and selection in the field conditions.

Somaclonal variation has been used for the genetic improvement of several agronomic characters. GCTCV-218, a somaclonal variant obtained from the giant Cavendish that shows resistance to Foc, has been registered under the trade name of FORMOSOMA (Hwang 2002). Other somaclonal variants of Cavendish called GCTCV-53 and GCTCV-119 have shown resistance to *Fusarium*wilt (Hwang and Ko 2004). With the knowledge of these techniques, an increase of the speed for improvement of this crop is expected over conventional breeding techniques.

Chemical mutagens have become an important tool to enhance agronomic traits of banana crops. They are being used to develop *Fusarium*-resistant lines in various susceptible banana cultivars. There are several mutagens like ethyl methane sulphonate (EMS) and sodium azide (NaN_3) available for banana crop improvement, and each mutagen has its own important role as inducers of positive or negative effects on growth and development of banana plants. Kishor et al. (2017) treated shoot tip culture with various EMS (0.30, 0.60, 0.90 and 0.12%) and NaN_3 (0.01, 0.02 and 0.03%) concentrations. The putative mutants obtained after in vitro rooting were subjected for the artificial inoculation of *Fusarium oxysporum* f. sp. *cubense*. In the screening of putative mutants it was observed that EMS-treated mutants were more susceptible compared to those from NaN_3 treatment. Treatment with 0.01% NaN_3 was found to produce three resistant lines during subsequent screening under greenhouse conditions.

Selection pressure can be applied also at the cell population level. The potential of an in vitro selection system is based on the fact that it is possible to screen a large population of cells and regenerated plants in small spaces and in a controlled environment.

Mutagenesis has been established as a very efficient tool for use in plant genetic improvement (Ahloowalia et al. 2004; Henikoff et al. 2004). However, this is a complex process to use in vegetatively propagated plants. Jankowicz-Cieslak et al. (2012) have developed a method for the treatment of meristematic apices in banana yolks with the chemical mutagen EMS and recovered high densities of GC-AT mutations together with showing that the genotype of the mutants is stable in successive generations. In addition, five banana lines resistant to *Fusarium*wilt were obtained in Brazil and identified by field evaluations after in vitro mutagenic treatment with EMS (Chen et al. 2013). Hence it is possible to adopt different techniques for the genetic improvement of bananas and obtain resistance to *F. oxysporum*. Considering these facts, the use of alternative techniques such as mutation breeding

among others (Arvanitoyannis et al. 2008; Chen et al. 2011) has been taken into account to induce and select desired mutants to Foc. This technique, along with other biotechnological methods, has been valuable tools for the genetic improvement in *Musa* spp., obtaining mutants with different level of resistance to *Fusarium*wilt after treatment of in vitro material with gamma radiation (Bermúdez-Caraballoso et al. 2002; Hegde et al. 2019) or with different chemical mutagens (Krishna et al. 2013; Saraswathi et al. 2016; Kishor et al. 2017).

Finally, in this section, according to the Mutant Variety Database (IAEA-FAO 2017), only three officially released banana cultivars generated from gamma irradiation treatment showed significant improvements in agronomic traits. These included cv. 'Al-Beely' with 30% yield improvement, cv. 'Klue Hom Thong KU1' with large cylindrical shape bunch and cv. 'Novaria' – an early flowering mutant with improved fruit quality.

Genetic Transformation

Banana was first transformed through protoplast electroporation (Sági et al. 1994). Since then, direct gene transfer by biolistic methods (Gene-gun) has been successfully applied in *Musa* spp. (Sági et al. 1995; Becker et al. 2000; Vishnevetsky et al. 2011) as well as *Agrobacterium tumefaciens*–mediated transformation of embryogenic cell suspensions (ECS) (May et al. 1995; Ganapathi et al. 2001; Khanna et al. 2004; Remy et al. 2005; Pérez-Hernández et al. 2006; Ghosh et al. 2009). Likewise, numerous advances have been made in the genetic transformation mediated by *Agrobacterium tumefaciens*, since the compatibility between the bacterium and several tissues of the plant was demonstrated during the chemotaxis and adhesion phases (Pérez-Hernández et al. 1999). This method has the advantages of simplicity and low cost, the insertion of a low number of copies of the transgene and the ability to transfer large segments of DNA with low occurrence of rearrangements (Gelvin 2003). Because of this, it is the most used method in genetic transformation of *Musa* spp. (May et al. 1995; Ganapathi et al. 2001; Khanna et al. 2004; Remy et al. 2005; Pérez-Hernández et al. 2006; Ghosh et al. 2009; Chong-Pérez et al. 2012). Currently, most of the protocols developed for bananas use embryogenic cell suspensions (ECS), and several parameters of the process have been optimised (Ganapathi et al. 2001; Khanna et al. 2004; Becker et al. 2000; Chong-Pérez et al. 2012; Concepción-Hernández et al. 2017). As explants for genetic transformation, ECS are considered ideal, since the resultant somatic embryos will have a unicellular or multicellular origin from few cells. Therefore, the possibility of obtaining chimeric plants is low (Grapin et al. 1996).

Genetic improvement through transformation is approached through different strategies, most of which include gene overexpression or suppression. However, gene overexpression or suppression often comes with a fitness cost associated with pleiotropy (van Schie and Takken 2014). In many cases, delayed growth, lower yield and loss of tolerance to other abiotic factors or pathogens have been reported.

A common strategy to diminish pleiotropic effects is the control of the timing and location of gene expression through the use of regulatory sequences like inducible promoters. However, reported events of transformation in banana make use of constitutive promoter sequences that come from bacteria or virus, and in a few of the cases, from other plant species. The availability of an increasing number and quality of genomes from banana and its pathogens should favour the identification of more suitable regulatory sequences. Genes most commonly engineered act in recognition of pathogens and its effectors, defence signalling and regulation, or as susceptibility genes facilitating pathogen entry, its establishment and permanence in the host plant.

Due to its importance in plant-pathogen interactions, overexpression of genes coding for pathogenesis-related (PR) proteins have been widely used in resistance breeding. PR proteins comprehend a group of diverse plant proteins classified in 17 families which are elicited as a result of pathogen invasion and environmental stress (Sels et al. 2008). As a strategy for fighting *Fusarium* wilt in banana, plants overexpressing β-1,3-glucanase (*PR-2*), chitinase (*PR-3*) and thaumatin-like (*PR-5*) genes have been used with different results (Mahdavi et al. 2012; Maziah et al. 2007; Sreeramanan et al. 2006). β-1,3-glucanase alone or in combination with chitinase has been successfully used to impart resistance against fungal pathogens. Sreeramanan et al. (2006) stacked different chitinase and β-1,3-glucanase genes in cv. 'Rasthali' to assess their effect against Foc race 1, while Maziah et al. 2007 used a β-1,3-glucanase from soybean. The potential of these combinations should be further evaluated in pot bioassays and field trials against the pathogen. Additionally, Hu et al. (2013) obtained banana plants from cv. 'Furenzhi' (*Musa* AA) transformed with the endochitinase gene (*chit42*) from *Trichoderma harzianum*. In this study, three of the obtained seven transgenic lines showed higher levels of resistance to Foc TR4 during in vitro and ex vivo disease assays. Also, a correlation was found between transgene expression level and resistance to the pathogen.

On the other hand, plant defensins (PR-12) are a group of anti-microbial peptides that inhibit the growth of numerous pathogens and have proven useful as anti-fungal genes (Portieles et al. 2010). In *Musa*, this strategy has been used to obtain transgenic plants constitutively expressing a magainin analogue (Chakrabarti et al. 2003), petunia floral defensins (Ghag et al. 2012), *Stellaria media* defensin (Ghag et al. 2014a) and Ace-AMP1 (Mohandas et al. 2013). Chakrabarti et al. (2003) used the magainin analogue MSI-99 from the African clawed frog in banana cv. 'Rasthali'. This peptide has a broad-spectrum activity against bacteria, fungi and protozoa, as well as anti-tumorigenic properties. The obtained transgenic plants were assayed against Foc race 2, and several lines showed a reduction of external symptoms and corm discoloration. Also, Ghag et al. (2012) introduced floral defensin genes (*PhDef1* and *PhDef2*) in plants from cv. 'Rasthali' and tested its tolerance against Foc 1 in a pot bioassay. Transgenic plants expressing each gene showed fewer internal and external symptoms than the control plants. Moreover, in 2014 Ghag et al. overexpressed a defensin gene (*Sm-AMP-D1*) from *S. media* in cv. 'Rasthali' (AAB). Purified Sm-AMP-D1 had displayed strong inhibitory activity when assayed in vitro against fungi and oomycetes, including *F. oxysporum* (Slavokhotova et al.

2010). Transgenic lines showed enhanced tolerance against Foc race 1 in a pot bioassay.

Ace-AMP1 is another protein with inhibitory activity against Foc that was isolated from onion (*Allium cepa*) (Cammue et al. 1995). Accordingly, plants from cv. 'Rasthali' with higher expression of *Ace-AMP1* gene showed a negative correlation between protein content and disease severity. This study identified six lines with enhanced tolerance to Foc race 1 over an exposure period of 6 months (Mohandas et al. 2013). On the other hand, in cv. 'Pei Chiao', overexpression of a ferredoxin-like protein (*pflp*) gene reduced the severity to Foc TR4 in a pot bioassay (Yip et al. 2011). The previous results indicate the potential for the use of this strategy against *Fusarium* and also the stacking of these genes to provide a broader and more durable resistance against the fungus. Thaumatin-like proteins (TLPs) belong to the PR-5 protein family and are elicited in response to pathogen attack, stress and developmental signals. Among their various properties, the ability to alter the fungal membrane's integrity causing lysis, spore lysis and the consequent reduction in spore number and germination, makes them appealing candidates for resistance breeding. Furthermore, TLPs have been successfully used in plants to engineer resistance to fungal pathogens (Liu et al. 2010). In banana, Mahdavi et al. (2012) introduced a rice *tlp*gene in cv. 'Nangka' (AAB) through particle bombardment. Transgenic plantlets were challenged against FocTR4 in a pot bioassay where most of the lines showed fewer external symptoms than the susceptible control plantlets.

In a different approach, overexpression of anti-apoptosis genes could hamper plant colonisation by necrotrophic pathogens. In effect, Dickman et al. (2001) engineered resistance in tobacco to a broad range of necrotrophic fungi through overexpression of various members of the anti-apoptotic *Bcl-2* gene family. In *Musa*, Paul et al. (2011) transformed plants from cv. 'Lady Finger' (*Musa* AAB) with *Bcl-xL*, *Ced-9*, and *Bcl-2* 3′ UTR anti-apoptotic genes. Seven transgenic lines (2× *Bcl*, 3× *Ced-9* and 2× *Bcl-2* 3′ UTR) showed promising reduction of external and internal symptoms compared to wild-type Lady Finger in a pot assay with Foc race 1. Further studies by Ghag et al. (2014b) proved that native cell-death-related genes can be used to develop transgenics with enhanced resistance to Foc race 1. These authors identified *MusaDAD1*, *MusaBAG1* and *MusaBI1*genes and overexpressed them individually in transgenic plants from cv. 'Rasthali'. The resulting transformants were challenged against Foc race 1 in a pot bioassay and plants overexpressing *Musa*BAG1 showed the best resistance as well as the highest relative overexpression of the transgene.

Resistance (R) genes play a significant role in preventing disease. Most of these genes encode intracellular nucleotide-binding leucine-rich-repeat (NB-LRR)-type resistance receptors for Avr molecules of pathogens. In *Musa*, a resistance gene analogue 2 (*RGA2*) was isolated from a seedling of *Musa acuminata* ssp. *malaccensis*, resistant to Foc TR4 (Peraza-Echeverria et al. 2009). Recently, transgenic plants from the economically important cv. 'Grande naine' overexpressing this gene showed promising results in a 3-year field trial against Foc TR4 (Dale et al. 2017). In this experiment, plants transformed with the *C. elegans* anti-apoptotic gene *Ced9* were also analysed for resistance. For both genes, several lines showed a slower

development of symptoms compared to non-transgenic plants. This study also reported the presence in susceptible *Musa* cultivars of *RGA* homologues which showed insufficient expression levels to influence resistance. This offers new opportunities for overexpression and the use of genome editing techniques to increase endogenous gene expression.

Nonetheless, the effectiveness of plant resistance genes is confronted by constant evolution of the pathogens. That is why other alternatives like RNAi-mediated host-induced gene silencing (HIGS) are being considered. Here, RNA interference is used to silence highly conserved pathogen genes required for morphogenesis or pathogenesis. Thus, Ghag et al. (2014c) reported the application of this technique against *Fusarium* wilt in cv. 'Rasthali' by targeting a common sequence of three *velvet* family genes (*VeA*, *VelB* and *VosA*) and the *Fusarium transcription factor 1* (*ftf1*). These genes are required for fungal morphogenesis, colonisation and the infection process. The resultant transgenic plants were assayed against Foc race 1 in greenhouse conditions, and 11 lines derived from the ihpRNA-VEL construct and 12 lines derived from an ihpRNA-FTF1 construct showed no internal or external symptoms after 6 weeks of inoculation. This study paves the way for future applications of HIGS against important banana pathogens.

Resistance based on single dominant genes is prone to be rapidly overcome by the pathogen. Stacking of several genes is an alternative to this while the use of susceptibility (S) genes might be another one. S genes facilitate the pathogenesis and may act in different stages of the infection like early pathogen establishment, modulation of host defences and pathogen sustenance (van Schie and Takken 2014). Mutations of S genes have been naturally selected by farmers and breeders performing mutagenesis and have been shown to be durable over the years (Jorgensen 1992). However, S genes play different roles in plant development; therefore their usefulness for resistance breeding must be assessed in every case. In addition, exciting genome editing techniques like CRISPR/Cas have been successfully used in *Musa* cv. 'Gonja Manjaya Rasthali' to inactivate the sequence of the endogenous banana streak virus BSV (Tripathi et al. 2019). This is an important step in the improvement of the B genome germplasm and its further use in the production of hybrids. It is only a matter of time before CRISPR/Cas applications extend to breeding banana against its major fungal pathogens. Finally, although the number of reports of transgenic bananas has increased over the last years, there are no commercial banana varieties resistant to Foc. Moreover, the majority of the reports on transgenic banana only count for greenhouse experiments. Therefore, it is difficult to establish whether these experiments will progress into field trials and the release of new varieties. More field trials are needed to evaluate the real potential of genetic transformation for reducing the impact of the devastating *Fusarium* wilt (Table 20.1).

Table 20.1 Genetic transformation in *Musa* spp. for resistance to *Fusarium oxysporum* f. sp. *cubense* (Foc)

Transgene	Origin	Transfer method/explant	Banana cultivar (genome)	Foc race/bioassay	Reference
chitinase and *β-1,3-glucanase gene*	Unknown	Biolistics/multiple bud clumps	Rasthali (AAB)	Foc 1/plantlets in pots	Sreeramanan et al. (2006)
chit42	*Trichoderma harzianum*	Agrobacterium-mediated transformation/ECS	Furenzhi (AA)	Foc 1/in vitro and pot bioassay	Hu et al. (2013)
Magainin analogue (*MSI-99*)	*Xenopus laevis*	Agrobacterium-mediated transformation/ECS	Rasthali (AAB)	Foc 2/pot bioassay	Chakrabarti et al. (2003)
PhDef1, PhDef2	*Petunia hybrida*	Agrobacterium-mediated transformation/ECS	Rasthali (AAB)	Foc 1/in vitro and pot bioassay	Ghag et al. (2012)
Sm-AMP-D1	*Stellaria media*	Agrobacterium-mediated transformation/ECS	Rasthali (AAB)	Foc 1/pot bioassay	Ghag et al. (2014a)
Ace-AMP1	*Allium cepa*	Agrobacterium-mediated transformation/ECS	Rasthali (AAB)	Foc 1/pot bioassay	Mohandas et al. (2013)
pflp	*Capsicum annuum*	Agrobacterium-mediated transformation/bud slices from bud clumps	Pei Chiao (AAA)	Foc TR4/pot bioassay	Yip et al. (2011)
tlp	*Oryza sativa*	Biolistics/multiple bud clumps	Nangka (AAB)	Foc race 4/plantlets in pots	Mahdavi et al. (2012)
Bcl-xL, Ced-9 and *Bcl-2*	Animal	Agrobacterium-mediated transformation/ECS	Lady finger (AAB)	Foc 1/pot bioassay	Paul et al. (2011)
MusaDAD1, MusaBAG1 and *MusaBI1*	*Musa acuminata*	Agrobacterium-mediated transformation/ECS	Rasthali (AAB)	Foc 1/pot bioassay	Ghag et al. (2014b)
RGA2 and *Ced9*	*Musa acuminata* and *Caenorhabditis elegans*	Agrobacterium-mediated transformation/ECS	Grande naine (AAA)	Foc TR4/contained field trial	Dale et al. (2017)

(continued)

Table 20.1 (continued)

Transgene	Origin	Transfer method/ explant	Banana cultivar (genome)	Foc race/ bioassay	Reference
siRNAs targeting Foc *velvet* and *ftf1*	*Fusarium oxysporum* f.sp. *cubense*	Agrobacterium-mediated transformation/ ECS	Rasthali (AAB)	Foc 1/in vitro fungal inhibition assay and pot bioassay	Ghag et al. (2014c)

ECS Embryogenic cell suspensions

Biocontrol

Antagonistic microorganisms are those that have a negative effect on the growth of others (Chowdhury et al. 2015a), so they can be very useful in the integrated management of various diseases that affect crops of economic importance (Melnick et al. 2008). Its most important characteristics are related to its genetic stability, effectiveness at low concentrations, ability to be use different nutritional sources, survival in adverse environmental conditions, effectiveness against a wide variety of pathogens, ecological feasibility, resistance to fungicides and its compatibility with other commercial procedures (Berg 2009). Among the bacteria that have been studied for the control of *F. oxysporum* are the genera *Pseudomonas* (44%), *Bacillus* (13%) and *Streptomyces* (9%) (Antoun and Prévost 2005; Lecomte et al. 2016). Several genera and bacterial species have been described as antagonists specifically against Foc, for example, strains of *Pseudomonas* spp. (Sivamani and Gnanamanickam 1988; Sun et al. 2011; Sekhar and Thomas 2015), *Bacillus* spp. (Sun et al. 2011; Hadiwiyono and Widono 2013; Xue et al. 2015) and *Burkholderia* spp. (Ho et al. 2015).

Pan et al. (1997) noted that *Burkholderia* sp. can colonise the mycelial surface of Foc and cause deformations in the morphology of the fungus such as terminal swelling and intercalation in the hyphae. Also, Simonetti et al. (2018) demonstrated the ability of *Burkholderia ambifaria* T16 to inhibit the growth of several *Fusarium* species and that, in addition, it possessed the extraordinary ability to use the fungus' mycotoxins as energy sources. On the other hand, Sun et al. (2011) pointed out the antagonistic effect of a strain of *P. fluorescens* against Foc in addition to its remarkable growth promotion effects on the banana plant. Sekhar and Thomas (2015) also noted the significant anti-fungal properties of an endophytic *P. aeruginosa* isolate from the 'Grande naine' (AAA) cultivar against Foc. However, it is important to note that both this bacterial species and *Burkholderia* spp. have negative effects on immuno-compromised patients, which makes their use difficult (Kumar et al. 2013; Andueza et al. 2015; Lecomte et al. 2016).

In general, Zhang et al. (2013) concluded that the use of rhizospheric or endophytic strains of *Trichoderma, Pseudomonas,Streptomyces* and non-pathogenic strains of *Fusarium* has not yielded the desired results in allowing effective management of *Fusarium*wilt. Numerous species of *Bacillus*, in particular, have been widely studied as biological control agents due to their multiple mechanisms of

antagonistic action, such as anti-biosis, competition and the induction of systemic resistance in plants (Chowdhury et al. 2015a; Fan et al. 2017). In addition, this genus is distinguished from other bacterial genera such as *Pseudomonas* by forming endospores that allow them to survive for long periods of time under unfavourable environmental conditions (Radhakrishnan et al. 2017). This feature allows biofertilisers based on *Bacillus* spp. to be more active, since it extends the viability of the cells within the formulations (Gang et al. 2013).

Bacillus spp. as a Biological Control Agent

The genus *Bacillus*, belonging to the *Bacillaceae* family, is one of the most studied species in relation to its potential as a plant growth promoter (Radhakrishnan et al. 2017). Thanks to this, many of its species (*B. subtilis, B. brevis, B. cereus, B. pumilus, B. licheniformis* and *B. amyloliquefaciens*, among others) constitute ideal candidates as MBCA for numerous plant diseases (Chowdhury et al. 2015a; Tan et al. 2016). Within the species of phytopathogenic agents against which *Bacillus* spp. is employed as MBCA are *F. oxysporum*, *Fusarium roseum* (Link emend.) Sny. & Hans. 'Graminearum', *Rhizoctonia solani* (J.G. Kuhn) R.T. Moore, *Phytophthora capsici*Leo, *Phytophthora cactorum* (Leb. & Cohn) Schröeter, *Alternaria solani* (Cooke) Wint., *Alternaria alternata* (Fr.) Keissl., *Sclerotium cepum* and *Uromyces appendiculatus* (Link) Unger, and *Pseudocercospora fijiensis* Morelet, among others (Baker and Cook, 1974; Sun et al. 2011; Cruz-Martín et al. 2017).

Pseudomonas spp. and *Bacillus* spp. have been recognised as the plant growth promoting bacteria (PGPB) with more predominance in the soil (Kang et al. 2015). However, species of the genus *Bacillus* distinguish themselves from species of the genus *Pseudomonas* by forming endospores that allow them to survive for long periods of time under unfavourable environmental conditions (Radhakrishnan et al. 2017). Haas and Defago (2005) indicated that this characteristic allows biofertilisers based on *Bacillus* spp. to be more active than those based on *Pseudomonas* spp. since it extends the viability of the cells within the formulations. In addition, processing costs and storage and transportation conditions are very reasonable (Gang et al. 2013). According to Kilian et al. (2000), the first commercialised bacterial fertiliser (Alinit) was obtained from *Bacillus* spp. In the aforementioned study, it was shown that with the use of this bioproduct an increase of crop yields of up to 40% was obtained. Subsequently, other bioproducts have been commercialised from *Bacillus* spp. These include Quantum-400 (*B. subtilis* GB03), Rhizovital® (*B. amyloliquefaciens* FZB42), Serenade® ASO (*B. subtilis* QST713), YIP (*Bacillus* sp.) (Radhakrishnan et al. 2017) and Kodiak® (*B. subtilis* GB03) (Brannen and Kenney 2017), which have been developed with the purpose of increasing production in crops of high economic importance.

In studies conducted by Leyva et al. (2017) with bacteria of the genus *Bacillus*, it was possible to verify that of 17 strains evaluated, 9 showed in vitro anti-fungal activity against *F. oxysporum* race 1. Within these nine were species such as *B.*

subtilis, *B. licheniformis* and *B. amyloliquefaciens*. On the other hand, it is noteworthy that six of the nine strains that showed anti-fungal activity against this pathogenic fungus were isolated from Musaceae and demonstrated anti-fungal activity against *P. fijiensis* (Cruz-Martín et al. 2016). These results constitute a positive criterion for the use of these strains as possible biological controllers in this crop and show the potential of the natural microbiota to defend plants against phytopathogenic agents. Several studies suggest that native bacterial strains of certain ecosystems, compared to exotic strains, may have greater efficiency such as PGPB and biocontrol agents (Hernández-Rodríguez et al. 2010).

Among *Bacillus* species, several strains of *B. amyloliquefaciens* have been studied for antagonistic potential against Foc (Cao et al. 2011; Xu et al. 2013; Yuan et al. 2015; Leyva et al. 2017). In this sense Xue et al. (2015) showed that the NJN-6 strain of this bacterial species, in combination with compost, produced a significant decrease in the colonisation of the fungus in the rhizosphere of banana plants, an aspect of great interest in the management of the disease.

Mechanisms

Bacillus spp. are considered optimal microorganisms as MBCA for the following reasons: their ubiquity in the soil; production of spores resistant to desiccation, heat, ultraviolet irradiation and organic solvents; their induced resistance system and promotion of plant growth (Li et al. 2014). Likewise, their production of enzymes (chitinase, β-1,3 glucanase, xylanase) (Kaur et al. 2016) and antibiotics (iturines, surfactins and fengycins) are also relevant (Liu et al. 2014).

Numerous studies have focused on efforts assessing the anti-fungal potential of MBCAs with the aim of finding new strategies for the management of diseases caused by phytopathogenic agents in crops of economic interest (González et al. 2010; Campuzano et al. 2017). Anti-biosis is one of the mechanisms by which MBCAs inhibit other microorganisms and is mainly based on the production of secondary metabolites (El Arbi et al. 2016). Anti-biosis is defined as the inhibition or destruction of a microorganism by the metabolic products of others, generally by the action of antibiotics (volatile and diffusible) and lytic enzymes, which generally act at low concentrations (less than 10 ppm) (Vudem et al. 2011). Mo et al. (2013) pointed out that some microbial antagonists only produce this type of substance under low nutrient conditions. Consequently, they are not viable as MBCAs directly but may be indirectly suited due to the use of their metabolites.

The studies conducted by Cao et al. (2011) and Xu et al. (2014) suggest that the antagonistic effect of *B. amyloliquefaciens* SQR9 against several fungi is due to its ability to produce secondary metabolites with anti-microbial activity. In addition, it has been described that *Bacillus* spp. have the potential to produce volatile and widespread anti-fungal compounds that inhibit the growth of Foc (Wang et al. 2013). On the other hand, Chen et al. (2008) report that *B. subtilis* can produce more than 24 anti-fungal substances with a wide variety of molecular structures,

including volatile growth-inhibiting compounds and inhibitors of germination of fungal spores. All these examples demonstrate the importance of secondary metabolites as one of the main mechanisms by which *Bacillus* spp. exerts antagonism against phytopathogenic agents. Knowing the chemical nature of these metabolites and their mode of action would allow the design of better strategies for their use in the management of plant diseases, such as *Fusarium* wilt.

Lytic Enzymes

It has been described that Bacillus spp. has the ability to produce chitinase and β-1,3-glucanase type enzymes with important lytic activity on the cell wall of many fungi (Leelasuphakul et al. 2006). Chitin is a polysaccharide composed of monomer units of N-acetylglucosamine linked together by β-1,4 glycosidic bonds and is the second most abundant polymer in nature after cellulose. This molecule forms the basis of the integrity of the cell wall of most fungi (Das et al. 2010). A wide variety of bacteria produce chitinases to process and digest macromolecules and convert them into sources of nutrients (Cohen-Kupiec and Chet 1998). Among these bacteria are genera such as *Aeromonas, Bacillus, Clostridium, Klebsiella, Pseudomonas, Serratia, Streptomyces* and *Vibrio* (Deshpande 1986). In particular, the genus *Bacillus* stands out for its great chitinolytic capacity (Vudem et al. 2011), which can be a very important mechanism for the control of phytopathogenic fungi. Among the strains of *Bacillus* described with chitinolytic capacity are *B. amyloliquefaciens* and *B. subtilis* (Shafi et al. 2017). In addition, according to these authors, due to the characteristics of the plant cell wall (they lack chitin), the use of chitinase-like enzymes is more effective than glucanases to control phytopathogenic agents.

Lipopeptides

Lipopeptides constitute one of the most important known biosurfactants. These peptides are usually classified into three families: fengycins, surfactins and iturines (González-Jaramillo et al. 2017). Among the microorganisms that have the capacity to produce them are the bacteria of the genus *Bacillus* spp. (Falardeau et al. 2013; Singh et al. 2014; Jin et al. 2014), highlighting the species *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis* and *B. brevis* (Pretorius 2014). These lipopeptides are synthesised by a non-ribosomal multi-enzyme complex and are structurally formed by a hydrophobic lipid tail attached to a cyclic oligopeptide. The amphiphilic nature of these molecules gives it anti-microbial activity due to its great tendency to interact with the phospholipids of microbial membranes, particularly in fungi (Eeman et al. 2014; González-Jaramillo et al. 2017).

Numerous investigations have highlighted the anti-fungal activity of bacillomycin D against species of the genus *Fusarium*. For example, it was reported that

B. amyloliquefaciens SQR9 has a significant anti-fungal compound against *F. oxysporum* f. sp. *cucumerinum* and *Fusarium solani* (Xu et al. 2013; Liu et al. 2014), while in *B. amyloliquefaciens* FZB42 it turned out to be one of the most powerful anti-fungal metabolites in in vitro conditions (Chowdhury et al. 2015b). According to a study conducted by Li et al. (2014), this compound was the one that contributed most to the antagonistic effect of *B. amyloliquefaciens* SQR9 against *F. oxysporum*, which coincides with that described by Wang et al. (2014) who demonstrated that iturin and bacillomycin D have strong anti-fungal activity against Foc.

The genetic representation of secondary metabolites in the bacterial genome is relatively high. Some authors (Chen et al. 2009; Borriss 2013) have reported that in strain *B. amyloliquefaciens* FZB42 up to 11 groups of genes (representing more than 9% of their genome) are involved in the synthesis of secondary metabolites, including lipopeptides with anti-microbial effects. Three of the respective groups of genes are responsible for the synthesis of surfactin, fengycin and bacillomycin D. In other studies, it has been explained that this strain produces these metabolites in response to signals emitted by Foc and other fungi (Cawoy et al. 2015). Similarly, its beneficial effects in promoting plant growth have been widely documented as well as its usefulness in the suppression of diseases in tomato, cucumber (*Cucumis sativus* L.), cotton (*Gossypium* spp.), tobacco (*Nicotiana* spp.) and lettuce (*Lactuca sativa* L.) (Borriss 2011; Chowdhury et al. 2013, 2015a).

The application of biological control agents that are safe for the environment and mankind has become an important strategy for the integrated management of pests and diseases in plants. According to Bettiol et al. (2013), large agrochemical companies are entering with great force in the market for the development and commercialisation of new bioproducts. According to these authors, the world market for bioproducts was estimated to reach 6.1 billion dollars by 2022, but it is currently being reviewed and an estimated 25 billion is now estimated by 2030. In this regard, several studies suggest the potential of *Bacillus* spp. as a biological control agent due to its antagonistic capacity against several phytopathogenic agents. However, it is necessary to conduct studies of characterisation of the anti-fungal activity of strains of this bacterial genus in order to determine their potential as biological control agents against certain phytopathogenic organisms of agricultural interest.

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Chapter 21
Integrated Pest Management of Whitefly Crop: Free Periods Can Reduce *Begomovirus* Transmission in Tomato

Margarita Palmieri, Martha Patricia Herrera, and Ana Lucía Dubón

Abstract Tomato is an important crop for exportation and internal consumption in Guatemala. Growers almost abandoned the production of this crop due to the presence of *Begomovirus* infections in the field and to the high populations of *Bemisia tabaci*. The crop was rescued by applying new technology and concepts of integrated pest management. This article describes the techniques used to determine and establish a host crop–free period to greatly reduce whitefly presence and thus virus transmission. We identified species of whiteflies in order to find possible vectors of *Begomovirus*, determined the monthly percentage of infected female *Bemisia tabaci* and that of *Trialeurodes vaporariorum* occasionally. *T. vaporariorum* was studied because it is more abundant than *B. tabaci* principally at high altitudes. We also obtained knowledge of the insect's life cycle with respect to climate variables, the virus effect on crop, the effect of weeds on virus availability for transmission, we established a calendar with planting dates and effective communication with growers and grower organizations concerning host-free periods as well as promotion of concepts of whitefly management. This chapter includes information of different aspects of our large program on *Begomovirus* and whiteflies.

Keywords *Begomovirus* · Tomato · Guatemala · Whitefly · Crop-free periods

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Introduction

Tomato is an important crop in Guatemala that was almost eliminated by *Begomovirus* transmitted by whiteflies, especially *Bemisia tabaci* (Gennadius) during the last years of the twentieth century and the beginning of the twenty first. The crop was rescued by applying new technology and concepts of integrated pest management (IPM). This chapter describes the techniques and factors used to determine and establish a crop-free period to greatly reduce whitefly presence and thus virus transmission. These involved determining the percentage of whiteflies infected with the virus, knowledge of the insect's life cycle with respect to climate variables, determining the virus effect on the crop, determining the effect of weeds on virus availability for transmission, establishment of a monitoring program and effective communication with growers and grower organizations concerning crop-free periods. Growers also learned IPM such as cultural techniques (planting trap crops and eliminating residuals), genetic approaches (resistant varieties, certified seeds), biological control (use of natural enemies, entomopathogenic fungi) and others, including chemicals when really needed or as a last resort. We determined which whitefly biotypes were present and distinguished between natives and aliens. We also determined how *Bemisia* populations varied with the presence of *Trialeurodes vaporariorum* (Westwood), the greenhouse whitefly. This last species of whitefly is important because it can acquire the *Begomovirus* but is not capable of transmitting it. Nevertheless, it is a vector of other viruses and can cause physical damage to the crop.

Begomovirus and Whiteflies

In the Central American Region, one of the most important crops is tomato. When tomato production almost disappeared from the Guatemalan market at the turn of the twenty-first century, due to high earnings, tomato growers did not want to change crops, so they began cultivating it at an altitude above 1000 m where supposedly the virus vector, *Bemisia tabaci*, did not occur. Since the end of the twentieth century, a Guatemalan study on *Begomovirus* and whiteflies, principally *Bemisia tabaci*, was implemented using biotechnology to help growers start planting when the percentage of virus infected whiteflies in fields is at a minimum. The concepts of host-free periods and integrated pest management were principally introduced to the growers of two regions: Salamá, Baja Verapaz and Santa Rosa, Santa Rosa. This approach is important because it is based on the biology of the virus vector, climate and the poor capability of weeds to contain sufficient virus to allow easy transmission of virus from weeds to crop. Advances in technology have brought an easy and sensitive technique to identify the virus in the vector. By calculating the percentage of infected whiteflies in a field, the grower can anticipate the problems he will have

during the season and implement cost-effective solutions, such as eliminating weeds that host whiteflies or the use of fast-growing tomato varieties or postponing the planting date.

The use of the relationships among planting dates, percentage of viral infection of the whitefly and its life cycle to determine the duration of the host free period was very important. The life cycle of whitefly varies according to temperature, humidity and light. In places with high temperatures, it can be as short as 15 days or at low temperatures as long as 1 month, depending on the species of host plant and its physiological state. We believe that 2 months of not planting hosts of *Begomovirus* was enough to assure that the new generation of whiteflies is not infected if alternative hosts, weeds and/or lonely plants (voluntary plants) of the crop are not present in the field.

Begomovirus belong to the Geminiviridae and are viruses of single-strand DNA. There can be viruses of one ring (monocatenary) or bipartite with two DNA rings. They usually cause diseases of plants in tropical, subtropical and temperate regions. The first type of *Begomovirus* was initially found only in Old World viruses and the last type (two-DNA-ring viruses) in New World viruses, even though now we can find viruses from the Old World here. These viruses are transmitted and dispersed principally by its vector *Bemisia tabaci* in Guatemala (Palmieri et al. 1999). The transmission of infection of this kind of virus in its vector is in a persistent manner where the virus is acquired but the vector cannot transmit it until sufficient time has transpired that allows the virus to reach the salivary glands. After that, the whitefly can transmit the virus for the rest of its life though because the virus does not replicate inside the whitefly, the level of transmission is reduced with time as the number of particles does not increase inside the host. The diversity of *Begomovirus* is very high, for example, for tomato *Bemisia tabaci* can transmit at least 8–12 species of *Begomovirus* and can cause mixed infections with more than two different *Begomoviruses*. This high *Begomovirus* biodiversity in host plants, and the large populations of *Begomovirus* present, can result in recombinations. High biodiversity and large populations of *Begomoviruses* allow the production of new variants with a modified host range and with new symptoms or diseases that can bypass previous resistance genes. This is a major reason to detect and characterize the different virus populations, their hosts and principally their vectors.

The project involved almost all regions of tomato producers but in this chapter, we will emphasize two departments: Santa Rosa and Baja Verapaz. Santa Rosa is localized in Eastern Guatemala, and Baja Verapaz is in the North Central region of Guatemala. Salamá is the capital city of Baja Verapaz. We studied different valleys and localities with each one divided into fields and each field was a sampling point. Salamá and Santa Rosa are towns in two valleys and were divided into three fields in each selected locality. Sampling was done monthly from March 2006 to September 2008. The general procedure that the project followed is presented in Fig. 21.1.

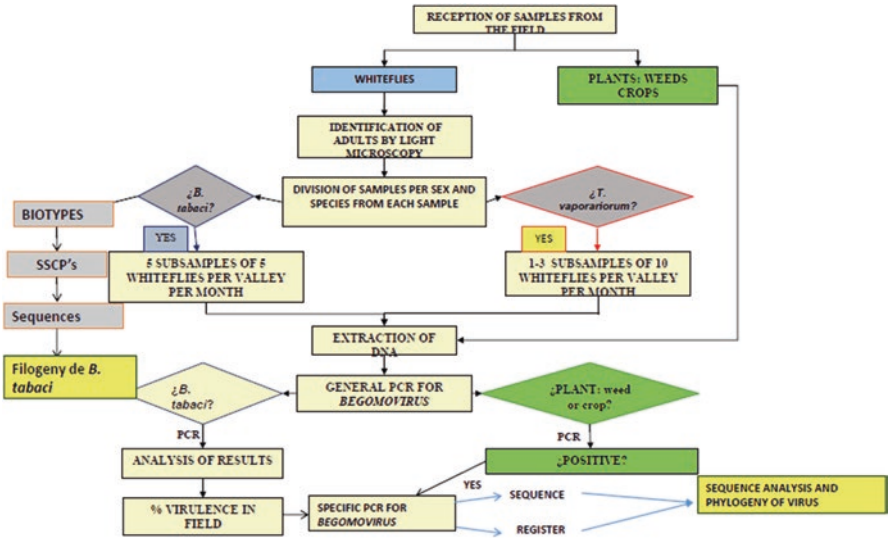

Fig. 21.1 Procedures followed in the project with the whitefly samples and the tissue samples of the crop

Nymphs

Samples of five whole leaves per plant were collected with whitefly nymphs attached from each of five plants per field. Leaves of each plant were placed in small Ziploc bags, identified and stored in a cooler. All samples were taken to the laboratory and stored at 4 °C to preserve the DNA and to prevent the emergence of adults.

To identify species of whiteflies we used nymphs of the fourth instar. To calculate the population density on the crop, we measured 1 square inch in the part of the leaf with highest concentration of nymphs for each of the five different leaves and counted the nymphs present. The rest of the nymphs that were present in the tissue were stored at 4 °C in 80% ethanol. For identification, all nymphs were mounted on slides, stained with Wilkey's triple stain (Hodges and Evans 2005) and identified using the keys of Rafael Caballero (1992, 1994). The most important criteria used for the identification of nymphs were: the form of the vasiform orifice, operculum, lingula, tracheal grooves, caudal and dorsal setae. Figure 21.2 shows the most common species found in the different fields and the color that the preparations showed.

After counting all the nymphs present monthly in the samples from each site, the percentage of each species for each month was calculated (Figs. 21.3 and 21.6).

Salamá has a cold to temperate climate and is located at 940–1000 m altitude. Three species were present in Salamá: *Bemisia tabaci*, *Trialeurodes vaporariorum* and *Trialeurodes abutiloneus*. *T. abutiloneus* was present during April and May 2006, then in October 2006, January, February, March, July and August 2007 and finally in January 2008. This species was present during the first months of the year and the last ones, except in 2007 that presented a high population in August. During

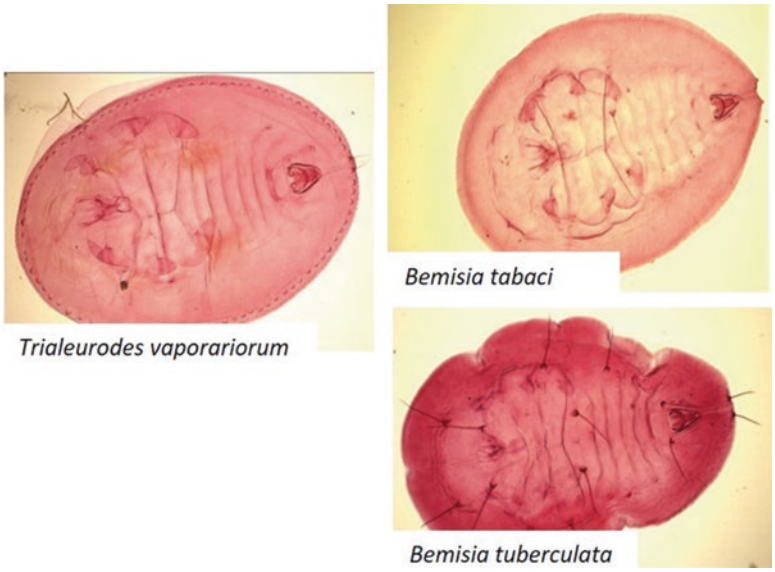

Fig. 21.2 Nymphs of the fourth instar stained with Wilkey's triple stain

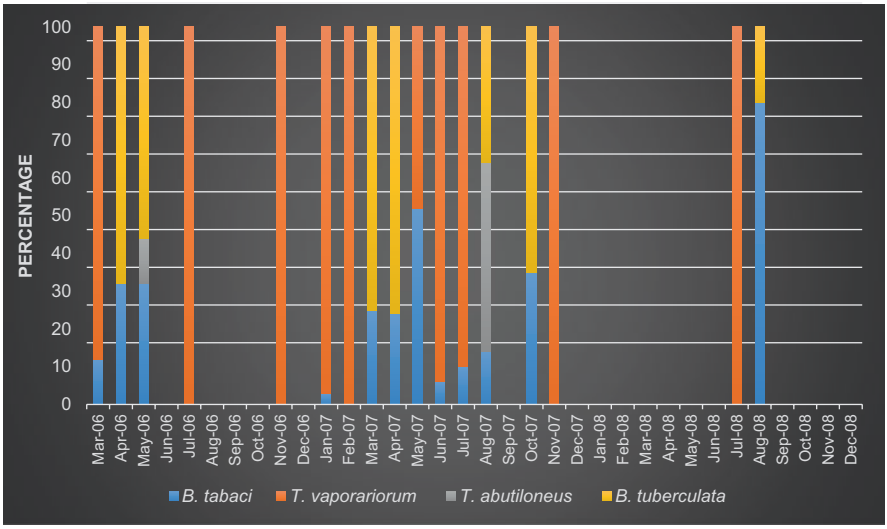

Fig. 21.3 Populations of each species of whiteflies from 2006 to 2008 in different fields of Salamá, Baja Verapaz

the first and last months of the year usually there is no rain. The temperatures are usually lower than the ones in the rainy season. It will be interesting to compare whitefly presence with climate variables during those dates in order to make specific conclusions about the presence and behaviour of these species, especially to explain

the greater populations in August 2007. *T. vaporariorum* and *B. tabaci* were present in almost all months of the years studied, principally *T. vaporariorum*. It had greater populations than *B. tabaci* in almost 55% of the months. *T. vaporariorum* showed higher population during the last and first months of the year more constantly than *B. tabaci*. These are the coldest months of the year. *B. tabaci* instead tends to be more abundant during the warmest months: April to August. It will be very important to compare these percentages to rain amounts to see if these populations were high even with rain. We can only say that both species are very important but in tomato in Guatemala, *B. tabaci* has been the one that transmits *Begomovirus* even with very low populations (Palmieri et al. 1999). The fact that *B. tabaci* is smaller and more active than *T. vaporariorum* making it more difficult to capture thus affecting the relative number of captures of each species. We tended to capture more *T. vaporariorum* than *B. tabaci*, especially when both species were present. When the population of *Trialeurodes* is present at 100%, we cannot be sure if *Bemisia* is absent; we can only say that the proportions of it were small or extremely low. Figure 21.4 presents the percentages of the three species found in Salamá.

Santa Rosa is a department in Guatemala and its capital is Cuilapa. The samplings were in localities between 1001.25 and 1270 m altitude, not specifically in Cuilapa. The climate is temperate. In Santa Rosa, the same three species were present, and *T. vaporariorum* was the most abundant as Fig. 21.5 shows.

As in Figs. 21.3 and 21.6 below show the monthly percentages of each species of nymphs collected in Santa Rosa during the study period.

T. abutiloneous was present in May 2006 and August 2007, months of high temperatures and rain frequently begins May, but no specimens were collected in other

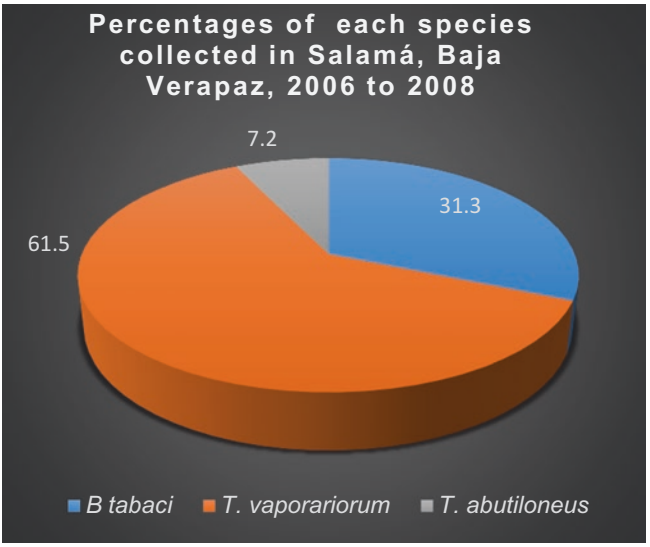

Fig. 21.4 Populations of *B. tabaci*, *T. vaporariorum* and *T. abutiloneus* identified in Salamá, Baja Verapaz, 2006–2008. Total of individuals classified: 2226

months. Capture levels in these localities were lower than in Salamá even though it was the same team that collected the samples in both departments. We need to analyze more years, more data of other communities and to correlate different

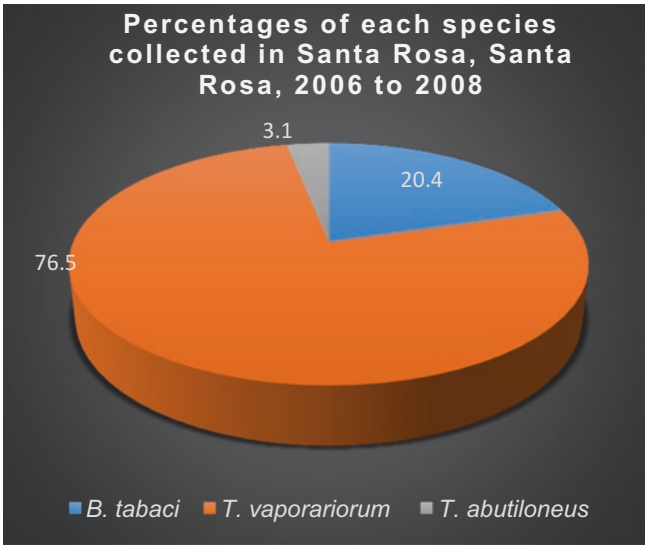

Fig. 21.5 Populations of *B. tabaci*, *T.* vaporariorum and *T. abutiloneus* identified in Santa Rosa, Santa Rosa, 2006–2008. Total of individuals identified: 609

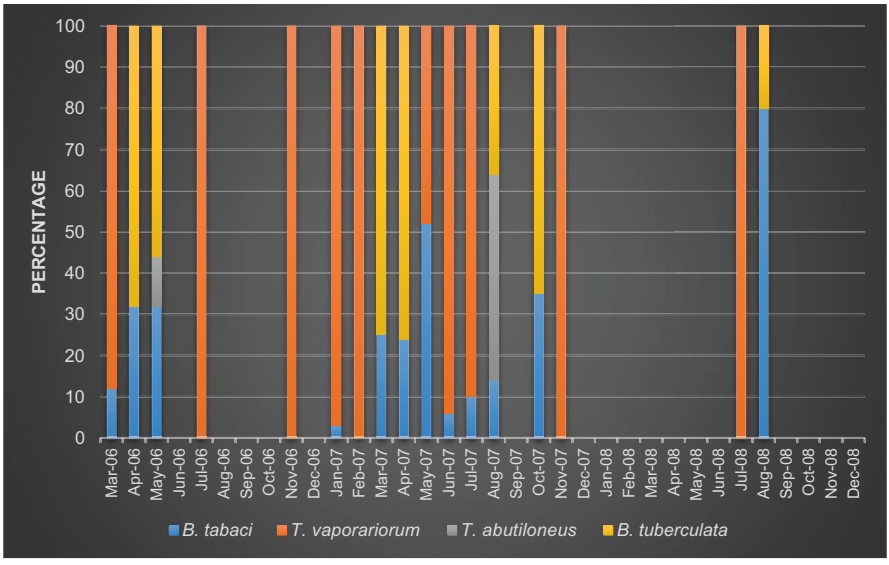

Fig. 21.6 Populations of each species of whiteflies from 2006 to 2008 in fields of Santa Rosa, Santa Rosa, Guatemala

species to altitude because Santa Rosa is a little higher than Salamá. This high altitude also explains that *T. vaporariorum* is more abundant than in Salamá; *B. tabaci* instead is less abundant than in Salamá compared in percentages, but even then, *B. tabaci* was the only species captured in November 2006. *B. tabaci* was present in 2006 and 2007 during March, April and May. In 2007, its presence extended to October. The species found in all sites of the study are *Bemisia tabaci*, *Trialeurodes vaporariorum*, *Trialeurodes abutiloneus*, *Bemisia tuberculata* Bondar, *Trialeurodes variabilis* (Quaintance). Others were occasionally present but not related with virus transmission, such as *Aleurothachelus socialis* Bondar, *Aleurocanthus woglumi* Ashby and *Aleuroglandulus melangae* Russel.

Adult Whiteflies

Samples of adult whiteflies were captured in each field monthly with an aspirator and were stored in 80% ethanol in 50 ml tubes. They usually contained around 100 whiteflies per sample. Adult whiteflies were separated into species according to the type of union of the upper and lower sections of the compound eye, which is composed of many units (ommatidia). We wanted to separate adults of the species that could be vectors of *Begomovirus*. In Fig. 21.7, we present the different dispositions and number of the ommatidia joining the upper and the lower section of the compound eye used to determine the species of the adult whiteflies. The disposition of the ommatidia also can be correlated to other characters such as presence or absence

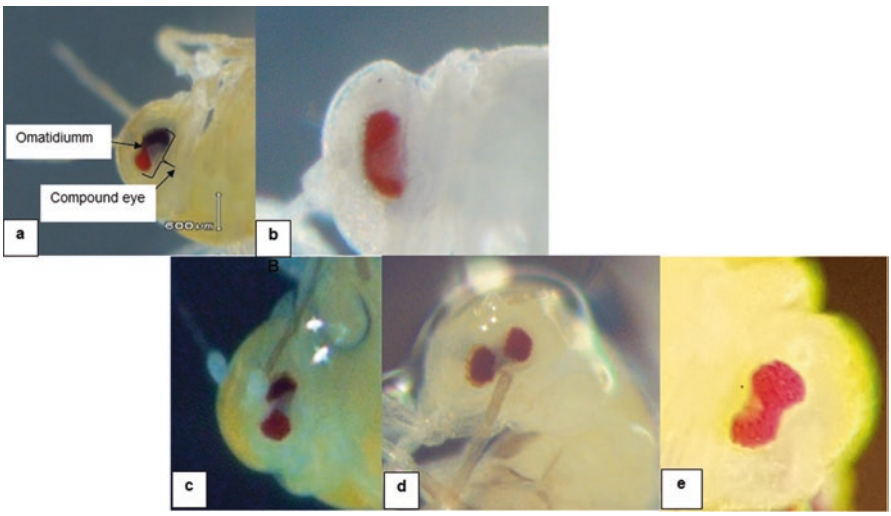

Fig. 21.7 Different types of union between the two groups of ommatidia of the upper and lower sections of the compound eyes of adult whiteflies. (**a**) *Bemisia tabaci*, (**b**) *Bemisia tuberculata*, (**c**) *Trialeurodes vaporariorum*, (**d**) *Trialeurodes abutiloneous* and (**e**) *Trialeurodes variabilis*

of dark spots on the wings or body. We separated the males from the females using the following characters: (1) the females are larger than the males and (2) the form of the last segment of the male is like a clip or tweezer that is formed by the clasper, edeago, supragenital plate and vasiform orifice. The posterior segment of the female is rounder than that of the male and forms a triangle composed of the ventral and dorsal valves, the intervalvular membrane and in the upper part, the vasiform orifice.

We observed that *Bemisia tabaci* has only one ommatidium joining the upper and lower section of the compound eye, and many times one section of the eye can be of different colour, one black and the other red. *Bemisia tuberculata* generally has the two sections of the compound eye connected by two lines of ommatidia; in *Trialeurodes vaporariorum* and *Trialeurodes abutiloneous* the upper and lower sections are not joined but the difference between the two species is that *T. abutiloneous* has black bands on the wings. *Trialeurodes variabilis* can be separated from the others because the females have four lines of ommatidia between the upper and lower sections of the compound eye. *T. variabilis* is one of the species in which the male has a different type of union than the female (Calvert et al. 2001). Calvert et al. (2001) used similar criteria to identify whiteflies for that study. After separating and identifying the whiteflies species, we proceeded to do the polymerase chain reaction (PCR) for five unmixed groups, each with five adult females of *B. tabaci* and/or *T. vaporariorum* from each field from each valley. *B. tabaci* is a known vector of *Begomovirus*, and *T. vaporariorum* is known to be infected but does not transmit the virus. In case *T. vaporariorum* was present in the field and we detected the virus in it; we used it as an indicator of the presence of the virus in that field. To detect *Begomovirus* we used denatured primers 504 and 1048 (Brown et al. 1994; Wyatt and Brown 1996) that amplify the central or core region of the coat protein gene of the whitefly transmitted *Begomovirus* with the DNA ring A. The amplified fragment of DNA has an approximate size of 544 base pairs. This is a highly conserved region. The presence of *Begomovirus* and the percentage of infection were determined for each sample point, every month of the study, and results for each field were sent to the growers in no more than 2 weeks so they could use them as a guide in the management of their crops. Also graphs of monthly collections in both Salamá, Baja Verapaz and Santa Rosa were done to determine the dynamics of infection in each area as shown in Figs. 21.8 and 21.9.

These data show the periodicity of infection in *Bemisia tabaci* at different times of the crop cycle in both localities. Figure 21.8 shows data from infection in *Bemisia tabaci* whiteflies in Salamá in the Northern Central part of Guatemala. After the analysis of the data from PCR to detect the presence of virus infection, we found that the infection was persistent almost all year long but was worst during the dry months and at the beginning of the rainy season, from April to June. That is why we thought, based on data from 2007 (Fig. 21.8), that a host-free period during December and January was needed, in which no planting of tomato, no crop hosts or weeds hosts of *Bemisia tabaci* and *Begomovirus* are present, so in February the crop could begin to be planted again and in March, the young susceptible plants will be able to grow without virus transmission due to lack of reservoirs, and the few whiteflies present probably will not have the virus. Figure 21.9 shows the same data

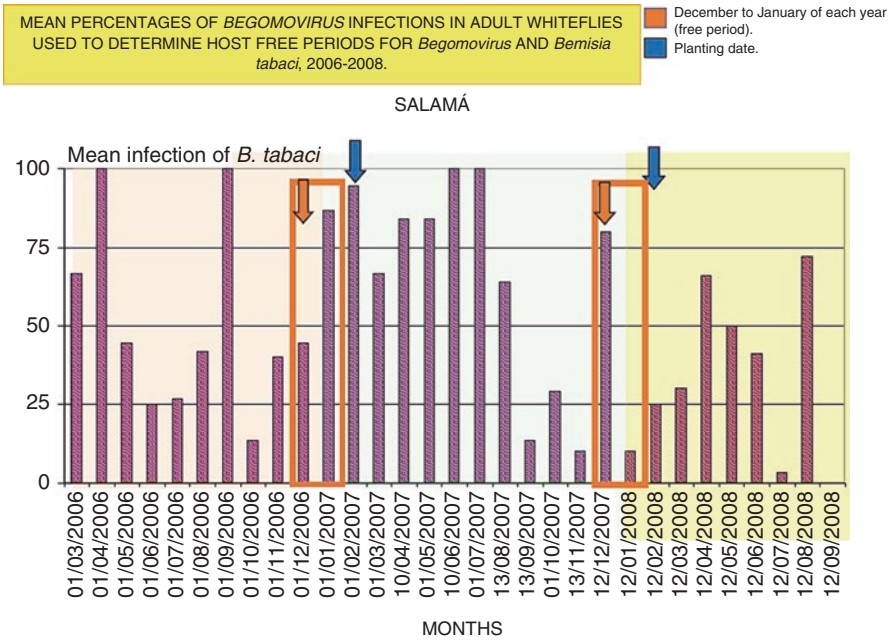

Fig. 21.8 Percentage of *Bemisia tabaci* females infected from March 2006 to August 2008 in Salamá, Baja Verapaz and possible host-free periods of solanaceous plants and *Begomovirus* hosts

but from Santa Rosa, and we can see that the best time for the free period for this region is June and July. Those months are the ones in which the rains have begun, and the climate is warmer. After those months, the rain is heavier, and it helps to maintain the whiteflies controlled longer.

The evaluation of infection in *Trialeurodes vaporariorum* was not done during the whole study period. We only evaluated infection during March 2006 to February 2007 in both localities. The mean percentages of infection of *Begomovirus* for *Trialeurodes vaporariorum* and *Bemisia tabaci* Figs. 21.10 and 21.11. They are presented together to facilitate comparison of data.

The results from Salamá, Baja Verapaz (Fig. 21.8), show that infection of *B. tabaci* by *Begomovirus* during that period (March 2006 to February 2007) was present in different percentages all the months of the study. According to these data, infection in the Valley of Salamá never ceases. The infection was higher than 60% in this whitefly species during March, April, September 2006 and January and February 2007 and no lower than 13% in the rest of the study period. Infection in *T. vaporariorum* was present in almost all the months of the study except in July 2006. Remember that this species of whitefly has the capability of excreting the virus and not transmitting it.

In Santa Rosa (Fig. 21.9), infection of *Begomovirus* in *B. tabaci* persisted all through the study period. The months with higher infection (higher than 60%) were March, April, May, July, August, September, November 2006 and January and

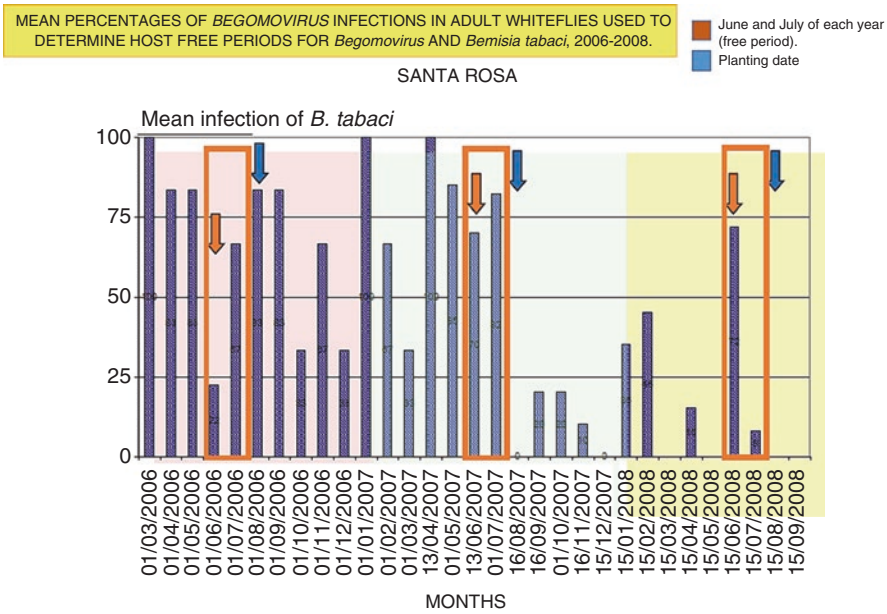

Fig. 21.9 Percentage of *Bemisia tabaci* females infected from March 2006 to August 2008 in Santa Rosa and possible host-free periods of solanaceous plants and *Begomovirus* hosts

February 2007. Only in 3 months was infection lower than 50%. Infection in *T. vaporariorum* was higher than 60% only in March 2006 and February 2007; the rest of the months it was between 0% and 50%. The purpose of including this species of whitefly (*T. vaporariorum*) was to see if we could use it as an indicator of the presence of *Begomovirus* in the fields. In valleys such as Santa Rosa and Salamá, the detection of virus in *T. vaporariorum* is not useful because populations of *B. tabaci* are usually present and usually infected during the entire study period. From the results of other valleys at higher altitudes (>1500 m altitude) and where populations of *B. tabaci* are low, it was useful to know if presence of *B. tabaci* was possible through the identification of *Begomovirus* in *T. vaporariorum*. If virus is found in *T. vaporariorum*, it had to be obtain from an infected plant. The only way that plant could be infected is via *B. tabaci* implying the presence of this species. At times sampling will indicate there is no or very low population of *B. tabaci* in the fields. This is because it is not easy to capture them without the adequate equipment and excellent *the* ability of the technician. So even if the sampling indicates no *B. tabaci* present, it may be there and thus allow transmission of the virus. In these cases the presence of *T. vaporariorum* can be used to indicate the presence of the vector *B. tabaci* in the field allowing transmission and explaining the presence of the virus in the crop. *B. tabaci* even with very low populations can transmit the virus.

The experience of using host-free periods by the growers continued after this study ended; so we kept sampling longer. Growers had less problems when they

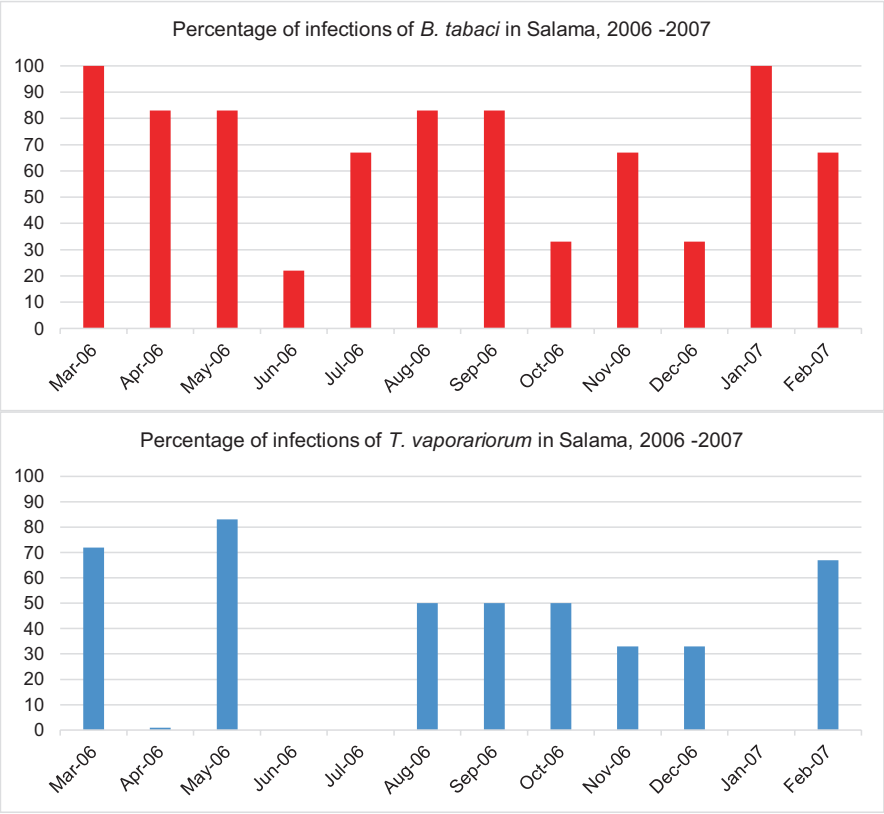

Fig. 21.10 Percentages of *Begomovirus* infection in *B. tabaci* and *T. vaporariorum* in Salamá, 2006–2007

used host-free periods, but one critical point was that populations of whiteflies can be carried by the wind many kilometers, and because fields are at different altitudes, these vectors were carried from higher altitudes to lower ones and contaminate fields of growers using host-free periods. For the host-free periods to work, an agreement needs to be established between the higher and lower growers to respect the host-free periods. If no agreement is obtained, law enforcement is necessary.

Specific Identification of *Begomovirus*

From samples of *Begomovirus* detected in the whiteflies, we sequenced samples selected through the single-strand conformation polymorphism (SSCP) technique. This technique gives us patterns of bands related to the different conformations that the DNA fragment can acquire while migrating in an acrylamide/bisacrylamide gel

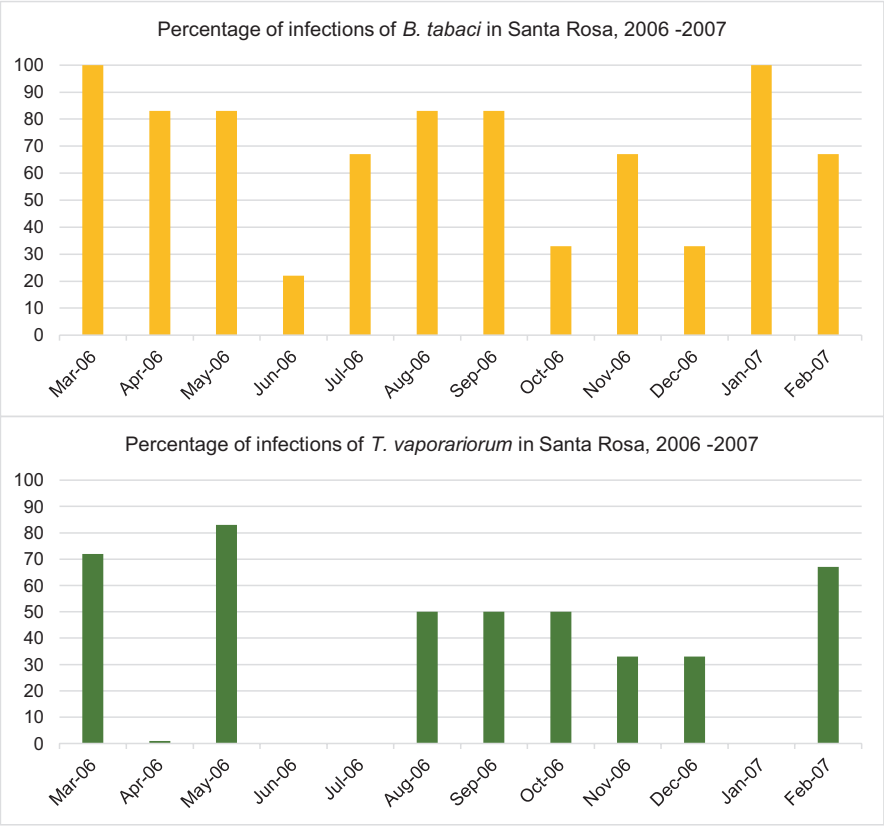

Fig. 21.11 Percentages of *Begomovirus* infection in *B. tabaci* and *T. vaporariorum* in Santa Rosa, Santa Rosa 2006–2007

in a vertical electrophoresis chamber with electrical voltage. It can detect up to one mutation. We selected three samples of different patterns and ran them with a specific pair of primers for eight different *Begomovirus* (Nakhla et al. 2005). The *Begomovirus* analysed were tomato severe leaf curl virus (ToSLCV), tomato golden mottle virus (ToGMoV), tomato mild mottle virus (ToMiMoV), tomato yellow mottle virus (ToYMoV), pepper golden mosaic virus (PepGMV), tomato mosaic Havana virus (ToMHV), tomato leaf curl Sinaloa virus (ToLCSinV) and pepper Huasteco yellow vein virus (PHYV). All these primers amplify fragments of 400 bp except PHYV that amplifies a fragment of 700 bp. The results from the PCR experiments with specific primers for *Begomovirus* are shown in Table 21.1.

Of the eight species of Begomovirus for which we tested, only four were detected. Most of the viruses found were more abundant in Salamá than in Santa Rosa, except for TSLCV. PepGMV virus presented the greatest difference in quantity of virus between the two sites.

Table 21.1 *Begomovirus* incidence in Salamá and Santa Rosa, Guatemala from 2006 to 2008

Name of *Begomovirus*	Salamá (%)	Santa Rosa (%)
Pepper Huasteco yellow vein virus (PHYV)	66.3	33.7
Pepper golden mosaic virus (PepGMV)	86.5	13.5
Tomato mosaic Havana virus (ToHMV)	57.0	43.0
Tomato severe leaf curl virus (TSLCV)	44.0	56.0

Tomato Leaf Curl Virus (TYLCV)

In 2007, a new symptom was found in the field in plantations of tomato in Salamá. The leaves presented a very striking yellow mosaic and were curled upward, had interveinal chlorosis, were crumpled, and the plants and leaves were smaller than normal (dwarf). These symptoms were similar to those reported from the Dominican Republic (Salati et al. 2002) for a new *Begomovirus*, transmitted by *Bemisia tabaci* and called tomato leaf curl virus (TYLCV).

Studies on the detection of TYLCV were implemented to see if it were present in the tomato areas in Guatemala and from when it started in those valleys. This virus was very important to detect because it could cause losses of 100% as seen in the Dominican Republic. As with other *Begomovirus*, TYLCV has a persistent way of transmission, and the tomato plant needs between 4 and 8 weeks to build a sufficient viral concentration that affects the plant (Salati et al. 2002). This *Begomovirus* apparently is a recent arrival from the Old World.

Two graduation projects complemented this study: the first was the detection of TYLCV in Guatemala in the departments of Baja Verapaz (Salamá), El Progreso (Sanarate) and Jutiapa (Asunción Mita) (Solares 2007). The second project concerned the detection of TYLCV in different regions of Guatemala (Morales et al. 2010). For these studies, we used TYLCV primers from Nakhla et al. (2005) (PTYIRv21 and PTYIRc287) which amplify a fragment of approximately 311 base pairs. According to Solares (2007), TYLCV has been present in Salamá since August 2006 in 25% of *B. tabaci* even though we tested collections of previous months (from March 2006). Figure 21.12 shows the results of these analyses.

Since the detection of TYLCV in August 2006, the percentages of *Begomovirus* in *B. tabaci* fluctuated from month to month. In some months (September, October and December 2006, January, February, May to August 2007) TYLCV appears to dominate, but in August and November 2006 and March, April and September 2007, other *Begomovirus* were also present, sometimes in lower percentages and sometimes dominating. These results include mixed infections among other *Begomovirus* and TYLCV.

Figure 21.13 shows a phylogenetic tree of TYLCV using maximum parsimony analysis which is based on the intergenic region (Brown 1997), and a sequence of 266 nucleotides was generated. The purpose of this was to try to group by genetic similarity; the sequences of TYLCV found in Baja Verapaz, one sequence of TYLCV from Salamá (2006) and one from Purhulá (2009) were included in this

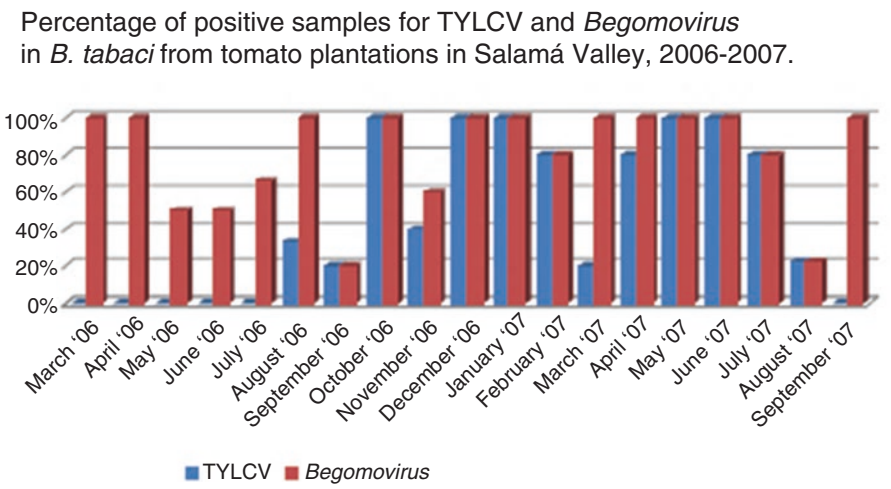

Fig. 21.12 Percentages of females *B.tabaci* infected with *Begomovirus* and TYLCV in Salamá, Baja Verapaz, 2006–2007

tree. This phylogenetic analysis was done to compare the Guatemalan sequences with the ones found in other places.

Purhulá is a small town in Baja Verapaz that is at 50.4 km or 1 hour and 2 minutes distance by car from Salamá. The samples from Guatemala are marked in red and the one marked in black was a sample from Salamá deposited in Genbank by Dr. RL Gilbertson in 2009. The tree has three principal clades, the first two clades include all TYLCV sequences, and the third clade contains the outgroup, a one ring *Begomovirus* (sweet potato leaf curl virus – SPLCV from Bengal). From this phylogenetic tree, we can see that the Puruhlá sequence 2009 (Morales et al. 2010) and the Salamá sequence 2006 (Solares 2007) are found close to each other in the same clade and are related to sequences found from the Caribbean (principally Jamaica and Florida) and México and California. We can also see that the complete sequence of *Begomovirus* sent by Dr. Gilbertson to Genbank in 2009 was grouped in another clade, together with sequences from the Dominican Republic and United States (South Carolina, Florida and Texas). These may mean that TYLCV could come from different places but the first one from Salamá of 2006 probably came from México or Jamaica as well as the one from Puruhlá in 2009. We cannot conclude this because we do not have sequences from other countries in Central America to confirm. TYLCV was not found in Santa Rosa during the period of the study (2006–2007).

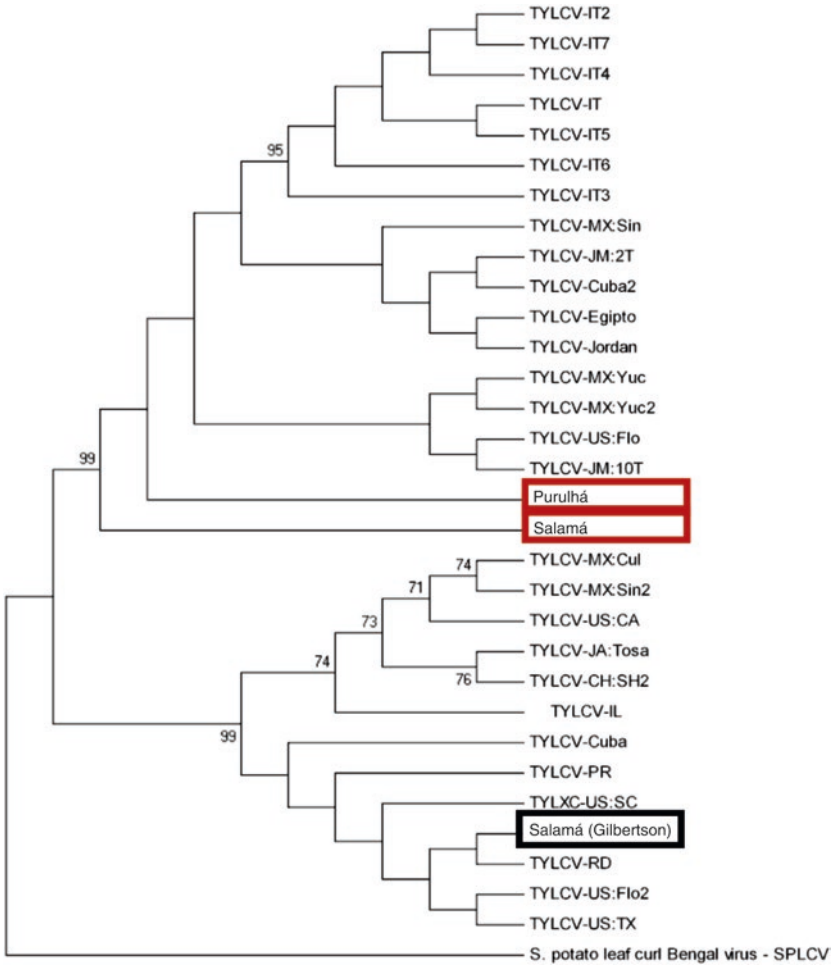

Fig. 21.13 Maximum parsimony analysis of TYLCV from Guatemala compared with TYLCV from other regions

Biotypes of *Bemisia tabaci*

Another graduation project was done for the detection of *Bemisia tabaci* biotypes (Font 2008). The detection of biotypes was very important for the growers and us because not all the biotypes have the same behaviour and the same host range. We wanted to know if all were the same biotype or if Biotype B was already present and causing problems that will have to be taken in account in an IPM program. Its presence is also important because this biotype B can reproduce resulting in high populations principally by parthenogenesis, it has a wide host range and its efficiency in the transmission of *Begomovirus*. For this study, we used mitochondrial cytochrome

oxidase I gene (mtCO1) as a marker to determine the biotypes (Brown 2001; Brown et al. 1995; Chu et al. 2006; Frohlich et al. 1999).

The samples were whiteflies, females of *Bemisia tabaci*. DNA was extracted and PCR analyses were done. The results were sequenced, and the sequences were analysed with the software DNA Star Lasergene version 8. A phylogenetic tree was built with the sequences using parsimonial analysis. The software to edit, align and run the tree was DNA Star Lasergene version 8.0. Results are presented in Fig. 21.14. Here we have a phylogenetic tree of samples of *Bemisia tabaci* collected from 2006 to 2008 in Salamá and Santa Rosa and compared with specimens from other countries marked with yellow boxes. Biotype B was not found during these samplings. Since no Old World biotypes were found, we are presenting only the New World clades of *B. tabaci* and use as an outgroup *B. tabaci* biotype A from Arizona that has an orange box in the tree. The samples at the bottom of the tree that have yellow boxes are samples from other places. The samples with the black box are samples from Salamá, Baja Verapaz (BajVsal1572) and from Santa Rosa, Santa Rosa (SROS313).

The tree shows that the New World clade has different groups; the bottom one is from samples not from Guatemala but from places close to Guatemala. Then we can see the different groups in which the *Bemisia tabaci* collected in Guatemala is

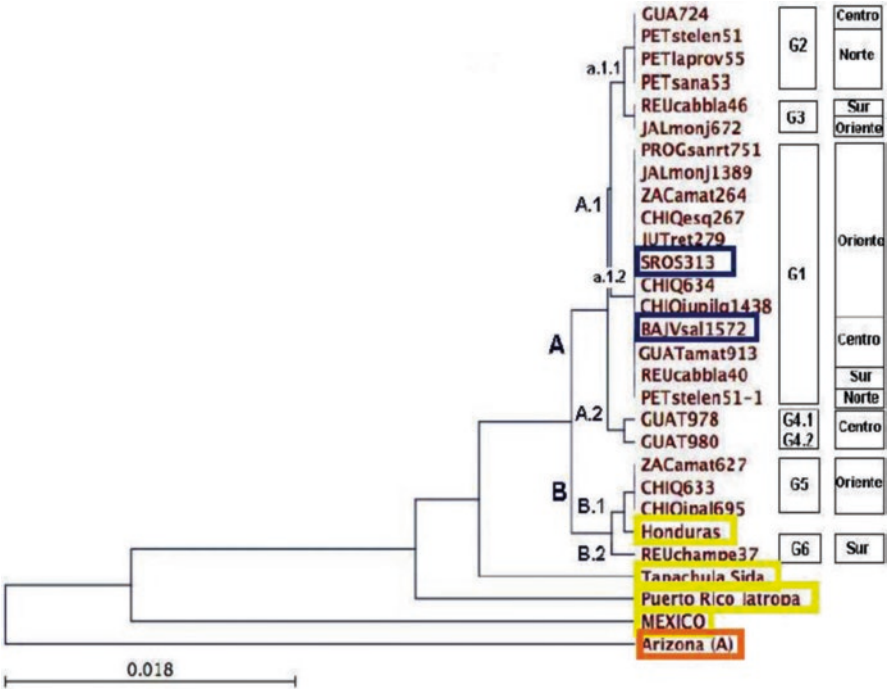

Fig. 21.14 Phylogenetic tree of New World clade of biotypes from the different samples of Guatemalan *B. tabaci* analysed with CO1 (Font 2008)

distributed. We found six different groups (G1–G6) with very little distance among them and their distributions in the country. We can see that group G4 divides in two subgroups: G4.1 and G4.2. The biotype most widely distributed in the country is the G1 (a creole one) biotype. The samples from Salamá and Santa Rosa belong to this group. We only found one biotype there. Font (2008) concludes that this G1 biotype is not only the more prevalent but is more adapted to any environmental or altitudinal change and affects tomato and pepper principally. This biotype is more abundant at altitudes between 500 and 2000 m (Font 2008).

Actual Situation and Recommendations

At this point we can ask ourselves, "Now what?" With the monitoring of *Bemisia tabaci* principally because it was found to be the only vector of *Begomoviruses* in those valleys, growers were happy because it was easy to collect whiteflies and send them to a laboratory and then have a result that will help them decide if they could plant tomato or not. During the project the analyses were financed by the project. When the project ended, the growers had to finance their own sample analysis, which some could not afford. Others just did not want to pay for the analysis, even though these first analyses with the project allowed them to establish their planting and host-free period calendar. However, not all the growers were convinced with the host-free period as well as having to clean the fields of alternate hosts and voluntary crop plants, or to plant a new short-cycle crop with economical value and especially not being the host of *Begomoviruses* during the host-free period.

Many actors participated in this project. Some were institutions that gave us some funds to do the project such as CONCYT through its FODECYT line, CATIE, IPMCRSP through the International Plant Virus Disease Network managed by Sue Tolin and IPM in Latin America and the Caribbean managed by Jeff Alwang. Agropecuaria Popoyán and a very active vegetable growers association called Federation of Agricultural Associations of Guatemala (FASAGUA) had important roles in the fieldwork. We contributed with trainings, publicity, posters, radio spots, advertisements, field days and other activities that helped improve some growers' associations, including the provision of one meteorological station and other contributions (Figs. 21.15 and 21.16).

What was very important was to obtain the agreement of tomato producers of many valleys to participate in the project. Some valleys were still using the host-free period years after the project was terminated. The basic impacts of this project on the growers were understanding of the concept of host-free periods, learning to use a good tool to verify the amount of virus circulating in the fields (detection of virus in whiteflies), learning to monitor their crops, learning that they have different alternative crops to grow in the host-free period and its requirements, learning new IPM technologies to manage the crop (they reduced pesticide usage by up to 70%) and with all of these, they obtained more and better production, increasing it up to 80% (FASAGUA 2005; http://www.fasagua.com/node/46).

Fig. 21.15 Radio spots, publicity posters, equipment and field days to growers

Fig. 21.16 Training growers and technicians in the field

FASAGUA, Popoyán and Universidad del Valle de Guatemala made efforts to increase acceptance and implementation of the 2-month host-free period and integrated pest management (IPM) using the results of the project. To be successful in this effort we needed the help of the ministry of agriculture (MAGA) to enforce the host-free period because not all the growers were cooperating, and this strategy must be a group or valley wide effort to be successful. One result of our efforts was that MAGA decreed in The Agriculture Ministerial Agreement, No. 21-2008 on 11 January 2008 that reinforced the host-free period. We submitted a more challenging agreement in 2009, but this has not been resolved.

After that, the tomato growers that were following the host-free period obtained tomatoes of good quality as in Fig. 21.17 compared with the tomatoes obtained by growers that did not follow the host-free period. In Fig. 21.18, we can appreciate the change in the appearance of the fields of a grower following the host-free period and one that did not. The growers that implemented the host-free period and other IPM recommendations increased their production and quality of their tomatoes. These also helped them to obtain the authorization to export tomatoes and peppers to the United States. The first year (August 2007–August 2008), they sold the equivalent of $6 million, exceeding their expectations. During this period, they sent approximately 4.9 million pounds of tomatoes and 1 million 875 pounds of pepper to the United States (FASAGUA 2005; http://www.fasagua.com/node/46).

The importance of this study is that it is one of the few reported for the management of *Begomovirus* and is dealing principally with biology and is based on the life cycle of the vector, the method of transmission of the virus and the capability of the crop to accumulate an adequate concentration of the virus to be affected (Salati et al. 2002). One has to keep in mind that the strategy needs the collaboration of all growers in the valley in keeping the host-free period. This is a group strategy. Growers must remember that during the host-free period, no voluntary plants nor alternative hosts must be present in the fields because some are symptomless and can be reservoirs of the virus. Monitoring frequently the field is one of the best methods to see any change in the plantation and take timely action.

This strategy of host-free periods for *Begomovirus* and whitefly hosts has given good results in the management of *Begomovirus* and TYLCV in Guatemala and has reduced insecticide contamination. Further research should be done on whitefly biology to be integrated into an IPM program, especially because recently it was found that TYLCV is also transmitted transovarially and can be transmitted through seeds (Kil et al. 2016). Research should be done to determine the response of the whitefly-tomato ecosystem to global warming and climate change.

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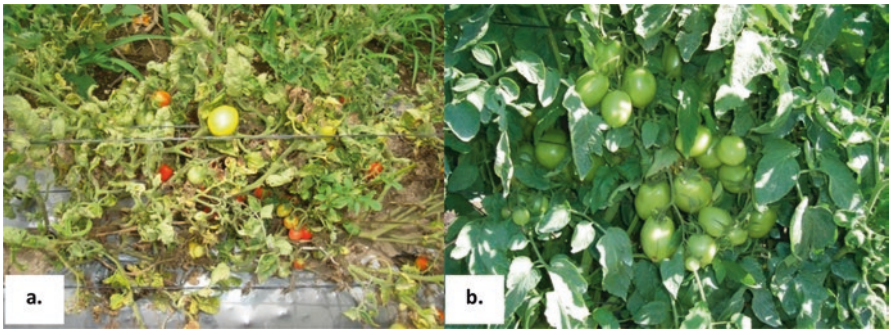

Fig. 21.17 (**a**) Tomato quality of a grower not following the host-free period and (**b**) quality of the fruit of one grower following it

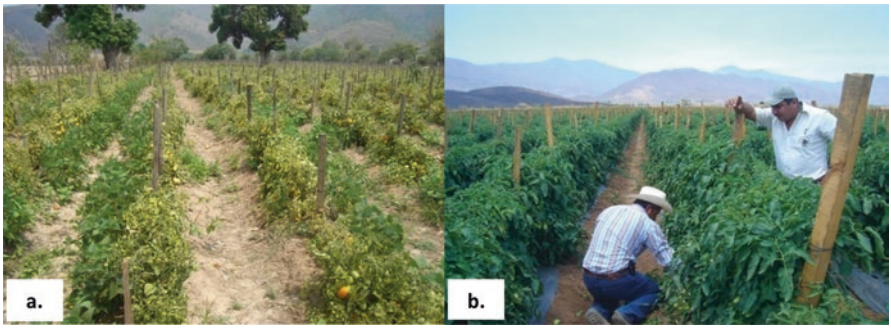

Fig. 21.18 (**a**) Field of a grower not following the host-free period and (**b**) field of a grower following it

America and the Caribbean managed by Jeff Alwang, CATIE, CONCYT through its FODECYT line, to Aproecuaria Popoyán principally to Francisco Viteri, to FASAGUA principally to Eddie Martínez and to the growers that participated in the study. Also, we thank the persons that did the field and laboratory work from plant protection laboratories from Universidad del Valle de Guatemala principally Elena Dardón and Andrés Ávalos, and the students who contributed with their graduation projects: Mónica Morales, Estuardo Solares and Arnoldo Font, and also to the technicians. Finally, and nonetheless, to Dr. Robert Gilbertson from University of California, Davis and to Dr. Judith Brown from the University of Arizona who were our guides and counsellors in all the work done.

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Index

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