Bryan A. Bailey · Lyndel W. Meinhardt
Editors

Cacao Diseases

A History of Old Enemies and New Encounters

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Preface

Chocolate! My first memories of chocolate have nothing to do with science, but rather the simple pleasure of eating sweet milk chocolate. It seems chocolate and holidays in the United States have gone hand in hand for many years and I cannot remember a holiday passing in which chocolate was not included. Chocolate cake was a standard for my birthday, chocolate eggs and rabbits were a staple at Easter, chocolate combined with nuts and/or caramel were highly sought on Halloween, and chocolate in just about every form was fair game at Christmas. To be sure, it did not take a holiday to encourage my consumption of chocolate, as I was and still am willing to eat chocolate any day of the year. My love of milk chocolate has not diminished with age. Fortunately my wife, Susan, in whom I always depend, is almost always willing to aid this pseudo dependency by providing me chocolate in some form, chocolate pie, chocolate ice cream, and chocolate delight being my personal favorites.

Although the interaction between man and the cacao tree has continued for at least 3000 years, it is in relatively recent times that chocolate as we generally know it, a sweet/semisweet dessert, has become a global food phenomenon. The extensive global interest in chocolate has coincided with the establishment of organized farms, some large but mostly small, tended by the often overlooked but critically important cacao farmers. Having met some cacao farmers, yet never enough, I have found them to always be keenly interested in the crop they produce, primarily because, in most cases, they depend on it for basic necessities needed for supporting their families and improving their lives. The benefits of chocolate to farmers are not limited to cacao farmers only, since a multitude of other agricultural commodities are incorporated into chocolate dishes/products: peanuts, coconut, various other "nuts" like almonds and my personal favorite pecans, fruits, various flours, fats/oils, and of course sugar, not to mention more unique combinations involving meat products like bacon and spices like pepper. A surprise to me is the common occurrence of farmers having never tasted chocolate made from their own cacao trees. To me this is like a tomato farmer never tasting the tomatoes he grows. The fact is many cacao farmers seldom consume chocolate of any kind. Unfortunately, you cannot pick chocolate bars straight off the tree like a tomato from a vine,

although if that were the case I would probably live in a tropical climate. Fortunately, we have the chocolate industry, employing many people in its many forms, which assembles chocolate products, in their many forms, providing us with the endless pleasure of consuming old and new chocolate products whenever we can create/fabricate a good enough reason to celebrate. To me that means waking up and taking a deep breath or in other words being alive.

Now I realize my exposure to chocolate was not the same as for everyone else in the world. As I have traveled to different countries and learned about their cacao/cocoa cultures, I am still surprised when I find out consumption of chocolate in many countries falls far below that in the United States, not that the United States is the largest consumer. Growing up I had no idea of the history of chocolate, how chocolate was made, where cacao was grown, or of the many problems cacao farmers faced when growing the crop. It seems from the time (perhaps before) a cacao seed is planted in the soil until a mature cacao pod is harvested in the field, the crop is faced with continuing obstacles, some of the most important being the plant diseases we describe in the book. Chocolate may be the "food of the gods," but cacao, the tree whose seeds (cacao beans) are used to make chocolate, is also the food of many pathogens which cause disease and limit its production. Plant diseases cause significant losses almost everywhere cacao is grown and have encouraged the global migration of cacao to the point where most production occurs outside the areas where the crop originated. In these new areas, new plant diseases have emerged and serious crop losses continue to limit supplies of that most precious commodity, the cacao bean. In South America, where cacao evolved, two major pathogens, *Moniliophthora roreri* (frosty pod rot) and *Moniliophthora perniciosa* (witches' broom), severely limit production, each being capable under the right conditions of completely destroying the crop. In west Africa, *Phytophthora megakarya* (black pod rot) and *cacao swollen shoot virus* (CSSV) are serious and expanding threats to production, and in the island nations of southeast Asia, *Ceratobasidium theobromae* (vascular streak dieback) along with *Phytophthora palmivora* (causing the global threat of black pod rot) continually threaten production. This is not meant to underemphasize the many other recognized pathogens of cacao which cause serious losses locally or may have the potential for causing expanding losses in the future.

Globally, cacao researchers have not been standing by allowing these disease threats to continue unchallenged. Scientists are using new and traditional technologies to understand the processes leading to disease in cacao, developing new tools for managing cacao diseases, and selecting cacao trees that continue to yield well despite disease pressures. It should be comforting for the cacao farmer and chocolate consumer to know that, in locations all over the world, scientists are investing their time and efforts combating all of these diseases. Part of the impetus for assembling this book was the recognition that such excellent science is being carried out in these efforts and that assembling much of this information, past and current, into one place would be of value to those interested in cacao, cocoa, and chocolate. Cacao being produced all over the world complicates the sharing of

information between scientists and others interested in the crop. Researchers working in one part of the world are often literally half a world away from other areas where similar research is being carried out. Despite the availability of modern electronic technologies, much research remains out of reach to researchers due to barriers like publishing rights, perceived limitations to local interests, and in some cases a general lack of awareness to look and see what is going on in other areas. An obvious observation derived while editing this book was that a great deal of research is tied up in local institute/agency reports greatly reducing the impact of the research and, in many cases, leading to its loss from the institutional memory of global cacao researchers. This lack of communications of the most basic experimental results limits potential sharing of ideas and their resulting synergies, results in redundancy of effort, and prevents the recognitions of common themes that might accelerate the derivation of solutions to the problems being addressed, global losses to cacao diseases. The many excellent authors associated with this book have done their best to assemble and highlight the current state of knowledge of cacao diseases and their impact on production. Perhaps most importantly, authors have also been encouraged to provide direction, as they see it, to future work sharing their ideas where gaps in our understanding of cacao diseases occur and how the gaps might be filled leading to improved management of cacao disease. It is through these efforts that we hope to maintain and improve cacao production by limiting losses to disease and insure an ample supply in the future for all the world's chocolate fans.

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Contents

Part I
The Crop and Its Pathogens

Chapter 1
Origin, Dispersal, and Current Global Distribution of Cacao Genetic Diversity

Dapeng Zhang and Lambert Motilal

Abstract Cacao (*Theobroma cacao* L.) is native to tropical South America, but as the unique source of cocoa butter and powder for the 200 billion USD global confectionery market, it is cultivated globally. Despite its economic importance, cocoa was, and continues to be, predominantly produced in low-input and low-output systems. Production constraints, including depletion of soil fertility on cacao farms, increasing damage due to diseases and pests, and expanding labor costs, limit cacao sustainability. Therefore, instead of increasing yields, the predominant contributing factor that keeps up with the rising demand for cocoa products has been expansion to new production regions. The future of the world's cocoa economy depends significantly upon using germplasm with a broad genetic base to breed new varieties with disease and pest resistance, desirable quality traits, and the ability to adapt to changing environments. Cacao differs from major field crops with regard to the untapped wild populations, which are still abundant in the Amazon region where they are coevolving with the pathogens. Moreover, in the absence of reproductive barriers, these wild populations could be readily crossed with cultivated crops. Yet only a very small fraction of the wild germplasm, mostly represented by a small number of clones in the so-called Pound collection, has been used for breeding since the 1940s. Contributions from this small set of clones have made tremendous impacts in disease resistance and adaptability. However, breeding efforts in the past 70 years have been reshuffling this small fraction of genetic diversity, with little addition of new variation. The on-farm genetic diversity in Southeast Asia and West Africa is low and cannot meet the challenge of the mounting pressure from diseases and pests. New breeding strategies are needed to combine more disease resistance genes/alleles from untapped wild germplasm and provide farmers with enhanced genetic diversity.

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1.1 Introduction

Theobroma cacao L. is an important tropical rainforest tree, previously classified in the Sterculiaceae and presently recognized as a member of the family Malvaceae (Bayer and Kubitzki 2003) that originated in tropical South America (Bartley 2005; Cheesman 1944; Wood and Lass 2001). The tree is commonly known as cacao, while the term cocoa is reserved for the products made from the dried and fermented seeds. *T. cacao* encompasses many morphologically variable populations with a marked potential for inter- and intra-matings (Bartley 2005; Cheesman 1944). Although self-fertilization is possible in self-compatible plants, outcrossing is the predominant strategy.

Early use of the plant concentrated on the pulp and beans, and the former may have been the first factor that led to cacao farming. Archeological studies, in the Ulua Valley in Honduras, showed that the Olmecs fermented the sweet cacao pulp to make an alcoholic drink at least 3000 years ago, well before the practice of grinding the bitter seeds to produce a chocolate drink (Henderson et al. 2007; Powis et al. 2011). Since the cacao bean was additionally used for monetary, cultural, and political reasons, the tree was widely grown in Mesoamerica before the Spanish arrived (Bergmann 1969; Young 1994). However, the earliest people carried only a single strain of cacao out of the Amazon into Mesoamerica, where cacao was cultivated deliberately. Later on, the European colonists introduced a couple of other strains from the Amazon and transferred these traditional varieties to their newly won lands and colonies. Therefore, the cacao economy in the colonial time was based on a very narrow genetic background (Dias 2001a; Bartley 2005). On the other hand, little difference exists between cultigens and wild cacao in terms of their morphological characteristics and agronomic traits. It is still common today for the Amerindian to directly take native trees found in nature and adopt them as a crop, with little deliberate changes to their phenotypic features. Therefore, unlike many other domesticated field crops, cacao has the advantage that wild germplasm can be directly used in breeding or commercial production (Bartley 2005; Dias and Resende 2001; Eskes and Efron 2006).

The "wild relatives of cacao" include two types of germplasm. The first type is the large spectrum of wild populations that spontaneously grow in the Amazonian rainforest, from French Guiana to Bolivia. The second type of germplasm refers to the 22 related *Theobroma* species (Cuatrecasas 1964; Zhang et al. 2011), which have made negligible contribution to cacao improvement, due to interspecific crossing barriers. So far, conservation efforts have focused on the wild *T. cacao* populations. The main exception is *T. grandiflorum* (cupuassu), which is considered an important fruit crop in various Amazonian countries. Research studies on cupuassu have included breeding and germplasm collection (Alves, et al. 2007); germplasm characterization, interspecific hybridization, and product development (Silva et al. 2001); and phylogenetic studies (Silva et al. 2004; Silva and Figueira 2005).

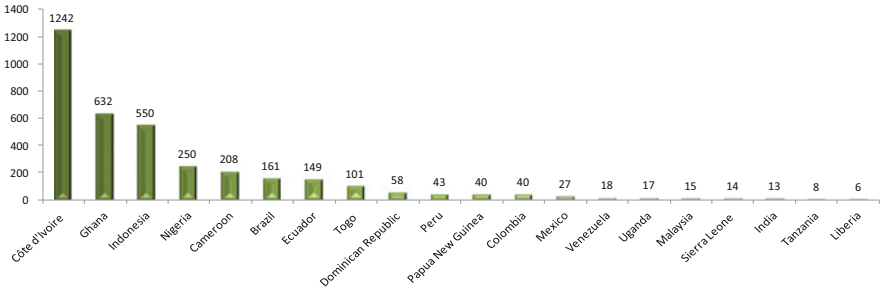

Fig. 1.1 Top 20 cacao-producing countries with a total output of 4,309,000 tons of cacao beans in 2010–2011 (ICCO, 2012). http://www.icco.org/about-us/international-cocoa-agreements/cat_view/30-related-documents/45-statistics-other-statistics.html

Today, cacao is cultivated extensively as the unique source of cocoa butter and powder for the confectionery industry. According to the World Cocoa Foundation (WCF), the production of cacao takes place mainly on small-scale farms in developing countries across Africa, Asia, and Latin America. The number of cacao farmers, worldwide, is 5–6 million, and the number of people who depend upon cacao for their livelihood is 40–50 million worldwide (World Cocoa Foundation 2012). The majority of cacao farmers employ a low-technology and low-finance approach, bordering on subsistence agriculture.

The annual worldwide production of cacao is estimated at 4.3 million tons for 2010–2011 (International Cocoa Organization 2012). During the past 100 years, there has been an average increase in demand of 3 % per year. The last 10 years have witnessed an increasing geographical concentration in cacao growing, with the African region now firmly established as the top supplier (Fig. 1.1). Increased demand has been met by expansion in production, mainly in the major West African cacao-producing countries. The demand for cacao is estimated to exceed supply with cocoa consumption increasing in emerging middle-income countries, including Brazil, China, Eastern Europe, India, Mexico, and Russia. Revenue derived from the sale and export of cacao provides crucial support to livelihoods of farmers and landholders throughout the tropics.

1.2 Agronomy of Cacao

Cultivation

Cacao is cultivated within 20° of the equator (Toxopeus 1985) with the major producers having easy access to supplies of low-cost labor and forest land (Woods 2003). Irrigation is rarely applied, but may be undertaken in countries with a dry season (rainfall less than 100 mm/month) to prevent drought stress. Cacao can be

propagated from seeds, rooted cuttings, or grafted or budded plants. Many cacao farmers have seedling trees on their farms. Seeds can be obtained from open-pollinated or biparental crosses (sometimes called "hybrid" crosses) carried out in seed gardens or from their own farm or local community. Vegetative (or clone) propagation, by budding or grafting onto rootstocks or by cuttings, is increasingly practiced in Asia and Latin America (Maximova et al. 2005; Wood and Lass 2008). Large-scale vegetative propagation of planting materials by somatic embryogenesis is being undertaken in Indonesia and Côte d'Ivoire. Clones propagated by orthotropic rooted cuttings of somatic embryo-derived plants are not different from clones propagated by grafting in performance and bean quality traits (Goenaga et al. 2015).

In most countries, the average planting density is 1000–1200 trees ha^{-1} with a minimum of 600 trees ha^{-1}. In the high-yielding and high-input system in Malaysia, the planting density can reach 3333 trees ha^{-1} and optimal planting densities are highly variable for different clones (Lockwood and Pang 1996).

Pruning increases the productivity of a tree because it can optimize the structure of tree canopy, improve photosynthesis activity, facilitate pollination of the flowers and strengthen the formation of new leaves and growth of the pods. Pruning is also an effective measure to control cacao diseases and insect pests. All dead branches and chupons (new branches that grow upward out of the trunk) need to be removed. As they grow, trees should be pruned to control both height (3–4 m is ideal) and shape of the tree, which expedites maintenance and harvesting (Wood and Lass 2008).

Phytosanitation is one of the most cost-effective method for reducing pests and disease for small-holder farmers. It refers to the removal and burial of diseased cacao pods, branches, leaves, and weeds. Field trials in Peru found that weekly removal of pods infected with black pod reduced incidence of the disease by 35–66 % and improved yield by 26–36 % (Soberanis et al. 1999).

Cacao farms can become significantly depleted of nutrients, due to the many years of low or no fertilization input (Baligar and Fageria 2014; Wood and Lass 2008). Soil nutrition deficits are a critical hindrance to cacao productivity in most areas. The current level of soil fertility on cacao farms in West Africa averages less than 10 % of what is necessary for productive crops and soil (Cocoa Fertilizer Initiative, http://www.idhsustainabletrade.com/Fertilzer). Cacao responds well to fertilization, especially on farms where harvested or pruned plant material is not left in the field to decompose (Baligar and Fageria 2014). For each 1000 kg of dry beans harvested, about 20 kg N, 4 kg P, and 10 kg K is removed from the soil. If pod husks are also removed from the field, the amount of K removed increases to about 50 kg (Puentes–Páramo et al. 2014). About 200 kg N, 25 kg P, 300 kg K, and 140 kg Ca are needed per hectare to grow the trees prior to pod production (Moriarty et al. 2014). Fertilization is also believed to extend the productive life of trees. It is estimated that fertilizer alone may be sufficient to increase yield by 500 kg to 1 ton/ha (Moriarty et al. 2014). Nonetheless, few smallholders use agrochemicals because they lack the funds to purchase them at the time they need to be applied (Cocoa Fertilizer Initiative, http://www.idhsustainabletrade.com/Fertilzer).

Fruit and Harvest

Cacao trees require approximately 3–5 years to bear their first fruits, commonly known as "pods," and can remain productive for several decades. The cacao flowers develop as compact inflorescences, directly on the woody tissue of suitable physiological age, throughout the trunk and canopy of the tree, and are pollinated by small flying insects. The pods take approximately 5–6 months to develop and mature, and once ripe, each consists of a thick husk enclosing some 40–50 seeds that are surrounded by a semisweet acidic pulp. The average yield is 450 kg ha^{-1} (Food and Agricultural Organization 2014), but yields of up to 3000 kg ha^{-1} are possible under good agricultural practices that combine management practices, pest control, improved plant material, and appropriate fertilizer application (Maharaj et al. 2005; Pang 2006). Low farm gate prices, lack of access to farming inputs/fertilizers, and finance are the main barriers to high-yielding cacao production. In many cases, farmers have limited knowledge of improved production techniques and farm management skills. The participation of new or traditional farmers who use suboptimal farming practices also contributes to poor production and a low-grade product.

Harvesting pods from cacao trees is labor-intensive and occurs within a short season. Harvesting varies by area and climatic conditions with the first harvest typically falling between April and June and a second harvest around October. Harvested pods are cracked open by hand, and the pulp and seeds are manually separated from the husks. The bulked cacao seed mass is generally fermented in simple heaps covered by banana leaves, resulting in variable cacao bean quality. However, a better practice is fermentation in wooden boxes fitted with drainage holes. This is increasingly present in central facilities, cooperatives, or on large farms. The fermentation period is variable and depends primarily on the type of cacao, generally taking 3–8 days. Cacao with high Criollo ancestry typically has a shorter fermentation period than Forastero or Trinitario cacao. A critical mass of fermenting beans is required to achieve the temperatures necessary for ideal fermentation. After fermentation, the beans are commonly sun-dried to reduce the moisture content, ideally to 7.5 %. The fermentation process initiates the formation of flavor precursors which are only fully developed following drying and roasting. The dried cacao beans are usually bagged on farm and transported to the ports for export or local processors. Before making cocoa and chocolate products, the beans are roasted, usually by the manufacturer, to develop the final chocolate flavor. Then the shells are removed from the roasted beans and the cocoa nibs are treated with alkalizing agent (usually potassium carbonate), to modify the flavor and color. The nibs are then further milled to create cocoa liquor, which is used to make chocolate paste, cocoa, cocoa butter, and chocolate (International Cocoa Organization 2013).

1.3 History of Cacao

Ancient Cacao Agriculture and Traditional Variety Names

Cultivation of cacao started in Mesoamerica, where cultural elaboration and use of cacao can be traced back more than 3000 years (Gómez-Pompa et al. 1990; Henderson et al. 2007; Powis et al. 2011). The ancient cultigens that were deliberately planted and utilized by Amerindian civilizations including the Mayas (300–900 AD) and the Olmecs (400–1200 BC) (Henderson et al. 2007) became known as Criollo cacao ("Creole" in Spanish). Cacao depictions in Mayan artifacts provide supporting evidence for the deliberate planting by early peoples in Costa Rica, Belize, El Salvador, Guatemala, Honduras, Mexico, and Nicaragua (Wood 1985a; Coe and Coe 1996; Dias 2001a).

"Criollo" is frequently used in contrast to the later introductions called "Forastero" in the literature. Preuss (1901) and Bartley (2005) indicated that the word Criollo means native or first variety cultivated outside the indigenous range of the species, thus distinguishing it from later introductions, whereas Forastero or Forestero means "of the forest" or foreign, i.e., not among the first cultivated or indigenous variety of a region. The names applied to local variations in the Criollo group are often of a descriptive nature, usually referring to fruit characters, including Porcelana, Pentagona, Angoleta, and Cundeamor. In spite of the morphological variations, Criollo cacao is self-compatible and is nearly fully homozygous as revealed by SSR and SNP markers (Motilal et al. 2010; Ji et al. 2013).

In the Amazonian rainforest, the evidence of cacao cultivation by different indigenous groups has been minimal (Bartley 2005; Dias 2001a; Sánchez et al. 1989). It was suggested that Amazonian tribes might not have had the need to formally cultivate a tree that occurred in abundance (Bartley 2005; Dias 2001a). Clement (1999), based on the ease with which cacao survives in abandoned humid forest ecosystems, classified cacao as a crop with semidomesticated populations. Furthermore, cacao was probably not grown in the Caribbean islands during pre-Columbian times (Wood 1985a).

In addition to Criollo, the Nacional and Amelonado groups were classed in the category "traditional cultivars," which was interpreted to represent some degree of domestication (Clement et al. 2010). These authors indicated that the results of Motamayor et al. (2008) show that Criollo and Nacional cacaos group together as an Ecuadorian assemblage of western Amazonia, whereas Amelonado groups with French Guiana cacao, indicating a possible eastern Amazonian origin. Bartley (2005) opined that it was likely that the Nacional cacao of Ecuador existed for several centuries prior to the arrival of the Europeans. However, Loor Solorzano et al. (2012) suggested that *T. cacao* and its products were part of the pre-Columbian culture around 2000 BC, a controversy that was not resolved, although the center of origin of Nacional cacao was suggested, based on microsatellite evidence, to be in the southern Amazonian area of Ecuador. Patiño (2002 as cited in Clement et al. 2010) argued that the Amelonado cacao in eastern Pará of Brazil was from ancient cultivation.

Similarly, Barrau (1979 as cited in Clement et al. 2010) suggested, based on ethnographic observations, that cacao had long been cultivated in French Guiana by the native peoples. Drawing on the opposite extremes of cacao distribution in the Americas for Criollo and Amelonado, and the low number of private alleles, Clement et al. (2010) reasoned that Amelonado cacao should be considered to be at least incipiently domesticated in eastern Amazonia.

Amelonado cacao, so called because of the fruit shape (Spanish for "melon shaped"), is another ancient variety. Based on the pod characteristics, this variety has been called Indio, Amelonado, Calabacillo, Matina, Común, Catongo, and Pará (Bartley 2005; van Hall 1932). The Amelonado cacao is more widespread than Criollo in Mesoamerica and the Caribbean, has better adaptability than the ancient Criollo cacao, and, thus, is either replaced or hybridized with Criollo in many places. The earliest time period for cultivating Amelonado (Lower Amazon Forastero cacao) in Mesoamerica and the Caribbean is not clear.

Colonial History

The catastrophes (Table 1.1) of cacao production in the last four centuries, often caused by diseases and pests, were the main force driving cacao dispersal and the shift of production centers. Severe cacao disasters, generally known as blasts or blights, occurred in the early colonial plantations in Latin America and the Caribbean (Motilal and Sreenivasan 2012 and references therein). Historical records have shown that the "blasts" that occurred in several Caribbean countries had different origins (Motilal and Sreenivasan 2012). Cacao production in Martinique experienced severe disasters in 1671 and 1727 (Quesnel 1967), which decimated entire plantations and almost ruined the cacao industry of the island (Kimber 1988). However, the Amelonado cacao that survived was likely transferred to surrounding islands, which had undergone similar devastation events. Direct introduction from South America into cacao plantations of the Caribbean islands also occurred (Preuss 1901; van Hall 1932; Bartley 2005).

During the Spanish colonial rule, Trinidad cacao planters grew mostly Criollo cacao. Prior to, and even more so after, the 1725 destruction of the majority of the cacao crop by a trifecta of climatic, agronomic, and genetic causes (Motilal and Sreenivasan 2012), Forastero material was introduced, most likely from Brazil (Shephard 1932; Joseph [1838] 1970), Hispaniola (Bartley 2005), and Venezuela (Bartley 2005). Several resultant natural hybridization events (Motilal et al. 2010; Motilal and Sreenivasan 2012; Yang et al. 2013) led to the Trinitario germplasm, which is noted for its fine flavor (Toxopeus 1985). Heterosis (hybrid vigor) resulted in vigorous planting material, which was then reintroduced to Venezuela. The term Trinitario probably accompanied the transferred germplasm and has since been used to describe these cacao types, arising as products of hybridization and recombination through various generations, which are now known in the trade for their floral/fruity flavors. Much later, at the then Imperial College of Tropical Agriculture

Table 1.1 Catastrophic cacao events caused by diseases and pests

Country	Period	Attributed cause	Decline	Reference
Venezuela	Mid-1630s	Blight "alhorra"	>50 % trees lost	Ferry (1989)
Cuba, Hispaniola, Jamaica	1660s–1670s	Drought, "blasts of unknown origin," "blasting," heat, and drought	General failure—all trees	Joseph ([1838] 1970), Historicus (1896), Johnson (1912)
Santo Domingo in Hispaniola	1715	Malicious destruction by residents of Martinique	100 % trees lost	Shephard ([1831] 1971), Southey ([1827] 1968)
Martinique	1718	Wind, hurricane	100 % trees lost	Shephard ([1831] 1971)
Trinidad	1725	Trifecta of climatic, agronomic, and genetic causes	General total failure	Motilal and Sreenivasan (2012)
West Indian islands from Trinidad to Jamaica	1727	"Blasts" of unknown origin [these were probably due to the same trifecta of causes given above and reported in Motilal and Sreenivasan (2012)]	General total failure	Bartley (2005), Quesnel (1967)
Martinique	1727–1732	Two "plagues": root fungus, leaf-eating caterpillar	~95 % trees lost	Revert (1949) cited in Kimber (1988)
Ecuador	1920s	Witches' broom disease and frosty pod	60–70 % drop in production; reduced numbers of Nacional trees	Wood (1985b)
East Java	1936	Cocoa pod borer	All trees removed	Wood (1985b)
Ghana	1930s–1980s	Cocoa swollen shoot disease	185.5 million trees removed; 31 million trees still affected	Lass (1985) and references therein
Valle, Cauca (Colombia)	1950s	Ceratocystis wilt	>50 % trees destroyed	Barros (1981)
East coast of Peninsular Malaysia	Late 1950s–1970s	Vascular-streak dieback	Badly affected; 30–50 % disease	Lass (1985), Chan and Syed (1976)
Togo	1955–1977	Cocoa swollen shoot disease	3.5 million trees destroyed	Lass (1985) and references therein
Fiji	1969	Phytophthora canker	45 % of trees in one estate	Firman and Vernon (1970)
Bahia (Brazil)	1970	Phytophthora canker	Significant loss of trees	Rocha and Ram (1971)
Bahia, Espirito Santo (Brazil)	1979	Phytophthora canker	Eight million trees affected	Pereira et al. (1980)
Brazil	1989–1999	Witches' broom disease	70 % drop in production	

in Trinidad, an extensive survey of the Trinitario population was conducted, resulting in the selection of approximately 100 Imperial College Selections (ICS) clones, which were selected principally for yield characteristics (Pound 1932, 1934).

Ancestry of Trinitario

The genetic composition of Trinitario cacao has been further dissected to clarify whether their ancestry includes only Criollo and Amelonado (Johnson et al. 2009; Motilal et al. 2010; Yang et al. 2013). Recent SSR analysis showed that the genesis of Trinitario cacao was when a limited population of Criollo × Forastero hybrids emanated from the introduced Forastero population of Trinidad (Motilal et al. 2010). The multi-lineage origin of modern Trinitario is also supported by analysis based on plastidic single nucleotide polymorphisms (cpSNPs) and polymorphic simple sequence repeats in plastids (cpSSRs) (Yang et al. 2013). Three cpSNP haplotypes were revealed in the Trinitario cultivars sampled in Trinidad, each highly distinctive and corresponding to reference genotypes for the Criollo, Upper Amazon Forastero, and Lower Amazon Forastero varietal groups. These three cpSNP haplotypes likely represent the founding lineages of cacao in Trinidad and Tobago. The cpSSRs were more variable with eight haplotypes, but these clustered into three groups corresponding to the three cpSNP haplotypes. The most common haplotype found in farms of Trinidad and Tobago was Amelonado, followed by Upper Amazon Forastero and then Criollo. The authors concluded that the Trinitario cultivar group was of complex hybrid origin and was derived from at least three original introduction events.

Out of America: Dispersal of Cacao to the Old World

In spite of the complex requirement of planting materials, environmental factors, and management practices, cacao was, and continues to be, dominantly produced in low-input, low-output, and high-risk systems. Sustainability is limited by many factors, which constrain production, including the depletion of soil fertility on farms and the legions of pests causing damage and diseases. Cacao agriculture provides a prime example of the continued confrontation of crop production with new and recurrent epidemics (Table 1.1). As the center of cacao production shifted from place to place, a small fraction of the cultigens were transported to the new production sites. Genetic diversity represented in these cultigens is actually a tiny fraction of available genetic diversity in the primary gene pool of cacao in South America. The low level of genetic diversity in cacao farms could not meet the challenge of mounting pressure of diseases and pests, and expansion to new

production regions has been essential to keep up with the world demand for cocoa products.

The first contact that Europeans had with the crop was attributed [by Oviedo y Valdez (1855) as cited in Bartley (2005)] to Alonso Pinzón in 1510 in southern Yucatan. In the sixteenth century, the Europeans started to cultivate cacao in Asia and Africa where the Criollo, Amelonado, and the Trinitario hybrids started their route of dispersal from the Americas to the old world.

The first shipment of cacao germplasm to Southeast Asia was recorded in 1560, when the Dutch introduced cacao that was believed to be the fine flavor variety "Venezuelan Criollo" into Celebes, Indonesia (van Hall 1932). Cacao production started in northern Sulawesi where cacao was processed and consumed only locally (van Hall 1932). Another introduction to this region in 1670, believed to be a Criollo variety from Mexico, was via the Acapulco-Manila galleons (Bartley 2005). Around 1770, the Dutch introduced cacao to Peninsular Malaysia (Thong et al. 1992), and fruiting cacao was subsequently found in Malacca [Koenig (1894) as cited in Thong et al. (1992)]. In 1798, the British took cacao to Madras, India, from the island of Amboina, and it was introduced into Ceylon (now Sri Lanka) from Trinidad at about the same time (Ratnam 1961; Wood 1991). From Ceylon, cacao was subsequently transferred to Singapore and Fiji (1880), Samoa (1883), Queensland (1886), and Bombay and Zanzibar (1887). Cacao was also grown in Malaysia as early as in 1778 and in Hawaii by 1831 (Bartley 2005). Remnants of the ancient Criollo, Amelonado, and Trinitario populations can still be found in Asia and Pacific regions, such as Indonesia (Susilo et al. 2011), South Pacific (Fiji and Samoa), and Madagascar (Zhang et al., unpublished data).

With the establishment of chocolate manufacturing in Europe in the second half of the eighteenth century and the increase in chocolate consumption in North America, there was an explosion in demand, requiring yet more cacao to be produced. Commercial cultivation started in Africa after the Portuguese introduced Amelonado cacao into Principe in 1822. By the 1850s, cultivation of cacao spread to the main island of Sao Tome, where the Amelonado cacao ("Común" in Bahia, Brazil) became known as Sao Tome "Creoulo." This self-compatible variety was then brought by Spaniards into the island of Fernando Po (now Bioko), Equatorial Guinea, and repeatedly introduced into the mainland West Africa (Bartley 2005). The limited genetic diversity within the initial foundation of West Africa Amelonado was also mentioned by van Hall (1932). During the late nineteenth century, the colonial administration also introduced some red-pod cacao materials from British West Indies into botanical gardens established in Aburi (Ghana) and Lagos (Nigeria) (Toxopeus 1964). Consequently, the bulk of cacao grown on farmers' plantation must have consisted of a mixture of these earlier varieties, with the self-compatible "West African Amelonado" type dominating the production at the beginning of the twentieth century.

The shift in the world's center of cacao production followed a boom-and-bust pattern, from Mesoamerica to Venezuela, from Venezuela to Ecuador, from Ecuador to Brazil, and from Brazil to West Africa (Ruf and Schroth 2004). As new

countries/regions adopted the crop, the previous production centers collapsed. Production shifts from one country to the next were reproduced by similar cycles on a subnational scale (Ruf and Schroth 2004). Among the many factors contributing to this boom-and-bust cycle, the impact of biotic constraints, due to the limited on-farm genetic diversity, apparently played a key role. Subsequently, it was only a matter of time before coevolved fungal pathogens moved—naturally or human assisted—from their forest hosts into the cacao plantations. The various catastrophic "blasts" that occurred in the last 400–500 years suggest that disease was the main force that drove cacao dispersal and the shifting of production centers. Therefore, a brief review of the cacao primary gene pool is essential for improving our understanding about future sustainability of cacao production.

1.4 Upper Amazon: Cacao's Primary Gene Pool

The term "Upper Amazon" has been used to describe the location of most of the known wild cacao populations from the "Alto Amazonas," a region from the start of the Marañón River in Peru to the frontier of Brazil. In this region, a series of major river systems in Peru, Ecuador, Colombia, and Brazil flow into the Marañón and Amazon rivers. Wild cacao populations are found in these river basins in both spontaneous (without human interference) and subspontaneous forms (wild cacao trees exploited by man) prior to European occupation (Almeida 2001; Bartley 2005). Wild cacao germplasm samples from the expeditions in the Amazon were predominantly collected along the banks of navigable rivers (Pound 1938; Lachenaud and Sallée 1993; Lachenaud et al. 1997; Almeida 2001). Each natural cacao population has a narrow genetic base and is thought to have been founded by a limited amount of reproductive materials (Pound 1945; Bartley 2005).

Genetic diversity of natural cacao populations is generally stratified by the major river systems in the Amazon (Pound 1938; Almeida 2001; Bartley 2005). Within each river basin, wild cacao is usually grouped in patches and separated by large spatial distances between patches. It is hypothesized that gene flow in cacao is limited and mating is likely confined within patches (Chapman and Soria 1983), due to the short distance of seed dispersal by rodents and monkeys and short-distance pollen dispersal by insects, including midge species (*Forcipomyia* spp.) as well as other insect vectors.

A significant departure from the Hardy-Weinberg equilibrium (HWE) was detected in the French Guiana wild populations (Lachenaud and Zhang 2008). In addition to the likely short-distance gene dispersal, cacao has a gameto-sporophytic self-incompatibility system (Cope 1962), which works in a quantitative manner (Lanaud et al. 1987). Self-compatibility in some genotypes is partially responsible for the high fixation index in several natural populations. Indeed, fully homozygous

genotypes were frequently found in the populations from French Guiana (Lachenaud and Zhang 2008). Some wild cacao trees are found in the form of single plants, but the majority will form a "clump" (several trunks at different development stages and overlapping generations at one growing site). This apparent generation overlap within a patch is another likely factor contributing to mating between relatives, thus increasing the level of inbreeding. The multiple trunks can also come from self-propagation by chupon production, which increases the chance of inbreeding by self-mating as opposed to inbreeding by mating between family members.

Despite the commonly perceived short-distance gene flow and limitation in effective population size in wild cacao, isolation by distance was detected only over a long geographical range (e.g., a few hundred kilometers) and not in a local basin or short distance (Zhang, et al. 2006). Sereno et al. (2006) reported that in the natural or seminatural populations sampled in four regions of Brazil (Acre, Rondonia, Lower Amazon, and Upper Amazon), most of the genetic diversity was allocated within populations rather than between populations, indicating a typically high gene flow. Therefore, some of the apparently isolated populations may actually belong to the same metapopulation in terms of gene dispersal, which impacted their genetic differentiation. A study on the cacao mating system at the hierarchical levels of fruits and individuals showed that the cacao population was spatially aggregated, with significant spatial genetic structure up to 15 m. Mating was correlated within, rather than among, the fruits, suggesting that a small number of pollen donors fertilized each fruit (Silva et al. 2011). A similar study in the northeast lowlands of Bolivia revealed 7–14 % self-pollination in wild cacao populations. Cacao pollen was transported up to 3 km, with an average of 922 m, suggesting pollination distances larger than those typically reported in tropical understory tree species (de Schawe et al. 2013).

Using SSR markers, Motamayor et al. (2008) genotyped 1241 cacao accessions existing in most of the ex situ germplasm collections in Latin America. The result led to the identification of 10 genetic clusters in the Forastero cacao, which was proposed as a new classification of the cacao germplasm (Fig. 1.2).

Thomas et al. (2012), using the same SSR data set, reanalyzed the spatial pattern of intraspecific diversity of cacao in Latin America. Grid-based calculations of allelic richness, Shannon diversity, and Nei's gene diversity and spatial cluster analysis suggested the highest levels of genetic diversity were observed in the Upper Amazon areas from southern Peru to the Ecuadorian Amazon and the border areas between Colombia, Peru, and Brazil (Fig. 1.3). Simulation modeling suggests that cacao was already widely distributed in the western Amazon before the onset of glaciations. During glaciations, cacao populations were likely to have been restricted to several refugia where they presumably underwent genetic differentiation, resulting in a number of genetic clusters which are representative of, or closely related to, the original wild cacao populations. The analyses also suggested that genetic differentiation and geographical distribution of a number of other clusters seem to have been significantly affected by processes of human management and accompanying genetic bottlenecks.

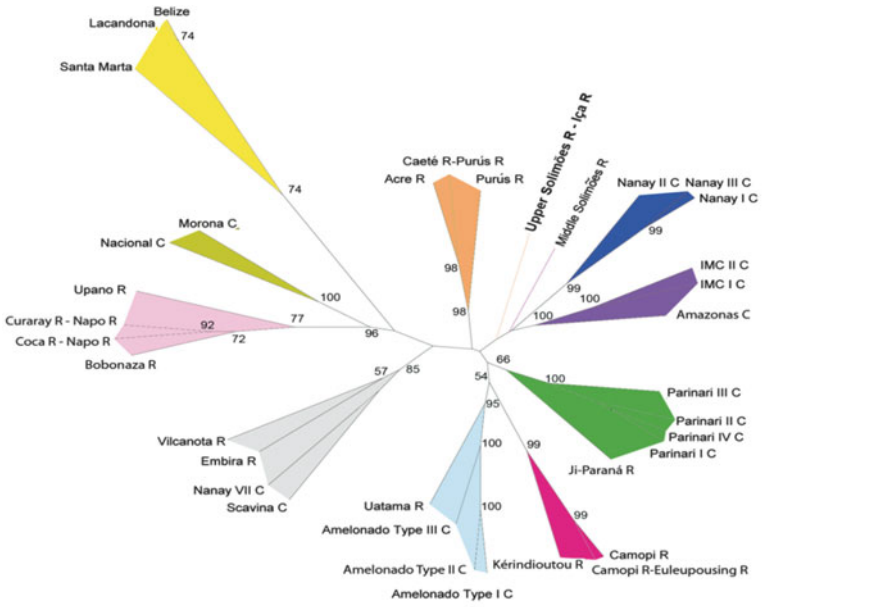

Fig. 1.2 Neighbor joining tree from Cavalli-Sforza and Edwards genetic distance [16] matrix among the 36 subclusters identified using structure (559 clones). Motamayor, et al. (2008) Geographic and Genetic Population Differentiation of the Amazonian Chocolate Tree (*Theobroma cacao* L). PLoS One 3(10): e3311. doi:10.1371/journal.pone.0003311

Since 2008, a series of collecting expeditions have been launched to survey the full cacao genetic diversity in the Peruvian Amazon (Fig. 1.4a). The expeditions were aimed at areas lacking representation in the ex situ cacao germplasm collections. The first geographical focus was the major tributaries of Rio Marañón, including Rio Santiago, Rio Pastaza, Rio Nucuray, Rio Urituyacu, Rio Tigre, Rio Ucayali, Rio Madre de Dios, and Rio Putumayo. Within each subbasin, the identification of collecting sites was assisted by GPS mapping tools. Habitat descriptions were examined and the target area was chosen based on the potential for complementary diversity. The expeditions were supported by the Peruvian government and by the USDA. To date, a total of 520 wild trees have been collected, representing 19 river basins. Preliminary characterization using SNP markers showed a significant amount of diversity complementing the existing national and international ex situ collections. The living trees were propagated in the facilities of Tropical Crop Institute (ICT—Spanish acronym) in Tarapoto, Peru (Fig. 1.4b). These trees are currently being evaluated for agronomic traits with the emphasis on resistance to diseases and bean quality and flavor. Next-generation sequencing (NGS) genotyping of these trees is being planned, together with all the wild trees

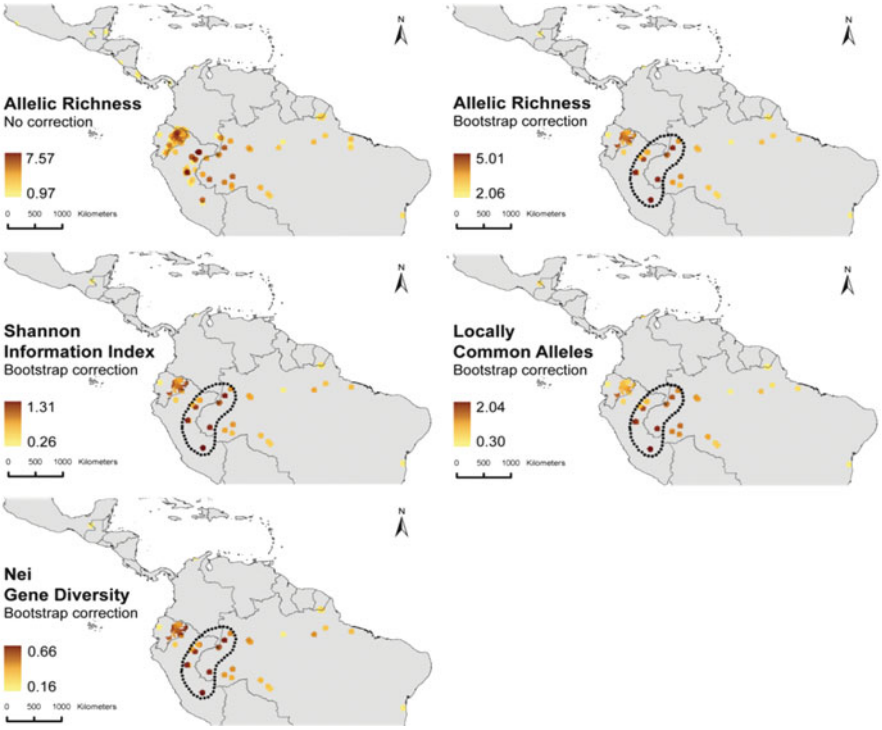

Fig. 1.3 Spatial variation of different genetic parameters, represented at a resolution of ten-minute grid cells and a circular neighborhood of 1 degree. Highest values are consistently observed in the extensive bean-shaped Amazonian area covering both the Peruvian-Brazilian border and the southern part of the Colombian-Brazilian border, as well as Amazonian Ecuador. Thomas et al. (2012). doi:10.1371/journal.pone.0047676.g001

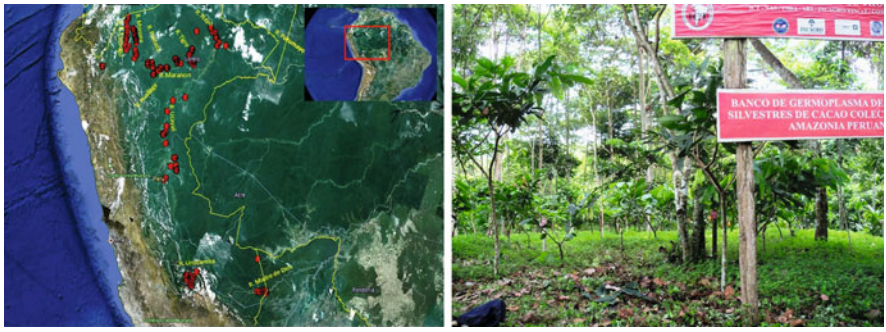

Fig. 1.4 (**a**) Ongoing new collection expeditions in Peruvian Amazon since 2008. Each *red dot* represents a collecting site. (**b**) Field genebank of the ICT wild cacao collection at Tarapoto, Peru

from the International Cocoa Genebank, Trinidad (ICG, T), and other major ex situ collections, to provide a comprehensive overview of the diversity distribution in the cacao primary gene pool.

1.5 The "Pound Collection"

Collection from Wild Cacao Germplasm

The outbreak of witches' broom disease (WBD) in Trinidad in the late 1920s led to the search for genetic resistance in the Upper Amazonian region. During the 1930s–1940s, wild germplasm was collected from the Upper Amazon basin of Ecuador and Peru (Pound 1938, 1945; Wood and Lass 2008). To date, the germplasm that has made the most fundamental contribution to the modern cacao breeding programs is, by far, the Pound collection (named after the collector F. J. Pound). This collection results from the first cacao germplasm collecting expedition into the Upper Amazon, and the collecting sites included part of the tributaries of Rio Ucayali, Rio Morona, and Rio Marañón (Pound 1938, 1945; Bartley 2005; Zhang et al. 2009; Fig. 1.5).

This led to the establishment of the "Pound collection" in Iquitos, Peru. Pound's expeditions were aimed at searching for genotypes resistant to WBD, caused by the fungus *Moniliophthora perniciosa* (Stahel) (Aime and Phillips-Mora 2005), after the outbreak of witches' broom disease (WBD) in Trinidad in the late 1920s. These germplasm accessions have henceforth served as the foundation for breeding programs around the world for resistance to WBD. The Pound collection was primarily comprised of five germplasm groups: "Iquitos Mixed Calabacillo" (IMC), "Morona" (MO), "Nanay" (NA), "Parinari" (PA), and "Scavina" (SCA) (Pound 1938, 1943, 1945). An unknown number of pods (fruits) were collected from trees without any symptoms of WBD. The 25 half-sib families yielded 250 fruits (Lockwood and End 1993; Motilal and Butler 2003). The seeds were then bulked and sent to Barbados where approximately 2500 seedlings were raised (Toxopeus and Kennedy 1984). These germplasms were transferred to Trinidad in the form of bud wood and vegetatively propagated onto rootstock at Marper Estate in Plum Mitan, Manzanilla, Trinidad, as 486 accessions (Motilal and Butler 2003). In addition to the 25 half-sib families from the five accessions groups, Pound's collection also includes 32 clones, which Pound collected in 1943 when he revisited the same sites where the NA, IMC, and SCA were previously collected. These accessions were collected as bud woods and were referred as "Pound clones" or "P clones."

In addition to these five groups collected from Peru from 1938 to 1943, Pound also collected 80 half-sib families from western Ecuador. The 80 half-sib families yielded 1185 Ecuadorian Refractario that are in the ICG, T (Fig. 1.6a, b) and were the result of the first expedition (Lockwood and End 1993).

Among the 80 or so different germplasm groups held in the ICG, T, those in the Pound collection are among the most widely distributed germplasm, due to their valuable agronomic traits and their potential for resistance to WBD (Lockwood and End 1993; International Cacao Germplasm Database, http://www.icgd.reading.ac.uk/) (Table 1.2). In many cacao-producing regions around the world, the Pound selections of Upper Amazonian cacao are either adopted directly as clones or used

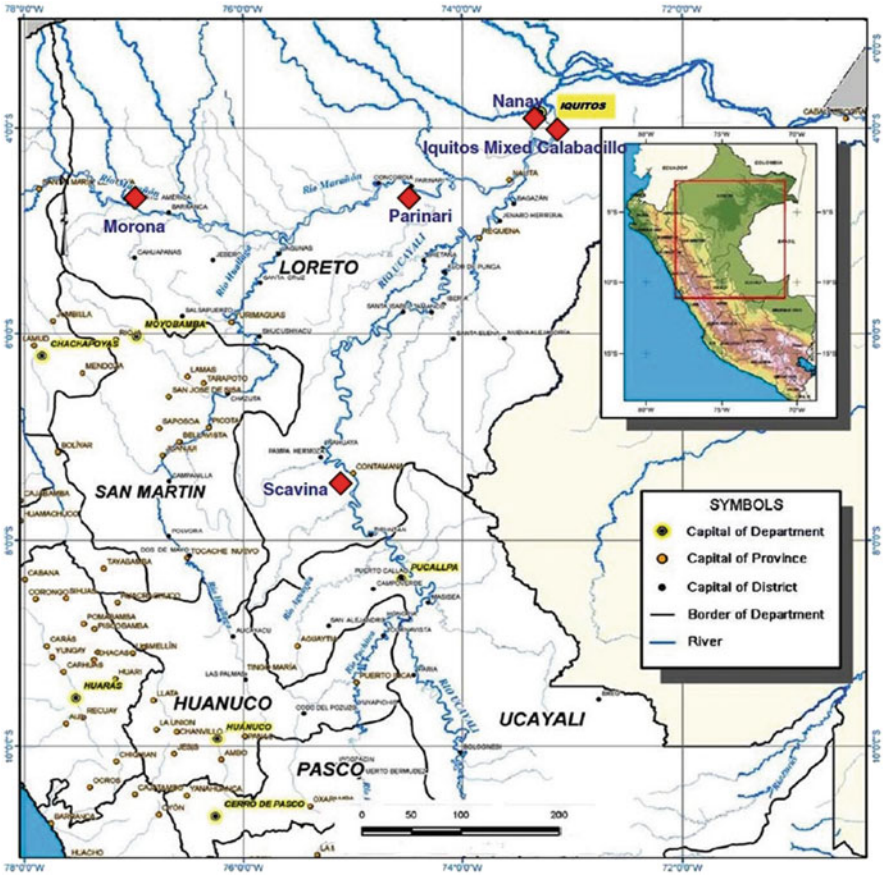

Fig. 1.5 Geographical region where Pound's collection was taken in Peruvian Amazon, 1938–1943

Fig. 1.6 (**a**) ICGT looking north, Field 5B on *left*, Field 6B on *right*, Field 5A at sign on *upper left*, and Field 6A at sign on *upper right*. Observer has walked away from southern T junction between 5B and 6B of Google map. Photo courtesy Lambert A. Motilal. (**b**) Aerial view of ICGT from Google Earth. ICGT bordered on north by Caroni River. Field to *right* of 6B is Field 7 with individuals from recent crosses

Table 1.2 Summary of cacao germplasm accessions collected by F. J. Pound (1938–1945) and their distribution status since 1945–1998

Accession group	No. of mother trees	No. of progenies	No. of internationally distributed individuals 1945–1998	No. of crosses served as parental clones in breeding programs
IMC	2	61	5	68
Morona	2[a]	24	3	N/A
Nanay	17	223	5	57
Parinari	20[a]	116	4	31
Scavina	2	15	2	68
Pound	32	N/A	4	40
Refractario	80	800	4	N/A
Total	>133		27	

[a]Estimation based on reconstructed sibships using SSR markers

as parents for the production of seed families. This collection is, by far, the most widely used germplasm for cacao breeding in the world (Bartley 1994, 2005; International Cacao Germplasm Database, http://www.icgd.reading.ac.uk; Posnette 1986).

Numerous additional collecting expeditions took place in the Amazon rainforest after Pound. Among these expeditions, Table 1.3 lists the significant ones for their geographical location, genetic diversity, and potential breeding value for cacao improvement. Nonetheless, a majority of these collections are either not in the international collections or were not well distributed internationally for the time being.

Although the international and national collections in total contain a substantial amount of genetic diversity, recent expeditions have discovered novel variability not contained within existing collections. This points to the importance of filling the diversity gaps in the ex situ collections and the need to systematically sample areas, with the objective of capturing novel variability existing in the primary gene pool. A number of expeditions to sample the variability within the Amazonian home of cacao are presently underway in Brazil, Ecuador, French Guiana, and Peru.

Impact of "Pound Collection" in Cacao Breeding

Breeding programs started in the 1920s in the major cacao-growing countries with phenotypic selection of locally available germplasm, but the large genetic diversity present in the wild Amazonian populations is yet to be widely exploited and utilized in cultivated varieties (Bartley 2005). Among the wide range of Trinitario varieties, the group of clones that has most influenced cacao

Table 1.3 Cacao germplasm groups from major cacao collecting expeditions in the Amazon

Major collecting expeditions	River basins	No. of mother trees
Refractario collection (1937)	Upper Amazon in Ecuador	80
Pound collection (1938–1943)	Morona, Nanay, Ucayali, Maranon	32–48
Anglo-Colombian collection (1952–1953)	Apaporis, Caquetá, Caguán, Cauca, Inírida, Negro, Putumayo, Vaupés	191
IBPGR-Bolivian collection (1974)	Rio Beli	21–43
Brazilian collection (1965–1967; 1976–1991)	36 river basins including Jari, Amapá, Maicuru, Pará, Jamari, Rondônia, Ji-paraná, Acre, Iaco, Tarauacá, Purus, Japurá, Amazonas, Solimões, Baixo Japurá	144 940 (and 1817 from seedlings)
French Guinea (1990, 1995)	Oyapok, Camopi, Euleupousing, Tanpok, Yaloupi	187
Chalmers collection (1968–1973)	Curaray, Coca, Napo, Putumayo	184
LCT EEN collection (1979–1987)	Curaray, Coca, Napo, Putumayo	255
ICA and IBPGR Colombia collection	Colombia	151
UWE Guyana collection (1998)	Guyana	31
Peruvian collection (1987–1989)	Ucayali	51
ICT (Peru)/USDA collection (ongoing since 2008)	Santiago, Morona, Madre de Dios, Pastaza, Nucuray, Nanay, Napo, Urituyacu Chambira, Putumayo, Tigre, Nanay, Napo, Urituyacu, Madre de Dios, Ucayali	540
Cacao ancestors of the Nacional variety collection (CAN) (2010) (Silvestres Aromáticos)	Southern Ecuadorian Amazonia	71

breeding undoubtedly came from Trinidad. These ICS accessions were used at different times in various countries.

The WBD outbreak in Trinidad in the late 1920s exposed the deficiency in the ICS clonal selection, which was primarily based on productivity rather than disease resistance. The agronomically important TSH hybrids, developed in Trinidad over 60 years of breeding, were mostly based on four clones: SCA 6, IMC 67, POUND 18 and ICS 1 (Gonsalves 1996), of which ICS 1 was a descendant of the hybridization that occurred in Trinidad post-1725.

The outbreak of cocoa swollen shoot disease in the 1930s in Ghana, Togo, and Nigeria almost destroyed the cacao industry due to the lack of resistance in the West Africa Amelonado germplasm. The demand for new genetic variation resulted in the first large-scale dissemination of UAF germplasm when Dr. A. F. Posnette visited Trinidad in the early 1940s. Posnette studied the incompatibility reactions with the newly arrived germplasm collected by Pound and produced seed progenies. A total of 121 cacao pods were introduced from Trinidad. After being

Table 1.4 Germplasm from Pound Collection used in generating "T clones" for West Africa in 1945

Name	Country of origin	Other parents used in cross
AM 2/28	Ecuador	OP
AM 2/38	Ecuador	OP
CLM 19	Ecuador	OP
IMC 24	Peru	OP
IMC 47	Peru	PA 7
IMC 53	Peru	OP
IMC 60	Peru	NA 32, NA 33
IMC 76	Peru	NA 32
JA 79	Ecuador	OP
LZ 2	Ecuador	OP
LV 36	Ecuador	OP
M 8	Surinam	OP
M 253	Surinam	OP
MO 12	Peru	OP
MO 14	Peru	OP
MOQ 6/12	Ecuador	OP
NA 32	Peru	IMC 60, IMC 76, NA 33, PA 7, PA 35, M253
NA 33	Peru	NA 32, NA 34, PA 35, IMC 60, OP
NA 34	Peru	IMC 60
NA 43	Peru	OP
NA 60	Peru	OP
PA 103	Peru	OP
PA 35	Peru	NA 32, NA 33, PA 7
PA 37	Peru	OP
PA 7	Peru	IMC 47, NA 32, PA 35
SCA 12	Peru	OP
SCA 6	Peru	OP

quarantined in Accra, Ghana, the shipment reached the West African Cocoa Research Institute headquarters in Tafo, Ghana, and Ibadan in Nigeria (Toxopeus 1964). Each pod was numbered serially with the prefix T (Trinidad), resulting from either open pollination (T1–T59) or from open pollination (T60–T121) for which both parents are known (Table 1.4).

The resultant plants (about 3000) were planted out at Tafo, Ghana, in 1945. In 1948, the precocity and generally superior performance of these progenies of Upper Amazon parentage were definitely acknowledged. For the purpose of breeding for resistance to cocoa swollen shoot virus (CSSV) disease, cuttings of the parental clones have been introduced since the 1950s. Out of the progenies, a small fraction of the elite selections were used to produce second and third generations of Amazon known as "F3 Amazon" or "Mixed Amazon" in Ghana (Aikpokpodion 2012). By

early 1960, the "F3 Amazon" or "Mixed Amazon" had been widely distributed to farmers in Ghana and Nigeria to cope with CSSV. In Ghana, seed production plots planted with selected Amazon × Amelonado hybrids and Inter-Amazon hybrids are the main sources of materials for farmers. In a recent survey, Edwin and Masters (2005) showed that released hybrids accounted for 42 % higher yields obtained by Ghanaian farmers. In Cote d'Ivoire, the 12 selected Upper Amazon-derived hybrids (Besse 1975) played a very significant role in the phenomenal increase in cacao production. In Nigeria, the Old Western Region, comprised of the present Ondo, Oyo, Ogun, Ekiti, and Osun states, had functional Cocoa Development Units (CDU) or Tree Crop Unit (TCU), responsible for distributing seeds of the "F3 Amazon" and the WACRI Series II varieties from seed gardens (Toxopeus 1964; Aikpokpodion et al. 2009).

1.6 Current On-Farm Diversity in West Africa and Southeast Asia

Surveys of planting materials in West Africa have shown that a mixture of Amelonado landraces and Upper Amazon-derived cacao hybrids distributed by the government are present in farmers' fields (Aikpokpodion et al. 2009, 2012; Edwin and Masters 2005). Among the parental germplasm groups from the Pound collections, the Parinari group had the greatest impact on the farmer's selections (Aikpokpodion et al., 2012). Unlike the traditional local Amelonado, which has only one peak of fruit production, this Amazon cacao has year-round production, high-yielding potential, and a shorter juvenile period. These are important criteria used by local farmers for selecting "improved varieties." The traditional West African Amelonado, on the other hand, are appreciated for their low vigor, high bean-to-pod volume ratio (less mucilage and placenta content), and less susceptibility to black pod disease (Aikpokpodion et al. 2012).

Farmers in Côte d'Ivoire largely used their own planting materials to establish new plantations in new cacao-growing regions. However, many of the planting materials were originally introduced by farmers from Ghana (Pokou et al. 2009). Assessment of on-farm genetic diversity in six producing regions of Côte d'Ivoire was reported by Pokou et al. (2009). Based on farmer selections sampled from 280 farms, open-pollinated seed progenies were collected from 561 trees. Twelve microsatellite markers were used to assess parentage and genetic diversity. Most of the farm accessions appeared to be hybrids between Upper Amazon (UA) and Lower Amazon (LA, Amelonado) or African Trinitario parental genotypes. However, a certain percentage of accessions appeared to be fairly pure UA or LA types. The best accessions for black pod resistance appear to be mostly hybrids between Upper Amazon and Amelonado.

A survey of 400 farm accessions in Cameroon, based on 12 microsatellite loci, suggests that 25.5 % of the farm accessions are still closely related to the traditional

Amelonado variety called "German cocoa" by the farmers (Efombagn et al. 2006, 2008). Another 46.3 % of the farm accessions were found to be direct descendants (20.8 % first-generation (F1) hybrids and 25.5 % selfed genotypes) from 24 parental clones used in bi-clonal seed gardens (BSGs) established in the 1970s in southern and western Cameroon. Furthermore, 28.3 % of farm accessions appeared to descend from uncontrolled pollination events in cacao farms, which could be related to a common practice of cacao growers to use seeds collected from their own farm for new plantings.

A survey of ancestry and parentage in farmers' selections in Sulawesi, Indonesia, using SSR markers generated similar result as those observed in West Africa. Sulawesi farmer selections are mainly comprised of hybrids derived from Trinitario and two Upper Amazon Forastero groups. Trinitario made the largest ancestral contribution to these farmer selections, with an average population membership (Q value) of 51.0 %. The second largest contributor was from the Parinari group, which explained 27.5 % of the ancestry. The Nanay group accounted for 12.6 % of the admixture, and the group of Morona/Scavina and Iquitos Mixed Calabacillo only explained 4.3 and 4.7 % of the assigned membership of the tested farmer selections (Diny Dinarti and Dapeng Zhang, unpublished data).

The low level of on-farm diversity in Asia and Africa is also demonstrated in the seed gardens that provide planting materials for the next several decades in West Africa. Cacao seed gardens are considered an efficient and dynamic seed production and distribution system in West Africa, because they play a significant role in replacing the aging cacao trees in this region. There are a total of 47 seed gardens in Cameroon (3), Ghana (26), and Nigeria (18), which are predominantly run by the state at subsidized prices. These seed gardens produced approximately five to six million hybrid pods in 2008 (Asare et al. 2010). However, parental clones used in these seed gardens reflect a narrow genetic background. The total number of parental clones currently used in seed gardens for production of hybrid pods is approximately 50 (Table 1.5). However, pedigrees from the Upper Amazon Forastero germplasm can be traced back to no more than 10 clones from the Pound collection (e.g., NA 32, PA 7, PA 35, SCA 6, SCA 12). This is the same small set of Upper Amazon Forastero that was introduced into Africa 70 years ago. Since then, no new genes or alleles have been added to the breeding pool.

The series of on-farm diversity surveys in West Africa and Southeast Asia, as mentioned above, showed that the current level of functional allelic diversity is low in these major cacao-producing countries. The available resistances to cacao disease and pests in the major producing countries have been dominantly based on no more than 10 mother trees from Pound collection, which were introduced from Trinidad in the mid-1940s. As a result, the breeding efforts since then have been reshuffling these limited genetic variations, despite the superficially high level of heterozygosity and gene diversity (typical of an outcrossing species) in farmer selections and breeding lines. This small set of Pound accessions, which form the foundation for current on-farm diversity in Asia and Africa (and to large extent in Latin America as well), represents only a tiny fraction of the existing genetic diversity in the primary gene pool of cacao. Cacao is very different from any other major

Table 1.5 Parental clones currently used in seed gardens in West Africa for production of hybrid seedlings

Cameroon		Ghana		Nigeria	
Parental clones	Pedigree	Parental clones	Pedigree	Parental clones	Pedigree
SNK 13	Trinitario	C20	PA 35 × NA 32	C14	Amelonado
SNK 64	Trinitario	C27	PA 7 × NA 32	C18	Amelonado
SNK 10	Trinitario	C42	PA 7 × NA 32	C24	Trinitario
SNK 16	Trinitario	C67	NA 32 × PA 7	C 20	PA 35 × NA 32
SNK 109	Trinitario	C69	NA 32 × PA 7	C25	Trinitario
IMC 67	IMC 67	C74	PA 35 × NA 32	C 27	PA 7 × NA 32
UPA 134	F2 of T clones	C75	PA 35 × NA 32	C69	NA 32 × PA 7
UPA 143	F2 of T clones	C77	IMC 60 × NA 34	C74	PA 35 × NA 32
SCA 6	SCA 6	C84	IMC 60	C75	PA 35 × NA 32
SCA 12	SCA 12	C85	PA 7	C77	T85
T79/467	NA 32 × PA7	PA7	PA7	NA32	NA32
T79/501	NA 32 × PA 7	PA150	PA150	P7	P7
SNK 413	Trinitario			PA150	PA150
				PA35	PA35
				T10/15	M 80
				T22/28	JA 3/11
				T60/887	PA7 × NA 32
				T65	PA 7 × IMC 47
				T86/2	PA 35 × PA 7
				T9/15	M 116

Source: Asare et al. (2010)

field crop in terms of the amount of available wild populations, which still exist in the Amazon and are coevolving with the pathogens. Moreover, these wild populations can be readily crossed with cultivated varieties without reproductive barriers. Recent QTL and functional diversity studies have showed that different germplasm groups harbor different genes/alleles of disease resistance, as reviewed by Lanaud et al. (2009). A major change must be taken in cacao breeding in order to cope with these threats. New breeding approaches should take full advantage of the available diversity in wild populations. An important strategy is long-term germplasm enhancement through pre-breeding, i.e., introgression of exotic germplasm to improve population diversity, which has generally been recommended for most domesticated crops (Stander 1993), as well as for cacao in particular (Dias 2001b; Eskes 2011; Surujdeo-Maharaj et al. 2001; Tahi et al. 2000). Pre-breeding programs allow accumulation of resistance genes/alleles from different origins, thus increasing future on-farm genetic diversity.

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Chapter 2
The Impact of Diseases on Cacao Production: A Global Overview

Randy Ploetz

Abstract Cacao (*Theobroma cacao*), one of the most important tropical crops, is responsible for a multibillion-dollar confectionary trade. It originated in the neotropics, but most production now occurs outside its native range. Diseases are significant constraints in commercial production and reduce yields by a conservative total of 20 %, or a projected 1.3 million tons of beans in 2012. As it was moved outside the neotropics, cacao was released from its two primary American enemies, *Moniliophthora roreri* (cause of frosty pod) and *M. perniciosa* (witches' broom). The diseases they cause would devastate production in West Africa and Asia if they were reunited with their cacao host. Diverse pathogens impact production in other areas. Newly encountered pathogens in West Africa (*Cacao swollen shoot virus* and *Phytophthora megakarya*) and Asia [*Ceratobasidium* (aka *Oncobasidium*) *theobromae*] are serious problems in those areas, as are other fungi and stramenopiles with wider geographic distributions. The ranges of the major pathogens and the impacts and losses that result from the diseases they cause are considered below. The potential impacts of the geographically restricted problems are assessed to evaluate the vulnerability of this crop. In a worst-case scenario, global production would be impossible in the presence of all of the major pathogens.

2.1 Harold Charles Evans: A Pioneer in Cacao Pathology

As a fitting introduction to this chapter, Harold Charles Evans is profiled as a pioneer in cacao pathology research. Anyone who has worked on cacao diseases will know Harry, or will certainly recognize his name. Harry was born in Liverpool, shortly after members of the Beatles were born in that city. He received all of his secondary education in the UK (B.Sc. in Botany, University of London, 1965; M.Sc. in Plant Pathology, Exeter University, 1966; Ph.D. in Mycology, Keele

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University, 1969), as well as a D.Sc. in Applied Mycology (University of London, 1999), which was awarded on the basis of his published work.

Harry began his international work on this crop at the Cocoa Research Institute of Ghana (CRIG) in 1969. During a 4-year secondment from the UK Ministry of Overseas Development [ODM, which subsequently became the Overseas Development Administration (ODA) as it was subsumed into the Foreign Office], he assisted breeding efforts and examined interactions between virus and fungal pathogens of this crop. Perhaps, his most notable work there was on the epidemiology of black pod. He identified invertebrate vectors of the causal agent(s) and studied their potential contributions to the movement and development of this important disease (Evans 1971, 1973). This work coincided with investigations on the biological control of arthropod pests (Evans 1974). Biological control of arthropods and subsequently of plant diseases and weeds became some of Harry's predominant areas of research and remain significant topics of his professional work.

Harry's assignment in Ghana was followed by another 4-year secondment to the Tropical Experimental Station (INIAP) in Pichilingue, Ecuador, beginning in 1973. His responsibilities there as a Technical Assistance Officer were to investigate the biology and control of major diseases of cacao and to train fellow scientists in the Plant Pathology Section. His time in Ecuador was significant as it introduced him to the frosty pod and witches' broom diseases on which he has made major contributions over the last four decades (Fig. 2.1). While at INIAP, he made fundamental advances in understanding the causal agents of these devastating diseases. In addition to a comprehensive monograph on frosty pod (Evans 1981), he published, with coauthors from the Netherlands and USA, the first evidence that the frosty pod agent (known at the time as *Monilia roreri*) was a basidiomycete (Evans et al. 1978). On the basis of in vitro and in vivo observations, they indicated that the fungus was a hemibiotroph and suggested that a new genus, *Moniliophthora,* be used to name the pathogen. *Moniliophthora roreri* (Cif.) H. C. Evans, Stalpers, Samson, and Benny remains the binomial that is used for this pathogen (Evans et al. 2013). Harry also described the hemibiotrophic nature of the witches' broom pathogen (known then as *Crinipellis perniciosa*) while on a subsequent assignment in Brazil from 1977 to 1980. While at the Special Amazonian Cocoa Research Department of the Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC) in Belém, Pará, he observed microscopic and karyotypic changes in the fungus in vitro and in vivo (Evans 1980). These studies began a career-long investigation of the biology, ecology, and pathology of these pathogens and facilitated subsequent advances by others on these fungi (Aime and Phillips-Mora 2005; Griffith et al. 2003; Phillips-Mora et al. 2007a). While in Brazil, he also advised on disease management and quarantine issues and trained counterpart scientists in disease identification and control. He published on Ceratocystis wilt in the Brazilian Amazon (Bastos and Evans 1978) and continued work on witches' broom (Evans and Bastos 1980a, b).

In a subsequent 4-year assignment, Harry served as a Forest Pathologist on a research project funded by ODA at the Commonwealth Mycological Institute at

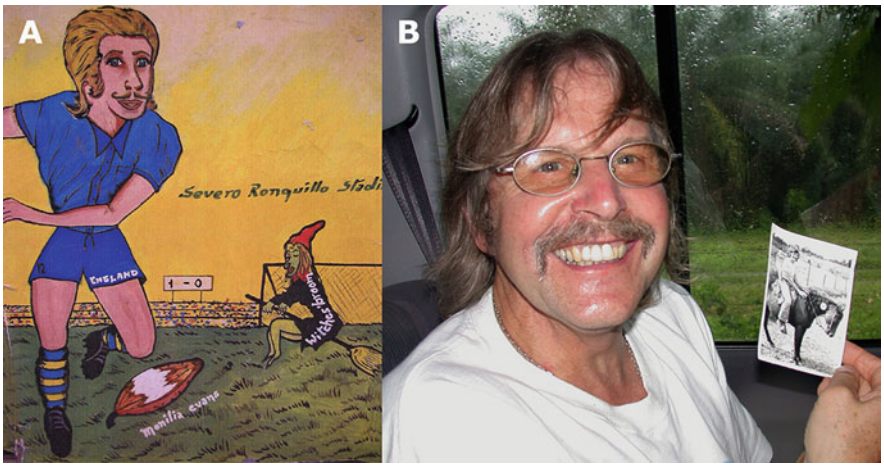

Fig. 2.1 (**a**) Harry Evans as a footballer dealing with the two disease threats he confronted in Ecuador in the early 1970s, frosty pod ("Monilia Evans") and witches' broom (artist unknown). (**b**) Harry in 2004, showing a photo of himself in 1974 on the way to cacao research plots in Ecuador (Photo: R.C. Ploetz)

Kew (CMI) and located at the Forestry Research Institute in Honduras. While there, he surveyed and inventoried fungal diseases of Central American pine species, published a series of papers on fungal pathogens of insects (e.g., Evans 1982) and on new diseases of pine and a monograph on *Dothistroma* pathogens of pine (Evans 1984), as well as continued his output of papers on pathogens and diseases of cacao.

Harry then returned to the UK. From 1984 to 2006, he was a Principal Scientist and Senior Pathologist at CABI Bioscience at Ascot, UK (formerly International Institute of Biological Control, IIBC), where his principal responsibilities were to initiate and coordinate research projects in the Biological Control of Weeds and Plant Diseases Program. This period witnessed a significant increase in the publications Harry wrote on the biological control of plant diseases (Evans et al. 2003b; Holmes et al. 2004), of insect pests (Evans 1988; Samson et al. 1988), and of weed pests (Barreto and Evans 1997; Evans 1995, 2002a; Evans and Ellison 1990). In addition, he continued to write about the cacao pathogens and diseases on which he had started his career in the tropics (Evans and Barreto 1996; Evans 2002b; Evans et al. 2002, 2003a).

From 2006 to 2007, Harry was a Visiting Professor at the Universidade Federal de Viçosa in Minas Gerais, Brazil, and since 2008, he has been an Emeritus Fellow at CAB International, at the Europe-UK Centre in Egham, Surrey, UK. While in Viçosa, Harry taught and supervised postgraduate students in the Department of Plant Pathology and conducted research on cacao and coffee diseases, much of which was with his long-time colleague Robert Barreto. He continued his work on tropical rust fungi, in particular the coffee rust pathogen, *Hemileia vastatrix* (Fernandes et al. 2009; Carvalho et al. 2011, 2014), and, with Robert, investigated the origins of *M. perniciosa* in the Amazon basin. Upon "retirement," Harry has

continued to publish regularly on all of the above topics, including the cacao problems on which he began his career (Evans 2007, 2012; Thomas et al. 2008; Evans et al. 2013).

In summary, Harry is an authority on tropical mycology and plant pathology. He has published over 200 research publications and continues to work on a diverse range of basic and applied topics. Harry's intellectual curiosity, frequent assignments overseas, fluency in Spanish and Portuguese, and willingness to conduct work under a range of conditions have been instrumental in the seminal contributions he has made to mycology and plant pathology and, in particular, to our understandings of cacao pathogens and the diseases that they cause on this important crop.

2.2 Introduction

Cacao, *Theobroma cacao*, is one of the most important tropical crops. Cacao seeds (aka beans) are the source of chocolate, cocoa butter, and powder and are a significant internationally traded raw product (George 2013). Currently, the global chocolate industry is valued at US$80 billion year^{-1} (The Guardian 2015). Over 5 million metric tons of beans were produced in 2012 (Table 2.1), and the trade in beans was worth US$9 billion (FAOSTAT 2014; ICCO 2010).

Cacao originated in the headwaters of the Amazon River (Bartley 2005). After Europeans understood its commercial potential, it was harvested from largely wild stands for another 200 years. Cacao was the "foundation of the economy of the Amazon Region. . .until the late 19th century" (Bartley 2005), and until well into the nineteenth century, virtually all production came from this area (Fig. 2.2). In 1900, 80 % of the global output came from the Western Hemisphere (Bradeau 1969), but by the turn of the twenty-first century, the region had become a relatively minor producer. In 2012, about 86 % of production came from the Eastern Hemisphere, and the top five producing nations, all of which were from the east, produced about 82 % of the total (FAOSTAT 2014) (Table 2.1).

Destructive diseases in the Americas that did not occur in the Eastern Hemisphere, notably frosty pod and witches' broom, were prime reasons for these major shifts in production (Evans 2002b, 2007). Frosty pod and witches' broom are examples of the "enemy release" phenomenon (Evans and Ellison 2004; Hallett 2006). Enemy release occurs when coevolved pathogens are not disseminated with plants when they are moved outside their native ranges (Ploetz 2007b). Crop plants are often most productive when they are separated from their coevolved enemies, and cacao in West Africa and Asia is a good tropical example of a crop that is produced most productively outside its native range in the absence of damaging coevolved enemies (Evans 2002b; Ploetz 2007b).

Cacao is a volatile commodity, and prices for beans routinely fluctuate through bust and boom cycles (George 2013). For example, a 27-year low of US$714 metric ton^{-1} occurred in 2000, but by 2011, this unit price had risen to a 32-year high of

Table 2.1 Cacao bean production in the leading countries in 2012

Countries	Bean production (MT)	% of global total
Côte d'Ivoire	1,650,000	33.0
Indonesia	936,300	18.7
Ghana	879,348	17.6
Nigeria	383,000	7.7
Cameroon	256,000	5.1
Brazil	253,211	5.1
Ecuador	133,323	2.7
Mexico	83,000	1.7
Dominican Republic	72,225	1.4
Peru	57,933	1.2
Colombia	49,509	1.0
Papua New Guinea	38,700	0.8
Togo	34,500	0.7
Venezuela	20,000	0.4
Sierra Leone	18,000	0.4
Totals		
Africa	3,289,192	65.7
Americas	708,324	14.2
Asia	960,407	19.2
Oceania	45,289	0.9
Global	5,003,211	

Data are from FAOSTAT (2014)

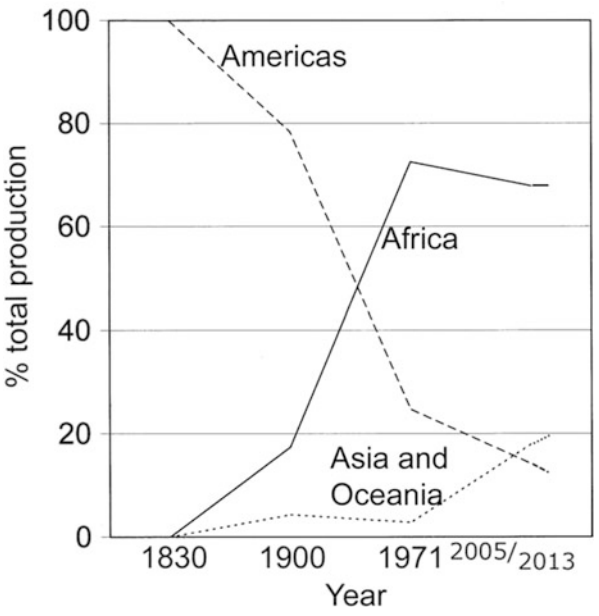

Fig. 2.2 Regional trends in global cacao production since 1830. Data are from Bradeau (1969), FAOSTAT, and Lanaud et al. (2003)

$3775 (The Guardian 2015). Diverse factors are responsible for this volatility. A global glut in production reduced prices in 2000, political instability in Côte d'Ivoire fueled doubts about global bean supplies in 2011, and recent concerns over the Ebola crisis have resulted in speculation about ongoing production in West Africa (George 2013; The Guardian 2015).

In a somewhat different manner, cacao diseases play decisive roles in bean production. Diseases are the most important biological constraints in the production of this crop, probably exceeding the combined impacts of arthropod and vertebrate pests (Bowers et al. 2001; Ploetz 2007a). However, despite consensus on the importance of diseases, estimates for the magnitude of losses that they cause are inconsistent. The variable figures that are available reflect the complex nature of loss assessment and multitude of known and unknown factors that are involved. Good estimates are available for very few crops, and almost none of these are grown in the tropics under the range of conditions that are used to produce cacao (James 1974; Savary et al. 2012). Nonetheless, estimates that have been made are useful as they provide relative references for the importance of the various problems.

In 2001, disease losses in cacao represented a conservative 20 % of potential yield (losses of 810,000 tons and a total harvest of 3,218,281 tons) (Bowers et al. 2001; FAOSTAT 2014). Although the rank order of diseases in Table 2.2, which is based on these figures, has probably not changed, the gross losses that they cause surely have. From 2001 to 2012, global production increased by 1.8 million tons (a factor of 1.56). Although the disease-resistant germplasm that has been deployed conceivably reduced losses in some areas (de Albuquerque et al. 2010; Medeiros et al. 2010), global losses due to diseases would have undoubtedly been higher in 2012 than in 2001, perhaps 1.3 million tons based on the above figures. In Table 2.2, losses for the top five diseases in 2012 (black pod, frosty pod, witches' broom, cacao swollen shoot, and vascular streak dieback) were estimated by first calculating the increase in production in the areas affected by each of the diseases from 2001 to 2012. For example, an increase of 1.56× was used for the pantropical black pod, whereas a 1.52× increase was estimated for the areas that are affected by witches' broom. These figures were then used to project the losses a given disease caused in 2012, using the 2001 figures as a baseline; for example, in 2012 black pod losses were estimated at 700,000 tons (1.56 × 450,000), whereas witches' broom losses were 380,000 tons (1.52 × 250,000).

Greater losses have been projected by others. Ten Hoopen et al. (2012) indicated that the top five diseases reduced production by 40 % (2.5 million tons in 2012?), and much higher maximum losses have been indicated for each of these diseases: cacao swollen shoot (100 %), witches' broom (100 %), frosty pod (90 %), black pod (90 %), and vascular streak dieback (70 %) (Bowers et al. 2001; Evans 2002b; Gotsch 1997).

Regardless of the estimates that are used, it is clear that diseases are prime reasons for lost production. Cacao swollen shoot and vascular streak dieback are debilitating diseases that can kill trees, whereas black pod, frosty pod, and witches' broom, which are usually not lethal, directly impact yields by rendering beans in

Table 2.2 The major diseases of cacao, *Theobroma cacao*

Disease	Annual losses[a]	Impact[b]		Agent(s)	Geographical distribution	References
		Current	Future			
Black pod	450 (700)	***	***	*Phytophthora capsici*	Brazil, El Salvador, Guatemala, India, Jamaica, Mexico, Trinidad, Venezuela	Bowers et al. (2001), End et al. (2014), Erwin and Ribiero (1996), Evans (2007), Guest (2007)
				P. citrophthora	Brazil, India, Mexico	
				P. heveae	Malaysia	
				P. megakarya	Cameroon, Côte d'Ivoire, Fernando Po (aka Bioko), Gabon, Ghana, Nigeria, São Tomé (islands of Principe and São Tomé), and Togo	
				P. megasperma	Venezuela	
				P. nicotianae var. *parasitica*	Cuba	
				P. palmivora (also as *P. arecae*)	Pantropical	
Witches' broom	250 (380)	***	***	*Moniliophthora perniciosa* (previously *Crinipellis perniciosa* and *Marasmius perniciosa*)	Bolivia, Brazil, Colombia, Ecuador, French Guiana, Grenada, Guyana, Panama (east of the Panama Canal), Peru, Trinidad, St. Lucia, St. Vincent, Suriname, and Venezuela	End et al. (2014), Evans (2002b, 2007)
Swollen shoot	50 (76)	**	***	*Cacao swollen shoot virus*	Benin, Côte d'Ivoire, Ghana, Liberia, Nigeria, Sierra Leone, and Togo	Bald and Tinsley (1970), Brunt et al. (1996), Lockhart and Sackey (2001), Thresh and Owusu (1986)
Frosty pod (moniliasis)	30 (53)	**	****	*Moniliophthora roreri*	Belize, Bolivia, Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama, Peru, and Venezuela	Phillips-Mora et al. (2007a, b); personal communications, Carmen Suarez-Capello and Wilberth Phillips-Mora

(continued)

Table 2.2 (continued)

Disease	Annual losses[a]	Impact[b]		Agent(s)	Geographical distribution	References
		Current	Future			
Vascular streak dieback	30 (76)	**	**	*Ceratobasidium* (*Oncobasidium*) *theobromae*	Burma, China (Hainan Island), southern India, Indonesia, Malaysia, Papua New Guinea (main island, New Britain and New Ireland), the Philippines, Thailand	Guest and Keane (2007), Keane et al. (1972)
Ceratocystis wilt (mal de machete)	n/a	**	**	*Ceratocystis cacaofunesta* (*C. fimbriata*)	Brazil, Colombia, Costa Rica, Ecuador	Engelbrecht et al. (2007)
Cushion (green-point) gall	n/a	*	*	*Fusarium decemcellulare*	Western Hemisphere, Africa, and Sri Lanka	Ploetz (2006)
Black (Rosellinia) root rot	n/a	*	*	*Rosellinia bunodes*	Tropical America, India, Indonesia (Java and Sumatra), Malaysia (peninsular), Philippines, Sri Lanka	Ploetz (2007a), ten Hoopen and Krauss (2006)
				R. paraguayensis	Grenada	
				R. pepo	Central America, West Indies, West Africa	

[a]Diseases are listed in order of current loss estimates; figures are estimates from 2001 in 1000 s of metric tons or are not available (n/a) (Bowers et al. 2001). Parenthetical estimates are for 2012 and were calculated based on the proportional increase in production in the affected areas from 2001 to 2012, multiplied by the 2001 loss estimates (see text)

[b]Relative impact is: * = low; ** = moderate; *** = high; **** = very high. Future impact is based on the potential destruction a given disease would cause if it were disseminated outside its current geographic range

affected pods commercially useless. The latter diseases are referred to below as the "big three," due to their pronounced, direct impact on yield and their current and potential effect on global production (Fulton 1989; Evans 2007). Ironically, their potential impact on yield is inversely related to their current effect on production (Table 2.2). As summarized by Evans (2007), the #1 disease, black pod, became a minor problem in Bahia after the #2 disease, witches' broom, arrived in that production area, and the #4 disease, frosty pod, became the principal constraint in Peru after it moved into areas affected by both black pod and witches' broom.

2.3 The Major Cacao Diseases

Purdy et al. (1998) listed several dozen cacao diseases, most of which are caused by fungi. Less common pathogens include alga, bacteria, nematodes, parasitic plants, stramenopiles, and viruses. Those that are considered below are listed in order of their current impact (Table 2.2). The most dangerous diseases are frosty pod and witches' broom, American problems that are caused by related hemibiotrophic basidiomycetes, *Moniliophthora roreri* and *M. perniciosa*, respectively (Aime and Phillips-Mora 2005; Evans 2002b).

Frosty pod is twice as damaging as black pod and more difficult to control than witches' broom (Evans 2002b). It is responsible for lower losses than the most destructive diseases only because it is restricted to areas in which comparatively little cacao is produced (Fig. 2.3a). After frosty pod, witches' broom is the next greatest threat worldwide (Evans 2002b; 2007). Importantly, witches' broom also has a narrow geographic distribution (Fig. 2.3b) (Evans 2002b). Disastrous losses would occur if either frosty pod or witches' broom spread to major production centers in Africa and Asia (Evans 2002b).

Other pathogens in Table 2.2 that have narrow geographic distributions would also cause serious problems if they spread to new production areas. For example, the newly encountered pathogens *Cacao swollen shoot virus* (Fig. 2.3c) and *Phytophthora megakarya* (Fig. 2.3d) would cause serious problems if moved outside West Africa, as might *Ceratobasidium theobromae* (Fig. 2.3e) if it moved outside Asia and Melanesia. Ongoing production will, to a large extent, depend on keeping the distributions of these pathogens restricted. In this regard, it is imperative that the dissemination of cacao germplasm be made safely (End et al. 2014).

The relative impacts of the various diseases vary considerably among and within the different production regions. For example, Duguma et al. (2001) reported that disease losses varied from 10 to 30 % in Côte d'Ivoire, 30 to 50 % in Ghana and Togo, and 50 to 80 % in Cameroon. This chapter outlines in general terms the most important diseases. Their histories and the centers of origin and current geographic distributions of the causal agents are discussed below, as are the nomenclature and taxonomy of the pathogens and the epidemiology and management of the diseases that they cause. For the most serious diseases, an attempt is made to predict how they would affect production were they disseminated to new areas. Overall, a

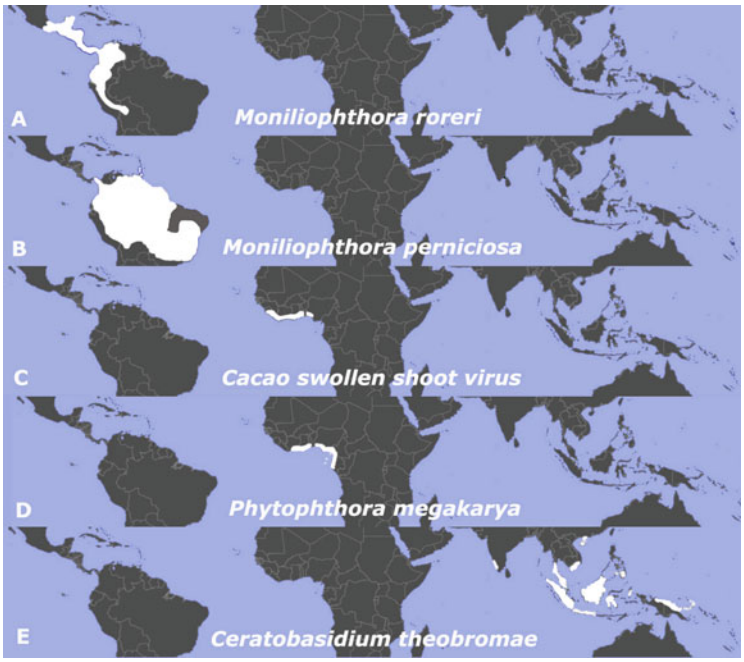

Fig. 2.3 Geographic distributions of the major pathogens of cacao: (**a**) *Moniliophthora roreri* (frosty pod), (**b**) *Moniliophthora perniciosa* (witches' broom), (**c**) *Cacao swollen shoot virus* (cacao swollen shoot), (**d**) *Phytophthora megakarya* (black pod), and (**e**) *Ceratobasidium theobromae* (vascular streak dieback). Note the narrow distributions for these pathogens. Cacao is extremely vulnerable to their movement and establishment in new areas

synthesis is presented for the past, current, and future impact of these important problems in cacao production.

Black Pod

Black pod is the most widely distributed of the diseases that are considered below. Black pod directly impacts production by reducing the harvest of commercially acceptable beans, but the black pod pathogens also cause trunk and branch cankers that can kill or debilitate trees. Despite their importance, cankers are often overlooked as yield-limiting factors in cacao production (Appiah et al. 2004; Guest 2007). These pathogens also infect cacao roots, which are important reservoirs for these pathogens in cacao plantations (Guest 2007; Luz 1989). Considering the negative impacts of other *Phytophthora* root pathogens (Erwin and Ribiero 1996), the decrease in healthy root biomass that these pathogens cause on cacao may also be significant. Seven species of *Phytophthora* are involved, but *P. capsici*,

P. citrophthora, *P. megakarya*, and *P. palmivora* are the most important (End et al. 2014; Guest 2007).

The most damaging species is *P. megakarya* (Akrofi et al. 2015; Guest 2007; Ndoumbe-Nkenga et al. 2004). It was first identified in Cameroon and Nigeria in 1979 (Brasier and Griffin 1979), where it was originally confused with *P. palmivora*, to which it bears a close resemblance. *Phytophthora megakarya* is still spreading in West Africa and is now recognized in Cameroon, Côte d'Ivoire, Equatorial Guinea, Fernando Po (aka Bioko), Gabon, Ghana, Nigeria, São Tomé (islands of Principe and São Tomé), and Togo (Fig. 2.3d) (Akrofi et al. 2015; End et al. 2014; Andrews Akrofi, personal communication). It has not been reported in Benin (note the gap in Fig. 2.3d), presumably due to the unimportance of cacao in that country.

Pods that are infected by *P. megakarya* develop symptoms earlier and produce inoculum (primarily sporangia) earlier and more prodigiously than when infected by the other species (Guest 2007). The pathogen is heterothallic. Its A1 and A2 mating types are found in its two putative centers of origin, Cameroon and the border between Cameroon and Nigeria, and oospores are produced when isolates of the mating types are paired. However, the pathogen reproduces primarily in a clonal manner, and sporangia and zoospores are the main infective propagules. The biologies of the other black pod pathogens are similar.

Although *P. megakarya* probably originated in West Africa, its original host (s) is(are) unclear. In cacao plantations, the primary reservoir for *P. megakarya* inoculum is the cacao root system, although alternative hosts of *P. megakarya* have been recognized for some time; they include *Cola nitida* (Nyassé et al. 1999), *Irvingia* sp. (Holmes et al. 2003), *Dracaena mannii*, *Funtumia elastica*, *Ricinodendron heudelotii*, and *Sterculia tragacantha* (Opoku et al. 2002). Recently, Akrofi et al. (2015) recovered the pathogen from asymptomatic roots of additional species in cacao plantations, including *Ananas comosus*, *Athyrium nipponicum*, *Carica papaya*, *Colocasia esculenta*, *Elaeis guineensis*, *Mangifera indica*, *Musa* spp., *Persea americana*, and *Xanthosoma sagittifolium*. They suggested that the pathogen's relatively wide host range could play a role in the difficulties that West African producers have had in managing *P. megakarya*-induced black pod.

In terms of global losses, the most important black pod pathogen is *P. palmivora* (End et al. 2014; Erwin and Ribiero 1996; Guest 2007). This pathogen was first reported on cacao in 1909 and currently causes more damage on cacao than any of the other species. *Phytophthora palmivora* is found in virtually all cacao production areas and has a very wide host range, numbering in excess of 200 species (Erwin and Ribiero 1996; Guest 2007). Flood et al. (2004) indicated that it was responsible for worldwide pod losses of 20–30 % and tree mortality as high as 10 %.

Phytophthora capsici and *P. citrophthora* also have wide host ranges, although their geographic distributions on cacao are narrower than that for *P. palmivora*. On this crop, *P. capsici* and *P. citrophthora* are found mainly in the Western Hemisphere (End et al. 2014; Erwin and Ribiero 1996).

Integrated management of this disease that utilizes more than a single approach is most effective (Guest 2007). Sanitation (removal of affected pods) is one of the

more useful cultural tools. However, its efficacy depends on economics (whether labor costs offset corresponding increases in yield), frequency (shorter pod removal intervals are more effective), and the causal agent (Guest 2007; Ndoumbe-Nkenga et al. 2004; Soberanis et al. 1999). For example, in Peru, where *P. palmivora* was responsible for the disease, weekly pod removal was more effective than fortnightly removal (i.e., reduced black pod by 66 % vs. 35 %) (Soberanis et al. 1999). In contrast, in Cameroon, where *P. megakarya* was involved and pods were removed on a weekly basis, reductions of 22 and 9 % were observed over 2 years in one site and 31 and 11 % in another (Ndoumbe-Nkenga et al. 2004). Although direct comparisons of data from different areas can be problematic, the above differences may reflect the greater virulence of *P. megakarya* compared to *P. palmivora*, a greater and more rapid production of inoculum that occurs on pods affected by *P. megakarya*, and the comparatively greater soilborne reservoir of *P. megakarya* inoculum; all of these factors would make management of black pod damage via sanitation more difficult when caused by *P. megakarya* than by *P. palmivora* (Appiah et al. 2004; Guest 2007).

Management with pesticides (copper, metalaxyl/mefenoxam, and phosphonates) is also useful, but this option faces logistical and environmental obstacles (Guest 2007). Importantly, the expense of pesticides reduces their adoption by resource-poor smallholders who produce much of this crop. Smallholders are risk averse; in their minds, disease management practices may not be justified, given the volatile and unpredictable nature of bean prices (Bateman et al. 2005).

Given the labor-intensive nature of sanitation and barriers that can exist to pesticide usage, it is not surprising that neither of these tools are used to any great extent to manage this (or other) disease(s). In the long term, resistance holds the greatest promise for sustainably managing black pod. Gutiérrez et al. (2015) summarize progress that has been made to produce black pod resistant genotypes.

Caution should be used when transporting budwood from areas that are affected by *P. megakarya*. End et al. (2014) recommended intermediate quarantine of such materials before they are disseminated. Although other means by which cacao pathogens are moved, such as in infected pods or seeds, are not great problems with these pathogens, germplasm dissemination via these routes is never a good idea and should be avoided regardless of which pathogens are thought to present the greatest risk.

Witches' Broom

Although witches' broom was first studied in Suriname in 1895, its symptoms were first described in Amazonia in 1785 (Meinhardt et al. 2008; Purdy and Schmidt 1996). Thus, witches' broom appears to be the first disease to be recognized on this crop, preceding the first descriptions of frosty pod by some 50 years (Phillips-Mora et al. 2007b).

Moniliophthora perniciosa (previously *Crinipellis perniciosa* and *Marasmius perniciosus*) probably evolved with cacao in the Amazon basin, but remained restricted to that region until cacao was moved to and produced in other areas (Evans 2002b; Griffith et al. 2003; Meinhardt et al. 2008; Purdy and Schmidt 1996). The pathogen and disease are now present in Bolivia, Brazil, Colombia, Ecuador, French Guiana, Grenada, Guyana, Panama (east of the Panama Canal), Peru, Trinidad, St. Lucia, St. Vincent, Suriname, and Venezuela (Fig. 2.3b) (End et al. 2014). Notably, Brazil, Ecuador, and Peru are the only affected countries that are among the top 10 producers (Table 2.1).

Wherever witches' broom is present, it dramatically reduces production (Evans 2002b; Griffith et al. 2003). Shortly after the disease arrived in Bahia, it resulted in 200,000 lost jobs, a soaring crime rate, and extensive rural depopulation (Griffith et al. 2003; Pereira et al. 1996). Clearly, production in the affected areas would be higher if witches' broom were not present, and the disease would have a greater impact if it were more widely spread among the leading producers.

Cacao (C) and solanaceous (S) biotypes of *M. perniciosa* affect, respectively, plants in the Malvaceae (*T. cacao*, *T. sylvestris*, *T. obovata*, *T. grandiflorum*, *T. bicolor*, and *Herrania* spp.) and the Solanaceae (several *Solanum* and *Capsicum* spp.) (Evans et al. 2013; Griffith et al. 2003). In addition, an L biotype affects bignoniaceous lianas and an H-biotype, *Heteropterys acutifolia*. Although only the C biotype affects cacao, increased understandings of the origins, phylogeny, and host range of the fungus have come from comparative studies of the C and other biotypes (Griffith et al. 2003; Meinhardt et al. 2008). An ever-widening host range for the fungus is evident from recent work, but much remains to be learned about this pathogen (Evans 2007).

Basidiospores of *M. perniciosa*, which infect meristematic tissues of cacao and are important for the spread of witches' broom in and among plantations, are sensitive to desiccation and UV light and are, thus, not suited to long-distance dispersion; they probably spread the disease no more than 60 km (Frias et al. 1991; Griffith et al. 2003). Long-distance spread can occur when latently infected pods are moved, and it assumed that this was responsible for most large jumps in the disease's distribution (Evans 2002b). In addition, intentional movement of the pathogen has also been reported (Caldas and Perz 2013; Evans 2007; Junior 2006).

Maddison et al. (1993) reviewed a series of experiments in tropical America in which sanitation (removal of brooms) was examined for reducing disease and increasing yields. They indicated that the method's success depended on its thorough implementation, which was not possible in plantations with large trees and inaccessible or hidden brooms. Furthermore, the efficacy of this measure was eroded when sanitation was not practiced in adjacent plantations. Both factors may have influenced results from a recent study in which broom sanitation was marginally effective (Medeiros et al. 2010), since it was conducted in an abandoned orchard with unclear parameters for when, and how frequently and thoroughly, brooms were removed and destroyed in the study area or in surrounding areas. Previously, Rudgard and Butler (1987) had shown that removing 95 % of the brooms in an experimental plot resulted in only a 50 % reduction in pod loss.

Sanitation is usually advisable wherever one or more of the "big three" diseases are present. When all three were present in Peru, Soberanis et al. (1999) demonstrated the profitability of weekly sanitation. Although sanitation increased labor costs from US$125 to US$293 ha^{-1} $year^{-1}$, net profits after sanitation increased from US$83 to US$650 ha^{-1} $year^{-1}$.

Contact (e.g., copper and chlorothalonil) and systemic (e.g., triazoles) fungicides are effective against this disease (Laker 1991; Laker and Ram 1992; Medeiros et al. 2010; Meinhardt et al. 2008). Although they can reduce broom formation and increase pod production, they are most effective when integrated with sanitation. Despite the potential benefits of using integrated cultural and chemical measures, these practices are seldom used by smallholders (Medeiros et al. 2010).

There has been considerable work conducted on the biological control of cacao diseases, especially witches' broom and frosty pod. Biocontrol can circumvent environmental concerns that are associated with fungicidal control, and for witches' broom and frosty pod, it addresses particularly challenging control targets. In general, endophytic strains of *Trichoderma* have received the most attention, as they comprise significant portions of the native mycoflora in cacao trees and have provided significant experimental control of these diseases (Crozier et al. 2006; de Souza et al. 2006; Holmes et al. 2004; Krauss et al. 2010; Meinhardt et al. 2008). However, despite the promise this approach holds, it has been largely unrealized. More information is needed for how endophyte communities could be manipulated to reduce the impact of these important problems.

Pound began the search for resistance to witches' broom in the 1930s, when he collected cacao germplasm in the headwaters of the Amazon basin (Bartley 2005). Cacao evolved in this region with *M. perniciosa*, and considerable variation in the host is evident in different tributaries of the Amazon for witches' broom tolerance and other traits (Bartley 2005). Pound returned to Trinidad with two highly tolerant accessions from the Rio Ucayali basin in Peru, Scavina 6 and Scavina 12. They became parents of the Trinidad Selected Hybrids (TSH), lines that were ultimately responsible for reducing the impact of witches' broom in Trinidad (de Albuquerque et al. 2010). The Scavina parents also appear in the backgrounds of other important breeding lines and hybrids that have been released in different areas (Lopes et al. 2011). Unfortunately, Scavina-based tolerance to witches' broom has eroded in the Brazilian Amazon, Ecuador, and Peru, due possibly to variants of the pathogen. Recently, de Albuquerque et al. (2010) reported new sources of resistance from the Acre, Jamari, Purus, and Solimões river basins, which could be used to introduce into advanced lines other sources of resistance to this disease.

Cacao Swollen Shoot

Symptoms of cacao swollen shoot were first recognized in 1922 in present-day Ghana and in neighboring countries soon after the viral nature of this disease was understood in the 1940s (Lockhart and Sackey 2001; Oro et al. 2012). The disease is

now found in Côte d'Ivoire, Ghana, Liberia, Nigeria, Sierra Leone, and Togo (Fig. 2.3c) (Brunt et al. 1996; Lockhart and Sackey 2001; personal communication, E. Muller). Reports from outside West Africa (e.g., Malaysia, Papua New Guinea, Sri Lanka, Sumatra, and Trinidad) require confirmation (Ameyaw et al. 2014; Bald and Tinsley 1970; Brunt et al. 1996; Kouakou et al. 2012; Lockhart and Sackey 2001; Thresh et al. 1988; personal communication, E. Muller).

Cacao swollen shoot virus (CSSV), a member of the genus *Badnavirus*, causes this disease (Lockhart and Sackey 2001). CSSV is genetically variable, and different strains cause an array of symptoms depending on host genotype (Ameyaw et al. 2014; End et al. 2014; Lockhart and Sackey 2001; Oro et al. 2012). Unlike the previous two diseases, swollen shoot indirectly reduces yield. Symptoms include leaf and pod chlorosis, transient red leaf veins and mottling, root atrophy and stunting, and/or root and stem swelling. Highly pathogenic strains cause severe leaf chlorosis, defoliation, and rapid deterioration and death of cacao trees (Ameyaw et al. 2014; Brunt and Kenton 1971; Oro et al. 2012).

CSSV is moved in vegetative materials and can latently infect cacao cuttings that are used for clonal propagation for up to 20 months. Thus, it is imperative that postentry quarantine be used when germplasm is moved from the affected countries (End et al. 2014). CSSV is transmitted naturally by at least 14 species of mealybugs (*Homoptera*: *Coccidae*), but is not sap-transmissible, nor is it seed transmitted, in cacao (End et al. 2014). About 40 species in the *Malvaceae* are infected and develop symptoms, many of which are indigenous to West Africa (Brunt and Kenton 1971).

The origin(s) of CSSV is(are) not clear. Because the original reports were from West Africa and several relatives of cacao are alternative hosts in the region, it is "generally accepted that infection in these species antedated that in cacao" in West Africa (Brunt and Kenton 1971). However, Tinsley (1971) and Lockhart and Sackey (2001) indicated that the supporting data are equivocal. The only species that is commonly infected by CSSV in nature, *Cola chlamydantha*, is found only in Western Ghana. Based on historical evidence and infectivity assays, Bald and Tinsley (1970) suggested that *C. chlamydantha* was not a primary host but was, in fact, affected by CSSV that originated in cacao plantings. The possible but unconfirmed presence of CSSV in Asia, where West African cacao germplasm has not been introduced, also argues against a West African origin for CSSV. Until additional data are available from outside West Africa, only circumstantial evidence would appear to exist for a non-African origin for this virus (personal communication, E. Muller).

Extreme, but effective measures were developed to manage swollen shoot several decades ago (Dzahini-Obiatey et al. 2010; Thresh and Owusu 1986). In summarizing 40 years of research, Thresh and Owusu (1986) concluded that coppicing affected trees was ineffective and that removing only symptomatic trees was effective during the early stages of an epidemic. Once the disease was established, it was necessary to remove at least symptomatic and the adjacent asymptomatic plants. In newly affected areas in Nigeria, effective management was achieved when symptomatic trees and asymptomatic trees were removed

within 4.6, 9.1, and 13.7 m of outbreaks containing 1–5, 6–50, and 51–200 symptomatic trees, respectively. In serious outbreaks in which the disease had not been managed properly, it was necessary to remove entire blocks and replant with CSSV-free trees.

Thresh et al. (1988) considered these eradication efforts to be the most ambitious and costly ever used to control a virus-induced plant disease. The campaign in Ghana began in the 1940s, and to date over 200 million trees have been destroyed (Dzahini-Obiatey et al. 2010; Lockhart and Sackey 2001). Unfortunately, these programs have met periodic opposition from producers and have been administered inconsistently (Padi et al. 2013). Although the battle continues, "the disease is more prevalent now than ever before" (Domfeh et al. 2011).

Although modern techniques (e.g., ELISA, ISEM, PCR, and virobacterial agglutination methods) can detect CSSV (Hughes and Ollennu 1993; Muller et al. 2001; Sagemann et al. 1985), no technique detects all strains of this variable virus. Thus, visual indexing for infection is still recommended (End et al. 2014). Budwood should be tested for the presence of CSSV by grafting it to West African Amelonado seedlings, which develop conspicuous symptoms when infected.

West African Amelonado varieties, which originated in the Lower Amazon basin of Brazil, are extremely susceptible to this disease (End et al. 2014). Upper Amazon germplasm was reported to possess greater resistance to the disease and experience slower deterioration and lower yield losses after infection (Brunt 1975). Thus, Upper Amazon varieties were recommended as replacements for the Amelonado varieties in the hope that production could be increased in the impacted areas. Unfortunately, gains that were expected did not occur after these replacements were made (Thresh and Owusu 1986; Domfeh et al. 2011).

A recent evaluation of resistance to this disease revealed that Lower Amazon male parents were actually some of the most tolerant lines when crossed with specific Upper Amazon varieties (Padi et al. 2013). The authors suggested changes for evaluating disease response in new hybrids. They also reported slight "genetic gains for resistance. . .over the past seven decades" (Padi et al. 2013). Whether new hybrids could significantly reduce the spread and development of this disease in West Africa seems doubtful, unless they are used with the above cultural measures. In general, all of the diseases that are highlighted in this chapter are managed most effectively if multiple approaches are used.

Frosty Pod

Frosty pod (aka moniliasis, Quevedo disease, and podredumbre-acuosa) causes far lower losses in the world's cacao production areas than the above diseases, but it is potentially the most destructive of all of the diseases that affect this crop (Evans 2002b). All wild and cultivated species of *Herrania* and *Theobroma* (i.e., *T. cacao* and *T. grandiflorum*) are susceptible (Phillips-Mora et al. 2007a; Phillips-Mora and Wilkinson 2007).

Frosty pod was recognized as early as 1817, when symptoms of the disease were reported in northeastern Colombia (Department of Norte de Santander); this report preceded by 34 years the frosty pod outbreak in the lower Cauca Valley of Colombia and by 100 years the appearance of the disease in Ecuador (Phillips-Mora et al. 2007b). This sequence of events agrees with the idea that the causal agent, *Moniliophthora* (previously *Monilia*) *roreri*, originated in central/northeastern Colombia. Phillips-Mora et al. (2007a) indicated that the pathogen's genetic diversity was greatest in this area and that strains of *M. roreri* from the area were related to and probably responsible for subsequent outbreaks of frosty pod elsewhere in the region. They suggested that *M. roreri* originated on wild hosts (*Herrania* and *Theobroma* spp.) or in small patches of cultivated cacao in Colombia, Ecuador, and perhaps Venezuela. As cacao cultivation increased in the eighteenth century, so did the opportunities for the pathogen's spread. Its movement from these initial locations probably occurred over a long period of time. Based on the early records and genetic data, ancient spread of *M. roreri* among wild hosts followed by its subsequent anthropogenic dissemination on cacao is possible (Phillips-Mora et al. 2007a, b). The disease remained on the western side of the Andes until 1977, when the disease crossed this barrier and spread into the Amazon region of Ecuador (Carmen Suarez-Capello, personal communication). In 1988, it appeared in the Bagua Grande area in Peru and in 2011 was confirmed in the Alto Beni region of Bolivia, causing great concern in Brazil and placing officials at Porto Velho on high alert (Carmen Suarez-Capello, personal communication).

Frosty pod is currently found in Belize, Bolivia, Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama, Peru, and northwestern Venezuela (Fig. 2.3a) (Phillips-Mora et al. 2007a; Phillips-Mora and Wilkinson 2007; Carmen Suarez-Capello and Wilberth Phillips-Mora, personal communication). As noted previously for witches' broom, none of the leading producing nations are affected by frosty pod; only Ecuador (7th), Mexico (8th), and Peru (10th) are among the top 10 (Table 2.1). Why frosty pod does not have a wider geographic distribution is a puzzle, given the environmental resilience of its spores and ease with which they are spread; the production of billions of spores on affected pods; the cryptic, latent infection of pods which can be transported great distances before conspicuous symptoms develop; and the great susceptibility of cacao and other host species (Evans 2002b; Phillips-Mora and Wilkinson 2007).

A significant reason for the importance of frosty pod is that it is more difficult to manage than any of the other diseases. In general, biological and chemical measures that have been developed for frosty pod management have not been widely adopted by smallholder producers for much the same reasons that the same measures are not widely used for black pod and witches' broom. In addition, the chemical and biological measures that have been developed for frosty pod "invariably" depend upon concomitant pod sanitation, which automatically increases the expense of management (Krauss et al. 2010). The intensive measures that are required to adequately control this disease are simply beyond the means of most smallholder producers (Bateman et al. 2005).

In general, scant resistance exists to this disease (Phillips-Mora et al. 2005). Nonetheless, the program at CATIE has made significant progress in developing frosty pod-tolerant lines, and two of the exceptionally tolerant hybrids, CATIE-R4 and CATIE-R6, also possess excellent tolerance to black pod (Phillips-Mora et al. 2013). Considering the severe impact of the disease and the above difficulties in its management, frosty pod-tolerant lines hold the greatest promise in regions affected by this disease.

Vascular Streak Dieback

Vascular streak dieback is a destructive disease of cacao in much of South and South East Asia and Papua New Guinea (Guest and Keane 2007). The disease was first recognized in Papua New Guinea in the 1960s. Keane et al. (1972) were the first to correctly identify the causal agent (previously thought to be *Botryodiplodia theobromae*) and clearly distinguish it from other nonpathological problems on cacao.

Vascular streak dieback is caused by an unusual basidiomycete, *Oncobasidium theobromae*, which systemically infects host xylem (Guest and Keane 2007). Based on rDNA sequences, it was shown recently to reside in the anamorphic genus *Ceratobasidium* (Samuels et al. 2012). Phylogenetic analysis of ITS sequences placed *O. theobromae* sister to *Ceratobasidium* anastomosis groups AG-A, AG-Bo, and AG-K, and the anamorph was named *Ceratobasidium theobromae*. Although the fungus probably evolved on an indigenous, Far Eastern host, that host is unknown.

The pathogen produces infective basidiospores on leaf scars. Basidiospores are dispersed by wind and initiate infection during periods of high moisture. The pathogen's low rate of sporulation and sensitivity to desiccation limit its dispersion, and the disease is most common where rainfall exceeds 2.5 m year^{-1}.

As its common name suggests, the disease affects the vascular system of cacao and its only known other host, avocado (Guest and Keane 2007). Chlorosis and defoliation are typical symptoms, and discolored vascular elements are evident in affected leaf scars or as discolored sapwood when the bark is removed. Tree death can occur if the main trunk is affected, and young seedlings are most sensitive as a single infection of such plants can be lethal. Overall, the disease debilitates the host and can reduce yields significantly.

Despite its new encounter nature, there is considerable variation among different cacao backgrounds for response to this disease. For example, where diverse Upper Amazon, Trinitario, and Amelonado types were introduced in Sulawesi, the disease has not been as damaging as it was in Papua New Guinea in the 1960s (Guest and Keane 2007). In Papua New Guinea, germplasm that resists the disease has been developed and deployed (Efron et al. 2002; Epaina 2012). The segregating hybrids SG1 and SG2, which were derived from Trinitario × Upper Amazon crosses, were released in the 1980s but are susceptible to pests and diseases and suffer variable

and declining yields within 4–5 years (Epaina 2012). Although improvement of these lines is needed, it is clear that sufficient resistance exists in cacao to improve commercial lines there and elsewhere in the region.

2.4 How Would the Major Diseases (Pathogens) Impact Global Production?

Destructive pathogens of this crop would cause even greater damage if they were widely distributed. That the worst cacao pathogens have relatively narrow geographic ranges is fortunate, as production of this crop would be most difficult in the presence of a fuller complement of the damaging agents.

The following evaluations provide global perspectives on the most destructive diseases. Although this discussion is limited to the top five diseases, other diseases can be equally destructive in some situations. For example, Ceratocystis wilt has a long and worsening history in South America (Engelbrecht et al. 2007), and Rosellinia root rot, which can interact with Ceratocystis wilt, has an equally long history in Latin America and the Caribbean (ten Hoopen and Krauss 2006). Verticillium wilt is a serious problem in Brazil and Uganda (Resende et al. 1994), and the cushion gall disorder was a major problem in some American and African production areas (Ploetz 2006). Although these and other diseases that are currently considered less important are not covered below, it should be noted that diseases other than the top five constrain production of this crop.

*Black Pod (*Phytophthora megakarya*)*

Black pod causes more damage than any of the other cacao diseases, with estimated losses totaling 450,000 tons in 2001 and 700,000 tons in 2012 (Table 2.2) (Bowers et al. 2001; this chapter). The spread of the most damaging black pod agent, *P. megakarya*, to Asia or the Americas could have serious consequences. Holmes et al. (2003) summarized data from Ghana that indicated an approximate 70 % decrease in production in areas previously affected by *P. palmivora* after the invasion of *P. megakarya* (pre- and post-invasion losses were 4.9–13.5 % and 60–100 %, respectively). Although a 70 % decrease in production seems improbable in areas that are already affected by frosty pod, witches' broom, or vascular streak dieback, it is clear that this pathogen would become a serious problem, since genotypes that are produced in the unaffected areas are generally susceptible and environmental conditions in these areas are typically conducive to disease development.

Excluding *P. megakarya* is an important goal in unaffected areas. In the meantime, it is imperative that new and productive genotypes that resist this and the other black pod pathogens be developed and deployed.

*Witches' Broom (*Moniliophthora perniciosa*)*

Witches' broom caused estimated losses of 250,000 tons in 2001 and 380,000 tons in 2012 (Table 2.2) (Bowers et al. 2001; this chapter). Were it to spread, it has the potential to cause much greater losses. Shortly after it appeared in Guyana and Suriname, witches' broom caused the decline and eventual abandonment of cacao production there (Evans 2002b; Evans et al. 2013). Production in coastal areas of Ecuador was halved after the disease's arrival there, and within a decade of the Bahia outbreak, production fell from 400,000 to 150,000 tons year^{-1} (Evans 2007; Evans et al. 2013; Griffith et al. 2003). Brazil then became a net importer of beans and fell to its current sixth in importance worldwide, after it occupied the first or second position for much of the twentieth century (Table 2.1).

If the reductions in Bahia and Ecuador are reasonable benchmarks, additional losses of ca 500,000 tons might be expected if the disease spreads to Asia (where *P. palmivora*-induced black pod and vascular streak dieback exist) and 1.5 million tons if the disease spreads to Africa (where *P. megakarya*-induced black pod and swollen shoot exist).

*Cacao Swollen Shoot (*Cacao Swollen Shoot Virus*)*

Cacao swollen shoot is a serious constraint to cacao production in Ghana and Côte d'Ivoire and is the most damaging disease in Togo (Oro et al. 2012). Losses were estimated at 50,000 tons in 2001 and 76,000 tons in 2012 (Table 2.2) (Bowers et al. 2001; this chapter). Although one need only recognize the ongoing devastation that this disease causes in Ghana to understand its potential impact, predicting its influence in new areas is difficult. The disease is still spreading in West Africa, and although there should be areas in which pre- and post-invasion yield data are available, no reports are evident in the literature. Most importantly, it is not clear how rapidly and effectively the disease would spread in new areas. The roles and efficacy of transmission by endemic species of mealybug, the unclear roles that alternative hosts would play as inoculum reservoirs, and how cacao genotypes that are grown in the different areas would respond to strains of CSSV that might be disseminated are all important but imperfectly understood factors. Better information is needed for the epidemiology of this complex pathosystem.

*Frosty Pod (*Moniliophthora roreri*)*

As discussed above, frosty pod is the most serious disease on this crop in terms of potential loss and the difficulty with which it is managed (Evans 2002b; 2007). About 30,000 tons were lost in 2001 and 53,000 tons in 2012 (Table 2.2) (Bowers et al. 2001; this chapter). Wherever susceptible clones are grown, it has been responsible for abandoned production or conversion to other crops. In Ecuador, production declined from 50,000 to 30,000 tons after the appearance of frosty pod, and although declining production in Costa Rica probably has several causes, including low bean prices, the precipitous drop from 12,000 tons in 1962 to 708 tons in 2001 was certainly influenced by the disease.

Throughout frosty pod's range, it has caused losses between 20 and 90 % (Evans 2002b; Gotsch 1997; Wilberth Phillips-Mora, personal communication). If losses at least as great as those proposed above for witches' broom are reasonable, one might expect losses of at least 500,000 tons in Asia and 1.5 million tons in Africa if it reached those production areas.

*Vascular Streak Dieback (*Ceratobasidium theobromae*)*

Vascular streak dieback caused an estimated loss of 30,000 tons in 2001 and 76,000 tons in 2012 (Table 2.2) (Bowers et al. 2001; this chapter). Despite these significant losses, it would seem to be the most manageable and least threatening of the top five diseases. Even where it has had a great impact in Papua New Guinea, it can be managed culturally, with host resistance and proper nursery practices (Guest and Keane 2007).

Based on production figures in Asia in 2001, the total losses that were caused by this disease were something less than 5 % of total production in the region. Given the diversity of germplasm that is grown elsewhere and the variable impact of the disease on the same, maximum losses in the Americas and Africa might total 30,000 and 150,000 tons were the diseases moved to those areas.

2.5 Summary

In summary, several diseases have the potential to devastate global cacao production. When they are combined, the losses that they could cause total more than 100 %. Clearly, diseases cannot cause more than a total loss. However, what these evaluations do suggest is that cacao production would not be possible in the presence of all of the major pathogens.

Future production of cacao will depend on observing current recommendations and quarantine regulations regarding the safe movement of germplasm

(End et al. 2014), continued improvement of the crop for disease resistance (Gutiérrez et al. 2015), and dissemination of the improved genotypes to local producers. Failure in any of these areas could have dire consequences for the future production of this important commodity and the production of one of the world's most popular foods.

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Part II
Moniliophthora roreri and *Moniliophthora perniciosa*: History and Biology

Chapter 3
Frosty Pod Rot (*Moniliophthora roreri*)

Harry C. Evans

Abstract The history of frosty pod rot is chronologed and divided conveniently into periods or eras to better reflect the state of knowledge of the disease at the time. During the first period (1917–1975), the causal agent was ascribed to a species of *Monilia* (Ascomycota), but knowledge of the life cycle—including the mechanics of dispersal and infection—was wanting. In the second period (1975–1987), details of the life cycle emerged which cast doubts on the phylogeny of the fungus: specifically, as a sophisticated hemibiotroph with two genetically and morphologically distinct nutritional phases—parasitic, monokaryotic; saprophytic, dikaryotic. This led to a critical examination of its morphology which showed that the fungus has a unique form of sporogenesis and a hyphal structure characteristic of the Basidiomycota. The new genus *Moniliophthora* was erected to accommodate this species, considered to be the asexual morph of an unknown basidiomycete. In the present molecular period, DNA sequencing has revealed that the pathogen is a sister species of the witches' broom fungus (see Chap. 5) and both pertain to a unique clade within the Marasmiaceae. In addition, cytological evidence indicates that the purported conidia (mitospores) have a sexual function since meiotic events have been documented. The biology and evolution of the fungus is discussed, with particular reference to another variety occurring on wild *Theobroma* hosts in the forests of western Ecuador and Colombia. Finally, past and present disease management strategies are reviewed.

3.1 General Introduction: History

Thorold (1975), in his magnum opus on "*Diseases of cocoa*"—the first book devoted solely to the subject—included chapters on "Black Pod Disease" (19 pages), "Witches' Broom Disease" (15 pages), and "Monilia Disease" (5 pages). The decreasing pagination mirrors their economic importance and, therefore, the research inputs into and knowledge base of each disease. Not surprisingly, for a disease confined at that time to north-west South America, the information on

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"Monilia Disease" was sparse and, unfortunately, mostly erroneous. No blame rests with the distinguished author, who—from his time spent in West Africa and Trinidad—was well acquainted with and did much valuable research on the management of the major diseases of cacao, especially black pod and witches' broom, but was not familiar with this relatively minor and geographically isolated disease. He was merely interpreting the limited literature available, most of which was in Spanish in obscure journals or in unpublished reports. Thus, since the first scientific report of the disease—nearly a century ago—its history can conveniently be delimited into time frames, based on our increasing knowledge: starting with the initial 60 years, the "Monilia" Period, as summarized by Thorold (1975); the post-Thorold years (1975–2000) or Pre-Molecular Period; and, of course, the present or Molecular Period.

The "Monilia" Period

With the benefit of hindsight, it would be easy to criticize the science and research undertaken during this period, but it is now evident that we are dealing with a complex and highly evolved tropical forest pathogen that, coincidentally, bears more than a passing resemblance—both in morphology (spores in monilia-like chains) and symptomatology (mummified fruits with whitish spore blooms)—to a well-documented and economically troublesome but completely unrelated group of fungi attacking stone fruits in temperate climes (Agrios 2005). Thus, when specimens of a novel cacao disease, which threatened the large plantations on the coastal plain of Ecuador, were dispatched in 1917 to an international expert on fruit diseases, Professor Ralph E. Smith at the University of California (USA)—following a meeting organized by the Union of Farmers (Asociación de Agricultores del Ecuador)—the answer came back as a species of the genus *Monilia*, close to *M. fructicola* (Wint.) Honey, the causal agent of a serious disease of temperate orchard crops, notably of *Prunus* species (Agrios 1997, 2005). Several Ecuadorian academics who made presentations at the farmers' meeting had correctly identified the disease as cryptogamic in origin—rather than the popular assumption that it was due to edaphic or climatic factors—but considered the pathogen to be a species of *Phytophthora* (Martínez 1916). Although there is some confusion here, since—according to Jorgensen (1970)—the name *Monilia* had been used in an earlier report to the Asociación de Agricultures (Campos 1916), and there can be little doubt that this distinctive disease had been around for some considerable time. For example, in a book chapter reviewing cacao cultivation in Ecuador, van Hall (1914) stated that: "*A rather sudden decline of temperature brings on what is called 'helada'* [frost] *of the pods, which causes an abnormal growth of the pods and beans*." It can safely be concluded, however, that what van Hall was describing was the same disease: not, in fact, caused by plummeting temperatures but named after the frosty appearance of infected pods. Jorgensen (1970) reported an even earlier and credible record of the disease from 1895 in the diary of a plantation owner:

"*most of the pods become white while maturing on the trees and on opening the inside is watery*"; both the external and internal symptoms match those of the so-called "Monilia" disease. However, it would also seem that the same disease was rife in the Antioquia region of north-west Colombia even earlier in the nineteenth century. Research on the cultivation of cacao in Colombia (Holliday 1953) unearthed a study on the colonization of the region which revealed that cultivation of the crop had expanded rapidly in the 1830s but, in 1851—according to "*the old writings*"—much of the crop was destroyed by "*a virulent velvety fungus growth developing to an impalpable dust and attacking the fruit only*": a remarkable interpretation for the time because the germ theory of disease was still being developed (Agrios 2005).

James B. Rorer—an American mycologist based in Trinidad and contracted by the farmers' union to investigate the disease—arrived in late 1917 and, after an intensive survey involving almost 20 cacao estates or haciendas, he concurred with the earlier identification and concluded that the fungus represented a new taxon within the genus *Monilia* (Rorer 1918, 1926). In fact, although his illustration purports to show "monilioid" spore chains, there is a clear indication that development is basipetalous (youngest spores at the base; Fig. 3.1a), rather than acropetalous (spore development from the apex), as typified by *Monilia*. And, here is where the problem began for the common name of the disease—as well as for the technical name of the causal agent—which would have a direct bearing on how the disease was classified and, more practically, how the biology of the fungus was interpreted.

Since the pod symptoms are so prominent—especially the white mat or pseudostroma covering the surface, bearing a creamish-brown and powdery spore bloom—the disease had a number of descriptive local Spanish names, in addition to helada: hielo (ice), ceniza (ashes), mal paludico (malaria), pasmo (wilt due to frost), polvilho (powder), whilst other names described the internal liquefaction of the pod, such as podredumbre or pudrición acuosa (watery rot) or, as favored by Rorer (1918), enfermedad acuosa (watery disease). By far the most frequently used vernacular in the Spanish literature during this period was "la Monilia" or "la Moniliasis" (Ciferri and Parodi 1933; Naundorf 1954, 1955; Sepulveda 1955; Desrosiers and Diaz 1956; Diaz 1957; Franco 1958; Barros 1966, 1975; Meza Sanoja 1973), but pudrición acuosa has also been employed (Barros 1977), whilst the English publications overwhelmingly favored "Monilia pod rot" (Desrosiers et al. 1955; Orellana 1956; Ampuero 1967; Jorgensen 1970; Desrosiers and Suárez 1974). Both, of course, are corruptions of the Latin name, rather than descriptive or diagnostic of the actual disease, which more befits a common name since this can be understood and interchanged by farmers and scientists alike without ambiguity. In fact, Holliday (1989) has detailed four principles that should guide compilers of plant disease names: it is the disease not the pathogen that is being named; a name should be one the grower uses, a farmer is concerned with the disease not the pathogen; a name should be taken from the most conspicuous abnormality [or symptom] caused; and, finally, the introduction of any part of the pathogen's binomial is undesirable, because this may be subject to taxonomic change. Clearly,

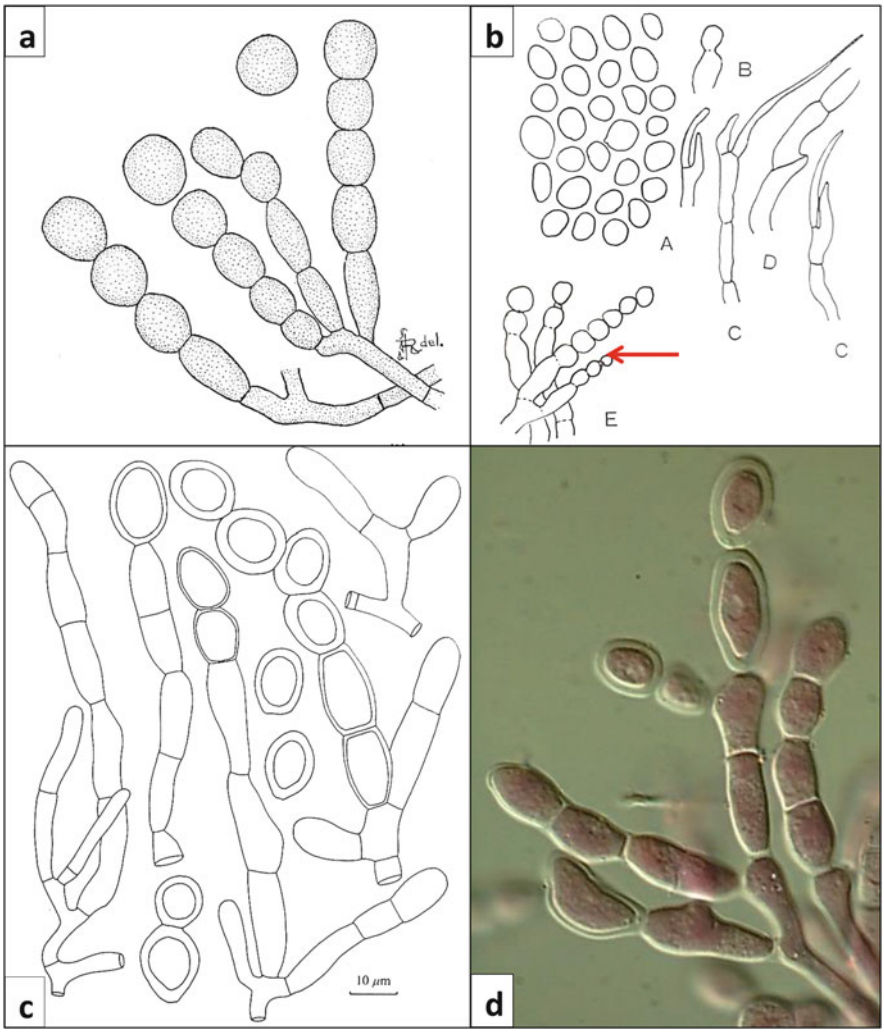

Fig. 3.1 Sporogenesis in *Moniliophthora roreri* as interpreted by light microscopy. (**a**) Illustration from Rorer (1918), showing the basipetalous chains, older spores at the apex, rather than acropetalous typical of *Monilia*. (**b**) Illustration from Ciferri and Parodi (1933), ambiguously depicting mixed basipetalous and monilioid (*arrow*) chains. (**c**) Illustration from Evans et al. (1978), showing the basipetalous formation of predominantly globose, thick-walled spores [reproduced in Evans (1981) by permission of the National Research Council of Canada]. (**d**) Phase contrast microscopy, to emphasize the branching sporophores and wall-thickening process

the use of Monilia or Moniliasis as a common name contradicts all of these principles; even more confusing, not to say embarrassing, is that moniliasis is synonymous with candidiasis in the medical literature (Kirk et al. 2008). And, of course,

the last principle is especially prescient since the ensuing taxonomic changes have been radical, to say the least.

The final report compiled by Rorer (1918) formed the knowledge bank of the disease for the next 60 years. However, it took another 15 years before the fungus was named formally and described as *Monilia roreri* Cif. (apud Ciferri and Parodi 1933; Fig. 3.1b), by Raffaele Ciferri: "*a versatile and prolific Italian mycologist and plant pathologist*" (Holliday 1989); "*a most versatile mycologist who made notable contributions to tropical mycology*" (Kirk et al. 2008). Nevertheless, both he and Rorer failed to determine if the fungus was the causal agent of the new disease: Rorer, because—as he concluded, somewhat prophetically—that at least 3–4 months were needed to complete the inoculation experiments and both his visits to Ecuador were of shorter duration; Ciferri, because he worked only with herbarium material sent to him in Italy from Ecuador. It was not until the 1970s that definitive confirmation of pathogenicity, as well as elucidation of the infection process, was forthcoming. In fact, doubts had been raised earlier about the pathogenic status of *M. roreri*, after extensive laboratory tests undertaken in Colombia—using various inoculation methods and thousands of detached pods—led to the conclusion that the fungus was a saprophyte and not the causal agent (Bastidas 1953). Of course, as with the negative results reported by Rorer (1918), pathogenicity screening with detached pods will always be inconclusive because of the inherent biotrophic and systemic nature of the pathogen. However, Naundorf (1954) working with attached pods recorded low levels of infection (8–17 %), but infection rate increased significantly (>60 %) when stink bugs (*Mecistorhinus tripterus* F., Pentatomidae) were present. The idea that these insects facilitated entry of the fungus, and even transmitted the disease, gained momentum in Colombia when Sepulveda (1955) and then Franco (1958) corroborated the results, although the latter also showed that infection was not dependent on the presence of wound entry points. Earlier, Diaz (1957) in Ecuador had linked infection to prior pod damage when he recorded increased infection rates with artificially wounded pods compared to unwounded controls. Doubtless, this line of research stemmed from the much earlier observations of Rorer (1918) who conjectured that certain insects (treehoppers, Membracidae), commonly associated with cacao pods in some of the outbreak areas of the disease, may be involved in its transmission.

Rorer (1918) also recorded the disease on native *Theobroma bicolor* Humb. & Bonpl. and *T. balaensis* Preuss (now assigned to the related genus *Herrania*; Schultes 1958), whilst another endemic species, *T. gileri* Cuatr., was considered to be a wild host of the disease in Colombia (Baker et al. 1954; Holliday 1957).

Thus, the state of knowledge of the disease and its causal agent in publications up to the mid-1970s can be summarized:

The disease is caused by the ascomycete fungus, Monilia roreri, *although it is questionable if* "*Koch's postulates have been carried out as critically as possible*" (Holliday 1971);

The fungus evolved in north-west Ecuador or Colombia, *possibly on native* Theobroma *or* Herrania *species*;

"*The life cycle is so imperfectly known that were it not for some familiarity with it in the field I might find insufficient justification for labeling it a major pathogen*" (Holliday 1971);

"*It is not known how M. roreri is spread naturally; conidia are probably airborne, and insects may carry them*" (Thorold 1975);

"*Insect punctures may facilitate entry of germ tubes*" (Thorold 1975), *with the implication that sucking insects* (Membracidae; Pentatomidae) *also act as disease vectors*, *with even a control recommendation of applying a fungicide together with an insecticide* (Avila 1966).

The Pre-molecular Period

Although the paucity of information on the fungus was highlighted in these pre-1975 publications, particularly relating to its pathogenicity, studies had been ongoing in Ecuador since the early 1960s, but, unfortunately, these were not in the mainstream literature and thus were overlooked, being in Spanish and mainly in unpublished theses. Bejarano (1961), for example, was the first to demonstrate that prior pod wounding was not necessary for infection, although the results were inconsistent and infection rates were relatively low (38–47 %). Later, Sotomayor (1965) achieved consistently high infection (91–100 %) by spraying pods with concentrated spore suspensions, whilst Suárez (1971) investigated the infection process and showed that fresh spores germinate rapidly (within 3 h) and penetrate the pod at all stages of development, either directly through the epidermis or via stomata.

Building on this body of work, research continued in Ecuador to fill in the blanks concerning the biology and life cycle of the fungus. By using a simple inoculation method—cotton-wool pads dipped in spore suspensions—large numbers of pods could be inoculated in order to determine the viability of spores and to record disease symptomatology. Spores from soil or pods on the soil had reduced viability (1–3 months), compared to those from mummified attached pods (~9 months), enabling them to survive the extended dry season of western Ecuador and suggesting a potential method of reducing disease losses through good crop sanitation, especially paying attention to removing all hanging pods during the dry season to reduce inoculum sources (Evans et al. 1977). It was also established that pods are most at risk during the first 3 months of development and that the incubation or systemic phase, from infection to necrosis, varies from 45 to 90 days and that spores of *M. roreri* are a common component of the air spora throughout the year, within and outside cacao farms, as measured using a volumetric spore trap (Evans et al. 1977; Evans 1981). Details of the life cycle based on these studies were presented in later reviews (Evans 1981, 1986).

During this period, laboratory, greenhouse, and field studies were slowly building up a profile of the fungus which did not match that of the genus *Monilia*. The earlier study of Suárez (1971) had revealed that there is a sophisticated systemic

colonization of the pods with the formation of intercellular, convoluted, parasitic hyphae. Later, it was reported that the same swollen (4–7 μm diameter) hyphae could be found in cacao seedlings inoculated with the fungus (Evans et al. 1977), growing between the cortical parenchyma, "*in the same manner and with the identical appearance of the mycelium in living tissues colonised by* C. [Crinipellis] perniciosa" (Evans 1981). In the dead or dying stem tissues, however, different intracellular hyphae were observed, similar to the saprophytic mycelium (thin, 1.5–5 μm diameter) produced in culture. From such observations, it was concluded that "*the fungus is not congeneric with* Monilia fructigena *Pers. ex Fr., the type species of the genus* Monilia" (Evans et al. 1978). Basidiomycete affinities were suspected, based on similarities in pod symptoms with the agaric fungus *Crinipellis perniciosa*—a fact noted by Rorer (1918), six decades earlier—and, more intuitively, because infected pods have "*a musty odor reminiscent of mushrooms*" (Evans et al. 1978). Because of the apparent absence of a sexual stage or morph, and the lack of clamp connections on the saprophytic mycelium—a diagnostic feature of basidiomycetes—mycelial morphology and spore ontogeny of the fungus were studied in more detail, using both transmission and scanning electron microscopy, in order to try to determine its phylogeny. Thus, it was revealed that the septum in the vegetative mycelium is a complex, barrel-shaped structure, or dolipore, found only in dikaryotic basidiomycetous hyphae (Fig. 3.2d; see Moore and McLear 1962), whilst the spores are formed in basipetalous rather than monilioid (acropetalous) chains (Figs. 3.1c, d and 3.2a–c). On this basis, the new genus *Moniliophthora* H.C. Evans, Stalpers, Samson & Benny was erected, and the new combination *M. roreri* (Cif.) H.C. Evans et al. was made, on the assumption that it represented the asexual stage or morph of an unknown basidiomycete fungus (Evans et al. 1978), belonging to the phylum Basidiomycota and not to the Ascomycota, as classified previously. However, the following rider was added:

> Any attempt to further classify *M. roreri* within the Basidiomycetes must of course be speculative. However, it may be relevant to consider the similarities that it shares with the agaric *Crinipellis perniciosa* (Stahel) Singer, the causal agent of witches' broom ('Krulloten') disease of cocoa. Both fungi are indigenous to tropical South America attacking *Theobroma* and *Herrania* species. Symptoms in cocoa pods, which in themselves are complex and diverse, are almost identical up to the appearance of the pseudostroma and conidia. The formation of swollen or deformed pods suggest a common ability to upset the host's hormonal balance. *Moniliophthora roreri* is able to invade the stems of cocoa flushes and seedlings, occasionally resulting in hypertrophy and abnormal growth responses, although it never provokes gross hyperplasia and the subsequent development of witches' brooms which is so characteristic of *C. perniciosa*. Distinct biotrophic and necrotrophic stages occur in the life cycles of *M. roreri* and *C. perniciosa* and they would both appear to qualify as hemibiotrophs. Detailed biochemical analyses may reveal an even closer relationship between these two important cocoa pathogens (Evans et al. 1978).

When the last statement was made, there was no indication that the face of taxonomy could be changed so drastically and that the science of systematics would advance so dramatically to allow unequivocal proof of this relationship: welcome to the molecular period.

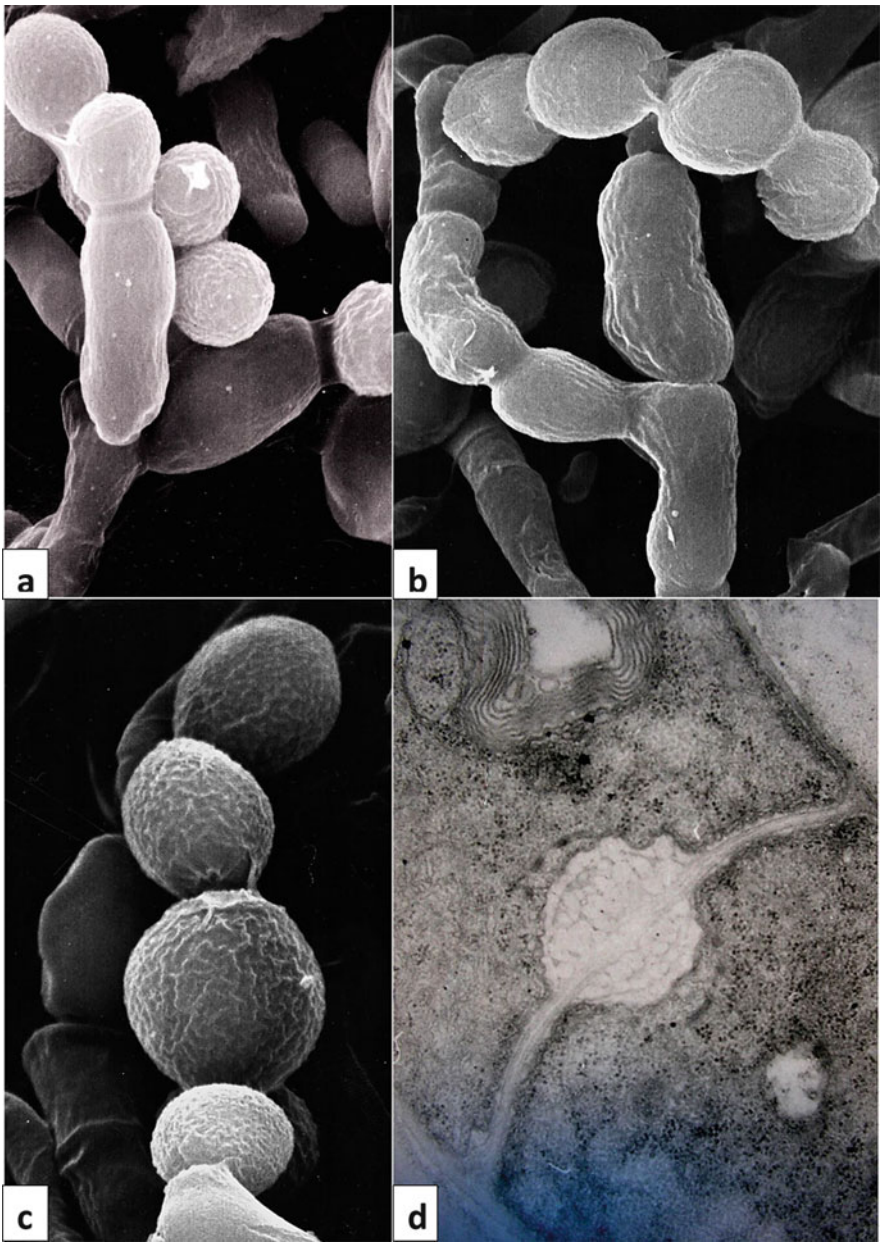

Fig. 3.2 Scanning (SEM) and transmission (TEM) electron microscopy to show critical taxonomic details of *Moniliophthora roreri* (**a**)–(**c**) SEM of sporogenesis, showing basipetal spore chains in various stages of development, from (**a**) differentiation of the initial ellipsoidal spore at the base, and (**b**) fracturing outer wall, (**c**) becoming wrinkled in the older chains consisting of spores of variable sizes. (**d**) TEM of dolipore septum in vegetative hypha, diagnostic of basidiomycete fungi

The Molecular Period

Prior to the generally accepted practice of using molecular techniques to help resolve taxonomic affinities, our knowledge on the identity of the causal agent—as well as on the mechanics of the disease—was summarized by Evans and Prior (1987), thus:

The causal agent is now considered to be the asexual state of an unknown Basidiomycete (sexual structures have never been found) and has been placed in a monotypic genus of the Fungi Imperfecti as *Moniliophthora roreri*;

In the field, *M. roreri* attacks only pods and has been recorded on a range of *Herrania* and *Theobroma* species. Like the witches' broom pathogen, it probably evolved on these trees in primary forests, possibly on the western slopes of the Andes, leading to the speculation that these two fungi are more closely related than their taxonomy suggests;

The fungus is a sophisticated pathogen with a dual life cycle: a biotrophic phase, involving non-recognition of the invading mycelium by the host, and a necrotrophic phase, with rapid tissue death and subsequent mummification as the saprophytic mycelium develops;

The dry asexual spores (conidia) are readily dislodged from the pods and carried in air currents to susceptible pods (up to 3 months of age);

Mummified pods in the canopy can retain viable conidia for prolonged periods and constitute the main interseasonal or intercrop carryover.

Note, however, that the new identity of the fungus was overlooked in general plant pathology publications, such as the standard reference work by G.N. Agrios, the fourth edition of which included tropical plant diseases for the first time and flagged Monilia pod rot as a disease likely to cause severe losses in the future, placing it firmly within the Ascomycetes related to the brown rot diseases of stone fruits (Agrios 1997).

The first molecular proof of identity and phylogenetic relationships came somewhat later: "*The theory that* M. roreri *and* C. perniciosa *are closely related*, *if not actually two evolutionary branches of the same taxon*, *is nearer to being proved*; *preliminary molecular data show them to be very similar*, *based on their rRNA*" (Evans 2002). Moreover, when ITS and small mitochondrial sequences from *M. roreri* and *C. perniciosa* were compared, there was an extremely close match (Evans et al. 2002). This was further elaborated upon by Griffith et al. (2003)—after they had included the rRNA genes of *M. roreri* in their extensive phylogenetic analyses of *C. perniciosa* in order to determine its affiliation within the Basidiomycetes—who "*were surprised that these two cocoa pathogens appear to be quite closely related*....and *that* M. roreri *may even lie within the* Crinipellis *clade*". Earlier, Evans et al. (2002) had actually opted to transfer the fungus to the genus *Crinipellis*, relegating *Moniliophthora* to synonymy, and proposing the new combination *Crinipellis roreri* (Cif.) H.C. Evans. This was based not only on molecular but also on cytological evidence. The unusually high levels of genetic variability of

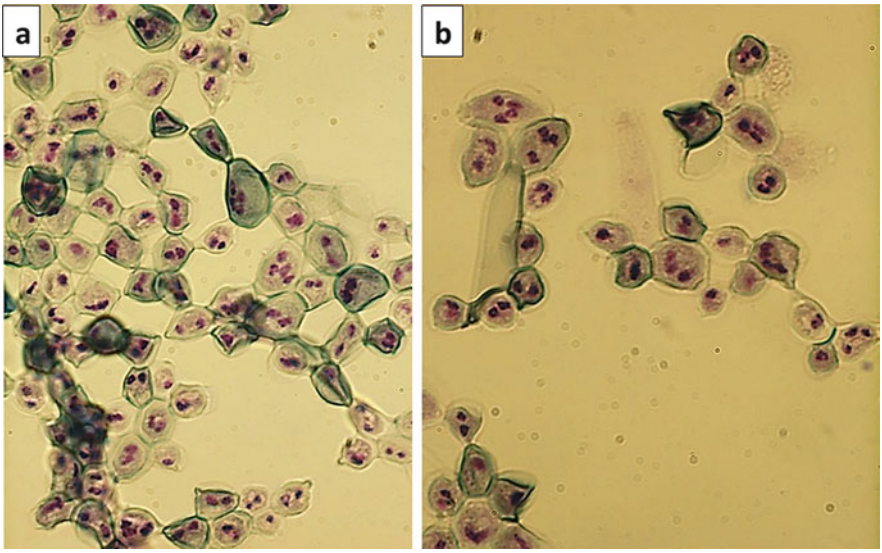

Fig. 3.3 Cytological evidence for meiosis in spore chains of *Moniliophthora roreri*. (**a**) *M. roreri* var. *roreri*, the cacao pathogen, with predominantly globose spores in different stages of development, showing 1, 2, or 4 nuclei. (**b**) *M. roreri* var. *gileri*, with larger, more ellipsoidal spores

geographic isolates—especially, the ITS sequences—suggested that genetic recombination is commonplace in the fungus and, therefore, that meiosis must occur in the fungal life cycle. Nuclear staining revealed that meiotic events do indeed take place during sporogenesis and spore germination and, therefore, that the spores are not asexual (conidia or mitospores) but sexual (meiospores) structures (Fig. 3.3a). The meiospore was interpreted to be a basidium, radically modified and multifunctional, with sexual, dispersal, and survival roles (Evans et al. 2002). Subsequently, this hypothesis was validated in a study on a "wild" isolate of the fungus from an indigenous forest tree, *Theobroma gileri*, in Ecuador (Evans et al. 2003a; Fig. 3.3b). In addition, this isolate could be separated from cacao isolates from both Ecuador and Peru—based on morphology, incompatibility, and nucleotide sequence data—and the new variety *Crinipellis roreri* var. *gileri* was proposed, whilst BLAST searches showed that the closest match for the ITS sequences from both varieties was with *C. perniciosa*. Conclusive proof came from much more extensive phylogenetic analyses involving five nuclear gene regions which confirmed that the frosty pod rot and witches' broom pathogens are sister taxa within the family Marasmiaceae (eugarics), forming part of a separate and distinct lineage (Aime and Phillips-Mora 2005). This history and the repercussions for the systematics of the fungus are summarized in the following section.

Note again, that—even after this flurry of publications on the phylogeny of the fungus—the updated edition of Agrios' classic text on plant pathology gives a confused picture of its true identity and affiliation, since it remained in the family Sclerotiniaceae (Ascomycota), being described erroneously as "*Monilia pod rot of*

cacao, caused by the fungus Moniliophthora roreri, *anamorph* Monilia roreri" (Agrios 2005). More unfortunate still are the images of the disease symptoms which purport to show an early stage of pod infection, as well as severely infected older pods: unequivocally, the latter are papaya fruits covered by a white spore bloom (Evans 2007), possibly caused by a mildew, while the former is not diagnostic of frosty pod rot. Subsequently, Rossman and Palm-Hernández (2008) compounded the confusion by stating that the genus *Moniliophthora* was established on the basis that the spores are sexual (basidiospores) rather than asexual (conidia) propagules. However, as detailed above, the genus was erected purely on the basipetalous sporogenesis and, at the time, the spores were still interpreted to be the result of a mitotic rather than a meiotic division and, therefore, that it represented the asexual morph of an unknown basidiomycete fungus (Evans et al. 1978).

3.2 Major Harry A. Dade (1895–1978), Pioneer in Applied Mycology and Diseases of Cacao

I was torn between Major Dade (Fig. 3.4)—as he was always known—and Charles A. Thorold (1906—1998) for my choice of pioneer of cacao diseases. I never knew the former personally, while, on various occasions, I enjoyed the hospitality of the latter at his rambling house on the banks of the river Thames at Twickenham, near London. Animated discussions on my latest research—which he hoped to incorporate in an update of his book (see Chap. 5 and Thorold 1975)—took place in his "garret," lined with books, documents, and files all relating to his passion: cacao and cacao diseases. However, since I can find no details of his life or obituary in scientific journals, apart from his listing in *Burke's Peerage*, as the son of Leofric de Buckenhold Thorold—presumably, because he outlived all his contemporaries (or, in this particular case, his lordly peers!)—I have chosen Major Dade.

Dade achieved the Field rank of Major during the First World War and was badly wounded on the Somme in 1916. After the war, he graduated from the Royal College of Science (London) and joined the Colonial Service in 1921 as a government mycologist in the Gold Coast (now Ghana): coincidentally, almost 50 years later, I followed an identical path, being seconded by the UK Ministry of Overseas Development as mycologist to the Cocoa Research Institute of Ghana! This is where I discovered his beautiful photographic plates and watercolor drawings of cacao diseases languishing in a storeroom. In hindsight, I should have salvaged them for some future publication, because I have never seen them in print. During his 15 years in the Gold Coast, he published widely on cacao diseases (e.g., Economic significance of cacao pod diseases and factors determining their incidence and control: *Bulletin of the Department of Agriculture* 6, 1–58, 1927), and plant diseases, in general. He was recalled to the Gold Coast in 1937 from his position at the Imperial Mycological Institute (IMI, then CMI, now part of CABI) to advise on a new disease, "Swollen Shoot and Die-back." In his meticulous report

Fig. 3.4 Major Harry A. Dade (*right*) with HRH the Duke of Edinburgh at the opening of the new Commonwealth Mycological Institute building in 1955 [Published with permission of CAB International]

(*Swollen Shoot of Cacao*, Government Printer, Accra, 1937), he concluded that "*the extensive loss of trees is mainly due to drought* die-*back, the result of environmental conditions*"—because—"*We have found no parasitic organism in association with the swellings, nor are the symptoms consistent with those of diseases caused by fungi, bacteria or nematodes*." In this interpretation he was correct, of course, but in later years he received criticism for not identifying the virus causal agent but at that time this was still a relatively new field, especially for tree crops. In mitigation, his report also recommended that "*The affection appears to be systemic. . .. The possibility of a virus disease cannot at this stage be excluded . . .. I regard the hypothesis of a virus origin as an unlikely one*, *but it should be tested*."

During his time at IMI (1935–1960)—of which he became Assistant Director—he established the world-renowned culture collection and undertook pioneering research on spoilage and industrial fungi, as well as developing an interest in the educational side of mycology, which concluded with the publication *Classwork with Fungi* (1966). If this were not enough, he was also a talented draughtsman and expert microscopist—he was President of the Queckett Microscopical Club from 1955 to 1958—and an early experimenter with epoxy resins (*New Techniques for Mounting Fungi*, 1949): I have even used some of his exquisite, epoxy-sealed slides for demonstration purposes! He was also an enthusiastic bee-keeper and, around the time of his retirement, he produced the wonderfully illustrated book, *The Anatomy and Dissection of the Honey Bee* (1960). In 1962, he followed his son to Australia and continued with his other passion, coprophilous fungi, collecting and illustrating Australian specimens which were the subject of a recent CBS monograph (A. Bell, *An Illustrated Guide to the Coprophilous Ascomycetes of Australia*, 2005), dedicated to his memory.

3.3 Taxonomy

Thus, as graphically illustrated above, the taxonomic history—as well as the nomenclatural changes, both formal and informal—has been eventful with dramatic changes since the first tentative identification almost a century ago, culminating in the quandary faced by Aime and Phillips-Mora (2005) on the correct generic placement for the fungus. Technically, the genus *Moniliophthora* was erected for anamorphic or asexually reproducing fungi, hence the proposal to move the supposed sexually reproducing frosty pod pathogen to the genus *Crinipellis* alongside its sister species *C. perniciosa* (Evans et al. 2002, 2003a). However, the work of Aime and Phillips-Mora (2005) had shown that these two species form a monophyletic clade within the Marasmiaceae, clearly separated from the genus *Crinipellis*—typified by saprophytic, litter-degrading species (Singer 1942, 1976)—and, therefore, that they should be accommodated in another genus. The decision was taken not to create a new genus but to transfer *C. perniciosa* to *Moniliophthora* and to treat the former asexual (anamorphic) genus as pleomorphic (Aime and Phillips-Mora 2005). This is in accordance with the *International Code of Nomenclature for algae, fungi, and plants* (ICN)—by keeping name changes to a minimum—and the recent, fundamental modification of Article 59 in order to regulate the separate naming of different morphs of pleomorphic fungi: the one fungus-one name concept (Hawksworth 2011). However, this has been considered to be an inadequate compromise, since the generic description was not amended from the original diagnosis (Evans et al. 1978), to include species producing basidiomata ("mushrooms") and clamp connections, such as "*C. perniciosa*." Subsequently, the generic diagnosis was amended to better accommodate both species (Evans et al. 2013), and this is summarized here:

Moniliophthora H.C. Evans, Stalpers, Samson & Benny in *Can J Bot* 56: 2530 (1978) emend. H.C. Evans, J.L. Bezerra & R.W. Barreto in *Plant Pathol* 62: 734 (2013)

Basidiomycota (Phylum), Agaricomycetes (Class), Agaricales (Order), Marasmiaceae (Family)

Mycelium of two types: intercellular, swollen, convoluted, lacking clamp connections, monokaryotic; intracellular, narrow, straight, usually with clamp connections, dikaryotic. Basidiomata either agaricoid or pseudostromatal; on living or dead host tissues. If agaricoid, pileus small, convex; pileal surface an open network of thin-walled hyphae encrusted with pigment; pileal hairs or setae short, strigose, generally crowded at centre and with membrane pigment; stipe short, cylindrical, fleshy, with bulbous base. Gills distant, thin, white, fleshy; basidia clavate, 4-spored; basidiospores ellipsoid, hyaline, thin-walled, inamyloid; spore print white; cheilocystidia clavate to fusoid, hyaline, thin-walled. In non-agaricoid forms, basidiomata reduced to a pseudostroma with modified basidia, producing spores in basipetal chains; meiospores globose to subglobose, sub-hyaline, thick-walled, powdery.

Note: Compare with the generic description in Seiffert et al. (2011), who still include *Moniliophthora* in the anamorphic or asexual fungi (Hyphomycetes), under genera with single-celled conidia (ameroconidia) produced by thallic-arthric conidiogenesis.

Type species: *Moniliophthora roreri* (Cif.) H.C. Evans, Stalpers, Samson & Benny (1978)

= *Crinipellis roreri* (Cif.) H.C. Evans in Evans, Holmes, Phillips & Wilkinson, *Mycologist* 16: 151 (2002)
= *Monilia roreri* Cif. in Ciferri & Parodi, *Phytopath Z.* 6: 542 (1933)

The following descriptions are abridged from Evans et al. (1978) and Evans (1981)

In vivo: *appearing as a snow-white, tough pseudostroma, composed of hyaline, thick-walled, non-septate, skeletoid hyphae, on necrosing pods* (Fig. 3.5a)*; rapidly turning creamish- to pinkish brown, as the dense spore masses develop* (Fig. 3.5b, c); *hyphae lacking clamp connections but with dolipore septa. Spores (meiospores) holoblastic, produced basipetally in chains, following the terminal swelling of a fertile hyphal extension and the development of the first or primary holoblastic spore and successive swellings below as further spores are differentiated, varying from 4 to 10 in a chain, enclosed or enveloped within the original cell wall; spores usually round-off with age, becoming globose to sub-globose, (6.5–) 8–15 µm, occasionally ellipsoidal, 8–20 × 5–14 µm, sub-hyaline to brown and thick-walled (up to 2 µm*; Fig. 3.5d*); liberated by fracture of the cell wall, remnants of which may adhere to the spores.*

In vivo: *Growth on malt extract agar slow, colony attaining a diameter of 8–15 mm after 2 weeks; margin appressed to slightly raised, even. Mycelial mat woolly to felty; pale ochraceous salmon to pinkish buff, at first; becoming cinnamon to apricot buff* (Fig. 3.5e). *Odor indistinct, colony reverse unchanged; tetragonal crystals produced in agar; laccase and tyrosinase confirmed; optimum growth temperature 25–26 °C, maximum 33 °C.*

Based on morphological, as well as on molecular evidence (Evans et al. 2003a; Phillips-Mora et al. 2007), isolates of frosty pod rot from the indigenous forest tree *Theobroma gileri* in north-west Ecuador can be separated readily from all cacao isolates. Thus, a new variety has been proposed:

Moniliophthora roreri var. *gileri* H.C. Evans in Evans, Bezerra & Barreto, *Plant Pathol* 62: 734 (2013)

= *Crinipellis roreri* (Cif.) H.C. Evans var. *gileri* H.C. Evans & K.A. Holmes in Evans, Holmes & Reid, *Plant Pathol* 52: 485 (2003)

Meiospores in chains, ellipsoidal to globose, 8.0 – 16.0 (–22.0) × 5.5 – 11.0 µm, often thin-walled.

Holotype: IMI 389647, from diseased pod of *Theobroma gileri* (Malvaceae) in primary sub-montane forest, collected in Guadual-Lita, Esmeraldas Province, Ecuador, 650 m a.s.l., 14 Sept. 1999, H.C. Evans.

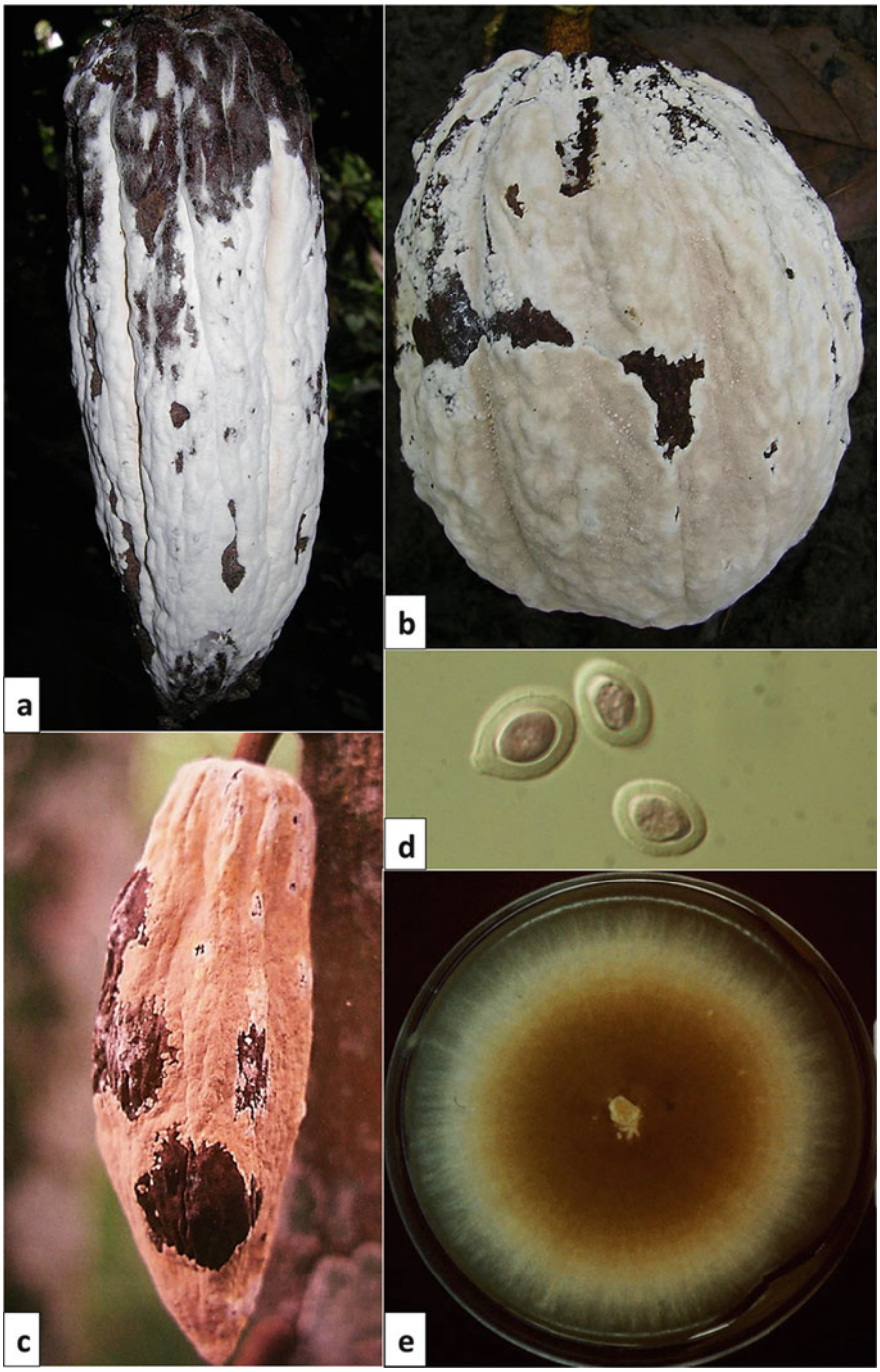

Fig. 3.5 Morphological characteristics of *Moniliophthora roreri*. (**a**) White mycelial mat (pseudostroma) developing rapidly on the necrosing pod. (**b**) Pseudostroma coalescing to form a thick skin over the pod surface and developing brown hues as sporulation is initiated. (**c**) Dense,

This variety is distinguished morphologically from the cacao isolates, *Moniliophthora roreri* var. *roreri*, by the significantly greater proportion of thin-walled, ellipsoidal spores, which raises the mean length from ~8.0 μm in var. *roreri* to ~11–12 μm in var. *gileri*, as well as DNA sequences and non-pathogenicity to cacao (Evans et al. 2003a).

3.4 Disease Cycle

Suárez (1971, 1972) was the first to investigate the mechanism of infection and it was shown that the spores germinate on and penetrate directly through the pod epidermis, or via stomata, colonizing the tissues intercellularly without inducing an antagonistic reaction, such as cell necrosis. In the field, only young or meristematically active (expanding) pod tissues are susceptible, but infection of cacao seedlings and flushes has also been demonstrated experimentally in the greenhouse (Evans 1981). However, the fungus fails to sporulate on these tissues and this infection process is considered to be artificial and not relevant to the disease cycle in the field. Nevertheless, it has been suggested—and circumstantial evidence has been presented—that the fungus can invade the cacao flower cushion via the infected pod and remain systemically within the tissues, potentially infecting pods borne subsequently on that cushion (Naundorf 1954; Evans 1981). More studies are needed to support this hypothesis.

Pods are most susceptible to infection during the initial 3 months of development. One of the characteristics of frosty pod rot, which it shares with the witches' broom pathogen (see Chap. 5), is the long incubation period from penetration to the appearance of external symptoms, although this is modified to some extent by pod age and provenance. The results of pod inoculation experiments can be summarized, thus [modified from Desrosiers and Suárez (1974) and Evans (1981)]:

Pods inoculated at 20–60 days of age; the entire process from penetration to intercellular colonization and intracellular invasion (necrosis) occurs within 40 days; young pods (cherelles) may be grossly swollen (Fig. 3.6a).

Pods inoculated at 60–80 days of age; the process is usually completed within 2 months—with the range from 55 to 75 days—and may be accompanied by premature ripening or pod deformation (Fig. 3.6b), prior to visible necrosis.

Pods inoculated at 120–160 days of age; the infection process is slower and may be restricted to small localized lesions often without endocarp penetration, resulting in healthy beans within the pod chamber.

Fig. 3.5 (continued) pinkish-brown, powdery spore bloom covering the pseudostroma; note the absence of sporulation on the conspicuous pod swelling [from Evans (1981), published by permission of CAB International]. (**d**) Phase contrast microscopy and acetocarmine staining to show the thick, sub-hyaline spore wall. (**e**) Culture of *M. roreri* after 1 month on malt extract agar

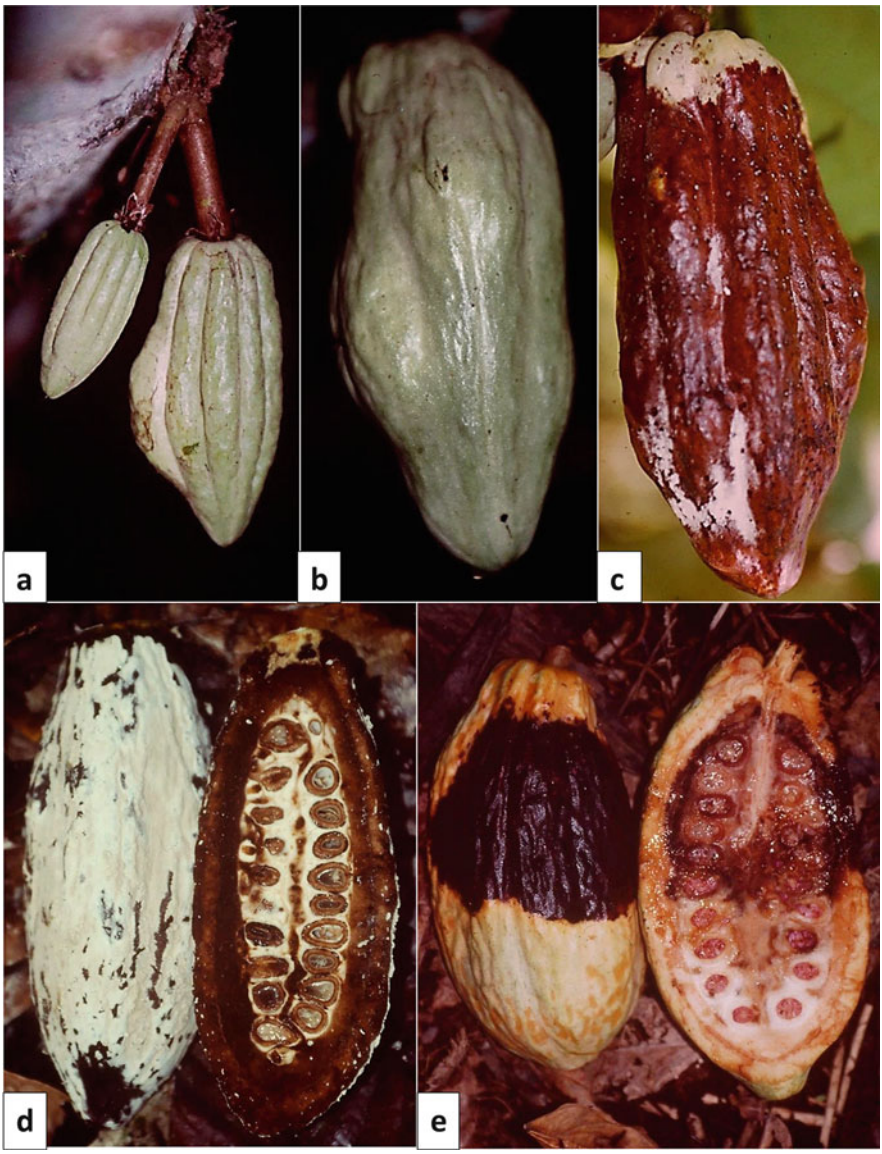

Fig. 3.6 Symptomatology of *Moniliophthora roreri* in biotrophic and early necrotrophic phases. (**a**) Young pod or cherelle (~1-month-old) with gross swelling; note smaller healthy cherelle on same cushion. (**b**) Swollen and deformed green pod, 2–3 months old. (**c**) Pod, 3–4 months old, showing swellings and initial phase of necrosis. (**d**) Pod with developing pseudostroma, harvested to show internal necrosis with compaction and complete destruction of the bean mass. (**e**) Atypical black external lesion—more typical of infection by *M. perniciosa*—lacking the diagnostic external pseudostroma, but with internal moist or watery rot; specimen from Costa Rica, where witches' broom disease is absent

Typically, the first external symptom is the sudden appearance of chocolate to dark brown, irregular lesions which rapidly coalesce to cover the whole pod surface (Fig. 3.6c). Some 3–8 days later, a white mycelial mat or pseudostroma envelops the pod (Fig. 3.6d), gradually turning cream, to pinkish, or tan brown as the spores develop and mature. It has been estimated that sporulation density can reach 44 million spores per cm^2 with a mature pod producing over 7 billion spores (Campuzano 1976), and it is difficult to mistake this stage of infection with that of any other pod pathogen: the "frosted" pod covered by a dense layer of powdery spores (see Fig. 3.5c). Internal symptoms also vary with pod age and variety. Pods infected at the cherelle stage show failure of bean development, the tissues being disorganized and gelatinous, hence the name watery pod rot. In later infected pods, there is gross hyperplasia of cells resulting in tissue compaction in the pod chamber and malformed beans stuck together and to the pod wall, and only a moist or even a dry rot (Fig. 3.6d). Such pods are significantly heavier than healthy ones of a similar age. The cut surfaces of infected pods quickly become covered by the tough, white fungal pseudostroma—frequently imparting a mushroom-like odor—followed by a dense brownish spore bloom, making it easily distinguishable from other pod diseases, notably witches' broom, in the occasional absence of external sporulation, which may be related to ambient conditions or pod resistance (Fig. 3.6e). The talcum-powder-like spores are dislodged by any air or tree movement—water, as raindrops or canopy run-off, is also effective in releasing small clouds of spores—and subsequently dispersed by the convection currents within cacao farms: readily verifiable by blowing on or touching a sporulating pod and observing the spore cloud swirl upwards into the tree canopy (Evans 1981).

The fungus appears to be a common component of the air spora wherever frosty pod rot occurs, as evidenced by the regular collection of spores in a volumetric spore trap, situated ~1 km from the nearest cacao plantation, and that they are present throughout the year. These findings from western Ecuador indicate that the highest frequency is in the wet season between 11.00 and 18.00 h, and it has been posited that this is due to increased wind velocity prior to the frequent afternoon rains (Evans 1981). There was also a marked peak at the start of the first heavy rains, following the prolonged dry season, with the suggestion that these were old spores released from mummified pods by the physical action of rain (Evans 1986).

Spores which are released during the first few days of sporulation may be less resistant to abiotic factors (irradiation, desiccation), since these tend to be pale-colored and thin-walled. However, with time the wall thickens and the spores develop a darker coloration and can survive for up to 9 months on hanging pods (Evans 1981). Those spores deposited onto the soil, or on discarded pods on the soil or leaf litter, survive for much shorter periods and infectivity tails off after 1 month. Infected pods rapidly lose moisture following fungal colonization, becoming mummified and woody and, if not harvested, remaining on the tree for several years. Gradually, the spore bloom is eroded by weathering and ultimately only the flaking pseudostroma persists. The fungus, therefore, has a mechanism for long-term survival

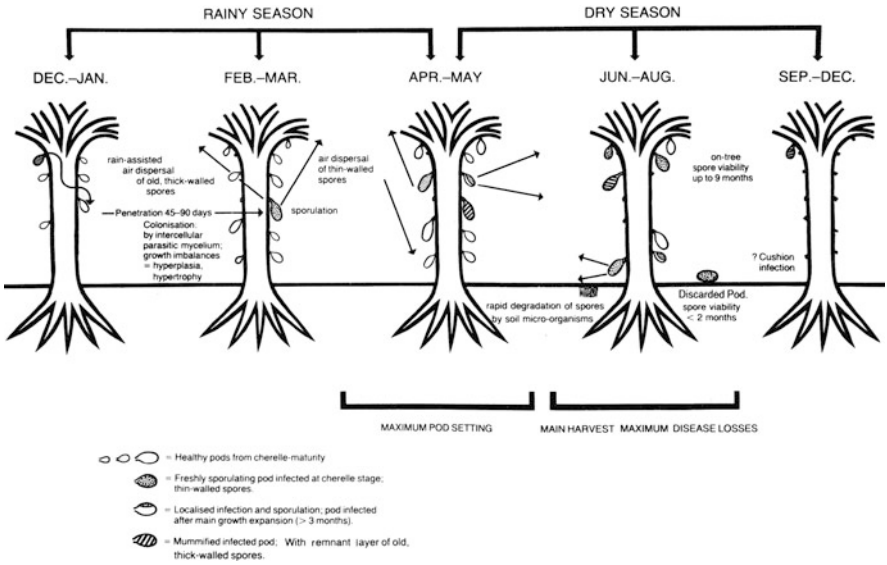

Fig. 3.7 Schematic disease cycle of *Moniliophthora roreri* in a cacao farm in western Ecuador, where there is an extended dry season from May to December

between crops or to "over-summer" during extended dry periods, such as the 5- to 6-month dry season experienced in western Ecuador. A schematic representation of the disease cycle in this region is presented in Fig. 3.7.

3.5 Host Range

As mentioned previously, Rorer (1918) reported frosty pod rot on species of both *Theobroma* and *Herrania* in western Ecuador, and Baker et al. (1954) found it on wild *T. gileri* in north-west Colombia (Fig. 3.8c), despite later reviews stating that only cacao is attacked (Ampuero 1967). Subsequently, Evans (1981) recorded it on all species of these two genera held in an extensive germplasm collection in an experimental station in western Ecuador, following natural or artificial infection, and it was concluded that the potential host range extends to all species of *Herrania* and *Theobroma*. The deeply fluted pods of *Herrania* have a less compact bean chamber compared to cacao, and it is not unusual to find necrotic *Herrania* pods bearing little or no evidence of external sporulation and a densely sporulating pseudostroma on the inner wall of the bean chamber (Fig. 3.8b).

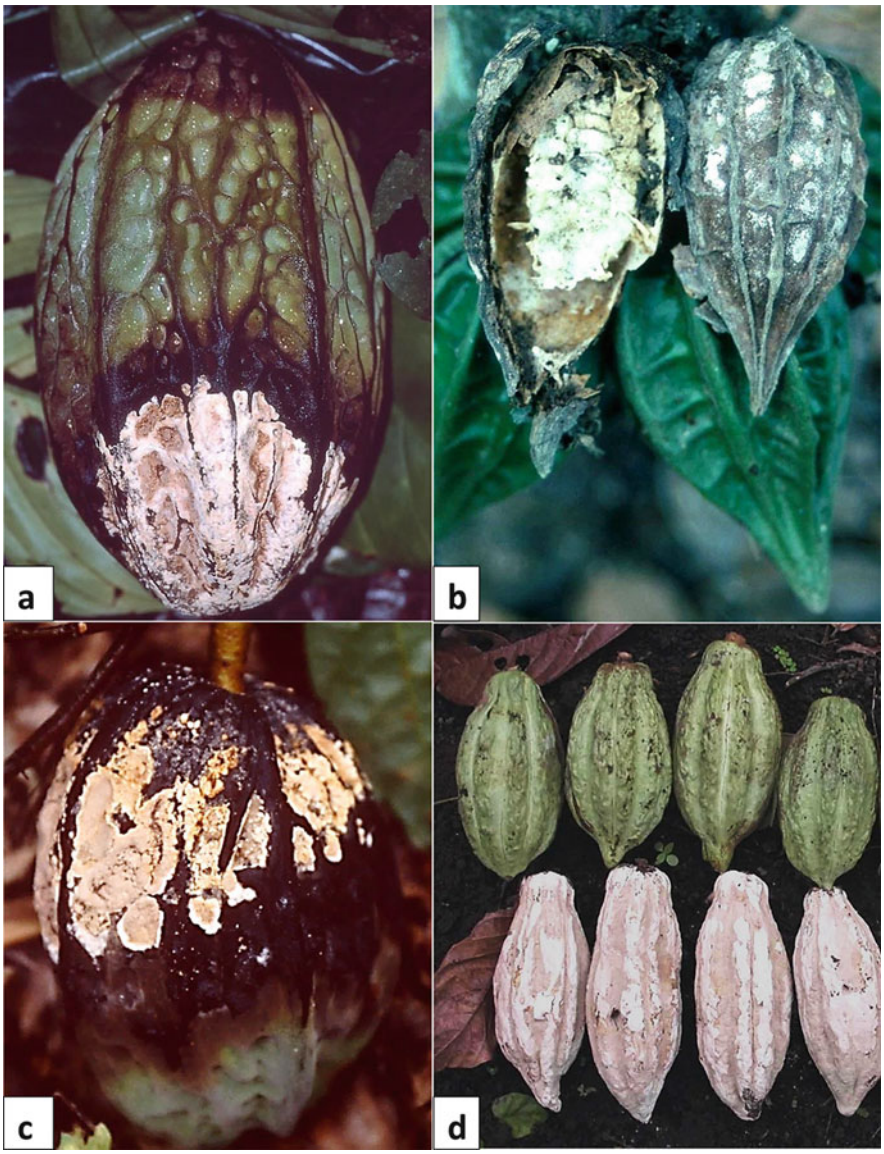

Fig. 3.8 Host range of *Moniliophthora roreri*. (**a**) The cacao pathogen, var. *roreri*, on pod of *Theobroma bicolor* in eastern Ecuador. (**b**) The cacao pathogen, var. *roreri*, on old mummified pod of *Herrania balaensis* in western Ecuador; note the restricted external sporulation contrasting with the internal bean chamber, completely enveloped by sporulating pseudostroma. (**c**) Pod of *Theobroma gileri* colonized by var. *gileri* in sub-montane forest of north-west Ecuador; note the pseudostroma being eroded by natural enemies. (**d**) Field experiment showing diseased cacao pods inoculated with var. *roreri* (*lower row*), and healthy pods inoculated with var. *gileri* (*upper row*)

3.6 Origin, Present Distribution, and Economic Impact

Based on his findings, and the apparent absence of the disease from other cacao-growing countries, Rorer (1918) considered the fungus to be endemic to Ecuador, notably on *Theobroma bicolor* (Fig. 3.8a), specifically, to the coastal or western region. The report of the disease attacking an indigenous *Theobroma*, *T. gileri*, in central Colombia (Baker et al. 1954) led Holliday (1970, 1971) to propose that this is the original wild host and, therefore, that this region is the center of origin of *M. roreri* from where it spread to attack cultivated cacao. However, the type locality of this *Theobroma* species is north-west Ecuador (Cuatrecasas 1953)—in the sub-montane forests of the highly diverse and unique Chocó Refugium (Gentry 1982), which extends along the western seaboard from northern Colombia to northern Ecuador—and the original botanical reports by the collectors indicated that the pods were destroyed by a disease matching the description of frosty pod rot: "*Almost all fruits we saw suffer a disease which hardens the mucilaginous tissue making it compact and hardening the seeds*" [in Cuatrecasas (1964)]. Surveys revealed that the disease is, indeed, caused by *M. roreri*, but that the fungus differs morphologically—as well as genetically, based on DNA sequences—from the pathogen of cacao, and, even more surprisingly, it proved to be nonpathogenic to cacao (Evans et al. 2003a; Fig. 3.8d). The hypothesis that the fungus "*invaded the burgeoning cacao plantations of coastal Ecuador from these forest loci, on the lower, western slopes of the Andean cordillera, sometime during the nineteenth century*" (Evans 2002), was no longer tenable.

So, if not in Ecuador on *T. gileri*, where did the cacao pathogen originate and on what host did it evolve? Phillips-Mora et al. (2007) employed AFLP and ISSR profiling of DNA samples from a wide geographic range, and they concluded that whilst Ecuadorian isolates exhibited relatively low genetic diversity, the Colombian isolates had the highest diversity levels and, therefore, that the center of origin or diversity lies in Colombia, specifically in what they designated as the central-eastern region. This embraces the locality, Villa Arteaga, in north-west Antioquia Department, where frosty pod rot has been recorded on wild *Theobroma*: "*The fungus* Monilia roreri *Ciferri was found infecting fruit of this species*" (Baker et al. 1954). Subsequently, this was identified as *T. gileri* by Cuatrecasas (1964), who classified the region as western rather than central Colombia and where, purportedly, the first records of frosty pod rot disease emanated from in the 1850s (Holliday 1953, 1971; Thorold 1975; Phillips-Mora et al. 2007), following expansion of cacao cultivation in the 1830s. It has been conjectured that this may, in fact, represent an undescribed species of *Theobroma*—rather than *T. gileri* from Ecuador—since an examination of specimens held in the Kew Herbarium (BGD Bartley, personal communication 2006, in Evans 2007) showed significant differences in leaf size and fruiting habit (Evans et al. 2013). Moreover, the Colombian records are from much lower altitudes (~200 m a.s.l.; Baker et al. 1954) than the Ecuadorian records which are confined to a narrow altitudinal range of 500–700 m (Evans 2002; Evans et al. 2003a, b). Whatever the situation, it appears certain that *M. roreri* is

endemic on wild species of *Theobroma* in the forests of north-west Ecuador and western Colombia and that the cacao strain or variety evolved in the latter region, whilst the Ecuadorian strain (var. *gileri*) never escaped from the sub-montane forests, being nonpathogenic to cacao. Interestingly, an abandoned experimental plot of cacao less than 20 km from the type locality of *T. gileri* was found to be heavily attacked by witches' broom disease—although, notably, this disease was absent from the wild species—but with no evidence of frosty pod rot (HC Evans, personal observation 1999). The nearest commercial, frosty pod-infected cacao plantations were some 200 km to the south-west, the forests on the lower slopes of the Andes seemingly acting as an effective barrier or buffer.

It is probable that cacao—originally brought from its center of origin in the Upper Amazon—has been cultivated by the indigenous peoples of Antioquia, Colombia, for several thousand years (Bartley 2005), and thus the crop has intermingled with endemic *Theobroma*, as well as with *Herrania* species, and it is this region where the frosty pod pathogen seems to have adapted to and evolved on cacao hosts. Commercial cacao plantings in the nineteenth century would have been at risk from the pathogen, hence the historical and catastrophic disease outbreaks reported in the 1850s (Holliday 1953, 1957; Baker et al. 1954; Thorold 1975; Phillips-Mora et al. 2007). It also seems highly likely that the switch from the indigenous Nacional variety of western Ecuador to the more productive Forastero during the latter part of the nineteenth century—as reported by Rorer (1918)—led to the accidental importation of the exotic variety of frosty pod rot from Colombia with disastrous consequences for the plantation industry and to the gradual abandonment of large-scale production of the crop and its replacement by bananas (Evans 1986). Rorer (1926) presents compelling data of the demise of a small Forastero plantation where the annual yield fell from just under 30 metric tons in 1916 to a little over 10 metric tons in 1918, plummeting to around 1.6 metric tons the following year and to its subsequent abandonment. Typically, cacao cultivation became a smallholder activity and a socioeconomic survey has revealed that pod losses due to *M. roreri* can range from 20 to 43 %, with annual variations directly affecting the living standards of the peasant farmers (Aragundi 1974).

The fungus was confined to the western regions of Colombia—with the disease accounting for an estimated annual revenue loss of over US$ 20 million in the 1970s (Evans 2002)—and Ecuador for many decades after its original discovery, and thus was regarded as a geographically isolated pathogen of minor economic importance globally (Holliday 1971), although posing a very real threat to other cacao-growing countries in Latin America, as well as a potential threat to the Old World (Thurston 1973; Evans 1981). The disease spread eastwards into Zulia State of western Venezuela, probably in the 1940s (Malaguti and Diaz 1958; Thorold 1975; Evans 1981), but it has yet to reach the more important cacao-growing regions of eastern Venezuela (Capriles de Reyes 1979; Phillips-Mora and Wilkinson 2007), or the nearby cacao-rich island of Trinidad, although, recently, its presence on the island was reported erroneously (CABI 2011a), rapidly followed by a redaction (CABI 2011b). The latter also lists an unconfirmed report from Cuba, but this must be considered as a highly dubious record. It then moved northwards

into Central America, firstly into eastern Panama in the 1950s (Orellana 1956) and thence to Costa Rica in the late 1970s (Enríquez and Suárez 1978), where over a 5-year period production fell from just over 10,000 metric tons to below 2,000 (Ram et al. 2004). Dissemination into Central America may have been by airborne spores, although accidental introduction by man is considered to have been more likely (Orellana 1956). Further spread northwards to the cacao-growing countries of Mesoamerica has been relatively slow and there were unconfirmed reports of its arrival in Guatemala and Honduras in the late 1990s (Evans 2002), and these have since been confirmed (Phillips-Mora and Wilkinson 2007), as have reports of its presence in Belize and Mexico in 2005 (Phillips-Mora et al. 2006a, b).

It was concluded that natural dispersal of the fungus had reached its limit in western Ecuador and that the Andean cordillera was an impassable barrier to further spread south and eastwards but that "*with the increasing agricultural development in this area* [Amazonian Ecuador, where colonists were following the new oil pipeline roads] *chances of introduction of the fungus by man are high*" (Evans 1981). This proved to be the case and by the mid-1980s, *M. roreri* was well established in the Napo basin of the Oriente or eastern region of Ecuador (Evans 1986). From there, the pathogen dispersed southwards along the eastern slopes of the Andes to reach northern Peru in 1988 (Hernández et al. 1989) and by stepwise movements has invaded all the cacao-growing areas of the country, arriving in the southern valleys of the Apurimac and Ene rivers by 1995 (Evans et al. 1998), and eventually reaching the most southerly region of Cuzco (Evans 2002; Phillips-Mora and Wilkinson 2007). Recently, the fungus has been reported in Bolivia (Phillips-Mora et al. 2013, 2015). Although Evans (1981) listed much earlier reports of the occurrence of *M. roreri* in both Peru (McLaughlin 1950)—later promulgated by Reynoso (1965), Ampuero (1967), and Thurston (1973)—and Bolivia, these were treated with caution and, based on observations in the Bolivian Alto Beni region, it was concluded that the disease was due to witches' broom and not to frosty pod (Evans 1981). Disease impact in Peru has been severe and, reportedly, this has resulted in the abandonment of some plantations—with overall pod losses of 40–50 % (Evans et al. 1998)—constituting over 50 % of the cultivated area (Phillips-Mora and Wilkinson 2007).

Once *M. roreri* had crossed the Andes into the Ecuadorian Oriente region in the early 1980s, it was adjudged to be "*only a matter of time before the pathogen reaches the more extensive plantings in the Brazilian Amazon*" (Evans 1986)—notably, in Amazonas and Pará States—although the rider was added that dispersal could be slow because of forest barriers and low host density in the headwaters of the Amazon basin, along the Napo and Solimões river systems. Nevertheless, after more than 30 years, there have been no confirmed records of its occurrence in Brazil (Ram et al. 2004; Phillips-Mora and Wilkinson 2007). Brazil is ringed by countries (Colombia, Ecuador, Peru, and now Bolivia) where *M. roreri* is established along the eastern borders and it is puzzling, therefore, why this efficiently dispersed airborne fungus has not arrived in the lower Amazon region where both cacao and a related species *Theobroma grandiflorum* (cupuaçu) are grown on a commercial scale.

A distribution map of *M. roreri* has been published recently (CAB International 2011b). However, there is an ambiguous footnote to the effect that, although the

presence of the pathogen has not been confirmed in Brazil, it has been detected close (<50 km) to the cacao-growing region of Pará State: geographically, this makes absolutely no sense, since Pará at the mouth of the Amazon abuts the Brazilian States of Amazonas to the west and Amapá and the Guianas to the north.

3.7 Biology

Like its sister species *M. perniciosa* (see Chap. 5), *M. roreri* is a sophisticated pathogen with two morphologically, physiologically, and genetically distinct phases in the life cycle. This pleomorphism is manifested in the parasitic phase within the actively growing host tissues followed by the necrotrophic phase in the dying and dead host tissues and, thus, both species can be classified as hemibiotrophs: organisms which start their association with the host as a biotroph but later revert to a well-defined saprophytic phase (Cooke 1977; Evans 1980, 1981). The parasitic mycelium grows between the host cells without eliciting a response and in this intercellular, systemic phase the hyphae are swollen (4–7 μm in diameter) and convoluted and there is no evidence of feeding structures—such as the haustoria in rust fungi—within the plant cells, and it is considered that nutrients are obtained by a process that changes the permeability of the host cell wall, possibly involving the release of specific enzymes, allowing cell contents to pass into the intercellular spaces. Among the contents of the invaded meristematic tissues would be auxin precursors and growth hormones which could disrupt normal growth resulting in hypertrophy and hyperplasia and the gross abnormalities associated with pod infection by *M. roreri* and, of course, with the formation of witches' brooms in *M. perniciosa*. Remarkably, this vitally important aspect of the parasitism of both pathogens is still poorly understood and has yet to be addressed in any depth. Based on circumstantial evidence, it has been postulated that the symbiosis may be tritrophic involving a third organism, possibly a virus-like particle, vectored into the host by the fungus and which is responsible for the growth abnormalities (Evans et al. 2013). This hypothesis has yet to be tested rigorously, but it offers some explanation as to how and why these fungi, in sharp contrast to other members of the typically saprophytic order Agaricales (Money 2007), have become such sophisticated plant pathogens.

It has also been confirmed that the parasitic mycelium is monokaryotic (Griffith et al. 2003)—as reported for *M. perniciosa* (Evans 1980)—and that this genetic state is maintained as long as host nutrients are freely available. Once these become limiting, as the pod matures and the physiology changes, dikaryotization occurs along with the formation of the thin-walled, narrow, intracellular saprophytic mycelium that results in tissue degradation and pod death. This necrotic phase is extremely rapid, occurring within days after the extended asymptomatic biotrophic phase, manifested by irregular and coalescing chocolate brown lesions on the pod surface and the formation of the white pseudostroma, sporophores, and spores. The systemically colonized pod has been interpreted as a giant fruiting body, producing

billions of thick-walled and long-lived meiospores, in which the thick, fleshy pseudostroma represents the vestiges of the pileus or cap of the mushroom, and the sporophores are modified basidia (Evans et al. 2013). From light microscopy and SEM examination of sporogenesis, it is possible to follow the delimitation of basidium-like structures—with cytological studies showing meiotic events during this process—and the rounding off of these "basidial units" into spores: globose, thick-walled types being dominant in the cacao variety, whilst there is a preponderance of ellipsoidal, thin-walled spores in var. *gileri* (Evans et al. 2003a).

How did this bizarre sequence of events occur, with the loss of the agaricaceous basidioma and the replacement of the hyaline, thin-walled, ephemeral basidiospores by sub-hyaline to pigmented, thick-walled, long-lived, multifunctional meiospores? It has been hypothesized (Evans 2007; Evans et al. 2013) that this is linked with the uplift of the Andean cordillera which created the Chocó refuge (Gentry 1982) and separated it from Amazonia with its qualitative and quantitative richness of *Herrania* and *Theobroma* species (Schultes 1958; Cuatrecasas 1964; Evans 1998; Bartley 2005). Thus, in this now isolated forest ecosystem, straddling the western slopes of the northern Andean chain, with its paucity of suitable hosts, there would have been evolutionary pressures to increase spore production. Pods, rather than stem tissues, would have provided the means of achieving this goal and perhaps, therefore, *M. perniciosa* put "all its eggs into one basket" with the gradual erosion of ability to invade and penetrate shoot meristems and eventually morphed into the obligate pod pathogen *M. roreri*. Conversely, these two cacao pathogens may have evolved separately from a common and benign endophytic, *Moniliophthora* ancestor: *M. roreri* to the west of the Andes as a pod pathogen on endemic *Herrania* and *Theobroma* species; *M. perniciosa* to the east in Amazonia not only on Malvaceae hosts, but also on hosts in the Bignoniaceae, Malpighiaceae, and Solanaceae, on which its forms the diagnostic brooms and the typical agaric mushrooms (Evans 2007; Evans et al. 2013; see Chap. 5). It has been proposed that all the species assigned to the section *Insignes* of the genus *Crinipellis* by Singer (1976) belong to *Moniliophthora* and live as cryptic endophytes of forest trees in the Neotropics (Evans et al. 2013). Evidence of the inherent ability of *M roreri* to colonize woody tissues endophytically and asymptomatically comes, indirectly, from inoculation experiments with cacao seedlings and flower cushions (Evans 1981, 1986), as well as directly, when it was isolated from healthy stems of *Theobroma gileri* trees in primary forest (Evans et al. 2003b). It is further presupposed that this forest isolate (var. *gileri*) is the progenitor of the cacao pathogen (var. *roreri*), which moved from an endemic *Theobroma* species and adapted to cultivated cacao in western Colombia. In the process, the spores became predominantly globose and thick-walled—rather than thin-walled and ellipsoidal in var. *gileri* (Evans et al. 2003a)—probably to ensure survival in the drier ecosystems of cacao farms compared to the canopied primary forest habitats of var. *gileri*. Indeed, germination of spores of the cacao pathogen under laboratory conditions is often erratic and problematic (López 1954; Evans 1981) and suggests that a dormancy mechanism is in operation—certainly in the older, thick-walled spores—and that germination may be triggered by host elicitors.

3.8 Disease Management

Cultural Control

Fulton (1989) suggested that control of frosty pod "*would appear to be a piece of cake*" since the infective stage is limited to spores produced on the pod surface and, therefore, good crop sanitation would be the best option for disease management. As discussed previously (Evans 1981, 1986), cultural control through regular harvesting and the removal of infected pods before sporulation should effectively reduce inoculum sources and thus decrease disease incidence. This has been demonstrated to be effective in large-scale field trials in both Ecuador (Desrosiers and Suárez 1974), as well as in Colombia (Barros 1966; Cubillos and Aranzazu 1979), and more recently in Costa Rica (Porras et al. 1990) and Peru (Soberanis et al. 1999). In particular, emphasis should be placed on judicious sanitation during the interseasonal or intercropping period (Leach et al. 2002), with particular emphasis on removal of hanging mummified pods in order to disrupt the disease cycle at the beginning of the rainy season (see Fig. 3.7). However, as pointed out by Evans (2002), "*erratic cropping cycles, poor follow-up sanitation and contamination from badly managed neighboring farms can all prejudice cultural control.*"

Chemical Control

Rorer (1926) concluded that many applications of fungicides at frequent intervals are necessary in order to maintain protection during the highly susceptible period when the pods are actively growing and the surfaces are expanding rapidly, and that "*the cost of the work was absolutely prohibitive*." Nevertheless, in more recent times with more heavily cropping varieties, copper-based protectants have proven to be cost-effective in Ecuador, particularly when combined with cultural practices (Cronshaw 1979), although not in Costa Rica (Gonzales et al. 1983). It would be fair to say that little progress has been made on the chemical control front since Rorer's damning assessment.

Biological Control

Focus has turned to biological control only recently, using mycoparasites singly or in "cocktails," with positive results if linked with cultural control and cropping patterns in Peru (Krauss and Soberanis 2001a, b, 2002). Similar potential has been demonstrated in Costa Rica with mixtures of *Clonostachys* and *Fusarium* mycoparasites (ten Hoopen et al. 2003), although application frequency and formulation issues still need to be addressed before commercialization can be considered.

In these studies, much was made of the use of local or native strains of mycoparasites from Peru and Costa Rica, but, since *M. roreri* is an invasive alien organism in these countries, this is inundative biological control using opportunistic rather than specialist antagonistic fungi. A much more structured and, potentially, more lucrative approach is the classical biological control strategy or the exploitation of coevolved natural enemies from the center of origin or diversity of the target pest, which has been employed with considerable success against invasive alien insects and weeds (Mack et al. 2000; Evans 2013). As detailed previously, for *M. roreri* this lies in the sub-montane mesic forests of the Chocó phytogeographic region of western Ecuador and Colombia, where it evolved on endemic species of *Theobroma*. Surveys were conducted in the north-west region of Ecuador, specifically in the type locality of *T. gileri* ("chocolatillo"), not only looking for natural enemies feeding directly on or parasitizing the pathogen on diseased pods, but also investigating the hidden biocontrol potential of benign fungal colonizers (endophytes) within healthy pods and the host tree itself. Populations of the understory tree *T. gileri* were located in and restricted to a narrow belt of forest, between 550 and 700 m a.s.l. An eclectic range of mycoparasites was found colonizing the pseudostroma on *M. roreri*-infected pods—accounting for approximately 20–30 % of pod production—dominated by the sexual fruit bodies of *Nectria* and their *Clonostachys* asexual states (Evans 2002; Evans et al. 2003b), which appeared to impact on and reduce sporulation. In addition, not only is there a guild of novel mycoparasites colonizing the pathogen, but also a suite of unusual invertebrate natural enemies. One of these, a bizarre dipteran larva, or fungal gnat (Mycetophilidae), equipped with a hooked abdomen to cling onto the substrate as it feeds on spores of *M. roreri* on pods high in the canopy, proved to be difficult to identify. Attempts to follow the life cycle, in order to obtain adult flies for further taxonomic studies, were thwarted since all the pupae were hyperparasitized by a hymenopteran parasitoid belonging to a genus, *Acidopsilus* (Diapriidae), described over a century ago but not collected since (A. Polaszek, Natural History Museum, London, personal communication 2003). Other diseased pods were colonized by fungal-feeding nematodes belonging to the genera *Bursaphelenchus* and *Neodiplogaster*, both thought to be undescribed species (D. Hunt, CABI-UK, personal communication 2003). No equivalent colonization of *M. roreri*-infected pods by mycoparasites and invertebrates has been observed in the cacao plantations lying some 200 km to the south-west on the coastal plain, or, indeed, in any other cacao-growing country where frosty pod disease occurs. Thus, there is a unique mycobiota and fauna associated with and feeding on spores of *M. roreri*—together with their own natural enemies, forming a complex trophic mosaic—in its center of origin on the lower slopes of the western Andean cordillera. These natural enemies seemingly appear to keep the host–pathogen system in balance, so that fungal inoculum is at a level which does not act as a constraint to reproduction, since 70–80 % of the pods seemed to escape infection. By comparison, commercial cacao plantations in

western Ecuador with little or no disease management could be expected to experience 100 % pod loss.

Moreover, tissue samples taken from healthy pods and stems of *T. gileri* revealed a rich diversity of endophytic fungi, with many representatives in the Hypocreales, including new species and a novel endophytic clade of the mycoparasitic genus *Trichoderma* (Evans et al. 2003b; Samuels 2006; Samuels and Ismaiel 2009), as well as numerous basidiomycete morphospecies in the Agaricales and Polyporales (Thomas et al. 2008). Laboratory screening of both mycoparasitic and endophytic isolates using the pre-colonized plate method revealed a high level of antagonism to *M. roreri*, notably by a complex of *Clonostachys* and *Trichoderma* species (Evans et al. 2003b). The endophytic isolates of *Trichoderma* were shown to have the ability to colonize cacao seedlings without triggering host defense mechanisms, and to reach the apical meristems. Finally, it was suggested that these could act as mutualistic symbionts by increasing host resistance to pathogens such as *M. roreri* (Evans et al. 2003b). Ongoing studies are showing this to be a promising area of research with evidence of antibiosis and mycoparasitism in these endophytic *Trichoderma* species (Bailey et al. 2008), as well as other benefits, such as promoting growth and increasing drought resistance (Bae et al. 2009).

Resistance

During his visits to Ecuador, Rorer (1918, 1926) noted that the imported Forastero varieties were heavily attacked by *M. roreri*, but, in sharp contrast, disease incidence was low in the local cacao Nacional: "*This variety of cacao apparently has a great amount of natural resistance*." Sotomayor (1965), and later Evans (1981), confirmed this resistance in inoculation studies where all the material tested was highly susceptible apart from clone EET-233, a Nacional cross, in which lesion development and sporulation were restricted. Nacional has recently been identified as belonging to a distinct genetic cluster group (Motamayor et al. 2008). Little evidence of resistance has been reported since, with all cacao genotypes, as well as all germplasm collections of *Theobroma* and *Herrania*, proving to be susceptible (Evans 1981, 2002). More recently, a comprehensive screening study with a range of cacao genotypes and Colombian isolates of *M. roreri* demonstrated a high level of resistance in ICS-95, a genotype from the Trinidad collection (Phillips-Mora et al. 2013), which had also been reported as promising in Peru (Evans et al. 1998).

Resistance reported in the so-called Refractario trees of western Ecuador has been identified as being due to disease escape through a cropping pattern in which the main harvest develops during the extended dry season when conditions are unfavorable for infection (Evans 2002). Based on this principle, crop manipulation in which flowering is stimulated during the dry season has been shown to hold some promise (Evans et al. 1977).

3.9 Summary

Frosty pod rot of cacao is caused by *Moniliophthora roreri* (Basidiomycota, Agaricomycetes, Agaricales, Marasmiaceae), a hemibiotrophic pathogen with two trophic phases—the parasitic, biotrophic monokaryophase, and the saprophytic, necrotrophic dikaryophase—which can be separated morphologically, physiologically, and genetically: an analogous pleomorphic life cycle is shared with its sister species *Moniliophthora perniciosa*, the causal agent of witches' broom disease (see Chap. 5).

A variety nonpathogenic to cacao, *M. roreri* var. *gileri*—distinguished by morphology and molecular profile—occurs on endemic *Theobroma* in the forests of north-west South America and, possibly, is the progenitor of the cacao pathogen.

The cacao pathogen, *M. roreri* var. *roreri*, attacks all species of the genera *Theobroma* and *Herrania*; within *T. cacao* there is little or no evidence of genetic resistance.

Pods are most susceptible to infection during their first 3 months: with external symptoms absent or manifested as swellings and distortion of the green pods during the extended (averaging 6–10 weeks) parasitic, systemic phase, which is followed by rapid pod necrosis and external development of the diagnostic white hyphal mat (pseudostroma) and profuse sporulation.

Sporogenesis: holoblastic, spores in basipetal chains, ellipsoidal to globose; older spores rounding off, becoming thick-walled and pigmented, imparting pale tan to brown hues to the pseudostroma.

Cytological evidence has revealed meiotic events during sporogenesis and these sexual propagules (meiospores) are interpreted as modified basidia and the pseudostroma as a vestigial pileus.

Meiospores powdery, readily released by physical action—wind and raindrops—and dispersed by convection currents; long-lived on mummified pods (~9 months), probably with an in-built dormancy mechanism.

The fungus originated in the forests of north-western South America (Ecuador, Colombia) and has since spread north to western Venezuela, and all the cacao-growing countries of Central and North America, and south to Peru and Bolivia.

Frosty pod rot poses a real threat to Brazil and to the countries of the Guiana Shield, including eastern Venezuela, as well as a potential threat to all cacao-growing regions of the Paleotropics.

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Chapter 4
Moniliophthora roreri Genome and Transcriptome

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Abstract Frosty pod rot disease of cacao is one of the most destructive diseases of cacao and at this time is limited to regions in South America and Central America. Frosty pod rot is caused by a fungal pathogen *Moniliophthora roreri*, a basidiomycete that is closely related to another cacao pathogen that causes the witches' broom disease, *Moniliophthora perniciosa*. Combined these two pathogens are the leading causes of cacao yield losses in the Americas. Both pathogens are unique in that they have long biotrophic phases after infection as the disease progresses. In this chapter, genomic and transcriptomic sequencing will be used to corroborate and hypothesize various mechanisms of the molecular interactions of the host and pathogen during the disease interaction of frosty pod rot. The systematic timing of fungal and plant gene regulation in this pathosystem appears to be a key component of this plant disease resulting in specific molecular and cellular interactions. When this coordinated gene regulation is disrupted, for example, in a resistant plant variety, the disease interaction fails.

4.1 Taxonomy

Moniliophthora roreri (Cif) H.C. Evans, Stalpers, Samson & Benny (Evans et al. 1978) causes frosty pod rot (FPR) disease of *Theobroma cacao* L. (cacao). While reports of a similar disease can be traced back to 1817 in Colombia (Phillips-Mora 2003), the first verified observation of the disease caused by *M. roreri* was observed on cacao in Ecuador at the end of the nineteenth century (van Hall 1914; Rorer 1918). Although the fungus was initially misidentified and classified as *Monilia roreri*, an Ascomycete because of similarities to species of *Monilia*, the lack of a sexual stage, and fruiting bodies (Ciferri and Parodi 1933), it was reclassified as *Moniliophthora roreri*, a basidiomycete, when dolipores and septal caps were identified (Evans et al. 1978). In 2005, *M. roreri* and a sister taxon

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Moniliophthora perniciosa, the causal agent of witches' broom disease (WBD) of cacao, were grouped together into the Agaricales family Marasmiaceae based on genetic similarities (Aime and Phillips-Mora 2005). Genomic and mitochondrial gene comparisons further support the common lineage of these two fungal pathogens (Aime and Phillips-Mora 2005; Formighieri et al. 2008; Costa et al. 2012; Mondego et al. 2008; Meinhardt et al. 2014).

4.2 Origins

Frosty pod rot disease of cacao is one of the most destructive pathogens of cacao and is limited to the Andean region of South America and all of the cacao-growing region of Central America (Phillips-Mora et al. 2006a, b, 2007; Phillips-Mora 2003). This disease is found on all *Theobroma* and *Herrania* plant species. From the very beginning, FPR was thought to have originated on wild cacao species in Ecuador based on the existence of the disease in these wild hosts (Rorer 1918). Speculations that wild *Theobroma* and *Herrania* species found in north-western Colombia were the source of the disease were developed by Holliday, with *T. gileri* being one of the primary wild species (Holliday 1957, 1971). Evans (1981) expanded the origin concept to the North-western part of South America based on *M. roreri* being indigenous on wild hosts in the region and due to the fact that some cacao types were thought to have originated in this region, Nacional from Ecuador and Criollo from Colombia, and possess some resistance to FPR (Evans 1981). Amplified fragment length polymorphism and inter simple sequence repeat marker analysis carried out by Phillips-Mora et al. (2007) has also showed higher genetic diversity in Colombia compared to Ecuador. More recently based on single nucleotide polymorphism analysis, Ali et al. (2015) predicted the upper Magdalena Valley of Colombia as the possible center of origin for *M. roreri*.

4.3 Disease Process

In the countries of South America where frosty pod rot, witches' broom, and black pod diseases occur together, most of the production losses are the result of frosty pod rot. *Moniliophthora roreri* is a hemibiotroph and causes disease only on cacao pods, during the natural infection process. Artificial infections have been achieved on seedlings, flushes, and flower cushions using high inoculum levels, but the affected tissues failed to sporulate (Evans 1981). During the extended biotrophic phase of the disease interaction no major disease symptoms are observed (Fig. 4.1). The biotrophic phase of the susceptible disease interaction can last up to 45 days prior to chlorosis and the formation of necrotic (necrotrophic phase) lesion development (Fig. 4.1). The only symptoms observed during the biotrophic phase are pod malformations (Desrosiers and Suárez 1974); however, this doesn't occur in all

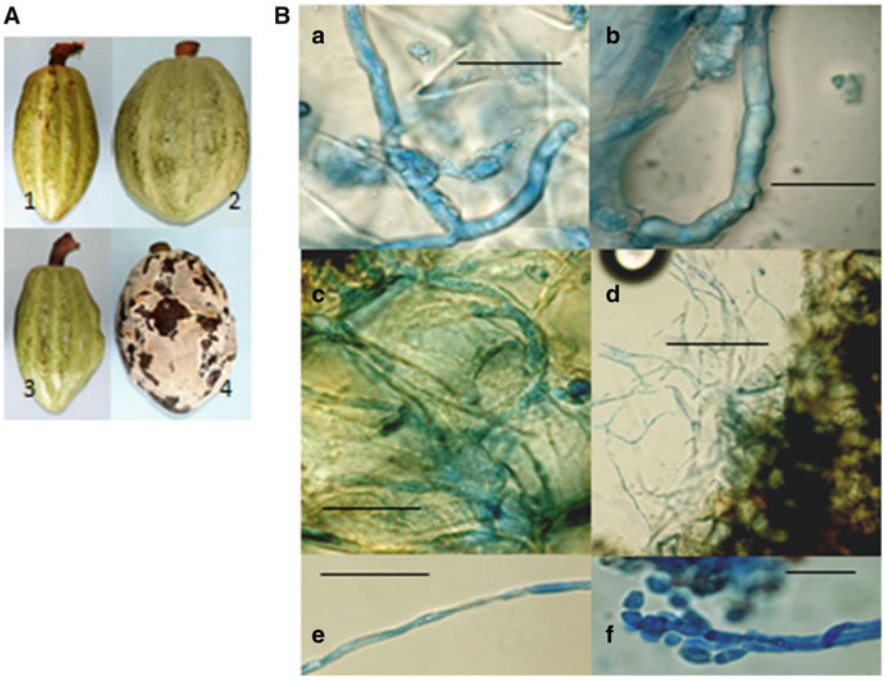

Fig. 4.1 Panel (**A**): Photos 1 and 2 are of health pods, photo 3 shows a malformation pod (biotrophic phase of *Moniliophthora roreri*), and photo 4 shows a sporulating pod (necrotrophic phase of the fungus). Panel (**B**): Photos (**a**), (**b**), and (**c**) show the biotrophic mycelia from malformed green pods (Panel A #3); (**d**) and (**e**) show the necrotrophic mycelia from a necrotic sporulating pod (Panel A #4); photo (**f**) shows the meiospores from the sporulating pod

infected pods. The necrotrophic phase of the disease normally starts with premature ripening or a chlorotic spot that rapidly turns into a necrotic lesion (Fig. 4.2). The chlorosis spreads rapidly ahead of the development of a single necrotic lesion that will eventually cover most of the pod surface. White sporulating mycelia form quickly in the center of the necrotic lesion and continue to expand rapidly until the entire lesion is covered (Fig. 4.2). The spores are readily dislodged from the mycelial mass by wind and rain and can form dense spore clouds that transport the spores to new infection sites. The sporulating pod will remain attached to the tree (Suárez 1971). Most of the spores are produced during the first sporulation cycle while the infected pod dries and mummifies. These mummified pods can release spores for several seasons (Evans 1986).

At the microscopic level the biotrophic and necrotrophic phases have distinct differences as well. In a compatible disease causing interaction the germinating spores penetrate the pod's epidermis through stomatal openings and grow intercellularly into the mesophyll levels of the pod. During this stage the fungal mycelia are pleomorphic and have a large diameter. The fungal cells are limited to the pectin- and hemicellulose-rich middle lamella layer between the plant cells of

Fig. 4.2 *Left panel* shows the premature ripening of a cacao infected with *Moniliophthora roreri*; note the infect pod cavity. The *middle panel* and *right panel* show sporulating Mr-infected pods

the pod. If the pod is cut open at the end of the biotrophic phase very small necrotic lesions may be observed in the infected tissue. These may be the result of localized shifts in the disease interaction, biotrophic to necrotrophic. In necrotic tissue where the fungus is in the necrotrophic phase, the mycelia are smaller in diameter and have a regular mycelia growth pattern. Clamp connections are not normally observed in *M. roreri*.

In comparison *Moniliophthora perniciosa*, the causal agent of WBD of cacao, infects cacao when germ tubes from basidiospores enter the stomatal openings of meristematic tissues such as shoots, flowers, and young developing fruits (Evans 1980; Frias et al. 1991). The fungal mycelia grows intercellularly, producing large convoluted hyphae that lack clamp connections (Evans 1980). The mycelia at this stage in the WBD cycle are uninucleate (Calle et al. 1982). During this biotrophic phase, the fungus grows slowly between the plant cells and after about 21 days begins to produce the distinctive WBD symptoms in shoots: hypertrophy and hyperplasia of the plant tissues distal to the initial infection site, loss of apical dominance with an abnormal proliferation of the axillary shoots, and formation of a vegetative broom structure, which is called a green broom (Holliday 1980). Infection of the cauliform flower cushions can produce vegetative cushion brooms or small parthenocarpic fruits (Holliday 1980; Meinhardt et al. 2008). These developmental alterations that occur during the biotrophic phase of *M. perniciosa* infection typically continue for 1–2 months before these tissues undergo necrosis and death. Necrosis and death of the plant tissue is associated with a change in the fungal morphology, with mycelia becoming narrower, having fewer convoluted cells and possessing clamp connections, and becoming binucleate or dikaryotic. These changes are indicative of the necrotrophic phase of the WBD interaction. The necrotic tissue desiccates while the fungus colonizes the mummified tissues that remain attached to the tree. Unlike *M. roreri*, *M. perniciosa*-infected tissues do not sporulate immediately, requiring several months of repeated wetting and drying

cycles before producing small pink-colored basidiocarps or mushrooms. In addition, *M. perniciosa* will also infect pods at all stages of development causing a watery pod rot in mature pods or resulting in aborted pods when young pods are infected (Meinhardt et al. 2008; Maddison et al. 1995).

In this chapter, we will discuss importance of the fungal genomes and how genomic information can direct our understanding of the host/pathogen interaction that occurs in this pathosystem. Comparisons will be made with what is known about *M. perniciosa*. After an introduction to the *M. roreri* genome, we will follow the disease interaction and overlay gene expression and function at various stages of compatible and incompatible disease interactions in an attempt to elucidate the molecular steps that are involved in the frosty pod rot disease of cacao.

4.4 The *M. roreri* Genome

Genome Structure

The *Moniliophthora roreri* draft genome was completed in 2014 (Meinhardt et al. 2014). Along with the genome survey, several transcriptome analyses have been conducted. The estimated genome size for *Moniliophthora roreri* is 52.3 million base pairs (Mbp), which is 7.7 million base pairs larger than the genome of *Moniliophthora perniciosa* (estimated at 44.6 Mbp) (Table 4.1). This increased genome size, however, does not equate to a similar increase in the number of genes. Of the 17,921 coding sequences found in the *M. roreri* genome, 16,713 genes (93 %) have homology with 15,674 or 92 % of the genes in the genome of its sister taxon *Moniliophthora perniciosa*. This gene comparison across both genome sequences of *M. roreri* and *M. perniciosa* categorically confirms the relatedness of these two fungal species.

Synteny analysis of contigs over 50 kbp in length showed that 222 *M. roreri* contigs were highly similar to 207 contigs from the *M. perniciosa* genome demonstrating that the two genomes have large segments of their genomes that are homologous. In spite of the fact that *Moniliophthora roreri* fails to produce a basidiocarp or mushroom, many of the genes found in this pathogen have homologs in the genome of *Moniliophthora perniciosa* and other basidiomycetes, further supporting their relatedness. When compared to *Moniliophthora perniciosa*, *Laccaria bicolor*, *Coprinopsis cinerea (Coprinus cinereus)*, and *Ustilago maydis*, 3048 genes were found to have high homology (E-value of E-04).

The average GC content of the coding sequencing for *M. roreri* is 49.55 % which is very similar to the 49.88 % for *M. perniciosa* (Table 4.1) (Meinhardt et al. 2014). Also found in the *M. roreri* genome were 670 tRNAs on 331 contigs which is 120 more than found in *M. perniciosa*. The average gene density for *M. roreri* (0.42) was lower than *M. perniciosa* (0.51), which is due to genome size/gene number ratio. There are 9078 coding sequences in the *M. roreri* genome encoding

Table 4.1 Genome comparisons of *M. roreri* and *M. perniciosa*

	Genome size (bp)	Total number contigs	Avg (G+C) CDS %	Total tRNA	Avg gene density	Total CDS	Maximum CDS size (bp)	Median CDS size (bp)
M. roreri	52,334,075	3298	49.55	670[a]	0.42185[b]	17,921	15,081	1023
M. perniciosa	44,661,472	3087	49.83	550	0.51058[b]	17,008	15,048	1095

[a]Default parameters for tRNAscan yielded 331 contigs that have tRNA. The total number found in the genome is 670 tRNAs
[b]CDS bases/total genome bases

hypothetical proteins with no known function. At least 3113 of these sequencing encoding hypothetical proteins were not expressed under the transcription conditions evaluated in our studies.

Resequencing of multiple clones of *M. roreri* has shown that each clone has a homogenous genetic background, suggesting that they are homokaryons with limited anastomoses and cellular fusion of dissimilar nuclear types. Previous experiments have shown this fungus goes through meiosis (Evans et al. 2002), which is supported by 10 genes in the *M. roreri* genome encoding for meiosis or meiotic specific proteins (Meinhardt et al. 2014). The result of meiosis is the production of meiospores that germinate into haploid mycelia. Like *M. perniciosa*, *M. roreri* is thought to have haploid mycelia during the biotrophic phase, and at the beginning of the necrotrophic phase, a nuclear duplication occurs resulting in a dikaryotic state. Further studies are needed to confirm the nuclear state of the fungus and determine exactly when these changes occur. However, to maintain a homokaryotic nuclear condition *M. roreri* must have a way of preventing dissimilar nuclei from inhabiting the same mycelia cells. There are multiple genes in the genome associated with het-c and het-e proteins that are involved in heterokaryon incompatibility (Saupe et al. 2000) and these could prevent vegetative heterokaryons from forming. Also found in the genome are multiple NWD2 genes that are similar to STAND/prion-like proteins that have been found to function in non-self recognition cell death (Daskalov et al. 2012) and may be involved in the het-s vegetative incompatibility mechanism (Paoletti and Clave 2007).

4.5 The *M. roreri* (Mr) Transcriptome

As part of the genomic analysis of frosty pod disease, various transcriptome libraries were sequenced from infected cacao pods at different time points and with susceptible and tolerant plants. RNAseq analysis was conducted on these infested materials and transcriptomic analysis revealed differential gene expression (Table 4.2).

Several gene families were highly represented in the genome. With regard to carbohydrate utilization in the fungus, 288 genes corresponding to 40 different glycoside hydrolase families were found in the genome. Glycoside hydrolases (GH) are expected to be important for breaking down complex sugars (Table 4.3), including plant cell wall components, or in the modification of fungal cell walls. Sixty glycosyltransferase genes were found representing 19 different families. These enzymes transfer carbohydrate moieties to various acceptor molecules such as complex carbohydrates, nucleic acids, lipids, proteins, or fungal cell walls (Bowman and Free 2006).

For detoxification in the fungus, 43 epoxide hydrolases, 296 cytochrome P450 genes, and 9 metallo-beta lactamases were identified. Epoxide hydrolases detoxify lipid-derived epoxides and are involved in lipid metabolism (Morisseau 2013),

Table 4.2 Summary of transcriptome libraries

Transcriptome library	Total number of genes evaluated	Number of genes expressed under the test conditions	% expressed of the total genes	Number of genes not expressed under the test conditions	% non expressed of the total genes
7–60 2013[a]	17,919	8464	47.2 %	9455	52.8 %
30–60 MPP 2014[b]	17,921	14,582	81.4 %	3339	18.6 %
30–60 BMC 2014[c]	17,921	13,761	76.8 %	4160	23.2 %

[a]Unpublished data, susceptible disease interaction libraries at 7 and 60 day post infection (DPI)
[b]Published data, susceptible and resistant disease interaction libraries at 30 and 60 DPI (Bailey et al. 2014)
[c]Published data, susceptible disease interaction libraries at 30 and 60 DPI (Meinhardt et al. 2014)

while cytochrome P450 proteins have a wide diversity of biological functions from metabolism to detoxification (Chen et al. 2014). Metallo-beta lactamases are normally associated with antibiotic resistance in bacteria (Wright and Sutherland 2007); however, their role in this pathosystem is not apparent. There are 42 hydrophobin genes that are associated with fungal cell surfaces such as appressoria formation and attachment to hydrophobic surfaces (Talbot et al. 1996), and the formation of aerial hyphae and fruiting bodies (Wessels et al. 1991). Like *M. perniciosa* (Mp), the *M. roreri* (Mr) genome also contained plant-like pathogenesis-related (PR-1) proteins (Teixeira et al. 2012). There are 12 genes in the *M. roreri* genome compared to the 11 in *M. perniciosa*, and 9 of these genes are homologous between the two genomes (Meinhardt et al. 2014). This family of genes is thought to be involved in neutralization of plant defense mechanisms, to have antimicrobial activity, and to be involved in fruiting body physiology (Teixeira et al. 2012).

The *M. roreri* genome contains 164 genes encoding retrotransposable element 155 kDa proteins, 21 retrotransposon nucleocapsid coding sequences, 13 reverse transcriptase ribonuclease H genes, and 212 reverse transcriptase-RNase H-integrase sequences. The higher number of transposable elements (TEs) and associated genes account for most of the size difference between the *M. roreri* and *M. perniciosa* genomes (Meinhardt et al. 2014). The genome expansion due to TEs that occurred in *M. roreri* appears to have happened after the speciation event that separated the Moniliophthora species. The rapid expansion of TEs in M. *rore*ri may have provided the allelic divergence necessary for sympatric speciation (Fournier and Giraud 2008; Giraud et al. 2008); however, further studies are needed to fully characterize the evolutionary role of TEs in *M. roreri*.

Expression Analysis in Susceptible Hosts

Expression studies of 30 days after infected (DAI) and 60 DAI pods were analyzed along with the genome study (Meinhardt et al. 2014). Additionally, expression analysis was conducted on susceptible and tolerant *Theobroma cacao* clones to assess the differences in the disease interaction (Bailey et al. 2014; Ali et al. 2014). Genomic and expression studies reveal coordinated gene expression that is highly orchestrated around the fungal–plant interaction during the different fungal growth phases. For susceptible host interactions the 30 and 60 DAI analysis at a fivefold induction level for genes with known functions and a 15-fold level for hypothetical genes were used for this discussion. The expression patterns evaluated at 30 days represent the biotrophic phase and the 60-day evaluation depicts the necrotrophic phase of the disease cycle.

Differential gene expression of the fungus in susceptible hosts appears to mirror the physical growth demands and challenges associated with the fungal–plant interaction. Expression patterns in the biotrophic and necrotrophic phase not only revealed different sets of genes being upregulated but also differential gene expression within the same family of genes. These gene families, which are sorted into groups as defined below, suggest that during the disease process the fungus is (Group 1) utilizing or modifying a diverse set of carbohydrates and protein substrates; (Group 2) is interacting and breaking down plant cell wall components and causing plant cell death; (Group 3) is being subjected to plant defenses that require detoxification or require protection mechanisms, (Group 4) is metabolizing alcohol, possibly associated with lignin breakdown; (Group 5) is undergoing fungal metabolisis; and (Group 6) is undergoing unspecified functions. Common gene families that have different members upregulated in both growth phases include (Group 1) GH family 16 proteins (cleaves β-1-4 and β-1-3 glycosidic bonds in various glucans and galactans) (Eklöf and Hehemann), GH family 18 proteins (chitinase) (Davies et al.), GH family 28 proteins (polygalacturonases) (Pickersgill), GH family 5 proteins (endoglucanases, endomannanases, exoglucanases, exomannanases, β-glucosidases, and β-mannosidases) (Davies), GH family 76 proteins (endo-acting α-mannanases) (Williams), hexose transporters, class II aldolase adducin domain proteins, NAD-dependent epimerase dehydratase family proteins, GABA permeases, phosphoglycerate mutases, serine threonine-protein kinases; (Group 2) laccases, copper radical oxidases, amino acid transporters, MFS transporters, cerato-platanins; (Group 3) hydrophobins, benzoate 4-monooxygenase cytochrome p450s, cytochrome p450s, 2oG-Fe oxygenases, aldehyde dehydrogenases, MFS multidrug transporters, tPA: cytochromes; (Group 4) NADP-dependent alcohol dehydrogenases, GMC oxidoreductases, short chain dehydrogenases; (Group 5) mitochondrial cytochrome, C_2H_2 conidiation transcription factors, fruit body-specific genes, metal-dependent phosphohydrolase, carbonic anhydrases, choline dehydrogenases, high-affinity nicotinic acid transporter, geranylgeranyl pyrophosphate synthases, *S*-adenosyl-L-

methionine-dependent methyltransferases, terpenoid synthases; and (Group 6) PR-1-like proteins, phenylacetyl-CoA ligases, and 260 hypothetical proteins.

Upregulated Genes in the Biotrophic Phase

Utilizing the differentially expressed genes we can correlate fungal activities during the susceptible disease interactions. In the biotrophic phase, there are fewer upregulated genes, which may be associated with the limited fungal biomass found during this stage.

While the fungus is growing in between cells and breaking down the cell wall components, it remains outside of the plant cell. Access to the plant nutrients is vital to the growth and development of the fungus and *M. roreri* does not develop specialized structures such as haustoria and intracellular mycelia (Perfect and Green 2001). Studies have shown that *M. roreri*-infected cacao pods in the biotrophic phase have reduced levels of glucose, asparagines, and phenylalanine (Bailey et al. 2013). An upregulated gene in *M. roreri*, pleurotolysin B, which is a pore-forming cytolysin (Schlumberger et al. 2014), could cause cell membrane leakage, thus providing the fungus access to the nutrients without killing the plant cell.

Two xylanase A and three GH family 43 genes are upregulated in this stage, which suggests the fungus is utilizing the hemicelluloses in the intercellular middle lamella region through the breakdown of xylan. One additional group of hydrolases, GH family 76, is also upregulated in the biotrophic phase. This family contains enzymes that are endo alpha mannanases and could be functional on the galacto-gluco-mannan hemicellulose chains (van den Brink and de Vries 2011). The upregulation of an acetyl xylan esterase would also increase the accessibility of the xylan groups in hemicelluloses to the other xylanase enzymes (Zhang et al. 2011). Another component of hemicelluloses, arabinans are also potential targets for cleavage by an upregulated endo-1,5 -alpha-L-arabinanase, a GH family 28 gene (Pickersgill), and two galactan beta-galactosidases, which may be active on both hemicelluloses and pectin (Lazan et al. 2004) Pectin in the cell walls is subject to the actions of an upregulated gene in the carbohydrate esterase (CE) family 8, a pectin methyl esterase (van den Brink and de Vries 2011). There is also a cell wall glycosyl hydrolase that has homology to GH family 105 that could function as an unsaturated rhamnogalacturonyl hydrolase to break down pectin. Cellulose activity is also upregulated with two GH family 5 genes that could function as endo-glucanases (van den Brink and de Vries 2011) and a cellobiohydrolase ll gene (Kleman-Leyer et al. 1996).

Another issue facing the fungus in the biotrophic phase is how to avoid the plant defense mechanisms. Hydrophobins are small ($\leq$20 kDa) secreted proteins with low sequence homology, but consisting of a unique conserved region comprised of 8 cysteine amino acids that forms 4 disulfide bonds (Bayry et al. 2012). These proteins are amphiphilic and can self-assemble into monolayers at hydrophilic/

hydrophobic interfaces. Of the 42 hydrophobins found in the genome four are upregulated in the biotrophic phase. Hydrophobins that cover fungal cells have been shown to mask the fungus from host detection (Aimanianda et al. 2009) and to be involved in the infection and colonization of susceptible host (Kim et al. 2005). In this case we can hypothesize that the hydrophobins are masking the *M. roreri* mycelia from plant defenses while promoting colonization. In addition to hydrophobins another class of small cysteine-rich proteins, cerato-platanins, are actively expressed during this phase. In *M. perniciosa* 12 cerato-platanin genes have been identified and found to be differentially expressed at differential phases of fungal development (Barsottini et al. 2013). Ten homologous cerato-platanin genes have been identified in *M. roreri* and have been given corresponding gene IDs. MpCP4 has no homologs in *M. roreri* and the MpCP1 homolog has multiple stop codes rendering the protein nonfunctional in *M. roreri*. This is of particular interest since MpCp1 is practically the only isoform found in basidiocarps and basidiocarp formation in *M. perniciosa* while *M. roreri* neither produces the MpCP1 homolog nor a basidiocarp. Additional investigation is needed to see if there is a direct correlation between the functionality of the MrCP1gene and the lack of mushroom formation in *M. roreri*. With the exception of MpCP4 all of the cerato-platanins (MpCP5, MpCP11, and MpCP12) upregulated in the biotrophic phase of *M. perniciosa* (Barsottini et al. 2013) have their homologous genes upregulated in *M. roreri* suggesting possible similar roles for these gene products during the biotrophic phase. One intriguing finding is that MpCP5 can bind chitin fragments which could mitigate chitinase-driven plant defense responses, thus protecting the fungus during the biotrophic phase of the disease (Barsottini et al. 2013). In addition to the hydrophobins and cerato-platanins, we also find genes encoding PR-1-like proteins from the fungus upregulated in the biotrophic phase. MrPR-1g, MrPR-i2, and MrPR-1n (Meinhardt et al. 2014) are expressed in the biotrophic phase of FPR. Only one of these *M. perniciosa* homologs, MpPR-1g, is highly expressed in the biotrophic phase of WBD (Teixeira et al. 2014), suggesting different mechanisms may be at work in this gene family with these different diseases.

Upregulated Genes in the Necrotrophic Phase

The necrotrophic phase is associated with changes in the nuclear content of the mycelia cells (monokaryotic to dikaryotic) and a rapid increase in fungal biomass accompanied by chlorosis, necrosis, and plant cell death. Previous studies have shown that plant nutrient reservoirs within the pod are typically exhausted at this stage (Bailey et al. 2013, 2014).

Several challenges face the fungus in this phase: (1) rapid growth, (2) acquisition and utilization of nutrients, and (3) pathogenicity and avoidance of plant defense mechanisms.

During the necrotrophic phase the fungal biomass is rapidly increasing and we find upregulated expression of two chitin synthase genes and a chitin synthase export chaperone protein gene. Also related to the fungal biomass expansion is the upregulation of an actin gene, an actin cortical patch component gene, and an actin filament organization transcript. *M. roreri* was also found to have two upregulated chitin deacetylase genes in the necrotrophic phase and this cell wall modifying activity may be similar to that found in the rust fungi *Puccinia graminis* f. sp. *tritici* and *Uromyces fabae*, where the chitin modifications protect the pathogen mycelial cells from host-derived chitinase activity.

Another event that is occurring during the necrotrophic phase is the active degradation of the pod lignin. From the transcriptome data we can identify upregulation of various lignin-degrading enzymes that appear to be working in concert. Laccases are digesting the pod lignin releasing methanol, water, and aromatic aldehydes and lignin monomers, while GMC oxidases and alcohol oxidases are breaking down the released alcohols into aldehydes and H_2O_2 (Hernandez-Ortega et al. 2012). The breakdown of aromatic aldehydes could be catalyzed by aryl-alcohol dehydrogenases resulting in the release of aromatic alcohol and NADH (Yang et al. 2012). The aldehydes are subject to decomposition by glyoxal oxidases that release oxalate or carboxylic acids and additional H_2O_2 (Kersten 1990). Finally, heme and fungal peroxidases are present that can react with the hydrogen peroxide to further depolymerize the pod lignin (Hofrichter et al. 2010). Oxalate produced by glyoxal oxidases can also be cleaved by the oxalate decarboxylate enzyme forming carbon dioxide and formate anion radicals that can directly oxidize lignin with oxygen (Hammel et al. 1994) or be converted into superoxide that can dismutate into H_2O_2 (ten Have and Teunissen 2001). Another upregulated enzyme associated with lignin degradation is methyl chloride transferase. This enzyme synthesizes the metabolite chloromethane that supplies the methyl group responsible for the methylation system that degrades lignin in bracket fungi (Harper et al. 1988; Harper and Hamilton 1988) and in white-rot fungi (Harper et al. 1990). Also upregulated in this phase are seven CE family four genes that include acetyl xylan esterases and chitin deacetylase. These enzymes could be involved in hemicellulose degradation and chitin modification, but, because of the number of these genes and the induction levels (7X to 120X), the function of this family of genes should be further investigated to demonstrate their functions. Another multifunctional enzyme found in this phase is a cell wall glucanase that could be acting on cellulose.

Gene expression patterns suggest that the fungus is utilizing the glyoxylate cycle during this phase, which has been found to be essential for pathogenicity in other fungi (Idnurm and Howlett 2002). While there is constitutive expression of the aconitase enzymes there is upregulation of an isocitrate lyase gene, a malate synthase gene, and two malate dehydrogenase genes. The resulting oxaloacetate product could then be acted upon by two oxaloacetate acetyl hydrolase genes that are upregulated to produce oxalate and acetate. Oxalate could then feed into the lignin breakdown mechanism discussed previously, while acetate could be converted to acetyl-CoA by acetyl-CoA synthetase, which is also upregulated in

this phase. Calcium oxalate crystals have been found during the biotrophic phase of WBD of cacao caused by *M. perniciosa*. The oxalate crystals found in WBD-infected plant tissues were derived from the fungal pathogen based on fungal gene expression patterns (Ceita et al. 2007; do Rio et al. 2008). Similar studies during the disease cycle of FPR need to be conducted to investigate the role of oxalate.

Numerous pathogenicity factors are upregulated in the necrotrophic phase. Genes associated with pathogenicity in other pathosystems such as cerato-platanins, necrosis inducing proteins (NEPs), salicylate hydrolases, and iron siderophores are all upregulated in the susceptible *M. roreri*/host interaction. Cerato-platanin and NEPs are fungal toxins that cause plant cell death (Cabrera et al. 2008; Garcia et al. 2007). Cerato-platanin genes have been shown to be differentially expressed in the necrotrophic phase of *M. perniciosa* (Barsottini et al. 2013). Among the five genes found in the Mp genome MpCP2, MpCP3, and MpCP8 are exclusively expressed in the necrotrophic phase while MpCP9 and MpCP10 are upregulated in this phase. Identical results were found for the *M. roreri* gene homologs during necrotrophic expression of these genes in FPR. While most cerato-platanin are associated with glycolytic activity, Bersottini et al. (2013) found no hydrolytic activity for MpCp1, 2, 3, and 5, suggesting that the cerato-platanins in the Moniliophthora genus may have alternative roles in these disease interactions.

There are three NEP genes in the *M. roreri* genome and two are upregulated in the necrotrophic phase. The highest upregulated gene is homologous to the MpNep2 gene, which has been shown to cause necrosis in tobacco and cacao (Garcia et al. 2007) and has the ability to recover its activity by refolding after heating (de Oliveira et al. 2012). The NEP genes are hypothesized to have been acquired in this genus by horizontal gene transfer from oomycetes (Tiburcio et al. 2010). The exact role of these proteins during this phase of FPR is not known, but they are thought to induce plant cell death causing a release of plant nutrients to support the rapid growth of the fungal biomass.

The salicylate hydrolases break down salicylic acid, which is a key plant defense hormone, and homologous genes have been found in *Ustilago maydis* (Rabe et al. 2013). This plant hormone is important in plant defense due to its function as a signal mediator of systemic plant defense mechanisms (Durner and Klessig 1995). The role of the upregulated salicylate hydrolase in FPR can only be hypothesized at this point, but it may be functioning to prevent salicylic acid-driven defense mechanisms.

Two groups of genes not typically encountered in fungal pathogens, PR-1-like proteins and thaumatin-like proteins, were found upregulated in the interactions. MrPR-1d, MrPR-1e, and MrPR-1o are the three PR-1 protein encoding genes upregulated in the necrotrophic phase. In addition to the PR-1 genes there are three thaumatin-like protein coding regions that are upregulated. Both of these gene families are normally associated with plant defense mechanisms that are used to combat plant diseases, but both have been found in the genus *Moniliophthora* (Teixeira et al. 2012; Liu et al. 2010). The exact functions of these genes during

frosty pod rot are unknown, but they could be interfering with the plant defenses or functioning to prevent secondary infection by other pathogens.

M. roreri is not known to produce mycotoxins; however, there is an upregulated gene for an ergot alkaloid biosynthetic protein A, in this phase, which could be functioning in toxin synthesis. In addition, 11 epoxide hydrolase genes were upregulated. Enzymes from this family of genes have been linked to potential involvement in toxin production in *Alternaria alternata* f. sp. *lycopersici* and *Hypocreales* (Pinot et al. 1997; Popiel et al. 2014).

During the necrotrophic phase, the fungus also appears to be under stress and must employ various mechanisms to detoxify and mitigate abiotic stress. There are 39 upregulated cytochrome P450 genes and a multidrug resistance protein, two MFS drug transporter proteins, two metallo-beta lactamases, and four small heat shock proteins. Heat shock proteins have been found to be expressed in another basidiomycete *Coriolus versicolor* when induced by hazardous chemicals (Iimura and Tatsumi 1997).

Also upregulated in the necrotrophic phase were transcripts for het c and het e proteins involved in heterkaryon incompatibility (Saupe et al. 2000), NWD2 genes that are STAND/prion-like proteins associated with non-self recognition cell death (Daskalov et al. 2012), metacaspase, anamorsin domain protein, fruit body-specific proteins, hydrophobins (Lacourt et al. 2002), and pheromone receptors (Kothe 2008). The het genes, prion, and NWD2 genes all appear to be involved in non-self recognition (Saupe and Daskalov 2012) while metacaspase is a cysteine protease involved in cell death pathways (Carmona-Gutierrez et al. 2010) and anamorsin proteins are involved in apoptosis (Song et al. 2014). Self-recognition and incompatibility are particularly important, since it has been observed that *M. roreri* is functionally clonal and shows very limited genetic polymorphism within the clonal lines (Phillips-Mora 2003; Phillips-Mora et al. 2007). These self-recognition and compatibility mechanisms may prevent heterokaryon formation during the necrotrophic phase when the fungal biomass is rapidly increasing and where different fungal spore lines caused by multiple infections are present to form mycelia anastomosis. The upregulation of the fruiting body-specific proteins, hydrophobins, and pheromone receptors all indicate the fungus is preparing for sexual reproduction in this phase, even though it has lost the ability to produce a basidiocarp. Figures 4.3 and 4.4 depict some of the genes upregulated during the biotrophic and necrotrophic phase, respectively, and their potential role in the disease process during that phase.

4.6 Sources of Resistance

Unfortunately, not many cacao lines in the international collections have been identified with resistance to *Moniliophthora roreri*. Additional surveys for frosty pod rot resistance should be made in the uncharacterized collections and from the remaining wild populations. The only known resistance to frosty pod rot is found

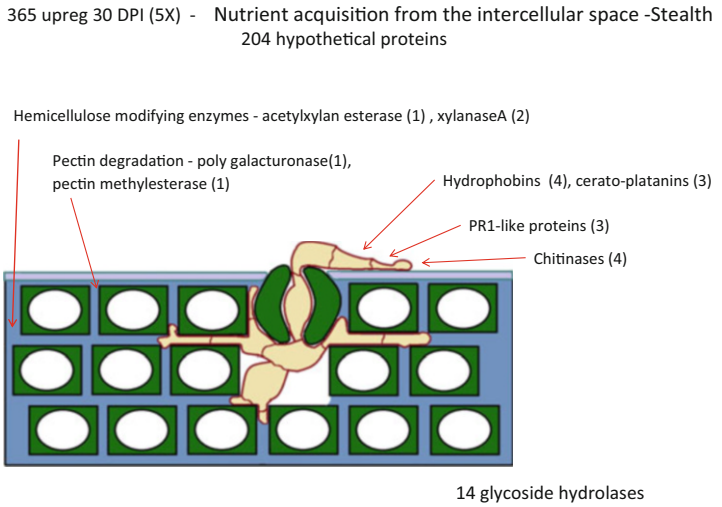

Fig. 4.3 Genes upregulated during the biotrophic phase of *Moniliophthora roreri*

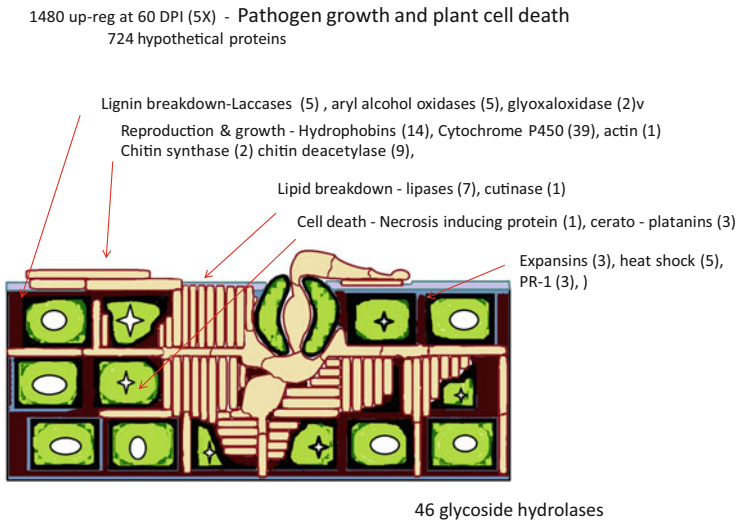

Fig. 4.4 Genes upregulated during the necrotrophic phase *Moniliophthora roreri*

in UF273 and PA169. UF273 is from the United Fruit Company collection, but the origin and location of the source is unknown. The United Fruit Company collection is derived from selections made in Central America, with some selections having their origin in Ecuador. The source of PA169 is the Rio Marañón, Parinari District, in the Loreto Department Peru, Coordinates 4.60 S 74.60 W. Both UF273 and PA169 have been used in the CATIE cacao breeding programs in Costa Rica and are responsible for resistance in the R4 and R7 cacao lines that have been planted

throughout Central America. ICS95 and SCC61 have also been identified as having resistance to FPR (Phillips-Mora 2003). ICS95 is a Trinitario X Criollo cross from Trinidad and SCC61 is a Colombian selection (Rondon Carvajal 1993). A search of the International Cocoa Germplasm Database (ICGD) found 8 additional cacao genotypes with resistance to FPR: CC246, CC252, EET75, EET183, ICS43, PMCT46, SC37, and UF712 (Turnbull and Hadley 2015). Four of these CC252, EET75, ICS43, and UF712 are being used in resistant breeding programs (Turnbull and Hadley 2015). Genetic diversity and parentage data are not available for all of these cacao lines so it is not possible at this time to determine if the resistance is derived from a single or multiple population groups. An important point to focus on is that some of the cacao resistance observed is due to a particular fungal isolate interaction. These variable interactions were noticed by Phillips-Mora (2003) and, within the ICG database, where some cacao clones had both resistant and susceptible evaluations. This raises two points, to make a valid resistance evaluation the host and pathogen genotypes must be identified and any breeding program must take into account not only the local fungal isolates but any isolates that could potentially enter into the region.

4.7 Gene Expression in Tolerant Versus Susceptible Cacao Clones

Utilizing a different set of host plants, additional studies were conducted with two tolerant and two susceptible hosts and those interactions utilized linear regression analysis and correlation analysis to identify key expression patterns (Bailey et al. 2014). Due to different response times and symptoms observed in resistant and susceptible host interactions, gene expression was evaluated based on comparable fungal biomass levels or fungal loads. Twenty-three transcripts were found to be differentially expressed in the susceptible and tolerant hosts. The fungal load and DAI would clearly put most of the interactions in the biotrophic phase; however, in tolerant cacao lines differences were observed. In tolerant interactions, it appears that the biotrophic/necrotrophic shift occurs rapidly, in a nonsystematic manner leading to plant cell death and rapid pod loss. There is more chlorosis of the infected pods with limited necrosis and either no sporulation or very limited sporulation. The rapid shift from the biotrophic to necrotrophic phase in tolerant interactions would greatly limit the colonization of the pod and severely restrict the access to plant nutrients when the pod undergoes senescence, thus inhibiting sporulation.

During an incompatible disease interaction, the biotrophic phase of the frosty pod rot disease is disrupted and the coordinated gene interactions found in susceptible hosts are not present. Many of the biotrophic gene interactions either don't happen or progress very rapidly to the necrotrophic gene interactions. Two of the *S*-adenosyl-L-methionine-dependent methyl transferases and a mycelial catalase that were highly upregulated in the biotrophic phase in susceptible hosts are

downregulated in the tolerant cacao lines. Additionally, genes for heat shock proteins, a cytochrome P450, and a glyoxylate dehydrogenase are also upregulated at the same time even though these genes are only upregulated in the necrotrophic phase of susceptible hosts. Other genes that are normally upregulated in the necrotrophic phase such as gmc oxidases, hydrophobins, and the hetc and pheromone receptor genes to name a few are upregulated in the early interaction with tolerant cacao lines.

In addition to finding genes upregulated or downregulated at the wrong time during the disease interaction, genes that are normally exclusively expressed in either the biotrophic or necrotrophic phases were found to be expressed at the same time in the tolerant cacao lines. A case in point are the cerato-platanins where in the tolerant interaction we find the biotrophic orthologs MrCP5, 11, 12 being downregulated at the early stages of infection at the same time that the necrotrophic exclusive genes MrCp2 and 8 are upregulated along with the MrCP10. This is further support for the conception that there is an early shift in the phases during the tolerant interactions where biotrophic phase expression is reversed and necrotrophic phase expression is turned on.

Expression of necrotrophic genes during the early infection process could be utilized as a screening tool to identify new source of resistance in cacao. Furthermore, screening based on the phenotypic reactions of these resistant lines could be used in regions where molecular screening is prohibited.

4.8 What Do We Still Need To Know?

Many of the purposed gene interactions suggested by the expression activity at the different phases require validation through wet chemistry, QPCR, and immunocytology. Furthermore, there is a large group of differentially expressed hypothetical genes that require in-depth analysis to understand their function.

While much of the disease process has been evaluated, information is still lacking on the initial stages of the disease, from the infection stage to very early growth in the pod. These early stage experiments are complicated by the slow growth of the fungus and the limited amount of biomass that limits DNA and RNA extractions. In addition our understanding of the resistance mechanisms in the different sources of the resistance/tolerance is lacking. Detailed studies with the different resistant lines would allow us to compare the mechanisms and identify variations that could be used to stack disease resistance. Finally, early screening for FPR in seedlings is an area that is unexplored and could greatly alter this line of research if mechanisms could be developed.

4.9 The Expression Levels of the Various Glycoside Hydrolase Families During FPR

Table 4.3 Glycoside hydrolase protein families of *Moniliophthora roreri*

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK92642.1	GH family1 protein	0	18.65	25.29	12.00	0.47	−1.08	0.27	1.00
ESK95676.1	GH family1 protein	0	10.69	11.49	9.88	0.86	−0.22	0.87	1.00
ESK93576.1	GH family2 protein	0	128.24	27.73	228.75	8.25	3.04	0.25	1.00
ESK97613.1	GH family2 protein	0	138.20	190.38	86.03	0.45	−1.15	0.20	0.98
ESK83675.1	GH family2 protein	0	5.89	4.49	7.29	1.62	0.70	0.75	1.00
ESK88175.1	GH family3 protein	0	1.58	1.40	1.76	1.26	0.33	1.00	1.00
ESK86003.1	GH family3 protein	0	76.43	25.42	127.44	5.01	2.33	0.02	0.36
ESK88732.1	GH family3 protein	0	315.47	248.61	382.34	1.54	0.62	0.60	1.00
ESK82144.1	GH family3 protein	0	59.75	58.50	60.99	1.04	0.06	0.90	1.00
ESK91328.1	GH family3 protein	0	58.80	22.06	95.54	4.33	2.11	0.06	0.72
ESK82910.1	GH family3 protein	0	46.65	18.76	74.53	3.97	1.99	0.07	0.76
ESK86004.1	GH family3 protein	0	42.08	18.23	65.92	3.62	1.85	0.07	0.76
ESK88409.1	GH family3 protein	0	33.87	39.00	28.74	0.74	−0.44	0.58	1.00
ESK84343.1	GH family3 protein	0	19.85	18.53	21.17	1.14	0.19	0.80	1.00
ESK85620.1	GH family3 protein	0	14.29	16.54	12.04	0.73	−0.46	0.94	1.00
ESK90260.1	GH family3 protein	0	13.44	11.78	15.11	1.28	0.36	0.53	1.00
ESK90734.1	GH family3 protein	0	8.43	5.97	10.88	1.82	0.87	0.74	1.00
ESK90244.1	GH family3 protein	0	3.64	4.89	2.39	0.49	−1.03	1.00	1.00
ESK98422.1	GH family3 protein	0	2.82	3.39	2.26	0.67	−0.59	0.64	1.00
ESK91329.1	GH family3 protein	1.00E-91	0.23	0.00	0.46	Inf	Inf	1.00	1.00
ESK93811.1	GH family3 protein	6.00E-89	0.12	0.00	0.23	Inf	Inf	1.00	1.00
ESK93154.1	GH family3 protein	0	10.68	8.58	12.78	1.49	0.57	0.92	1.00
ESK93201.1	GH family5 protein	0	2079.92	3971.14	188.71	0.05	−4.40	0.00	0.00

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK83001.1	GH family5 protein	0	2052.13	3387.89	716.37	0.21	−2.24	0.01	0.30
ESK96962.1	GH family5 protein	0	95.21	27.05	163.37	6.04	2.59	0.01	0.20
ESK85926.1	GH family5 protein	0	61.36	10.81	111.92	10.35	3.37	0.00	0.01
ESK92044.1	GH family5 protein	0	27.96	7.10	48.81	6.87	2.78	0.02	0.35
ESK89845.1	GH family5 protein	0	15.23	4.60	25.86	5.62	2.49	0.16	0.94
ESK95331.1	GH family5 protein	0	2.29	0.70	3.89	5.55	2.47	0.58	1.00
ESK92719.1	GH family5 protein	0	513.86	744.28	283.44	0.38	−1.39	0.10	0.83
ESK83705.1	GH family5 protein	0	145.96	118.90	173.01	1.46	0.54	0.55	1.00
ESK83665.1	GH family5 protein	0	79.77	98.57	60.97	0.62	−0.69	0.35	1.00
ESK94242.1	GH family5 protein	0	60.12	29.90	90.33	3.02	1.59	0.08	0.76
ESK92278.1	GH family5 protein	0	51.55	46.72	56.39	1.21	0.27	0.59	1.00
ESK89493.1	GH family5 protein	0	44.40	64.46	24.34	0.38	−1.41	0.09	0.81
ESK94612.1	GH family5 protein	0	29.21	39.14	19.29	0.49	−1.02	0.35	1.00
ESK91213.1	GH family5 protein	0	24.99	22.99	26.99	1.17	0.23	0.89	1.00
ESK85922.1	GH family5 protein	0	22.78	20.07	25.50	1.27	0.35	0.80	1.00
ESK95295.1	GH family5 protein	0	20.67	8.18	33.16	4.05	2.02	0.35	1.00
ESK95303.1	GH family5 protein	0	16.59	20.91	12.28	0.59	−0.77	0.34	1.00
ESK83709.1	GH family5 protein	0	15.99	9.87	22.10	2.24	1.16	0.40	1.00
ESK89100.1	GH family5 protein	0	9.97	8.69	11.25	1.29	0.37	0.90	1.00
ESK84613.1	GH family5 protein	0	6.66	7.10	6.22	0.88	−0.19	0.80	1.00
ESK92210.1	GH family5 protein	0	6.61	3.50	9.71	2.78	1.47	0.75	1.00
ESK93934.1	GH family5 protein	2.00E-149	5.69	8.10	3.29	0.41	−1.30	0.61	1.00
ESK98068.1	GH family5 protein	0	5.02	6.37	3.66	0.57	−0.80	0.69	1.00
ESK88512.1	GH family5 protein	0	4.05	6.11	2.00	0.33	−1.61	0.78	1.00

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK91570.1	GH family5 protein	2.00E-112	2.95	4.60	1.30	0.28	−1.83	0.36	1.00
ESK84544.1	GH family5 protein	0	2.91	4.20	1.63	0.39	−1.37	0.40	1.00
ESK96875.1	GH family5 protein	0	2.03	1.29	2.76	2.14	1.10	0.76	1.00
ESK90427.1	GH family5 protein	0	0.58	0.00	1.16	Inf	Inf	0.84	1.00
ESK94672.1	GH family5 protein	0	0.23	0.00	0.46	Inf	Inf	1.00	1.00
ESK88117.1	GH family5 protein	0	0.23	0.00	0.46	Inf	Inf	1.00	1.00
ESK88516.1	GH family5 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK97575.1	GH family9 protein	0	36.75	10.35	63.15	6.10	2.61	0.01	0.26
ESK90601.1	GH family10 protein	0	2.65	0.70	4.59	6.56	2.71	0.52	1.00
ESK92006.1	GH family10 protein	2.00E-121	1.82	2.58	1.06	0.41	−1.28	0.88	1.00
ESK93958.1	GH family10 protein	0	12.60	13.37	11.84	0.89	−0.18	1.00	1.00
ESK94267.1	GH family10 protein	0	7.60	11.67	3.52	0.30	−1.73	0.26	1.00
ESK94266.1	GH family10 protein	0	3.51	2.10	4.92	2.34	1.23	0.97	1.00
ESK88650.1	GH family10 protein	9.00E-72	0.90	1.80	0.00	0.00	(-)Inf	0.59	1.00
ESK87274.1	GH family10 protein	0	11.64	0.70	22.57	32.25	5.01	0.04	0.55
ESK95708.1	GH family10 protein	0	0.18	0.00	0.37	Inf	Inf	1.00	1.00
ESK95656.1	GH family10 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK82333.1	GH family12 protein	4.00E-154	8.09	9.79	6.39	0.65	−0.62	0.52	1.00
ESK94127.1	GH family12 protein	3.00E-156	399.01	579.20	218.81	0.38	−1.40	0.23	1.00

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK90292.1	GH family12 protein	9.00E-152	160.41	201.88	118.95	0.59	−0.76	0.41	1.00
ESK82472.1	GH family12 protein	1.00E-146	38.24	55.90	20.59	0.37	−1.44	0.11	0.85
ESK94630.1	GH family13 protein	0	4.60	7.66	1.53	0.20	−2.32	0.26	1.00
ESK94488.1	GH family13 protein	0	103.69	87.05	120.34	1.38	0.47	0.56	1.00
ESK93520.1	GH family13 protein	0	44.04	52.61	35.47	0.67	−0.57	0.79	1.00
ESK89076.1	GH family15 protein	0	33.74	37.31	30.17	0.81	−0.31	0.79	1.00
ESK89077.1	GH family15 protein	2.00E-115	32.49	27.29	37.70	1.38	0.47	1.00	1.00
ESK96359.1	GH family16 protein	2.00E-153	754.35	1276.75	231.95	0.18	−2.46	0.01	0.19
ESK91860.1	GH family16 protein	0	0.76	1.29	0.23	0.18	−2.47	0.84	1.00
ESK91855.1	GH family16 protein	0	0.76	1.29	0.23	0.18	−2.47	0.84	1.00
ESK91106.1	GH family16 protein	0	101.05	3.50	198.60	56.75	5.83	0.00	0.03
ESK95838.1	GH family16 protein	0	20.09	3.98	36.20	9.09	3.18	0.01	0.27
ESK98039.1	GH family16 protein	0	18.76	3.98	33.54	8.43	3.07	0.02	0.36
ESK85953.1	GH family16 protein	0	17.46	0.70	34.22	48.89	5.61	0.00	0.04
ESK95791.1	GH family16 protein	0	14.06	4.60	23.52	5.11	2.35	0.11	0.86
ESK95686.1	GH family16 protein	0	8.89	0.70	17.08	24.41	4.61	0.02	0.36
ESK91795.1	GH family16 protein	0	3.59	0.00	7.18	Inf	Inf	0.06	0.69

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK87930.1	GH family16 protein	0	200.83	80.61	321.05	3.98	1.99	0.04	0.62
ESK91623.1	GH family16 protein	0	123.32	72.36	174.28	2.41	1.27	0.19	0.97
ESK92778.1	GH family16 protein	0	92.57	96.52	88.61	0.92	−0.12	1.00	1.00
ESK91620.1	GH family16 protein	0	32.86	50.00	15.72	0.31	−1.67	0.24	1.00
ESK96764.1	GH family16 protein	0	29.97	11.60	48.35	4.17	2.06	0.15	0.92
ESK95066.1	GH family16 protein	0	28.38	26.44	30.32	1.15	0.20	0.84	1.00
ESK88894.1	GH family16 protein	0	26.56	14.69	38.42	2.61	1.39	0.39	1.00
ESK86240.1	GH family16 protein	0	24.38	9.76	39.00	3.99	2.00	0.06	0.72
ESK83935.1	GH family16 protein	0	18.86	17.68	20.05	1.13	0.18	1.00	1.00
ESK90838.1	GH family16 protein	0	14.99	0.00	29.99	Inf	Inf	0.00	0.01
ESK86385.1	GH family16 protein	0	14.14	13.15	15.13	1.15	0.20	1.00	1.00
ESK96360.1	GH family16 protein	1.00E-147	9.35	10.38	8.32	0.80	−0.32	0.74	1.00
ESK85956.1	GH family16 protein	0	8.54	7.69	9.38	1.22	0.29	0.89	1.00
ESK82846.1	GH family16 protein	0	7.65	7.48	7.82	1.05	0.06	0.67	1.00
ESK85464.1	GH family16 protein	0	4.36	2.10	6.62	3.15	1.66	0.73	1.00
ESK91882.1	GH family16 protein	0	1.51	0.70	2.32	3.32	1.73	0.94	1.00
ESK96457.1	GH family16 protein	0	1.21	0.00	2.43	Inf	Inf	0.51	1.00

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK92442.1	GH family16 protein	0	0.95	0.70	1.20	1.71	0.77	1.00	1.00
ESK91847.1	GH family16 protein	0	0.67	0.00	1.33	Inf	Inf	0.76	1.00
ESK92446.1	GH family16 protein	7.00E-171	0.47	0.70	0.23	0.33	−1.59	0.77	1.00
ESK91857.1	GH family16 protein	6.00E-136	0.18	0.00	0.37	Inf	Inf	1.00	1.00
ESK84358.1	GH family16 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK81085.1	GH family16 protein	1.00E-172	0.00	0.00	0.00	NA	NA	NA	NA
ESK94013.1	GH family17 protein	0	288.06	47.47	528.64	11.14	3.48	0.15	0.92
ESK95077.1	GH family17 protein	0	127.92	67.58	188.27	2.79	1.48	0.16	0.94
ESK89643.1	GH family18 protein	0	6686.06	12684.55	687.56	0.05	−4.21	0.00	0.00
ESK94160.1	GH family18 protein	0	3537.13	6125.47	948.80	0.15	−2.69	0.00	0.14
ESK83904.1	GH family18 protein	0	2581.75	4897.09	266.40	0.05	−4.20	0.00	0.00
ESK93128.1	GH family18 protein	0	291.80	527.31	56.29	0.11	−3.23	0.00	0.05
ESK97512.1	GH family18 protein	0	62.11	17.99	106.22	5.90	2.56	0.00	0.11
ESK89485.1	GH family18 protein	0	7.67	2.10	13.23	6.30	2.66	0.44	1.00
ESK85470.1	GH family18 protein	0	3.34	0.00	6.68	Inf	Inf	0.07	0.74
ESK96283.1	GH family18 protein	0	2.53	0.00	5.05	Inf	Inf	0.18	0.96
ESK93101.1	GH family18 protein	0	7811.80	12287.00	3336.60	0.27	−1.88	0.02	0.42

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK98190.1	GH family18 protein	0	267.74	263.61	271.88	1.03	0.04	0.99	1.00
ESK95147.1	GH family18 protein	0	53.43	19.56	87.31	4.46	2.16	0.05	0.65
ESK90876.1	GH family18 protein	0	23.12	14.18	32.06	2.26	1.18	0.29	1.00
ESK94735.1	GH family18 protein	0	12.56	14.18	10.94	0.77	−0.37	0.74	1.00
ESK95097.1	GH family18 protein	0	11.80	8.21	15.39	1.88	0.91	0.42	1.00
ESK98490.1	GH family18 protein	0	10.43	12.08	8.78	0.73	−0.46	0.91	1.00
ESK96122.1	GH family18 protein	0	9.00	9.68	8.32	0.86	−0.22	0.86	1.00
ESK94384.1	GH family18 protein	0	5.39	1.99	8.79	4.41	2.14	0.31	1.00
ESK96512.1	GH family18 protein	0	2.83	1.80	3.86	2.14	1.10	0.48	1.00
ESK96510.1	GH family18 protein	0	2.05	1.40	2.70	1.93	0.95	1.00	1.00
ESK89292.1	GH family18 protein	0	1.92	1.29	2.56	1.98	0.99	0.80	1.00
ESK83912.1	GH family18 protein	5.00E-168	0.86	0.00	1.73	Inf	Inf	0.64	1.00
ESK91561.1	GH family18 protein	5.00E-79	0.83	0.70	0.96	1.38	0.46	0.98	1.00
ESK91727.1	GH family18 protein	5.00E-162	0.65	0.70	0.60	0.85	−0.23	0.87	1.00
ESK91562.1	GH family18 protein	4.00E-74	0.12	0.00	0.23	Inf	Inf	1.00	1.00
ESK86667.1	GH family18 protein	2.00E-77	0.00	0.00	0.00	NA	NA	NA	NA
ESK83911.1	GH family18 protein	8.00E-72	0.00	0.00	0.00	NA	NA	NA	NA

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK83478.1	GH family20 protein	0	46.85	13.77	79.92	5.80	2.54	0.15	0.93
ESK95657.1	GH family20 protein	0	35.82	47.56	24.07	0.51	−0.98	0.21	0.99
ESK96590.1	GH family23 protein	0	708.24	672.13	744.36	1.11	0.15	0.99	1.00
ESK96753.1	GH family27 protein	0	44.83	31.95	57.71	1.81	0.85	0.22	0.99
ESK91411.1	GH family27 protein	0	14.66	12.75	16.56	1.30	0.38	1.00	1.00
ESK90508.1	GH family27 protein	0	7.08	3.39	10.78	3.18	1.67	0.48	1.00
ESK92386.1	GH family28 protein	0	75.07	129.19	20.94	0.16	−2.63	0.01	0.32
ESK92263.1	GH family28 protein	0	4.48	0.70	8.25	11.79	3.56	0.19	0.97
ESK87991.1	GH family28 protein	0	60.86	87.44	34.28	0.39	−1.35	0.13	0.89
ESK87990.1	GH family28 protein	0	14.02	12.70	15.34	1.21	0.27	0.91	1.00
ESK89371.1	GH family28 protein	0	7.71	7.77	7.65	0.98	−0.02	1.00	1.00
ESK92247.1	GH family28 protein	0	6.08	5.27	6.88	1.31	0.38	0.88	1.00
ESK92387.1	GH family28 protein	0	2.25	0.00	4.49	Inf	Inf	0.19	0.97
ESK87993.1	GH family28 protein	0	0.30	0.00	0.60	Inf	Inf	1.00	1.00
ESK87992.1	GH family28 protein	0	11.14	9.39	12.88	1.37	0.46	0.88	1.00
ESK90546.1	GH family29 protein	0	14.58	11.45	17.70	1.55	0.63	0.50	1.00
ESK89470.1	GH family29 protein	0	10.49	13.07	7.91	0.61	−0.72	0.52	1.00

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK90547.1	GH family29 protein	0	1.21	0.00	2.43	Inf	Inf	0.51	1.00
ESK96732.1	GH family30 protein	0	39.70	11.67	67.72	5.80	2.54	0.04	0.56
ESK89906.1	GH family30 protein	0	17.77	0.00	35.54	Inf	Inf	0.00	0.16
ESK83394.1	GH family30 protein	0	1.18	0.00	2.36	Inf	Inf	0.47	1.00
ESK85316.1	GH family30 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK90799.1	GH family31 protein	0	16.12	19.47	12.78	0.66	−0.61	0.80	1.00
ESK87976.1	GH family31 protein	0	0.77	0.70	0.83	1.19	0.25	0.95	1.00
ESK89413.1	GH family31 protein	0	101.95	109.05	94.85	0.87	−0.20	0.68	1.00
ESK90779.1	GH family31 protein	0	11.83	11.16	12.51	1.12	0.16	1.00	1.00
ESK98043.1	GH family32 protein	0	3.78	2.50	5.05	2.02	1.01	0.59	1.00
ESK93618.1	GH family35 protein	0	33.98	9.17	58.79	6.41	2.68	0.21	0.98
ESK83683.1	GH family35 protein	0	9.86	0.00	19.71	Inf	Inf	0.00	0.06
ESK86579.1	GH family35 protein	0	81.32	33.92	128.71	3.79	1.92	0.04	0.61
ESK88391.1	GH family35 protein	0	74.42	65.75	83.10	1.26	0.34	0.81	1.00
ESK91113.1	GH family35 protein	0	34.38	42.85	25.92	0.60	−0.73	0.47	1.00
ESK87049.1	GH family35 protein	0	6.97	7.15	6.78	0.95	−0.08	1.00	1.00
ESK91112.1	GH family35 protein	0	3.63	3.09	4.16	1.35	0.43	0.71	1.00

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK87034.1	GH family35 protein	0	0.57	0.00	1.13	Inf	Inf	0.85	1.00
ESK82299.1	GH family35 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK93769.1	GH family35 protein	1.00E-105	31.67	7.99	55.35	6.93	2.79	0.13	0.88
ESK92520.1	GH family37 protein	8.00E-71	16.98	0.70	33.26	47.52	5.57	0.10	0.84
ESK87469.1	GH family37 protein	0	279.60	426.49	132.71	0.31	−1.68	0.06	0.71
ESK84799.1	GH family37 protein	0	26.37	29.51	23.24	0.79	−0.34	0.67	1.00
ESK96763.1	GH family38 protein	0	140.45	186.38	94.52	0.51	−0.98	0.22	1.00
ESK82845.1	GH family39 protein	9.00E-178	1.53	1.99	1.06	0.53	−0.90	0.80	1.00
ESK82851.1	GH family39 protein	2.00E-162	3.55	4.57	2.53	0.55	−0.86	0.77	1.00
ESK91698.1	GH family43 protein	0	144.29	234.21	54.37	0.23	−2.11	0.02	0.43
ESK91917.1	GH family43 protein	0	16.49	17.21	15.78	0.92	−0.13	0.72	1.00
ESK84267.1	GH family43 protein	0	4.29	4.68	3.90	0.83	−0.26	0.66	1.00
ESK86744.1	GH family43 protein	0	22.83	15.47	30.20	1.95	0.96	0.67	1.00
ESK88955.1	GH family43 protein	6.00E-166	4.73	4.90	4.55	0.93	−0.11	0.62	1.00
ESK88956.1	GH family43 protein	1.00E-94	0.12	0.00	0.23	Inf	Inf	1.00	1.00
ESK91370.1	GH family43 protein	0	2.21	1.40	3.03	2.16	1.11	1.00	1.00
ESK90496.1	GH family47 protein	0	83.97	117.61	50.34	0.43	−1.22	0.18	0.96

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK97770.1	GH family47 protein	0	33.92	43.63	24.20	0.55	−0.85	0.47	1.00
ESK93354.1	GH family47 protein	0	32.03	43.58	20.48	0.47	−1.09	0.18	0.96
ESK92882.1	GH family47 protein	0	24.31	15.25	33.36	2.19	1.13	0.44	1.00
ESK85836.1	GH family47 protein	0	13.04	20.30	5.79	0.28	−1.81	0.20	0.98
ESK87392.1	GH family47 protein	0	10.38	10.68	10.08	0.94	−0.08	1.00	1.00
ESK85837.1	GH family47 protein	0	9.94	10.06	9.81	0.98	−0.04	0.72	1.00
ESK85838.1	GH family47 protein	0	2.72	4.38	1.06	0.24	−2.04	0.68	1.00
ESK91159.1	GH family51 protein	0	357.50	571.78	143.23	0.25	−2.00	0.02	0.44
ESK87073.1	GH family51 protein	0	113.49	44.37	182.61	4.12	2.04	0.13	0.88
ESK92058.1	GH family53 protein	0	45.11	2.80	87.42	31.22	4.96	0.02	0.42
ESK95726.1	GH family53 protein	0	18.37	3.98	32.76	8.23	3.04	0.02	0.37
ESK94770.1	GH family53 protein	3.00E-129	1.71	1.29	2.13	1.65	0.72	0.84	1.00
ESK83301.1	GH family53 protein	3.00E-141	1.12	0.70	1.53	2.19	1.13	1.00	1.00
ESK95736.1	GH family53 protein	6.00E-163	0.30	0.00	0.60	Inf	Inf	1.00	1.00
ESK92869.1	GH family55 protein	0	25.34	29.43	21.25	0.72	−0.47	0.56	1.00
ESK90642.1	GH family55 protein	0	16.20	20.33	12.08	0.59	−0.75	0.65	1.00
ESK92896.1	GH family55 protein	0	5.90	3.20	8.60	2.69	1.43	0.52	1.00

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK93339.1	GH family61 protein	0	24.05	7.26	40.84	5.62	2.49	0.07	0.74
ESK86393.1	GH family61 protein	4.00E-135	21.54	0.00	43.07	Inf	Inf	0.01	0.20
ESK86392.1	GH family61 protein	1.00E-134	13.29	0.00	26.58	Inf	Inf	0.01	0.27
ESK84070.1	GH family61 protein	3.00E-124	3.53	0.00	7.06	Inf	Inf	0.07	0.73
ESK95156.1	GH family61 protein	5.00E-148	2.91	0.00	5.82	Inf	Inf	0.10	0.84
ESK93407.1	GH family61 protein	0	39.76	17.86	61.65	3.45	1.79	0.11	0.86
ESK95700.1	GH family61 protein	5.00E-73	7.93	10.43	5.42	0.52	−0.94	0.71	1.00
ESK95699.1	GH family61 protein	5.00E-135	5.00	5.49	4.52	0.82	−0.28	0.58	1.00
ESK94064.1	GH family61 protein	8.00E-137	4.57	4.89	4.25	0.87	−0.20	0.78	1.00
ESK89694.1	GH family61 protein	9.00E-143	4.36	5.86	2.86	0.49	−1.03	0.70	1.00
ESK86500.1	GH family61 protein	1.00E-124	3.80	6.29	1.30	0.21	−2.28	0.27	1.00
ESK88334.1	GH family61 protein	0	2.74	1.29	4.19	3.25	1.70	0.50	1.00
ESK97120.1	GH family61 protein	1.00E-142	2.18	3.20	1.16	0.36	−1.46	0.54	1.00
ESK91695.1	GH family61 protein	7.00E-145	1.63	2.10	1.16	0.55	−0.85	0.58	1.00
ESK85191.1	GH family61 protein	6.00E-144	1.05	2.10	0.00	0.00	(-)Inf	0.24	1.00
ESK95698.1	GH family61 protein	4.00E-137	1.00	0.00	2.00	Inf	Inf	0.56	1.00
ESK86397.1	GH family61 protein	2.00E-133	0.65	1.29	0.00	0.00	(-)Inf	0.57	1.00

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK86498.1	GH family61 protein	2.00E-130	0.28	0.00	0.57	Inf	Inf	1.00	1.00
ESK92657.1	GH family61 protein	2.00E-136	0.12	0.00	0.23	Inf	Inf	1.00	1.00
ESK92658.1	GH family61 protein	2.00E-150	0.12	0.00	0.23	Inf	Inf	1.00	1.00
ESK86499.1	GH family61 protein	5.00E-125	0.12	0.00	0.23	Inf	Inf	1.00	1.00
ESK83440.1	GH family61 protein	3.00E-145	0.00	0.00	0.00	NA	NA	NA	NA
ESK88398.1	GH family61 protein	0	43.55	13.48	73.62	5.46	2.45	0.04	0.61
ESK97413.1	GH family63 protein	0	45.77	40.00	51.55	1.29	0.37	0.78	1.00
ESK98183.1	GH family72 protein	0	181.86	72.70	291.02	4.00	2.00	0.03	0.47
ESK83724.1	GH family74 protein	0	7.19	5.30	9.07	1.71	0.78	0.83	1.00
ESK92894.1	GH family74 protein	5.00E-115	1.22	0.00	2.43	Inf	Inf	0.48	1.00
ESK83499.1	GH family76 protein	0	7.03	12.67	1.39	0.11	−3.18	0.13	0.89
ESK87721.1	GH family76 protein	0	3.65	6.70	0.60	0.09	−3.49	0.11	0.85
ESK95306.1	GH family76 protein	0	2.28	3.87	0.70	0.18	−2.47	0.70	1.00
ESK88190.1	GH family76 protein	0	6.49	0.70	12.28	17.55	4.13	0.11	0.87
ESK93152.1	GH family76 protein	0	5.02	1.29	8.74	6.78	2.76	0.13	0.89
ESK87723.1	GH family76 protein	0	7.96	4.79	11.13	2.32	1.22	0.69	1.00
ESK84823.1	GH family76 protein	0	4.80	7.80	1.79	0.23	−2.12	0.19	0.97

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK90026.1	GH family76 protein	0	4.04	4.79	3.29	0.69	−0.54	0.46	1.00
ESK85221.1	GH family76 protein	0	3.94	2.10	5.78	2.75	1.46	0.80	1.00
ESK84824.1	GH family76 protein	0	2.53	3.20	1.86	0.58	−0.78	0.78	1.00
ESK84833.1	GH family76 protein	0	1.62	1.80	1.43	0.79	−0.33	0.94	1.00
ESK87722.1	GH family76 protein	0	1.13	0.70	1.56	2.23	1.16	1.00	1.00
ESK90021.1	GH family76 protein	0	0.90	1.80	0.00	0.00	(-)Inf	0.59	1.00
ESK83076.1	GH family76 protein	0	0.82	0.70	0.93	1.33	0.41	0.94	1.00
ESK85560.1	GH family76 protein	0	0.70	0.00	1.40	Inf	Inf	0.74	1.00
ESK89511.1	GH family76 protein	0	0.65	0.70	0.60	0.85	−0.23	0.87	1.00
ESK83075.1	GH family76 protein	0	0.35	0.70	0.00	0.00	(-)Inf	0.62	1.00
ESK90370.1	GH family76 protein	0	0.18	0.00	0.37	Inf	Inf	1.00	1.00
ESK90017.1	GH family76 protein	0	0.18	0.00	0.37	Inf	Inf	1.00	1.00
ESK84821.1	GH family76 protein	0	0.18	0.00	0.37	Inf	Inf	1.00	1.00
ESK91118.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK90374.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK90375.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK89893.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK88859.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK87807.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK87810.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK87139.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK86993.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK86835.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK85703.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK83500.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK83436.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK83346.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK83137.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK82968.1	GH family76 protein	0	0.00	0.00	0.00	NA	NA	NA	NA
ESK87374.1	GH family78 protein	0	12.54	5.97	19.11	3.20	1.68	0.28	1.00
ESK86421.1	GH family78 protein	0	2.14	1.29	2.99	2.32	1.21	0.67	1.00
ESK87372.1	GH family78 protein	0	1.12	1.40	0.83	0.59	−0.75	0.66	1.00
ESK84757.1	GH family79 protein	0	4.17	0.00	8.34	Inf	Inf	0.06	0.69
ESK89020.1	GH family79 protein	0	24.51	25.71	23.31	0.91	−0.14	1.00	1.00

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK94670.1	GH family79 protein	0	15.29	19.70	10.87	0.55	−0.86	0.32	1.00
ESK95563.1	GH family79 protein	0	6.98	3.79	10.17	2.68	1.42	0.29	1.00
ESK88247.1	GH family79 protein	0	6.56	7.07	6.06	0.86	−0.22	0.95	1.00
ESK93591.1	GH family79 protein	0	1.89	0.00	3.79	Inf	Inf	0.24	1.00
ESK87768.1	GH family85 protein	0	22.66	28.16	17.16	0.61	−0.71	0.56	1.00
ESK94258.1	GH family88 protein	0	107.91	144.22	71.61	0.50	−1.01	0.26	1.00
ESK94259.1	GH family88 protein	0	22.62	28.51	16.74	0.59	−0.77	0.43	1.00
ESK94271.1	GH family88 protein	0	40.11	49.50	30.72	0.62	−0.69	0.42	1.00
ESK97557.1	GH family88 protein	0	24.92	32.81	17.02	0.52	−0.95	0.45	1.00
ESK83461.1	GH family92 protein	0	1.41	0.00	2.83	Inf	Inf	0.39	1.00
ESK92641.1	GH family92 protein	0	3.72	0.00	7.45	Inf	Inf	0.05	0.66
ESK84677.1	GH family92 protein	0	1915.31	2935.90	894.72	0.30	−1.71	0.05	0.62
ESK91898.1	GH family92 protein	0	53.30	72.90	33.71	0.46	−1.11	0.32	1.00
ESK92075.1	GH family92 protein	0	39.27	35.28	43.25	1.23	0.29	0.71	1.00
ESK89472.1	GH family92 protein	0	20.54	14.66	26.42	1.80	0.85	0.38	1.00
ESK84879.1	GH family92 protein	3.00E-54	0.00	0.00	0.00	NA	NA	NA	NA
ESK84880.1	GH family92 protein	6.00E-44	0.00	0.00	0.00	NA	NA	NA	NA

(continued)

Table 4.3 (continued)

Gene ID	Gene annotation		Base mean	Base mean 30 days	Base mean 60 days	Fold change	log2 Fold Change	pval	padj
ESK91919.1	GH family95 protein	0	51.38	41.77	60.99	1.46	0.55	0.55	1.00
ESK93723.1	GH family105 protein	0	4.98	0.70	9.25	13.22	3.72	0.14	0.92
ESK94701.1	GH family105 protein	0	2.13	0.70	3.55	5.08	2.34	0.65	1.00
ESK85073.1	GH family105 protein	0	10.12	9.90	10.34	1.04	0.06	0.82	1.00
ESK93603.1	GH family105 protein	0	3.14	1.29	4.99	3.86	1.95	0.38	1.00
ESK93295.1	GH family115 protein	1.00E-104	4.43	0.00	8.86	Inf	Inf	0.27	1.00
ESK93287.1	GH family115 protein	0	43.42	20.15	66.69	3.31	1.73	0.08	0.79

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Chapter 5
Witches' Broom Disease (*Moniliophthora perniciosa*): History and Biology

Harry C. Evans

Abstract The history of witches' broom disease is cataloged with special reference to its socioeconomic and ecological impact on the countries and regions affected. In particular, focus is placed on the Brazilian States of Rondônia and Bahia and the disastrous political events that have shaped their histories. The changing taxonomic status of the fungal causal agent—especially, its current placement in the genus *Moniliophthora* and its relationship to *M. roreri*—is documented and discussed. Like the frosty pod rot pathogen, *M. perniciosa* is hemibiotrophic with well-defined parasitic and saprophytic phases: separated morphologically, physiologically, and genetically. The symptomatology is described and illustrated in cacao and its relatives in the Malvales, as well as in the plant orders in which other hosts of the pathogen have been confirmed (Malpighiales and Solanales). The occurrence of a nonpathogenic biotype in the Bignoniaceae (Lamiales) is discussed in relation to the evolution of the pathotypes from benign to malign endophytes of woody plants. The etiology of the disease remains a matter of conjecture and there is circumstantial evidence indicating that the fungus may have developed a symbiotic association with another microorganism to facilitate invasion and disrupt host physiology. The only infective spore is the sexual basidiospore produced in the basidioma (mushroom) and spores are forcibly released and air dispersed over considerable distances as the temperature falls and the humidity rises during the night. The full life cycle of the cacao pathogen is described and illustrated schematically, and compared to that of the nonpathogenic L-biotype on Bignoniaceae. Finally, the areas of research that need to be addressed are prioritized.

5.1 History of the Disease

This section charts the early history of the disease in the leading cacao-producing countries at the time and reviews its subsequent impact on cacao cultivation, as well as the wider socioeconomic and ecological implications, for each country.

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Surinam

"*The severe loss which the witch-broom disease has caused to the cacao industry of Dutch Guiana, coupled with the fact that, though very probably of fungous origin, it has never been produced by artificial inoculation, make its study one of the most interesting of tropical pathological problems, from both the practical and scientific standpoints*" (Rorer 1913). This situation has continued until the present day in all the countries afflicted by this enigmatic disease.

Unlike frosty pod rot, witches' broom disease of cacao coevolved with its host in the Amazon basin. And, as stated by Rorer (1913)—who was, of course, historically linked with both diseases (see Chap. 3)—the causal agent remained a mystery for some considerable time after it became problematic in the plantations of Surinam in north-east South America in 1895, which led to the first scientific investigation of the disease (Ritzema Bos 1900, 1901). The early history of the new malady of cacao—known locally as "Krulloten" disease—that was sweeping through the plantations inducing gross distortion and malformation of the meristematic tissues, and resulting in the diagnostic witches' brooms (see Fig. 5.1), was documented by van Hall (1914). Initially, expert help was sought from abroad, and samples were sent to mycologists in the Netherlands, the UK, and the USA, but opinions about the identity of the causal agent were conflicting: *Fusarium* (Howard 1901), *Lasiodiplodia* (Charles 1906), and a new species of the ascomycete genus *Exoascus*, provisionally named as *E. theobromae* (Ritzema Bos 1900, 1901). The Dutch mycologist F.A. Went visited Surinam and published the first illustrations of the symptoms of the witches' brooms, as well as of the diseased or indurated pods (previously considered to be due to *Phytophthora* or black pod disease). Detailed drawings of the mycelium in green brooms clearly show intercellular colonization by the highly specialized (monokaryotic, endophytic) parasitic mycelium (Went 1904; see Figs. 5.2d and 5.3)—a defining characteristic of the pathogen which was not investigated until many decades later (Evans 1980)—although this differed radically from the mycelium obtained in pure culture and which failed to induce symptoms when inoculated into cacao plants both in the field in Surinam and in the greenhouse in the Netherlands. This was followed by a more sustained study over several years by scientists resident in Surinam whose field observations indicated that an anthracnose was consistently associated with diseased tissues, especially the pods, and that "Colletotrichum *fruiting bodies were the only fructifications formed in cultures of the witch-broom fungus*" (van Hall and Drost 1909). The new species *Colletotrichum luxificum* was described and proposed as the causal agent in two comprehensive publications, including full disease symptoms—as well as the economic impact (van Hall and Drost 1907, 1909)—despite the fact that Koch's postulates were never proven. Moreover, evidence from histological studies revealed colonization of the living brooms and pods by the distinctive intercellular mycelium, previously reported by Went (1904), which is not typical of essentially necrotrophic fungi, such as *Colletotrichum*, although van Hall and Drost (1909) assumed that this belonged to *C. luxificum*. The disease was also linked with an

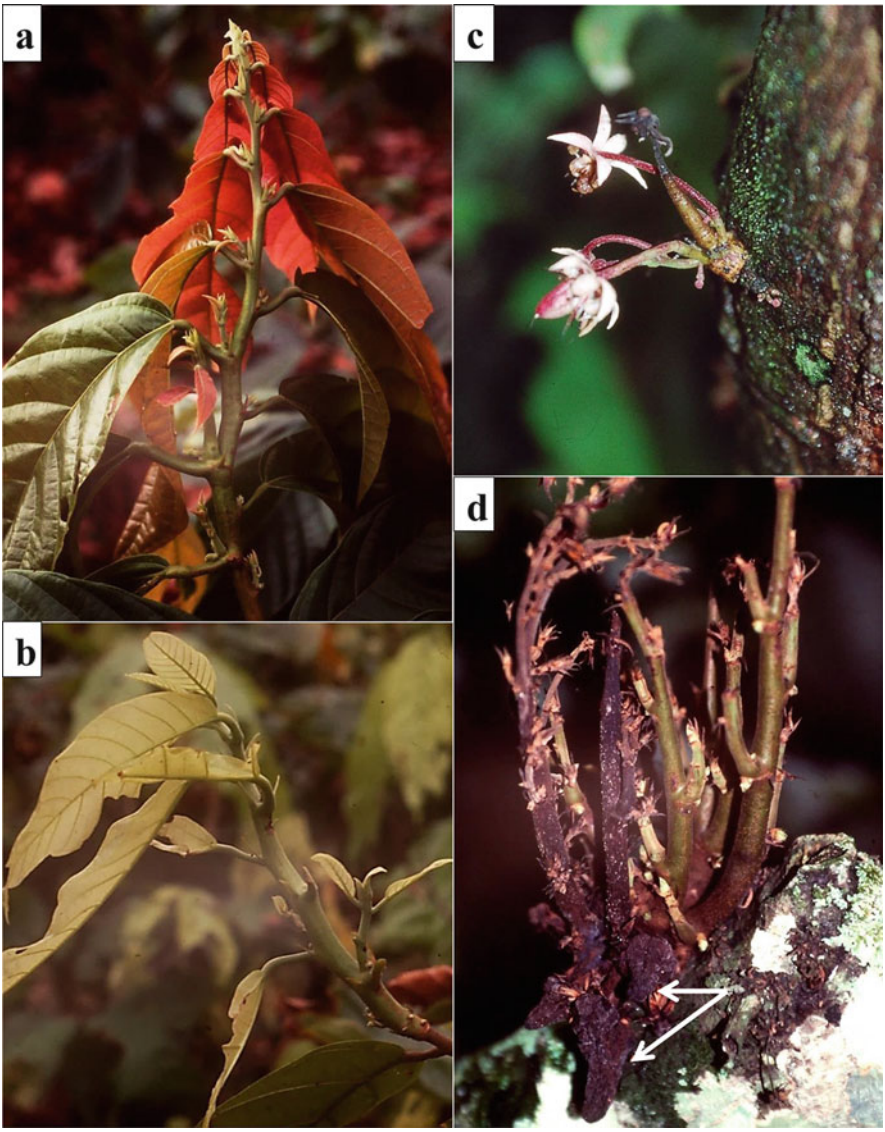

Fig. 5.1 Infection on cacao shoots and flower cushions. (**a**) Terminal broom with pigmented (anthocyanin) flush leaves. (**b**) Lateral broom showing non-pigmented flush leaves of "mutant" Catongo clone. (**c**) First symptoms on flower cushion, showing swollen flower stalk amongst healthy flowers. (**d**) Multiple cushion brooms in varying stages of necrosis with necrotic, strawberry-shaped parthenocarpic pods in foreground (*arrows*)

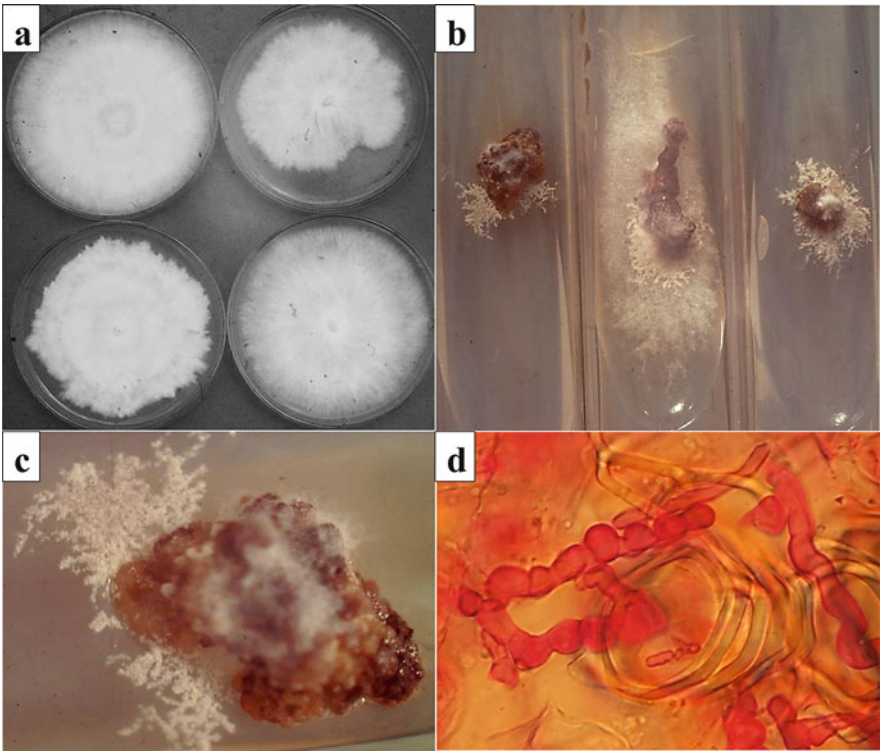

Fig. 5.2 Pleomorphism of *Moniliophthora perniciosa*. (**a**) Dikaryotic, saprophytic phase in culture, isolated from necrotic brooms of various *Theobroma* species; note considerable variation in colony morphology (compare also Fig. 5.4e). (**b**) Monokaryotic, parasitic phase produced on cacao callus—3 months after inoculation with basidiospores—note the callus in the central tube is less active (aging) and the dikaryotic mycelium is colonizing and overrunning the callus and spreading rapidly onto the agar. (**c**) Detail of monokaryotic mycelium spreading around callus, superficially on agar surface. (**d**) Swollen, aseptate, intercellular monokaryotic mycelium from squash of internal tissues of green, infected cacao pod

indigenous forest tree, and van Hall (1910) concluded that "*the wild* Theobroma speciosum *is still more liable to the disease than the ordinary cacao*," further noting that "*the mycelium of the* Colletotrichum luxificum *was found easily on microscopical examination*." Subsequently, Massee (1910) included a full description of *Colletotrichum luxificum* as the pathogen responsible for "*witches' brooms of cacao*" in his book, *Diseases of Cultivated Plants and Trees*. However, it was obvious that this diagnosis was not accepted universally since investigations continued, leading Rorer (1910) to test the pathogenicity of a species of *Colletotrichum* cf. *luxificum* isolated from "*abnormal growths*" on cacao in Trinidad, with negative results. Later, Rorer (1913) visited Surinam and availed himself of the opportunity to isolate from cacao brooms and pods. He noted that the hyphae in cultures from diseased tissues (brooms and pods) consistently produced clamp connections—

found only in the dikaryotic mycelium of the Basidiomycota (Kirk et al. 2008)—and, therefore, that the true pathogen must be an unknown basidiomycete fungus. Nevertheless, inoculations with this mycelium, as well as with spores of *Colletotrichum luxificum*, failed to reproduce the disease symptoms.

Rorer's supposition proved to be correct when Gerold Stahel in 1915—who had arrived in Surinam only the year before—found that if necrotic witches' brooms, bearing pink to crimson mushrooms (basidiomata; see Figs. 5.3, 5.4, and 5.5), were suspended above cacao seedlings, typical disease symptoms developed. Basically,

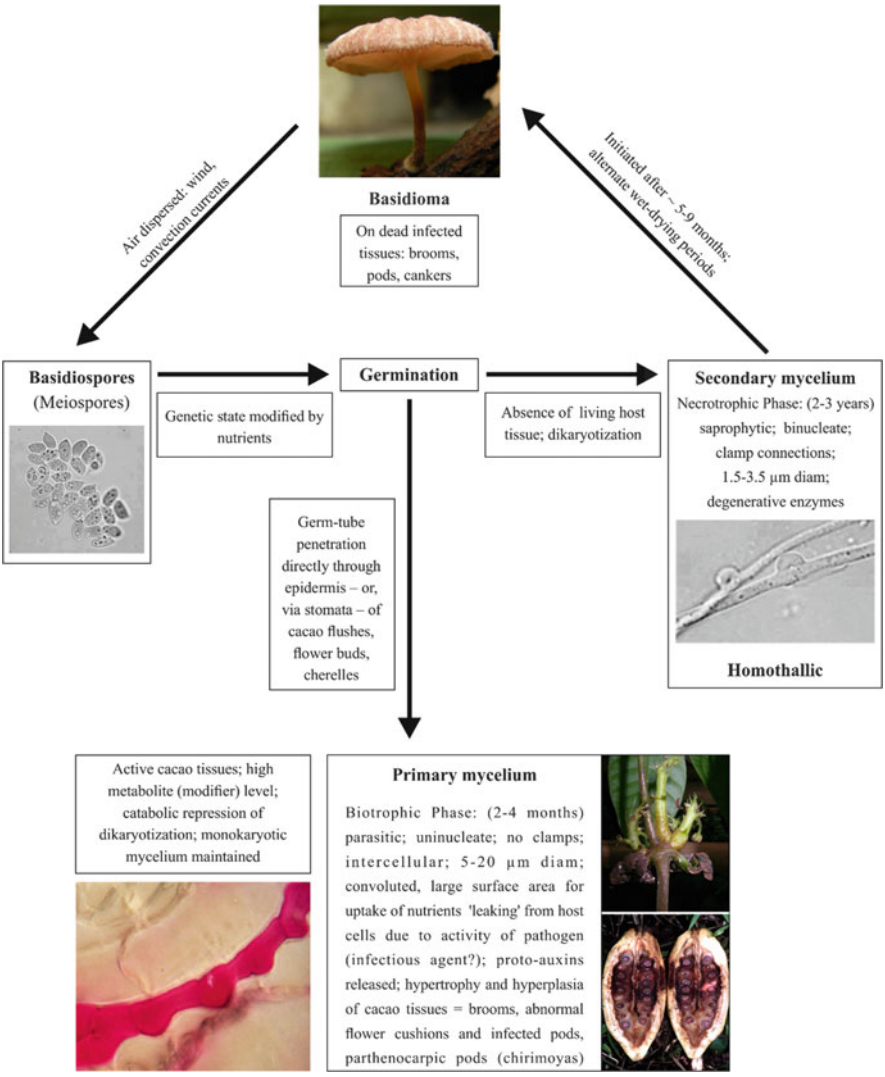

Fig. 5.3 Schematic life cycle of *Moniliophthora perniciosa* on cacao [Modified from Evans (1980)]

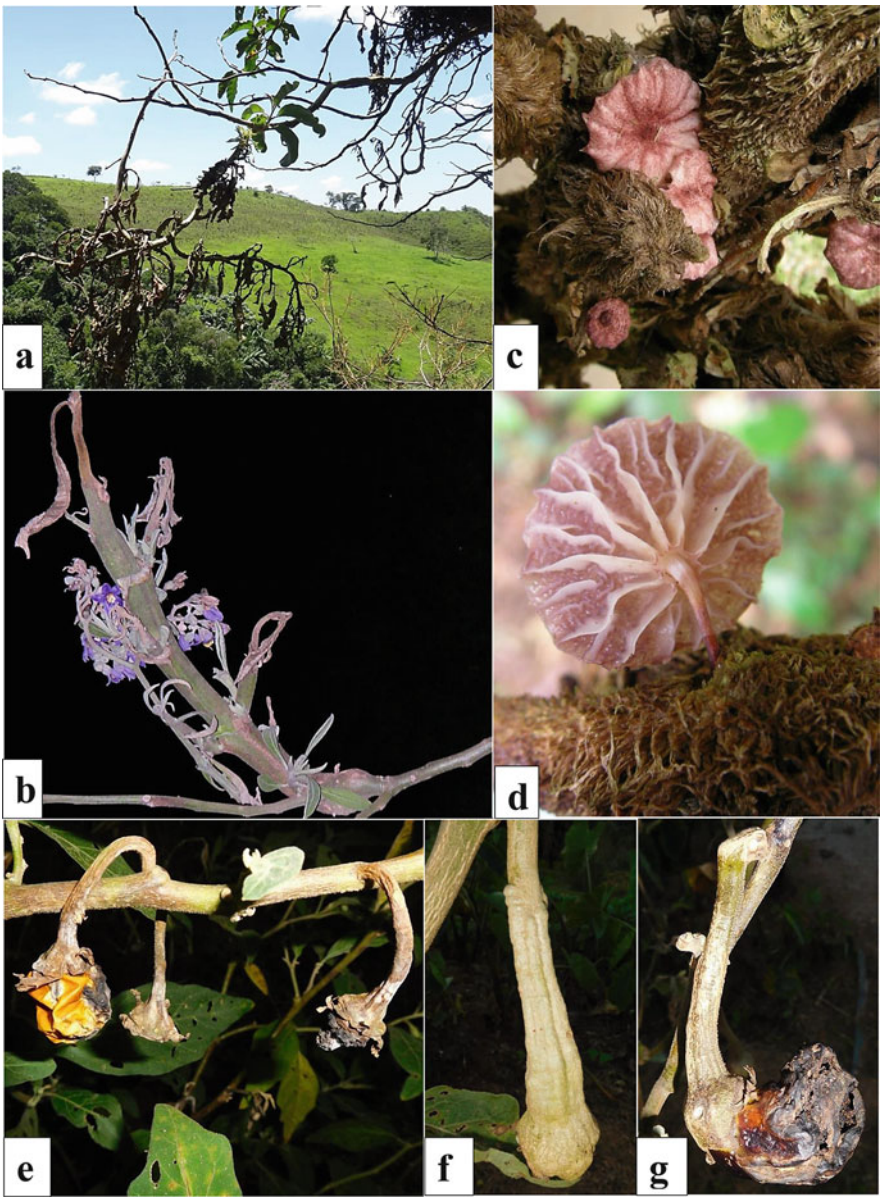

Fig. 5.4 *Solanum* hosts. (**a**) Large pendulous brooms on *Solanum lycocarpum* ("lobeira") in pasture, Minas Gerais, Brazil. (**b**) Flowering broom on *Solanum mauritianum*, in secondary forest, Minas Gerais, Brazil. (**c**) Typical pink mushrooms (basidiomata) on hirsute broom of *Solanum cernuum*—"braço-de-preguiça," so-called because it resembles a sloth's arm—in Atlantic rainforest, Minas Gerais, Brazil. (**d**) Lower surface of basidioma on *S. cernuum*; note the hirsute broom surface. (**e**) Group of naturally infected parthenocarpic fruits (chirimoyas) of *S. gilo* in vegetable plot, Minas Gerais, Brazil. (**f**–**g**) Naturally infected fruits of *S. melongena* (aubergine), showing swollen woody petiole or fruit stalk (**f**), and a parthenocarpic fruit (**g**), same habitat as above (**e**)

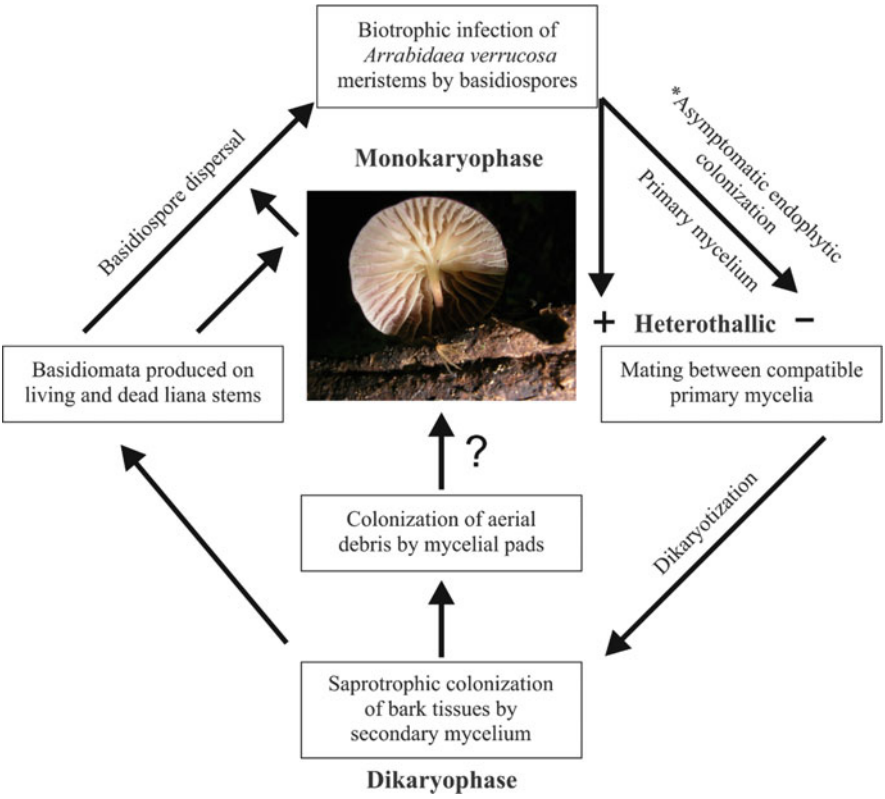

Fig. 5.5 Schematic life cycle of *Moniliophthora perniciosa* L-biotype, on Bignoniaceae [Modified from Griffith et al. (1994)]. *Note: Griffith et al. (1994) described this phase as "Saprotrophic growth of primary mycelia in liana bark tissues." However, there is no doubt that this is, in fact, the monokaryotic, intercellular mycelium living in a "benign"—rather than a malign (parasitic)—endophytic association with its host. At that time the concepts of endophytes and the status of such symbiotic associations were still poorly understood (Rodriguez et al. 2009)

Stahel had completed the first part of Koch's postulates, but he would have needed to wait another year before the fruiting bodies appeared on the inoculated material in order to complete the postulates. Nevertheless, he named the causal fungus *Marasmius perniciosus* (Stahel 1915): a novel agaric species producing its mushrooms only on the dead host tissues, including necrotic pods, as well as on the brooms. Later, Stahel (1919, 1932) published a more comprehensive account of the disease—with recommendations for its management and details of its economic impact—and of the biology of the causal agent, as well as the completion of Koch's postulates, which has been little bettered since.

In addition, Stahel (1915, 1919) also recorded the disease on other species of *Theobroma* (*T. bicolor* and *T. speciosum*), confirming the earlier observations of van Hall (1910), and he concluded that these wild populations—including

T. cacao—were the original hosts of witches' broom disease, although, following inoculation, he considered that *T. bicolor* showed resistance in the form of hypersensitivity (Stahel 1919). Later, Myers (1930) found wild populations of cacao heavily infected with witches' broom disease, deep in the hinterland of Surinam.

When witches' broom disease first appeared in Surinam, the cacao industry was vibrant and in expansion, but the subsequent decline in yields led to the abandonment of some plantations and diversification into alternative crops, such as banana and coffee, and, according to Holliday (1952), to the extinction of the industry. However, other factors may have played a part and, in fact, cacao cultivation has survived and has even been considered as a viable commercial prospect with improved control methods and new varieties (Stahel 1945; Thorold 1975).

Trinidad

Witches' broom did not reach Trinidad until the late 1920s (Stell 1928), when cacao exports were around 30,000 metric tons; however, within a decade, these had fallen to less than 8000 tons (Padwick 1956). This coincided with the depression years and plummeting prices for cacao, so that the growers failed to implement the emergency control measures recommended shortly after the arrival of the disease (Briton-Jones and Cheesman 1931), which, in fact, had proved to be ineffective as well as uneconomical (Baker and Holliday 1957). Thus, began an intensive period of research on all aspects of the disease—in order to improve its management—but, in particular, to breed for disease resistance, culminating in a series of publications, summarized by Baker and Crowdy (1943, 1944). The focal point of this research was the cacao selections brought back from collecting trips to the Iquitos region of Peru and the Napo-Putumayo river system of Ecuador (Pound 1938, 1943). Since Frederick J. Pound had encountered an increasing diversity of cacao varieties—as well as evidence of resistance to witches' broom disease—as he surveyed westwards in the Amazon basin, Cheesman (1944) posited that the Upper Amazon region is the center of origin of cacao and that natural selection for disease resistance evolved here. Indeed, a high level of resistance was demonstrated in these Imperial College Selections (ICS) when they were tested in Trinidad, most famously in the Marper (derived from: *Marasmius perniciosus*) farm. Immune or resistant selections, most notably the Scavinas from around Iquitos, formed the basis of new plantings in Trinidad, and, much later, in the ill-fated colonization program in the Brazilian Amazon (see below). This resistance held up in Trinidad, but the golden age of the big cacao estates was over as the country moved from an agricultural- to an oil-based economy. Nevertheless, these collections have continued to supply and be pivotal to cacao breeding programs across the tropics.

Ecuador

During most of the nineteenth and up to the early part of the twentieth century, Ecuador was the foremost producer of cacao, famed for its high quality—the so-called Arriba cacao, based on the indigenous variety Nacional, from the upper Guayas basin in Los Rios Province—which was much sought after on the world market (Bartley 2005). Vast haciendas of almost contiguous Nacional were established under thinned forest. However, from the 1890s onwards other cacao types, predominantly Trinitarios, were introduced and integrated with the Nacional and a hybrid complex resulted (Evans et al. 1977). This was the heyday of the cacao millionaires—who, allegedly, even sent their laundry to Paris—as manifested by a smaller replica of the Eiffel Tower which dominates the center of the quietly decaying town of Vinces. Tragically, the introduction of the Trinitario varieties also coincided with the appearance of frosty pod disease (see Chap. 3), and to the start of the decline of the cacao industry, hastened by the arrival of witches' broom disease in the early 1920s (Rorer 1926). It is now beyond doubt that both diseases were accidental introductions by man: frosty pod from northern Colombia (Phillips-Mora et al. 2007) and witches' broom from the Amazonian or Oriente region of Ecuador, and it has been speculated that it was transported with planting material along a land trail from the Napo River in the Oriente over the Andean cordillera to the Pacific coastal region of Balao in south-western Ecuador, where the disease was first reported (Baker and Holliday 1957). J.B. Rorer, of course, was familiar with the latter disease (see above), and, during his later visit to Ecuador in 1918, he described for the first time frosty pod disease (Rorer 1918), but made no mention of witches' broom. Clearly, it was not present then—at least, not in the main cacao-growing region of Los Rios—and it must have arrived somewhere between 1918 and Rorer's subsequent visit in 1925 (Rorer 1926). The combined impact of the two diseases led to the abandonment of many estates and to diversification into other plantation crops, notably banana. It has been estimated that pre-1915, Ecuador exported over 40,000 metric tons of high-quality Arriba cacao per annum—with yields approaching 1000 kg/ha—but this fell to less than 15,000 metric tons by 1925 (Pound 1938; Evans 2002). Cacao grown under traditional management systems is said to produce between 150 and 300 kg/ha (Evans et al. 1977).

Cacao clones resistant to witches' broom under Trinidadian conditions were imported over the following decades, but resistance quickly broke down, supporting the conclusion reached earlier by Stahel that the fungus in Ecuador is different from that in north-eastern South America, noting distinctive morphological traits, in particular, the darker red color of the mushrooms, and he erected a new variety, var. *ecuadoriensis* (Stahel 1924). Cacao cultivation is now mainly in the hands of smallholders, with mixed cropping systems and subsistence farming, but also with some investment in plantation cacao using new clones resistant to witches' broom, as well as material derived from the local Nacional cacao. Nacional has always exhibited some degree of tolerance to frosty pod disease, which is probably associated with the thick pod husk (Bartley 2005).

Brazil

Viera (1942) and Silva (1987) presented compelling evidence that witches' broom disease had been described much earlier in cacao farms established by Portuguese colonists in the Brazilian Amazon, more than a century before the outbreaks reported in Surinam. The account of the disease by the natural historian Alexandre Ferreira—following a 2-year exploration of the lower Amazon and the Rio Negro, from 1785 to 1787—matches that of witches' broom, which garnered the local name "lagartão," based on the sinuous or lizard-like nature of the green brooms. The disease was said to be common and a serious constraint to cacao production in the upper Rio Negro, but not along the lower Amazon. This historical view of witches' broom disease as a limiting factor to the cultivation of cacao in Amazonia continued into the late twentieth century, and all the efforts of the autonomous cacao organization CEPLAC (Commissão Executiva do Plano da Lavoura Cacaueira) were channeled into the Bahian region of eastern Brazil. This resulted in a highly successful industry—producing ~ 400,000 metric tons of cacao per annum, making it second in the world producer's list—centered on a high-profile, well-funded research infrastructure, with an efficient extension service and its own port for cacao exports. In Bahia, the rustic or "cabruca" cacao-cultivation system (Rice and Greenberg 2000)—complementing the Atlantic rainforest under which the cacao was grown—benefitted the ecology of the region, as well as offering a highly lucrative crop for the owners of the large "fazendas" and a stable socio-economic climate, enhanced by the many CEPLAC outreach programs in education, health, and general agriculture. Bahia was far removed from the witches' broom hotspot of Amazonia—buffered by thousands of kilometers of the arid to semiarid, spiny forests of the unique Cerrado and Caatinga ecosystems—and subject "only" to the toll taken by black pod disease.

This was the "idyllic" situation until the early 1970s, when politics intervened, in more ways than one. The first political maneuverings were in 1970 when the Brazilian President, General Emilio Medici, shocked by the poverty in the north-east brought about by persistent droughts, decreed that land would be freed up for colonization and farming by construction of a road bisecting Amazonia from east to west—thus, the ill-fated Trans-Amazonian Highway was conceived and implemented as a matter of national urgency. Cacao, because of its ecological, economic, and sustainability credentials, was chosen as a key crop for the immigrant farmers, mainly from north-east Brazil: on the assurances, of course, that CEPLAC could supply material resistant to witches' broom from its extensive germplasm collection in Bahia. The grand scheme was for Brazil to become the biggest producer of cacao in the World, achieving one million metric tons per annum: 600,000 from Amazonia and 400,000 from Bahia.

Pods from witches' broom "resistant" clones and hybrids—mostly derived from the Pound collections in Trinidad (see above)—were transported to cacao centers (Polos Cacaueiros) established by CEPLAC, typically on some of the best soils ("terra roxa") in the Amazon basin, along the new Highway, often taking up to a

week to reach the remotest settlements. The pods were pretreated with systemic fungicides to eradicate any diseases—notably black pod—and, on arrival, the massive task of setting up nurseries to supply the farmers was coordinated through CEPLAC and the colonization organization, INCRA (Instituto Nacional de Colonização e Reforma Agrária). Initially, the cacao thrived on the rich soils and production started at a very early stage, within 2–3 years. However, thereafter, many of the "Polos Cacaueiros" were ravaged by witches' broom disease, affecting the cacao canopy, flower cushions, and pods—with up to 90 % crop losses (Evans and Prior 1987)—although, for a long period, the pod disease was ascribed to *Phytophthora palmivora* (Evans 1981a). It now became a case of "fire fighting," to mitigate the impact of the disease—and, especially, to motivate the colonists, who were unfamiliar with the crop and might be tempted to abandon it and resort to slash-burn subsistence crops: ecologically and economically disastrous. There was also discontent among the cacao farmers of Bahia who feared that the opening up of Amazonia, and the increased levels of witches' broom from the new plantations, would increase the chances of the disease reaching the region. Thus, from 1978, a cordon sanitaire was put into operation, involving all the airports and roads out of Amazonia with specially trained CEPLAC personnel in place. Such was the concern of the threat posed not only to Bahia, but also to the world's cacao market, that an international research program was established to investigate methods of managing the disease: with the rider that "*If this disease would ever hit Bahia's cacao area its production would decline severely in the following 5 years. This forecast might well cause the market to explode*" (IOCC 1984).

In 1989, witches' broom disease was first reported in Bahia—apparently confined to a single farm (Pereira et al. 1989)—"*the second largest concentration of cacao in the World*" (Pereira et al. 1990), with the implication that this was accidentally human assisted. "*However, a well-defined strategy has been planned to cope with localized introduction and establishment of the pathogen: its eradication is now in progress by mobilizing personnel to collect and remove brooms and destroy them in the field with herbicides and fungicides and by burning infected plantations*" (Pereira et al. 1990): alas, to no avail. The disease spread rapidly through the almost contiguous 600,000 ha of cacao, seemingly from multiple foci, and within several years plantations were being abandoned and the socioeconomic, as well as the ecological, landscape of the region had changed irrevocably, with annual cacao production plummeting from 400,000 to somewhere around 150,000 metric tons in less than a decade (Pereira et al. 1996), as predicted by IOCC (1984). In fact, with the increasing chocolate consumption in the country, it has been claimed that Brazil is now a net importer of cacao (Meinhardt et al. 2008). Apart from the local economy and the livelihoods of thousands of cacao workers, the impact has also been felt on the ecology of the region and the unique but fragile Atlantic rainforest. The "cabruca" system protected the forest and provided "green corridors" for movement of the fauna; however, with the abandonment of plantations and their replacement by subsistence farms, this buffer has been lost (Alger and Caldas 1994; Saatchi et al. 2001; Donald 2004; Rolim and Chiarello 2004).

As Evans (2002) concluded: "*There can be no doubt that the arrival of witches' broom disease was human-assisted. Whether it was an accidental introduction on infected planting material…or deliberate, as popular belief would have it, will always remain a mystery*." Not so, the conclusion was premature because, shortly afterward, a local man came forward to admit to his part in bringing sacks of witches' brooms from Amazonia on several occasions and distributing them through cacao plantations (Junior 2006a). This was the second political maneuvering, since—as it turned out—the motive was to destroy the economy and to break the stranglehold of the powerful right-wing cacao barons in favor of the opposition left-wing "Petista" party. The rest, as they say, is history! The plan worked and the Petistas swept to power in Bahia, and in Brazil, in general. There was a subsequent redaction of the events (Junior 2006b), but the story held up, especially as the molecular analysis proved that the introductions matched the strains of the fungus present in Rondonia: the area from where the brooms were claimed to have been smuggled into Bahia (Bastos 1990; Andebrhan et al. 1999). This is now considered to be one of the first examples of agro- or bioterrorism for political rather than financial gain (Evans 2007; Caldas and Perz 2013).

Many of the big plantations in Bahia no longer exist, but cacao cultivation has continued on a smaller scale with cacao production stabilizing thanks to a CEPLAC-driven renovation campaign, involving an integrated approach to the management of witches' broom disease based on improved planting material, supplemented by cultural as well as biological control. However, it would be fair to say that the Amazonian cacao program has been an unmitigated disaster, especially in Rondonia, due to the impact of witches' broom disease and, to be blunt, to the arrogance and ignorance of the plant breeders who failed to take into account the concept of pathogen variation over a wide geographic range. If cacao can vary so much, from the western to the eastern extremes of the Amazon basin, then why should the fungus be any different? Most of the colonists either switched to subsistence crops or abandoned the land—which was subsumed into increasingly large cattle ranches—adding an ecological dimension to the socioeconomic misery. Nevertheless, it should be noted that in parts of the Amazon—notably, in western Pará State—the same cacao material that was virtually wiped out in Rondonia has performed relatively well (Author, personal observation). It must be concluded that, either resistance has held up—due to a less virulent pathotype of the fungus—or, different ecosystem components are in operation, such as natural-enemy pressure. Significantly, a potent antagonist of *M. perniciosa*, the mycoparasite *Trichoderma stromaticum*, was collected from this region (Samuels et al. 2000).

Concluding Remarks

If this history shows us anything—and, it is crucial to analyze the past in order to improve the future—it is that the plantation system for cacao is unlikely to be successful when faced by a disease such as witches' broom. This is because it

attacks not only the shoots and pods but all of the active growing points and to protect such infection sites—especially over large or contiguous expanses of cacao—is a logistical and, therefore, a financial nightmare. The premise behind the colonization scheme in the Brazilian Amazon in the 1970s—and for similar ambitious new plantations in Ecuador around the same era (Evans 1981a)—was that the planting material, based on Trinidad selections, was resistant to witches' broom disease, and, therefore, it could be grown like an "orchard" crop: without shade or shelter belts and in monocultures of genetically uniform clones or hybrids, often with incompatibility issues. For a pathogen of meristematic tissues—tuned into the phenological patterns and physiological activity of its host—the demise of these plantations was immediate and predictable, given that the resistance was based not on local isolates or pathotypes of the fungus but on geographically distant ones. Thus, because the disease is favored by increased tree vigor, unshaded plantations that flush and flower more intensely or frequently than shaded cacao would be particularly vulnerable. Absence of shade also promotes rapid drying of the dead brooms which reduces invasion by secondary organisms, further increasing the amount and duration of mushroom production (Evans and Solórzano 1982). Furthermore, unshaded cacao grown in blocks without windbreaks creates turbulence which means that air currents are continually cycling from the ground into the open and back into the canopy (van Arsdel 1967), favoring infection of flower cushions and pods, as well as the foliage. Moreover, in such monocultures, there are few breeding sites for pollinating insects and self-incompatible selections are especially at risk from flower cushion infection due to excessive or continuous flowering. This was the situation in Rondonia, with unprecedented levels of pod and cushion infection, that led to the abandonment of many small farms in the colonization scheme, as well as to the final demise of the plantation system in western Ecuador (Evans 1981a).

5.2 Dr. Rolf Singer—Pioneer Agaricologist

It was difficult to decide between the two most influential scientists involved with the taxonomic intricacies of witches' broom disease of cacao: Gerold Stahel (1887–1955) and Rolf Singer (1906–1994). The former, a Swiss plant pathologist, seconded to Surinam in 1914 to investigate the disease, who first described and named the causal agent, and who contributed much, not only on diseases of cacao but also of other major tropical crops—in particular, coffee and rubber—during his 34-year residence in the country. However, my focus of attention will be on the latter (Fig. 5.6), not least because I had the privilege of meeting and working with him, albeit briefly, in the Amazon rainforest. Rolf Singer has been described as "*one of the most influential figures in the history of mycology*" (Chicago Field Museum website), and a scientist who "*revolutionized the way we classify Agaricales and how we view the role that they play in the environment, especially in the Neotropics*" (Mueller 1995). It was during one of his many sojourns in the Neotropics that

I first made his acquaintance in 1978 in Manaus, where he was based at the Instituto Nacional de Pesquisas Amazônicas (INPA). I was stationed at the Departamento Especial da Amazônia of the Brazilian cacao organization CEPLAC (Commissão Executiva do Plano da Lavoura Cacaueira) in Belém do Pará, and charged with trying to mitigate and manage the outbreaks of witches' broom disease decimating the new plantations of cacao along the Trans-Amazonian Highway. At that time, CEPLAC was a wealthy and dynamic organization under the charismatic Director of Research, Paulo de Tarso Alvim, which possessed the direction and resources to be innovative. Thus, an international conference was planned to highlight the witches' broom problem: initially, in the exotic and luxurious surroundings of the premier hotel in Manaus on the banks of the Amazon River, and then to transpose the entire conference, by jet and small charter planes, to the remote but pivotal cacao outstation in Ouro Preto do Oeste (Rondônia). Naturally, I took advantage of Singer's proximity to invite him to join the "jamboree," although we almost lost him en route, as I caught sight of him wandering across the tarmac in the stopover city of Rio Branco. I guided him back onto the airline and, after a change of planes in Porto Velho, we eventually landed on a muddy airstrip in the middle of the vast and relatively untouched forests of Rondônia and, thereby, to witness at first-hand the ravages inflicted by witches' broom in the colonists' cacao. Singer presented an erudite but taxonomically challenging overview of the fungus and its near relatives (see Singer 1978), which was lost on most of the audience, who also included CEPLAC field assistants, as well as cacao farmers. Unfortunately, as papers were presented and questions came and went, the subject was addressed always as *Marasmius perniciosus*. I could see Singer becoming more agitated, especially since he had already talked about its taxonomic position within the genus *Crinipellis*—published by him some 45 years earlier (Singer 1942)—so, I was forced to make a request that, out of respect to our distinguished guest and, of course, in the interest of science itself, the name *Crinipellis perniciosa* should be used, henceforth. This was no mere taxonomic nicety, or an etiquette issue, since it was misleading to continue using the name *Marasmius*: a genus of leaf litter inhabiting mushrooms with no history of or capacity for a sophisticated plant parasitic lifestyle and, therefore, irrelevant within the context of witches' broom disease.

The following day, Singer was in his element—often whistling manically and distractingly—as we combed the fungal-rich forest looking for the pink mushrooms of *Crinipellis*, which we duly found on forest debris. In his subsequent report (Singer 1978), he chose to classify this species as *C. eggersii* whilst I considered it to be a nonpathogenic strain (Liana or L-type) of *C. perniciosa*, based on previous collections in Ecuador. Almost four decades on, we are still undecided about the taxonomic status of such non-cacao collections: however, more importantly, in his unpublished report—and, in subsequent landmark publications—he recognized the taxonomic and ecological uniqueness of this group of "*Crinipellis*" species, assigning them to a separate section. Now, of course, they merit separation at the generic level: a highly evolved group of taxa able to live endophytically within their plant hosts, in a benign or a malign symbiosis. As a bizarre example of his scholarship, it is fitting to end with a story of Singer's residence in Leningrad—

Fig. 5.6 Photograph of Dr. Rolf Singer in 1958 in Nevado de Toluca, Mexico

from where he fled shortly before the traumatic siege began—during which he noted the annual flushes of *Crinipellis* mushrooms on the trunk and in the canopy of a Brazilian forest tree (*Siparuna*), growing within a tropical greenhouse in the Botanical Garden, which he duly named *C. siparunae* (Singer 1942). He compared the new species to *C. perniciosa*—"*biologically and morphologically very closely related*"—and posited that the fungus must have been introduced with its host from South America. This suggests that he appreciated the endophytic habit, as well as the Neotropical origin, of the fungus—and, of course, of it being carried within the original planting material to Russia—and which he confirmed over 30 years later in his seminal work on the Marasmieae of the New World (Singer 1976).

5.3 Biology

Taxonomy

Nearly three decades after its original description by Stahel (1915), *Marasmius perniciosus* was transferred to the genus *Crinipellis* as *C. perniciosa*, within the section *Eu-Crinipellis*, subsection *Iopodinae* (Singer 1942), based on the tough,

thick-walled hairs or setae on the mushroom cap or pileus. Eventually, Singer (1976) recognized *Iopodinae* as a distinct section, placing *C. perniciosa* in the new subsection *Insignes*, noting, prophetically (see Sect. 5.1), "*The fact that the fungus is still frequently quoted as* Marasmius perniciosus*, 30 years after its transfer to* Crinipellis*, is a good illustration* of *the 'conservatism' of some phytopathologists and their reluctance to adopt the results of mycological work*." Perhaps, he was reflecting on the illogical statement by Baker and Holliday (1957) that because: "*The name* Marasmius perniciosus *is so well known… we do not recommend the general adoption of this transfer*." Prior to this, Dennis (1951) had accepted its inclusion in the genus *Crinipellis* in his revision of the Agaricaceae of Trinidad, and also listed *Marasmius scalpturatus* from Cuba, as a synonym. Evans (1977), on examining the type specimen held in the Royal Botanical Gardens (RBG) at Kew, considered that there was insufficient material for a positive identification, but noted that "*the twigs have all the appearance of small cushion brooms as seen on diseased cacao trees*." However, Singer (1976) in his monograph on Neotropical Marasmieae also examined a fragment of the type held at Harvard Museum (details in Singer 1978) and chose to place it in *Crinipellis siparunae*. Conversely, Pegler (1978) endorsed the earlier conclusion of Dennis (1951) that the Cuban material, deposited as *M. scalpturatus* in the Kew Herbarium on an unidentified woody plant, is *C. perniciosa*. Because no published description of *M. scalpturatus* could be found, the name was included as a *nomen nudum*. Further intrigue surrounds this record from the mid-nineteenth century because witches' broom disease has never been reported from Cuba. Indeed, the first official record from the Caribbean region was from Trinidad in the 1920s (Stell 1928). The mystery deepened still further when the host wood was identified at the Jodrell Laboratory (RBG) as *Theobroma* (Evans 1977; Pegler 1978). So, is this the earliest official record of the disease and was the fungus introduced accidentally with cacao by the Spanish during the colonial era, or is it the closely related *C. siparunae*, as suggested by Singer (1978), coincidentally growing on cacao? Pegler (1978) also recognized morphological differences in the mushrooms collected in Ecuador, based predominantly on variation in size and color intensity, and agreed with Stahel (1924) that they should be afforded varietal status, as var. *ecuadoriensis*, which has received further support since (Hedger et al. 1987; Wheeler and Mepsted 1988). In addition, another variety, var. *citriniceps*, was described from a single collection on a cacao broom from Ecuador with citron-yellow basidiomata. However, there is little doubt that the latter is based on an aberrant or mutant strain lacking red pigmentation, and there have been no subsequent records. Similarly, the status of var. *ecuadoriensis* still remains uncertain and the variety has not been validated within or transferred to the genus *Moniliophthora* (Evans et al. 2013).

Holliday (1971) described *C. perniciosa* as a unique species within the Agaricaceae because of its obligate pathogenicity. This was before the basidiomycete nature of the causal agent of frosty pod disease was revealed and its close relationship to *C. perniciosa* was posited (Evans et al. 1978). Nevertheless, in the absence of a sexual fruit body (basidioma) to prove this connection and to clarify its generic affinity, the frosty pod pathogen was considered to represent the asexual

stage of an unknown Basidiomycete, and the new monotypic genus *Moniliophthora* was erected to accommodate it. Subsequent cytological studies indicated that the purported asexual spores are, in fact, meiospores rather than mitospores (see Chap. 3). Citing these similarities in symptomatology and their shared and highly specialized hemibiotrophic, pleomorphic lifestyles, Evans et al. (2002) transferred *M. roreri* to *Crinipellis*, although, critically, the type species of the genus *Crinipellis* was not examined. In a subsequent study, Aime and Phillips-Mora (2005) undertook a comprehensive phylogenetic analysis—involving five nuclear gene regions—and confirmed that the two pathogens are, indeed, closely related; neither, however, is congeneric with *Crinipellis*, as exemplified by the type species, *C. stipitaria* (Singer 1942, 1986; Griffith 2004). They are, in fact, sister species and occupy a separate and distinct lineage within the family Marasmiaceae (Eu-agarics), which is not congeneric with any other genus previously included within the subtribe Crinipellinae. The sister genera *Chaetocalathus*, *Crinipellis*, and *Marasmius* comprise mainly saprotrophic litter fungi. Aime and Phillips-Mora (2005), rather than creating a new genus to accommodate these two cacao pathogens, transferred *Crinipellis perniciosa* to the existing anamorphic or asexual genus *Moniliophthora*, making the new combination *M. perniciosa*. The full citation is thus:

Moniliophthora perniciosa (Stahel) Aime & Phillips-Mora in *Mycologia* 97:1021 (2005)
= *Crinipellis perniciosa* (Stahel) Singer in *Lilloa* 8:503 (1942)
= *Marasmius perniciosus* Stahel in *Bull Dept Landbouw Suriname* 33:16 (1915)

Unfortunately, the generic description was not amended to include two species with radically different methods of sporogenesis. Recently, however, Evans et al. (2013) amended the description and provided a new diagnosis for the genus *Moniliophthora* (for more details, see Chap. 3).

Host Range

Malvales It is probable that all species in the genera *Theobroma* and *Herrania* (Malvaceae *s.l.*, formerly Sterculiaceae; Nyfeller et al. 2005) are actual or potential hosts of the fungus. Van Hall (1910) first drew attention to wild hosts, notably *T. speciosum*, as the potential source of infection for the cacao plantations in Surinam. Subsequently, this was endorsed by Stahel (1915, 1919), who also included *T. bicolor* as a host. *Theobroma speciosum* appears to be especially susceptible to cushion infection: "*Total cushion infection, extending from 1m up to 8 m above ground level, is not uncommon in this species*" (Evans 1977; see also Fig. 5.7e). Baker and Holliday (1957) reported an additional six *Theobroma* species in South America, based on the findings of an Anglo-Colombian cacao collecting expedition in the 1950s (Baker et al. 1954). The highly prized and cultivated cupuaçu (*T. grandiflorum*) is heavily attacked in the forests of the eastern Brazilian

Amazon (Pará State)—where it is known as the mother of witches' broom ("mãe da vassoura-de-bruxa")—and, as a consequence, it was considered to pose a threat to neighboring cacao farms (Gonçalves 1965). However, both Evans (1981a) and Bastos et al. (1988) reported negative results in cross-inoculation tests with fungal strains from cacao and cupuaçu, although, according to the latter authors, both infected four other *Theobroma* species, as well as a species of *Herrania*. Earlier, Baker and Holliday (1957) had stated that the fungus "*has never been found attacking any living plant outside of the genus* Theobroma." We now know that *M. perniciosa* has an extensive host range within the Solanales, Malpighiales, and Lamiales—as well as in the Malvales, including an isolated record in the Bixaceae: a small family in the Malvales typified by the plant *Bixa orellana* (urucu or achiote), the source of an industrial food coloring (annatto). Bastos and Andebrhan (1986) reported the occurrence of brooms on *Bixa* plants adjacent to a heavily infected cacao plantation in the eastern Amazonian State of Pará. The fungus was isolated in culture, but basidiomata failed to mature on the brooms. However, Koch's postulates were proven when inoculum from cacao isolates of *M. perniciosa* was shown to be pathogenic to *B. orellana* and the fungus was re-isolated from infected tissues. Nevertheless, there have been no further reports of its association with this widespread and commonly cultivated Amazonian plant. Spectacular terminal brooms sometimes occur on *Herrania*—a serious constraint to the growth of this single-stemmed tree—but, more usually, infection is limited to gross swellings of the leaf midrib, accompanied by distortion of the large palmate leaves (Evans 1981a; Author, personal observation).

Solanales Morphologically identical basidiomata were first reported from non-malvaceous hosts from both the western and eastern (Amazonian) regions of Ecuador in the late 1970s on unidentified lianas, without obvious disease symptoms (Evans 1977; Pegler 1978)—the so-called L-type to distinguish it from the cacao or C-type (Evans 1978)—and basidiospores proved to be nonpathogenic to cacao, inducing only a hypersensitive reaction. Subsequently, the fungus was found in the Brazilian Amazon on asymptomatic and unidentified liana hosts (Bastos et al. 1981), but also on two species of *Solanum*, *S. lasiantherum* and *S. rugosum*, associated with typical witches' brooms, as well as with parthenocarpic fruits (Bastos and Evans 1985). The characteristic intercellular mycelium was found in green brooms and basidiomata were induced on necrotic tissues. This was considered to represent a new biotype of *M. perniciosa* or S-type: essentially, nonpathogenic to cacao but capable of inducing growth abnormalities on stems of tomato (*Solanum lycopersicum*) and sweet pepper (*Capsicum annuum*), and necrosis of tomato fruits. Subsequently, non-pathogenicity of these *Solanum* isolates to cacao was confirmed (Bastos et al. 1988). Other *Solanum* hosts have been added steadily to the list, their geographic range stretching from the northern Amazon to western (Goiás, Mato Grosso do Sul), central (Distrito Federal), eastern (Bahia), and southeastern (Minas Gerais) Brazil: on *Athenaea pogogena* (Bastos et al. 1991); *Solanum paniculatum* (Silva et al. 1992); *S. cernuum* (Evans and Barreto 1996; Fig. 5.4c, d); *S. lycocarpum* (Barreto and Evans 1996; Resende et al. 1997; Fig. 5.4a); *S. gilo* and

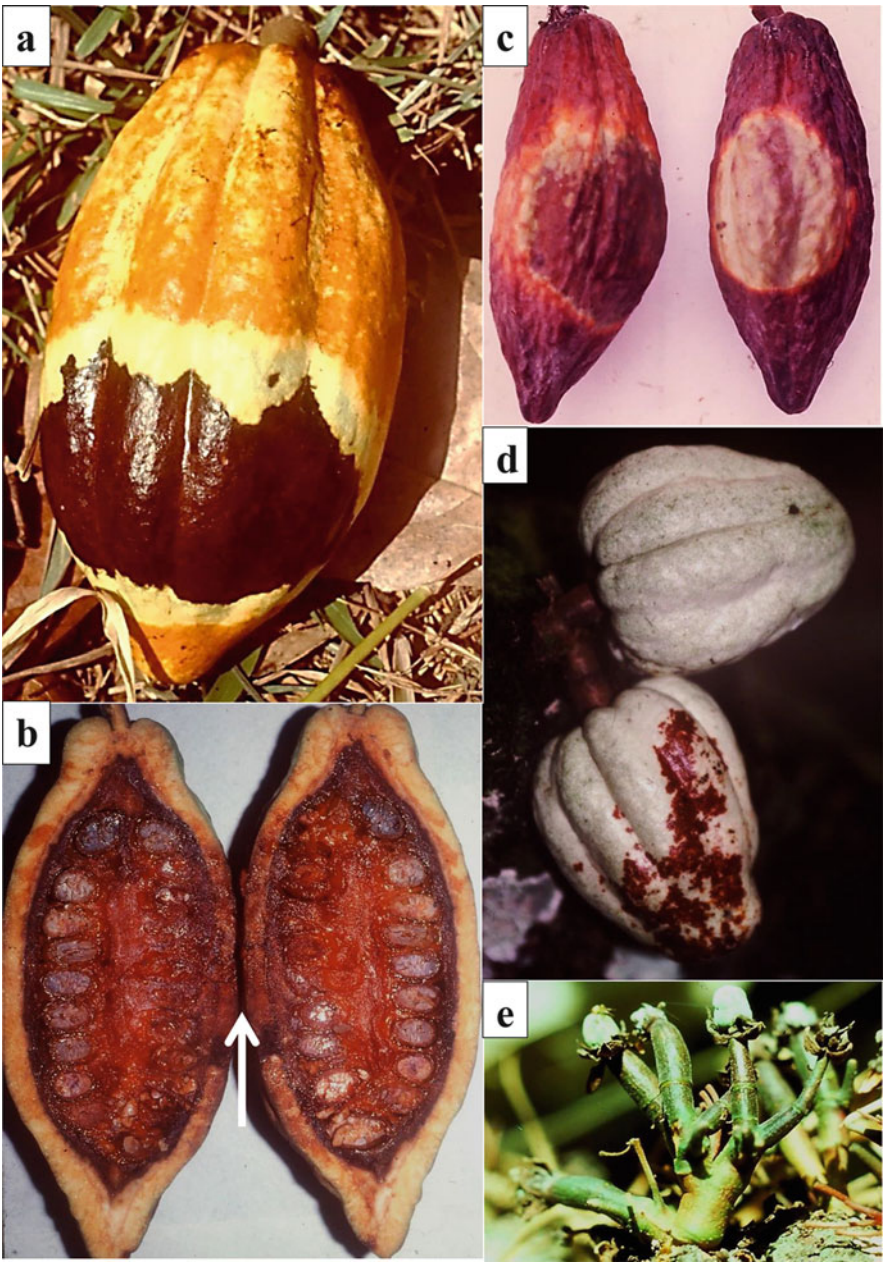

Fig. 5.7 Pod symptoms (**a**) Cacao pod, 4 months after inoculation, showing premature ripening and irregular, dark brown lesion, without sporulation, although older lesions often become indurated and colonized by *Colletotrichum* species. (**b**) Internal symptoms, with complete destruction of bean mass—indistinguishable from frosty pod rot (see Chap. 3)—but external necrosis restricted to small lateral lesion (*arrow*). (**c**) Young infected pods (cherelles) showing physiological wilt, but swollen area either remains "healthy" (*right*, green island), or becomes necrotic (*left*), appearing darker than the wilted area. (**d**) Abnormal parthenocarpic pods—termed "chirimoyas"

S. stipulaceum (Luz et al. 1997). *Solanum lycocarpum* ("Lobeira") is extremely common along roadsides and often invades pastures in Minas Gerais (Pereira et al. 1997), where, invariably, it is heavily attacked, producing exceptionally large brooms (see Fig. 5.4a), as well as conspicuous parthenocarpic fruits (see Evans et al. 2013). Luz et al. (1997) also reported *M. perniciosa* on various solanaceous crops—*S. melongena*, *Capsicum annuum*, *C. frutescens*, for example—in Bahia, but whether this was the result of natural or artificial infection is unclear. More recently, *S. cladotrichum*, S. *gemellum,* and *S. mauritianum* (Fig. 5.4b) have been confirmed as hosts in remnant Atlantic forest of Minas Gerais, and *S. grandiflorum* in the drier Cerrado vegetation of Goiás (HC Evans and RW Barreto, unpublished): regions where cacao or its relatives are not native and, traditionally, have never been cultivated. The boundaries of the endemic range of *M. perniciosa* have now been pushed even further south to the States of Rio de Janeiro and Rio Grande do Sul, where brooms have been recorded on *S. gemellum*, with a new species record on *S. schwartzianum* and a new genus record on *Cestrum* sp. (RW Barreto, personal communication, 2015). Natural infection of both *S. gilo* and *S. melongena* has been recorded recently in Minas Gerais (Author, personal observation, see Fig. 5.4e–g).

Tests of pathogenicity to cacao were conducted with some of these solanaceous isolates from Minas Gerais, but inoculation with basidiospores derived from infected *S. cernuum* and *S. lycocarpum* did not result in broom formation. However, cacao leaves in the former treatment occasionally showed vein-clearing symptoms, reminiscent of virus infection, whilst many plants in the latter treatment developed cankers on the upper and lower stems, with some evidence of dwarfing (HC Evans and RW Barreto, unpublished). An earlier study (Silva et al. 1992), however, demonstrated that an isolate from *S. paniculatum* in Bahia induced broom formation in cacao seedlings and, subsequently, it was shown, using DNA (RAPD) analysis, this strain of *S. paniculatum* clustered with nine isolates from cacao. However, the remaining solanaceous isolates from Bahia—which included three from *S. paniculatum* and one from *Athenaea*—grouped together but distinct from the *S. rugosum* isolate from Amazonas (Yamada et al. 1998). *Solanum paniculatum* was later included in a series of cross-inoculation experiments with two *Theobroma* species (*T. bicolor*, *T. grandiflorum*), in addition to cacao (Lopes et al. 2001). Results were somewhat ambiguous, with the isolate from *S. paniculatum* consistently forming brooms on both *T. cacao* and *T. bicolor* but not on *T. grandiflorum*; however, the symptoms were restricted to localized swellings when *S. paniculatum* was challenged with the same isolate.

A recent and more detailed analysis of the ITS sequences of isolates from a range of *Theobroma*, *Herrania*, and *Solanum* species concluded that they are not

Fig. 5.7 (continued) due to resemblance to fruits of custard apple—with lower pod beginning to show necrosis. (**e**) Infected flower cushions bearing aborted, parthenocarpic pods on wild *Theobroma speciosum* in the Brazilian Amazon

genetically distinct since they clustered together within the phylogenetic tree (Marelli et al. 2009). de Arruda et al. (2003), from a parsimony analysis of the intergenic spacer (IGS) region, agreed with these findings, whilst an even more comprehensive phylogeny—involving three DNA regions (ITS, IGS, RPB1)—has firmly placed isolates from *Theobroma* and *Solanum* in the same clade, but as distinct lineages (Tarnowski 2009).

Malpighiales *Mascagnia* cf. *sepium* (Malpighiaceae) was the first host record for *M. perniciosa* in this order, when brooms were found on this woody liana in the Brazilian Amazon (Bastos et al. 1998). Inoculum from the basidiomata produced on *Mascagnia* brooms proved to be pathogenic to cacao seedlings inducing swollen stems and stimulation of lateral branching, although there was no indication that distinct brooms were formed (Bastos et al. 1998). This was soon followed by reports of brooms on another woody malpighiaceous climber, *Stigmaphyllon* cf. *blanchetii*, in Bahia (Bezerra et al. 1998; Lopes et al. 2001; Braz 2011). Another woody liana host, *Heteropterys acutifolia*, was recorded, subsequently, in southern Minas Gerais (Resende et al. 2000). Isolates of which were also shown to be pathogenic to cacao, although, in these cases, it has been noted that only highly susceptible cacao varieties were tested (Griffith et al. 2003), and, moreover, there are doubts that full symptoms were expressed. Resende et al. (2000) stated, categorically, that the basidiomatal morphology of the *Heteropterys* (H-type) isolate matched, in all respects—including basidiospore size and shape of the cheilocystidia—that of the cacao isolate. However, a subsequent study—analyzing material from the same site in Minas Gerais—considered that there were sufficient differences in basidiospore size and cheilocystidia shape to merit separation of the isolate from *Heteropterys* as a distinct species, and this was supported by the sequence data of the ITS region: the name *Crinipellis brasiliensis* was proposed (de Arruda et al. 2005). It is noteworthy that an isolate of *M. perniciosa* from another species of *Heteropterys*—*H. nervosa*, a small tree, rather than a liana, from the semiarid Cerrado of the Distrito Federal—clustered in the cacao clade and was morphologically identical. An isolate of *M. perniciosa* from *H. acutifolia* in Minas Gerais has since been screened against the highly susceptible Catongo cacao variety—using a modified half-bean inoculation method—and, although classic symptoms of witches' broom were not observed, atypical dwarfing and rhizomania were induced (Evans et al. 2013). Similarly, Viana (2001) failed to achieve broom formation on cacao with this isolate. Interestingly, all the isolates from *H. acutifolia* used in these studies were collected from the same locality in gallery forest at Itumirim, southern Minas Gerais, where this riparian host is common along both banks of the Capivari river and is always heavily broomed (Fig. 5.8a, b).

In addition to these differences in host-range screening, further doubts about the validity of the morphological criteria employed to separate *C. brasiliensis* have also been raised by Tarnowski (2009). Nevertheless, the Malpighiaceae clade—including isolates from *H. acutifolia*, as well as those from unidentified malpighiaceous lianas collected in Minas Gerais, Goiás, and Mato Grosso do Sul (HC Evans and RW Barreto, unpublished)—received strong support in a multilocus phylogenetic

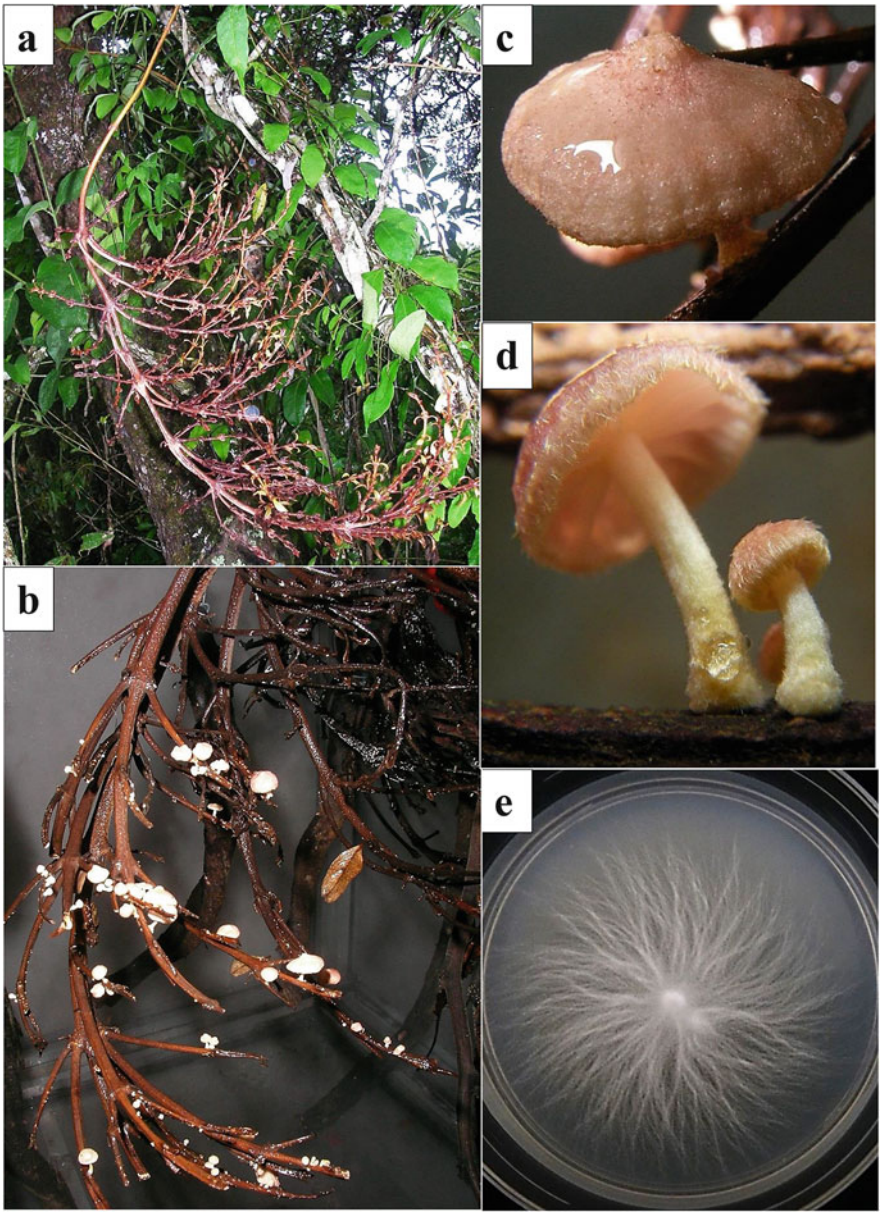

Fig. 5.8 *Moniliophthora perniciosa* on *Heteropterys acutufolia* (Malpighiaceae). (**a**) Large broom hanging down from liana host in tree canopy of riparian, gallery forest, Minas Gerais, Brazil. (**b**) Necrotic broom held in humid chamber ("vassoureiro"), and subjected to several months of alternate wetting–drying cycles, showing flush of basidiomata. (**c**) Expanding basidioma; note concentration of pigmented hairs around umbonate or raised central part of cap (pileus). (**d**) Maturing and emergent basidiomata with distinctive felty or hirsute caps and stalks. (**e**) Colony in culture, radically different from the isolates derived from cacao brooms (see Fig. 5.2a)

analysis, compared to the other clades. It has been conjectured that, although it may be appropriate to recognize the new species—and, thereby, to transfer *C. brasiliensis* to the genus *Moniliophthora*—additional molecular evidence was needed (Tarnowski 2009). However, in view of the contradictions highlighted here—both in morphology and pathogenicity—doubts about the taxonomic status of this species remain. Nevertheless, there do appear to be significant differences in basidiomatal morphology and colony characteristics (see Fig. 5.8c–e), which strongly indicate that the fungus on Malpighiaceae may, indeed, warrant separation as a distinct taxon.

A spectacular witches' broom has also been found on the malpighiaceous, woody, and hallucinogenic liana, *Banisteriopsis caapi* (Ayahuasca), in Amazonian Ecuador (Holmes et al. 2004). Normally, this robust climber is devoid of vegetation in the accessible lower understory: however, in this case, the vine had been cut, resulting in activation of the lateral buds and the formation of a massive broom (Author, personal observation). What actually happens in the "meristematically active" upper canopy is intriguing.

Lamiales As reported above, surveys in the lowland forests of western Ecuador, from 1974 to 1975, revealed that basidiomata characteristics of *M. perniciosa* were associated consistently with dead hanging lianas and also on fallen debris within the understory canopy (Evans 1977). There were no indications of growth abnormalities on these forest hosts and pathogenicity to cacao was not proven, and it was concluded that two distinct populations of the fungus exist in western Ecuador: the nonpathogenic liana or L-type and the pathogenic cacao or C-type (Evans 1978). These findings were confirmed later by Hedger et al. (1987), who misidentified the host as a leguminous climber (*Entada gigas*). Subsequently, Griffith (1989) studied the forest system in more depth and found that, invariably, the basidiomata were associated with asymptomatic living and dead stems of the woody climber *Arrabidaea verrucosa* (Bignoniaceae, Lamiales)—an endemic in the forests of the western Andean foothills (Griffith et al. 1994)—and not, as previously reported, a liana belonging to the Fabales (Leguminosae).

In a series of papers, the genetics and breeding strategies of the C-, S-, and L-types of *M. perniciosa* were investigated (Griffith and Hedger 1994a, b; Griffith et al. 1994). The conclusion was reached that the L-type on Bignoniaceae represents the ancestral form, being out-crossing or heterothallic, as well as a benign endophyte (see Fig. 5.5), whilst the pathogenic or malign C- and S-types are of more recent origin, being in-breeding or homothallic. Similarly, asymptomatic collections of dead lianas bearing basidiomata of *M. perniciosa*—some identified as Bignoniaceae hosts—have been reported consistently from the Amazon Basin (Evans 1977, 1978, 1981a; Bastos et al. 1988), as well as from Atlantic rainforests in south-east Brazil (Evans and Barreto 1996; Evans 2007; Tarnowski 2009). However, the placement of such liana hosts—typically, occurring as decaying stems, hanging in the litter of the lower canopy as well as on the ground—within the Bignoniaceae is tentative, at best, and should be treated with caution, since a

recent molecular analysis grouped some purported bignoniaceous isolates within the Malpighiaceae clade (Tarnowski 2009).

Concluding Remarks It seems likely, therefore, that *Moniliophthora perniciosa* pathosystem represents a series of host jumps to plants in several different orders, rather than a coevolution with cacao, as often stated (Purdy and Schmidt 1996; Meinhardt et al. 2006). In fact, according to Tarnowski (2009), the most recent divergence appears to be that of the *Theobroma* and *Solanum* lineages: those that diverged earlier have mostly malpigheaceous and bignoniaceous hosts. Interpretation of these phylogenies suggests that the homothallic, highly specialized pathogen could have evolved from a heterothallic, saprotrophic, or endophytic ancestor, and subsequent host jumps resulted in the lineages recently identified in *M. perniciosa* (Tarnowski 2009). There appears to be no obvious phylogenetic patterns in these host jumps: apart from the families Malvaceae and Bixaceae both of which occur in the Malvales, and, moreover, the *Bixa* record could be viewed as a "one-off-event," perhaps due to close proximity to a high inoculum source. The Malvales are now placed in the superorder Rosanae, along with the Malpighiales which cluster in a separate clade ("fabids"), rather than the "malvids," whereas the Lamiales fall into a separate superorder, the Asteranae, where they share a direct linkage with the Solanales within the phylogenetic tree (Chase and Reveal 2009). However, more collections are needed and more genes should be sequenced before a more meaningful phylogenetic tree can be constructed. This needs to be done in conjunction with a well-structured, host-range screening program in order to clarify the inconsistencies, discrepancies, and ambiguities detailed above for previous host-range tests.

Current Distribution

The first section of the chapter details the early history and anthropogenic spread, accidental or deliberate, of the cacao pathogen from its presumed Amazonian origin to western Ecuador (~1920), Trinidad (1928) and Tobago (1939)—and, subsequently, to Grenada (1948; Baker and Holliday 1957)—as well as to the Bahia region of eastern Brazil (1989). The latter authors reported that the other Caribbean islands were disease free and that it was still absent from Central America. Later, it reached the Dominican Republic, Saint Vincent, and Grenadines (Evans 2002), and, only recently, the first report of witches' broom on cacao in Saint Lucia was confirmed (Kelly et al. 2009). In Panama, the disease was first reported in 1978 in the Darien Province adjacent to the Colombian border and, apparently, this outbreak was contained by a buffer zone, with later claims that the disease was eradicated (Pereira et al. 1996). A second outbreak was recorded in central Panama, in Colon Province, in 1988 (Espino 1989). However, it has not moved further north into the isthmus of Central America, where it poses a threat not only to the main cacao-growing region of Panama (Bocas del Toro Province) but also to other

Mesoamerican cacao-producing countries. There is a record of the occurrence of *M. perniciosa* in Belize (CABI 2002): however, this cannot be substantiated and, therefore, it must be considered as erroneous.

Etiology

When the concepts of the genus *Moniliophthora* are better defined—after more comprehensive collections and molecular analyses have been undertaken—it is highly probable that all of the species assigned to the genus will be found to be sophisticated endophytes of plants, predominantly of tropical trees, such as those taxa moved by Singer (1976) into the new subsection *Iopodinae* of the genus *Crinipellis* (Evans et al. 2013); the greater majority living cryptically and benignly within their hosts. The nature of this symbiotic association is still unclear: the fungus gains protection and nutrients from the host plant, but how the plant benefits, if at all, remains to be established. Recent studies show that the stems of wild *Theobroma* and *Hevea* species harbor a highly diverse endophytic mycobiota in which basidiomycetous fungi, especially Agaricomycetes, are well represented (Evans et al. 2003; Crozier et al. 2006; Thomas et al. 2008; Martin et al. 2015). It must be assumed, therefore, that such cryptic basidiomycete colonizers must be beneficial in some way to the plant—or, at least, pose no threat to it, living in a form of commensalism—because they are associated with seemingly healthy, asymptomatic plants and are considered to represent latent saprotrophs (Oses et al. 2008; Hibbet et al. 2014). However, *Moniliophthora perniciosa*, and its sister species *M. roreri* (see Chap. 3), have "broken this mold," which led Money (2007) to ponder the question—in relation to witches' broom disease—"*But how and why did* Crinipellis *evolve as a cacao pathogen?*" In other words: how did a benign colonizer become a malign invader and cause such a debilitating disease of cacao, which is now one of the main constraints to production in Latin America? Basically, members of the Agaricomycetes are not listed as major plant pathogens (Agrios 2005), and their ecological impacts are overwhelmingly as wood-decay fungi and ectomycorrhizal symbionts (Hibbet et al. 2014).

Evans et al. (2013) provided circumstantial evidence to support the hypothesis that a complex tritrophic relationship, or a three-way symbiosis, could be the reason why this fungus, as well as the closely related *M. roreri*, became pathogenic. It is posited that somewhere along the evolutionary trail, these fungi acquired an infectious agent which possibly linked into the fungal genome. Cross-infectivity studies using the half-bean inoculation method (Posnette 1947), with basidiospores of *M. perniciosa* from witches' brooms collected on non-cacao hosts—in the Bignoniaceae, Malpighiaceae, and Solanaceae—revealed a range of symptoms on a disease-susceptible cacao variety (Catongo, see Fig. 5.1b). Although no true brooms were formed with these non-cacao inoculum sources—in sharp contrast to the cacao control—there was consistent evidence of growth abnormalities, including leaf chlorosis, mottling, and vein banding with some of the solanaceous

isolates. However, the most striking and consistent symptoms occurred with the inoculum derived from the malpighiaceous host *Heteropterys acutifolia* (see Fig. 5.8), which were manifested by severe stunting or dwarfing of both the upper and lower stems with leaf deformation (Fig. 5.9a), accompanied by a highly abnormal proliferation and swelling of the root system ("rhizomania"), resulting in plant death (Fig. 5.9b). These were interpreted as "virus-like events," which was enforced by the failure to detect the diagnostic intercellular primary mycelium of *M. perniciosa* (see Fig. 5.2d), in any of the affected tissues. When sap from the abnormal leaves was extracted and the filtrate was inoculated using the half-bean method, the presence of an infectious agent was demonstrated and Koch's postulates were proven: the radicle became grossly swollen, the plumule failed to open, and all the seedlings died within a few weeks (Fig. 5.9c, d). As previously observed, the presence of the monokaryotic mycelium was not detected and the fungus was not isolated from the dying plants. Similar symptoms had been reported previously with *Moniliophthora roreri*, when pre-germinated cacao beans were exposed to extremely high inoculum levels, but no satisfactory explanation was forthcoming, at that time (Evans 1981b).

As interpreted by Evans et al. (2013), when a non-host species—or, in the case of the pod pathogen *M. roreri*, a non-susceptible cacao tissue—is challenged with an artificially high inoculum level, host defenses act to prevent significant establishment of an infection by the intercellular monokaryotic phase. Various levels of localized symptoms may be expressed—depending on the fungal pathotype—such as hypersensitivity; abnormal bark; minor stem swellings; and necrosis or cankering (Evans 1981a, b). However, in certain cases, it is posited that the infectious agent is released from the fungal genome into the conducting tissues of the cacao seedlings and, thereby, is transmitted throughout the plant. Thus, tissues not normally colonized by the fungal mycelium—roots, leaf lamina—are invaded, with atypical symptoms being expressed, such as "rhizomania"; stem dwarfing; and mottling, crinkling, or deformation of the leaves. The latter symptoms, in particular, would suggest the presence of virus-like particles, but this remains to be proven. However, recent studies have demonstrated the occurrence of just such a tritrophic relationship between a plant, an endophytic fungus, and an RNA virus which conferred thermotolerance and enabled the plant to colonize hot springs: this ability was lost in endophyte-free plants or when the virus was removed from the endophyte (Márquez et al. 2007). This association is beneficial to all "parties"—mutualism or reciprocal altruism—in sharp contrast to witches' broom disease, which is an example of antagonistic symbiosis. The infectious agent vectored by the fungus induces abnormalities in the meristems—possibly by interfering with the permeability of the cell membranes—releasing nutrients for the intercellular mycelium but also plant growth hormones, causing unregulated cell growth and resulting in hyperplasia and hypertrophy. Thus, more tissues are created for colonization by the fungus and its symbiotic partner—to the detriment of the plant host—and, subsequently, for the production of basidiomata.

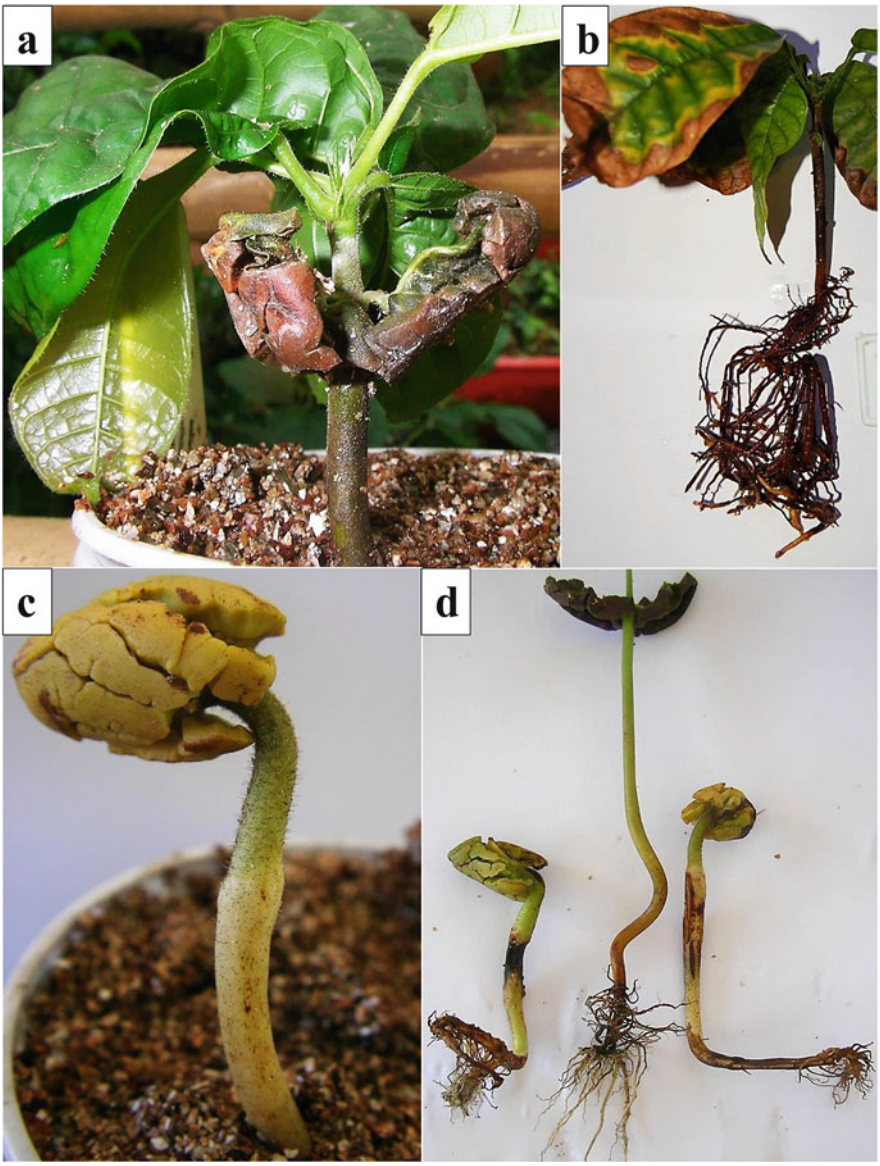

Fig. 5.9 Evidence of infectious agent within a tritrophic association. (**a**) Dwarf cacao plant, 3 months after inoculation of half-bean with basidiospores from mushrooms produced on brooms of *Heteropterys acutifolia* (see Fig. 5.4), showing stunted and swollen lower and upper stems and normal-sized but distorted, rugose leaves. (**b**) Wilting 4-month-old plant, excavated to reveal grossly swollen and abnormally profuse root system ("rhizomania"). (**c**) Cacao seedling with swollen plumule, 7 days after inoculation of half-bean with sap extract from distorted leaves (**a**). (**d**) Stunted and dying cacao seedlings, 3 weeks after inoculation; note central healthy control plant

Symptomatology

The full range of symptoms of witches' broom disease on vegetative shoots, flower cushions, and pods of cacao was illustrated beautifully and comprehensively by Stahel (1919) in Surinam and later by Baker and Holliday (1957) in Trinidad. However, because these publications are not readily accessible, it is considered appropriate here to describe and depict the most common symptoms of the disease, on cacao as well as on other hosts.

Vegetative brooms—the most typical infection type from whence the common name is derived (Krulloten, escoba-de-bruja, vassoura-da-bruxa)—are formed on the active shoots or flushes and their occurrence is related to the flushing patterns of the cacao tree: their size depends on tree vigor and variety; typically, stems are grossly swollen and axillary buds are activated, whilst leaf petioles and pulvinii may also be hypertrophied (see Fig. 5.10c, d), but, characteristically, the lamina is unaffected (Fig. 5.1a, b). Necrosis and death occur some 3–4 months after infection.

Cushion infection varies from a single swollen flower stalk (Fig. 5.1c) to prominent clusters of lateral brooms (Fig. 5.1d); in addition, infected flowers develop abnormally into pods of varying form—lacking seeds and assumed, therefore, not to have been pollinated—and such parthenocarpic pods can be carrot shaped, strawberry shaped (Fig. 5.1d), or shaped like custard apples (chirimoyas, Fig. 5.7d); all necrose rapidly, becoming woody, hard, and mummified. In cacao, there appears to be a genetic link to susceptibility to cushion infection which may be linked to flowering pattern or vigor and incompatibility. It is not uncommon to see wild trees in the Upper Amazon where this is the only disease symptom manifested (Author, personal observation), and spectacular infection is often associated with the heavily flowering *Theobroma speciosum* (Fig. 5.7e).

Pod infection, resulting from penetration of the young pod or cherelle, rather than systemic infection of the unpollinated flower (as above), has been confused in the past with black pod and frosty pod diseases. It can easily be distinguished from the former Phytophthora disease since the infected pods usually exhibit varying degrees of hypertrophy—depending on the age at infection and the cacao variety—and necrosis is delayed for up to 2–3 months, rather than a matter of days. Up to this point, both external and internal symptomatology are indistinguishable from frosty pod disease, but the irregular chocolate to dark-brown lesions—relatively restricted in size and often associated with chlorosis or premature ripening—remain free of mycelium and sporulation (Fig. 5.7a), unlike frosty pod (see Chap. 3). Frequently, these lesions become sunken or indurated and colonized by saprophytic fungi, such as *Colletotrichum*; eventually, the whole pod mummifies. Internally, however, pods with premature ripening and restricted lesion development show complete destruction of the bean chamber (Fig. 5.7b), varying from a watery or mucilaginous tissue breakdown to a dry compacted bean mass. Older pods (>3 months) are often "tolerant" or "resistant" and, in some instances, the infection is restricted to necrotic speckling of the outer and inner pod walls and the beans can be saved, whilst in others, infection in the form of compacted beans on the inner pod wall is localized

Fig. 5.10 Spore dispersal experiment, Ecuador. (**a**) General view of experimental design 1, showing necrotic brooms (inoculum source) suspended from canopy and radiating rows (16 main compass points) of cacao seedlings every meter for 100 m, isolated in the center of extensive

within the pod chamber. This is not genetic resistance per se, but linked to the physiology of pod growth, specifically to meristematic activity which decreases significantly after 3 months. As noted by Baker and Holliday (1957), cherelles undergoing physiological wilt retain metabolic activity around the swollen infected areas, with the appearance of "green islands" (Fig. 5.7c): a characteristic of obligate biotrophs, such as rust fungi, and linked to increased cytokinin activity (Walters and McRoberts 2006). The term "green bionissia" has been proposed recently as a general descriptor for this symptom in biotrophic fungi (Walters et al. 2008).

Hidden infection—used by Evans (1981a) to emphasize this cryptic or hidden inoculum source—as the name suggests, is easily overlooked but may be the most common infection event and, therefore, critical to successful management using cultural control. The importance of this was highlighted during an epidemiology experiment with cacao seedlings, when only 17 %, of more than 3000 infection events, were vegetative brooms: most were restricted to swellings on the green shoot, or on the leaf pulvinus and petiole (Fig. 5.10c, d), or associated with discrete cankers on the hardening shoots (Evans and Solórzano 1982). It was concluded that most infections occur on the expanded but unhardened flush: basidiospore penetration being common on the leaf petiole, pulvinus, and the stem adjacent to the leaf base. In the following flush, 70 % of these leaf base-stem swellings developed into terminal or lateral brooms (Fig. 5.10e), but only 19 % of the leaf infections formed brooms: the majority (>80 %) underwent necrosis and produced cryptic cankers. Such cankers and leaf symptoms are frequent on wild *Theobroma* and *Herrania* species—especially those with unbranched single stems—in Amazonian forest ecosystems (Evans 1981a).

Epidemiology

The fungus is wind dispersed and natural spread and infection is only by airborne basidiospores (Baker and Holliday 1957). Studies by Baker et al. (1941) in Trinidad indicated that most infection occurs within 100 m of the infection source, although longer-distance dispersal was also acknowledged. However, no quantitative data were provided. Later, Evans and Solórzano (1982) attempted to map the movement of basidiospores—using cacao seedlings as living spore traps, as well as mechanical spore traps—from different inoculum sources in order to compare their importance as initiators of infection and thus to improve disease management. The experimental design was based on the premise that heavy infection is limited to within

Fig. 5.10 (continued) pasture. (**b**) General view of experimental design 2, as above but inoculum source (necrotic brooms) on ground beneath canopy and rows extending to 50 m only. (**c**) Symptoms of natural infection, predominantly restricted to swellings on central leaf vein (causing distortion), and pulvinus. (**d**) Gross swelling of leaf pulvinus, petiole, and leaf-stem base (*arrow*). (**e**) Proliferation of lateral brooms from infected leaf-stem base

100 m of the inoculum and, therefore, that the trial sites situated in the middle of an extensive pasture system (Fig. 5.10a, b)—some 800–1500 m from the nearest cacao plantations—would be buffered from infection. However, before sporulation was recorded in the inoculum sources—as evidenced by mushroom (basidiomata) production on the dry brooms—almost 20 % of the 2400 trap plants had become infected, often with multiple infections in the form of discrete swellings of the petioles, pulvinii, and leaf base, rather than brooms per se (Fig. 5.10c, d). Although the immediate aims of determining infection gradients were invalidated, the experiment demonstrated that there is a high level of background contamination of basidiospores over considerable distances from inoculum sources—in contrast to previous suggestions (Baker et al. 1941; Baker and Holliday 1957)—which was further endorsed by the data generated by the volumetric spore traps. These data also provided valuable information on patterns of spore dispersal: basidiospores were recorded throughout the year, but the greater majority of spores were trapped in the wetter months during the hours of 22.00 to 04.00. An analysis of the meteorological data (see Fig. 5.10a, b), revealed that a rise in relative humidity combined with a fall in temperature favors basidiospore release: the greater the difference between relative humidity and temperature, the greater the rate and duration of spore liberation. Thus, the spores are programmed for release during the late evening and early morning, presumably to escape the effects of sunlight and low humidity which would rapidly kill the hyaline, thin-walled basidiospores (see Fig. 5.3). It was estimated that the spores would remain viable under normal conditions for ~12 h, but for much longer periods on overcast days, and that given an average wind speed in the tropics of 4–5 km/h, the spores would have a median range of 70–80 km, and up to a maximum of ~15 km (Evans and Solórzano 1982). As discussed above, this is a critical consideration in epidemiology and disease spread where natural barriers, such as mountain ranges and semiarid ecosystems, would prevent long-distance dispersal.

In addition, this study highlighted the dangers of leaving pruned brooms on the ground—rather than removing and destroying them—as was the general recommendation at that time. Although suspended brooms produced three times more basidiomata (>4000 at 11/broom) than those on the ground—rapid decomposition accounted for the greatly reduced sporulation potential—the latter produced basidiomata much later in the season and, significantly, during the dry season following extended periods of mist and heavy dew. In sharp contrast, the suspended brooms dried rapidly, preventing colonization by secondary fungi and delaying decomposition, and, thereby, increasing the life span of this inoculum source. As discussed earlier, this has implications for disease management since brooms require alternating periods of drying and wetting for maximum sporulation (Rocha and Wheeler 1985; Wheeler and Suárez 1993; Purdy and Schmidt 1996), and this cycle is much reduced in buffered shaded cacao—or in tropical forest ecosystems—compared to unshaded cacao plantations. Undoubtedly, such phenological and climate patterns also govern synchronous flushing events in cacao, and, of course, when the two combine, massive infection will result (Evans 1981a).

Life Cycle

Holliday (1971) described the devastating impacts of the disease on cacao production in those countries where it had invaded, and added that: "*If discovered early enough, I consider witches' broom of cacao to be a disease which could be eradicated without too much difficulty*." This was based on the seemingly simplistic life cycle of the fungus, or what was known about it at that time: and, "*Without a complete knowledge of the fungal life cycle, it is difficult to improve on present control measures*" (Evans 1980). Thus, began a study of the life cycle and an attempt to clarify how the pathogen operates in two separate phases—parasitic and saprotrophic. Delgado and Cook (1976) had argued that the monokaryophase is ephemeral and that host penetration occurs only after formation of the dikaryon and, therefore, that this is the dominant phase and the principal nuclear condition in the life cycle, contradicting the hypothesis put forward earlier by Pegus (1972)—in an unpublished thesis—who proposed a dual-mycelial theory which posited that the monokaryotic mycelium represents the extended biotrophic or parasitic phase, whilst the dikaryotic mycelium corresponds to the necrotrophic or saprophytic phase of the life cycle. This begs the question, of course: how were two diametrically opposed ideas on the nature of the life cycle of *M. perniciosa* conceived? The evidence cited in support of the former theory was generated from in vitro studies, critically, on basidiospore germination experiments which showed that the monokaryotic (uninucleate) mycelium is short-lived (<24 h), with "*the secondary mycelium persisting thereafter in the dikaryotic state*" (Delgado and Cook 1976).

Evans (1980) confirmed later that, indeed, when basidiospores were allowed to germinate in water, or on standard culture media, a fast-growing colony developed, typical of isolations from diseased cacao tissues (Fig. 5.2a), composed of a fine, binucleate mycelium with prominent clamp connections (see Fig. 5.3). In sharp contrast, however, in the presence of living cacao tissues, germination was modified such that a grossly swollen, convoluted, uninucleate mycelium, lacking clamp connections, slowly colonized the substrate. This was achieved by using actively growing cacao callus (Evans 1980), resulting in the formation of extremely slow-growing cerebriform colonies on, but not in, the agar around the central callus (Fig. 5.2b, c). An identical mycelium is always present in green brooms and infected young pods, growing intercellularly (Fig. 5.2d). As these tissues mature, and the cacao callus loses viability, this mycelium is overrun by the fast-growing dikaryotic mycelium (see Fig. 5.2b). The dual mycelium hypothesis had been proven and it was posited that modifying metabolites present in active cacao tissues delay or inhibit dikaryotization and thus promote the systemic or endophytic parasitic phase (Evans 1980). In a parallel study, Evans and Bastos (1980) further confirmed the presence of such a modifier, or promoter of the monokaryophase, in extracts of cacao flushes, using germinating basidiospores as a means of identifying and assessing this association. Significantly, greater modifier activity was identified in susceptible cacao clones compared to resistant ones and, presumably, the latter either contain less of the promoter or, alternatively, this is overridden by an

inhibitor: a bound monophenol—found in the resistant Scavina clones—has been suggested as the inhibitory secondary metabolite (Evans and Bastos 1980).

A potential modifier has now been identified (Thomazella et al. 2012), following the detection of a mitochondrial alternative oxidase (AOX) gene implicated in biotrophic development. A novel mechanism has been proposed for *M. perniciosa* in which AOX plays a key role in biotrophy by regulating the transition to the necrotrophic phase. Evans (1980) elected to classify *M. perniciosa* in the hemibiotrophs—parasitic organisms which start their association with the host as a biotroph but later revert to a well-defined saprotrophic phase—based on the mode of nutrition and the latest concepts of symbiosis at that time (Lewis 1973; Luttrell 1974; Cooke 1977). In the case of witches' broom disease of cacao, the biotrophic phase is significantly longer than any of the other fungi included in this group, reflecting, perhaps, a more advanced or coevolved association with its host.

The modifier of the monokaryophase must operate soon after the basidiospore lands on and penetrates the host meristematic tissue—typically, directly through the epidermis or via the stomata (Cronshaw and Evans 1978)—otherwise, the germ tube would undergo dikaryotization (Evans 1980; Evans and Bastos 1980). Critically, this is the phase when susceptibility or immunity to infection "kick in" and, probably, depends not just on genetic resistance—absence of a modifier; presence of an inhibitor—but also on the nutritional status or vigor of the host. In susceptible encounters, the monokaryotic intercellular parasitic mycelium develops from the germ tube and then invades and colonizes the meristematic tissues, causing the growth abnormalities associated with the disease. A schematic representation of the full life cycle is shown in Fig. 5.3 for witches' broom disease of cacao. By way of contrast, the life cycle of the nonpathogenic form or L-biotype of *Moniliophthora perniciosa* on Bignoniaceae hosts is outlined in Fig. 5.5.

5.4 Conclusions

As stated at the beginning of this chapter, witches' broom of cacao is an enigmatic disease and, although we are gradually stripping away the layers to reveal the true identity of the causal agent(s)—exactly a century after it was provisionally identified as being caused by the basidiomycete fungus *Marasmius perniciosus* (Stahel 1915)—much is still hidden and we need to dig deeper. In other words, this chapter remains to be completed and can only serve as an aide mémoire for future research. This also applies, of course, to the closely related and equally enigmatic frosty pod rot (Chap. 3). This is best achieved by summarizing "what-needs-to-be-done" in a series of bullet points, in order to focus attention on and, hopefully, to stimulate new investigations into these perplexing areas which, essentially, adhere to the research priorities identified by Evans et al. (2013).

Proof of Pathogenicity

The conjecture is that the genus *Moniliophthora* may contain many species, the majority of which are cryptic but widespread endophytes of tropical trees with two well-defined nutritional forms: the monokayotic intercellular mycelium, with a haustorial-like function; and the dikaryotic intracellular mycelium, with an extracellular enzyme-feeding strategy on naturally senescing host tissues. Somewhere along the evolutionary trail, both *M. perniciosa* and *M. roreri*—or their common progenitor—acquired the ability to alter host growth, creating additional tissues on which to propagate: spectacularly so, in the case of witches' broom disease. Circumstantial evidence indicates that an infectious agent may be incorporated within the fungal genome and that is released under stress to cause symptoms not normally associated with witches' broom disease, or frosty pod rot (Evans et al. 2013). This proposed mechanism of pathogenicity needs to be investigated in more depth and, if proven to be erroneous, then alternative mechanisms should be explored in order to establish how these two species diverted from the typical endophytic pathway of commensalism or mutualism to become parasitic and major plant pathogens. The key may lie with the nonpathogenic and heterothallic bignoniaceous form or biotype of *M. perniciosa* endemic to the forests of western Ecuador. Is there an equivalent nonpathogenic biotype on the eastern side of the Andes and, if so, is this the progenitor of witches' broom disease, and the western biotype that of frosty pod rot? And, of course, is there a link between the switch from hetero- to homothallism and pathogenicity? It would seem also that both *M. perniciosa* and *M. roreri* can sometimes occur as asymptomatic endophytes: the former has been routinely isolated from healthy tissues of cacao (Lana et al. 2011), whilst the latter has similarly been found as an endophyte in its wild host, *Theobroma gileri* (Evans et al. 2003).

Genetic Variation

This, of course, overlaps with and follows on from the above. Measuring genetic variation will be particularly daunting for *M. perniciosa* because, literally, the natural geographic, as well as the host range of this fungus, is growing by the day. However, there is so much conflicting data on the pathogenicity and cross-infectivity of the geographic isolates from cacao and the isolates from host families outside of the Malvaceae that there is no clear picture of intra- and interspecies specificity. In addition, the morphological variation is high in the biotypes from cacao from eastern to western South America, with specific varieties being delimited—as well as considerable differences in pathogenicity, both within cacao and between other *Theobroma* hosts—but neither has been quantified. Clearly, there is a need for more rational and intensive collecting from across South America to fully establish the natural host range and to assemble these

isolates for molecular analysis and pathogenicity screening. The latest interpretation of phylogeny indicates that the ancestral form evolved in the lianas of the family Bignoniaceae (Lamiales), probably as nonpathogenic endophytes, and—since the Lamiales share a close relationship with the Solanales, within the superorder Asteranae (Chase and Reveal 2009)—it seems logical that the first "pathogenic jump" was to this related order. The host jump into the Malvales and Malpighiales, within the superorder Rosanae, however, is difficult to explain, especially since the isolates from Solanaceae and Malvaceae cluster in the same clade, albeit as distinct lineages, whilst those from Malpighiaceae fall into a distinct clade, possibly representing a separate taxon.

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Chapter 6
Genomics, Transcriptomics, and Beyond: The Fifteen Years of Cacao's Witches' Broom Disease Genome Project

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Abstract Cacao production in Brazil was severely affected by the outbreak of witches' broom disease (WBD) in the late 1980s. WBD is caused by the basidiomycete fungus *Moniliophthora perniciosa*, a hemibiotrophic pathogen that displays an uncommonly long-lasting biotrophic stage during which the host cacao suffers intense morphologic alterations in the infected shoots, giving rise to "green brooms." Two months after infection, the fungus becomes necrotrophic resulting in the necrosis and destruction of the infected tissues that turn into a "dry broom." During the last 15 years, the knowledge of this devastating and intriguing disease has been growing due to initiatives such as the WBD genome project. By using genomics and transcriptomics as tools to obtain insights about this disease, the WBD project has been elucidating the biochemistry and physiology of both plant host and pathogen, paving the way for practical applications to combat the fungus. In this chapter we present an overview of progress in the understanding of *M. perniciosa* genetics and the molecular mechanisms governing WBD, provide a model for the *M. perniciosa*–cacao interaction, and point to new directions to fight this disease.

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6.1 Introduction

The impact of a plant disease on society can often be measured by the economic importance of the infected crop, by the losses suffered by farmers and by the supply chain, and by the damage to the environment. Considering these three factors, witches' broom disease (WBD) of cacao (*Theobroma cacao* L.), caused by the fungus *Moniliophthora perniciosa*, can be considered one of the most important phytopathological problems in the Americas. Most of the Brazilian cacao plantations are located in the southern region of Bahia, a state situated in the northeastern region of the country. Cacao originated in the Amazon basin and was introduced into Bahia in the middle of the eighteenth century. The climate, similar to Amazonia, made southern Bahia an optimal environment for cacao plantations. For many years, Brazil was one of the main exporters of cacao beans. However, with the introduction of WBD into Bahia in 1989, Brazil went from being the second highest producer of cacao in the world to being a net importer. By the late 1990s, the consequences of the WBD outbreak in Bahia were catastrophic. Cacao trees were devastated by the pathogen; farm owners abandoned their plantations, increasing rural unemployment, or switched to alternative crops (e.g., oil palm) and livestock. This change in the rural landscape caused serious ecological damage due to the deforestation of the "cabruca", an agroecological system where cacao trees are grown under the shade of the Atlantic Rainforest trees (Pereira et al. 1996).

The social–economic situation in the south of Bahia, and the subsequent losses to the Brazilian chocolate supply chain, needed a fast and proactive initiative from the Brazilian government, farmers, agronomists, and scientists to find new ways to combat the pathogen. In 2000, The Witches' broom genome project (www.lge.ibi.unicamp.br/vassoura) was launched by Dr. Gonçalo Pereira at the State University of Campinas (UNICAMP). This initiative brought together several Brazilian university laboratories and scientific institutions conducting cacao breeding (e.g., CEPLAC and EMBRAPA). The focus of the initiative was to collaboratively explore the genome of *M. perniciosa*, with the goal of providing clues to the fungal biology that could aid cacao crop management and the design of fungicides against the pathogen. The multidisciplinary approach of the team, comprised of expertise in cacao breeding, plant physiology, mycology, biochemistry, genomics, gene expression, and structural biology, has been essential in the advancement of our understanding of WBD. The initiative, led by Dr. Gonçalo Pereira at UNICAMP and Dr. Julio Cascardo (in memoriam) from the State University of Santa Cruz (UESC), has provided the scientific basis supporting practical field applications. This chapter aims to give an overview of the advances in our understanding of WBD, reviewing the results obtained through the use of genomic and transcriptomics techniques and connecting these results to the biochemistry and physiology of both plant and pathogen.

6.2 Prof. Julio Cezar de Mattos Cascardo, A Pioneer in Cacao Plant Pathology

Julio (Fig. 6.1) was born on May 29, 1964, in Rio de Janeiro, where he spent the first half of his life. He received his bachelor's degree in Agronomy from the Federal Rural University of Rio de Janeiro in Seropedica in 1987. He then moved to Minas Gerais state to obtain a M.Sc. degree from the Federal University of Lavras in 1991. His research focused on the ecophysiological aspects of the rubber tree (*Hevea brasiliensis*) (Cascardo et al. 1993a, b). Before continuing his education, Julio received a fellowship to conduct research at the EMBRAPA Genetic Resources and Biotechnology Center in Brasilia, where he began developing his skills in molecular biology and biochemistry on a project investigating the phylogeny and regulation of tuberization of cultivated cassava (*Manihot*). In 1993, Julio was hired by Mars Inc. as a research scientist at the Mars Center for Cocoa Health Science in Barro Preto, Bahia. This position introduced him to cacao and its pathogens and definitely changed his career and life. During this period, he defined his passion for his future wife Dr. Fátima Alvim (Fafá), *Theobroma cacao* (cacao), and southern Bahia (Ilhéus), the region he adopted as his final home. In 1995, Julio started his PhD at the Federal University of Viçosa (UFV), working with BiP proteins in soybeans (Cascardo et al. 2000, 2001; Alvim et al. 2001; Buzeli et al. 2002). He had considered going abroad for his degree, but the fact that Fafá was an undergraduate student at Viçosa was a determinant factor for his final decision! After graduation in 1999, Julio joined the State University of Santa Cruz (UESC) in Ilhéus, where he developed his brilliant and inspiring scientific career, becoming a full-time professor, and established an important and productive research group. He became the Provost for Science and Graduate Studies of UESC in 2007 and helped to catalyze the transformation of a new and incipient teaching institution into a relevant producer of science and technology in Brazil.

Julio had a very productive scientific career, publishing over 60 articles in important high-impact journals in various scientific fields, including the

Fig. 6.1 Prof. Julio Cezar de Mattos Cascardo (1964–2010)

Proceedings of the National Academy of Sciences of the United States of America, *Plant Physiology*, *The Journal of Biological Chemistry*, *Journal of Bacteriology*, *Molecular Plant-Microbe Interactions*, and *BMC Genomics*, to name a few. His publications ranged from agronomic aspects of tropical crops, such as the characterization of the existing genetic diversity for cacao seed quality traits, including fat and cocoa flavor (Figueira et al. 1997; Pires et al. 1998), to genomics (de Almeida et al. 2003; Vasconcelos et al. 2005) and structural biology (Monzani et al. 2010; Galante et al. 2012; Santos et al. 2011; de Oliveira et al. 2012a), his major focus later in his career.

In the early 2000s, genome sequencing initiatives were launched in Brazil, and Julio joined a federal consortium involved in sequencing two bacterium genomes (*Chromobacterium violaceum* and *Mycoplasma synoviae*) and fully explored the opportunity and established modern laboratory facilities at UESC. At the same time, Prof. Gonçalo Pereira launched the project to sequence the genome of *Moniliophthora perniciosa* (Mondego et al. 2008), the causal agent of the witches' broom disease of cacao together with Julio. The *M. perniciosa* genome project received support from national and state funds and stimulated the development of the Foundation for Research Support of the state of Bahia (FAPESB). Julio wisely used these opportunities to participate in other national and state research consortia in genomic, proteomic, and structural biology that were established at that time in Brazil. Along with obtaining resources and facilities, Julio was able to convince many of his former graduate colleagues from UFV to join him at UESC and to attract many foreign scientists from distinct fields to UESC, such as the microbiologists Prof. Martin Brendel (Germany) and his wife Cristina Pungartnik and the bioinformaticians Dr. Diego Gervásio Frias (Cuba) and Dr. Nicolas Carels (France), and to establish a successful cooperation with CIRAD (French Agricultural Research Centre for International Development) to support resident scientists at UESC, Dr. Fabienne Micheli and Dr. Dominique Garcia.

The genome initiatives included the Genolyptus project, which developed and sequenced cDNA libraries from *Eucalyptus* under different biological conditions, particularly during xylogenesis and wood development. Julio's outstanding bench skills to perform wet experiments were remarkable as he was able to extract and purify RNA and proteins from difficult tissue samples! He would repeat his motto: "Have I ever let you down?" followed by successfully obtaining good-quality products from the most challenging samples. During this time, he revisited some of his previous work with *Manihot* to molecularly describe a sugar-storing cassava landrace (Carvalho et al. 2004; Souza et al. 2004). A shrimp genome sequencing initiative included his lab as a partner, and the biofuel boom after 2006 caught his attention on the novel oil crop *Jatropha curcas*. He started a project on sequencing expressed genes in developing *Jatropha* seeds (Gomes et al. 2010). His cooperation with French researchers included projects studying the interaction between *Hevea* and *Microcyclus ulei* (South American leaf blight) (Garcia et al. 2011; Salgado et al. 2014). Julio also was involved in projects on metagenomics, searching for biotechnological processes and products from the microbiome of the Atlantic Rainforest and the Brazilian semiarid region (Duarte et al. 2013). Later in the

decade, his interest shifted to participate in a National Proteomics Network and in structural biology to characterize structure and activities of some of the proteins he identified in his many projects.

His major contributions were related with the work on the *Moniliophthora perniciosa* × *Theobroma cacao* interaction (Mondego et al. 2008; Micheli et al. 2010). In the late 1990s, we demonstrated that the disastrous invasion by *M. perniciosa* of southern Bahia in 1989 most likely occurred by two independent invasions events (Andebrhan et al. 1999). Together with Prof. Pereira's group, Julio's lab characterized the necrosis- and ethylene-inducing protein (NEP) of *M. perniciosa* (Garcia et al. 2007; de Oliveira et al. 2012a; da Silva et al. 2011), developed and compared transcriptomes from inoculated resistant and susceptible cacao genotypes (Gesteira et al. 2007; Lima et al. 2010; da Hora et al. 2012), investigated the occurrence of programmed cell death of infected cacao tissues, and proposed the role of calcium oxalate degradation in the pathogenesis (Ceita et al. 2007; Pirovani et al. 2010; da Silva et al. 2011; Dias et al. 2011) and dsRNA-induced gene silencing in *M. perniciosa* (dos Santos et al. 2009), among other achievements (Pires et al. 2009; Britto et al. 2013).

Julio was a friendly, very likable, and enthusiastic person, with a sophisticated sense of humor, bringing laughs and joy to those who were close to him. He could easily turn his scientific collaborators into close friends, building strong and lasting friendships. He was passionate for food, particularly Italian, and cooking, and his house in Ilhéus was always open for cookouts and dinners. He practiced an open-door policy in his lab, where everybody was welcomed to use his most sophisticated pieces of equipment, which he was very proud of. He was very creative and pursued the newest technologies and the latest scientific developments and was always thinking about the future, which helped him identify new opportunities. At UESC, he played an important administrative role attracting new funds and defining scientific policies for the university and participating in committees at the state level to support large projects in Bahia state. He was an inspiring leader that brought confidence for his colleagues to strive for bigger, better, and more difficult challenges.

Julio passed away on August 4, 2010, and he was survived by his wife Fafá and his son Tomás. His legacy has been celebrated by institutions and colleagues: a building at UESC was named after him (2012), and a molecular biology laboratory (2010) at the Brazilian National Laboratory for Scientific Computing in Petropolis, Rio de Janeiro, carries his name (https://www.youtube.com/watch?v=0XvJC5GmbVA). During the declaration of the completion of a draft genome sequence of cacao in 2010, the coordinators of the International Cocoa Genome Sequencing Consortium dedicated the announcement to "our colleague Dr. Julio Cascardo, who would have relished this moment with all of his enthusiasm and joy." It becomes clear how much Julio's life has impacted and inspired colleagues, students, scientific communities, and institution during his brief journey among us (written by Antonio Figueira, Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Brazil).

6.3 An Overview of WBD

Witches' broom disease is caused by the hemibiotrophic basidiomycete *Moniliophthora perniciosa* (Stahel) Aime and Phillips-Mora (2005), previously known as *Crinipellis perniciosa* (Tricholomataceae, Agaricales, Marasmiaceae). In general, hemibiotrophic pathogens cause a brief asymptomatic biotrophic stage that precedes the destructive necrotrophic phase, wherein the disease symptoms are evident. However, *M. perniciosa*'s biotrophic stage is unusually long lasting compared to most hemibiotrophic (e.g., *Magnaporthe oryzae* and *Colletotrichum* spp.) and even biotrophic pathogens (e.g., *Ustilago maydis* and *Cladosporium fulvum*).

The fungus produces small (2–3 cm wide) basidiocarps (Fig. 6.2a) with a pink fan-shaped cap, which releases infective hyaline basidiospores. *M. perniciosa* spores infect meristematic cacao tissues including flowers and young fruits causing swelling and anomalous ripening followed much later by death of the infected tissues. Also, parthenocarpic pods (Fig. 6.2d) are induced by the infection on flower

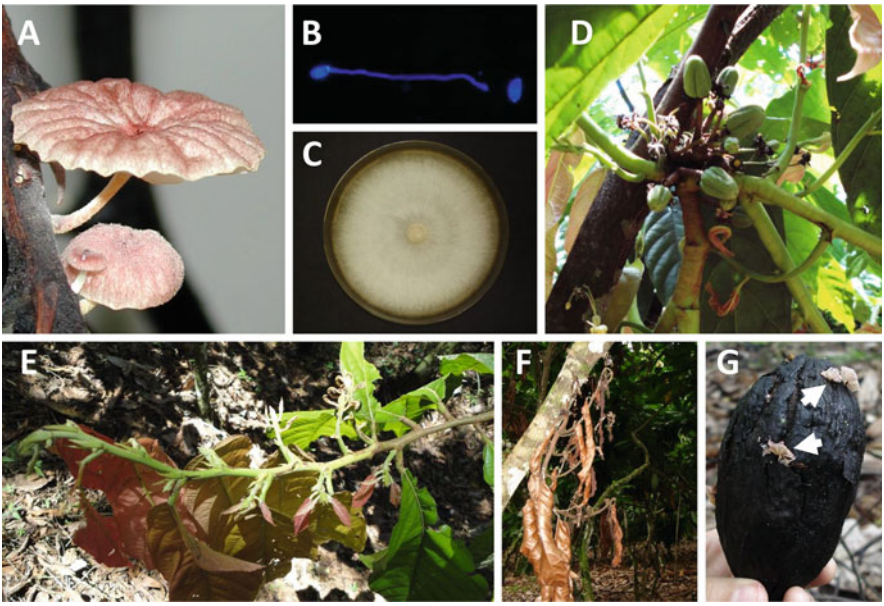

Fig. 6.2 *Moniliophthora perniciosa* developmental stages. (**a**) As a basidiomycete, *M. perniciosa* produces pink mushrooms (basidioma), where basidiospores are formed. (**b**) Germinating basidiospores originate the biotrophic mycelium, which is usually ephemeral under in vitro conditions. (**c**) Following the biotrophic stage, the mycelium becomes necrotrophic. A petri dish of the necrotrophic mycelium is shown. This fungal stage is easily cultivated under axenic conditions. (**d**) The biotrophic stage causes drastic symptoms in the plant, which include the formation of parthenocarpic fruits (when flowers are infected) and (**e**) the development of abnormal stems called green brooms (when apical or lateral meristems are infected). (**f**) After 30–90 days after biotrophic growth, green brooms become necrotic and the fungus grows as a necrotroph. (**g**) Lastly, mushrooms originate from the dead cacao tissues, thus reinitiating the disease cycle

cushions (Meinhardt et al. 2008; Melnick et al. 2012). However, the typical symptoms of the disease are the consequence of infection of shoot meristems. Briefly, the germination of basidiospores (Fig. 6.2b) and the consecutive development of germ tubes occur between 6 and 12 h after spores contact the plant (Silva et al. 2002; Sreenivasan and Dabydeen 1989; Kilaru and Hasenstein 2005). After that period, germ tubes from germinating spores penetrate meristematic tissues through stomata or through wounds in cacao tissues. The first visible symptom of infection is a slight swelling of the plant apical meristems, which is detected 15–20 days after infection (dai). Within 30 dai, the shoots become swollen and hypertrophic and lose apical dominance, containing leaves that display intense chlorosis. These abnormal structures are called "green brooms" (Fig. 6.2e). During this disease stage, the fungus thrives in the apoplast as a biotrophic pathogen, being found at very low density within the infected tissues. After a remarkable period of approximately 60 days in the biotrophic phase, WBD enters the necrotrophic phase of development, resulting in the necrosis of the infected tissues that turn into a "dry broom" (Fig. 6.2f). Intriguingly, dead infected tissues do not form abscission layers, remaining attached to cacao trees. Following alternating wet and dry periods, necrotrophic mycelia produce basidiocarps (Fig. 6.2g) and subsequent spore formation occurs, thus completing the disease cycle (Evans 1980; Meinhardt et al. 2008). A peculiar feature of WBD is that its disease stages parallel the pleomorphic switch of *M. perniciosa* hyphae inside cacao tissues. The biotrophic stage is restricted to the apoplast and is characterized by convoluted extracellular monokaryotic hyphae, whereas necrotrophic stage (the sexual phase) is distinguished by thin dikaryotic hyphae containing clamp connections, which intensively grow and invade cacao cells.

M. perniciosa and the phylogenetically related fungus *Moniliophthora roreri*, the causative agent of frosty pod rot (FPR) of cacao (Evans 2007), are phytopathogens that are part of the Agaricales family, in which the great majority of species are saprotrophic litter fungi. The acquisition of phytopathogenicity by these two Moniliophthora was studied by Tiburcio et al. (2010), which found evidence that pathogenic traits of *M. perniciosa* and *M. roreri* are not only the result of the adaptation of ancestral gene families but may have resulted from the acquisition of genes through horizontal gene transfer (HGT). Among these genes are those encoding NEPs (necrosis- and ethylene-inducing proteins), which are extracellular proteins that have the ability to induce necrosis in plants, including cacao (Garcia et al. 2007; Zaparoli et al. 2011), and are found in many phytopathogens (Gijzen and Nurnberger 2006). Strikingly, *M. perniciosa* NEPs seem to be acquired from the oomycete *Phytophthora* spp. which also causes disease (black pod rot) on cacao (Evans 2007). Double infections with Phytophthora and *M. perniciosa* are commonly seen in cacao fruits (Flament et al. 2001), which may facilitate the interchange of genetic material between these species. Therefore, the acquisition of NEPs through HGT could be an important factor in the acquisition of phytopathogenicity by *M. perniciosa* and *M. roreri*.

6.4 The Intriguing Biology of *Moniliophthora perniciosa* Biotypes

Although *M. perniciosa* is best known for causing witches' broom disease in cacao, this fungus is also able to infect other unrelated plants. Based on host adaptation, the species *M. perniciosa* is currently subdivided into three biotypes (C, S, and L). The C-biotype infects Theobroma and the closely related genus *Herrania* and is responsible for WBD in cacao. The S-biotype is adapted to solanaceous plants and was first isolated from brooms developed in native *Solanum* spp. in the Amazon basin (Bastos and Evans 1985). Later, infected solanaceous species were also described in Minas Gerais (Brazil), where cacao has never been grown (Evans and Barreto 1996; Pereira et al. 1997). Finally, the L-biotype was isolated from liana vines of the Bignoniaceae family in western Ecuador, but does not induce symptoms on the host and seems to develop as an endophyte (Evans 1978; Hedger et al. 1987; Griffith 1994; Griffith and Hedger 1994).

The host diversity and the geographic distribution of the different biotypes is an intriguing aspect of *M. perniciosa* biology. To date, very little is known about evolutionary relationships among these biotypes, and the genetic basis of host specificity remains elusive. Infection of tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) with the S-biotype results in symptoms similar to the cacao WBD (Bastos and Evans 1985; Marelli et al. 2009; Deganello et al. 2014), indicating that these more tractable Solanaceae plants might be used as model systems to investigate WBD. Interestingly, multiple studies showed that the C and S biotypes of *M. perniciosa* are genetically very similar (de Arruda et al. 2005; Marelli et al. 2009; Deganello et al. 2014). Even so, the C-biotype cannot infect tomato and the S-biotype cannot infect cacao (Bastos and Evans 1985; Deganello et al. 2014), which suggests that small differences existing in the genomes of these biotypes may be associated with host adaptation. In contrast, the L-biotype is evolutionarily more divergent from the C and S biotypes and seems to present higher levels of genetic diversity (Griffith 2004). This observation is in accordance with the different crossing strategies exhibited by these biotypes: isolates from the L-biotype are heterothallic, meaning that basidiocarp production requires crossing of mycelia from different mating types. On the other hand, the C and S biotypes are homothallic, and mycelia developed from single spores are able to develop reproductive structures (Delgado and Cook 1976; Griffith 1994). Because homothallic species may have evolved from heterothallic ancestors (Lin and Heitman 2007), and homothallism is uncommon among Agaricomycetes (James 2007), it is likely that C and S biotypes (or their ancestor lineage) gained the ability of self-crossing during evolution. Since both C and S biotypes are pathogenic, we can hypothesize that homothallism could have favored their pathogenic lifestyles. Being homothallic, the fungi do not have to encounter another compatible individual that coinfected the same stem to reproduce and complete their life cycles. In this scenario, the fungus ensures plant colonization without depending on the presence of a mating partner.

Despite being homothallic, *M. perniciosa* C-biotype has a tetrapolar mating-type genetic structure (i.e., A and B mating-type loci are unlinked), which is a characteristic of the heterothallic fungi (Kües and Navarro-González 2010). Interestingly, a retrotransposon insertion was found in the A locus (Barau and Almeida, personal communication) positioned between the homeodomain transcription factors genes HD1 and HD2 that control the expression of pheromones and pheromone receptors in locus B (Ni et al. 2011). Whether this transposon insertion influences the reproductive strategy of *M. perniciosa* remains to be elucidated.

Given the complexity of the *M. perniciosa* biology and our relatively low understanding of it, comparative genomics of isolates representing all three biotypes constitutes a promising and exciting strategy to explore the evolution of pathogenesis in this species. This might shed light on genetic signatures associated with host specialization as well as provide a catalog of the genetic variability within *M. perniciosa* populations.

6.5 Genomics and Transcriptomics of *M. perniciosa* and WBD

As mentioned above, the rationale of the WBD genome project was based on the genome analysis of *M. perniciosa*. Indeed, the knowledge about genome sequences of the pathogen was important to give insights about the cacao–*M. perniciosa* interaction. In 2008, a genome survey based on whole genome shotgun (WGS) and Sanger sequencing of the *M. perniciosa* C-biotype isolate CP02 was released (Mondego et al. 2008). Despite the estimated low coverage (1.9× at that time), the strategy, which combined ab initio gene prediction (using gene predictors) and extrinsic gene prediction (using BLAST search in public gene banks) in the draft genome, supported by the alignment of ESTs from in vitro-grown *M. perniciosa* mycelia (Rincones et al. 2008) and basidiocarps (Pires et al. 2009), was shown to be efficient in identifying genes putatively involved in disease progression. We could infer that the *M. perniciosa* genome had around 39 Mbp, a number that was similar to that inferred by Rincones et al. (2008) through pulsed-field gel electrophoresis (PFGE) followed by Feulgen image analysis.

Many other interesting results have come from the genome assembly initiative. First, an approach for normalization of the number of gene family members using incomplete genome data was developed. This was an important tool for genome draft annotation during the time-and-money "expensive" Sanger sequencing era. Second, the assembly of the mitochondrial genome of *M. perniciosa* was carried out (Formighieri et al. 2008), resulting in a large mtDNA with 109,103 bp. One uncommon feature of this mitochondrial genome is the stable integration of a linear plasmid similar to pKalilo-like plasmids (Formighieri et al. 2008), which have a typical invertron structure (with long inverted repeats) and are known to affect the

lifetime of their hosts, likely by disturbing mitochondrial stability (Bertrand et al. 1985; Griffiths 1992).

During the annotation of WGS sequencing of *M. perniciosa*, the WBD genome project team could not imagine the forthcoming impact of next-generation sequencing (NGS) methodologies in high-throughput analyses of genomes, especially for bacterial and fungal genomes. During the last few years, the team has used NGS to sequence *M. perniciosa* C-biotype and the other biotypes of the fungus. The latest assembly of the *M. perniciosa* C-biotype genome has 44.6 Mbp, a more accurate prediction than the 39 Mbp of the original draft genome (Mondego et al. 2008). This recent genome prediction was performed during a comparative genome analysis between *M. perniciosa* and *M. roreri* (Meinhardt et al. 2014). These authors compared the genomes of both *Moniliophthora* species and detected that, despite their number of genes being very similar, their genomes have different lengths. *M. roreri* has a larger genome (52 Mbp), a difference compared to the *M. perniciosa* genome mostly due to the higher prevalence of repetitive elements, including long terminal repeat–transposable elements (LTR–TEs). In the current genome assemblies, TEs correspond to 1 % and 7 % of *M. perniciosa* and *M. roreri* genomes, respectively. The presence of active, transcribed retrotransposons in *M. perniciosa* was reported (Rincones et al. 2008; Pereira et al. 2013, 2015), indicating that these genetic elements may be involved in the generation of *M. perniciosa* genetic variability, through ectopic recombination and in the modulation of gene expression.

Along with genomics, transcriptomics data has been showing to be extremely important in the unraveling of WBD. Three transcriptomic initiatives gave the first insights into molecular mechanisms of *M. perniciosa* development and pathogenesis. Rincones et al. (2008) used a combination of DNA microarray, expressed sequence tags, and real-time reverse-transcription polymerase chain reaction (RT-qPCR) to address the molecular differences between the monokaryotic biotrophic-like mycelia and the later dikaryotic necrotrophic phase in vitro. Pires et al. (2009) analyzed genes involved in basidiocarp development through the evaluation of selected EST genes from a non-normalized cDNA library. Leal et al. (2010) built a *M. perniciosa* cDNA library obtained by suppression subtractive hybridization enriched for genes expressed under nitrogen limitation, a condition thought to mimic the host environment colonized by biotrophic pathogens, inducing the expression of pathogenicity genes (Bolton and Thomma 2008). These analyses, though essential for the onset of more specific analyses of proteins hypothetically involved in WBD, were based on artificial conditions and, consequentially, did not assess the cacao side of WBD interaction.

Regarding gene expression of cacao plants infected with *M. perniciosa*, three initiatives provide interesting results comparing genes involved during the response to pathogen infection in resistant (R) and susceptible (S) genotypes of cacao. Gesteira et al. (2007) constructed two cDNA libraries corresponding to the R and S plants infected with *M. perniciosa* spores. Leal et al. (2007) used a similar approach but applied a suppression subtractive hybridization (SSH) strategy. Finally, da Hora et al. 2012 used DNA macroarray methodology to speculate

about resistant and susceptible cacao–*M. perniciosa* protein interactions through bioinformatics analyses. These three works had as a main goal the identification of resistance mechanisms, giving less attention to the susceptible interaction.

The more recent initiative concerning WBD transcriptomics is based on RNA-seq methodology. Using powerful high-throughput NGS technologies, RNA-seq can give a dual portrait of a biotic stress interaction, providing transcriptomes from both host and pathogen (Westermann et al. 2012). Teixeira et al. (2014) focused their RNA-seq analyses of WBD on the green broom stage, with a deep and unparalleled evaluation of the biotrophic interaction between *M. perniciosa* and cacao. In addition to the evaluation of the green broom transcriptome, the authors initiated the analysis of the WBD Transcriptome Atlas (www.lge.ibi.unicamp.br/wbdatlas), a database consisting of RNA-seq transcriptomes of a collection of *M. perniciosa* developmental stages and stress responses of the fungus under in vitro, and also during in planta, interaction with cacao. Throughout the following sections of this chapter, we will compile the data from the genomics and transcriptomics results regarding *M. perniciosa* and its cacao host, connecting them to biochemical and physiological studies and updating a biological model for WBD.

6.6 Producing the Brooms: Hormones in Action

The conspicuous phenotype of green brooms is one of the main characteristics of WBD. The building of swollen, etiolated stems without apical dominance, which hallmarks the biotrophic phase of the interaction, suggests hormonal imbalances during WBD. Many plant pathogens produce phytohormones that lead to morphological alterations in the host (Navarro et al. 2006). Searching for genes related to phytohormone production, genome annotation of *M. perniciosa* revealed the presence of genes encoding putative proteins involved in gibberellin biosynthesis (Mondego et al. 2008). In addition, a putative biosynthesis pathway of auxin indole-3-acetic acid (IAA) was found in the *M. perniciosa* genome (Mondego et al. 2008), which confirms the observation of IAA production in *M. perniciosa* reported by Kilaru et al. (2007). Extending the analysis of the participation of phytohormones in WBD, Teixeira et al. (2014) used the HORMONOMETER software (Volodarsky et al. 2009) to evaluate whether green brooms present transcriptional signatures that are indicative of hormonal imbalances. The authors verified that *M. perniciosa* infection altered the signaling of phytohormones in infected cacao, especially for the hormones gibberellin, auxin, cytokinin, and ethylene. Cacao genes related to gibberellin biosynthesis and perception were upregulated, while no expression changes were found for auxin biosynthesis (Teixeira et al. 2014). Nevertheless, plant auxin-responsive genes were upregulated. One can speculate that IAA-like substances produced by *M. perniciosa* could be responsible for the upregulation of auxin-responsive genes in infected cacao plants. The expression of gibberellin- and auxin-responsive

genes supports the observations of intense hypertrophy and stem elongation in green brooms (Ross et al. 2000). Accordingly, Teixeira et al. (2014) found that genes encoding cell elongation/expansion proteins were expressed in the green broom stage. Interestingly, some of these genes such as XTHs (xyloglucan endotransglycosylase/hydroxylases), expasins, and pectinesterases are considered gibberellin-responsive genes, connecting the green broom phenotype with the presumptive phytohormone imbalance during WBD.

In agreement with Scarpari et al. (2005) who detected ethylene (ET) production in infected cacao plants, transcripts of genes required for ethylene biosynthesis accumulate in green brooms (Teixeira et al. 2014). Ethylene stimulates tissue elongation at low concentrations, but in high doses promotes senescence and cell death (Pierik et al. 2006). Thus, depending on its concentration in the brooms, ethylene may have a dual action in WBD progression, stimulating cell elongation of the green broom during the biotrophic phase and making part of the biochemical mechanism that causes the death of dry brooms during the necrotrophic phase. Finally, genes related to the degradation and inactivation of cytokinins (CKs) are upregulated in green brooms (Teixeira et al. 2014). It is likely that this may be the result of a negative feedback from the CKs present at earlier stages of infection (Orchard et al. 1994). It is proposed that the modulation of CKs is responsible for the loss of apical dominance of the green brooms (Teixeira et al. 2014).

Recent studies have elucidated the interplay between plant hormones and plant defense responses. For instance, the production of auxins by phytopathogens is associated with the suppression of the salicylic acid (SA) response against biotrophs (Wang et al. 2007). Ethylene, together with jasmonic acid (JA), mediates defense responses against necrotrophs, which can counteract plant defense against biotrophic pathogens (Zhu 2014). Gibberellins and cytokinins seem to play ambiguous roles in the plant defense network, inducing susceptibility or resistance to (hemi)biotrophic pathogens depending on which plant is infected, and on spatial and temporal conditions (De Vleesschauwer et al. 2014). Therefore, the phytohormone portrait of cacao's WBD suggests that in the first stages of the infection, IAA and ethylene mediate a downregulation of defense against biotrophic mycelia, allowing pathogen development in the apoplast.

6.7 The Arms Race in Cacao Witches' Broom Disease

Several genes encoding defense-related proteins were highly expressed in green brooms, demonstrating that the plant perceives the infection and mounts an immune response against the pathogen (Teixeira et al. 2014). Transcripts of genes encoding immune receptors (receptor-like kinase, receptor-like proteins, and NB-LRR) accumulate in infected plants. In addition, genes that belong to the pathogenesis-related (PR) superfamily such as PR-1, PR-2 (β-1-3-glucanase), PR-3, PR-4, PR-8, PR-11 (chitinase), PR-5 (thaumatin), PR-6 (protease inhibitor), PR-9 (peroxidase), and PR-10 (ribonuclease) were upregulated in infected plants. The production of

secondary metabolites seems to be significant in green brooms, since genes related to the biosynthesis of alkaloids, flavonols, anthocyaninins, and terpenoids were strongly induced in infected plants during the green broom stage of the disease. In fact, alkaloid content was higher in green brooms than in noninfected plants (Scarpari et al. 2005). Other transcriptomic evaluations of WBD indicated the upregulation of plant defense-related genes. For instance, Leal et al. (2007) evaluated the expression of cacao defense genes during the very early stages of infection (24, 48, 72, 120 and 240 h after inoculation). Plant defense-related genes from susceptible cultivar "ICS 39" showed a stronger expression at 120 and/or 240 h after inoculation, while these same genes from resistant genotypes, "CAB 214" and "CAB 208," displayed an earlier induction at 48 and 72 h. Such quantitative differences of expression were reported in other pathosystems (Benitez et al. 2005; Fernandez et al. 2012), suggesting that resistance against pathogens may be a consequence of faster activation of host gene defenses that halt pathogen development. Indeed, restricted colonization of infected tissues was reported in resistant genotypes, based on optical or electron microscopy (Silva and Matsuoka 1999; Wheeler 1985).

Some of the defense-related proteins of cacao found to be expressed in green brooms during compatible and incompatible interactions have been characterized. Pirovani et al. (2010) observed that cystatins, which are cysteine proteinase inhibitors, showed in vitro inhibitory activities against *M. perniciosa*. In addition, the same research group found that a cystatin highly expressed in incompatible interactions (TcCys4) halted the progression of cell death in tobacco plants triggered by MpNEPs expressed by *M. perniciosa* during WBD (Santana et al. 2014), suggesting that this cystatin may hamper plant necrosis elicited by *M. perniciosa* (see below). A gene encoding a ribonuclease (TcPR-10) was identified from a cacao–*Moniliophthora perniciosa* compatible interaction cDNA library (Gesteira et al. 2007). TcPR-10 showed antifungal activity against *M. perniciosa* by inhibiting mycelial growth and basidiospore germination (Pungartnik et al. 2009a). Interestingly, TcPR-10 was internalized by yeasts and *M. perniciosa* hyphae and inhibited the growth of the fungus. This transport seems to be mediated by a high-affinity copper transport and by a Snq2 export permease (Pungartnik et al. 2009a). In addition, Britto et al. (2013) and Menezes et al. (2014) reported that a β-1,3 glucanase (TcPR-2) and a chitinase/Barwin-like protein (TcPR-4) also impaired *M. perniciosa* mycelial growth, respectively.

Looking toward the compatible interaction, even though defense-related proteins are highly expressed by cacao plants infected by *M. perniciosa*, this response against the pathogen is ineffective. Consequently, *M. perniciosa* succeeds over cacao by using efficient mechanisms to manipulate host metabolism and to resist defensive proteins. As mentioned above, the production of phytohormones by *M. perniciosa* can be an important strategy to suppress plant defenses. Additionally, genes encoding proteins associated with fungal pathogenesis participate in the development of WBD. For instance, four genes encoding cerato-platanin proteins (MpCPs) are highly expressed in green brooms (Teixeira et al. 2014). *M. perniciosa* CPs were thoroughly analyzed by de Barsottini et al. (2013). The authors found that

MpCP2 aggregates were able to promote cellulose fragmentation, suggesting a role in plant cell wall loosening during fungal colonization. Furthermore, MpCP5 binds N-acetylglucosamine (NAG) and seems to block the plant defense response elicited by the chitin fragment NAG6. NAG6 is a component of the fungal cell wall, suggesting that MpCP5 can suppress the plant immune response (de Barsottini et al. 2013). Other proteins involved with fungal protection are chitin deacetylases. These enzymes defend the fungal cell well from plant chitinases through the modification of chitin to chitosan (El Gueddari et al. 2002). Teixeira et al. (2014) found four chitin deacetylases overexpressed in green brooms, reinforcing the idea that *M. perniciosa* may be able to surpass chitin-triggered immunity.

The CAP protein superfamily comprises cysteine-rich secretory proteins, antigen 5-like proteins, and plant pathogenesis-related proteins PR-1. These proteins have been recently associated with nematode and fungal pathogenesis (Lozano-Torres et al. 2014; Prados-Rosales et al. 2012). Choudhary and Schneiter (2012) found that *S. cerevisiae* CAP proteins bind to and export sterols and also have a protective effect against the plant alkylbenzene eugenol, which affects fungal membrane integrity. Genome and transcriptome annotation revealed the presence of 11 copies of genes from the CAP superfamily in *M. perniciosa* (Teixeira et al. 2012). Gene expression analyses showed that four MpPR-1 genes were preferentially expressed in green brooms and that two other MpPR-1 are highly expressed in both green brooms and germinating basidiospores (Teixeira et al. 2012, 2014). Moreover, recent data support that at least five MpPR-1s may have sterol binding properties (Darwiche and Schneiter, personal communication). Thus, it is possible that *M. perniciosa* PR-1 proteins may protect fungal membranes through the detoxification of plant hydrophobic compounds with antimicrobial properties. Knowing that plant PR-1 proteins are markers of plant defense response, the expression of PR-1-like proteins from both cacao and *M. perniciosa* during green broom development is intriguing and suggests that some MpPR-1s could act as competitive inhibitors of the plant PR-1, modulating cacao immunity (Teixeira et al. 2012). Nevertheless, the functions of plant PR-1s in immune responses remains elusive.

As a fungus that inhabits the extracellular space between cacao cells, *M. perniciosa* has to deliver its proteins toward the apoplast, which then becomes a battlefield between plant and pathogen. Therefore, one trait that is used to identify putative pathogen effectors is the presence of signal peptides. In addition, most of these proteins are compact and rich in cysteine and lack clearly characterized homologues in other organisms. Mondego et al. (2008) discovered genes encoding putative secreted polypeptides rich in cysteine during *M. perniciosa* genome annotation. Teixeira et al. (2014) improved these analyses, using hierarchical clustering analysis of the *M. perniciosa* transcriptomic atlas. The authors inspected the genes from a green broom-specific cluster and detected that 33 genes (7.4 %) encoded candidate secreted effector proteins (CSEPs). In an additional analysis, Teixeira et al. (2014) explored the expression profile of the complete set of the 247 *M. perniciosa* CSEPs by performing a CSEP-specific clustering analysis. Three clusters were associated with cacao infection (green brooms, germinating

basidiospores, and green brooms + germinating basidiospores), in total 61 CSEPs are probably involved with *M. perniciosa* pathogenicity. Curiously, *M. perniciosa* CSEPs did not have motifs found in effectors of other fungal pathogens (e.g., RSIDELD, CHXC, and Y/F/WXC motifs). An evolutionary analysis showed that most CSEP genes have multiple copies in the genome and exhibit high dN/dS (ratio of nonsynonymous and synonymous substitution rates), which are features of fast evolution (Teixeira et al. 2014). These results are consistent with the hypothesis that these proteins indeed act in pathogenesis, given that pathogen effector proteins must rapidly evolve to escape from the pressure imposed by the plant immune system.

6.8 Oxidation, Starvation, and Senescence: Stress as a Trigger of the Biotrophic to Necrotrophic Switch in *M. perniciosa*

Oxidative and Nitrosative Stress in the Green Broom

The production of reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet -}$), hydroxyl radicals (OH), and hydrogen peroxide (H_2O_2), has beneficial effects to plants acting as defense signaling molecules and combating fungal development (Apel and Hirt 2004). The role of ROS as inhibitors of pathogen infection is well documented, mainly in the hypersensitive response (HR) against biotrophs, which have their development halted by the programmed cell death (PCD) triggered by the ROS (Wu et al. 2014). It was found that H_2O_2 was produced during the progression of WBD (Ceita et al. 2007; Dias et al. 2011). Ceita et al. (2007) made a microscopic analysis during the *M. perniciosa–T. cacao* interaction, focusing on the cellular modifications during disease progression. In this work, the authors provided molecular evidence of PCD in WBD through the detection of DNA fragmentation and TUNEL analysis that showed augmentation of apoptotic nuclei during the progression of WBD, even before the switch to the fungal necrotrophic stage. In addition, calcium oxalate crystals (COCs) were found in tissues of infected susceptible cacao genotypes. The number of COCs increased during the development of the green broom, abruptly decaying before the onset of the broom necrosis (Ceita et al. 2007). Although *M. perniciosa* was found to produce COCs in vitro (Rio et al. 2008), the origin of the COCs in the brooms is not completely understood. The decrease of COCs may be caused by its degradation by germin oxalate oxidase, producing H_2O_2, (Ceita et al. 2007). In fact, Ceita et al. (2007) detected H_2O_2 accumulation in sites adjacent to COCs. Interestingly, only resistant cacao genotypes accumulate H_2O_2 during the early stages of the infection, while H_2O_2 was not found in susceptible clones during this stage (Dias et al. 2011). Conversely, more COCs were observed in the susceptible varieties in comparison to the resistant genotypes in more advanced stages of the disease (Ceita

et al. 2007; Dias et al. 2011), suggesting that the higher the production of ROS by infected plants, the less chance there is to stop the progression of WBD.

The production of H_2O_2 in green brooms clearly indicates that cacao creates an oxidative environment throughout WBD, aiming to block pathogen development. However, *M. perniciosa* contains a plethora of genes encoding ROS decomposing enzymes such as catalases, superoxide dismutases, peroxiredoxins, thioredoxin system enzymes, and manganese-dependent peroxidases (Mondego et al. 2008). The ability to resist high concentrations of H_2O_2 was tested in vitro (de Oliveira 2012) and was affirmed by the expression of a series of anti-oxidative enzymes during green broom development and under other stress conditions (Teixeira et al. 2014). Therefore, *M. perniciosa* may use an opportunistic strategy regarding highly oxidative environments by protecting itself using anti-oxidative enzymes and taking advantage of the harmful effects of ROS in the plant. The excess of ROS may lead to PCD, which provide nutrients to the invasive necrotrophic stage of *M. perniciosa*.

Apart from ROS, plants produce nitric oxide (NO), a reactive nitrogen species (RNS), which is a well-known inhibitor of the cytochrome-dependent respiratory chain (CRC, complex IV) resulting in oxidative stress (Maxwell et al. 1999). However, animal pathogens (e.g., *Trypanosoma brucei*) use an enzyme named as alternative oxidase (AOX) to alleviate the ROS/RNS stress during host infection (Brown 1999). AOX is a mitochondrial protein that characterizes an alternative respiratory pathway to CRC. Therefore, the activity of AOX circumvents a possible inhibition of CRC, thus averting oxidative/nitrosative stress. An alternative oxidase gene (MpAOX) was identified in the *M. perniciosa* genome (Mondego et al. 2008; Thomazella et al. 2012). Moreover, gene expression evaluation revealed that MpAOX was upregulated in green brooms. Strikingly, elevated amounts of NO were found in green brooms, and MpAOX expression was induced by the NO donor NOC-5 in vitro, indicating that MpAOX may participate in fungal avoidance against plant nitrosative stress during WBD (Thomazella et al. 2012).

Energetic Status and Fungal Starvation

MpAOX was found to be preferentially expressed in the monokaryotic biotrophic hyphae in relation to dikaryotic necrotrophic hyphae (Thomazella et al. 2012). Strikingly, the application of CRC inhibitors in monokaryotic mycelia prevented transition to dikaryotic mycelia. These results show that these two life stages apply different respiratory pathways in their development and suggest a connection between energetic status and morphogenetics of *M. perniciosa*. A considerable reduction in cell energy (ATP) production is correlated with AOX activity in relation to CRC (Elthon and McIntosh 1987). Therefore, MpAOX expression in monokaryotic mycelia can be one important factor of slow growth of biotrophic hyphae in green brooms. Based on MpAOX data, Thomazella et al. (2012) hypothesized that *M. perniciosa* biotrophic mycelia may decrease its growth rate centering

its metabolism in ROS/RNS detoxification and that AOX may be the switch from biotrophy to necrotrophy. MpAOX would be "switched on" by plant NO production during early stages of infection and "switched off" when NO is reduced by the death of plant tissues, therefore increasing the cytochrome/AOX ratio providing a larger amount of ATP for intense pathogen growth during the necrotrophic phase (Thomazella et al. 2012).

M. perniciosa is a fungus devoid of specialized feeding structures (i.e., haustoria) forcing it to feed in the apoplast, a relatively nutrient-poor environment (Wilson et al. 2012). The evidence that some kind of "nutrient restriction" could be one of the factors that sustain the long-lasting biotrophic stage of WBD emerged during the first efforts to obtain the biotrophic-like mycelium in vitro. This mycelium rapidly shifts to the necrotrophic-like phase when grown in nutrient-rich media (Fig. 6.2c). However, the use of "poor"-nutrient media containing glycerol as the sole carbon source sustained the biotrophic-like mycelia growth in vitro for a longer period of time (Meinhardt et al. 2006). Interestingly, Pungartnik et al. (2009b) suggested that the interaction of both glycerol and the H_2O_2 could be a signaling component of dikaryotization, because micromolar amounts of H_2O_2 induced the formation of clamp connections in biotrophic-like mycelia grown in glycerol media. Therefore, oxidative stress, energetic status, and the nature of carbon sources available for the monokaryotic mycelium could regulate the extended biotrophic development inside the green broom.

In planta biochemical studies were used to explore whether the energetic status and the nature of carbon metabolites might be related to the fungus's biotrophism in the early stages of WBD and its transition to necrotrophism. In a seminal report, Scarpari et al. (2005) provided a wide characterization of the biochemical alterations in infected tissues during WBD development. The authors found higher amounts of glycerol in green brooms in comparison to noninfected plants and dry brooms (Scarpari et al. 2005). These authors also found an increase in starch concentrations 21 dai and sucrose and glucose concentrations 35 dai (Scarpari et al. 2005). In an early stage of WBD, high starch levels suggest the mobilization of soluble sugars. However, the starch that was stored is remobilized to glucose in a later stage. This data was confirmed by a microscopic analysis 30 dai that showed the depletion of starch grains in green brooms when compared to healthy plants and was associated with the upregulation of genes encoding alpha-amylases in this stage of the infection (Teixeira et al. 2014). Teixeira et al. (2014) detected the high-level expression of a cell wall invertase, which breaks sucrose into fructose and glucose, in green brooms, and Barau et al. (2015) found that this enzyme is active during the first stages of the infection. Curiously, alpha-amylases and invertases are upregulated by gibberellins and auxins, respectively (Gubler et al. 1995; Tymowska-Lalanne and Kreis 1998), two phytohormones that probably act in WBD development. Additionally, genes encoding sugar transporters from both plant and pathogens were found to be expressed in the green broom (Teixeira et al. 2014). These results seem to indicate that plant hexoses accumulate in green brooms and that they can be absorbed by the pathogen as soluble carbon sources in the apoplast.

In a recent work, Barau et al. (2015) went further and characterized the biochemical changes during WBD by assessing the presence of soluble carbon sources in the apoplast and the interface of fungus and plant during the green broom component of WBD. The authors asked the question of whether extracellular carbon availability is related to the long biotrophic phase of WBD. They found that the dynamic of soluble sugars in the apoplast is completely altered during green broom development in relation to healthy plants, leading to the loss of rhythmic growth patterns observed in noninfected plants (Barau et al. 2015). Compared to control plants, glucose and fructose were found to be higher in the apoplastic fluid of *M. perniciosa*-infected plants in the first 25 dai of *M. perniciosa*, coinciding with the onset of morphogenesis of green brooms. Recently, Mason et al. (2014) established that sugar availability is essential for axillary bud release from apical dominance. Therefore, together with hormonal imbalance, the higher levels of hexoses in proliferative meristems may lead to the loss of apical dominance displayed by green brooms.

Barau et al. (2015) manipulated apoplastic fluid sugar availability artificially to test if the fungal sensing of carbon starvation could interfere with the progression of WBD. The infiltration of a carbon solution through petiole feeding assays led to a delay of the later necrotic process when compared with untreated controls. Therefore, it seems that soluble carbon sources may be important for the maintenance of the biotrophic phase through the avoidance of fungal starvation. In the same manuscript, *M. perniciosa* was found to suffer autophagy derived from in vitro carbon starvation and that rapamycin, an inhibitor of carbon-sensing TOR (target of rapamycin) kinases, circumvents nutrient availability signals, activating an autophagic response in *M. perniciosa* even in the presence of adequate amount of carbon (Barau et al. 2015). Since plant TOR kinases are reported to be insensitive to rapamycin, the authors added rapamycin to carbon source solutions to test whether the delay of plant necrosis by carbon feeding was caused by the delay of fungal starvation or due to an increase in plant tissue survival. These plants presented a necrosis progression more similar to that verified in controls (non-carbon source fed), in comparison with those infiltrated only with carbon source solutions. Clearly, rapamycin inhibited the delayed necrosis phenotype of carbon source-infiltrated plants. These results support the idea that fungal starvation is a factor in triggering the shift from biotrophy to necrotrophy.

Accelerating Plant Senescence

The onset of chlorosis in green brooms is one of the most peculiar traits of WBD. In fact, different approaches indicate that photosynthesis is arrested in green brooms. A decrease in chlorophyll b levels was observed soon after infection (Scarpari et al. 2005). Accordingly, RNA-seq data indicate that photosynthesis genes are downregulated in green brooms (Teixeira et al. 2014). It is proposed that free soluble sugars diminish photosynthesis activity through a negative feedback that

is a property of sink tissues (Berger et al. 2007). The combination of reduced photosynthesis and increased concentration of soluble sugars is observed in plants during the onset of senescence (Jongebloed et al. 2004) and parallels green broom biochemical data such as increases in ethylene biosynthesis. Barau et al. (2015) verified that cacao orthologs for genes previously characterized as markers of carbon starvation in plants were upregulated in dark-treated plants and in green brooms. Curiously, chlorotic young leaves in the distal parts of mature brooms were found to accumulate more soluble sugars, express a higher amount of transcripts of senescence associated genes, and display necrotic symptoms initiated at the leaf tips (Barau et al. 2015), correlating photosynthesis arrestment and senescence to WBD progression.

In fact, the RNA-seq analysis of Teixeira et al. (2014) strongly suggests that the green broom is a tissue with all the signs of a premature senescence event. The authors found that genes from the ethylene biosynthesis pathway and fatty acid β-oxidation pathway (in which fatty acids are catabolized to produce energy) and from the glyoxylate (GLOX) cycle are overexpressed in the green broom. Curiously, the GLOX cycle is one of the hallmarks of physiological plant senescence, participating in the reuse of nutrients (Buchanan-Wollaston 1997). The overexpression of lipases and the fatty acid β-oxidation pathway enzymes correlate with the increase of glycerol and malondialdehyde (MDA) in the green broom (Scarpari et al. 2005). In addition to lipids, amino acid compounds were found to be catabolized in green brooms (Scarpari et al. 2005; Teixeira et al. 2014). The catabolism of amino acids produces ammonium, a toxic compound that must be detoxified. The upregulation of an asparagine synthetase (asn), which mediates the incorporation of ammonium into aspartate to produce asparagines, may be a strategy to avoid the harmful effects of ammonium in the green broom (Teixeira et al. 2014). Indeed, asparagine was found to accumulate in green brooms (Scarpari et al. 2005). Strikingly, asn is induced in senescing tissues and in plants treated with photosynthesis inhibitors (Fujiki et al. 2001). In general, starch, lipids, and amino acids appear to be catabolized in the green broom, causing a carbon deprivation that culminates with the broom senescence. Apparently, this assumption contradicts the sink-like development of green brooms. However, the presence of structures similar to a callus at the base of the brooms may impair the communication between healthy and infected parts of the plant (Barau, personal communication), resulting in the complete consumption of the nutrients and the consequent senescence.

Destroying the Green Broom

Although the accelerated senescence process seems to be the trigger of green broom cell death, the active participation of the fungus in the death of host tissues has been thoroughly inspected by the WBD project. Five genes encoding NEPs (necrosis- and ethylene-inducing proteins; see above) were identified in the genome of *M. perniciosa* (Garcia et al. 2007). The protein MpNEP2 was further studied, and

its application in plants led to the necrosis of cacao leaves. Curiously, MpNEP2 was not expressed during the early death of infected plants (Zaparoli et al. 2011). During the disease progression, MpNEP2 expression increases, reaching its highest level of expression at the advanced necrotic stage (Zaparoli et al. 2011). In vitro analysis showed that carbon starvation conditions upregulated MpNEP2 expression (Barau et al. 2015). In addition, transcripts of MpNEP2 diminished in green brooms after the artificial application of carbohydrates (Barau et al. 2015). However, the earlier expression of MpNEP2 treated with rapamycin did not advance the onset of necrosis. Therefore, despite the active participation of *M. perniciosa* in death of plant tissues by expressing MpNEP2, this protein is not the unique agent causing necrosis. In fact, the secretion of necrosis inducing proteins in vitro was found to be modulated by different carbon sources. Interestingly, secretion of necrosis inducing proteins in vitro was promoted by glycerol, the non-fermentable sugar which accumulates in green brooms (Alvim et al. 2009). Hence, the sensing of the green broom collapses, and senescence of the tissues by the fungus may be the trigger for MpNEP2 to actively destroy the remaining cacao tissues. Consequently, the fungus spreads through the broom and speeds up the necrosis of host cells, which provide soluble nutrients to the necrotrophic hyphae.

Together with MpNEP2, *M. perniciosa* expresses a myriad of cell wall-degrading enzymes, which can contribute to the spread of the fungus in the broom and to the ultimate breakdown of the plant cell wall. A comparative analysis between *M. perniciosa* and other fungi that interact with plants demonstrated that the WBD causative agent presents an arsenal of pectin-degrading enzymes that is similar to the ones of other hemibiotrophic fungi, such as *Fusarium graminearum* and *M. oryzae* (Mondego et al. 2008). *M. perniciosa* colonizes the apoplast and has to breach the middle lamella, which is rich in pectin. In addition, pectins are cross-linked with calcium (Ca^{2+}) that may be released during pectin degradation. It is hypothesized that this Ca^{2+} can be scavenged by oxalate, forming calcium oxalate crystals (de Oliveira et al. 2012b), which were detected in vitro during *M. perniciosa* mycelial development (Rio et al. 2008) and in vivo (Ceita et al. 2007) during green broom development. Immunofluorescence detection of the pectic homogalacturonan domain in infected and noninfected cacao seedlings shows a reduction in the level of pectin methyl esterification in infected cacao seedlings (de Oliveira et al. 2012b), suggesting fungal methyl esterification of plant pectin. Furthermore, *M. perniciosa* polygalacturonase activity and transcription were found to be upregulated by fermentable carbon sources, such as galactose, glucose, and fructose (Santos Carvalho et al. 2013a, b). Pectin degradation releases methanol, which was found to be metabolized by *M. perniciosa* (de Oliveira et al. 2012b). The authors showed that *M. perniciosa* is able to grow on methanol as the sole carbon source and has genes involved in the metabolism of methanol, including methanol oxidase (MOX), formaldehyde dehydrogenase, and formate dehydrogenase. Even though MOX is considered a peroxisomal enzyme, *M. perniciosa* possesses an extracellular methanol oxidase (de Oliveira et al. 2012b), indicating that this fungus starts the methanol metabolism in the apoplast. Along with the pectin degradation apparatus, the *M. perniciosa* genome

contains genes encoding enzymes involved in the degradation of cellulose and hemicelluloses, including β-1,4 cellulases, exocellobiohydrolases, endo-beta-1,4-xylanases, and endoglucanases (Mondego et al. 2008). In addition, the fungus presents genes encoding lignolytic enzymes such as multicopper polyphenol oxidases (laccases) and manganese-dependent peroxidase (Mondego et al. 2008), thus containing a complete arsenal of plant cell wall-degrading enzymes.

Giving a summary of the participation of stress events in the pleomorphic switch of *M. perniciosa*, we can speculate that on the first days of the disease, *M. perniciosa* slowly grows in the low energetic environment of the apoplast combating the effects of defensive nitrosative stress in fungal CRC by expressing AOX. The fungus builds the green broom by producing or modulating plant hormones that give rise to the remarkable expansion of stems. Those phytohormones also modulate plant defenses that favor pathogen development. As a consequence, cacao plants expend carbon resources to support the intense growth of the broom, producing hexoses that together with phytohormones lead to loss of apical dominance. To sustain its development, the fungus feeds on these sugars and on other resources, such as methanol. However, the sugar accumulation appears to trigger the downregulation of photosynthesis, a symptom characteristic of senescent tissues. The continuously increasing production of ROS and ethylene during the green broom stage tends to accelerate a PCD/senescence in the brooms. The exhaustion of nutrients in the green brooms causes carbon starvation that is sensed by the fungus in the apoplast, which produces MpNEP2 that accelerates tissue necrosis, with the goal of accessing intracellular plant resources. Tissue death reduces the production of NO, switching on the fungal CRC, supplying the energy needed for intense pathogen growth during the necrotrophic phase.

6.9 Living in the Ruins and Breeding a New Generation: The Fungus in the Dry Broom

To finish its development, *M. perniciosa* must inhabit the dry brooms, an environment consisting of dead plant cells depleted of nutrients by the intensive degradation of necrotrophic mycelia. In this ecological niche primarily composed of decaying wood, *M. perniciosa* must expresses plant cell wall degradation enzymes, especially those related to the digestion of wood constituent polymers (cellulose and lignin; see above). In addition, the fungus may have to deal with other competing wood-decaying organisms. During *M. perniciosa* transcriptome and genome annotations, genes encoding enzymes with antifungal properties were identified. For instance, the *M. perniciosa* necrotrophic mycelium expresses KP4 toxins (killer toxin proteins) that are polypeptides produced by viruses inhabiting fungal genomes (Rincones et al. 2008). These proteins are produced by the fungus to kill competing fungal strains, even from the same species, by creating pores in targeted cell membranes (Clausen et al. 2000). Other *M. perniciosa* proteins that

have antifungal properties are thaumatin-like proteins (TLPs). Mondego et al. (2008) reported that *M. perniciosa* has a genome expansion of TLPs. Recent transcriptomic data indicate that this fungus contains 13 TLPs and that 4 are expressed in dry brooms infected by *M. perniciosa* (Franco et al., 2015), in agreement with its putative role against competitors.

Another strategy to avoid competition in the dead tissues is related to one of the most intriguing features of WBD, the lack of abscission layers in the dry brooms (Purdy and Schmidt 1996). As discussed by Barau et al. (2015), the persistent attachment to the cacao trees can prevent their contact with leaf-litter decomposers, giving preferential access of dry broom resources to *M. perniciosa*. Additionally, the presence of brooms in the canopy may be a way to produce basidiospores near shoot meristems increasing the chances of their infection. Curiously, as far as we know, *M. perniciosa* and *M. roreri* are the only Agaricomycetes that infect aerial parts of the plants (i.e., shoots and fruits).

The formation of basidiocarps in dry brooms is one of the most remarkable events in the WBD cycle. In order to obtain insights about this stage of development, Pires et al. (2009) provided a description of primordium development of *M. perniciosa* basidiocarps using an in vitro approach. Using optical and electronic microscopy, the authors give a detailed timecourse of events concerning basidiocarp development. The events of fusion of hyphae, the formation of hyphal nodules, aggregation, initial primordium, and differentiated primordium were observed and correlated with transcriptome analyses from mycelium in different stages of basidiocarp formation. During in vitro growth, mycelia are subjected to intermittent watering, mimicking dry broom environmental conditions at which basidiocarp production is observed (Pires et al. 2009; Rocha and Wheeler 1985). The transcription of a glucose transporter gene (MpGLU) increased in mycelium until water stress was applied, suggesting that glucose reduction in the medium must be important for the fructification of basidiocarps (Pires et al. 2009). Pungartnik et al. (2009b) found that Atg8, a key gene of autophagy and nutrient starvation in fungi, is expressed before in vitro basidiocarp production. These results suggest that the depletion of nutrients in dry brooms may trigger the mushroom production from necrotrophic mycelia. As mentioned above, *M. perniciosa* mitochondria contain a pKalilo-like plasmid, which is related to fungal senescence in *Neurospora* (Bertrand et al. 1985). In vitro-grown *M. perniciosa* necrotrophic mycelia display symptoms that characterize fungal aging, such as a decrease in growth rate, an increase in pigmentation, and a reduction in the formation of aerial hyphae (Formighieri et al. 2008). Interestingly, recent studies on pathogenic yeasts have shown a positive correlation between aging cells and resistance to host-derived stresses during chronic diseases (Bouklas and Fries 2013).

6.10 Development of New Agrochemicals Against Witches' Broom Disease

The use of agrochemicals in agriculture is currently essential to maintain crop productivity and, consequently, to meet the increasing global demand for food and energy resources from renewable sources. However, in recent years, there is a general concern on the sustainable use of these compounds. In this context, the study and development of safer and more effective agrochemicals are needed and might have notable consequences, reducing the areas required for cultivation and preventing water and energy waste.

WBD is one of the most important phytopathological problems affecting South America in recent decades. Despite its importance, the available fungicides are ineffective or insufficient to control WBD. Therefore, there is great motivation for the discovery/development of new compounds that target well-defined fungal molecules and successfully impair *M. perniciosa* development.

Based on the results generated along the fifteen years of the WBD genome project, we are now able to develop more successful molecules to control WBD. As a starting point, the mitochondrial enzyme AOX, which has been proven to be important for *M. perniciosa* development, will be one of our initial targets. As discussed before, Thomazella et al. (2012) verified that *M. perniciosa* is resistant to an important class of mitochondrial fungicides called strobilurins, which specifically block fungal respiration by inhibiting the mitochondrial respiratory chain. However, AOX bypasses the mitochondrial respiratory chain, thus allowing fungal respiration to continue in the presence of these chemicals. Remarkably, the use of strobilurins along with AOX inhibitors seems a promising strategy to combat WBD (Thomazella et al. 2012). However, this strategy depends on the use of AOX inhibitors, which are not sufficiently stable under field conditions.

Recently, based on the 3D structure of the *Trypanosoma brucei* AOX (Shiba et al. 2013), the 3D structure model of the *M. perniciosa* AOX was produced (Guido, R., unpublished data). Combining results of molecular docking and AOX inhibition assays, we expect to develop new compounds that can block AOX activity and display high stability under field conditions. Moreover, given the rapid development of fungicide resistance, the 3D structure of many other fungal proteins with a potential role in *M. perniciosa* pathogenicity has been solved. In the upcoming years of WBD research, we aim to design and develop a set of highly stable molecules that specifically and effectively prevent WBD progression under field conditions.

6.11 Combining Science and Field Management to Combat Witches' Broom Disease

The development of strategies to combat WBD based on the accumulated knowledge on the *M. perniciosa* pathogenic mechanisms is still in progress; however, the battle against WBD is ongoing in southern Bahia. Farmers are aiding in the development of field management solutions for combating WBD, which is helping in the recovery of cacao plantations. One example of observations of WBD development in the field that have led to new strategies to combat the disease was the initiative of an agronomist and farmer Edvaldo Sampaio (in memoriam). He fused tradition with innovation, by applying conventional agronomical techniques for managing cacao, by changing the time of these management activities contrary to the traditions established in Bahia. For instance, pruning of cacao was moved from January to March, when the peak of *M. perniciosa* sporulation occurs, to September to November. Pruning of cacao is usually followed by a flush of new growth. By delaying pruning, newly released basidiospores are unable to find new meristems to infect. Strikingly, the farms that apply such modifications have a drastically reduced incidence of WBD. Although these empirical observations have not been substantiated scientifically, we believe that this modification in cacao handling causes a desynchronization between the fungus and the plant development. Due to their daily contact with WBD, we believe that the farmers are the infantry in the frontline of this battle, and we hope to provide them with new weapons, such as safer agrochemicals, and new insights to extend their knowledge base to help combat this disease.

6.12 Future Research

Important advances in the understanding of witches' broom disease have been achieved during the 15 years of the WBD genome project. Even so, a number of relevant aspects of this disease still need to be addressed. Future research on WBD will focus on the following topics:

Development of genetic manipulation techniques for both cacao and *M. perniciosa*
Identification of resistance genes in cacao varieties that are resistant to WBD
Investigation of the mechanisms responsible for the maintenance of the long biotrophic stage in *M. perniciosa*
Investigation of the mechanisms involved in host adaptation in *M. perniciosa*
Characterization of drug resistance strategies in *M. perniciosa*

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Part III
Phytophthora spp. Causal Agents of Black Pod Rot

Chapter 7
Black Pod and Other *Phytophthora* Induced Diseases of Cacao: History, Biology, and Control

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Abstract The Oomycete genus *Phytophthora* is one of the most destructive plant pathogens. Over 100 *Phytophthora* species are described to date, but only a few are of major economic concern to the cacao industry. The history, biology, and control of *Phytophthora* spp. affecting cacao worldwide are examined. The genetic diversity, symptomatology, control measures, and screening for resistance of these pathogens are reviewed. The *Phytophthora* species attacking cacao were distributed across four of the ten clades recognised, and of these *P. palmivora* is ubiquitous to the cacao-growing regions. Although highly destructive, the genetic diversity within each species that attack cacao appeared to be limited, with many clonal lineages being recognised. Free water is needed for dispersal, infection, and completion of the life cycle of *Phytophthora*. The typical life cycle of *Phytophthora* is elucidated and illustrated. Infection on cacao is usually by motile, biflagellate zoospores which are liberated from stalked sporangia. Hyphae grow inter- and intracellularly and result in necrosis of host tissues. Various methods devised for the control of the disease and their relative merits are discussed. Screening methods for the identification of resistance are evaluated and their importance to future breeding programmes to generate stable resistant cultivars is discussed. Reliable high-quality disease resistance data for genomic applications will be increasingly needed. This data is dependent on robust screening methods and guidelines for future direction of research are also outlined.

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7.1 Introduction

Phytophthora: The Great Destroyer

'Phytophthora' was coined by Heinrich Anton de Bary, the founding father of plant pathology and modern mycology in 1876, and literally means *phyto* (plant) and *phthora* (destroyer) in Greek. *Phytophthora* encompass a large genus of plant-pathogenic Oomycetes in the lineage of Stramenopiles (Gunderson et al. 1987; Thines 2014) and are extraordinarily important plant pathogens. The Irish potato famine, for example is one of the most recognised plant pathogen-incited human disasters. Potato blight, incited by *Phytophthora infestans*, caused severe famines in the years 1845–1846, and is attributed to Ireland losing around a quarter of its population to starvation and emigration (Ristaino 2002; Ribeiro 2013). This disaster highlights the risks of genetic uniformity and overdependence on a few food crops and the increased potential for emergent diseases with widespread distribution of non-indigenous crops (Schumann and D'Arcy 2012). This situation in cacao can be severe also as, under the right conditions and with the right pathogen/cacao gemplasm combinations, cacao yields can be devastated.

Evolution and Divergence

Although they were once considered as fungi, *Phytophthoras* are more closely related to diatoms and brown algae (Gunderson et al. 1987; Thines 2014). The Oomycetes are suggested to have evolved from simple holocarpic marine parasites with hyphal growth and oogamous sexual reproduction probably developing after migration onto land (Beakes et al. 2012). Like fungi, *Phytophthoras* have a thallus or mycelium consisting of branched, tubular structures (hyphae; singular hypha). *Phytophthora* and other Oomycetes, differ from true fungi in having cellulose cell walls with variously linked β-glucans (Bartnicki-Garcia 1969; Tokunaga and Bartnicki-Garcia 1971). *Phytophthora* species are diploid in their vegetative stage, unlike true fungi that are usually haploid. *Phytophthora* species do not synthesise sterols and require an exogenous source of β-hydroxy sterols for sporulation (Hendrix 1970; Elliot 1983). The similarities between *Phytophthoras* and true fungi are examples of convergent evolution which leads to organisms from different evolutionary lineages sharing similar features/habits.

The knowledge about the genus *Phytophthora* has expanded dramatically in the past 139 years and over 100 species are now recognised (Érsek and Ribeiro 2010). In the last decade, the number of identified *Phytophthora* species has nearly doubled (Kroon et al. 2012). Its species diversity and vast host range make

Phytophthora one of the most devastating pathogens on plants including the chocolate tree—cacao (*Theobroma cacao* L.) (Zentmyer 1983).

Phytophthora Diseases of Cacao: Importance and Distribution

The chocolate industry is valued at 110 billion USD annually (Percival 2015). The cacao crop for 2015 is forecasted at a world total of 4,168,000 tonnes with an approximate value of 12.5 billion USD (ICCO 2015). Annual losses from *Phytophthora* diseases were estimated at 30 % of the crop (Guest 2007) which translates to approximately 3.8 billion USD loss to the cacao farmers worldwide. The Phytophthora diseases, most notably black pod rot and stem canker, are ubiquitous wherever cacao is grown (Thorold, 1975; Despréaux, 2004).

Although a number of *Phytophthora* species are reportedly infectious on cacao, only a few species, *P. palmivora*, *P. megakarya*, *P. citrophthora*, and *P. capsici*/*P. tropicalis*, are considered as commercially important, causing serious crop losses in different parts of the world. At present, only *P. palmivora* is found in every country where cacao is grown (Brasier and Griffin 1979). *P. megakarya* has been reported only in cacao crops in West Africa (Brasier and Griffin 1979). *P. capsici* on cacao is commonly encountered in the Americas and the Caribbean area (Erwin and Ribeiro 1996; Lass 2001; Guest 2007) but has also been found in Cameroun (Bakala 1981; Zentmyer et al. 1981). *P. citrophthora* has been reported as economically important on cacao in Brazil (Lawrence et al. 1990) and is also known to affect cacao in Indonesia (Appiah et al. 2004) and India (Chowdappa and Chandramohanan 1995, 1996, 1997). Presently, while not all species exist in all cacao-growing areas, there is a serious threat of introductions due to human involvement. Due to the importance of *P. megakarya* in the largest cacao-producing region (West Africa; World Cocoa Foundation 2012), a separate chapter (Chap. 8) has been devoted to this species. This chapter will therefore focus on diseases caused by the other *Phytophthora* species.

7.2 History and Taxonomy of *Phytophthora* on Cacao

Although black pod disease was recognised on cacao for many years, Carruthers (1898) was the first to record a fungus-like organism associated with black pod rot of cacao but misidentified the causative fungus as *Peronospora*. Massee (1899) studied the disease on cacao pods received from Trinidad and identified the causal agent as *Phytophthora omnivora* de Bary. Butler (1907) described a 'fungus', designated as *Pythium palmivorum*, causing bud rot of coconut, palmyra, and areca palm in the Godavari River Delta in India. Von Faber (1907) described the

fungus associated with black pod rot in Cameroun applying the generic name *Phytophthora* sp. to his isolate. The cacao pathogen was given the species name *Phytophthora faberi* by Maublanc in 1909. Butler (1919) and later Ashby (1929) established that the *Phytophthoras* isolated from cacao, rubber, palmyra, coconut, and cotton were closely related. Butler (1919) renamed the pathogen *Phytophthora palmivora*. From that time until the 1970s, black pod rot throughout the world was generally considered to be caused by this single species (Brasier and Griffin 1979). At least eleven species of *Phytophthoras* have been associated with diseases of cacao including *P. palmivora* (Gregory 1974), *P. megakarya* (Djiekpor et al. 1981; Dakwa 1988), *P. capsici* (Lawrence et al. 1982), *P. citrophthora* (Campêlo et al. 1982), *P. heavae* (Lozano and Romero 1984), *P. katsurae* (Liyanage and Wheeler 1989), *P. meadii* (Baker 1936; Sreenivasan 1975), *P. botryosa* (Chee and Wastie 1970), *P. parasitica* and *P. megasperma* (Zentmyer 1988a), and P. *tropicalis* (Aragaki and Uchida 2001). Only four species (*P. palmivora*, *P. capsici*/*P. tropicalis*, *P. megakarya*, *P. citrophthora*) are of commercial importance. The uncertainty of the status of *P. capsici* and *P. tropicalis* will be discussed later.

Morphological and Cytogenetic Differentiation of Phytophthora *Species*

A universally acceptable classification system to differentiate various species within the genus *Phytophthora* has been sought for a long time. Prior to the development of molecular tools, morphological aspects of the various species/isolates were primarily used to designate *Phytophthora* species. *Phytophthora* produces zoospores in structures called sporangia held on a sporangial stalk or pedicel. The sporangia are hyaline to light yellow in colour and vary in shape and size. The sporangial shape (spherical, subspherical, ovoid, obovoid, ellipsoid, limoniform, pyriform, obpyriform, turbinate, obturbinate) is often species specific (Brasier and Griffin 1979). Pedicel length, caducous or persistent sporangia, the presence or absence of conspicuous or inconspicuous papillae, and sporangial stalk morphology were also useful descriptors (Waterhouse 1963, 1974a, b; Blackwell 1949; Newhook et al. 1978; Stamps et al. 1990; Zentmyer 1976; Zentmyer et al. 1977; Kaosiri 1978; Kaosiri et al. 1978). Morphological identification of *Phytophthora* was recently reviewed (Martin et al. 2012).

Current Taxonomy

Morphological taxonomy has traditionally been used to separate species and keys to facilitate identification have been published (Rosenbaum 1917; Tucker 1931;

Leonian 1934; Waterhouse 1963, 1970; Newhook et al. 1978; Stamps et al. 1990) with six morphospecies groups (Group I–Group VI) established (Waterhouse 1963, 1970; Newhook et al. 1978; Stamps et al. 1990). Other than *P. megakarya*, four species of *Phytophthora* are of importance in causing black pod disease of cacao and can be differentiated as follows:

Phytophthora palmivora

Synonyms of *P. palmivora* include *P. omnivore* de Bary (1881), *Pythium palmivorum* Butler (1907), *P. faberi* Maublanc (1909), *P. theobromae* Coleman (1910), *P. palmivora* var. *piperis* Muller (1936), and *P. palmivora* var. *theobromae* (Coleman) Orellana (1959), *as well as Kawakamia carica* Hara (1915), *P. fici* Hori (1915) and *P. carica* Hara (1916) as found at http://www.*Phytophthora*db.org/species and in Tanaka (1920).

Zentmyer (1976) divided *P. palmivora* isolates into four groups according to sporangial stalk characteristics: (a) Sporangia with a round base and shed with short thick stalks < 5 μm in length; (b) Sporangia with a round base and with thin stalks 10 μm in length; (c) More elongated sporangia with 'sloping shoulders' and a platform base shed with a long fairly thick stalk > 15 μm in length; and (d) Isolates with persistent sporangia.

At a cacao *Phytophthora* workshop held at Rothamsted in 1976, a general consensus was arrived to temporarily group the different forms of *P. palmivora* on cacao into four morphological forms (Griffin 1977):

MF 1: Cultures on carrot agar stellate/striate (smooth-combed) with sharp well-defined edges and aerial mycelium usually sparse. Sporangia with rounded bases shed with a short broad occluded pedicel (<5 μm in length). Cosmopolitan. Predominantly of the A2 compatibility type. 9–12 small chromosomes. Subsequently retained as *P. palmivora*.

MF 2: Cultures were not available at the time of the workshop. However, cultures were apparently similar to MF1 having a stellate/striate appearance. Sporangia have a rounded base, short occluded pedicel (<5 μm).

MF 3: Cultures on carrot agar with no distinctive colony pattern, with plentiful cotton wool-like aerial mycelium and with the leading edge less well defined than for MF 1 isolates. Sporangia with rounded bases shed with a thin stalk of length > 5 μm but < 15 μm containing cytoplasm. Mainly restricted to the Nigeria/Cameroun/Gabon area. Predominantly of the A1 compatibility type. $n = 5$–6 large chromosomes. Subsequently renamed as *P. megakarya*.

MF 4: Cultures on carrot agar with a petaloid pattern and with a moderate amount of aerial mycelium. Elongated sporangia with 'sloping shoulders' and a platform across the base are shed with fairly thick long stalks > 15 μm in length. Predominantly found in South American countries. Chromosome type unknown.

Subsequently renamed as *P. capsici* and now known to be similar in many ways to *P. tropicalis*.

Out of the four morphological forms, only MF 1, MF 3, and MF 4 were examined in the workshop. Cytological examinations by Sansome et al. (1975) revealed the presence of two distinctive chromosome types in *P. palmivora* infecting cacao in West Africa. One group (MF 3) showed 5–6 large (L) chromosomes and the other group (MF 1) showed 9–12 small (S) chromosomes at metaphase. Culture studies distinguished the L and S types when they were grown in the dark on carrot agar medium. Cultures of the S type produced a stellate pattern with very little aerial mycelial growth diagnostic of MF 1, while the L type produced profuse aerial mycelium without the formation of any definite pattern diagnostic of MF 3.

Brasier and Griffin (1979) published a detailed study involving roughly 950 *Phytophthora* isolates from cacao with respect to morphology, physiology, chromosome type and number, culture characters on carrot agar, cacao pod lesion characteristics, sporangial morphology, compatibility type, growth on synthetic medium, response to *Trichoderma*, and temperature relations. The results of this study showed that the majority of isolates of *P. palmivora* from cacao belonged to one of the three main morphological forms, S (MF 1), L (MF 3), and MF 4. The authors proposed that the S type should be referred to as *P. palmivora* superseding the term MF 1. The L (MF 3) type was described as a new species, *P. megakarya*.

Phytophthora capsici/Phytophthora tropicalis

Leonian (1922) reported a stem and fruit blight disease of pepper (*Capsicum annum*) and identified the causal agent as *P. capsici*. Baker (1936) isolated a new *Phytophthora* from cacao pods and identified it as a *Phytophthora* sp. *arecae-meadii*. Sreenivasan (1975) reported the isolation of a *Phytophthora* from infected cacao pods in Trinidad which resembled an isolate from cacao described by Baker (1936). This isolate was assigned to the MF 4 group of *P. palmivora* at the Cocoa *Phytophthora* workshop at Rothamsted in 1976. Kaosiri et al. (1978) noted the similarities between *P. palmivora* MF 4 isolates and *P. capsici*. Brasier and Medeiros (1978) determined the chromosome type and number of three Brazilian isolates (MF 4). These isolates showed 9–12 small type (S) chromosomes. Zentmyer et al. (1981) presented further evidence to show the close similarity between *P. capsici* and MF 4 of *P. palmivora* and suggested that the MF 4 of *P. palmivora* be designated as a form of *P. capsici*. Synonyms of *P. capsici* include *P. hydrophila* (Curzi 1927), *P. parasitica* var. *capsici* (Leonian) (Sarejanni 1936), and *P. palmivora* MF4 (Griffin 1977).

Resolution of P. tropicalis *from* P. capsici *on Cacao*

Previously, *P. capsici* and *P. tropicalis* were thought to be conspecific (Mchau and Coffey 1995; Oudemans et al. 1994). Both species produce sporangia with similar characteristics (deciduous oblong shaped with prominent apical papillae on long pedicels). Both cause tropical diseases on Macadamia (*Macadamia* spp.), cacao (*Theobroma cacao* L.), and eggplant (*Solanum melongena* L.) (Erwin and Ribeiro 1996). *P. tropicalis* was given a separate species taxon from *P. capsici* in 2001 based on morphological characteristics, absence or minimal growth at 35 °C, and having weak or no pathogenicity on pepper (*Capsicum annum* L.) (Aragaki and Uchida 2001; Leonian 1922; Enzenbacher et al. 2015). Furthermore, their separation into two distinct species is supported by genetic and phylogenetic analyses (Donahoo and Lamour 2008a; Quesada-Ocampo et al. 2011; Zhang et al. 2004).

P. capsici are commonly recovered from vegetables, whereas *P. tropicalis* is more often recovered from woody perennials such as cacao. Donahoo and Lamour (2008a) stated that in Tennessee, *P. tropicalis* was routinely isolated from nursery-grown ornamentals, but never from nearby vegetable production fields. The converse was also true, as *P. capsici* isolates were never recovered from nurseries. At least one in vitro cross has been successful between *P. capsici* and *P. tropicalis* (although many were tried that were unsuccessful), and this led to oospore progeny with alleles from both parents (Donahoo and Lamour 2008b). *P. tropicalis* continues to be misidentified as *P. capsici* in the literature as a result of their morphological similarities and the presence of mislabelled sequences in GenBank. Clearly, historical references in the literature to *P. capsici* isolated from cacao must be considered possibly, if not likely, to be *P. tropicalis*.

Phytophthora citrophthora

This species was first described by Smith and Smith (1906) causing disease of lemon and other citrus fruits in California, USA. Although they named the fungus *Pythiacystis citrophthora* R.E. Sm and E.H. Sm., Leonian (1925) noted the similarity of *Pythiacystis* to *Phytophthora* and proposed the current name *Phytophthora citrophthora*. Synonyms of *P. citrophthora* include *Pythiacystis citrophthora* Smith and Smith (1906) and *P. imperfecta* var. *citrophthora* (Smith and Smith) Sarejanni (1936). Mchau and Coffey (1994a) in summarising what was known about *P. citrophthora* found that the species existed throughout the world on a broad range of hosts. Confusion in the older literature exists since diametric descriptions were often given: caducous or non-caducous sporangia; and sterile or heterothallic. Initially, chlamydospores were not reported (Smith and Smith 1906; Leonian 1925), but was later observed in old cultures (Tucker 1931). Kellam and Zentmyer (1986) reported the formation of chlamydospores by *P. citrophthora* isolated from cacao in Brazil. Brasier and Hansen (1992) hypothesised that *P. citrophthora* may have originated as a hybrid species in situations like cacao in Brazil where several

Phytophthora spp. coexist. *P. citrophthora* isolated from cacao in Brazil appears to be atypical of the species. Goodwin et al. (1990) reported that cacao isolates of *P. citrophthora* from Brazil were non-reactive to *P. citrophthora*-specific DNA probes developed using sequences from isolates from other hosts. Similarly, in an analysis of mtDNA restriction fragment length polymorphisms, isolates from cacao in Brazil were separated from those from citrus (Förster et al. 1990). Oudemans and Coffey (1991b) were able to divide *P. citrophthora* isolates into two subgroups based on isozyme analysis: CTR1 composed of isolates from a wide host range and CTR2 consisting of only Brazilian isolates from cacao.

7.3 General Life Cycle of *Phytophthora* Species

Knowing the life cycle (Fig. 7.1) is critical for understanding pathogenic variation and implementing appropriate control measures. The genus *Phytophthora* is closely related to the genus *Pythium*, both of which are water moulds. The disease caused by *Phytophthora* is favoured by free water in soil and on foliage. *Phytophthora* has coenocytic mycelium with no, or few, septa and under suitable conditions will asexually reproduce. Sporangia borne on sporangiophores release uninucleate reniform zoospores (Fig. 7.1) each with one whiplash and one tinsel flagellum (Desjardins et al. 1969; Hemmes 1983). Some morphological characteristics used to define particular species include the physical appearance and dimensions of the sporangia, presence or absence of conspicuous or inconspicuous papillae, and the

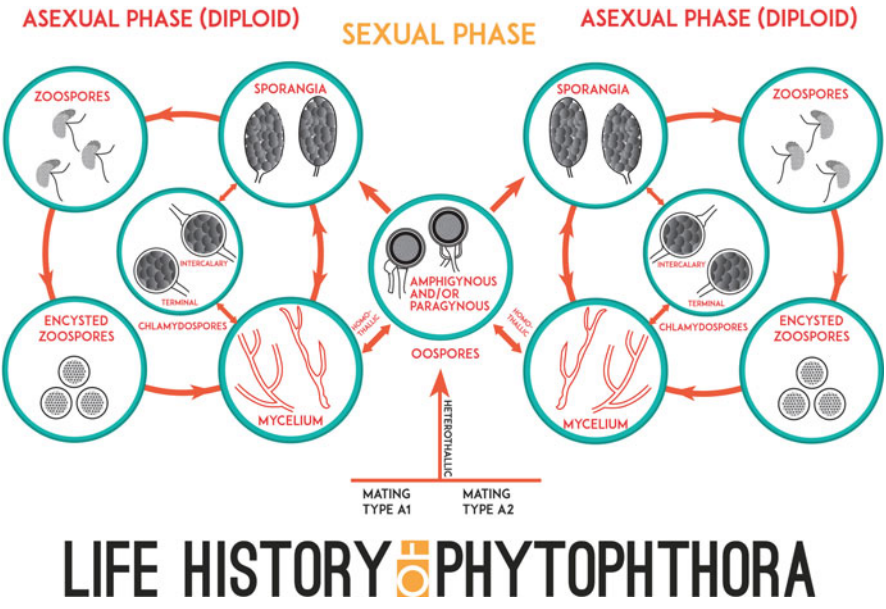

Fig. 7.1 Life cycle of *Phytophthora*

number of zoospores (Blackwell 1949; Waterhouse 1963; Newhook et al. 1978; Stamps et al. 1990).

The sporangium either germinates (a) by the production of a germ tube that usually, but not always, emerges from the tip of the sporangium (direct germination) or (b) by the production of zoospores from within the sporangium (indirect germination). Zoospore formation takes a few minutes and is considered to be one of the fastest developmental processes in biological systems (Walker and van West 2007). Zoospores are considered to be major infectious propagules. Zoospores can swim for hours (Bimpong and Clerk 1970) but eventually stop, round up, and within 10 min develop a cell wall (Bartnicki-Garcia 1973) becoming a cyst. Eventually the flagella are shed (Bimpong and Hickman (1975) or retracted (Van der Molen 1971). Encystment is induced artificially by shaking or naturally by zoospores coming in contact with solid surfaces. Cysts germinate by producing germ tubes (Fig. 7.1). Additionally, hyaline or deep brown, spherical—oval chlamydospores with a thin or thick (about 0.5—1.5 μm) wall are produced terminally or intercalary under undesirable conditions.

Some species of *Phytophthora* are homothallic (self-fertile), whereas others are heterothallic (self-sterile) (Savage et al. 1968). Oomycetes reproduces sexually by the production of oospores from fusion events involving oogonia (female) and antheridia (male) structures. In heterothallic species, the gametangia are produced only in the presence of both mating types (A1 and A2) due to the fact that a growth regulator produced by one thallus stimulates the other to produce gametangia. Meiosis occurs in the coenocytic antheridium and in the oogonium. A fertilisation tube from the antheridium ruptures the oogonial wall and deposits the antheridial protoplasm. The single oospore that forms within the oogonium is globose and characteristically develops a thick (0.5–6 μm) inner wall (Waterhouse 1963). The outer oospore wall is electron dense and about 20 nm thick while the thicker (0.7–1.0 μm) endospore wall that forms within the oospore is not electron dense. Oospores function in the homothallic species as persistent propagules in diseased plant material (Slusher and Sinclair 1973; Stack and Miller 1985). The diploid oospore germinates by production of single or multiple germ tubes at the tips of which sporangia may or may not form. Prior to germination of the oospores, the haploid nuclei from the antheridium and oogonium fuse to form the diploid nucleus. Sexual reproduction between mating types can result in genetic recombination and the production of novel races and more virulent biotypes.

Zentmyer and Mitchell (1970) and Zentmyer et al. (1973) reported detailed studies on mating types and their worldwide distribution based on the extensive *Phytophthora* culture collection at the University of California, USA. The compatibility types in *P. palmivora* were designated as A1 (= rubber) and A2 (= cacao) following the studies of Gallegly and Galindo (1958) on mating types and oospores of *P. infestans*. According to Waterhouse (1974b) although *P. palmivora* existed in two distinct morphological forms (MF 1 and MF 2) each of these forms contained A1 and A2 compatibility types. Even though A1 and A2 compatibility types were recorded for *P. palmivora* (Massee 1899; Ashby 1920; Venkatarayan 1932; Thomas et al. 1948; Chowdappa and Chandramohanan 1997), *P. megakarya*

(Zentmyer 1988b), and P. *capsici* (Kamjaipai and Ui 1978; Ristaino 1990; Chowdappa and Chandramohanan 1997), one type attains dominance over the other in distribution. Thus, in *P. palmivora*, the A2 compatibility type is predominant throughout the world (Turner 1961; Zentmyer 1974), except Jamaica, where the A1 type was reported to be prevalent. Contrariwise, in *P. megakarya*, the A1 compatibility type was reported to be widespread in distribution with only one A2 type being reported (Brasier and Griffin 1979). Likewise, the A1 compatibility type is predominant in *P. capsici* (Chowdappa and Chandramohanan 1997).

7.4 Genetic Diversity

The six morphological groups described earlier do not represent natural groupings (Cooke et al. 2000). Genetic diversity in *Phytophthora* is due to the interaction of many factors including mutation, mitotic recombination, parasexual recombination, interspecific hybridisation, migration (predominantly indirectly) by human action, genetic drift, selection, and mating systems (Goodwin 1997). Genetic diversity can be revealed by protein profiles (Erselius and De Vallavieille 1984), but mitochondrial and nuclear polymorphisms are increasingly used. Although these studies capture part of the diversity, accurate delineation is easily compromised by ascertainment bias. Genetic diversity of *Phytophthora* on cacao was reviewed by Ducamp et al. (2004) and for *Phytophthora* in general by Martin et al. (2012).

Species Differentiation Using Molecular Markers

Nomenclature issues have reduced the number of species considered pathogenic on cacao. *P. faberi* was found to infect both coconuts and cacao in the Philippines and was initially suggested to be different from *P. palmivora* based on non-production of antheridia and oogonia (Reinking 1923) but later declared to be synonymous (Ashby 1929). *P. arecae* was suggested to be conspecific with *P. palmivora* (Oudemans and Coffey 1991b; Mchau and Coffey 1994b; Martin and Tooley 2003) and was later confirmed by Cooke et al. (2000) and Kroon et al. (2004).

Phytophthora genomes contain many repetitive sequences (Kamoun 2003) and abundant transposable-like elements (Kamoun et al. 1999). Isozymes (18 loci) were able to distinguish among several *Phytophthora* spp. (Oudemans and Coffey 1991a, b) and supported the synonymy of *P. arecae* with *P. palmivora* (Oudemans and Coffey 1991b). Faleiro et al. (2004) using 191 polymorphic bands from seven RAPD primers distinguished *P. citrophthora*, *P. capsici*, and *P. palmivora* from each other. Restriction digests of mitochondrial DNA were able to clearly distinguish six *Phytophthora* species, including *P. capsici*, *P. citrophthora*, *P. megakarya*, and *P. palmivora*, from a wide range of host plants and geographic areas from each other. Microsatellite polymorphisms such as the mitochondrial

$(A)_n$ loci could differentiate some but not all of the 10 species that were studied (Dobrowolski et al. 1998). Eight clades were initially recognised based on genetic markers (Cooke et al. 2000) and at present ten clades (Fig. 7.2) are accepted (Kroon et al. 2004; Blair et al. 2008; Martin et al. 2014).

Taxonomic relationships among the *Phytophthora*s were best revealed by using multiple loci from conserved regions. Cooke et al. (2000) characterised 50 *Phytophthora* species at the ITS1 and ITS2 rDNA sequences into eight main lineages (Fig. 7.2). Clades 1–5 were mainly comprised of species with papillate caducous sporangia that exhibited aerial dispersal whereas Clades 6–8 were mainly non-papillate with a soil-borne root-infecting habit.

Martin and Tooley (2003) constructed a phylogenetic tree based on sequencing of mt Cox I. Kroon et al. (2004) provided a more detailed taxonomy based on nuclear and mitochondrial sequences (Tef, α 1 tubulin, β-tubulin, mitochondrial Cox II, mitochondrial nad 11) but keeping the same number of clades. Blair et al. (2008) using 8700 nucleotides over seven nuclear loci (28S ribosomal DNA, 60S ribosomal protein, β-tubulin, elongation factor 1, enolase, heat shock protein 90, TigA gene fusion protein) separated all 84 *Phytophthora* species into ten clades (Fig. 7.2). The main species attacking cacao were retained in the same clades as Cooke et al. (2000). *P. megakarya* and *P. palmivora* are in clade 4 and *P. capsici*, *P. tropicalis*, and *P. citrophthora* are in clade 2 (Cooke et al. 2000).

Appiah et al. (2004) characterised 140 isolates of *P. capsici*, *P. citrophthora*, *P. megakarya*, *P. nicotianae*, and *P. palmivora* spp. from cacao in cacao-growing countries worldwide and 21 isolates from other plants using restriction digests of the ITS nuclear RNA. These species were all distinguished from each other and comprised 10 RFLP groups. However, the isolates of *P. palmivora* were predominantly from Ghana, while *P. capsici* isolates from cacao originated from Brazil, Ivory Coast, and India, but only one each of *P. citrophthora* (cacao, Indonesia) and *P. nicotianae* (non-cacao, Malaysia) were present. Sequence analysis of the ITS1/5.8S/ITS2 regions and short termini of the small and large subunits of 18 isolates from five species (*P. capsici*: Brazil, Ivory Coast, India; *P. citrophthora*: Indonesia; *P. nicotianae*: Malaysia; *P. palmivora*: Costa Rica, Ghana, Indonesia, Taiwan; *P. megakarya*: Cameroon, Ghana, Togo) covering the 10 RFLP groups revealed highly conserved regions with minimal (1–3) base changes within the ITS1, ITS2, and 5.8S subunit. This study found three main groups: *P. nicotianae* in one group; *P. palmivora* and *P. megakarya* were sister groups; and *P. capsici* and *P. citrophthora* in a separate group. These studies offer a good glimpse into the interspecies diversity that exists in *Phytophthora* but are often compromised by the limited number of isolates used per species which may skew the phylogenetic results.

P. tropicalis and *P. capsici* were reported as conspecific species (Appiah et al. 2004; Bowers et al. 2007). Bowers et al. (2007) showed the integration of a type specimen of *P. tropicalis* into a cluster of 41 *P. capsici* isolates based on AFLP and ITS sequence polymorphisms. They also showed that *P. tropicalis* nested within *P. capsici* based on Cox II sequence polymorphisms and RFLP digests of 30 *Phytophthora* species. In contrast, Donahoo et al. (2006) provided ITS sequence

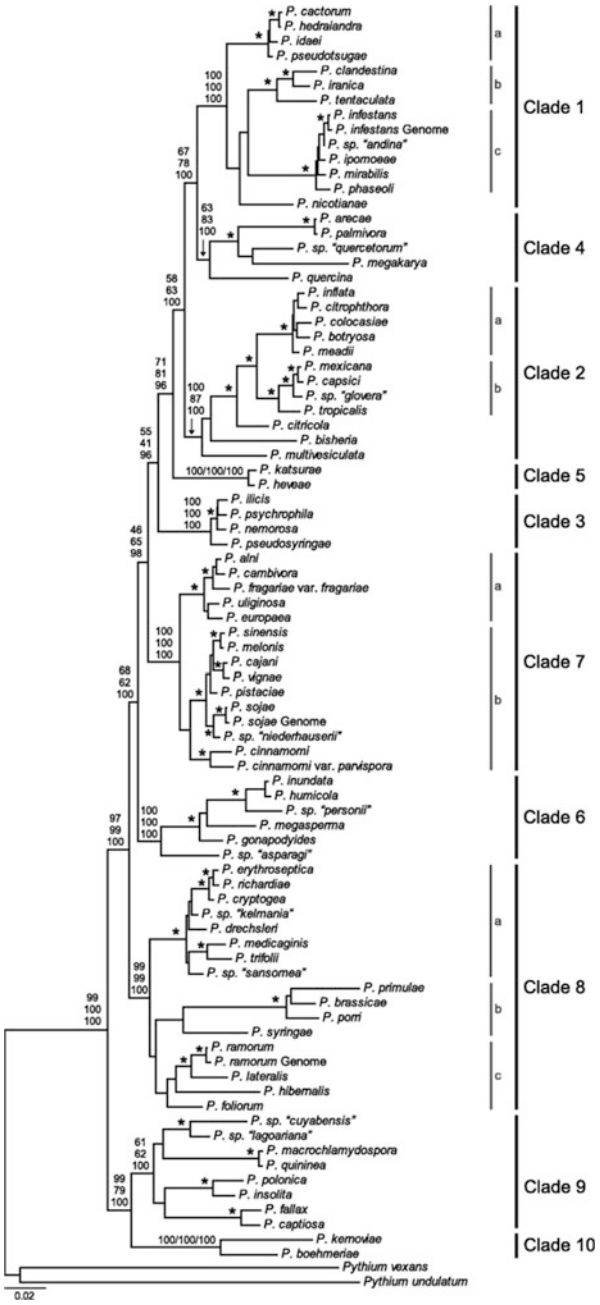

Fig. 7.2 A genus-wide phylogeny for *Phytophthora* using seven nuclear loci (_8700 nucleotides). Maximum likelihood branch lengths are shown. Numbers on nodes represent bootstrap support values for maximum likelihood (*top*) and maximum parsimony (*middle*), and Bayesian posterior probabilities presented as percentages (*bottom*). Nodes within clades receiving unambiguous (100 %) support in all three analyses are marked with an *asterisk*. *Scale bar* indicates number of substitutions per site (reprinted from Fig. 1 Blair et al. 2008)

polymorphisms that indicated that these two species although close could be differentiated from each other and from 61 other species (Donahoo et al. 2006). Similarly, Blair et al. (2008) clearly differentiated *P. tropicalis* from *P. capsici*. The ITS sequence polymorphisms of Abad et al. (2008) located *P. tropicalis* next to but different from a subcluster containing *P. capsici*. *P. tropicalis* has also been separated from *P. capsici* on morphological (Aragaki and Uchida 2001) and ITS polymorphisms (Zhang et al. 2004). The latter has suggested that *P. tropicalis* is a transitional form captured during species evolution. Defined true-type isolates of *P. tropicalis* should therefore be used to determine whether this species is really pathogenic to cacao and hence validate early studies or whether the early reports had inadvertently used a closely related species.

P. arecae and *P. palmivora* are reportedly conspecific species (Oudemans and Coffey 1991b; Mchau and Coffey 1994b; Martin and Tooley 2003; Cooke et al. 2000; Kroon et al. 2004), which is supported from the results of Förster et al. (1990). In the latter work, a single *P. arecae* isolated from the palm *Chamaedorea* was present and was contained within one of the two subgroups of *P. palmivora* being more closely related to the *P. palmivora* isolate from Florida (isolated from milkweed vine, *Morrenia odorata*). A cladogram, based on concatenated β-tubulin–EF-1a sequences, indicated that while *P. arecae*, *P. botryosa*, and *P. palmivora* formed a subgroup that was positioned close to *P. megakarya*, *P. arecae* was closer to *P. botryosa* and differed from *P. palmivora* (Donahoo et al. 2006). In contrast, the use of Cox II sequence polymorphisms positioned *P. arecae* within *P. palmivora* isolates although *P. botyrosa* was absent from this cladogram (Donahoo et al. 2006). The close relationship of *P. arecae* with *P. palmivora* and positioning of *P. botryosa* closer to *P. citrophthora* were supported by sequence polymorphisms (Cooke et al. 2000; Donahoo et al. 2006; Blair et al. 2008).

Abad and Coffey (2008) developed a morphological and molecular key for the identification of *Phytophthora* species. The use of multiple loci from nuclear and mitochondrial genomes should offer a better picture of the inter- and intraspecific relationships and diversity of *Phytophthora* isolates (Ivors et al. 2004; Kroon et al. 2004; Donahoo et al. 2006; Villa et al. 2006; Schena and Cooke 2006; Blair et al. 2008). The use of DNA probes to identify pathogens provides a highly specific tool and this technology is rapidly becoming a new frontier for research on detection and identification of *Phytophthora* species and biotypes which may help not only to differentiate species with similar morphology but also to detect species in roots and soil with more precision (Erwin and Ribeiro 2005). Additionally, high-throughput sequencing offers another complementary approach: direct comparison of the genomes of the species. However, cost and the number of genomes sequenced per species may be limited. The possibility of a skewed relationship may also exist, but if the type isolates are chosen carefully, the polymorphisms over the entire genome should reflect true relationships.

Molecular Species Variation

In *Phytophthora*, approximately 58 % of the taxa are homothallic, 38 % heterothallic, and 4 % sexually sterile (Cooke et al. 2000; Blair et al. 2008) and it is suggested that homothallism is the ancestral condition (Kroon et al. 2004). Factors that affect detection of intra-species diversity include techniques; choice of polymorphism and the number of polymorphic loci used; and number of isolates used per species, per geographic area, and per host plant. Though interspecific hybridisation as a concept is generally well known, it may not be common in *Phytophthora*. Boccas (1981) indicated that interspecific hybridisation was unlikely to be a major source of variability in *Phytophthora* finding self-fertilisation to be the rule. However, he found a single oospore from a successful interspecific hybridisation between *P. capsici* and *P. palmivora*, but it was suspected by Donahoo and Lamour (2008a) that the *P. palmivora* isolate might have been *P. tropicalis*. Waterhouse (1963, 1970) found that many hybrid oospores were unable to germinate.

Intraspecific Genetic Diversity of P. capsici

Although *P. capsici* has been found to have high levels of sexual recombination in North America (Ristaino 1990; Lamour and Hausbeck 2001), clonal lineages have been observed along the Peruvian coast by Hurtado-Gonzales et al. (2008) and in Argentina by Gobena et al. (2012). A sample of 79 isolates of *P. capsici* had a low expected heterozygosity ($H_e = 0.20$) and high fixation index ($F = 0.91$) although the taxa is heterothallic (Goodwin 1997). Five isolates from four different locales in India had identical protein profiles (Chowdappa and Chandramohanan 1995). Forty-six isolates of *P. capsici* (22 from chilli and 24 from black pepper) in Vietnam were genetically distinct from each other based on random amplified microsatellites and repetitive extragenic palindromic DNA fingerprinting analysis (Troung et al. 2010). Forty-one isolates from pepper and pumpkin from 11 sites in Argentina, were found to be of the same mating type and were separated into four groups based on eight polymorphic SNPs (Gobena et al. 2012). A strong predominance of one SNP genotype was present and ten random isolates within this group had identical AFLP profiles further confirming the clonal lineage (Gobena et al. 2012). In China, although one clonal lineage was dominant, there were two mitochondrial SNP haplotypes and 97 multilocus SNP genotypes (Hu et al. 2013). Temperate and tropical isolates of *P. capsici* were differentiated based on AFLP and ITS polymorphisms but were not as well resolved with COX II probes (Bowers et al. 2007). In addition, the tropical isolates that were grouped into three clades, had the most variability, and two of these clades were affiliated with the temperate isolates (Bowers et al. 2007). The 41 *P. capsici* isolates were all different from each

other based on AFLP profiles (Bowers et al. 2007) but were not as fully discriminated with ITS or Cox II sequence polymorphisms.

In contrast, *P. capsici* from cacao has exhibited greater diversity among isolates. AFLP data supported the existence of two subgroups of isolates from cacao in India (Chowdappa et al. 2003). Faleiro et al. (2004) found that eight isolates from different parts of the cacao plant and from different collection sites in Brazil fell into three subgroups and were apparently distinguishable from each other based on RAPD profiles. High mitochondrial and nuclear DNA diversity were found by Förster et al. (1990). However, although the latter was more pronounced and some isolates had identical mitochondrial DNA patterns, subgroups could not be correlated with host or site origin under either marker system (Förster et al. 1990). The data of Förster et al. (1990) indicated that five subgroups were present and that the *T. cacao* isolates were differentiated by geographic location. Interestingly, the three Brazilian isolates showed intra-country variation and were clearly demarcated into two groups. In contrast, Oudemans and Coffey (1991b) found three subgroups and one of these (CAP3) was only obtained from diseased cacao in Brazil and was monomorphic over the isozymes studied. *P. capsici* isolated from cacao could, however, be allocated into at least two subgroups (Oudemans and Coffey 1991b). Appiah et al. (2003) working on seven isolates from five countries showed that colony patterns on V8 agar differed within and between country isolates. Sporangial patterns were also able to delineate the isolates by country origin (Appiah et al. 2003). Appiah et al. (2004) separated eight isolates into three groups from RFLP-ITS digests. Cacao contained all three RFLP groups and the isolates were separated by geographical site (Brazil/Ivory Coast, India, Mexico). Sequencing of the rDNA-ITS region of isolates from Brazil, Ivory Coast, and India confirmed this separation, but unfortunately the isolate from Mexico was not included (Appiah et al. 2004).

Intraspecific Genetic Diversity of P. citrophthora

P. citrophthora relies exclusively on asexual means (Erwin and Ribeiro 1996; Beakes and Sekimoto 2009). Goodwin (1997) indicated a low H_e of 0.20 but a moderate fixation index (0.47) for this heterothallic species suggesting a mixed mating system. Protein profiles separated five isolates from four locales in India into two subgroups (Chowdappa and Chandramohanan 1995). Two subgroups based on isozyme analysis were found and one of these (CTR2) was only comprised of isolates from diseased cacao in Brazil (Oudemans and Coffey 1991b). Förster et al. (1990) found that *P. citrophthora* were subdivided into two clusters and had a host origin (*T. cacao* vs. *Citrus*) based on RFLP digests of mtDNA. The data of Förster et al. (1990) suggested that geographic clustering was absent. However, the data was confounded since all the cacao isolates originated from Brazil although the citrus isolates were collected from three disjoint sites (Australia, California, and South Africa). RAPD profiles separated nine isolates from cacao in various

locations in Brazil into three sister groups (Faleiro et al. 2004). Hulvey (2010) recognised 8 genotypes based on AFLP polymorphisms from 18 isolates in Tennessee, USA.

Intraspecific Genetic Diversity of P. katsurae

Goodwin (1997) indicated a low H_e of 0.11 and a high fixation index (1.0) compatible with the homothallic nature of this species. The pathogen was reported on coconut and cacao in tropical areas, Hawaii and Ivory Coast (Liyanage and Wheeler 1989). Lee et al. (2009) found high sequence similarity (≥96 %) among isolates of *P. katsurae* obtained from chestnut, coconut, and cacao among others. Twenty-two isolates of *P. katsurae* from Korea were identical and *P. heveae* was located as a subcluster within the *P. katsurae* clusters (Lee et al. 2009). The possibility of the development of one of these species from each other has been suggested (Ko et al. 2006) and is supported by the high ITS sequence similarity (Lee et al. 2009) and similar morphological characteristics with *P. heveae* but differed in its verrucose oogonial wall (Stamps et al. 1990; Ho et al. 1995). The usefulness of this verrucose wall as a taxonomic character has been questioned (Ko et al. 2006). *P. katsurae*, as well as *P. palmivora*, was reportedly isolated from diseased coconut trees with bud rot in Jamaica (Steer and Coates-Beckford 1991). However, *P. katsurae* from cacao in Jamaica has not been reported in the literature. This pathogen was first described as *P. castaneae* by Katsura and Uchida as it caused the trunk rot of Asian chestnut (Oh and Parke 2012). However, Ko and Chang (1979) deemed *P. castaneae* invalid and renamed the pathogen *P. katsurae*. Nomenclature investigations revealed that *P. katsurae* is an illegitimate superfluous name and *P. castaneae* is considered as the original and legitimate nomenclature (Pennycook 2012). Eight *P. castaneae* isolates were arrayed as three subgroups and only two of these were not differentiated from each other using eight sequenced genes (Weir et al. 2015).

Intraspecific Genetic Diversity of P. palmivora

Two morphological forms of *P. palmivora* (MF1 and MF2) have been advocated (Waterhouse 1974b) although the justification of MF2 has been challenged (Brasier and Griffin 1979). Two mating types (A1 and A2) are present with a preponderance of the latter in cacao (Turner 1961; Zentmyer 1974). Appiah et al. (2003) sorted 89 *P. palmivora* isolates from 12 countries worldwide into eight colony patterns on V8 agar with the 70 cacao isolates having six different colony patterns. The data of these workers showed that the most samples were collected from Ghana and Indonesia and five and six colony patterns were obtained respectively although Indonesia had a 3.5-fold lower number of samples.

Goodwin (1997) indicated a low H_e of 0.08 but a moderate fixation index (−0.39) for this heterothallic species. However, the majority of the heterozygosity was at one locus and a fixation index of 0.35 was obtained when that locus was discounted. RFLPs of mitochondrial DNA showed limited variability in *P. palmivora* isolates from a variety of seven hosts and six disparate geographical sites (Förster et al. 1990). Two subgroups were present and there appeared to be geographic effects for isolates from the same host and host effects for the same geographic site. Three *T. cacao* isolates, one each from Costa Rica, Nigeria, and Jamaica, were arrayed as two subgroups with the Jamaican isolate being the most different (Förster et al. 1990). In the absence of convergent morphological variation, the data may support the transport of fungal types during human movement between Costa Rica and Nigeria.

In a larger study involving 18 isozyme loci, 100 *P. palmivora* strains from varied hosts and wide geographic sites were shown to be relatively homogenous with only two loci detecting differences among the strains (Oudemans and Coffey 1991b, c). In Indonesia, AFLP analysis delineated coconut and cacao isolates within a broadly homogenous population (Smith and Flood 2001). Chowdappa et al. (2003) provided ITS and AFLP evidence that isolates from coconut were identical to those from cacao in India. Appiah et al. (2004) using RFLP-ITS digests separated 88 isolates from at least 37 distinct areas worldwide into two groups with the majority as one form and only one isolate from *Citrus* in Taiwan being different. However, sequencing of the rRNA-ITS region of select members of the uniform RFLP group demonstrated differences among the isolates. One isolate from Ghana was similar to an isolate from Costa Rica. The RFLP distinct isolate from *Citrus* in Taiwan was similar to cacao isolates from Ghana and Indonesia (Appiah et al. 2004). Faleiro et al. (2004) found that isolates from Brazil (3) and Peru (2) were subdivided into two subgroups based on RAPD fingerprinting, with one isolate from Peru being distant from the other subgroup of four isolates.

Maora (2008) studied 263 *P. palmivora* isolates from 40 farms throughout Papua New Guinea using four microsatellite primers. Only two primers were polymorphic and generated only six polymorphic loci. Under this system, seven clonal lines were found without any geographic separation and the genetic phenotypes were similar over 16 years. Overall, although *P. palmivora* isolates from cacao exhibited low genetic diversity (Turner 1961; Förster et al. 1990; Appiah et al. 2004), isolates among countries and within a country could be differentiated from each other based on DNA polymorphisms.

7.5 Case Study: *Phytophthora* in Trinidad and Tobago

Only two species, *P. capsici* and *P. palmivora*, are reportedly present in Trinidad and Tobago, on cacao (Iwaro et al. 1998). One isolate of each species was tested on ten accessions drawn from the Nacional (MO 9), Nanay (NA 90, 186, 286), and Refractario (CLM 91, JA 5/39, JA 10/12, SLC 18, SLC 19, SJ 2/22) populations and

it was found that *P. capsici* was less aggressive than *P. palmivora* when inoculated in wounded detached fruits. Surujdeo-Maharaj et al. (2001) showed that isolates collected from Trinidad (8) and Tobago (2) differed in aggressiveness, with a sixfold range at the pre-penetration (no wounding; number of lesions used as response) stage and a fourfold range at the post-penetration (wounding; lesion size used as response) stage. Despite the range of aggressiveness, these authors found an absence of host genotype × isolate interaction indicating that the rating of cacao accessions would remain unchanged regardless of the isolate that was used. They also found that the aggressiveness of the isolates at the penetration stage was highly correlated ($r = 0.8$) to the post-penetration stage even though the number of lesions and lesion size were poorly correlated ($r = 0.3$).

Johnson et al. (2006) investigated the intraspecific diversity of *P. palmivora* and worked with 13 isolates from different areas in Trinidad, two isolates from Tobago, 11 isolates from Costa Rica, four isolates from Colombia, two isolates from Nicaragua, and one isolate each from Colombia and Panama. These authors demonstrated the similarity in: sporangial dimensions over all isolates; mating type (A2); in PCR product sizes from ITS amplifications. However, two isolates (one from Costa Rica and one from Nicaragua) were found to liberate zoospores *en masse* instead of individual free zoospores. Johnson et al. (2006) further examined sequence polymorphisms of five genes (COX I, ITS, EF1α, NADH1, β-tubulin) for a subset of seven isolates (one each from Colombia, Costa Rica, Ecuador, Nicaragua, Panama, Trinidad, Tobago) with the aberrant type coming from Nicaragua. Interestingly, 100 % sequence similarity was obtained over isolates and even with the other *P. palmivora* sequences in GenBank. This study highlights the need for more markers in assessing intraspecific genetic diversity of *Phytophthora* species as the aberrant phenotype, if not a vagary of inconsistent incubation conditions which seems to have been excluded in this case, was not differentiated at the molecular level. The aggressiveness of the isolates from Trinidad and Tobago was not investigated in this study.

Preliminary experiments by Mata (2014), investigating the intraspecific diversity of *P. palmivora* using 27 isolates throughout Trinidad and one from Tobago, found significant differences in colony growth on V8 agar with responses after six days of growth being the most discriminatory among the isolates. In contrast to Johnson et al. (2006), Mata (2014) found significant differences for sporangial length, sporangial width, and pedicel length among the isolates although these parameters had low discrimination indices. The presented values were within the ranges published for *P. palmivora* (sporangia length 35–70 μm, breadth 20–40 μm, and pedicel length <5 μm) (Brasier and Griffin 1979). The data from Mata (2014) was reassessed for growth rate (mm day^{-1}) as: (Growth at Day 6 − Growth at Day 1)/(5 × Growth at Day 1). Re-examination of the growth rate using this parameter indicated that the 24 isolates could be grouped into three growth classes (Fig. 7.3) and that the isolates from the low and high growth classes differed significantly (*t*-test, 11 d.f., $P < 0.001$; Mann–Whitney *U* test, $P < 0.01$) from each other. Enríquez and Soria (1967) suggested that a 10 % coefficient of variation was sufficient for a character to be confidently used as a descriptor. Therefore, growth rate as calculated

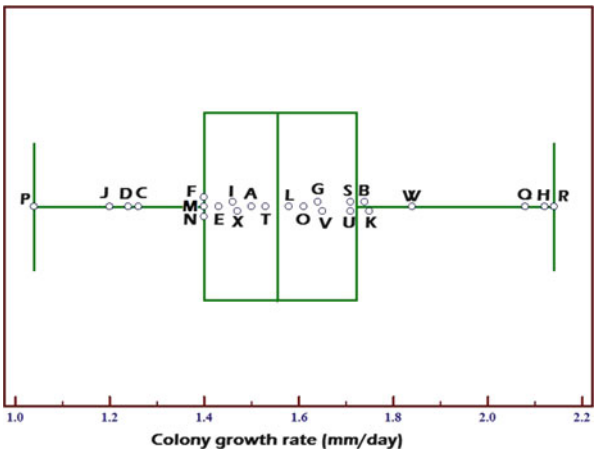

Fig. 7.3 Colony growth rate (mm day^{-1}) on V8 agar of *Phytophthora palmivora* collected throughout Trinidad. Isolates were obtained from East Central Trinidad (A, Biche; B, Charuma; C, D, Rio Claro), North Central Trinidad (E, Aripo; F, La Chaguramas), North East Trinidad (G, H, Cunapo; I, Cunaripo; J, Matura; K, Sangre Grande), North West Trinidad (L, Maracas; M, Morne Diablo; N, Santa Cruz), South Central Trinidad (O, P, Moruga; Q, R, Penal), South West Trinidad (S, T, Cedros; U, V, Gran Couva; W, Tortuga) and Tobago (X, Richmond). Colony growth rate determined as: (D6-D1)/5D1 where D1 = Colony diameter at Day 1; D6 = Colony Diameter at Day 6. Confidence limits (95 %) for mean = 1.42 – 1.71. Isolates with low growth rate (C, D, F, J, M, N, P) were significantly different ($P < 0.01$) than isolates with high growth rate (B, H, K, Q, R, W)

here, with a comparative approximate twofold increase (relative standard deviation 17.9 %), suggests that this parameter may be useful in discriminating isolates.

7.6 Biology of *Phytophthora*

The biology of *Phytophthora* has been described in detail by Hemmes (1983). In summary, the cytoplasmic organisation of nearly all the stages of the life cycle of *Phytophthora* has been described from thin sections and viewed under transmission electron microscopy. The cytoplasm contains a complement of eukaryotic organelles similar in morphology to those of plants and animals, including nuclei, mitochondria, rough ER, and dictyosomes. In addition, four organelles have been described that are peculiar to *Phytophthora* and most other oomycetes: peripheral vesicles, which eventually locate at the periphery of zoospores, where they are released during encystment; fingerprint vacuoles, which contain a reserve polysaccharide, mycolaminaran; bullet-shaped microbodies with crystalline arrays of fibrils; and clusters of microtubules contained within cisternae of rough ER, which are secreted as flagellar hairs.

In areas of cytoplasm actively engaged in wall deposition, such as at hyphal tips or along the periphery of developing sporangia or oogonia, the endomembrane system is active in forming wall-destined vesicles. The contents of these vesicles are not fully known but probably include wall precursors, as well as, wall-synthesising and wall-softening enzymes.

The most thoroughly studied example of wall deposition in *Phytophthora* is in the secretion of peripheral vesicles during zoospore encystment. Several investigations have documented the fusion of the 'peripheral' vesicles to the plasma membrane to form membrane bilayer diaphragms and the release of adhesive materials and wall-forming precursors to the exterior of the cell. The formation of the fibrillar architecture of the cyst wall has been followed by studying replicas of the cyst surface at various times during encystment (Bartnicki-Garcia and Wang 1983).

The early development of sporangia, chlamydospores, and oogonia of *Phytophthora* appears to occur largely by cytoplasmic inflow from the underlying mycelium. The spore wall remains thin during the expansion phase, whereas considerable wall thickening occurs after expansion. The cytoplasm developing chlamydospores and oospores, as well as the cytoplasm of aged hyphae and aged sporangia, undergoes a drastic change from a 'functional' cytoplasm filled with synthesising organelles such as nucleus, mitochondria, ER, and dictyosomes to a 'reserve' cytoplasm dominated by lipid-like bodies and fingerprint vacuoles. Upon germination, there is rapid conversion of the cytoplasm back to the 'functional' state. Hyphae will form haustoria and can penetrate plant tissues growing inter- and intracellularly via dissolution of host cell walls (Slusher et al. 1974).

7.7 Phytophthora Disease of Cacao: Symptomatology

Attacks on fruits are the most frequent signs of the disease, known as black pod or Phytophthora pod rot. However, stems and trunks can also be affected, with cankers occurring on the bark, and the disappearance of flower cushions. Infections have also been found on leaves and roots, though less frequently and with a much lower incidence.

Black Pod Disease

P. palmivora can attack pods at all stages of development (Thorold 1975). Infection could be initiated by sporangia, chlamydospores, or zoospores which germinate and penetrate the pod surface. Disease symptoms may appear within 3–4 days after infection has occurred and production of sporangia could begin on the night following the second day of appearance of visible symptoms (Spence 1959). In addition to the 'seaweed-like' odour which characterises pod rot by *P. palmivora* (Sreenivasan and Quesnel 1977), Adegbola (1981) described a typical black pod

symptom as a brown water-soaked lesion, which enlarges rapidly and concentrically to cover the whole pod surface as infection progresses. Infection is accompanied by a change in pod colour to dark brown or black (Thorold 1975) and pods could be rendered commercially useless in 10–20 days (Opeke and Gorenz 1974). However, Thorold (1975) indicated that beans in a mature pod may remain partly or wholly unaffected as the pathogen may not traverse the gap between the internal wall the mass of beans.

Disease severity by *P. palmivora* varies between localities, seasons, and years. Studies on disease epidemiology revealed that the situation could vary with the susceptibility of cacao types, occurrence of different strains of *P. palmivora*, and differences in cultural practices and environmental conditions, including temperature, rainfall, plant spacing, and shade (Gregory 1969; Adegbola 1981). The mass of sporangia on the fruit surface may serve as a source of primary inoculum for the spread of the disease under favourable environmental conditions, which are the same as those for the optimum growth of the cacao tree (Adegbola 1981). Field survey experiments by Thorold (1953) and Blencowe and Wharton (1961) showed that yield could play a significant role in the development of disease epidemics of black pod. Their results indicated that loss from black pod increases as yield increases. This trend is, however, different in the Ivory Coast where the percentage loss decreases with high yield (Adegbola 1981). A similar situation is reported in Ghana where percentage loss was almost constant (Adegbola 1981).

Following the pattern of infection in the field, Spence (1959) indicated that *P. palmivora* attack on pods of varying ages could be lateral, distal, or proximal. Proximal infection, according to Wharton (1957) and Spence (1959), may occur from the disease cushion or from water collected around the stalk. A high incidence of proximal infection was reported from Ghana, but was of less importance in Nigeria. Distal infection, on the other hand, was most common with young pods, as moisture is retained at the distal end. Most early infections were noted to start as either proximal or distal, but as inoculum builds up in the field lateral infection could occur. Infection of young pods (cherelle) is often confused with cherelle wilt. The latter is characteristically preceded by a preliminary yellowing followed by slow broadening from the tip, as described by Opeke and Gorenz (1974), which allows it to be distinguished from black pod.

Preliminary analysis of black pod in cacao fields in Trinidad during the rainy season of 2013 (June–December) indicates that the overall infection in fields at the International Cocoa Genebank, Trinidad (ICGT), was at 14 % with the percentage infection between mature and immature pods being 60 % and 40 % respectively (Umaharan 2014).

Stem Canker

Stem canker, in most cases, develops as a result of mycelial spread from infected pods along the stalk into the flower cushions and further along the stem to cause

more extensive damage (Firman 1974). On the other hand, infection could be initiated directly through wounds (Zaiger and Zentmyer 1965). The appearance of infected tissues as reported by Firman (1974) depends on the age of cankered tissue and the activity of the fungus (Okey et al. 1994). Stem canker is closely associated with black pod and cushion infection. Its importance was emphasised by Firman and Sundaram (1970), Vernon (1971), and Prior (1981). Severe losses due to stem canker were reported in Fiji and Papua New Guinea, where a number of trees died (Firman and Vernon 1970; Prior 1981 and Adegbola 1981). Canker disease could lead to 'sudden death' of trees, as reported in Papua New Guinea (Prior 1981), or in general unhealthy appearance of trees. The interference with the functions of the bark may cause either defoliation or death of twigs (Nowell 1923; Briton-Jones 1934; Urquhart 1961). Bark cankers also provide another location for the survival of the pathogen on the tree which can reinfect pods in subsequent seasons (Maddison and Griffin 1981).

Canker infection had reportedly declined significantly in most cacao regions, possibly due to the replacement of the susceptible Criollo cacao by more resistant Amelonado and Trinitario types (Urquhart 1961; Firman 1974). The upsurge in interest of fine flavour varieties which may have a high Criollo ancestry may therefore lead to an increased canker incidence. More recently there has been an outbreak of stem canker on a farm on the island of Tobago (S. Surujdeo-Maharaj and T.N. Sreenivasan, personal communication). In Tobago, several factors were observed to have contributed to the outbreak including poor phytosanitary measures, inadequate drainage, lack of pruning and weeding, and irregular shade management (S. Surujdeo-Maharaj and T.N. Sreenivasan, personal communication). A combination of these factors resulted in compromising the health of the cacao tree and consequently its ability to fight off infections.

Flower Cushion Infection

Although flower cushion has received little attention in comparison to pod rot and stem canker, its importance in the disease cycle is well emphasised by Rorer (1910), Dade (1927, 1928, 1929), Thorold (1955), Manco (1966), Wharton (1974), Henry (1977), Griffin et al. (1981), and Maddison and Griffin (1981). Their findings showed a close relationship between proximal infection of pod, stem canker, and cushion infection. Dade (1929) in his experiments involving artificial inoculation and serial dissection observed that the fungus passed through the stalk into both cortex and the medullary rays of the stele, reaching the swollen base (the spur) in about 16 days. Further spread of the fungus mycelium was identified to be via the cortical and cambial tissue.

Inoculation and re-isolation experiments by Manco (1966) and Okaisabor (1969) and observations made by Thorold (1955) have revealed that cushion infection could occur if infected pods are allowed to remain on trees for 2 weeks from the first appearance of pod rot. The interval could be shorter in the case of young pods

(cherelles) (Wharton 1974). However, Henry (1977) indicated that infection of the flower cushion and surrounding bark could take 3–4 months, as observed in Jamaica. This variation in time interval required for the initiation or infection might be due to difference in the prevailing microclimatic conditions under which experiments were conducted or the prevalence of different strains of the pathogen or varieties grown.

Propagules of *P. palmivora* can survive the dry season in infected cushions and will be reactivated when conditions become favourable (Wharton 1974). Manco (1966) indicated that cushion infection could have a significant direct effect on pod production, especially in old trees. Recent observations of a young cacao plantation in Tobago concurred with these findings where canker of trunks and subsequent death of trees occurred when infected pods were not removed from the trees (S. Surujdeo-Maharaj and T.N. Sreenivasan, personal communication).

Blight of Chupons, Seedlings, and Leaves

Blight of chupons, seedlings, and leaves of cacao has been reported in Suriname, Nigeria, Ghana, Costa Rica, and Brazil (Manco 1974). Although these diseases are considered of little importance in comparison to black pod and stem canker, Manco (1974) noted that blight and leaf infection could well be additional sources of inoculum, increasing pod infection, in addition to the damage they do to photosynthetic surfaces, thereby indirectly reducing pod production.

As with other diseases caused by *P. palmivora*, infection is favoured by high humidity under which the disease spreads rapidly. In the wet season of June to August in 1970 in Bahia, Brazil, Manco (1974) observed *P. palmivora* infection of mature leaves with conspicuous leaf fall and sporulation under field conditions. He thus reaffirmed his earlier observations (Manco 1966) that blight in canopy could be a source of inoculum to pods lower down the tree. Similar observations were made at the International Cocoa Genebank, Trinidad (S. Surujdeo-Maharaj, unpublished data). Studies by McLaughlin (1950) have also shown that blight of leaf flushes can cause dieback in old trees. The symptoms of blight of chupons and seedlings due to *P. palmivora* are all similar. The initial symptom, as described by Manco (1974), is characterised by the darkening of tissue followed by a browning. However, some differences may be observed in symptoms produced in young diseased plants, depending on the site of infection (leaves, stems, or petioles), age of tissues, and the relative humidity of the environment. In general, young leaves and young stem tissues were noted to be more susceptible to infection than the old leaves and mature stem tissue (Manco 1974; Iwaro 1995).

Root Infection

Root infection by *P. palmivora* was first reported in 1962 (Turner and Asomaning 1962). Very few reports are available on this aspect of *P. palmivora* infection, but the relationship between pod and root susceptibility and speculations that *P. palmivora* could be a root pathogen have led to further investigations on the significance of root infections (Gregory 1981).

Previous studies by Turner and Asomaning (1963), Amponsah and Asare-Nyako (1972), and Onesirosan (1971) revealed that root infection could result in retarded growth of seedlings. The growing points of the roots were identified as focal points of attack. Other experiments conducted by Amponsah (1970) and Amponsah and Asare-Nyako (1973) showed that root infection was positively correlated with pod infection and that, using a root inoculation method, it was possible to separate varieties that were truly resistant from those that merely escape infection in the field.

Gregory (1981) observed that the speculation by Turner and Asomaning (1963) on possible damage by the pathogen to old trees has been largely ignored over the past years. He suggested that a well-planned programme to monitor feeding roots throughout the season along with inoculations on both seedling roots and on feeding roots of mature trees be instituted to acquire more information on root infection.

7.8 Disease and Epidemiology

The epidemiology of root diseases caused by many soil-borne fungi is the single-cycle type because the inoculum that initiates disease carries over from the previous year but does not increase during the crop year (Van der Plank 1963); however, the inocula of *Phytophthora* species increase from low, often undetectable levels to high levels within a few days or weeks (MacKenzie et al. 1983; Weste 1983). Analyses of phenological (relationship of climate to periodic biological phenomena) changes indicate that the flux of the population density of the more ephemeral life form (e.g. sporangia and zoospores) rather than the survival propagule is more likely to determine the success of the organism. The increase of inoculum of *Phytophthora* is caused by the rapid production of sporangia and zoospores from infected plant tissues when environmental conditions, the most important of which is the presence of free water, are favourable. All the oomycetous microoganisms have a short generation time and great reproductive capacity (Dick 1992). For this reason, *Phytophthora*-caused diseases of fruit, foliage, crowns, and roots of plants are generally considered to be multicyclic (Fry 1982; MacKenzie et al. 1983) and include those of cacao.

Sources and Routes of Infection

In an attempt to reduce the rate of infection and to ensure the effectiveness of existing control methods, consistent efforts have been made to identify the initiators and sources of infection within plantations. As contribution towards this objective, a programme funded by the Cocoa Research Organization of the International Office of Cocoa and Chocolate was established in 1973 to identify possible sources and routes by which the fungus responsible for black pod disease reaches the pods.

Maddison and Ward (1981) indicated that rain splash from sporulating pods was the main route of pod infection. About 75 % of all infections were noted to come from this source which usually gives rise to clusters of diseased pods on individual trees. Trees bearing sequences of rain-splash infection were observed to occur irregularly in the plantation. Other possible routes of infection, including the dispersal of sporangia in dry still air as earlier reported by Thorold (1952, 1955), were tested in Nigeria. Both *P. palmivora* and *P. megakarya* were found to liberate sporangia into still air from infected pods about 10 mm above horizontal microscope slides in closed containers, but the number caught was small.

The search for sources of initial infection at Gambari Experimental Station in Nigeria showed that early season initiators were close to the ground and apparently a result of rain splash from the soil, or were associated with tents of soil built on trees by certain ants. Tests conducted by suspending healthy pods above bare soil and litter-covered soil, at varying distances, showed appreciable amounts of infection on pods hung over bare soil in comparison to those of litter-covered soil. A consistent steep gradient was observed over bare soil, with nearly 90 % of the infections occurring below 0.6 m above-ground level (Maddison and Griffin 1981). This result conformed with that of Okaisabor (1971) who showed that infections on attached pods above litter were fewer than those above bare soil.

Investigations on tent-building ants and rodents have also shown that these organisms could advance the spread of *Phytophthora* propagules from the soil to the canopy and between trees. Maddison and Ward (1981) observed that tent-building ants were unequally distributed in the plantation, but, where they occurred, disease was generally early and plentiful. Also, Gorenz (1968) reported that uninfected pods about 1.3 m above the ground on two infested trees (having ant-attended scale insects on pod stalks) developed black pod disease 2 weeks after placing infected pods, with litter cover, at their bases. These observations support the findings of Dade (1928) and Evans (1971) that ants (*Crematogaster* spp.) removed epidermis from cacao fruits for 'carton' construction. Healthy fruits may then become infected through contaminated mouth parts of ants.

The significance of abandoned husk as a source of infection was also investigated by Maddison and Griffin (1981). It was found that piles of fresh husk materials left in the plantation during the rainy season could increase the incidence of black pod disease on trees 2.5 m from sources, but no detectable effect was observed on trees 5.0 m away. Husk materials which had passed the dry season on the ground were also noticed to have a similar effect in some sites but not in all.

In Trinidad it was found that abandoned husk are moved around for great distances in cacao fields that occur in flat areas near river banks during flooding (S. Surujdeo-Maharaj, unpublished data). It is speculated that this can also be a means through which inoculum can move around from farm to farm within a flood zone.

Speculations about the effect of sporulating cankers and infected flower cushions as likely sources of inoculum were verified by Maddison and Griffin (1981) in a series of experiments involving trapping and baiting of stem flow water from trees noted for their sporulating cankers. Their results confirmed the availability of *Phytophthora* propagules from these sources. Positive baitings were more numerous from Amazon trees than from Amelonado, but, in general, stem flow water had intermittently a low level of infection. The unexpectedly high rate of losses from Sri Lanka has also led to speculation that rubber grown together with cacao could encourage more infection, as both are hosts of *P. palmivora* (Opeke and Gorenz 1974).

In several fields in Trinidad and Tobago, other pests may also act as transporters of propagules of black pod disease. Orange-winged parrots (*Amazona amazonica*) and rodents (mainly *Sciurus granatensis*) in addition to destroying pods directly (Mollineau et al. 2008) are capable of transporting inocula to healthy fruits.

7.9 Control of the Disease

Phytophthoras are unique and have special features that must be considered when planning experiments or control methods. Unfortunately, there is not a single solution or simple remedy to manage *Phytophthora* on tropical tree crops. The situation is complex due to (1) the biology of the pathogen; (2) economics; (3) politics; and (4) human behaviour (Drenth and Guest 2013). Traditionally four principles of control have been employed to manage plant diseases. These principles include exclusion (quarantine), eradication (phytosanitation/hygiene), protection (chemical), and immunisation (host resistance). However, this chapter focuses only on cultural, chemical, and use of resistant varieties with biological control being covered in Chap. 17.

Hygiene

The idea behind good agricultural practices is to employ aspects of farming that encourage healthy crop production, as well as hinder the growth and advancement of pathogens. In that regard, clean planting material, disease-free soil,

uncontaminated water, and nutrient management along with regular pruning, harvesting, sanitation, and hygiene all contribute towards good management practices on cacao farms. Clean planting material must be propagated in pathogen free substrates. Plant propagules can be obtained from disease-free cuttings or seeds from disease-free fruit; avoiding infection from rain splash by placing the pots at least 80 cm off the ground; and immediate removal of any infected material from nurseries to minimise spread to other plants.

To relieve the anaerobic stress from improper drainage at planting sites, drains may be placed both vertically (to a depth of 1.5 m) and horizontally, to avoid stagnation of water and subsequent proliferation of *Phytophthora* (McDonald et al. 2002). Before planting in orchards, drainage should be improved by building mounds such that newly planted seedlings are not flooded due to rain or irrigation.

Diseased fruits should not be left on trees as they serve as inoculum for future epidemics. The incubation period for infected cacao pods to be colonised completely by *P. palmivora* is about 4 days, so in order to reduce the inoculum load and the introduction of secondary inoculum it is necessary to harvest often and to remove all pods displaying the signs and symptoms of being diseased. Areas with rotting fruits are feeding grounds for vectors of *Phytophthora* to breed and with inoculum being transferred by flying beetles for example and should be buried or composted (Konam and Guest 2004).

Adequate ventilation reduces high humidity between growing trees on plantations and can be achieved through pruning, especially before the wet season. Periodically, soil organic matter should be supplemented with manures and composts. Where cankers may be present, they should be scraped and treated through application of copper and/or phosphine along with the removal and burning of dead trees.

Compelling evidence implicating the combined effect of shade, spacing, and fertilisation on the prevalence of black pod was gathered at Tafo during black pod experimental trials (Vernon 1966). Results from shade trials on Amazon cacao revealed that unshaded plots had 3.9 % of their trees with black pod, whereas plots with 40 % or 50 % shade had 11.2 % black pod incidence. More recently, casual observations were made in Trinidad and Tobago that plots with heavy shade had a higher than average black pod incidence (S. Surujdeo-Maharaj and T.N. Sreenivasan, personal communication). Further, increased infection is seen in fields with both heavy shading and close spacing and thus it is recommended that a reduction in shade with wider spacing, and fertiliser application can favour healthier trees (Drenth and Guest 2013). However, there are others who challenge this recommendation indicating that under high-density planting more healthy pods could be obtained per unit area (Russell 1953; Thorold 1959).

Early sanitation, removal of mummified pods, and continual harvesting of ripe pods during the season are recommended to reduce the rate of infection (West 1936; Owen 1951; Wharton 1962; Hislop 1964; Tondje et al. 1994). Other sources of inoculum include infected husks from post-harvest processing and it is suggested that this activity be carried out away from bearing trees (Maddison and Griffin

1981). In canker affected trees, pruning low branches to increase airflow along with complete removal of old cankered trees was recommended (Prior 1981).

Chemical Control

The use of fungicides has received a lot of attention over the years and appears to be the most popular method among farmers. However, Gregory (1969) noted that this method is costly and never completely effective. The need for repeated applications (8–10 per season) according to Fagan (1984) makes the practice uneconomical. Gregory (1969) indicated that trees averaging less than 10 or 12 pods annually, as well as plantations with too many unthrifty trees are not worth the cost of spraying. Nevertheless, application of fungicide is contended to be the most effective control method available now for *Phytophthora* diseases.

Presently, a number of fungicides are at the disposal of farmers. However, many prefer the traditional Bordeaux mixture and other inorganic copper formulations. Bordeaux mixture, in particular, was widely accepted as the most effective protectant in Nigeria (Filani 1973); Adegbola 1981), Costa Rica (Newhall 1968), and Trinidad (Lewton-Brain 1906; Nowell 1923 and Holliday 1960). In Fiji and Nigeria, Perenox was found to be equally as good as Bordeaux mixture (Chant 1957; Bowden 1961; Vernon 1977). Effective control of pod rot was also reported in Brazil following the use of Kocide (Rocha and Machado 1972; Aitken and Lass 1975). Other available chemicals that are effective against *Phytophthora* diseases include Brestan, Copper Sandoz, Tillex, Dexton, Plantvax, Ridomil, and zinc carbonate (Adegbola 1981; Laker 1982).

Besides the fungistatic or fungicidal activity of these chemicals, their success, when applied in the field, depended on the method of application, the prevailing environmental conditions, and the stage of development of the pod (Thorold 1975; Adegbola 1981). According to Adegbola (1981), the distribution of fungicide in the canopy and on pods depended on the type of spraying machine. He also indicated that chemicals with poor tenacity are strongly influenced by heavy rainfall and thus recommended that fungicide spraying be confined to sunny periods with a relative humidity of less than 80 %. Thorold (1975) also observed that the total pod surface doubles in about 10 days or about five times in 2 months. He indicated that this factor alone could reduce fungicide coverage significantly within 1 month. Other factors including the concentration, frequency of application, and the longevity of chemicals were considered of immense importance in fungicide application (Adegbola 1981).

Several methods of fungicide application have been developed with promising results. By testing the effectiveness of different doses of copper fungicide, Pereira (1985) observed that a quantity equivalent to a single season's spraying applied as a single dose was more cost-effective in the control of black pod than split applications. He therefore recommended a single application of 16 g of active ingredient (copper) in place of 3 or 4 applications at 30-day intervals. He also indicated that

two applications (8 g active ingredient/spray) could also be made per year at 60-day intervals.

A slightly modified method was implemented in Grenada where single application of copper fungicide was directed to the trunk and main branches of the cacao trees instead of the canopy application recommended by Pereira (1985). The fungicide application in Grenada was before the start of the rainy season on all of the cacao estates throughout the island. Black pod incidence was not observed during the cropping period of that year. Repeated yearly application over 3 years resulted in the elimination of black pod and further fungicide application was only applied if it was required (Sreenivasan, personal communication). The success of this disease control programme was directly attributed to the involvement of the Grenada Cocoa Association. Farmer involvement in Ghana was also recognised as a key component to controlling diseases in the cacao sector (Akrofi et al. 2003).

In Papua New Guinea, trunk injection of potassium phosphonate was recommended for the control of black pod and stem canker (Holderness 1992; Guest et al. 1994; Guest and Grant 1991). This method gave the highest healthy pod yield in a comparative trial with trunk paints, and foliar spray of potassium phosphonate and trunk injection of Aliette CA and Ridomil 250 EC (McGregor 1982; Prior and Smith 1982). The trunk injection of potassium phosphonate gave a consistent reduction of Phytophthora pod rot when applied annually at a 15 g active ingredient per tree per year. In addition, to the relatively low cost of application resulting from the relative small amount of fungicide injected directly into the tree, the effectiveness of this technique is independent of the seasonal timing of injection which is an advantage over conventional spraying techniques.

A collar method was recommended following a successful trial in Trinidad and Grenada (Sreenivasan 1981; Sreenivasan et al. 1990). This method involved the attachment of a water-permeable collar containing copper fungicide to the trunk and main branches of the cacao tree. The collar slowly releases copper into the run-off water flowing down the trunk and branches following rainfall. The treatment gave 100 % control of black pod on treated trees, while 60 % of pods were lost to black pod on untreated trees. The success of this method was attributed to a controlled distribution of fungicide down the trunk as rainwater flows through the collar. Further investigation over a period of 4 years showed a residual effect on treated trees which produced more healthy pods than the controls (T. N. Sreenivasan, personal communication). However, the branching pattern, especially on clonally propagated trees, may pose some difficulties, restricting the use of this method to trees derived from seedlings or chupons.

Polyene antibiotics suppress growth of most true fungi and not that of *Phytophthora*. Griffith et al. (1992) commenting on the mode of action of fungicides and the target sites of fungicides to control Oomycetous pathogens cogently take the stand that failure of plant pathologists (especially those conducting research on new approaches to fungicidal control) to recognise that the Oomycetes differ from the true fungi has retarded development of fungicidal control. Currently, there is no universal recommendation on chemical application for the control of *Phytophthora* infections due to differences in the level of success of the various

techniques under a range of environments, management conditions, and planting materials (Guest et al. 1994). In view of the high cost of fungicides and labour, as well as pollution to the environment, considerable effort has been made to identify sources of genetic resistance to *Phytophthora* among cacao varieties (Barreto et al. 2015).

7.10 Methods of Identifying Resistance (Screening)

With respect to field recordings of infected pods, several workers contended that the information that can be collected in the plantation was only an approximate indication of the true susceptibility of those accessions examined. Infection in the field is heterogeneously distributed and was influenced in part by genotype, microclimate, inoculum points, and planting density (Marticou and Muller 1964; Muller and Lotode 1971). It has been observed in Trinidad that seasonal variation during dry or rainy periods strongly influenced the distribution of disease incidence in fields at the International Cocoa Genebank Trinidad. Blaha (1974) suggested that the degree of susceptibility and the differences in the reaction of individual trees in the field be confirmed with the aid of artificial inoculation. Many artificial techniques have since been developed and used to assess the susceptibility of the different organs of cacao in the laboratory as well as in the field.

Experiments on pods were performed on detached and attached pods. Prior to 1998 at the initiation of the Common Fund for Commodities project, the type of inoculum and the mode of application differed among researchers at different experimental stations. Depending on the objective of the individual investigators, pods were either inoculated with or without wounding. The type of inocula varied from standard-size fragments of infected tissue adopted by Turner (1963a), mycelium agar discs applied by Rocha and Mariano (1969), and mycelium-sporangial suspensions used by Lellis and Peixoto Filho (1960) to application of zoospore suspensions of varying concentrations applied by Holliday (1954) and Wharton (1959).

Among the various forms of inoculum, zoospore suspensions are most commonly used, since this is regarded as the principal means of dissemination of *Phytophthora*. Moreover, it affords the experimenter the opportunity to calibrate and standardise inoculum density (Blaha 1974).

Inoculation of Pod Without Wounding

Inoculation of unwounded pods, either attached or detached, provides an ideal test for the assessment of pod resistance at the penetration stage. For the purpose of assessing resistance at the penetration stage, inoculation by spraying zoospore or sporangial suspensions on parts of or the whole pod (Lellis and Peixoto Filho 1960;

Medeiros and Rocha 1964, 1965; Rocha and Mariano 1969; Rocha and Medeiros 1969; Iwaro 1995; Iwaro et al. 2006; Nyadanu et al. 2012) is most widely used. However, some workers prefer immersion of the whole pod in zoospore suspensions (Wharton 1959; Toxopeus and Gorenz 1969). Both methods are effective, producing numerous lesions, but are often difficult to assess as the lesions coalesce rapidly. Various other techniques collectively known as point inoculation methods have been developed to minimise coalescence. Inoculum was applied to the pod surface on an area enclosed within a glass chamber (Holliday 1954) and sealed at the base with rings of modelling clay (Tarjot 1969), or petroleum jelly (Blaha 1967). Inoculated pods were then kept under high humidity in incubating boxes for 7–10 days. Lesions were counted after this period to account for the susceptibility of the different accessions tested. Where further assessments were made, as, in some cases, by measuring the size of lesions, resistance determined may be confounded with internal resistance. On attached pods, inoculation was effected using filter paper (Wharton 1959); 'Band Aid' plaster (Sreenivasan 1985; Iwaro 1995); modelling clay cups (Blaha 1967, 1971, 1972; Tarjot 1969; Blaha and Lotodé 1977); cotton pads (Toxopeus and Gorenz 1969; Gorenz 1971); and tubular devices (Adebayo 1971; Sreenivasan 1977) to localise inoculum and protect the inoculated site.

Laboratory-based detached pod inoculation could be more standardised and can permit a much wider field of investigation than those conducted in situ (Blaha 1974). However, some workers have shown that the susceptibility of detached pods was greater than that of attached pods. They recommended that efforts be intensified to test attached pods. But, Blaha (1974) indicated that the increased susceptibility of attached pods could be used in two practical ways: to estimate the virulence of zoospores by inoculating attached pods and detached pods at the same time (Medeiros and Machado 1967) and to predict the actual resistance of attached pods from the observed resistance of detached pods under laboratory conditions (Lellis and Peixoto Filho 1960).

Inoculation on Wounded Pods

Inoculation tests on wounded pods provide a suitable means of assessing internal resistance or post-penetration resistance. This inoculation technique was explored by a number of workers using different forms of inoculum. Wounded spots on detached pods were inoculated with mycelium agar discs (Prendergast 1965; Tarjot 1965; Prendergast and Spence 1967; Rocha and Mariano 1969) or mycelium-sporangial suspensions (Lellis and Peixoto Filho 1960; Medeiros 1967; Prendergast and Spence 1967). Similarly, on attached pods, wounded spots were inoculated with mycelium agar discs (Medeiros and Machado 1967; Akinrefon 1971; Rocha and Vello 1971) and zoospore or sporangial suspensions (Medeiros 1967; Medeiros and Rocha 1965; Partiot 1975).

The susceptibility of clones was assessed from the rate of progress of lesion size. In inoculation tests by wounding, some accessions originally classified as resistant

turned out to be very susceptible as observed by Oechsli (as cited by Thorold 1955) who reported in 1954 that the resistance of SIC 28 was lost following wounding. Prendergast and Spence (1967) also reported a similar occurrence in some clones. Consequently, it was concluded that wounded and unwounded pods should be tested separately to determine the form(s) of resistance inherent in the cacao cultivars for utilisation in breeding. This is at variance with recommendations by Orellana (1953), Tarjot (1967), and Sitapai (1989) that tests for resistance be limited to unwounded pods.

Growth of P. palmivora *in Extracts of Pod Husk*

The growth of *P. palmivora*, both germination of zoospores and mycelial growth, has been investigated on extracts of cortical tissue. Wharton (1954), Orellana (1954), and Prendergast (1965) have used this method and acknowledged that a reduction in mycelial growth corresponded with an increase in the resistance offered by extracts of the various clones tested. However, the results of Turner (1962, 1963b) showed no significant difference between clones which had earlier been selected in the field.

Leaf Inoculation

Interest in inoculation methods of cacao leaves has grown in the past two decades. Early investigations in this area have been conducted mostly on detached leaves or leaf discs as inoculation of leaves in the field was difficult. Furthermore, the results of leaf inoculation in the field were inconclusive and testing on detached leaves or leaf discs maintained under a saturated atmosphere was recommended (Lawrence 1978). Other leaf inoculation methods include spraying suspensions of sporangia on whole leaves and scoring the number of infected leaves and lesions (Siller and McLaughlin 1950; Holliday 1954 and Hansen 1961) and inoculation of leaf discs floating on sterile distilled water (Joseph 1985).

Joseph (1985) noted that the results from the inoculation of leaf discs were quite unsatisfactory, but thought that if the method was improved it might be useful for laboratory screening. Later, Iwaro (1995) found a close relationship ($r = 0.87$) between the reactions of detached leaves and attached leaves and suggested that detached leaves could be used to predict the post-penetration resistance of attached leaves. Since then, the leaf disc method was improved (Nyassé et al. 1995) and has been used as an early screening tool for the identification of foliar resistance to black pod in several labs.

Tahi et al. (2000) found good correlation using this method for resistance of leaves with field resistance in Côte d'Ivoire. However, Iwaro et al. (1997) opined that the poor correlation between resistance at the penetration and post-penetration

stages in both leaves and pods indicated that these two forms of resistance were independent and governed by different mechanisms. They also found that the correlation between resistance of leaves and pods was not significant at the penetration stage, whereas that of post-penetration resistance was positive and significant. This showed that internal or tissue resistance was common between leaf and pod and indicated that leaf resistance at the post-penetration stage of infection could be used to predict pod resistance. However, resistance of leaves at the penetration stage should not be used to predict penetration resistance in pods. A high positive correlation obtained between attached leaves and pods with their detached counterparts showed that detached organs could be used for the prediction of resistance in attached leaves and pods. These methods were later validated by Nyadanu et al. (2009).

Stem Inoculation

A common method of stem inoculation involves placing an agar disc of *Phytophthora* mycelium into a vertical slit on the stem and covering the slit with tape (Zentmyer 1968, 1969, 1972; Zentmyer et al. 1968; Lawrence 1978). Research conducted by Sreenivasan (1977), Joseph (1985) and Robertson (1986) substituted zoospore suspensions for the mycelium discs. Both forms of inoculum were equally effective for the initiation of stem cankers.

In general, assessments of cankers have been conducted by wounding, which is thought to mimic the entry of the pathogen through wounds in the field (Firman 1974). However, there is speculation that infection may occur without wounds (Prior and Sitapi 1980), but this hypothesis needs to be proven.

Several authors have suggested varying time durations for the assessment of cankers based on their experimental results. Zentmyer et al. (1968), Zentmyer (1972), and Lawrence (1978) reported 18 days after inoculations was sufficient, while Firman (1974) observed spread of lesions within 2 weeks of inoculation. Robertson (1986) found that the optimum time for canker observations was 9–12 weeks after inoculation. For comparisons to be made among clones, the age of material, type of inoculum, and the period allowed for lesion development needs to be standardised (Okey et al. 1994). Okey (1996) was able to place cacao clones into resistant, moderately resistant, and susceptible categories using standardised inoculation methods to evaluate stem resistance to canker after inoculation with zoospores.

Another method involved putting a mycelial disc on a wound created by a needle on stems or branches that were turning from green to brown (Despréaux 2004). Using this method, Tahi et al. (2000) found a strong correlation of twig resistance to field resistance. Furthermore, while the correlation was not as strong as that observed for leaves, the correlation was better for both organs when they were taken from nursery material rather than from trees in the field. Twig resistance was suggested to be better for predicting resistance to bark canker than to pod resistance

in the field (Tahi et al. 2000). Pinto et al. (2007) using the method of Lawrence (1978) found that cacao clones differed in resistance and that the three species (*P. palmivora*, *P. citrophthora*, and *P. capsici*) differed in aggressiveness for stem infection. Pinto et al. (2007) indicated that particular genotypes had consistent measures of both field and stem resistance while some genotypes had an inverse relationship. A similar effect was noted by Tahi et al. (2000) who suggested that discrepancies among the correlations were due primarily to IMC 67 which had higher resistance in twigs than the leaves or roots.

Root Inoculation

The chemotropic response of zoospores to cacao roots was demonstrated by Turner (1963b) and Zentmyer (1966). These studies led to the artificial inoculation of roots, as a test of susceptibility to *P. palmivora*. In Ghana, an analysis of plant growth after root infection was the basis of studies comparing cacao selections grouped as resistant and susceptible based on field observations (Asomaning 1964; Asomaning and Wharton 1963; Turner and Asomaning 1963). Prendergast (1965) inoculated seedlings in sterile soil and in aerated nutrient solution with mycelia fragments of *P. palmivora*, but no significant differences in seedling weight between test plants and control were observed. This led him to suggest that assessment of disease resistance could be based on the number of surviving plants rather than based on seedling weight.

Zentmyer (1968) inoculated seedlings in pots containing aerated modified Hoagland solution with fragments of *P. palmivora*. The percentage of decayed roots was assessed between 4 days and 5 weeks after inoculation. Zentmyer (1968) found a positive correlation between root reaction and pod resistance to *P. palmivora*.

Tahi et al. (2000) evaluated root infection after 16 days on 6-week-old rooted cuttings which were treated by pouring 50 mL of inoculum suspension (3×10^5 zoospores ml^{-1}) around the rooted cutting in the pot. Using this method, Tahi et al. (2000) obtained better correlation to leaf resistance than to twig resistance, and root resistance was also strongly correlated to pod resistance in the field. Despite the strong correlation, these authors advocated against this screening method as it was more laborious and destructive.

Present Situation with Screening Methods

Currently, several sources of resistance have been identified in cacao black pod disease, but there is still a lack of consensus in repeatably and reliably identifying durable sources of resistance for various organs of the cacao tree. Several researchers (Iwaro 1995; Nyassé et al. 1996; Nyadanu et al. 2009) have studied

the host–pathogen relationships, but the screening methods to date continue to yield inconsistent results (Thévenin et al. 2006). This was seen following a training workshop held at the Cocoa Research Institute, Ghana (CRIG) in October 2013, on standardising methodology for identifying resistance to *Phytophthora* species in cacao. It is imperative, therefore, to develop an early, reliable, and consistent screening method (Eskes 2001) capable of identifying the various levels of resistances that may exist in germplasm collections for both clonal and segregating populations. During the workshop a number of factors were identified for the lack of progress in identifying sources of black pod resistance. The following is a summary of what was presented. Together, we highlight the gaps and offer suggestions as to how advancement can be made in this area.

Suggestions for Improving Screening Methods

Standardising the factors of host, pathogen, and environment affecting infection success is critical to the development of a good screening method that is unambiguous in differentiating among various levels of resistance in different organs. Variation in factors (host, pathogen, and environment) can affect the sensitivity and reproducibility of the screening method and hence need to be optimised. A listing of these variables is provided below.

Host Factors Verification and resolution of host identity using molecular markers together with morphological based methods.

Tissue (Leaves, Pods, Stems; Attached vs. Detached; Wounded vs. Whole) Age (Mature, immature, pods, leaves, plants), Field vs. Lab evaluation, Shade/light, and their role in stomatal size and frequency.

Pathogen Factors Species, Isolates/geographical, Propagule (hyphae, zoospore, sporangia, chlamydospore; genetic variation), Culture medium (V8, Carrot, Potato, Cacao), Single spore culture vs. mixed spore cultures, Temperature for optimum production, Age of culture (day/light regime), Spore suspension preparation, Time lapse/interval between inoculation and spore preparation, Inoculum concentration, Inoculum volume, Reactivation regime, Length of time in storage, Correct identification of isolates, Culture viability before and following inoculation (adjustment of inoculum concentration to obtain 100 % infection in susceptible genotype), Pathogen aggressiveness.

Environmental Factors Incubation temperature, Relative humidity, Incubation period, Light.

Delivery System

Methods of introducing the inoculum to the plant play a crucial role in symptom occurrence and severity. An optimised inoculation method should be able to (1) simulate natural conditions as much as possible, (2) deliver the inoculum to the infection court in standard quantity, (3) standardised host, pathogen, and environmental conditions provided for infection establishment, (4) yield results that correlate well with field observations, (5) yield resistant indicators that are reliable, repeatable, quantitative, and accurate, (6) differentiate amongst various resistance levels, (7) allow early screening that is non-destructive in nature, (8) be cost-effective with respect to time, space, and labour.

Additional considerations regarding delivery systems include whether or not the method is destructive; using homogenised culture suspensions; high tech vs. low tech methods; and economic constraints.

Methods of Assessment

In assessing symptom severity, the following should be taken into account: qualitative vs. quantitative, manual vs. digital capture, and morphological vs. biochemical. The method chosen should be reliable, robust, easy to perform, and be able to discriminate clearly between resistant and susceptible varieties.

Breeding for Resistance

Only when sources of resistance for various species and isolates of *Phytophthora* have been identified can breeding for disease resistance be carried out in cacao to meet the specific local/global producer needs. The final chapter of this book deals exclusively with this aspect of disease management. Breeding programmes to date have been met with mixed success largely due to the difficulty in the identification of sources of resistance. When taking into account the designing of future disease resistance pre-breeding and breeding programmes for cacao, the importance of *Phytophthora* diversity, and understanding its biology in disease dynamics, careful considerations should be given to the recommendations made in this review.

7.11 Future Prospects

Significant advancements and achievements have been obtained to date in knowledge and understanding of the history, biology, and control of *Phytophthora* since it was first encountered. With the existence of over 100 species spanning the globe, they have certainly asserted themselves and gained our attention, recognition, and respect. We should not forget that *P. infestans* (late blight of potato) in Ireland contributed to a monumental social and historical period which gave birth to the science of Plant Pathology. *Phytophthora* is a genus that is mainly, if not entirely, parasitic on various plant hosts and a considerable amount of time, effort, and resources have been dedicated to understanding how they could be managed.

The role that *Phytophthora* pathogens have played in the economies of many countries for many crops (ornamental, horticultural, vegetable, and tree)—potatoes, avocados, and cacao, to name a few, can be considered equally as important in food and agriculture which ultimately impacts our survival. In the case of cacao, a new world orphan crop, the economic attractiveness is eclipsed by *Phytophthora* pathogens causing a number of diseases that manifest on all organs of the tree.

In being able to manage *Phytophthora* pathogens effectively on cacao farms, a number of factors need to be considered. Carefully planned management using various control approaches can be highly effective in reducing losses to the disease (Peter and Chandramohanan 2014). When deciding which strategy to adopt, careful attention should be paid to the resources available and local constraints to production. As such, the following strategies are suggested to develop an effective cacao *Phytophthora* disease management programme (Fig. 7.4).

Identification and Distribution

It is speculated from the presence of alternative hosts of *Phytophthora* in the different growing regions that more species may be present on cacao farms than have been reported. As such, it is suggested that cacao pathologists be aware of the

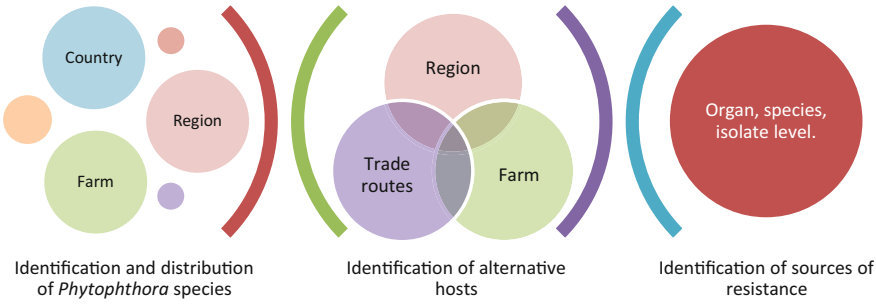

Fig. 7.4 Strategy for effective *Phytophthora* disease management in cacao

range of *Phytophthora* species, races, and spatial and temporal occurrence within the region they serve. Beginning with proper identification, many species have been identified and some resolved. There still exists a lack of information regarding the occurrence and distribution of *Phytophthora* species present in cacao-producing countries. This still remains the first and crucial step in being able to manage cacao diseases effectively and identification can be achieved easily through any one of the number of morphological or molecular techniques mentioned in Sect. 7.2. For example, several species are reported on cacao, but few cause significant disease. Also, species like *P. capsici*/*tropicalis* and *P. citrophthora* are little studied in cacao although they have at least been regionally important in causing disease in cacao in the past. Knowledge of their global distribution on cacao remains confused.

Identification of Alternative Hosts

It is important to identify alternative hosts that are present within a region that could harbour and perpetuate *Phytophthora* pathogens during unfavourable conditions in cacao fields. This can be achieved by using a proven screening method to establish Koch's postulates—cause–effect relationship of the host pathosystem. None of the *Phytophthora* species causing disease on cacao are host specific. Although progress is being made in identifying alternative hosts (there are many), their impact on disease development on cacao in the field is virtually unstudied.

Variability Within Species

Knowledge and understanding of the species variability should be investigated from a number of viewpoints such as morphological, biochemical, molecular, pathogenic (on main and alternative hosts), and their interaction with each other under field and laboratory conditions. Features of similarity or differences that may be peculiar to a habitat could provide useful information for managing a particular species of *Phytophthora*. There are no pathogen race designations for *Phytophthora* on cacao yet, but as resistance sources become better characterised, the possibility of developing race panels improves. This deeper understanding of pathogen variability will be critical to the sustainable use of resistance sources in the field.

Sources of Resistance and Host–Pathogen Interaction

Having identified the species, distribution, and isolates present along with alternative hosts, it is useful to know whether sources of resistance exist for different species in local germplasm collections. This can be done using screening methods that have been developed for identification of resistance in various organs (leaf, pod, stem, root) to study host–pathogen interaction. Coordinating the details of such screening methods across research programmes would greatly increase the usefulness of the results obtained to the global cacao research community.

7.12 Conclusion

The importance of *Phytophthora* diversity and biology in disease dynamics must be taken into account when designing future disease resistance pre-breeding and breeding programmes for cacao. These considerations should not be trivialised given that such programmes require considerable investment of resources in terms of time, financial commitment, and effort. Improved cultivars with *Phytophthora* resistance or control methods to increase productivity would only be realised if rigorous and reliable systems are implemented.

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Chapter 8
Phytophthora megakarya, a Causal Agent of Black Pod Rot in Africa

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Abstract In most parts of the world where *Theobroma cacao* is grown, *Phytophthora palmivora* is the major concern for causing black pod rot (BPR). *Phytophthora megakarya*, on the other hand, occurs only in Africa, but represents a major threat to cacao production, the countries of West Africa being the largest producers of cacao in the world. Since cacao did not originate in Africa, *P. megakarya* obviously only recently (likely prior to 1960) added cacao as a host and this new encounter has resulted in the most severe form of BPR worldwide. Although *P. megakarya* and *P. palmivora* are related, both being grouped in clade 4 in current *Phytophthora* diversity studies, *P. megakarya* has a distinct chromosomal composition and adaptations that make it particularly aggressive on cacao. *P. megakarya* has environmental requirements (temperature and rainfall) similar to cacao, and its ability to survive in soil and reinfect cacao pods through inoculum originating in the soil makes it particularly difficult to manage. Not only does *P. megakarya* survive in the soil for long periods, it also survives on the roots of cacao and other plant/tree species, many of which are cocultivated with cacao. Scientists have continued to make progress in understanding *P. megakarya* as a pathogen and have developed management tools for the disease it causes. Unfortunately, management tools such as fungicides and labor-intensive sanitation efforts, although effective, can be costly and, in some cases, difficult to obtain/maintain. As a result, farmers have difficulty justifying their use. Efforts toward breeding for tolerance in the crop to *P. megakarya*-induced BPR are making progress and in the future new planting materials should be able to greatly reduce disease losses if deployed with sound disease management practices. An improved understanding of the pathogen and its interaction with cacao at all levels, especially the molecular level, the deployment of tolerant cacao planting materials, the standardization of screening tools for developing tolerance to disease and determining pathogen diversity, and the refinement and more consistent employment by

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farmers of management tools already available will be critical for limiting losses due to *P. megakarya*-induced black pod rot in the future. The containment of *P. megakarya* to areas where it already exists is critical to cacao production around the world.

8.1 Introduction

Phytophthora megakarya (Brasier and Griffin 1979), one of several species of *Phytophthora* causing black pod rot (BPR) of cacao (*Theobroma cacao*), is a diploid heterothallic oomycete (Nyasse et al. 1999). Other *Phytophthora* species commonly causing BPR include *P. palmivora* (Butler) Butler (Chap. 7), *P. capsici*/*tropicalis* (Leonian emend.), and *P. citrophthora* (R.E. Smith and E.H. Smith). McMahon and Purwantara (2004) list four additional species of *Phytophthora* as having been isolated from cacao: *P. katsurae* (Ko and Chang), *P. arecae* (Coleman) Pethybridge, *P. nicotianae* (van Breda de Haan), and *P. megasperma* (Dreschler).

Although the oomycetes were for many years confused with filamentous fungi in their taxonomic designation, the use of molecular tools has made clear their phylogenetic relationship to diatoms and brown algae in the stramenopiles (Gunderson et al. 1987). The exact taxonomic designation of the genus *Phytophthora* remains a subject of intense discussion, but for our purposes here we will consider the following phylogenies: Kingdom: Stramenopila (sometimes spelled Straminopila)/Chromista; Phylum Oomycota/Heterokontophyta; Class Peronosporomycetidae/Oomycetes; Order Peronosporales; Family Peronosporaceae; Genus Phytophthora (Dick 2001; Thines 2014).

8.2 Pioneer in Cacao Plant Pathology Research, Dr. Clive Brasier

Great accomplishments sometimes have the oddest of beginnings and succeed only because of the imagination and tenacity of those involved, often under what some might consider difficult conditions. Dr. Paul Holliday (Fig. 8.1a) was on his way out the door, heading back to the West Indies, when he happened to mention to a young graduate student at Hull University something like "I have six isolates of *P. palmivora* from black pepper and one of *P. heveae* in my stock cultures. I think there is an issue with the sexual mechanism in *Phytophthora*. You might have a look at that. …" The relationship between that graduate student, Clive Brasier (Fig. 8.1b), and Dr. Holliday had begun when, as a second-year undergraduate student, Clive was fortunate to have Paul as a tutor and attend Paul's mycology lectures. Clive found Dr. Holliday's pioneering work, on cacao witches' broom disease and *Hemileia vastatrix* on rubber, inspirational. Clive's response to Dr. Holliday's suggestion undoubtedly had a huge impact on his exemplary career

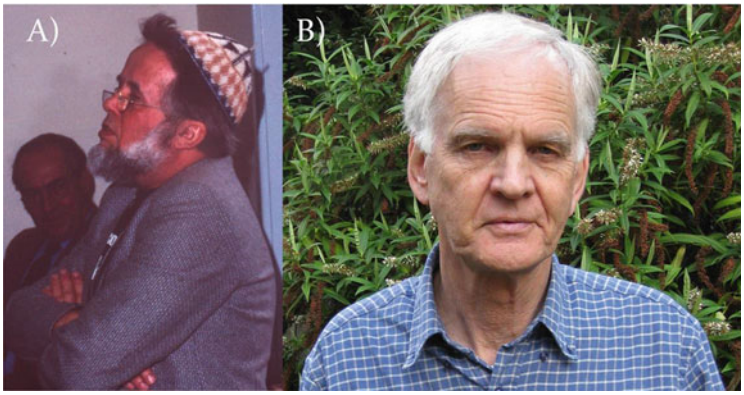

Fig. 8.1 (**A**) Dr. Paul Holliday in 1983 at his retirement and (**B**) Dr. Clive Brasier

and, more importantly for this presentation, cacao research. Clive headed off to the National Lending Library in Boston Spa, West Yorkshire, England, where for 2 weeks he photocopied and read every paper on *Phytophthora* sexuality he could get his hands on.

After Dr. Paul Holliday left for the tropics, Dr. Noel Robertson took over officially as Clive's supervisor, but Clive was given free reign as long as his proposals were reasonable. David Ingram was his contemporary, doing a Ph.D. on *P. infestans* (resistance of potato tissue cultures) under Dr. Robertson at the same time. Dr. Robertson also greatly stimulated Clive's interest in plant pathology, especially *P. infestans*. Similarly, Dr. Hugh Morgan, a lecturer and *Coprinus* geneticist, stimulated his interest in fungal genetics. So it seems two truths are evident: teaching instructors and supervisors can and do have a tremendous effect on the careers of their students, and good things result when a critical mass of good minds are brought together studying related subjects.

Clive received his B.S. degree in botany in 1963 and his Ph.D. degree in mycology in 1966, both from the University of Hull, England. His Ph.D. was on the physiology of reproduction in *Phytophthora*. Some of that research was published in Brasier (1969), an article entitled "Formation of oospores in vivo by *Phytophthora palmivora*." After working as a research fellow in the genetics department at the University of Birmingham (1966–1969), Dr. Brasier joined the British Forestry Commission's Research Division as mycologist, his research continuing from there.

For many years cacao researchers had noted the morphological differences among *Phytophthora* isolates causing disease on *T. cacao*, as well as differences in the geographical distribution, and aggressiveness. Clive's interest in cacao *Phytophthoras* initially grew out of his work on sexual mechanisms in heterothallic *Phytophthoras* and continued after he joined the Forestry Commission through his collaborations with Eva Sansome on *Phytophthora* karyotypes. This is evidenced by their coauthored publications including Sansome et al. (1975). It was there where chromosome differences among isolates from cacao in West Africa were first noted (the L-type had 5–6 large chromosomes and S-type had 9–12 smaller chromosomes)

and correlated with differences among morphological traits. Following Dr. Brasier's involvement with the "*P. palmivora*" chromosome studies, Dr. Philip Gregory connected Clive with the Cocoa, Chocolate and Confectionery Alliance, who provided Clive with a microscope for his chromosome and taxonomic work.

The stage having been set, Clive went about the detailed study of *P. palmivora* isolates causing disease on cacao. This study included *Phytophthora* isolates from diverse countries and provided intimate details of their morphological traits (compatibility type, colony morphology, sporangia structure, sexual structures) and chromosomal type. The correlations drawn provided ample support for the designation of a new species "*P. megakarya* Brasier & Griffin" causing disease on cacao, the publication of which (Brasier and Griffin 1979) is the foundation of research on this pathogen. Dr. Brasier has continued, since this early work, to have major impacts in forest pathology (especially with the *Phytophthoras* and Dutch elm disease) resulting in recognition for his efforts at many levels. But here we recognize the importance of that early work in cacao, where a new *Phytophthora* species became a part of the scientific discussion. This accomplishment was not achieved alone, Dr. Brasier having had the benefit of the instruction, experiences, advice, and discussions with many excellent scientists. Nevertheless, what is history may not have occurred had he not been curious and industrious enough to follow up on a simple suggestion "you might have a look at that."

8.3 History and Impacts of Disease

Undoubtedly the taxonomic designations of the *Phytophthoras* will be further clarified in the near future. The etymology (Widmer and Hebbar 2013) of "megakarya" is derived from "large" (mega) "karyotype" (karya) due to *P. megakarya* having much larger chromosomes compared to *P. palmivora*. BPR can be found wherever cacao is grown and was attributed to a single species (*P. palmivora*) until Brasier and Griffin (1979) using karyotyping identified *P. megakarya*. Although the name *P. megakarya* was proposed in Brasier and Griffin (1979), the differences in chromosome size and number between what would ultimately be *P. megakarya* and *P. palmivora* were noted earlier (Sansome et al. 1975). Sansome et al. (1975) described the L-types (ultimately *P. megakarya*) and S-type (remaining *P. palmivora*) and drew correlations between the L- and S-type with the N- and G-type described by Turner (1960). Ward and Griffin (1981) studied *P. megakarya* and *P. palmivora* survival in soil in 1974, although the species designation was not clarified in the literature until 1979. Clearly, researchers recognized the significant differences between the two species much earlier than 1979. Hislop (1963) studied the rubber group from cacao in Ibadan (Nigeria) based on oospore and sporangial dimension, the rubber group from cacao (now considered to have been *P. megakarya*) being "slow growing," and noted the greater virulence of the rubber type most common in Nigeria compared to cacao type from Ghana. The N-type was found in Nigeria, Southern Cameroon, Fernando Po, and Gabon (Turner 1960). Earlier, significant differences between

Phytophthora isolates in Nigeria and Ghana had been noted by Wharton (1958). It is also interesting that Thorold (1959) reported losses over a 9-year period in Nigeria at the government cacao farm near Ibadan, increasing from an average of 10.6 % between 1945 and 1948 to an average of 41 % between 1948 and 1951 and finally to 66.8 % between 1951 and 1954, perhaps an indication of the emergence of *P. megakarya* in the area. Although the details describing the differences between *P. megakarya* and *P. palmivora* can be found in Brasier and Griffin (1979), some of the more pertinent differences include the following:

P. palmivora-chromosomes ($n = 9$–12), sporangial pedicels (broad, short and occluded, length mostly 2–5 μm) (Fig. 8.2B), compatibility type (predominantly A2, A1 uncommon), cardinal temperature (min. 10–11°, op. 28–30°, and max. 34°), distribution on cacao (worldwide).

P. megakarya-chromosomes ($n = 5$–6 large), sporangial pedicels (narrow, medium length and not occluded; mostly 10–30 μm.) (Fig. 8.2A), compatibility type (predominantly A1, A2 rare), cardinal temperature (min. 10–11°, op. 24–26°, and max. 29–30°), distribution on cacao (western and central Africa).

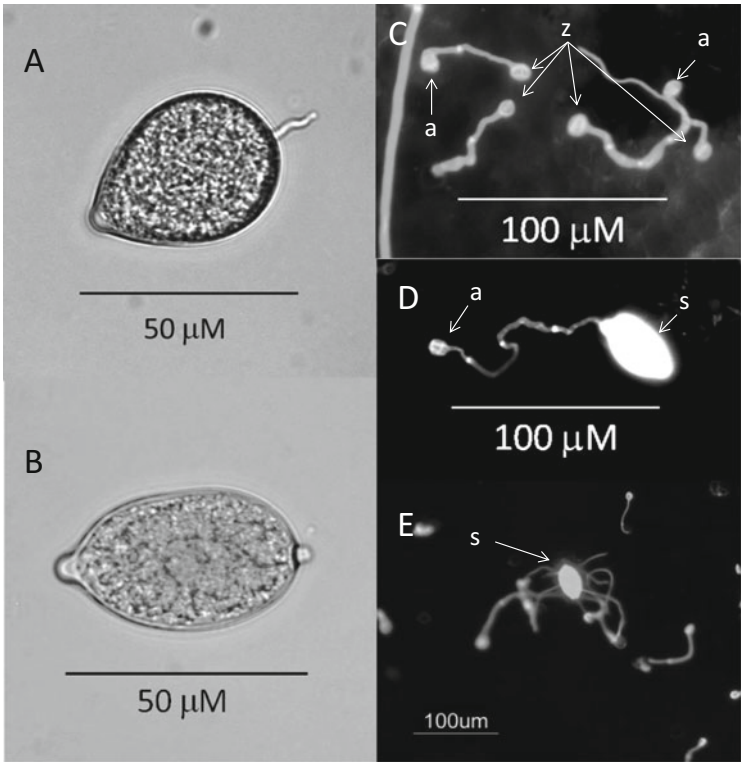

Fig. 8.2 Some structures involved in infection by *Phytophthora* species. (**A**) Sporangia of *P. megakarya*. Note narrow elongated pedicel. (**B**) Sporangia of *P. palmivora*. Note broad short pedicel. (**C**) Apressoria (a) formed by encysted and germinated *P. megakarya* zoospores (z). (**D**) Germinated *P. megakarya* sporangium (s) with apressoria (a). (**E**) Germtubes from *P. megakarya* zoospores which failed to release from the sporangium

The distinctions described above were some of the characteristics used in separating these species prior to the development of molecular tools. Appiah et al. (2003) found that pedicel length was the most consistent species-linked sporangial characteristic (Fig. 8.2A, B). The sporangial length/breadth ratios, when compared to the reciprocals of sporangial pedicel length, separated isolates into species groups (*P. capsici*, *P. citrophthora*, *P. palmivora*, and *P. megakarya*).

Both *P. megakarya* and *P. palmivora* are heterothallic with both A1 and A2 compatibility types. When the A1 and A2 types are grown together, oogonia and antheridia are produced abundantly (Brasier and Griffin 1979). Gametangia of *P. megakarya* are described in detail in Brasier and Griffin (1979), but generally of less diagnostic use in species identification among isolates causing disease on cacao. Proving sexual reproduction occurs in the field has been difficult, despite the occurrence of both compatibility types for *P. megakarya* in some areas (Yuen and Andersson 2013). The rarity of the A2 type limits the potential for sexual reproduction. Both compatibility types are found in Equatorial Guinea, Cameroon, Ghana, and Nigeria (Appiah et al. 2003). Nyasse et al. (1999) described two lines of evidence suggestive of *P. megakarya* undergoing sexual reproduction: (1) the nature of isozyme polymorphisms observed and the small genetic distance observed between A1 and A2 isolates in areas where both compatibility types occur, and (2) the occurrence of isolates with intermediate genotypes where otherwise geographically separated genetic groups overlap. Since both *P. palmivora* and *P. megakarya* occur together on cacao in Africa, the possibility of interspecific hybrids has been studied. Although this question does not appear to be settled, oospore products of crosses between A2-type *P. palmivora* and A1-type *P. megakarya* had low viability and were considered products of self-fertilization (Boccas 1981; Erselius and Shaw 1982). A viable hybrid between *P. megakarya* and *P. palmivora* has not been demonstrated in nature and products produced in the laboratory are of questionable origin. The large differences in the chromosomal sizes and numbers limit the possibility of fertile hybrids (Boccas 1981; Erselius and Shaw 1982).

P. megakarya occurs only in areas of western and central Africa. Since cacao is native to rainforest areas of South America (Motamayor et al. 2008), the interaction between cacao and *P. megakary*a necessarily represents a new encounter and undoubtedly a host jump from plant species native to Africa. Whereas *P. palmivora* is known to cause disease on over 1000 plant species (Widmer 2014), less is known about the host range of *P. megakarya*. *P. megakarya* is not known to cause significant disease on any plant species other than *T. cacao*. It was suggested by Nyasse et al. (1999), based on the areas of greatest genetic diversity, that the center of origin of *P. megakarya* lies in the primary forests of Central and West Africa in areas corresponding to ancient, Ice-Age forest refugia. Two highly differentiated genetic groups of *P. megakarya* were identified with isozymes and RAPDs, one located in Central Africa and the other in West Africa. Subsequently, Holmes et al. (2003) isolated *P. megakarya* isolate IMI 387090, identified by morphological traits and AFLP and ITS-RFLP characterization, from the necrotic areas of a fallen *Irvingia* sp. fruit. Isolate IMI 387090 was found in the Korup

National Park in western Cameroon, adjacent to the Nigerian border. Opoku et al. (2002) also isolated *Phytophthora* isolates from the roots of shade trees in cacao fields [Apocynaceae (*Funtumia elastica*), Sterculiaceae (*Sterculia tragacantha*), Agavaceae (*Dracaena mannii*), and Euphorbiaceae (*Ricinodendron heudelotii*)] that were pathogenic on cacao pods and morphologically indistinguishable from *P. megakarya*. In a more recent study *P. megakarya* was isolated, again principally from roots, from *Xanthosoma sagittifolium*, *Musa* x *paradisiaca*, *Elaeis guineensis*, *Persea americana*, *Carica papaya*, *Mangifera indica*, *Colocasia esculenta*, *Athyrium nipponicum*, and *Ananas comosus*, economic plants found on cacao farms in Ghana (Akrofi 2015). Although *P. megakarya* can be associated with the roots of many plant species, no significant symptoms on any of these species have been attributed to natural *P. megakarya* infection. The possibility exists that *P. megakarya* causes significant root pruning, but evidently not to the point that major macro-symptoms are observed.

8.4 A Case Study, the Movement of *P. megakarya* into Ghana

The history of movement of *P. megakarya* across western Africa is a story complicated by the emerging classification of a new species, varying abilities/availability of tools to identify this new species, sometimes-limited surveys due to time and distance, and shared borders and associated movement of plant materials between nations and regions. The first sign of the arrival of *P. megakarya* into a region is likely to be a significant increase in the losses to BPR as summarized in Akrofi and Opoku (2003). Keep in mind the arrival of *P. megakarya* occurs in a background of BPR caused by *P. palmivora* which also causes fluctuating levels of disease. For example, while the losses to *P. palmivora* in Ghana were reported as 19 % in 1961, losses were more than 60 % in areas where *P. megakarya* was first found (Dakwa 1987), and in some cases farmers were abandoning their cacao fields. As of 1979 (Brasier and Griffin 1979; Sansome et al. 1979), *P. megakarya* was not found in Ghana, Côte d'Ivoire, or Togo but was present in Nigeria, Cameroon, Fernando Po, and Gabon. *P. megakarya* was first identified in Ghana in 1985 (Dakwa 1988; Luterbacher and Akrofi 1994). Based on field studies, *P. megakarya* was in Togo in 1978–1979 (Djiekpor et al. 1982). Dakwa (1988) suggested, based on farmer interviews, that the epidemic reported in 1985 in the Akomadan District of the Ashanti region and attributed to *P. megakarya* (based on sporangial morphologies and thermal temperature death points) may have begun as early as 1980. Losses to *P. palmivora* were typically between 4.9 and 13.5 % (Dakwa 1984), whereas production in some areas was reduced 90 % in association with the *P. megakarya* outbreak. Opoku et al. (2000) further suggested *P. megakarya* may have existed in the Volta region of Ghana from the early 1970s, based on losses to black pod rot in that region and its shared border with Togo. *P. megakarya* continues to move into

new areas in West Africa. Though *P. megakarya* was reported to be on the Côte d'Ivoire border in 1993 (Luterbacher and Akrofi 1994), it was not reported to be in Côte d'Ivoire by Nyasse et al. (1999). However, it was reported present in the Eastern region (Abengourou Department) of Côte d'Ivoire by Koné (1999), obviously a difference in the isolates included in the individual studies. Once again its presence, this time in Côte d'Ivoire, was suggested due to increased losses to black pod (Koné 1999; Pokou et al. 2009).

The devastating effects of *P. megakarya* on cacao production in Ghana between 1988 and 1993 were quantified by Opoku et al. (2000). Over these 5 years, in nine districts associated with the arrival of *P. megakarya*, production dropped from 41,381 tons to 29,344 tons per year, whereas in nine districts with *P. palmivora* only, production increased from 38,041 tons to 51,205 tons per year. At the farm level, 40 farms in four districts with *P. megakarya* saw their production drop from 256.7 tons to 172.7 tons per year, while 40 farms in four districts with *P. palmivora* only saw their production increase from 362.1 tons to 567.5 ton per year. By the year 2000, 25 % of farms in Ghana were considered infested with *P. megakarya* and continued spread of the pathogen was expected. By 2006 *P. megakarya* was found in 5 out of the 6 cacao-growing regions in Ghana (Akrofi et al. 2012). A combined 1985–2012 survey indicates that *P. megakarya* has spread from where it was first reported in the Akomadan and Bechem districts in 1985 into 50 more districts, covering an area of 75,298 km^2 and including all six cacao-growing regions in Ghana.

The impact of *P. megakarya* on cacao production in Ghana is mirrored by the increase in research efforts directed toward BPR in Ghana as reported by the Cocoa Research Institute of Ghana (CRIG) in its annual reports of 1979–1994 (CRIG 1979–1994). In the CRIG annual report from 1979 to 1982 (Dakwa 1979–1982), a time period covering the realization that *P. megakarya* was a new species not yet found in Ghana, 3 pages were dedicated to BPR (not counting breeding efforts which were significant). Topics covered included regional disease incidence (maximum loss equaled 12.9 % Eastern region), subregional disease incidence (maximum loss 27.0 % in Kibi, Eastern region), and *Phytophthora* canker. The CRIG annual report from 1982 to 1985 (Asare-Nyako and Bruce 1982–1985) included 6 pages on BPR. Topics included fungicide trials (carried out in Tafo; maximum loss to BPR in one trial was 35.9 %) and a nationwide survey of BPR. The fungicide Ridomil Plus was highlighted in the fungicide trials. The 1985–1986 annual report included 5 pages but, more importantly, included the first report of *P. megakarya* in Akomadan, a district in the Ashanti Region, and the Bechem district, in the Brong Ahafo Region. A large reduction, over 85 % in some areas, in cacao purchases was reported for the Akomadan district between 1985 and 1986. Fungicide trials and *Phytophthora* canker were also discussed. By the year 1990 to 1991, 32 pages on BPR were included in the CRIG report (CRIG 1990–1991) under what was termed the "Black pod thrust" and *P. megakarya* was heavily emphasized. The topics discussed were extensive disease surveys, epidemiological studies, fungicide trials, and biochemical detection of *Phytophthora* species. The epidemiological studies included sources of infection, epiphytotic development, and epidemiology in soil.

Reports on fungicide trials from the Akomadan district were included. Disease surveys showed *P. megakarya* was in the Ashanti, Brong-Ahafo, and Volta regions. Overall, both *P. megakarya* and *P. palmivora* were recovered in similar frequencies in these regions, but when the focus was placed on local sites the tendency was to find only one species. It was hypothesized, based on disease surveys, that *P. megakarya* would become the dominant species once it entered an area, and since this time *P. megakarya* has been a major focus of CRIG research.

8.5 Molecular Identification of Species

Morphological characteristics are obviously very useful in identifying the species of *Phytophthora* causing disease on cacao having been used early on in identifying the new species *P. megakarya* (Brasier and Griffin 1979). For example, Appiah et al. (2003) were very successful in identifying to species level, isolates of *Phytophthora* causing disease on cacao, as described above. Despite successes, problems can arise in using strictly morphological traits for characterizing diversity between and within a species, sometimes due to the occurrence of rare phenotypes, overlapping ranges of morphological traits between species, the requirement for strict culturing conditions, and the necessity of specific training in the techniques used. As a result, simpler traits have been sought for characterizing *Phytophthora* genetic variation as distinguished from phenotypic variation. The ideal trait would be immune to technical error, simple, quick, and of low cost. Several approaches have been developed using molecular tools.

Studies using advanced molecular tools for identifying *Phytophthora* have generally been carried out in two patterns of effort. The most common pattern of effort has centered on identification at the species level, for example, separation of *P. megakarya* from all other *Phytophthora* species. In most cases these types of studies do not focus on *P. megakarya*. The second pattern of effort has focused on the characterization of *Phytophthora* species causing disease on cacao and highlights diversity within species in addition to diversity between species. The history of the use of these more advanced tools parallels the history of their general use in biology. The molecular techniques of polymerase chain reaction (PCR), restriction fragment length polymorphisms (RFLPs), and direct sequence analysis are commonly used. Mixed in with these efforts are studies at the protein/enzyme level.

Protein and enzyme patterns (Kaosiri and Zentmyer 1980) were one of the first biochemical markers used to separate *P. megakarya* (Morphological Form 3) from other *Phytophthora* species causing disease on cacao. Panabieres et al. (1989) used RFLPs to separate *Phytophthora* species, noting they were useful in separating the four species causing disease on cacao, including *P. megakarya*. The RFLPs were based on enzymatic digestions of genomic DNA and dependent on the presence of repetitive DNA. Nwaga et al. (1990) used total native and denatured proteins and isoenzyme marker esterases, malate dehydrogenases, and superoxide dismutases combined with polyacrylamide gel electrophoresis to characterize *P. capsici*,

P. cinnamomi, *P. cryptogea*, *P. megakarya*, *P. megasperma*, *P. nicotianae*, *P. palmivora*, and *P. citrophthora*. The interspecific variability observed was high. The extent of intraspecific mitochondrial DNA (mtDNA) diversity was investigated in isolates of *P. capsici*, *P. citricola*, *P. citrophthora*, *P. megakarya*, *P. palmivora*, and *P. parasitica* using RFLPs (Fo et al. 1990). Isolates of *P. megakarya* from Cameroon and Nigeria formed 2 distinct groups with unique mtDNA RFLP patterns. Cooke et al. (2000) sequenced the ITS regions of 50 *Phytophthora* species placing *P. capsici* and *P. citrophthora* in clade 2 and *P. palmivora* and *P. megakarya* together in clade 4, as part of a 10 clade phylogeny. Single-strand-conformation polymorphism (SSCP) of ribosomal DNA was used to separate 29 species of *Phytophthora* including *P. megakarya* (Kong et al. 2003). This somewhat unique PCR approach takes advantage of the changes in single-stranded DNA mobility introduced by conformational changes resulting from relatively simple sequence changes. The close genetic relationship between *P. palmivora* and *P. megakarya* (both clade 4) was confirmed by Kroon et al. (2004). A molecular phylogenetic analysis of the genus *Phytophthora* was performed based on regions of mitochondrial (cytochrome *c* oxidase subunit 1; NADH dehydrogenase subunit 1) and nuclear gene sequences (translation elongation factor 1α; β-tubulin) including 48 *Phytophthora* species. The *Phytophthora* species were distributed into 10 clades. Martin and Tooley (2004) developed polymerase chain reaction primers that amplified regions of mitochondrial DNA encoding *cox* I and II genes of 31 *Phytophthora* species. Digestion of the amplified fragments with restriction enzymes generated species-specific RFLP banding profiles that were effective for isolate classification to a species level. Once again, *P. capsici*, *P. citrophthora*, *P. palmivora*, and *P. megakarya* isolates were included in the study.

The internal transcribed spacer regions of *P. palmivora*, *P. megakarya*, *P. capsici*, and *P. citrophthora* were analyzed by polymerase chain reaction amplification and direct sequencing (Lee and Taylor 1992). Interspecific nucleotide differences were 0.3 %–14.6 %, and were useful in evaluating phylogenetic relationships. Both neighbor-joining and parsimony analysis of ITS variability supported a close relationship between *P. capsici* and *P. citrophthora* and a common lineage for *P. palmivora* and *P. megakarya*. It was suggested that the sequences provided might be used to develop "taxon-specific probes." Recently Ali et al. (2016) developed a set of four primer pairs that can identify and separate the four major species of Phytophthora causing disease on cacao: *P. megakarya, P. palmivora, P. capsici/tropicalis*, and *P. citrophthora*. Nyasse et al. (1999) used isozyme and RAPD markers to characterize genetic diversity and structure among isolates from the known distribution area of *P. megakarya* at that time (Ghana, Togo, Nigeria, Cameroon, Gabon, and Sao Tome). Isozymes and RAPD patterns were useful in separating the population into two genetic groups, Central Africa and West Africa. The *Phytophthora* species causing disease on cacao, *P. palmivora*, *P. megakarya*, *P. capsici*, and *P. citrophthora*, were easily distinguished by analysis of the internally transcribed spacer regions of the ribosomal RNA gene cluster by direct sequencing or by RFLP analysis of the PCR amplified ITS region (Appiah et al. 2004a). Appiah et al. (2004a) used the same set of 161 isolates of *Phytophthora* species causing BPR as was used in Appiah et al.'s (2003) study of diversity based on morphological traits. Sequence analysis grouped *P. capsici*

and *P. citrophthora* together and *P. palmivora* and *P. megakarya* together (Appiah et al. 2004a). Mfegue et al. (2012) identified microsatellite sequences in *P. megakarya* and the 12 most polymorphic and unambiguous loci were selected for microsatellite analysis of 652 isolates from Central and West Africa, with a focus on Cameroon. The number of alleles per locus ranged from two to nine. Among 66 pairwise comparisons of loci, 55 exhibited highly significant linkage disequilibrium consistent with a clonal reproduction of *P. megakarya*. In a clonal population, the occurrence of both phenotypic and genotypic variation may be attributed to mitotic and/or somatic recombination events (Ivors et al. 2006).

8.6 Characterization of *P. megakarya* and Its Interaction with *T. cacao*

Although we gain insight into population dynamics of the pathogen in the field by studying genetic diversity, we do not establish an understanding of what makes *P. megakarya* a pathogen on cacao, or what factors influence its aggressiveness or virulence. This continues to require molecular and biochemical analysis of the components and responses involved in the interactions. Although very little has been done studying the specific interactions between *P. megakarya* from the pathogen side of the molecular interaction, quite a few studies have focused on the plant-side reactions of the interaction. In general, most of the studies focus on anatomical/histological differences, enzymes, genes, and metabolites that are influenced by interactions between cacao genotypes varying in resistance to BPR (*P. megakarya*, *P. palmivora*, or both species).

Ndoumou et al. (1995) studied the activity of peroxidase, soluble phenol, and saccharide contents in crude extracts of the pod cortex and in seeds of cacao clones: SNK413 (most tolerant), SNKI0 (highly susceptible), and ICS95 (moderately susceptible) and in F1 hybrids. Their results suggested that phenolic compounds and peroxidase activity were positively correlated with tolerance to black pod disease. Crossing of clones reduced the phenol and saccharide contents compared to the most tolerant clone and was also associated with poor tolerance to black pod in the F1 hybrids. (Ndoumou et al. 1996) went further to show that the concentration of pod phenolics was higher in the more tolerant clones (SNK413 and ICS95) compared to the susceptible SNK10 clone, when infected *by P. megakarya* through wounds. Pods of all three clones could be infected, but lesions developed in line with the clones' levels of tolerance. Other factors, like increases due to infection in soluble amino acid content and reduction in soluble carbohydrate content, were associated with susceptibility. Wound inoculation with *P. megakarya* resulted in an increase in β-1,3-glucanase activity in the less susceptible clones and the appearance of an ionically bound isopoly-phenoloxidase A′2. The hypothesis was put forth that these enzymes might function in tolerance of cacao to *P. megakarya* through enhancement of lignification of pod cell walls (Omokolo et al. 2003).

Djocgoue et al. (2007) characterized tolerance to *P. megakarya* in wounded detached cacao leaves. Necrotic lesion development, phenolic content, and changes in phenolics were evaluated in ICS84 and ICS95 clones and their hybrids. Significant hybrid vigor and an additive genetic effect were observed, with 90 % of the hybrid progeny performing better than either parent for lesion size. Several progeny displayed a localized lesion reaction. A negative correlation ($r = -0.683$) was observed between development of necrosis and phenolic accumulation. Phenolic profiles were altered in response to infection, notably the accumulation of apigenin and luteolin derivatives. Similar results were obtained in leaves when the relationship between phenolic compounds and *P. megakarya* tolerance was evaluated in the parents SNK413 (tolerant) and SNK10 (susceptible) (Boudjeko et al. 2007). Again, a negative correlation was observed between development of necrosis and phenolic accumulation, and strong hybrid vigor was indicated. Qualitative analyses of phenolic compounds from the parents and progeny by high-performance liquid chromatography indicated accumulation of luteolin derivatives was dependent on the female parent (SNK413), suggesting some cacao resistance traits are of cytoplasmic origin. Louise et al. (2011), in further studies with SNK413 and SNK10, demonstrated a link between flavonoid content and resistance to *P. megakarya* using zoospore-inoculated wounded pods. HPLC analysis of pod extracts indicated epicatechin, procyanidin B2, and procyanidin C1 derivatives as potential markers for tolerance in cacao against *P. megakarya*. Phenolic content continues to be studied in association with tolerance to black pod in varying cacao genotype combinations. Simo et al. (2014) analyzed two hybrid families of cacao which are different in productivity and vulnerability to BPR. As seen in earlier studies, inoculation of detached pods with *P. megakarya* (mycelia to wounds) caused an increase in phenolic compound concentrations. Tolerant genotypes had higher phenol content. Ondobo et al. (2014) found similar results using a leaf assay and *P. megakarya* (wounded detached whole leaves inoculated with mycelia). Elevated phenolic content was negatively correlated with increased lesion size.

Arbuscular mycorrhizal fungi increased plant growth parameters and phosphorus uptake. A significant relationship was observed between arbuscular mycorrhizal fungi altered amino acids and disease severity (Tchameni et al. 2012). It was suggested that the synthesis of amino acids like arginine, cysteine, and glutamic acid in response to arbuscular mycorrhizal fungi colonization might be markers for resistance to infection by *P. megakarya*. Nyadanu et al. (2012) found anatomical/histological traits like the number of vascular bundles, epicarp thickness, and number of cells in epicarp, and cell width to be reliable markers for screening for resistance to black pod resistance. Resistance was evaluated by leaf disks and detached pod inoculations and examination of anatomical factors. The cells in the epicarp and mesocarp were found to be arranged more compactly in resistant than in susceptible genotypes. They suggested that the presence of an extra thickness of phloem fiber and its gritty nature in resistant genotypes may act as a strong mechanical barrier to penetration and absorption of sap from phloem. The cell walls of tolerant genotypes also had higher levels of lignin, based on results from phloroglucinol staining. Djocgoue et al. (2012) found that accumulation of proline

in response to infection (whole detached leaves, wounded and mycelia plugs) was associated with tolerance to infection by *P. megakarya*.

Nyadanu et al. (2012) further studied the importance of epicuticular wax in tolerance of cacao to Phytophthora pod rot, in 12 cacao genotypes. The wax layers were removed by washing leaves and pods in chloroform. Zoospores of *P. palmivora* and *P. megakarya* were used as inoculum. The level of resistance of cacao genotypes was higher in leaves and pods with a wax layer than in chloroform washed leaves and pods (wax removed). Genotypes with higher amounts of wax were more tolerant than genotypes with lesser amounts of wax. There was still a significant difference in lesion number and lesion size among genotypes after wax removal indicating tolerance of cacao against *Phytophthora* is influenced by factors in addition to wax layer thickness. As might be expected, a thick wax layer was associated with reduced water retention on the tissue surface.

As the cost of nucleic acid sequencing technologies continues to be reduced, we can expect a rapid expansion of our understanding of the *P. megakarya* transcriptome, as well as of the cacao transcriptome as expressed during their interactions. The genes involved and their regulation are the primary determinants of the interaction outcomes in tolerant and susceptible cacao clones. A better understanding of these interactions will be critical to the optimization of breeding efforts to develop and deploy cacao genotypes providing sustainable tolerance in the field to BPR. The responses of stress genes in *T. cacao* leaves to development, Nep1-induced stress, and a compatible infection by *P. megakarya* were studied (Bailey et al. 2005). Ten cacao genes were selected representing genes involved in plant defense, gene regulation, cell wall development, or energy production. Leaf development was separated into unexpanded, young red, immature green, and mature green. The results indicated that the constitutive defense mechanisms used by cacao leaves differ between different developmental stages and pointed out the importance of consistently synchronizing leaf stages when studying disease reactions in leaves. This is expected and likely contributes to the very refined leaf staging required if leaves are to be used for resistance screening. Leaves are usually staged as to age, but scientists require training if leaf disks are to be used efficiently. Factors such as the level of browning on the petiole, an obvious transient state, are considered critical.

NEP1 encodes the necrosis inducing protein Nep1 produced by *Fusarium oxysporum* (Bailey et al. 2005) and has orthologs in *Phytophthora* species (Bae et al. 2005). *P. megakarya* carries many copies of Nep1-related genes in a complex multigene family (Bae et al. 2005), a trait now known to be common for all *Phytophthora* species. The exact numbers vary for each species and perhaps for each isolate within a species. Nep1 caused necrotic lesions on cacao leaves and pods. Five of the six cacao genes that were responsive to Nep1 in leaves were also responsive to infection by *P. megakarya* zoospores. Susceptibility of *T. cacao* to *P. megakarya* includes altered plant gene expression, and phytotoxic molecules like Nep1 may contribute to susceptibility as they do in other plant–pathogen interactions (Oome et al. 2014). In a much broader study, four EST libraries from *P. megakarya*-infected tissues (3 leaf libraries and 1 pod library) were included in

an exhaustive examination of the cacao transcriptome by Argout et al. (2008). Naganeeswaran et al. (2012) analyzed data from these same four EST libraries, focusing on sequences derived from *P. megakarya*-infected cacao leaves and pods. Many cacao genes encoding enzymes or with putative defense functions were identified among the 1230 orthologous genes assembled. Although the use of molecular markers in cacao breeding is being covered in Chap. 18 of this volume, it is important to note here that tolerance to *Phytophthora* is considered a quantitative trait with evidence to support at least 13 consensus QTLs and 8 genomic regions being involved (Lanaud et al. 2009). Interest was expressed in verifying all QTLs that contribute to tolerance of all *Phytophthora* species. That is to say, resistance to one *Phytophthora* species contributes resistance to all *Phytophthora* species. Eventually, the genes associated with QTLs for tolerance to disease will be linked with specific genes encoding specific gene products involved in specific aspects of disease tolerance (like those discussed above), which should allow optimization of breeding programs that are screening for tolerance against BPR in cacao.

8.7 Culture Collection and Maintenance

Cultures of *Phytophthora* are easily recovered from infected pods. The practice is to cut open the pod with a large sterile knife and then remove a small section of internal tissues from the zone of lesion advance (necrotic tissue next to healthy tissue) with a small sterile blade and place the tissue on sterile water agar. Typically the interior tissues of healthy cacao have limited numbers of culturable organisms and a single colony type (the causal agent of the lesion) emerges from the tissue into the water agar. Using a low nutrient medium like water agar also suppresses bacteria growth. The emerging culture can usually be transferred sterilely to a nutrient medium like V8 agar as a pure culture. The cultures should be identified based on morphology (look for sporangia under described conditions) and molecularly by DNA sequencing or related techniques. If needed, cultures can be placed on an antibiotic medium like V8 PARP, 20 % V8 agar, containing antimicrobial amendments pimaricin, ampicillin, rifampicin, and pentachloronitrobenzene (Ferguson and Jeffers 1999) to limit bacterial and fungal contamination. Maintaining cultures of tropical *Phytophthora* species can prove difficult. We have found that cultures will last on active growth media for only 3 or 4 months, assuming the medium is not allowed to dry out. Incubating cultures on active growth media at 18 °C enhances the maintenance period. Our own efforts at keeping cultures at low temperature (−4 °C or lower) have failed. Presumably cultures can be stored in glycerol stocks under liquid nitrogen, but recovery from these stocks can be difficult. Another significant concern is infestation by fungal mites which consume and contaminate cultures. Clean laboratory techniques should be maintained and cultures routinely observed for mite contamination under a microscope.

Phytophthora species can be isolated from the soil by direct dilution plating on selective media or more commonly by baiting using pod pieces. Pod pieces are buried in soil and recovered at various time points. The pod pieces are observed for symptoms of BPR and tissues transferred to selective growth media like V8 PARP. Similarly, *Phytophthora* species can be isolated from roots and stems by surface sterilizing small segments and plating on selective media. Below we present some of the basic growth media used for isolating and maintaining Phytophthora cultures:

Water agar [adapted from Drenth and Sendall (2001)]. Add 15 g agar to 1 L distilled or deionized water. Autoclave, and add filter sterilized antibiotics if needed, once the media has cooled to 50–55 °C.

Clarified V8 juice Media [adapted from Drenth and Sendall (2001)]. Mix together 100 mL V8 juice with 1.0 g $CaCO_3$. Spin at 7000 rpm for 10 min. (Or spin at 4000 rpm and then filter through 2 layers of Whatman #1 with vacuum.) Divide supernatant into 50 or 100 mL aliquots and freeze at −20 until used.

V8 juice agar [adapted from Drenth and Sendall (2001)]. For routine *Phytophthora* cultures, preheat a large water bath to 55°. Add to a 1 L screw-cap bottle: Magnetic stir bar, 100 mL clarified V8, 8.5 g agar, distilled water to 500 mL. Mix well and autoclave for 30–40 min with the caps loosened. Gently mix media and place bottles in water bath to keep warm after autoclaving.

Carrot agar [Adapted from Drenth and Sendall (2001)]. Wash 200 g carrots and slice thickly. Autoclave carrots in 500 ml distilled or deionized water. Blend warm carrots in a blender at high speed for 40 s. Squeeze homogenate through 4 layers of muslin cloths. Make filtrate up to 1 L with deionized water. Add 15 g agar and autoclave. Gently mix media and place bottles in water bath to keep warm after autoclaving.

V8 PARP agar [adapted from Ferguson and Jeffers (1999)]. Prepare the antibiotics: Ampicillin (250 mg/mL in water. Filter sterilize and store at −20°), Rifamycin (10 mg/mL in methanol and store at −20° in the dark; no need to sterilize). Prepare the antibiotic and fungicide mixture by measuring into a 15 mL sterile Falcon tube (one tube per bottle of media): Delvocid (50 % Pimaricin, 5 mg), Terraclor (75 % PCNB, 33.4 mg), 500 μL of 250 mg/mL Ampicillin, 500 μL of 10 mg/mL Rifamycin, 1 mL sterile water. Vortex and hold on ice. To pour plates, vortex and then decant one 15 mL tube containing the additions into one bottle of V8 agar. Rinse the tube with another 1 mL of sterile water and add it to the bottle. Stir for 30–60 s to mix everything well. Pour plates, swirling gently after every 5 plates or so.

8.8 The Infective Units: Sporangia, Zoospore, Chlamydospores, and Mycelia

A typical method used for producing synchronized zoospores from axenic cultures is as follows (Opoku et al. 2002). Fresh cultures are started on V8 agar plates (other media like carrot agar are also commonly used) and incubated in the dark at 25 °C for 5 days. The 5-day-old cultures are transferred to the light and incubated for an additional 5 days. During this time sporangia will form and mature. The plate is flooded with cold, sterile distilled water (4 °C) and incubated for 20–45 min at 4 °C and then removed to 25 °C in the dark for 20–30 min during which time the zoospores are released. The zoospores are counted, typically by hemocytometer, and adjusted to appropriate concentrations. Inoculations are made using 50 μL drops. The exact incubation temperatures and times used are usually optimized by individual researchers according to their conditions and the specific isolates being studied. Similarly, the zoospore concentration and volume used is optimized for the experimental purpose. For example, when studying resistance mechanisms it is critical to use very consistent spore concentrations that do not overwhelm resistance levels.

Widmer and Laurent (2006) used very different conditions for producing zoospores. Isolates of *P. megakarya*, *P. palmivora*, and *P. capsici* were maintained on clarified V8 agar and zoospores were prepared by growing in clarified V8 broth. The V8 broth was removed after 4 days and the remaining mycelium was rinsed with sterile distilled water and then flooded with sterile distilled water. The cultures were incubated under continuous light at room temperature for 2 days before being placed at 4 °C for 30 min, after which the plates were moved to room temperature for zoospore release.

Since sporangia can germinate directly (Fig. 8.2D, E), they are sometimes used as quantitative inoculum for infecting plant tissues. This is particularly important for systems/isolates where getting zoospores to release from sporangia can be difficult. Sporangia have the added benefit that they are not dependent on motile zoospores which encyst, limiting the time allowed for their use in inoculations (approx. 30 min). When used, sporangia are counted as infective units the same way that zoospores are counted. The importance of direct sporangia germination by *P. megakarya*, which can occur as sporangia age, in establishing new infections is unclear. *P. megakarya* sporangia are caduceus and can be moved by rain drops. *P. megakarya* sporangia have not been heavily used as primary inoculum in cacao research but could provide a simple alternative to zoospores allowing more complex studies to be conducted.

Chlamydospores are basically rounded-off mycelia cells with thick cell walls. They are considered resting structures for long-term survival. Although survival of *Phytophthora* in soil as chlamydospores would seem to make sense, there is little evidence that chlamydospores function in the long-term survival of *P. megakarya* or *P. palmivora* in soil. For example, Chee (1973) found that chlamydospores of *P. palmivora* (pathogen of *Hevea brasiliensis*) were killed by air-drying and if

added to soil were not detectable in soil after 4 weeks, this despite *P. palmivora* continuing to be recovered from soil for up to 24 weeks. Simply inoculating soil with a mycelial suspension allowed for *P. palmivora* recovery for up to 32 weeks. For *P. megakarya*, chlamydospores have not been demonstrated to be the primary structure for longest term survival. Ward and Griffin (1981) did find chlamydospores in samples placed in soil for up to 8 weeks, but *P. megakarya* continued to be isolated from samples long after chlamydospore structures could no longer be found. The suggestion was that mycelial fragments or encysted zoospores might have persisted longer than chlamydospores. Considering the ability of *P. megakarya* to infect cacao roots as well as the roots of other trees, survival as a chlamydospore may be of less importance. Regardless, since chlamydospores can persist for long periods they still have ample opportunity to participate in initiating infections or in the movement to new areas in infested soil or infected plant material.

Similarly, mycelia fragments have potential as infective units. Mycelial plugs are efficient at establishing lesions through wounds and are sometimes used in studying resistance. In the end, any viable *P. megakarya* structure should be considered of potential importance in initiating disease, although obviously sporangia and resulting zoospores are of primary importance in the dynamics of a developing epidemic.

8.9 Epidemiology

Although encysted zoospores, chlamydospores and hyphae might serve as primary inoculum, active zoospores recently released from sporangia would appear to be the major primary inoculum source (Gregory et al. 1984). Sporangia require free water for zoospore release and zoospores move through water by their 2 flagella. The soil itself makes up a huge reservoir for *P. megakarya* and serves as a primary initiator of infection cycles. Infection cycles in cacao fields do not initiate from one or a few events in a localized area but instead begin from many initiation points and many sources throughout the production cycle. Although rain splash or contact with infected pods is the primary source of new infection (71.8 %) (Gregory et al. 1984), there are other significant sources like soil or litter contact, infected flower cushions of stem cankers, ant tents, and wounds due to insects and rodents. Rain splash can project droplets about 70 cm without the aid of wind (Gregory et al. 1984). Not all infection initiation points can be attributed to a known source, but Gregory et al. (1984) did draw a relationship between infection initiations from "no obvious" sources and spread through aerosols associated with water droplets. Research, with *P. palmivora,* at least suggests inoculum can be transported by invertebrates (Evans 1971). For example, tent-building ant species use various substrates in making their tents which can, depending on the species, include soil and debris from diseased pods. Insects including Coleoptera and Diptera species feed on disease pods and may vector BPR and, since these insects can fly, may serve

as long-distance vectors of the disease. The importance of invertebrates in the movement of *P. megakarya* inoculum needs further study considering it is still in the invasive stage in West Africa.

A significant contrast seems to exist between conditions required for zoospore production in the laboratory and zoospore production in the field. The cold temperatures used in optimizing *P. megakarya* zoospore production in the laboratory are never found in the field. This contrast is apparently due to the need in the laboratory to optimize zoospore production in time (all released at the same time in the lab versus continued release over hours, days, and months in the field) and space (concentrated production on a petri dish surface versus an entire three-dimensional field). The temperature optimums for *P. megakarya* have long been established and the differences between *P. megakarya* and *P. palmivora* likely contribute to their lifestyles. *P. megakarya* has the lower temperature optimum (24–26 °C) and maximum (29–30 °C). Although cooler temperatures are generally considered more conducive to BPR, information on the influence of temperature influencing the initiation of BPR epidemics by *P. megakarya* is limited. Ndoumbe-Nkeng et al. (2009) studied epidemics of *P. megakarya*-incited BPR at three locations and over 3 years in Cameroon. Over this time period and in these areas, the minimum air temperature under cacao trees was 17.3 °C and the maximum was 30.2 °C. The mean temperature varied between 19 °C and 26 °C and, although not always optimal, was within the growing conditions required for *P. megakarya*. Under these conditions it was rainfall that was critical to the development of epidemics and not temperature. These results were similar to those of Deberdt et al. (2008), also in Cameroon. *P. megakarya* is obviously adapted to many of the conditions under which cacao is highly productive; this attribute undoubtedly contributes to the severity of BPR it causes (Ndoumbe-Nkeng et al. 2009).

Phytophthora species vary in the conditions required for growth in the field, and small changes in the required conditions for sporulation in soil or on roots, stems, and pods could have a tremendous effect on inoculum loads and therefore disease epidemiology. High humidity is all that is required for sporangia production on pods; free water, typically from rain, is required for zoospore release and movement within the canopy. For zoospore release, after rain the conditions on wet surfaces are essentially microenvironments mimicking a flooded state, somewhat similar to that which occurs in soil after rainfall, or even during axenic culture methods used for zoospore production in the laboratory. As with soil moisture, more details of the exact humidity conditions required for sporangia are needed. The interactions between *Phytophthora* spp., soil, and soil moisture are complex. Sanogo and Ji (2013) reviewed the interactions between *P. capsici* and soil moisture. At least for *P. capsici*, sporangia are formed within 48 h in moist soil if other conditions are favorable. The influence of soil matric potential, an important component of soil water potential, on sporangium formation and zoospore release has been examined for some *Phytophthora* species. There are indications that sporangia formation benefits from fluctuating soil moisture levels, that is, low soil moisture (lower matric potentials) shifted to high moisture (higher matric potential) (Bernhardt and Grogan 1982). The limiting matric potential for *P. palmivora* was around

−10 bars when *P. palmivora* biomass was buried in soil, although optimum conditions for sporangia formation were around −5 mbars when biomass was on the soil surface and −10 mbars when biomass was buried in soil (Gisi et al. 1980). More research needs to be carried out looking at the conditions required for sporangia production by *P. megakarya* in soil and on roots.

Rainfall patterns in cacao-growing areas can be complex and variable between years. The study by Ndoumbe-Nkeng et al. (2009) included both bimodal (2 rainy seasons and 2 dry seasons) and monomodal (1 long hot rainy season and a short dry season) patterns. The minimum rainfall for cacao production was suggested at 1000–1200 mm, while the optimum rainfall was suggested at 1100–2000 mm. BPR was more severe with the monomodal rainfall pattern. Clearly details of rainfall patterns over time and in individual seasons will strongly influence the development of BPR. The importance of rainfall relative to BPR development is also highlighted in the study by Efombagn et al. (2004). In Cameroon, BPR due to *P. megakarya* on Clone UPA 134 varied from 13.1 % to 79.7 % over 3 years (1999–2001), principally due to increasing rainfall levels. Clone UPA 134 carries field resistance based on a shortened fruiting cycle. BPR in the susceptible clone SNK 10 varied from 52.7 % to 93.4 % over the same time period. This type of result at the same location makes it easy to see how a cacao clone/genotype might be highly tolerant of disease in one area but highly susceptible to disease in another without any change in the genetics of the host or pathogen.

Since rainfall/free moisture is so important to *P. megakarya* reproduction, the influences of dry season patterns on *P. megakarya* survival in soil and on plant tissues are of great interest. Both *P. palmivora* and *P. megakarya* can survive for extended periods of time on roots and in soil. *P. palmivora* persisted in a fiberglass matrix in the soil, including through the dry season, free of plant tissue for 10 months, while *P. megakarya* persisted for 18 months (Ward and Griffin 1981). Not only did *P. megakarya* survive longer in soil, but *P. megakarya* was also recovered more frequently than *P. palmivora*. Survival is likely dependent on the exact combination of conditions at any point in time. Once cacao is planted, *Phytophthora* populations increase in soil (Chee 1973; Ward and Griffin 1981) and are moderated by available moisture. Factors such as competition with other organisms and stress on the organism due to environment almost certainly will vary depending on soil moisture levels. The recovery of *P. megakarya* increased during the rainy season, suggesting the inoculum was being renewed.

Both *P. palmivora* (Turner and Asomaning 1962; in Ghana) and *P. megakarya* (Ward and Griffin 1981; in Nigeria) can be isolated from living cacao roots and soil. In fact both species can be isolated from the roots of other plant species cultivated with cacao (Akrofi et al. 2015), so why is the emphasis given to the soil phase for *P. megakarya* over *P. palmivora* when its impact is considered in epidemiology? Appiah et al. (2004b) carried out a study of canker on cacao caused by *Phytophthora* in two areas: one area only had *P. palmivora* while the other also had *P. megakarya*. In Ghana, *P. megakarya* was frequently isolated from flower cushions (Appiah et al. 2004b), showing that *P. megakarya* readily causes stem canker on cacao contrary to previous held views that *P. megakarya* is less able to

infect woody tissue (Gregory and Maddison 1981; Maddison and Griffin 1981). Cankers caused by *P. megakarya* were much more likely to occur near the soil line than cankers caused by *P. palmivora*, suggesting the soil is the primary source of inoculum for *P. megakarya*. At the same time, *P. palmivora* was more commonly isolated from cankers sourced from flower cushions.

In both Nigeria and Cameroon, *P. megakarya* has displaced *P. palmivora* as a significant causal agent of BPR. Why should this be the case? Both colonize roots and can survive in the soil for at least 10 months including a dry season. *P. palmivora*, although surviving in soil, does not appear to effectively infect pods from the soil. Dakwa (1974) observed that pods on or embedded in soil are often not infected by *P. palmivora*, whereas pods in contact with soil consistently become infected with BPR in *P. megakarya* areas. Both species sporulate heavily on infected pods although *P. megakarya* has the advantage in sporulating earlier and heavier (Akrofi et al. 2015). *P. megakarya* is more aggressive in general than *P. palmivora,* as demonstrated in several studies when compared side by side. The larger inoculum loads and greater virulence should insure that more pods are infected with *P. megakarya* each disease cycle, and a significant soil load contributing to new infection centers throughout the season would make sure this continues. *P. megakarya* is still expanding into new regions and fields within Africa. It remains to be seen if *P. megakarya* will continue to be favored to the virtual exclusion of *P. palmivora* in cacao as has occurred in Cameroon and Nigeria. Isolates of *P. palmivora* vary in their virulence and aggressiveness and, to that point, maintaining quarantine of *P. palmivora* as well as *P. megakarya* seems warranted. Will more aggressive *P. palmivora* isolates emerge under the severe selection pressure being placed on it in Africa? We have to wonder if such a severe selection pressure imposed by related pathogens should be expected to give results different from the imposition of severe host restrictions, as observed when resistant materials are misused.

So the life cycle of *P. megakarya* appears to follow that suggested by Gregory et al. (1984): that *P. megakarya* infects roots of cacao and other tree species with little or no disease consequence. Under high moisture conditions (temperatures typically appropriate), sporangia form from root infections and release zoospores. The zoospores actively migrate toward the soil surface (where they might also reinfect tree roots) and rainfall droplets splash the zoospores up and onto the cacao tree, with the pods being of principal importance. The height of splash can reach 70 cm, although aerosols can reach much higher. Starting around 2 days after infection, multiple translucent spots are seen, followed by production of sporangia on the pod surface starting 3 days after first symptoms appear (Akrofi 2015). These new zoospores/sporangia may be moved to secondary infection sites to continue the epidemic. In the absence of a dry period and with continued fruit presence, the epidemic could proceed unabated. Cankers on the trunk/stem or flower cushions could also serve as sources of primary inoculums.

8.10 Disease Management

Plant Density, Shade Trees, and Shade Management

It would seem that some of the most important decisions in managing BPR as well as other cacao diseases should be made prior to planting. Cacao being a long-lived perennial tree, the most basic decisions include what cacao germplasm is to be used, the number and positioning of trees within a field, shade trees and shade intensity in the field, and the height of cacao trees to be maintained. Although acquiring trees tolerant to disease is considered an optimal solution, these additional agronomic practices can contribute to long-term disease management in anticipation of changes in the pathogen and environment that might intensify disease pressure.

Tree spacing in Africa is typically 2.5 by 2.5 m to 3.0 by 3.0 m, although these patterns are severely disrupted by field limitations and the inter-planting of shade and other agronomic trees. As might be expected, spreading trees out can reduce disease incidence but at a significant cost to potential yield, thus the recommended tree spacing. Tree spacing, both cacao and other species, has a direct influence on shade level. Cacao can be grown without shade, which can reduce losses to BPR. In general less shade and more airflow through a field aids in drying, and humidity is reduced, reducing BPR losses. Growing cacao without shade trees can maximize yields (Cunningham and Arnold 1962), but there are significant problems with this practice. Trees grown without shade tend to lose productivity earlier than trees grown with shade. This may have more to do with the elevated productivity in full sun reducing soil fertility than the actual benefit of shade. Beneficial endophytes are considered reduced in full sun and, in fact, many may come from shade trees (Arnold and Herre 2003). And last and perhaps more important, insect pest and vectors of disease are more severe under conditions of full sun whereas shade reduces these pests. As a result, a moderate level of shade is often recommended. In Ghana, 15 to 18 shade trees/Ha are recommended. Unfortunately, in a farmer survey (Akrofi and Opoku 2003) in Ghana, it was observed that farmers tended to ignore recommendations and actually over planted shade trees averaging 39 to 42 trees/Ha, depending on the plot. On the other hand, farmers did a good job of planting the recommended number of cacao trees/Ha needed for optimum yields. Unfortunately none of the 22 farmers surveyed did anything to modify shade levels as recommended. Ten Hoopen et al. (2011) attributed locally higher BPR levels within a Cameroon field study to high relative humidity in a low heavily shaded part of the plantation bearing a small stream. Sanusi and Oloyede (2007) found that BPR incidence was reduced 4.01 % and yields increased 45.3 % after shade trees were removed.

In Africa, tree height ranges from 4 to 10 m, but the commonly used pneumatic knapsack sprayers reach only to a height of 3.5 m (extension lances could be used) from the ground (Opoku et al. 2007). At the two field sites used by Opoku et al. (2007), tree heights ranged between 7.5 to 12.8 m with means of 8.4 and 8.9 m. Fungicide sprays did reduce disease at all heights but generally to a lesser

extent in the canopy. More clearly stated, effective fungicide sprays reduced a greater percentage of the pods lost to *Phytophthora* infection on the tree trunk than they did in the canopy (Opoku et al. 2007). Disease in the canopy tended to benefit from reduced disease at lower levels, which can be a source of inoculum at higher levels. Keeping tree height within range of management, efforts like fungicide sprays, shade management, and sanitation/diseased pod removal can be important. It seems though that farmers have a hard time justifying the cost of the required labor, and in any event often look at pods in the canopy as potential yield regardless of their disease potential.

Symptoms of Disease and Evaluating Clone Tolerance

In a ring test involving 32 cacao clones and 10 countries, Cilas (2006) found a significant clone by country effect on resistance rankings in a leaf disk assay. Some of the clone by country effect was attributed to the isolate/species of *Phytophthora* being used in specific laboratories, but other additional laboratory practice issues were suggested. Also, questions arose concerning the exact identity of cacao "clones" used in different countries. Cacao planting material is often misidentified/mislabeled and should always be genotyped before studies are initiated. Clearly screening methods need to be standardized between research groups if research results are to be compared.

Although *P. megakarya* can attack and cause disease on all cacao plant parts if conditions are right, it is the disease on pods (BPR) followed by disease on the trunk/branches (canker) that are of primary concern (Fig. 8.3). When considering the interaction between plant and pathogen it is important to first understand the anatomy/structure of the tissues involved. The cacao pod is a complex structure consisting of the husk, seed cavity, and seed. The pod husk (pericarp) consists of several layers including the epidermal layer covered with wax and composed of stomata, glandular trichomes, glands, and parenchymal cells. Underneath the epidermis, the epicarp is 25 to 58 cells thick, which includes parenchymatous cells, vascular bundles, and large mucilage cavities. Next, is the mesocarp, which is relatively thin and lignified, adding hardness to the pericarp. Lignification of the meristematic band takes place toward the end of the transition period from the wilting to the non-wilting phase. The mesocarp includes a meristematic band. Below the mesocarp lies the endoderm layer, which browns quickly after exposure due the polyphenol oxidase system. An observation which may be of importance in the interpretation of the biochemical changes occurring in the developing pods is that the polyphenol oxidase activity at the cut surface of the inner pericarp diminishes as the pods get older. Inside the periderm, in the loculus or seed cavity (Nichols 1964) is the integument, providing nutrients to the developing seed. The seed are enveloped in what will become a sweet tasting pulp and nutritive matrix (Cheesman 1927). There are three connected vascular systems in the pod (Nichols

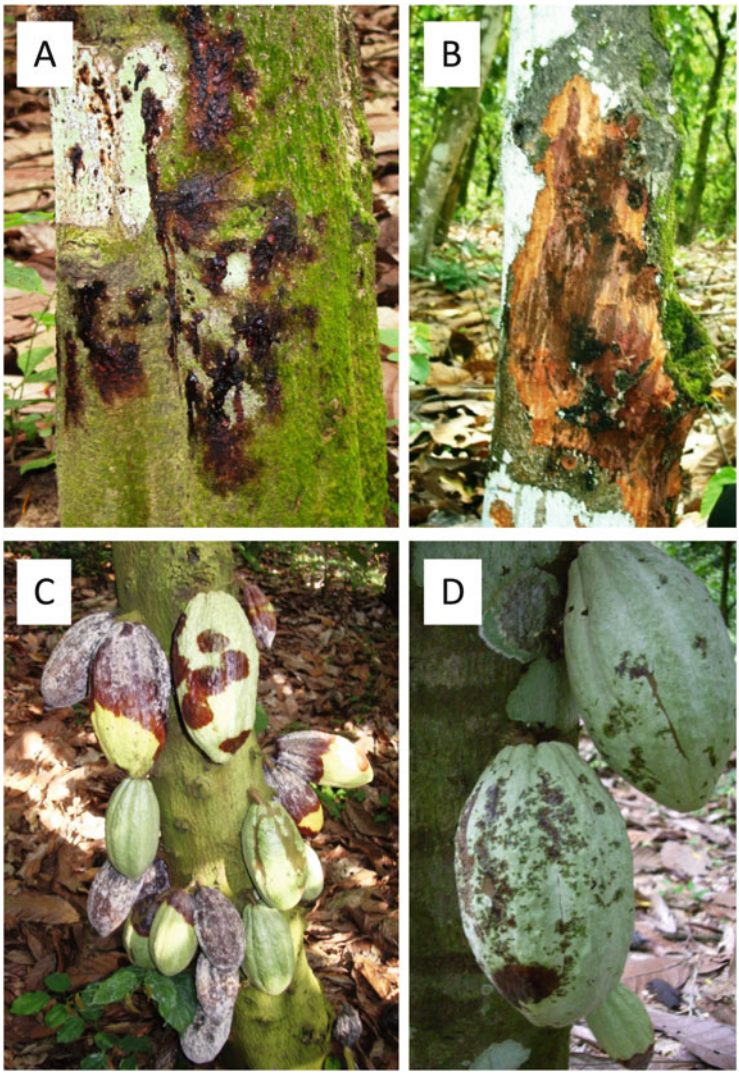

Fig. 8.3 Symptoms on cacao caused by *Phytophthora megakarya*. (**A**) Gummosis associated with *Phytophthora*-induced canker on cacao trunk. (**B**) Discoloration of woody tissues caused by *Phytophthora* after bark has been scraped away from a *Phytophthora*-induced canker. (**C**) Black pod rot of cacao caused by *P. megakarya*. Note the *powdery gray surface* associated with sporangia production uniformly spread of the entire necrotic surface of larger lesions. (**D**) Early symptoms of infection by *P. megakarya* on cacao pods. Note the multiple minute necrotic infection sites often associated with *P. megakarya* infections

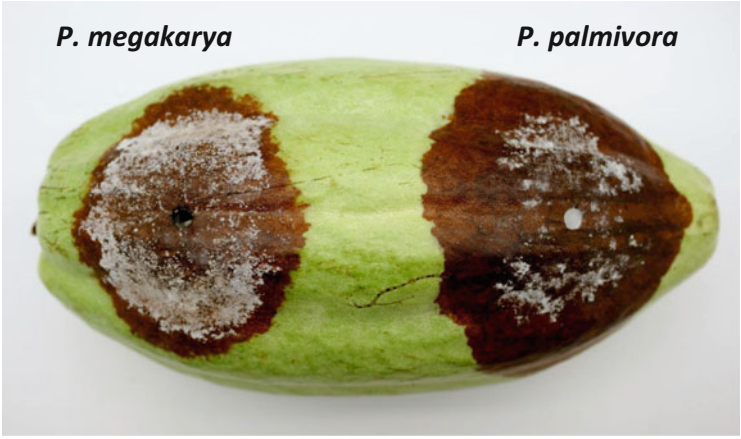

Fig. 8.4 Difference in black pod rot lesions on pods caused by *P. megakarya* and *P. palmivora* under similar conditions

1961): five bundles in the center of the seeds and pith, a middle ring of five bundles in the inner pericarp, and outer bundles next to the inside of the meristematic band.

P. palmivora lesions on pods were described as having "a sharp well-defined leading edge and only sparse fungal growth on the surface" (Brasier and Griffin 1979). Pod lesions produced by *P. megakarya* (Fig. 8.4) were described as having "a more diffuse leading edge and considerably more fungal growth on the surface often extending to within a few mm of the lesion edge" (Brasier and Griffin 1979). Lesions from *P. palmivora* tend to expand at a faster rate than those of *P. megakarya* when infections are through wounds (Brasier and Griffin 1979). This observation strengthens the ties between the N–G type isolates described by Turner (1960) and the L–S type isolates of Brasier and Griffin (1979), where it was also noted that lesions of the G-type (presumably *P. palmivora*) isolates expanded faster than those of the N-type (presumably *P. megakarya*) isolates. This seems somewhat counterintuitive since *P. megakarya* is the more aggressive of the 2 species in the field. Note *Phytophthora* species are hemibiotrophs with a short biotrophic phase (intercellular mycelia and haustoria invaginating the plant cell protoplast) followed by an extended necrotrophic phase. Wounding, whether using mycelia or zoospores for inoculums, may bypass/alter the biotrophic phase. Although *P. palmivora* seems to have an advantage when invading cacao pods through wounds and the necrotrophic phase, *P. megakarya* must have other advantages, either in the biotrophic interactions, perhaps a function of its effector pools (Stassen and Van den Ackerveken 2011), or in post-necrotrophic aspects such as sporulation timing and intensity or survivability when separated from cacao. Post-penetration tolerance is an easily quantifiable reaction (lesion size and spread). There is much less likelihood the *Phytophthora* infection will fail due to aspects outside the direct plant–pathogen interaction than when studying penetration resistance. This is undoubtedly due to the consistent wound environment which buffers

moisture and nutrient requirements. Also, whereas penetration resistance is ultimately determined over a short time period (it takes a few hours for *P. megakarya* to penetrate cacao tissues), post-penetration resistance can be evaluated over several days and benefits from compounding reactions. That is to say, the difference between slow spreaders and fast spreaders potentially increases over time. Studies by Akrofi and Opoku (2003) on *Phytophthora* invasion of cacao pods and beans determined the presence of *Phytophthora* in naturally and artificially infected cacao pod husk, bean, seed coat, embryo, and placenta by plating on *Phytophthora*-selective media. Results from that study showed that isolation of *Phytophthora* from these pod parts increased with the size of the lesion on pod surface and depth of penetration and that proximal infections resulted in earlier infection of beans than lateral or distal infections. Akrofi and Opoku (2003) and Awuah and Frimpong (2007) separately studied seed transmission of *Phytophthora* by artificially inoculating healthy beans from apparently healthy pods with *Phytophthora* inoculum and biopsying the fungus from the emerging seedlings. The results from both studies did not confirm seed transmission of *Phytophthora* reported by Kumi et al. (1996), but showed that the pathogen can be seed borne. The seed-borne nature of the pathogen may explain the long-distance spread of the pathogen on apparently healthy pods.

For penetration resistance to be studied, the inoculum and plant material must first be synchronized. Consistent inoculum (typically zoospores) of similarly aggressive isolates, and with similar concentrations and viability, must be produced so that results are comparable between experiments, laboratories, and on different germplasm sources. For any study, pods of similar ages (not difficult) or leaves at similar stages must be identified. The staging of leaves is particularly difficult since the developing leaf changes almost hourly from day to night and from immature (soft supple often red tented leaves depending on the clone) to mature (rigid highly lignified leaves) over a few days, and mature leaves can last on the tree for up to a year. These differences in leaf developmental stages can be significantly modified by the environment (light and humidity), nutrient status of the tree, and the endophyte population established on the leaf. It is the leaf assay, as used to measure resistance to *P. megakarya* and its correlation to pod resistance, that has been the topic of much discussion in the cacao research community. Obviously, we have presented several results that show good correlations between pod and leaf reactions to *P. megakarya*, and in fact field level tolerance. There may be a bias in the literature on this subject since publishing negative results is generally difficult. Iwaro et al. (1997a) found that, although leaf and pod resistance at the post-penetration levels were highly correlated, they were not correlated between clones at the penetration level. It was noted that stomatal frequencies were not highly correlated between leaves and pods, possibly explaining the disconnect when measuring penetration resistance. Stomatal development is incomplete in young leaves (deAlmeida and Valle 2007) and information on stomatal development in pods is difficult to obtain from the literature. The positive results seem to center around components shared by leaves and pods, most notably phenol reactions, lignifications levels, and wax layers. In the absence of any proven tissue specificity for these reactions, these are aspects of resistance that might be expected to show

consistent reactions. There may be an issue identifying aspects of resistance that are tissue specific (presently undefined), or if not related to these structural aspects of cacao tolerance, to infection (also undefined). Regardless, it seems progress might be made in the characterization of these demonstrated defense reactions so that uncontrolled variation might be detailed and limited. Proper and consistent training in the conditions used for bioassays across the cacao community would also be valuable in optimizing breeding efforts for black pod resistance as well as other diseases. An added issue for penetration resistance is the often common occurrence of divergent reactions. The same concentration of zoospores and the same pod tissue can give divergent reactions, from no infection to no inhibition and rapid spread. These divergent reactions may be influenced by the factors described above but also may be under genetic control. Simply put, having failed to reach a threshold, infection might be stopped, whereas having just exceeded a threshold, infection might be totally uninhibited. Having succeeded in overcoming penetration resistance, post-penetration resistance then comes into play which further complicates interpretation of the results. These issues are usually overcome by extensive sampling and the use of averages which must be carefully considered when results are compared.

For *P. palmivora*, lesion establishment (lesion frequency) and stomatal frequency and pore length are correlated relative to penetration resistance (Iwaro et al. 1997b). Essentially, larger pores contribute to susceptibility to *P. palmivora*. It is unclear if the same can be said for *P. megakarya*. Our own studies demonstrate (Ali et al., 2016) that *P. megakarya* zoospores also infect directly through appresoria (Fig. 8.2C). This ability might negate or limit the importance of stomatal frequency when studying resistance to *P. megakarya*.

Several studies have indicated that resistance in cacao to one *Phytophthora* species contributes resistance to all *Phytophthora* species. SCA6 is tolerant of *P. palmivora* and also contributes to tolerance against *P. megakarya*. Iwaro et al. (1997b) found that Sca6 (most resistant) and ICS1 were tolerant of *P. palmivora* (penetration resistance) and SCA6 and ICS95 were tolerant of *P. palmivor*a (post-penetration resistance). Post-penetration resistance could not be linked to pod morphological/physical traits and was attributed to "biochemical factors." Nyadanu et al. (2012) found that clones/genotypes did not change their tolerance ranking when either *P. megakarya* or *P. palmivora* was used in the evaluations. Cacao genotypes T60/887, Pa150, Sca6, and Pa7/808 were recommended as good general combiners for pod lesion number, pod lesion size, leaf disk score, and natural field infection to be incorporated into recurrent selection breeding programs. Here again SCA6 was a top performer regardless of the screening method used (leaf disk, detached pod, or field evaluation). Although the ranking of genotypes did not change between *Phytophthora* species, *P. megakarya* was always the more aggressive isolate regardless of the screen used. The limitation to this study is that only one isolate of each pathogen was used. It is unclear if more aggressive isolates might disrupt the relationship of resistance to one equaling resistance to both. In a similar study involving *P. palmivora* and *P. capsici*, Iwaro et al. (1998) found that cacao genotypes ranked

similarly regardless of species in a study of post-penetration resistance. At least for *P. palmivora*, the isolate used did not alter the ranking of genotypes relative to their tolerance, although the level of tolerance indicated did depend on isolate aggressiveness/virulence. Iwaro et al. (1998) found, as might be expected, that standardizing pod age and inoculation depth is important for obtaining consistent results.

Once/if lesions occur after zoospore inoculations, they appear to proceed at a rate similar/equivalent to that occurring with wounding. The critical difference appears to be the initial establishment of the short biotrophic phase of the infections cycle. Under our conditions it takes approximately 3 days for macro-symptoms to develop following zoospore inoculations of whole pods or pod pieces. Soh et al. (2013) found that fruits at the cherelle stage (less than 5 weeks old and up to 4 cm in length) were the most susceptible, after which pods shared similar levels of susceptibility as they developed. The orientation of the fruits on the tree did not influence their susceptibility. The practical impact of pod age at time of infection is that the younger the pod is, the more likely disease will advance to a stage where the pod is completely destroyed. The beans can often be rescued when pods are infected near maturity although the quality may suffer.

Fungicides

As with most plant disease interactions, fungicides are a major component of successful management programs for BPR. Due to the more severe losses caused by *P. megakarya*, fungicides are particularly important in its management. Fungicides incorporating copper compounds continue, as they have been from the beginning, to be used for managing BPR. As early as 1936, the Bordeaux mixture was recognized as a potential fungicide for managing BPR (West 1936); Thorold (1959) described the use of Peronox (50 % cuprous oxide) applied at 3-week intervals or 1 % carbide Bordeaux (a combination of copper sulfate and calcium carbide) at 3- or 6-week intervals to good effect. Losses were 61.6 % to BPR in the control plots and only 26.5 % or less in the fungicide-treated plots. Copper oxide and hydroxide fungicides were effective in reducing losses to BPR in Nigeria in studies from 1959 to 1961 that likely targeted *P. megakarya* (Hislop 1963; Hislop and Park 1962). In the 1963 report Hislop describes the "rubber"-type isolates of *P. palmivora* based on their morphology and notes the severity of disease with losses as high as 80 %. A significant issue with copper fungicides is that they act on the tissue surface and can easily be washed off by rains, a major factor in BPR disease development. Moreover, the continuous application of copper in agriculture is being questioned because of the ability of soils and plants to accumulate copper residues. For instance, the use of copper-based fungicides in treating BPR was reported to be mainly responsible for copper contamination in some cacao plantations in Nigeria (Aikpokpodion et al. 2010) and for copper contamination in cacao leaves, cacao beans, and soils in the Ahafo Ano North District of Ghana (Addo-Fordjour et al. 2013). The continuous accumulation of copper in soils could affect

soil fertility, while runoff from these soils could also introduce copper into nearby water bodies with serious ecological consequences. Metalaxyl, a systemic fungicide, was described as a treatment for *Phytophthora*-incited disease in 1977 (Ferrin and Mellinger 1977; Urech et al. 1977) and was used on cacao as early as 1981 (Djiekpor et al. 1981). BPR losses due to *P. megakarya* were reduced from 80 % to 3 % when treated with Ridomil 25 (metalaxyl) at 0.2 % at 3-week intervals. By 1980, the heavy use of metalaxyl in Ireland resulted in the emergence of resistant *P. infestans* strains that caused severe losses in potato. In order to reduce the potential for selecting for resistance in the field metalaxyl formulations incorporating copper were developed and tested against BPR (McGregor 1984). Fungicide residues are always of concern, especially with systemics like metalaxyl. As a result, mefenoxam was developed. Metalaxyl [methyl *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-DL-alaninate] is a racemic mixture of *R*- and *S*-enantiomers, while mefenoxam is the *R*-enantiomer of metalaxyl. Mefenoxam is effective at half the rate of metalaxyl. A list of the 2014 Ghana Cocoa Board approved fungicides is provided in Table 8.1. Note mefenoxam is recommended at half the rate of metalaxyl and these compounds are always mixed with copper. Although fungicides can be effective in managing BPR, the study by Sonwa et al. (2008) in Cameroon demonstrates some of the problems farmers face in using fungicides. In the absence of subsidies, over 50 % of farmers were cited as using chemical fungicides. Major limitations for farmers are cost and availability. As a result, farmers fail to apply chemicals at the recommended rate and frequency and the benefits promised based on research results are not obtained. Safety in the transportation, storage, handling, and application of fungicides continues to be an issue.

Pod Removal

It is logical to consider the removal of *P. megakarya*-infected pods from the field as soon as possible considering their potential as a source of secondary inoculum. *P. megakarya* is particularly known among *Phytophthora* species causing BPR for its propensity to rapidly and extensively sporulate on the surfaces of infected pods. Typically initial signs of what will be expanding black lesions appear 2–3 days after infection, and sporulation immediately begins 3–5 days after these first symptoms, forming as a confluent lawn of sporangia producing surface mycelia. Each infected pod can produce significant numbers of sporangia, estimated at 4 million each (Brasier et al. 1981) and, at least for *P. palmivora* (Dennis and Konam 1993), serve as a source of inoculum for years after they have mummified. The role of mummified pods in *P. megakarya* epidemics has received little attention (Akrofi 2015) and needs further study.

Yet Gregory et al. (1984) suggest "any return cycle of sporangia, zoospores, and old husk which add inoculum to the litter layer was of secondary importance compared with the active cycle on the roots." Essentially the argument is that more than enough inoculum exists in the soil for continued reintroduction to the

Table 8.1 Details of COCOBOD approved fungicides for use on cacao in Ghana as at 30th October, 2014

Fungicides				
Product	Active ingredient(s), Formulation type[a]	Dosage (g or mL) per spraying tank of 15 L	No. of sachets/ spraying tank	Maximum interval between sprays (weeks)
Kocide 2000 DF	53.8 % Copper (II) hydroxide, (35 % copper), DF	100 g	1	3–4
Funguran-OH	77 % Copper (II) hydroxide (50 % copper) WP	100 g	1	3–4
Champion WP	77 % Copper (II) hydroxide, (50 % Copper) WP	100 g	1	3–4
Fungikill 50WP	15 % metalaxyl + 35 % Copper (II) hydroxide, WP	75 g	1	3–4
Ridomil Gold 66 WP	6 % mefenoxam + 60 % Cupper (I) oxide, WP	50 g	1	3–4
Copper Nordox	86 % Copper (I) oxide, (75 % Copper) WP	75 g	1	3–4
Metalm 72WP	12 % metalaxyl + 60 % copper (1) oxide, WP	50	1	3–4
Sidalco Defender	Dicopper chloride trihydroxide, SC	100 ml	100 ml	3–4
Fantic	60 % Copper + 9 % Benalaxyl-M, WP	75 ml	75 ml	3–4
Metacide	12 % metalaxyl + 60 % Cupper (I) oxide, WP	50 g	1	3–4
Agro Commet 72WP	60 % Copper-1-oxide + 12 % Metalaxyl, WP	50 g	1	3–4
Kentan 40WG	40 % Copper (II) hydroxide, WG	100 g	1	3–4

[a]Formulation type: *DF* dry flowable, *WP* wettable powder, *SC* suspension concentrate, *WG* wettable granules

canopy to initiate and maintain an epidemic. Weekly removal of infected pods has been shown to be economically justified when managing frosty pod rot, witches' broom, and BPR in Peru (Soberanis et al. 1999). Yet in *P. megakarya* areas of Ghana (12 farms in the Hohoe district of the Volta Region and on 10 farms in the Bechem district of the Brong Ahafo Region), farmers surveyed between 1995 and

1997 did not even practice the removal of disease pods between growing seasons as recommended (Akrofi and Opoku 2003), principally due to the added labor cost. Yet Ndoumbe-Nkeng et al. (2004) demonstrated the weekly removal of diseased pods effectively reduced the black pod incidence by 9–11 % to 22–31 %, depending on the year. Although pods were eliminated/limited as sources of secondary inoculum throughout the season, under the conditions in Cameroon the epidemic intensified with the second rainy season suggesting other inoculums source were important, likely the soil as suggested by Gregory et al. (1984). Ward and Griffin (1981) found that soil populations of *Phytophthora* remained "abundant" 4 years after pods were removed as a source of re-inoculation. Even with pod removal BPR losses averaged between 41 and 69 %, demonstrating pod removal should be incorporated into broader disease management programs. Opoku et al. (2007) demonstrated exactly that. Sanitation practices, principally diseased pod removal, maintained net returns to the farmer, while reducing the number of fungicide (Ridomil 72 plus) applications required in a season from 6 to two or three.

Canker Treatment

Cankers can develop on the tree trunk and branches (more common on branches) and are often attributed to infections proceeding through the peduncle of infected pods and establishment in associated flower cushions (Appiah et al. 2004a). This appears to be more prevalent with *P. palmivora* but is common with *P. megakarya* also. *P. megakarya* is more likely to cause cankers on the main trunk at or near the soil line. The first signs of canker (Fig. 8.3) are well described by Akrofi (2015) as "a grayish brown or reddish-brown water-soaked lesion with dark brown to black margins on the bark; and exudation of reddish-brown resinous liquid (bleeding canker), usually through cracks in the bark." If the surface of the canker is scraped off, a spreading scarlet coloration can often be seen. The diseased area is confined to the bark and cambial layer. Expanding lesions can girdle the tree/branch and eventually kill the tissues above the canker site. Cankers are treated by scraping back the bark. The scraped surface can then be treated with fungicide (McMahon and Purwantara 2004). In addition to cankers initiated on flower cushions through diseased pods, essentially anything that causes a wound to the bark is likely to support canker development if inoculum is available and conditions are right.

8.11 A Way Forward

The genome sequences for both *P. megakarya* and *P. palmivora* should soon be available for general use by the research community (Bryan A. Bailey, personal communication). Although the web site has been established through GenBank (http://plantpathology.ba.ars.usda.gov/phytopthora/spcl/main.html), it is not due to

be made available until peer review is completed. Detailed analysis of the genomes and transcriptomes of the pathogen should allow us to greatly improve our understanding of the *Phytophthora*/cacao interactions and the interactions between *Phytophthora* and the environment.

The development of simple tools for determining genetic variation in the pathogen's population (for example, SNPs or direct sequencing) should allow us to understand factors influencing pathogen genetic diversity and pathogen epidemiology and contribute to the development and stable deployment of genetic based tolerance in cacao.

It should be possible to develop a direct PCR technique for assessing pathogen diversity from environmental samples, including infected pods, without obtaining pure cultures. This would eliminate the cumbersome task of obtaining and maintaining pathogen cultures.

By direct analysis of the pathogen transcriptomes and comparisons to other better studied species, it should be possible to identify the genetic components contributing to aggressiveness and virulence of *Phytophthora* against cacao.

The standardization of pathogenicity screens (leaves and pods, attached and detached) should improve the transference of research results between laboratories, countries, and pathogen species.

A series of verified standard clones (verified by SNPs) for screening pathogen isolates and identifying tolerant clones should be developed and shared between research groups and countries. If, as the data suggest, tolerance is shared between clones of *Phytophthora* species in general, this could greatly accelerate the identification and development of tolerant planting materials. This will require international agreement on identical materials which will need to pass through proper quarantine. By verifying the genetic identity of standard clones, cases where tolerance to a species might fail can be identified.

More detail is needed in understanding the variation in aggressiveness/virulence of *P. megakarya* isolates. What are the genetic bases of this variation and what are the consequences of this variation in the pathogens population dynamics and epidemiology.

What is the real potential of *P. megakarya* on other plant species? Does it cause disease on anything other than cacao? It is a threat to other crops?

Long-term demonstration plots should be established where optimum disease/insect management strategies are employed to determine their impact on disease epidemiology. Can you impact pathogen soil populations if management practices are consistently carried out over more than 4 years? What land area allows for optimum efficiency of disease/insect management?

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Part IV
Pathogens of Expanding Concern

Chapter 9
Vascular Streak Dieback (*Ceratobasidium theobromae*): History and Biology

Peter McMahon and Agus Purwantara

Abstract Vascular streak dieback (VSD) caused by the basidiomycete, *Ceratobasidium theobromae* (syn. *Oncobasidium theobromae*, *Thanatephorus theobromae*), is one of the most important diseases of cacao in the Southeast Asian/Melanesian region, causing branch dieback with infections capable of killing seedlings and mature trees of susceptible cacao varieties. The only known host is *Theobroma cacao*, but as a new encounter disease it is apparent that the fungus transferred to cacao from an original host, endemic to the region, which so far remains unidentified. Basidiospores that initiate infection are short-lived and dispersed by wind only for short distances. VSD is patchy in distribution but can be severe locally and, recently in Indonesia, has influenced farmers to convert to crops other than cacao. In Malaysia this disease, among other factors, influenced growers to replace cacao with oil palm. The causal pathogen and disease symptoms were first described in Papua New Guinea following a severe epidemic in the 1960s: a unique wind-dispersed basidiomycete pathogen was identified that infected young leaves of cacao seedlings and mature trees, colonizing the xylem and resulting, after about 3 months, in characteristic symptoms of leaf chlorosis, leaf fall, and branch dieback or seedling mortality. Sporulation was observed to occur on the monilioid hyphae emerging from leaf scars. Quite recently, within the last decade, symptoms of leaf marginal and tip necrosis, associated with a longer period of attachment before leaf abscission, have become dominant, replacing the formerly characteristic chlorotic symptoms. As a consequence of delayed abscission, sporulation now frequently occurs on hyphae emerging through cracks in the petiole or leaf mid-rib. The pathogen associated with the newer symptoms, *C. theobromae*, appears morphologically and genetically identical to the species previously described. However, haplotypes based on ITS sequences have been identified within the region, indicating that some regional genetic variability occurs. Just as in VSD-infected cacao displaying chlorotic symptoms, infections associated with the more recent (necrotic) symptoms spread from initially infected leaves via the

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xylem to neighboring leaves and, in more susceptible genotypes, reach the tip causing dieback. The two sets of symptoms (chlorotic and necrotic) may occur in the same area and even on the same tree, and their relative frequency is influenced by the season. At higher altitudes and in some genotypes the former symptoms of leaf chlorosis are predominant. Disease severity also decreases with altitude. It remains uncertain whether the recent increase in VSD severity in Sulawesi, East Java, and other areas in the region is linked to the new symptoms. Since the incubation period for the disease is long (3 months or more) it is important to keep seedlings in quarantine for about 6 months before distribution for planting in other locations. As *C. theobromae* is a vascular pathogen, control by fungicides is difficult, and although effective systemic triazole fungicides have been identified, they are generally too costly for smallholder farmers. The disease is best controlled by sanitation pruning and the adoption of resistant genotypes. Further work is under way to combine resistance with other characteristics such as superior cacao bean quality and yield.

9.1 Introduction

After Phytophthora pod rot (black pod), vascular streak dieback (VSD) caused by the tulasnelloid basidiomycete *Ceratobasidium theobromae* (syn. *Oncobasidium theobromae*, *Thanatephorus theobromae*) is the most important disease of cacao in the Southeast Asian/Melanesian region, with infections capable of killing seedlings and mature trees of susceptible cacao varieties. On less susceptible cacao the disease causes branch dieback that can severely compromise farm productivity. VSD generally spreads slowly, but in conducive weather conditions or when the causal pathogen infects susceptible cacao genotypes in new cacao plantings, the disease can become very severe locally. To date, the primary "control" of the disease has occurred through selection for resistance, whereby cacao genotypes with partial resistance are propagated, while susceptible types are either killed or removed from planting programs. This has involved a process of natural selection by farmers who, following devastating epidemics of the disease, have had no choice but to propagate from the surviving resistant individuals in genetically diverse cacao populations in the region.

9.2 Pioneers in Cacao Plant Pathology Research: Dr. Philip Keane

Philip J. Keane (Fig. 9.1) has spent most of his career introducing university biology and agriculture students to the stimulating fields of botany, mycology, and plant pathology. But earlier on, following undergraduate studies in agriculture at the Waite Agricultural Research Institute of the University of Adelaide where he specialized in plant pathology, he undertook PhD studies through the University of Papua New Guinea on a serious dieback disease that had devastated the cacao industry in the country since the early 1960s. A complex cause of the disease was suspected, involving infection of weakened stems by *Lasiodiplodia theobromae* (syn. *Botryodiplodia theobromae*) which was regularly isolated from branches suffering tip dieback. However, working at the Lowlands Agricultural Experiment Station at Keravat on the island of New Britain in Papua New Guinea, he realized that the first symptoms of the disease occurred well before the death of branches that were invaded by *Lasiodiplodia*. Keane and his supervisors, Ken Lamb of the University of Papua New Guinea and Noel Flentje of the University of Adelaide, identified *Rhizoctonia*-like mycelium invading xylem vessels of cacao branches and leaves in which leaves had begun to turn yellow and abscise. He later found that the fungus formed corticioid sporocarps when hyphae emerged from infected vascular traces caused by the fall of diseased leaves. The tulasnelloid fungus associated with the disease was named as a new genus and species, *Oncobasidium theobromae*, by Pat Talbot working at the Waite Agricultural Research Institute. The fungus formed sporocarps only during wet weather and sporulated at night after

Fig. 9.1 *Philip Keane* on a cacao smallholding in Sulawesi, Indonesia

being wetted by late afternoon rains. Later, he showed that the fungus was confined to the xylem and that young leaves of cacao seedlings could be infected with spores shed from sporocarps collected from natural infections in the field, resulting in symptoms identical to those observed on naturally infected cacao on farms. The fungus did not grow and sporulate in culture. In two key papers, Keane and his supervisors described a new pathogen species that was unique in being a wind-borne, xylem-invading basidiomycete pathogen only known in association with cacao. The disease was given the name vascular streak dieback (VSD) to distinguish it from other dieback conditions in cacao. The same disease was later identified in Malaysia, Indonesia, and several other countries in South East Asia, but has never been reported outside that region. Keane kept up his interest in pests and diseases of cacao, undertaking several consultancies for FAO and leading three projects for the Australian Centre for International Agricultural Research (ACIAR) in Indonesia which focused on field research on pest and diseases (including VSD and Cocoa Pod Borer), their management by smallholder farmers, and capacity building for local research institutions. Based at La Trobe University in Melbourne, Keane has supervised students conducting studies on a wide range of pathogens, from *Mycosphaerella* spp. on eucalypts, *Sclerotinia* on lettuces, and *Ascochyta* sp. on chickpeas to rusts on wheat. Keane has always advocated an integrated approach to pest and disease management to his students and anyone who will listen. Having recently retired from full-time work at La Trobe University, he will take up leadership of an ACIAR project on cacao development in Papua New Guinea, returning to where his career began more than 40 years ago.

9.3 History and Impacts of the Disease

Dieback diseases have long been noted as serious impediments to cacao production (Turner 1967). Dieback is differentiated from diseases such as sudden death or wilts caused by *Phytophthora palmivora* (stem canker), *Phellinus noxius* (and other root rot fungi), or *Verticillium dahliae*, as it is characterized by gradual leaf loss and branch tip death rather than by sudden death of the whole tree. Dieback of cacao is often caused by a complex of environmental factors such as nutrient stress, lack of shade (particularly in areas with a significant dry season), and insect damage, which provide conducive conditions for the invasion of facultative pathogens such as *Lasiodiplodia* (syn. *Botryodiplodia*) *theobromae*, *Fusarium decemcellulare* (anamorph of *Calonectria rigidiuscula*), and *Colletotrichum* spp. (Holliday 1980). In Papua New Guinea (PNG) in the early 1960s, a serious dieback disease, given the name "Botryodiplodia dieback," was attributed to infection by *Lasiodiplodia theobromae*, which was regularly isolated from diseased specimens (Shaw 1962; Bridgeland et al. 1966). Symptoms of vascular streaking, lenticel swelling, axillary bud growth, and bark sponginess were recorded. However, rather than infection beginning at the branch tip, this dieback was characterized by initial symptoms appearing on leaves behind the tip. Furthermore, unlike dieback occurring under

conditions of stress, this form of dieback disease attacked young healthy (but susceptible) trees. The causal pathogen was later identified as a basidiomycete fungus, which was demonstrated to be an aggressive pathogen that not only readily killed seedlings but also caused severe branch dieback of mature trees and even tree death (Keane et al. 1972). The disease almost destroyed the cacao industry in PNG in the 1960s, which only recovered as the most susceptible varieties died out, leaving cacao genotypes with partial resistance. These genotypes were propagated to allow cacao production to recover to its former levels (see Efron et al. 2002). The dieback disease was given the name "vascular streak dieback". Keane (1972) and Keane et al. (1972) demonstrated that infection was initiated by basidiospores shed onto seedlings from sporophores. Prior (1978) later collected basidiospores from natural sporophores and inoculated these onto healthy seedlings leading to disease development with symptoms identical to those observed in naturally occurring infections of cacao. The causal pathogen of the disease was described and named *Oncobasidium theobromae* (Talbot and Keane)—see below. However, isolation and growth of the fungus in axenic culture were difficult, as it could not be subcultured from the initial hyphae that emerged from infected stems onto agar media.

During the early decades of cacao planting in the 1950s and 1960s, Malaysia experienced a devastating epidemic of VSD, similar to the one in PNG (see Keane and Turner 1971; Keane 1981). Intensive hybrid screening programs that selected progeny initially for resistance to VSD, followed by performance testing for resistance against other pests and diseases and for yield and quality, led to the introduction of a number of resistant, high yielding clones now widely used in both Malaysia and Indonesia. In Malaysia, disease symptoms and the characteristics of the fungus isolated from diseased specimens were identical to those determined in PNG (Keane and Turner 1971).

While the disease is endemic to most cacao growing areas in the region, the impact of the disease is locally very variable. In addition to epidemics in PNG and Malaysia (see above), West Java, and, more recently, Sulawesi in Indonesia have experienced severe outbreaks of VSD. In contrast to the high impacts of diseases such as black pod (which is pantropical and causes losses of about 400,000 tons annually), VSD is reported as having a moderate impact with annual losses of approximately 30,000 tons, similar to the losses caused by *Moniliophthora roreri* (frosty pod) in South America, which, while extremely severe, also has a restricted distribution (Bowers et al. 2001; Ploetz 2007a). Nevertheless, VSD can cause severe losses locally: in Sulawesi, many farmers are converting their farms to other crops, especially maize, cloves, and oil palm and often refer to recent increases in VSD disease intensity as the main reason for this change. On some government estates in Java, VSD has affected production severely and has resulted in cacao plantings being replaced by sugarcane (Fig. 9.2). The market price of cacao has been high for the last few years (at least 3 USD per kg dry beans, of which Indonesian farmers receive a relatively high proportion), so the turn away from cacao is likely to be a result of factors other than pricing, especially pest/disease

Fig. 9.2 (**a**) Vascular streaking in a VSD-infected branch; (**b**) VSD-infected tree—East Java; (**c**) cacao cut down in preparation for sugar cane planting in East Java. Cacao production is no longer economically viable in some areas due to VSD. (**d**) Chlorosis on infected M01, Sulawesi; and (**e**) sporophore formed on leaf scar on infected stem

impacts (with VSD being a primary concern) and the high labor requirement of cacao production.

9.4 Taxonomy and Nomenclature

The causal pathogen of VSD was first described by Talbot and Keane (1971) as a Tulasnellales (now Cantharellales) species of Basidiomycotina in the family Ceratobasidiaceae. The ellipsoid basidiospores produced by adherent, corticioid

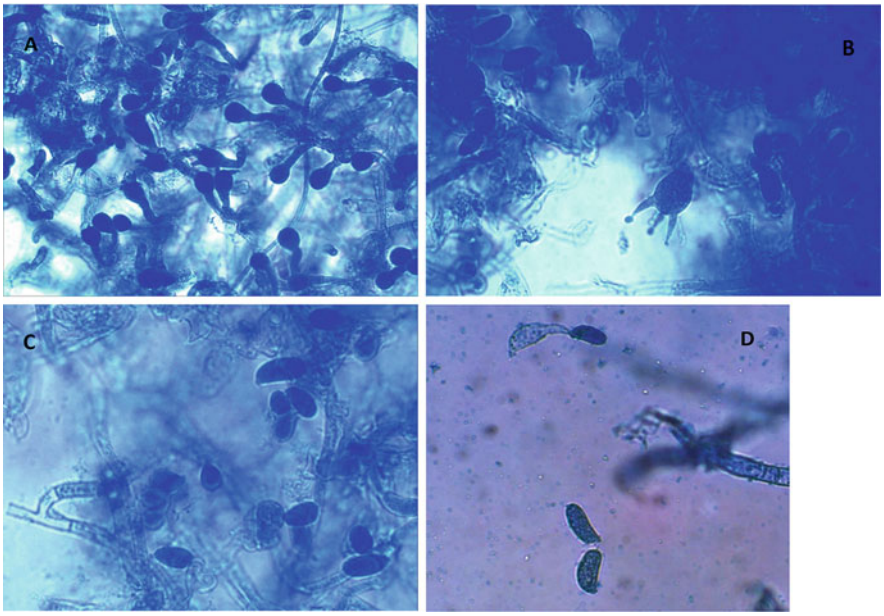

Fig. 9.3 (**a**) and (**b**) Basidia with spores forming on sterigmata; (**c**) loose spores; and (**d**) repetitive germination of *C. theobromae* basidiospores

sporophores are large (15–25 μm × 6.5–8.5 μm), hyaline, smooth, and thin walled and are approximately twice the length of the conically shaped sterigmata. Basidia are holobasidiate and swollen at the apex. Repetitive germination of the spores occurs (Fig. 9.3). The hyphal cells are binucleate, a characteristic of the genus *Ceratobasidium*, although the importance of this characteristic for taxonomical purposes has been questioned by Oberwinkler et al. (2013) (see below). The hyphal growth form is *Rhizoctonia*-like with aerial hyphae approximately 5 μm in width and basal hyphae up to 10 μm wide and dolipore septa near the junctions; clamp connections are absent. Anastomosis is not generally observed. Similarities in basidial morphology were found to those of the tropical web blight pathogen, *C. noxium* (syn. *Koleroga noxia*). However, Talbot and Keane (1971) reported that the fruit body is similar to that of *Thanatephorus* species, with ascending hyphae rather than having the web-like structure of some *Ceratobasidium* species, including *C. noxium*. Based on the growth habit of the fungus as a unique, xylem vessel-invading pathogen, and on the aerial dispersal of the spores (a different habit from other vascular pathogens which are soil-borne), a new genus was erected and the fungus given the name *Oncobasidium theobromae* (Talbot and Keane), inspired by the characteristically swollen apex of the basidium and its only known host, *Theobroma cacao*. As a near-obligate, xylem-invading pathogen, the species was clearly ecologically very different from most *Ceratobasidium* and *Rhizoctonia* species, which are true saprotrophs.

In a revision of the Rhizoctonia group, Roberts (1999) moved *O. theobromae* into the genus *Thanatephorus*, which Talbot and Keane (1971) had found had commonalities with *Oncobasidium*, despite the characteristic multinucleate state of *Thanatephorus* species. Roberts (1999), therefore, renamed the fungus *Thanatephorus theobromae*. While agreeing with Roberts that a sole genus for *O. theobromae* was not justified, Samuels et al. (2012), referring to phylogenetic analyses based on the ITS and LSU regions as well as the binucleate state of the species, argued that *O. theobromae* should be moved into the paraphyletic genus, *Ceratobasidium*, with a new name, *Ceratobasidium theobromae* (Talbot and Keane 1971; Samuels et al. 2012). This new positioning was challenged by Oberwinkler et al. (2013) who, referring to the paraphyletic nature of *Ceratobasidium*, provided morphological, ecological, and phylogenetic evidence that the genus should be restricted to its original type species, *C. calosporum* in addition to a few others, while the remaining species presently allocated to the genus, *Ceratobasidium*, belong rightfully in *Thanatephorus* (anamorph *Rhizoctonia*). In their proposed revision, *C. noxium* (syn. *Koleroga noxia*), the cause of tropical web blight, is moved into the *Thanatephorus* (*Rhizoctonia*) grouping. With regard to dependence on the number of nuclei in the hyphal cells of members of the group as a criterion for placement within *Ceratobasidium* (binucleate) or *Thanatephorus* (multinucleate), the authors suggested that this trait was likely to have evolved more than once and was inadequate as a taxonomically defining character. Furthermore, the authors suggested that the widely used name *Rhizoctonia* should be used in place of *Thanatephorus* and consequently suggested renaming *O. theobromae*, *Rhizoctonia theobromae* (Oberwinkler et al. 2013). Until these major revisions are accepted, the nomenclature suggested by Samuels et al. (2012) is perhaps most appropriate and useful.

9.5 Distribution and Host Range

VSD has been recorded on cacao in the Southeast Asian/West Pacific region from Java in the south (Turner and Keane 1985; Guest and Keane 2007) to Hainan Island, China, in the north (Turner and Keane 1982) and from Kerala, India, in the west (Abraham 1981; Peter and Chandramohanan 2011) to PNG and the island of New Britain in the east (see Keane 1974; Keane and Prior 1991). *Theobroma cacao* is the only known host of *C. theobromae*, although a disease with both symptoms and an associated fungus similar to those of VSD-infected cacao was reported on avocado seedlings in Karkar Island, PNG (Anderson 1989). There is thus strong evidence that VSD is a new encounter disease, whereby the causal pathogen has transferred to cacao from an indigenous host, in which it perhaps occurs as an innocuous endophyte or weak pathogen. The original host(s) of the fungus remains unknown. Since the pathogen has not coevolved with the host, new plantings of cacao in the region have been initially free of VSD (and other new encounter pests and diseases) enabling high productivity for a period of time (Ploetz 2007b; Keane 1992).

Typically, after this period, cacao production per unit area declines in the face of increasing impacts of local pests and/or pathogens. Such a typical pattern of boom and bust has been experienced by smallholder farmers in Sulawesi (Ruf and Yoddang 2000) and presently cacao production in many areas of the island has declined from almost a ton to 300–600 kg per ha. While the major cause of this decline was initially infestation with the cacao pod borer, exacerbated by poor management, VSD has become increasingly severe in many areas in the last decade and many farmers identify this disease currently as their major limitation to productivity.

The epidemiology of the disease is discussed further below, but it is evident that VSD has a patchy distribution. Even within a district, incidence can be high (exceeding 50 %) in one area of smallholder farms but difficult to detect in a nearby area only a few kilometers away. This is especially the case when the area of planting is quite small. As a "crowd disease" (see below), the infection agents (basidiospores) spread for short distances between branches and neighboring trees rather than over long distances; therefore, proximity of the host trees to one another is an important factor influencing disease spread. Additionally, other factors affect distribution. For example, the same or similar genotypes of cacao can carry heavy infections in lowland areas but be free of VSD at higher altitudes. It is uncertain whether this is an effect of temperature or another climatic factor or an effect of lack of inoculum production or a restricted distribution in a more heterogeneous habitat. For example, in government corporate plantations in Banyuwangi, East Java, VSD incidence and severity are high in lowland areas (under 60 m above sea level) but much lower at higher altitudes in the same district, particularly on steep slopes at over 250 m, where VSD is practically absent (Indah Anita-Sari, personal communication). However, VSD assessments in one cacao plantation established on a high altitude plateau (400 m) had a high incidence of the disease, although not as high or severe as in the lowlands. The factor(s) accounting for the low VSD incidence on the high altitude slopes, whether they are drainage, wind velocity, or soil properties, are still unknown. In East Java, Anita-Sari and Susilo (2014) found that the average number of stomata was lower in the same three genotypes grown at a relatively high altitude (over 250 m) where VSD incidence was very low, than at a lower altitude (around 40 m above sea level). Other research in West Java showed that VSD had a higher incidence at lower altitudes than in similar cacao, under similar rainfall conditions, at higher altitudes (400–600 m) (Pawirosoemardjo et al. 1990). It was shown that nocturnal temperatures were several degrees lower at the higher altitudes which could have a critical effect on the sporulation and infection processes of the pathogen that occur mainly at night (Keane et al. 1972).

While nearly all new infections result from disease spread between cacao trees (Keane 1981), it is possible that the fungus also transfers repeatedly from its indigenous host to cacao. Keane and Prior (1991) observed that cacao is often planted in the vicinity of areas of native forest or other vegetation. If the original host occurs in indigenous vegetation nearby to planted cacao, then *C. theobromae* might transfer to the cacao farm from its indigenous host. Since the disease is prevalent in mainland PNG and East New Britain, but absent from nearby New

Ireland, Bougainville, and the Solomon Islands, it is possible that the indigenous host is also absent from these islands (Keane and Prior 1991). In 1986, in a location in West Java where VSD had not previously been observed, a very high incidence of disease (30 %) was observed in seedlings planted adjacent to secondary forest. It appeared that this forest was the source of infection (Pawirosoemardjo et al. 1990).

VSD is not transmitted via seed (Prior 1985), but the disease could spread to other cacao-producing areas through the importation of infected material, especially seedlings. This is a major quarantine issue, since (as mentioned before) even within the Southeast Asian region, some areas remain apparently free of VSD. Since VSD symptoms do not become apparent to casual inspection for a number of months, Prior (1985) recommended that seedlings remain in quarantine for 6 months before distributing to other areas. Now that clonal propagation has increased dramatically, the question of whether infected budwood is a potential source of inoculum has become increasingly important. Prior (1985) found that disease did not spread from budwood to healthy seedlings, but that (rarely) the reverse occurred: VSD spread from infected seedlings into grafts. However, if a piece of budwood is infected with *C. theobromae* it is highly likely to be killed before it can grow or establish infection in the rootstock. Quarantine control will also be particularly important to prevent the pathogen spreading to cacao in other parts of the world.

9.6 Biology of the Pathogen

The pathogen, *C. theobromae*, is unique in that it is a wind-borne, xylem-infecting basidiomycete that has been found consistently associated only with cacao (*Theobroma cacao)*. The fungus is likely to be associated with an endemic plant species, possibly asymptomatically, from which it has transferred to cacao. Since Ceratobasidiaceae species have a wide host range and may be symbionts, saprophytes, or pathogens, the identification of the original host(s) of *C. theobromae* represents a considerable challenge.

Fungi related to *C. theobromae*, such as the pathogens *Thanatephorus cucumeris* (anamorph, *Rhizoctonia solani*) and *C. bicorne*, are soil borne and infect roots necrotrophically. Others form orchid mycorrhizae. While many of these fungi readily grow in culture, *C. theobromae* is a near-obligate pathogen. The fungus can be isolated from infected stems, leaves, or petioles and transferred to media such as Corticium Culture Medium (CCM, Kotila 1929), where it grows slowly producing a white-pale yellow mycelium, but the fungus cannot be maintained in subculture. Other fungi, including *Fusarium decemcellulare* (teleomorph *Calonectria rigidiuscula*), *Phomopsis* sp., *Eutypella* sp., *Lasiodiplodia theobromae*, *Meripilus giganteus*, *Ceriporia lacerata*, and *Lentinus squarrosulus*, also occur in the stems, petioles, or leaves (Keane et al. 1972; McMahon et al. Submitted) and some of these can rapidly overgrow *C. theobromae* in culture. *Guignardia mangiferae* was detected in leaves of VSD-infected cacao in Kerala. Surface

sterilization using up to 10 % sodium hypochlorite, in addition to 70 % ethanol (Keane et al. 1972), increases the chances of obtaining a pure *C. theobromae* culture (Amanda Firmansyah, personal communication). Cacao callus tissue will support growth of mycelia of *C. theobromae* up to the monilioid stage (Prior 1977). Sporulation cannot be induced routinely on artificial media. However, Lam et al. (1988) showed that by transferring the fungus from culture to a nutrient-poor medium and maintaining it in the presence of moist, moving air, the fungus sporulated, producing basidiospores of the same shape and dimensions as those described originally by Talbot and Keane (1971). Vadamalai (1999) used a similar method to induce spore formation. However, none of these studies has produced enough basidiospores to enable their use in pathogenicity tests.

Studies of the infection and growth of the pathogen in seedlings in PNG in the 1970s showed that the fungus grows in the xylem vessels of infected stems and leaves (see Fig. 9.4d); Keane et al. (1972) confirmed a *Rhizoctonia*-like fungus with dolipore septa located near the hyphal junctions (Fig. 9.4c) emerges only from the xylem of cut stems. The symptomatic streaking of the woody tissue (see Fig. 9.2a) and gum-like deposits are likely to be due to a host response, but the fungus can be detected microscopically in xylem vessels for up to 10 cm beyond the extent of the

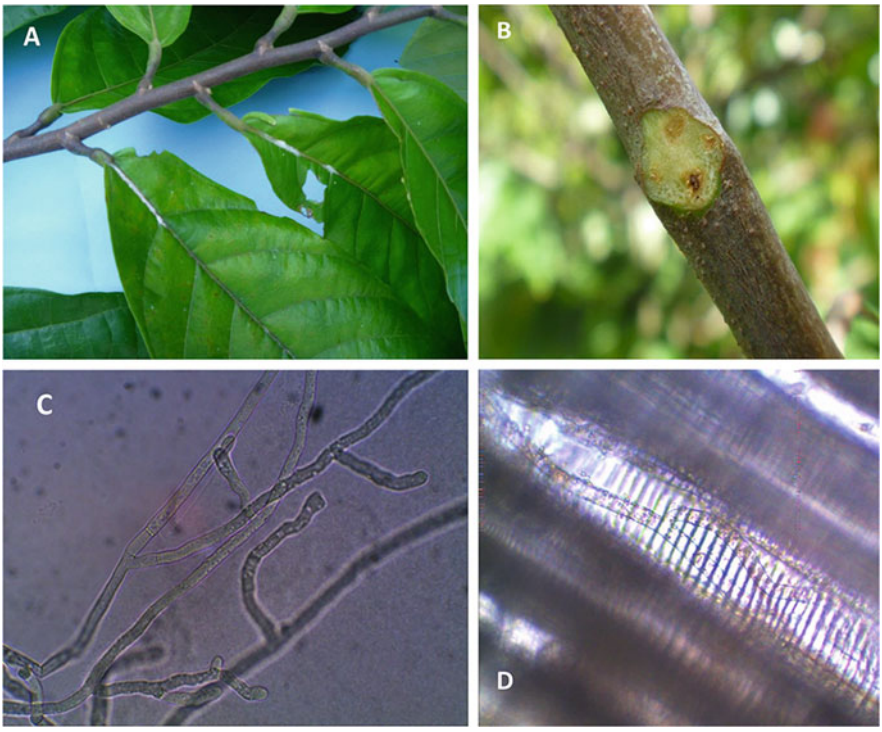

Fig. 9.4 (**a**) Sporulation on leaf mid-rib; (**b**) staining of vascular traces; (**c**) Rhizoctonia-like hyphae of *C. theobromae*; and (**d**) hyphae in xylem vessel

streaking (Keane et al. 1972). Sporophores (basidiomes) develop at points of emergence of the hyphae, such as the leaf scars left following abscission of infected leaves (Fig. 9.2e). Emergence of hyphae from leaf scars occurs only if leaf fall occurs during a period of very wet weather; otherwise the leaf scar dries off and seals over, preventing the emergence of hyphae (Keane et al. 1972). In particular, in conditions that maintain wetness of the sporophores, monilioid hyphae form basidia that produce basidiospores (Keane et al. 1972). In VSD-infected cacao associated with the recently observed necrotic symptoms (see below), sporulation takes place on fertile sporophores formed on cracks in the petiole or leaf mid-rib (see Fig. 9.4a). Spores are shed at night with peak numbers released between 1 and 3 am; by early next morning the basidia had collapsed (Keane et al. 1972).

Research conducted in PNG demonstrated that the short-lived, wind-borne spores of *C. theobromae* infect the soft young leaves at the branch tip, especially flush leaves in which the cuticle has not fully developed (Keane et al. 1972; Prior 1978, 1979, 1980; Keane 1981). Spores are transported by wind for only short distances of up to about 100 m. Keane (1981) observed that spores adhere strongly to leaves so are not readily washed off by heavy rainfall. Spores that reach unhardened leaves germinate, and some successfully penetrate the epidermis (Prior 1979). This is followed by colonization of the leaf xylem by the fungal hyphae although the route of entry has yet to be identified. From the leaf, the hyphae reach the stem through the petiole and spread via the xylem vessels in vascular tissue to other leaves, presumably infecting those leaves by growing out through the petiole into the leaves. This is supported by the ubiquitous occurrence of darkened vascular traces in petioles and leaf scars on VSD-infected branches (see Fig. 9.4b). An alternative scenario is that the branch tip with flush or unhardened leaves (with 5–10 occurring at a time at the branch tip see Fig. 9.5) receives multiple infections. Such a multiple infection of the flush leaves would account for the frequently observed appearance of initial VSD symptoms in two or more leaves on an infected branch. Monitoring of progress of infection in individual branches (see Fig. 9.5) in four clones suggested that the fungus moves from the initially infected leaf to leaves further up or down the branch, but sometimes leaves are bypassed so that healthy leaves remained in between the infected ones (unpublished data). It is clear, in any case, that transfer of the fungus occurs from the primary to secondary xylem, since streaking can be observed in the wood tissue (Prior 1979; and see Fig. 9.2). Abscission of the first infected leaves occurs about 3 months after initial infection followed by other leaves nearby (Keane et al. 1972; Keane 1981), which results in heavy leaf loss in the middle of the branch (see below). As the infection spreads, dieback of the whole branch can result, especially in more susceptible cacao genotypes. Branch death can occur within 6 months.

Fig. 9.5 Development of necrotic leaf symptoms in an infected branch of a locally selected clone in Sulawesi, Husbitori, from Day 0 (early symptoms of necrosis) to Day 90 (when the necrotic leaves have already fallen). Note the growth of flush leaves during the same period, indicating that the branch is outgrowing the infection

9.7 Characteristic Symptoms and Progress of Infection

In the studies in PNG referred to above, the most characteristic symptoms of VSD were recorded. While these symptoms are still currently prevalent, in the last decade or so new symptoms have emerged and in some areas have become predominant (see below). Nevertheless, the original symptoms may occur in the same genotype, even on the same branch, as the more recent, necrotic symptoms.

As described by Keane et al. (1972) in PNG, and observed also in Malaysia (Keane and Turner 1971), the most visible early symptom is chlorosis of the first infected leaf on the second or third growth flush behind the shoot tip, with this leaf becoming pale green then yellow with characteristic green spots or "islands" of tissue remaining more-or-less uniformly scattered over the chlorotic leaf. Some browning of lamina tissue may occur with advanced chlorosis. The leaf abscises followed by infection of neighboring leaves above and below the leaf scar of the initially infected leaf. These leaves also abscise within days of becoming chlorotic, resulting in infected branches that are leafless in the middle portion but still have leaves in the lower and upper parts of the branch. The position of the initially chlorotic leaf is linked to the timing of infection of young, unhardened leaves (see Sect. 9.6) since it takes 3 months in seedlings (and 3–5 months in mature branches) for the infected leaf to become symptomatic. In the meantime, one or two flushes have been added to the seedling apex or to the branch tip. Partial resistance in mature trees is characterized by the maintenance of disease-free tips so that growth continues as new flushes are added (McMahon et al. Submitted). However, in severe infections, especially if the host plants lack resistance, the tip of the branch dies resulting in branch dieback. While very wet conditions characteristic of the peak rainy season are required for sporulation and disease transmission, VSD symptoms become more severe in drier periods, most likely due to reduced water transport in the xylem.

In addition to leaf chlorosis and abscission, other symptoms occurring in VSD-infected cacao include swelling of lenticels (a common response to stress), darkly streaked vascular tissue and staining of the three vascular traces in the petiole (most likely a host response leading to deposition of polyphenols and other chemicals), the rapid browning of the cambium when exposed by stripping the bark (due to increased polyphenol oxidase activity), and the promotion of lateral bud growth, leading to growth of small branchlets, which can themselves become infected.

Recent Changes in Symptoms

From about 2004 it was evident that the characteristic VSD symptoms described by Keane et al. (1972) were no longer universal. A different set of symptoms had emerged in cacao infected with VSD and, in some areas, these were now more frequent than the original symptoms (Guest and Keane 2007). The occurrence of the new symptoms was apparently widespread, with observations in Malaysia, PNG, Vietnam, Sulawesi, Bali, and Java (Purwantara et al. 2009). However, in Kerala and West Papua the original (chlorotic) symptoms remained predominant (see Fig. 9.2d). The most obvious difference from the symptoms described originally is the development of necrotic lesions on the margins and tips of infected leaves. Rather than the pale or rusty brown coloration that occurs in leaves with advanced chlorosis, the necrotic lesions are dark brown to black. The boundary is well defined although a yellow halo may be present. The necrotic lesions expand over a period of weeks followed by leaf fall at an advanced stage of necrosis

(Fig. 9.5). Infection is still accompanied by vascular streaking, but in some cases this has been observed to be darker and more prominent than in infections associated with the original (chlorotic) symptoms. Additionally, as described in the earlier research in PNG, the disease progresses from an initially infected leaf along the branch to neighboring leaves both below and above it, in some cases reaching the tip causing branch dieback. Leaves near the branch tip that the fungus has not reached may also develop symptoms of chlorosis or necrosis (Parawansa 2013; and see Keane et al. 1972), possibly due to restricted transport of nutrients or production of toxins. Within the xylem of infected cacao showing new symptoms, hyphae have been observed to have the same *Rhizoctonia*-like characteristics as originally described (Keane et al. 1972).

Chlorotic symptoms may also be present on infected branches with leaves showing necrotic symptoms. Therefore, both sets of symptoms may occur on the same genotype, the same tree, or even the same branch. A recent study in Sulawesi demonstrated that the relative frequency of the symptoms varied between the months of March (wet season) and July (early dry season) with a higher proportion of chlorotic symptoms observed in July (Susanna Bryceson, Honors Thesis, La Trobe University, October 2014, Personal communication). In another study, the frequency of necrotic versus chlorotic symptoms was shown to be partly clone dependent, although even clones with a high frequency of chlorotic symptoms had predominantly necrotic symptoms (Parawansa 2013).

A common feature associated with the necrotic symptoms is that infected leaves remain attached to the branch for a period of a few weeks before falling off (Fig. 9.5). Leaf necrosis is sometimes associated with partial branch breakage and an extended period of attachment. Zaparoli et al. (2009) suggested that delayed abscission of leaves on broken branches occurs because transport in the vascular tissue (particularly the xylem) has become interrupted, preventing a signaling factor (s) that initiates the abscission process reaching the leaf. Leaves then become brown or necrotic. Abscission of senescent leaves is associated with increased levels of ethylene, which overcomes the effect of auxin produced at the leaf tip; transport of auxin to the abscission zone delays abscission. Chlorosis of leaves and leaf abscission have been linked to elevated ethylene levels and/or toxic effects by pathogens (Isaac 1992; Hodges and Campbell 1999). In the case of Verticillium wilt, ethylene may be produced by the pathogen (Tzima et al. 2010). If a signal sent to the leaf (perhaps via the xylem) is necessary to initiate chlorosis and/or abscission, interruption of this signal (due to xylem blockage) might delay the process, resulting in necrosis.

Plants respond to vascular pathogens by producing phenolic compounds, gels, gums, and tyloses, all of which could contribute to prevention of signaling compounds necessary for abscission being transported to the leaf. A similar process might occur in VSD-infected cacao, i.e., blockage of transport of an abscission factor(s) leads to the development of necrosis. If such a process accounts for the increased rate of necrotic symptoms in VSD-infected cacao in the last decade, the reason for this development remains unknown. Perhaps VSD has become more severe with heavier infections and increased quantities of hyphae in the xylem, or, alternatively, an adaptation in the host may have occurred increasing the resistance

response (which includes deposition of phenolics and gums and the production of tyloses) resulting in greater blockage of the xylem. The dark-colored vascular traces associated with necrotic symptoms suggest an enhancement in the host response.

The leaf necrosis of VSD-infected cacao might also be a result of the prevention of essential nutrients reaching the leaves. Keane et al. (1972) found that tip leaves on infected branches were free of fungal hyphae but showed interveinal necrosis, which, they suggested, might be caused by calcium (Ca) deficiency due to xylem blockage further down the branch. The recent, common symptoms of necrosis on leaf margins and tips in VSD-infected cacao are reminiscent of potassium (K) deficiency. Potassium, a macronutrient, is needed in large quantities for essential functions such as stomatal regulation and synthesis of compounds required for host defense. Potassium is also readily remobilized, and stress or senescence stimulates its redistribution from mature leaves to growing tissues or other plant parts (Himelblau and Amasino 2001; Jones 2013). Therefore, the necrosis observed in VSD-infected cacao might simply be a symptom of K deficiency interacting with the fungal infection. In East Java, leaf nutrient analyses of VSD-infected and healthy leaves collected from a range of clones indicated infected leaves had a 20 % lower K concentration on average, while the less mobile elements Ca and Mg appeared to accumulate and there was little difference in the leaf contents of other macronutrients (Abdoellah 2009). In Sulawesi, leaf tissue analyses of leaves showing necrotic and chlorotic symptoms in two clones indicated there were substantially lower concentrations of K in infected leaves (about 60 % the concentration of healthy leaves) suggesting substantial remobilization of this element occurs in response to VSD infection. In contrast, leaf contents of N, Ca, S, and Mg and a number of micronutrients were similar in infected and healthy leaves, while others, such as P, were lower in some samples but not others (unpublished data). However, since similar K decreases were observed in VSD-infected leaves with either symptom (necrotic or chlorotic), an additional factor(s) to K deficiency is needed to account for the specific and distinct necrotic lesions associated with the recent symptoms.

The production of necrosis and ethylene-inducing proteins (NEPs) and cerato-platanin (CP)-like proteins, small proteins resistant to heat and denaturation, has been demonstrated in a number of microorganisms, including basidiomycete fungi (Garcia et al. 2007; Zaparoli et al. 2009; de Oliveira et al. 2012). These proteins either cause necrosis by direct action or act as signals for cell apoptosis. In the basidiomycete, *Moniliophthora perniciosa*, the cause of witches' broom disease, it appears that these proteins are linked to pathogenic activity when *M. perniciosa* is in its biotrophic phase (Zaparoli et al. 2009). This raises the possibility that the enhanced necrotic response to VSD in recent years might be related to expression of related genes by the pathogen, *C. theobromae*. If such genes occur in the *C. theobromae* genome, they have yet to be identified. These two kinds of necrotic factors appear to act synergistically since if applied to leaves together they cause a necrotic reaction that is much more severe than if applied individually. They apparently initiate cell death in the host, but whether this is a strategy to increase nutrient availability to the pathogen remains unknown (Zaparoli et al. 2009).

Sporulation and the New Symptoms

Since VSD-infected leaves with necrotic symptoms remain attached for long periods (Fig. 9.5), alternative sites of sporophore formation, other than on leaf scars caused by the abscission of infected leaves, have become common. Basidiocarps now commonly form from mycelium emerging through cracks in the petiole or leaf mid-rib (see Fig. 9.4a), rather than only from leaf scars. In earlier reports, the only sporulation of the fungus observed was on leaf scars. Whether this change has increased or decreased the rate of sporophore formation is uncertain. However, as specified in previous accounts of the disease (Keane et al. 1972) the formation of basidiospores requires very specific conditions of rainfall and wetness (see Sect. 9.6) and many sporocarps are sterile, even though monilioid hyphae readily form.

In Sulawesi, spores and basidia associated with the new symptoms that have been collected from sporocarps formed on leaf and petiolar cracks are morphologically identical to those described originally in PNG and Malaysia (Talbot and Keane 1971; Lam et al. 1988). Despite the change in sporulation patterns and symptoms, the spore shape and dimensions are similar to those described in other studies (unpublished data). Samuels et al. (2012) identified the presence of *C. ramicola* on VSD-infected cacao leaves in Java using molecular methods. However, microscopic examinations of the Sulawesi collections have not revealed the presence of another *Rhizoctonia*-like species with spore dimensions differing from *C. theobromae*. If *C. ramicola* spores occur, they are likely to be smaller than *C. theobromae* spores: Tu et al. (1969) reported basidiospore dimensions of approximately 8.5×5.2 μm for *C. ramicola*, much smaller than the approximately 17.5×7.5 μm basidiospores in *C. theobromae* (Talbot and Keane 1971; Lam et al. 1988).

It remains uncertain whether the new symptoms can be explained by a change in pathogen strain or by the presence of another species. The presence of a secondary pathogen acting in concert with *C. theobromae* might play a role in producing the new symptoms, but there is little evidence for such an interaction. While a number of saprophytes and weak pathogens are known to be associated with dieback of cacao (Holliday 1980), DNA sequences of amplification products using the universal fungal primer pairs ITS1 and ITS2 and ITS5 and ITS4 consistently match those of *C. theobromae* in VSD-infected petioles and leaves collected in Sulawesi. The ITS region of DNA of *C. theobromae* amplified from samples of VSD-infected cacao displaying necrotic symptoms (collected in Sulawesi) and chlorotic symptoms (collected in Kerala, India) matched sequences previously published in GenBank (McMahon et al. Submitted). Both basidiomycotina-specific primers and primers designed specifically for *C. theobromae* (Samuels et al. 2012) produce PCR products with ITS sequences matching the submitted *C. theobromae* sequences. In longitudinal stem sections of VSD-infected cacao, hyphae of a *Rhizoctonia*-like fungus, presumably *C. theobromae*, are always observed in xylem vessels, while other types of hyphae have not been observed in the prepared

sections. Nevertheless, isolations onto media indicate that a number of species are associated with VSD-infected cacao, including the wood decay fungi *Ceriporia* and *Lentinus*, as well as *Fusarium* spp., and, consequently, the contribution of another fungus to the dieback symptoms observed on VSD-infected cacao cannot be ruled out completely.

Regional comparisons of ITS sequences of *C. theobromae* have indicated that populations in different regions are genetically identical or near identical (see Sect. 9.9). However, a change in another part of the genome could have influenced the pathogen's aggressiveness. A more aggressive genotype in which, for example, regulation of toxins has been altered could have spread through the region accounting for the widespread occurrence of the changed symptoms. The sequencing of the *C. theobromae* genome is under way at the USDA lab in Beltsville, and this will facilitate further exploration for pathotypes or variants (Bryan Bailey, personal communication). Alternatively, the widespread change in symptoms could be the result of an environmental change. The widespread occurrence of the new symptoms and their rapid emergence (prior to 2004 they had not been recorded) suggests that a change in an environmental factor(s) causing the change in symptoms must also have occurred on a region-wide basis. An obvious candidate is a climate change factor, such as rise in temperature or increased CO_2 levels. Alternatively, since large-scale cropping of cacao has occurred in the region for a similar period of several decades (especially in the main centers of production in Indonesia, Sulawesi and Sumatra, and more recently, in Vietnam), the new symptoms might be linked to exhaustion in the soil of a particular nutrient or to a general decline in soil fertility. Certainly, soils analyzed recently on cacao farms in Sulawesi have been shown to generally have a low pH with low concentrations of N and exchangeable cations and critically low organic matter content (authors' observation). The decline in soil fertility has been linked concomitantly to critical leaf nutrient concentrations, but a direct link between the newer symptoms and availability of nutrients has not been demonstrated. An intriguing possibility is that the large increase in the rate of burning during the dry seasons on farm land and forest in the region since the 1990s, especially with the expansion of oil palm plantations but also with the widespread burning of rice stubble, has led to increased localized concentrations of CO_2 or another haze-related component, which has interacted with the VSD pathogen triggering the change in symptoms (S. Bryceson, Honors thesis, 2014).

Evaluation of Disease Severity

VSD severity is usually evaluated in the field according to the visible symptoms of leaf infection, leaf loss, and other responses, such as lenticel swelling and growth of axillary shoots (Bong 1989). VSD can be evaluated for its impact on the whole tree or on individual branches. Diseased trees can be evaluated by scoring disease severity using an arbitrary scale based on the number of chlorotic leaves, number of leaves lost, etc., and/or impact on yield. Several scoring systems have been

developed, including a four-score system and a more detailed seven-score system. For example, in the four-score method, individual trees or branches are scored for disease severity as follows: (1) (no infection); (2) (light infection, with infected leaves or some leaf fall); (3) (moderate infection, high rate of leaf fall, with tip leaves remaining); and (4) (severe infection, substantial leaf loss with tip death, and/or branch dieback imminent). Disease severity is then calculated as:

$$(N_1 \times 1) + (N_2 \times 2) + (N_3 \times 3) + (N_4 \times 4)/N(\text{total})$$

[N (total) is the number of trees or branches evaluated, N_1, number of healthy or uninfected trees (or branches), N_2, the number of trees (or branches) scored as 2, etc.]. An average severity can then be obtained for the whole tree or plot.

9.8 Epidemiology

VSD spread is dependent on the close proximity of susceptible trees and is, therefore, a typical "crowd" disease that spreads slowly with increases at rates below 1 unit per month (Vanderplank 1948; Keane and Prior 1991). The infection agents, basidiospores of *C. theobromae*, which are released at night in particularly wet conditions, are short-lived and carried only short distances by wind. New infections are limited to a distance of 100 m or less from the origin of spore production (Keane 1981). Keane (1981) measured the rate of VSD disease increase in PNG at 0.3 units per month, a slow rate typical of systemic diseases, but more rapid than other systemic diseases such as Cacao Swollen Shoot Virus which increases at a rate of only 0.05 units per month.

Disease Spread in Cacao Plantings

VSD incidence is strongly correlated with rainfall (Keane 1981). New infections are initiated by basidiospores (the only known agents of infection), which are produced only when a high proportion of wet days occur in any given period and, importantly, when rain falls in the late afternoon or at night, resulting in sporophores remaining wet long enough during the night for basidiospore formation and spore release (Keane and Prior 1991). A study in PNG showed that spore release was closely linked to the number of consecutive hours that the sporophores remained wet (determined by conductance in mV). Sporophores that remained wet for less than 5 consecutive hours did not release spores, while a peak rate of spore release was reached in sporophores that were wet for 12 consecutive hours (Dennis et al. 1992). Spore release was found to be more closely correlated with the number of rainy days per month, than with the total amount of rainfall. Very rapid rates of disease increase have been reported on an unpruned, abandoned cacao

block on Bayabang Estate in West Java (average rainfall 2235 mm per year). Disease incidence was observed to increase from 30 % to 90 % within a year in 1986. During this period, even though the monthly rainfall was low (ranging from 83 to 181 mm), the number of wet days per month ranged from 20 to 23. In contrast, at Bunisari-Lendra Estate in West Java (average rainfall about 4500 mm per year), the proportion of infected trees increased from 0.5 % to 3.0 % at about the same time, and the disease incidence has always been observed to be much lower than at Bayabang Estate (Pawirosoemardjo and Purwantara 1992). It appears that the number of wet days per month may be more important than the monthly rainfall in determining the amount of infection. This further supports the hypothesis that infection may not be favored by excessively heavy rainfall as much as by lighter, more continuous drizzle.

The VSD pathogen survives in its vegetative form as hyphae in the xylem. During dry periods symptoms may become more severe as plants become water-stressed, but these symptoms are alleviated with the arrival of rains. Nevertheless, seasonal rainfall also stimulates the formation of sporophores on leaf scars (or on cracked petioles and mid-ribs associated with necrotic symptoms since 2004). Hence, while leaf loss and dieback are more apparent in drier times, disease spread occurs in the wet season.

Regional Spread and Quarantine

In Sulawesi, VSD has been recorded on cacao in the Kolaka and North Kolaka districts of Southeast Sulawesi province since the 1980s (Pawirosoemardjo and Purwantara 1992). These were among the first areas where cacao was brought from Malaysia by returning laborers: hence in North Kolaka some cacao trees are over 40 years old (about double the age of cacao in other provinces in Sulawesi). As observed by A. Purwantara, by the 1990s VSD was a common but relatively minor disease on cacao in Southeast Sulawesi. Evidently genotypes with a degree of resistance had been propagated. VSD had not been recorded as a problem in other Sulawesi provinces in the 1980s and 1990s. It was not until after 2002 that severe infections of VSD were observed in the provinces of South and Central Sulawesi, reaching epidemic levels in some areas (A. Purwantara's observation). Severe infections and tree death occurred in Pinrang District, South Sulawesi. More recently, in the same province, the disease has become severe in Bantaeng District, whereas it could not be found on cacao farms in the district before 2010. New plantings in Bantaeng, following a national government program implemented to improve cacao production, perhaps either resulted in the inadvertent introduction of the pathogen from other areas or else the area of continuous cacao plantings on smallholdings increased to a size where inoculum could build up and spread (Vanderplank 1948, 1963). The hilly terrain of Sulawesi may contribute to the

slow spread and patchy distribution of VSD. The virtual absence of the disease in many districts at higher altitudes over 250 m (see above, Distribution) could provide a barrier to spread of the disease. Keane and Prior (1991) suggested that some areas in West Java were little affected by VSD; they linked this to the very high rainfall (>4000 mm per annum) suggesting that excessive amounts of rainfall may in fact limit propagation of the disease.

9.9 Diversity of the Pathogen

Morphological Diversity

Studies so far have indicated little, if any, morphological diversity of the VSD pathogen within the region of endemicity. Keane and Prior (1991) refer to the identical nature of the VSD pathogen isolated in Malaysia and PNG. The identity of the Malaysian pathogen in VSD-infected cacao as *O. theobromae* was unambiguous. Furthermore, Lam et al. (1988) who produced basidiospores in culture reported similar dimensions and characteristics for the spores and basidia as those described by Talbot and Keane (1971). Collections of spores from VSD-infected cacao (including cacao with the more recent symptoms) in Sulawesi indicate that the same pathogen causes VSD of cacao on the island and, furthermore, it is associated with both kinds of symptoms (chlorotic and necrotic). In 2012, collections made from fertile sporophores from infected trees in West Sulawesi displaying the more recent symptoms of leaf necrosis revealed basidiospores with an average length of 17 μm, ranging from 15 to 22 μm in length and 7.5–8 μm in width. The spores had the same ellipsoid (slightly asymmetric) shape described by Talbot and Keane (1971) (see Fig. 9.3). In fertile sporophores collected in East Luwu, South Sulawesi, the conical sterigmata (see Fig. 9.3) were approximately half the length of the spores (consistent with the descriptions of Talbot and Keane). Hyphal characteristics both in sporophores and in culture were *Rhizoctonia*-like with most hyphae about 5 μm in width. Monilioid hyphae are also frequently observed in the sporophores on leaves and petioles. The consistent occurrence of a basidiomycete fungus in sporophores occurring on cacao which displays the new VSD symptoms and its identical morphology to that originally described in PNG and Malaysia suggest that the same pathogen, *C. theobromae*, is responsible for the infections associated with both kinds of symptoms. This suggests that the symptoms observed since 2004 are an effect of an environmental change, rather than a change in the pathogen. However, changes in the pathogenicity of *C. theobromae* cannot be ruled out as a factor influencing the changes in symptoms.

Genetic Diversity

The general uniformity of the pathogen in the region is suggested by its morphology despite the variation in symptoms (see above). However, a phylogenetic survey conducted by Samuels et al. (2012) indicated that some regional genetic variability occurs in the species. DNA sequences of the ITS region of *C. theobromae* in VSD-infected cacao tissues and isolates collected from different parts of the region, including Java, Bali, Papua, Sulawesi, Malaysia, and Vietnam, were subjected to parsimony analyses and a phylogenetic tree was generated, using a closely related Ceratobasidiaceae isolate as an outgroup. From the consensus tree, they identified three haplotypes, the Vietnam, Malaysia/Indonesia, and Papua haplotypes. These groupings were supported with bootstrap values higher than 70 %. Estimations of substitution rates enabled an estimate of the evolutionary time of separation of the haplotypes. The longest separation time was between the Vietnam haplotype (which clustered basally) and the Papua haplotype, estimated to be 3 million years.

9.10 Control of VSD

Cultural Methods

To reduce inoculum levels in mature trees, regular pruning of infected branches is effective. Pruning at monthly intervals has been shown to decrease disease to a greater extent than at intervals of 3 months (Jayawaderna et al. 1978; Prior 1980), although this frequency may be hard to achieve for many smallholders, who often rely on family labor. Branches should be pruned to well below the extent of infection of the branch (about 30 cm below visible staining of the vascular tissue). Prunings do not need to be removed since the disease is not propagated from cut branches (Keane and Prior 1991). Sanitation pruning has been implemented on most of the infected estates in Java. On Bunisari-Lendra Estate in West Java, monthly pruning carried out by a team of trained disease spotters in the late 1980s reduced disease to a very low level, despite the widespread occurrence of disease in the area since 1985 (Pawirosoemardjo and Purwantara 1992). In comparison, much higher infection rates occurred on the unpruned estate at Bayabang in West Java. On smallholdings it is unlikely that sanitation pruning will be as rigorously carried out as on an estate, and therefore much greater effort needs to be put into developing more resistant planting material for smallholdings (Pawirosoemardjo et al. 1990). If planting material with at least a moderate level of resistance is used, routine pruning will effectively control the disease on well-managed estates. In Integrated Pest and Disease Management (IPDM) trials in West Sulawesi, regular production pruning at intervals of 2 months significantly reduced the number of VSD-infected branches (authors' observation).

There is some evidence from Malaysia that flushing, stimulated by pruning, increases VSD incidence if other sources of inoculum remain in the vicinity (Zainal Abidin et al. 1984). In two clones in Sulawesi, monitored for 2 years, a positive correlation occurred between VSD severity and flush leaf production with a lag of 3–4 months, consistent with the time taken for clear symptoms to emerge after infection (McMahon et al. Submitted). Nutrient supply can also significantly affect VSD incidence and severity. An adequate nutrient input can improve performance against VSD since branch tips can grow at a greater rate than the spread of the fungus within the stem xylem, as long as the genotype has partial resistance. In Sabah, Malaysia, the widely planted clone PBC123 (partially resistant to VSD) is kept virtually VSD free with adequate application of fertilizer, even though unmanaged hybrid cacao in the same planting area is heavily infected with the disease (authors' observation). It is possible that supplying certain micronutrients may also increase resistance, but more studies are needed to verify this.

The basidiospore inoculum of VSD is only dispersed for short distances, generally less than 100 m (Keane 1981), but it is recommended that seedlings are raised a sufficient distance (at least 150 m) away from mature cacao trees in areas affected by VSD. Alternatively, seedlings can be raised in nurseries with protective cover. Young seedlings do not survive infections with VSD, and therefore, in areas with VSD infection, it is crucial to keep them under cover, which prevents spores falling onto the leaves of the seedlings, initiating infection. Also, free moisture is not retained on the leaves for the extended periods required for successful infection (Prior 1985; Keane and Prior 1991). In Malaysia, Sidhu (1987) exposed 500 young seedlings to nearby cacao infected with VSD resulting in a 67 % incidence of VSD infection, while incidence in seedlings under a polythene roof was only 5 %. As cacao is a shade-adapted species, seedling survival is increased under shade. Generally seedlings are raised in nurseries with a shading roof and open sides. The roof cover may consist of coconut fronds, but many farmers in Sulawesi and elsewhere now prefer UV-resistant plastic combined with shade cloth. This keeps free moisture out of the nursery, reducing infection not only by *C. theobromae* but also by *Phytophthora palmivora*, which causes seedling blight of cacao.

Chemical Methods: Fungicides

A wide range of chemical fungicides have been screened for control of VSD. Trials have shown that triazoles, organochlorides that inhibit ergosterol synthesis, are most effective in controlling the VSD fungus, although problems with phytotoxicity may occur (Holderness 1990). Singh (1989) tested 15 systemic fungicides against VSD in Malaysia and obtained good protection with Triadimefon applied as a soil drench, but not as a foliar application. Prior (1987) achieved disease reduction with propiconazole when it was applied on bark. Possibly, foliar application is less effective than application to roots or bark because the chemicals are not readily

redistributed via phloem to growing tissues (Prior 1987). Triazole fungicides are costly and are not usually an economic option for VSD control.

Selection for Host Resistance

Selecting for host resistance is the most promising strategy of control of VSD, which is a systemic disease that is difficult to control with either fungicides or cultural methods (see above). Resistance to VSD lowers the "proportion of newly infected branches and subsequent sporulation of the fungus" (Keane 1981). Control of VSD is especially achievable where partial resistance is combined with appropriate cultural management methods (Keane and Prior 1991; Guest and Keane 2007). Currently, in many parts of the region genotypes are propagated that have partial resistance to VSD. In PNG, Trinitario clones such as KA2-101 have shown durable resistance since the 1960s. Progeny of hybrid crosses with KA2-101 as a parent produced resistant progeny, in contrast to progeny of the more susceptible K-82 clone (Efron et al. 2002). In Sulawesi, the most widely propagated cacao clones, PBC123 and BR25, were originally developed in Malaysia. PBC123 was selected following extensive screening of progeny of hybrid crosses between parental clones with resistance and high yielding characteristics (Chong and Shepherd 1986). When grown in smallholdings in Sulawesi, the bean quality characteristics of PBC123 (also known as Sulawesi 1) are, however, often substandard with a high bean count. Local cacao genotypes with either high resistance to VSD or with good quality and yield characteristics have been selected and tested as clones on farms in Sulawesi (McMahon et al. 2010). A priority is to combine these traits in the same clone: large-scale crossing programs to achieve this end are under way in the region (see below).

The mechanism of resistance to VSD remains largely unknown. As a vascular pathogen it is likely that host responses in the xylem and adjacent parenchyma cells are critical: a number of compounds, and even elements, accumulate in vascular tissues following infection with vascular pathogens. For example, Williams et al. (2002) detected increased sulfur (S) deposition in the xylem of cacao in response to *Verticillium dahliae*. In addition, resistance could partly depend on the success of spore germination on the leaf surface and the successful penetration of the epidermis. Exudates produced by leaves could be important in this regard. Spore germination requires free water, which is provided by dew deposition on leaves, which releases exudates; these could potentially contribute to resistance. Prior (1979) found that free water containing exudates from leaves inhibited spore germination and recorded differential responses between clones, but these did not correlate with resistance in the field. Correlation between field resistance and the production of tyloses, which Emechebe et al. (1971) observed in cacao infected with *V. dahliae*, was also not detected by Prior (1979). In East Java, Anita-Sari and Susilo (2014) found that a VSD-resistant genotype, PA191, had a lower number of stomata per unit leaf area than two genotypes with higher susceptibility, suggesting

that transpiration rate may influence relative susceptibility to the disease in cacao genotypes.

Genotypes with partial resistance emerged from the VSD epidemic in PNG during the 1960s: they have retained their resistance to the present, in collections and on farms in PNG, indicating that the resistance traits are durable. Durable resistance is more likely to be associated with horizontal resistance since it usually involves the combined effect of a number of genes (Vanderplank 1963; Keane 2010, 2012). Resistance appears to limit the extent of infection along branches; thus while trees may still be infected with VSD, dieback and damage by the fungus are limited. In PNG, Tan and Tan (1988) evaluated a number of criteria of VSD resistance in the progeny of hybrid crosses. Most variance was attributable to the male and female parents and not the interaction between them, leading the authors to characterize VSD resistance as polygenic and additive with a high GCA (and low SCA). Efron et al. (2002) confirmed progeny of a resistant clone (KA2-101) as the male parent and KEE clones as the female parents had consistently greater resistance to VSD than the progeny of the more susceptible K-82 (also as the male parent) and crossed with the same KEE clones as female parents.

Evaluation of VSD resistance in hybrid crosses in East Java suggested that VSD resistance, identified as additive and quantitative by Tan and Tan (1988), might be linked to only a few genes. The progeny of reciprocal crosses conducted by the Indonesian Coffee and Cocoa Research Institute (ICCRI) between clones, including PBC123 (VSD resistant), TSH858 (moderately susceptible but with good yield and bean quality characteristics), and KEE2 (moderately resistant), were evaluated for VSD resistance in a randomized block design experiment. Mean resistance of the hybrid progeny of PBC123 and TSH858 was similar to that of the VSD-resistant parent, PBC123 in East Java (Susilo and Anita-Sari 2011) and in Sulawesi (S. Bryceson, Honors thesis, 2014). Furthermore, deviations occurred from expected segregation ratios of VSD resistance/susceptibility in progeny, which could be accounted for by major gene resistance involving two or a few genes (Susilo and Anita-Sari 2011). Horizontal resistance could be controlled by only a few genes—the main criterion is that overcoming resistance by genetic changes in the pathogen population takes longer or is more difficult. Certainly, the VSD resistance of clones such as PBC123 or KA2-101 has proved to be durable for decades.

9.11 Breeding for Resistance and Future Research

The most fruitful research aimed at improving the control of VSD is the selection and deployment of cacao genotypes with resistance combined with yield and quality characteristics. While a number of genotypes with promising resistance have been identified (see above), these are often susceptible to other pest/diseases or have low yield and bean quality, making the need for crossing resistance with other traits a priority. Efron et al. (2002) recommend a high planting density when

screening for VSD resistance. Programs are under way by the Indonesian Coffee and Cocoa RI in East Java, Mars Inc. in Sulawesi and Mondelez (formerly Cadbury), and Kerala Agricultural University in southern India (Koshy Abraham, personal communication). Since heritability estimates for bean quality (Lachenaud and Oliver 2005), and resistance to VSD, *Phytophthora* and other pest/disease problems of cacao are generally high (see Lockwood et al. 2007; McMahon et al. 2015, Submitted), one approach is to select for these traits at first, followed by screening for yield, which is extremely variable, even within a clone (Lockwood et al. 2007). More research is needed on the factors influencing the onset of the recent VSD symptoms: while observations so far suggest that they are due to environmental changes, evolution of greater virulence in the pathogen cannot be ruled out. In addition, further regional collections are needed to gain a better understanding of the genetic variability of the pathogen and to identify its potential original host.

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Chapter 10
Cacao Swollen Shoot Virus (CSSV): History, Biology, and Genome

Emmanuelle Muller

Abstract *Cacao swollen shoot virus* (CSSV) is the only virus disease of cacao that is prevalent and damaging. CSSV is a dsDNA virus of the genus *Badnavirus* and the family *Caulimoviridae* transmitted by several species of mealybugs. The historical emergence of the disease is closely associated with the establishment of cacao cultivation in West Africa as it appeared soon after the introduction of the cacao in West Africa and remains endemic to this area. The disease is likely due to several host shifts from indigenous hosts. We can additionally conclude from the high molecular variability of the virus that the disease consists of a complex of viral species. Although the disease spreads slowly, eradication campaigns have failed to contain the disease which continues to emerge in new West African regions.

10.1 Introduction

Currently, cacao swollen shoot disease (CSSD), caused by *cacao swollen shoot virus* (CSSV), can be regarded as the major viral disease on cacao and has been recognized as one of the most important diseases in West Africa limiting cacao production. However, the disease appeared only after the introduction of cacao in West Africa and is a particularly good example of emerging infectious disease following the introduction of plants into a new region. The incidence of this disease along the West African coast in the early thirties was literally responsible for initiating research into the productivity of the cacao tree. This led to the establishment of the West African Cocoa Research Institute (WACRI) in 1944 at Tafo, Ghana. A branch of the Institute was formally established in Nigeria in 1953. Extensive research on this emerging disease has been conducted in these institutes and elsewhere in virology, epidemiology, and genetic improvement of cacao tolerance to CSSD, and, yet, the disease has not been controlled and continues to spread into new areas.

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Based on the results of early investigations, it was obvious that a cure for the disease would be difficult to obtain, and the only measure of control adopted was to cut out the infected trees and their contacts. Even now, less destructive control measures have not been developed for CSSV, and priority should be given to the replanting of cacao varieties presenting a satisfactory level of resistance to the virus. Now that the disease has spread through most areas of West Africa where cacao is produced, it is urgent that we reevaluate the results of previous research and develop effective agricultural methods which can be disseminated to producers.

10.2 Mike Thresh, a Pioneer in Tropical Plant Virus Epidemiology

This chapter is dedicated to the memory of the distinguished plant virologist Professor John Michael Thresh who died at age 84 on 12 February 2015 (Fig. 10.1). Mike excelled at building small informal partnerships between scientists with complementary skills. Armed with a degree in botany, Mike's professional career took off in February 1954 when he began work at the West African Cocoa Research Institute, Nigeria, conducting field studies on the epidemiology of cacao swollen shoot disease, a disease caused by a complex of lethal and nonlethal viruses. He helped explain the behavior of the disease on farms and how effective control could be achieved.

Mike returned to the UK in 1960 and joined East Malling Research Station (EMRS) where he worked for the next 26 years on viruses of fruit crops and hops, research that earned him both Ph.D. and D.Sc. degrees. His work on hop nettlehead virus was recognized by a Queen's Award for Technological Achievement in 1979.

Mike helped found the Plant Virus Epidemiology Group of the International Society for Plant Pathology in 1978, was its chairman from 1979 to 2001, and

Fig. 10.1 Mike Thresh

remained a committee member throughout his life. He was President of the British Society for Plant Pathology (BSPP) in 1990 and was elected an honorary member in 2006. He also served on committees and councils of the Society for General Microbiology, the Biological Council, the Association of Applied Biologists, and the Federation of British Plant Pathologists (the forerunner of the BSPP).

Mike's hands on commitment to tropical plant virology resumed in 1971 with a 3-month assignment to Ethiopia and Kenya for FAO, which was followed by other overseas consultancies and advisory visits for several international sponsors. In 1986, he transferred to the Overseas Development Administration (ODA) Corps of Specialists advising on the virology of tropical crops, based first at EMRS and from 1991 at the Natural Resources Institute, Chatham. He contributed to numerous projects on the epidemiology and control of tropical plant virus diseases including rice tungro, rice yellow mottle, banana bunchy top, maize streak, cacao swollen shoot, cassava brown streak, and cassava mosaic in Asia and Africa. In recognition of his outstanding contribution to containing a devastating pandemic of cassava mosaic virus in East Africa, Mike became the first laureate of the Golden Cassava Award in 2012. Although past retirement age, Mike was made an Honorary Professor of Plant Virus Ecology in the University of Greenwich in 1998.

During his career, Mike worked in more than 40 countries, edited/coedited 11 books or conference proceedings, and published more than 200 scientific papers. His involvement with tropical plant viruses continued up to his death: he was a keynote speaker at cacao swollen shoot workshops in Ghana in 2011, Ivory Coast in 2013, and Brussels in 2014 and was a virology consultant for the International Cocoa Quarantine Centre, Reading University. Unpretentious and kindly, Mike's knowledge of plant virus diseases was encyclopedic. This accumulated wisdom he shared patiently and freely with local farmers and the science and business communities. He will be remembered with gratitude and sadly missed.

10.3 Historical Emergence of the CSSV

Whereas the cacao tree originated from the Western Hemisphere, the CSSD appeared in West Africa in 1922 (Paine 1945) and is today restricted to the Eastern Hemisphere (particularly in West Africa). The history of CSSV emergence is subsequently closely related to the history of the spread of cacao cultivation in West Africa. The installation of the first cacao plantations in modern times occurred in South America and in the Caribbean in the sixteenth and seventeenth centuries with Criollo cacao. During the eighteenth century, Forastero cacao began to be grown in Brazil and Ecuador (Wood and Lass 1987), and cacao of the Amelonado type (Forastero) was taken from Bahia to Principe (1822) and later the Sao Tome islands in 1852. From there, it was introduced to Fernando Po island (now Bioko island, part of the Equatorial Guinea) in 1855 (Wood and Lass 1987; Bartley 2005). By 1900, 20 % of all beans came from outside the Americas (Braudeau 1969), and by the turn of the twenty-first century, the Western Hemisphere had become a

relatively minor producer. In 2005, 86 % of the global cacao production came from the Eastern Hemisphere, 78 % of which originated in only four countries: Ivory Coast, Ghana, Indonesia, and Nigeria (FAOSTAT). The main reasons for this change in the primary centers of cacao production in the Americas were damaging diseases that do not occur in Africa and Asia. Now production areas in Africa are under significant disease pressure from *Phytophthora megakarya*, a causal agent of black pod rot, and CSSV, both pathogens that have jumped hosts.

Cacao cultivation in West Africa started in the Eastern Region of Ghana. In 1879, Tetteh Quarshie of the Gold Coast returning from Fernando Po brought with him some pods of cacao. He was reported to have started his cacao farm in Akuapim-Mampong in the Eastern Region. Other interested farmers bought pods from him, and consequently this led to the spread of cacao farms in the Eastern Region (COCOBOD Executive Diary 2007). Cacao cultivation subsequently assumed commercial dimensions and spread to all forest areas of the country particularly the Eastern, Ashanti, Brong Ahafo, Volta, Central, and the Western Regions (COCOBOD Executive Diary 2007). From Ghana, cacao cultivation subsequently spread to the neighboring countries, Ivory Coast in 1890 and in Togo and the West of Nigeria a few years later. The first wave of introduction in Eastern Nigeria occurred in 1874.

CSSD was first described in Ghana at Effiduase in the New Juaben district of the Eastern Region in 1936 (Steven) although the disease was probably present in the nearby Nankese township of Ghana from 1922 (Paine 1945). CSSD has devastated cacao farms in the Eastern Region, which was the most intensive cacao-producing area in Ghana until being surpassed by Ashanti in 1942. A large area of the Eastern Region was described as an area of mass infection (AMI) in 1985 (Hughes and Ollenu 1994). Another area surrounding the AMI was designated cordon sanitaire which was supposed to be an area under strict CSSD control to serve as a buffer between the AMI and other parts of the cacao-growing areas to check the spread of the disease (Hughes and Ollenu 1994; Dzahini-Obiatey et al. 2006). The Western Region remained relatively unaffected by CSSV for many years having a few scattered outbreaks; however, since the year 2000, this area is the most severely affected region in Ghana. The Brong Ahafo and Volta Regions are less infected with CSSV with 2.3 % and 1.7 %, respectively, based on the total infected cacao trees removed nationwide (Domfeh et al. 2011). Ever since CSSD was reported in 1936, nearly 200 million cacao trees have been cut out from about 130,000 ha of land (Ampofo 1997), and the disease continues to emerge in new areas.

The disease has spread widely in all cacao-growing areas in West Africa. Since its discovery in Ghana, CSSD has been subsequently discovered in the other West African countries where cacao was cultivated: in Côte d'Ivoire in 1943 (Burle 1961; Mangenot et al. 1946), in Nigeria in 1944 (Thresh 1959), in Togo in 1949 (Partiot et al. 1978), and in Sierra Leone in 1963 (Attafuah et al. 1963). In addition, West African Amelonado cacao, planted uniformly throughout West Africa, appeared to be highly susceptible and sensitive to CSSV which favored the rapid spread of the disease. CSSD has likely contributed to the reduction in cacao production in Ghana,

causing it to lose its ranking as the top cacao-producing country in the world to Ivory Coast in 1977.

In 1961, CSSV could be found in most of the cacao-producing areas in Côte d'Ivoire but with fewer outbreaks compared to the situation in Ghana (Meiffren 1953). The more virulent form of CSSV has only been described in the East of the Côte d'Ivoire, Kongodia (Renaud 1957; Burle 1961). The largest area of infection in Eastern Côte d'Ivoire corresponds to the area where larger-scale cacao production is conducted. Until 2003, the disease appeared to be confined to the Eastern Region without any particular problems in other regions. The recent discovery of new CSSV outbreaks in all of the cacao-producing areas of Côte d'Ivoire cast doubts on the sustainability of Ivorian cacao production (personal communication).

The first survey of Nigerian cacao to discover where CSSD was present in the country was completed in 1949 (Lister and Thresh 1957). Another survey in 1952 revealed that CSSD was only found in four provinces of the Western Region but not in the Eastern and Northern Regions of the country. So far, the severity of CSSV in Nigeria differs from that of Ghana (Lister and Thresh 1957). Despite the existence of another AMI in the Western production areas of Nigeria, similar to that described in Ghana, control measures have achieved some success (Lister and Thresh 1957). These controls have resulted in smaller yield decreases attributable to CSSV. Longworth (1963) showed that with adequate mirid control and good maintenance, infected farms can produce good yields. Since this report, no published research work has been conducted on CSSV in Nigeria. Most cacao trees in Nigeria are over 40 years old, and as such, leaf symptoms of the disease are masked except for stem swellings. The continuous loss of cacao trees by farmers in Nigeria is commonly attributed to old age and black pod diseases. Based on the economic importance of the disease in neighboring countries like Ghana and Togo, there is an urgent need for a reevaluation of the importance of the disease in Nigeria.

In a subsequent attempt to reestablish information on the presence of CSSV in Nigeria, Dongo and Orisajo (2007) detected CSSV in "symptomatic" cacao leaf samples from the Cocoa Research Institute of Nigeria, Ibadan, and neighboring farms using ELISA analyses. However, only a small sampling area was covered in their study. Work in progress indicates that the virus is now present in Nigerian cacao in the states of Oyo, Ondo, and Edo and between the Abia and Akwa Ibom borders (unpublished results, Obok and Wetten).

CSSD was observed for the first time in 1949 in Togo in the area of Agou, in the south of the country, and then spread within that area to contiguous areas only (Kloto). It was only toward the end of the 1990s that the disease was observed in the Wawa region, the main cacao-producing region in the Central-Western area of the country (Cilas et al. 2005). The disease is now endemic in the two major cacao-producing regions of Togo, Wawa and Agou-Kloto. A third smaller cacao production area, Amou around Atakpamé, in the Central-Eastern area of the country is now infected but in smaller proportions than the two major producing areas (Komlan Wegbe, unpublished results).

CSSD is endemic to West Africa and has never been reported in South America, the cacao tree's area of origin. Additionally, CSSV has not been reported in Sao

Tome or Fernando Po (Tinsley 1971) although these islands are the main crossroads of introduction of cacao from the American continent toward West Africa. A viral disease causing similar leaf symptoms was reported in Trinidad (Kirkpatrick 1953; Swarbrick 1961), but was not associated with swellings, and the disease has not been reported recently (Lockhart and Sackey 2001) as the result of an effective eradication campaign. The existence of a disease similar to CSSD in Malaysia, Indonesia, and Sri Lanka (Kenten and Woods 1976; Peiris 1953; Crop Protection Compendium 2002) has been mentioned but only as an attenuated form of CSSD, and there is no molecular data available on this viral disease. Additionally, in Malaysia, the disease is likely due to the importation of infected clones (Liu and Liew 1979). Swellings were only reported in Sri Lanka (Orellana and Peiris 1957).

Elsewhere in the Americas, viruses have been suspected in the Paria Peninsula of Venezuela and in the Dominican Republic, but no transmissions were made in Venezuela, and in the Dominican Republic, the symptoms may have been confused with a mineral deficiency (Swarbrick 1961).

10.4 Biology of the CSSV

It was not until 1940 that through experimentation, the causative organism of the CSSD as recognized by Steven (1936) was identified to be a virus. The disease was successfully transmitted from swollen shoots onto healthy stocks of cacao trees by bud grafting (Posnette 1940). This virus was subsequently named as the cacao swollen shoot virus (CSSV), and the name "swollen shoot" was adopted by the Imperial Mycological Institute (Wiltshire 1946).

Symptoms are mostly seen in leaves, but stem and root swellings are the more characteristic symptoms (Fig. 10.2). In some varieties of cacao, particularly Amelonado cacao, reddening of primary veins and veinlets in flush leaves is typical. This red veinbanding later disappears. There can be various symptoms on mature leaves, depending on the cacao variety and virus strain (Posnette 1947; Thresh and Tinsley 1959). These symptoms can include yellow clearing along main veins, tiny pinpoint flecks to larger spots, diffused flecking, blotches, or streaks. Chlorotic vein flecking or banding is common and may extend along larger veins to form angular flecks.

Stem swellings may develop at the nodes, internodes, or shoot tips and may be on the chupons, fans, or branches. Many strains of CSSV also induce root swellings. Infected trees may suffer from partial defoliation initially due to the incomplete systemic nature of the infection. Ultimately, in highly susceptible varieties, severe defoliation and dieback occurs leading to the death of the tree (Fig. 10.2).

Smaller, rounded to almost spherical pods may be found on trees infected with severe strains. Occasionally, green mottling of these pods is seen, and their surface may be smoother than the surface of healthy pods. A few avirulent strains occur in limited, widely scattered outbreaks, usually inducing stem swellings only, and having little effect, if any, on growth or yield. As for other perennial crops, we

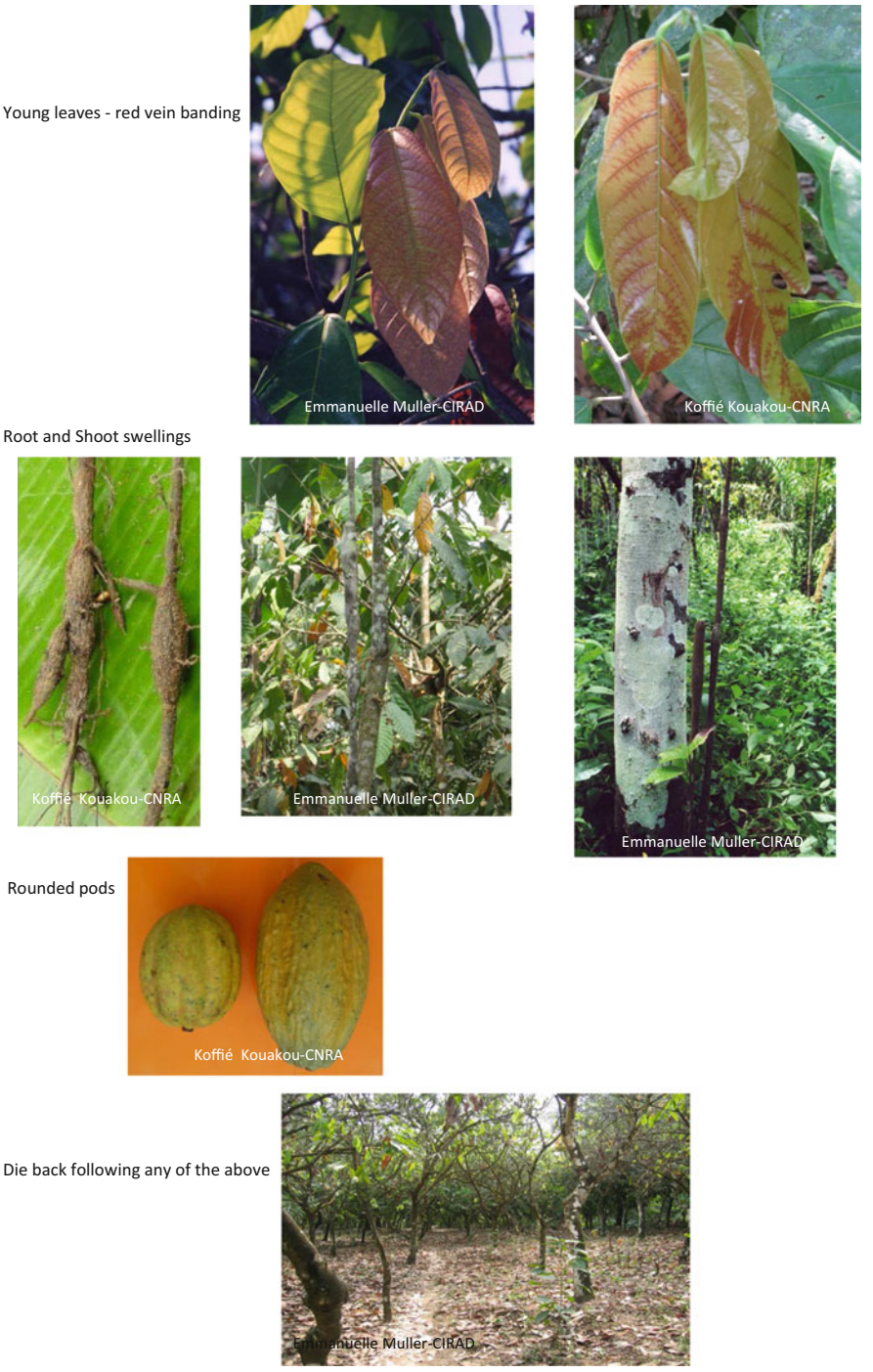

Fig. 10.2 Symptoms observed on cacao trees. (**a**) Red veinbanding on young leaves, (**b**) root, shoot and chupon swellings, (**c**) rounded pods, and (**d**) dieback resulting from previous specific symptoms. Photos by Koffie Kouakou, CNRA, and Emmanuelle Muller, CIRAD

observe a latency period between the time of infection and the time of symptom expression which complicates control measures. Moreover, there are periods of remission during which symptoms are not visible (Posnette 1947; Thresh and Tinsley 1959).

More than 100 isolates have been distinguished by the symptoms they cause in cacao, and this number would probably be increased if finer differences in symptom expression were considered. Posnette, in 1947, described the symptomatology and the geographical distribution in Ghana of four cacao virus isolates: A (New Juaben, Eastern Region), B (Bisa, Eastern Region), C (Kpeve, Volta Region), and D (Nkawkaw, Eastern Region). At that time, more than ten variants had already been distinguished (Posnette 1947). In 1971, Kenten and Legg described serological relationships between A, M (Mampong, Eastern Region), and Anibil (Western Region) isolates along with a C isolate also named cacao mottle leaf virus (Kenten and Legg 1971). However, symptoms are of limited value in indicating viral genetic relationships particularly with isolates from cacao. The results obtained from cross-protection tests between isolates inoculated successively on the same plant provide more reliable information on relationships between isolates. For example, two isolates from Offa Igbo and New Juaben cause similar symptoms on cacao yet do not cross-protect against each other, suggesting they are not closely related. By comparison, the mild and severe isolates from New Juaben cause dissimilar symptoms but show cross-complete protection (Thresh and Tinsley 1959). Currently, all of these forms of the disease are assigned to CSSV.

The structure of the virus was described after the virus was purified and observed by electron microscopy. The virus particles are rod shaped, and the mean dimensions were estimated at 120 × 28 nm (Brunt et al. 1964); they were later described as bacilliform and the virus later classified as a member of the genus *Badnavirus* (Fig. 10.3).

Jacquot et al. (1999) described several tissue modifications induced by CSSV after observing transverse sections of swollen cacao stem regions by light microscopy. CSSV-induced cell proliferation led to an increase in the number of cell

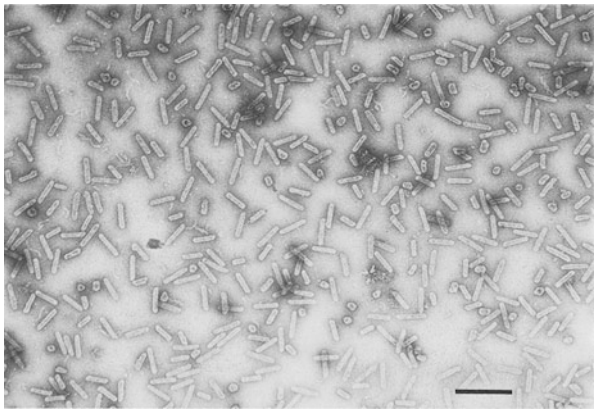

Fig. 10.3 Purified preparation of CSSV obtained after sucrose density gradient centrifugation. *Bar* represents 200 nm

layers in the xylem (threefold), in the phloem (twofold), and in the cortex (fourfold). The cambium of CSSV-infected tissues was restricted to a very few cell layers as opposed to the six cell layers in the normal stem. The radial organization of phloem fibers was disrupted. The cortex was devoid of mucilaginous cell cavities or canals, whereas the pith was not modified.

CSSV is naturally transmitted to cacao (*Theobroma cacao*) in a semi-persistent manner by several mealybug species, the vector of most badnaviruses. Fourteen species of mealybugs (*Pseudococcidae* spp.), including *Formicococcus njalensis*, *Planococcus citri*, *Planococcus kenyae*, *Phenacoccus hargreavesi*, *Pseudococcus concavocerrari*, *Ferrisia virgata*, *Pseudococcus longispinus*, *Delococcus tafoensis*, and *Paraputo anomalus*, have been reported to transmit CSSV (Dufour 1988). Only the nymphs of the first, second, and third larval stages and the adult females are able to transmit the virus (Brunt 1970). The young nymphs move about and so are more efficient vectors than the adults which are sedentary and inactive (Cornwell 1958). The virus does not multiply in the vector and is not transmitted to its progeny. CSSV is transmitted neither by seed nor by pollen (Posnette 1947) although the virus has been detected in embryos from pods collected from infected trees (Quainoo et al. 2008). CSSV can infect cacao at any stage of plant growth. The virus is transmitted experimentally to susceptible species by grafting, particle bombardment (Hagen et al. 1994), by agroinfection using transformed *Agrobacterium tumefaciens* (Jacquot et al. 1999), and with difficulty by mechanical inoculation (Brunt and Kenten 1962). Seedlings usually produce acute red veinbanding within 20–30 days and swelling on shoots and tap roots 8–16 weeks later (Fig. 10.4).

Fig. 10.4 Symptoms of swollen shoot observed on *Theobroma cacao* plantlets 4 months after agroinoculation with the Togolese Agou 1 isolate. From *left* to *right*: a plantlet inoculated with the wild strain *Agrobacterium tumefaciens* LBA4404 and two plantlets inoculated with the recombinant *A. tumefaciens* bacteria LBA4404 (pAL4404, pBCPX2) (Jacquot et al. 1999)

Accidental dissemination of the disease occurs by movement of infected pods after harvest and by the use of infected planting material. The virus is carried along by vectors surviving in the crevices of the pods and also in the budwood if it contains the virus in a latent or systemic phase (Adegbola 1971).

Susceptible alternative hosts were tested using mealybug transmission techniques. Of the tested plants which are indigenous from West Africa, Posnette et al. (1950) discovered seven species susceptible to cacao viruses (all assigned later to CSSV). All the CSSV-susceptible plant species were in the families Bombaceae, Sterculiaceae, and Malvaceae, from the order Malvales, which could help explain the origin of the disease in West Africa (discussed later). Tinsley and Wharton (1958) found 28 plant species of the 63 species tested were susceptible to CSSV. These were also in the same families Bombaceae, Sterculiaceae, and Malvaceae with the addition of five species in the family Tiliaceae. The indigenous species are more difficult to infect than cacao, and mealybugs do not become infective as readily when feeding on them as when feeding on infected cacao (Posnette et al. 1950).

Epidemiological characteristics of the disease derive directly from the natural transmission properties. The mealybug vectors (nymph of both sexes and adult females) spread the disease radially over short distances around the periphery of outbreaks by crawling through the canopy from infected trees to adjacent healthy trees. New outbreaks were shown to be associated with "jump spread" over greater distances by wind-borne viruliferous mealybugs or by the very active small first instar nymphs (Strickland 1950, 1951; Cornwell 1958; Thresh et al. 1988).

10.5 CSSV Genome

Badnaviruses are highly variable at both the genomic and serological level, a feature which complicates the development of both molecular and antibody-based diagnostic tests. Moreover, CSSV isolates were for a long time classified according to the variability of the symptoms expressed on *T. cacao*, and there is no obvious correspondence between this variability of symptoms and the intrinsic molecular variability of the virus. Molecular characterization of CSSV received a boost in 1990, thanks to the improvement of the purification techniques, and it was identified as a member of the genus *Badnavirus* (Lockhart 1990). CSSV possesses non-enveloped bacilliform particles, 130 nm × 28 nm in size (slightly different from the size established by Brunt et al. (1964) and a double-stranded circular DNA genome of about 7.5 kb (Lot et al. 1991).

The virus has been shown by dot-blot hybridization to occur in the cytoplasm of phloem companion cells and xylem parenchyma cells (Jacquot et al. 1999). The first complete sequence of a CSSV isolate (Agou1 from Togo) was determined in 1993 (Hagen et al. 1993). Five putative open reading frames (ORFs) are located on the plus strand of the 7.16 kb CSSV genome (Fig. 10.5). ORF1 encodes a 16.7 kDa protein whose function is not yet determined. The ORF2 product is a 14.4 kDa

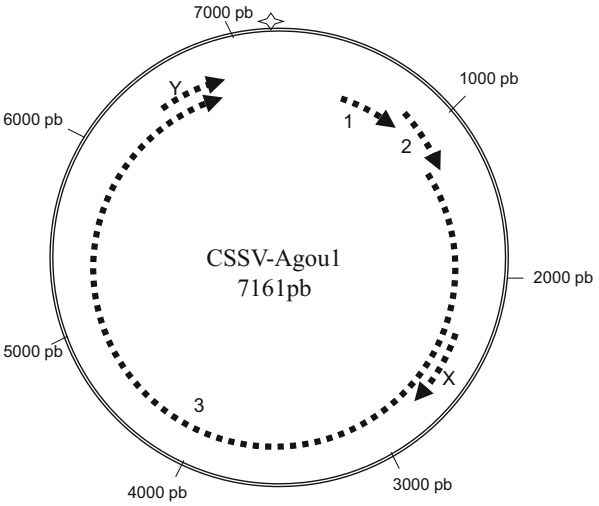

Fig. 10.5 Diagram of the CSSV genome (Agou 1 isolate). The circular double-stranded DNA genome (7161 bp) is represented by a *thin double circle*. Location of ORFs 1, 2, 3, X, and Y (*dotted arrows*) and the origin of replication (*diamond*) are indicated

nucleic acid-binding protein (Jacquot et al. 1996). ORF3, which is the largest, codes for a polyprotein of 211 kDa. The polyprotein contains, from its amino- to carboxyl-terminus, a consensus sequence for a cell-to-cell movement protein, an RNA-binding domain of the coat protein, an aspartyl proteinase, a reverse transcriptase (RTase), and a ribonuclease H (RNase H). ORFs X (13 kDa) and Y (14 kDa) overlap ORF3 and encode proteins of unknown functions. Comparison of the complete sequences obtained in 2005 shows that ORF-X is not conserved between isolates and may be fortuitous and without any biological signification, while ORF-Y is particularly conserved in size and nucleotide similarity (Muller and Sackey 2005).

Obtaining a more extensive study of the molecular aspects of CSSV variability is very relevant at this time for three reasons. Firstly, the knowledge of CSSV molecular variability will allow the improvement and the validation of a PCR diagnostic test (Muller et al. 2001) for better virus-indexing procedures. Secondly, the variability of the virus must be taken into account for resistance screening of new cacao varieties to CSSV. Lastly, a better understanding of the genetic diversity of CSSV in West Africa and elsewhere will in turn help to provide a better understanding of the development of epidemics, the evolution of viral populations, and their possible eradication.

Recently, the analysis of new complete sequences made it possible to gain a better idea of the variability of CSSV. The maximum nucleotide sequence variability between pairwise combinations of complete genomic sequences of CSSV isolates was 29 % (Muller and Sackey 2005; Kouakou et al. 2012). Phylogenetic relationships between Ghanaian and Togolese full sequences were more influenced by their geographical origin than on whether the sequences originate from mild or severe isolates. This confirmed the results reported before by Thresh and Tinsley (1959) showing that isolates producing identical symptoms are not necessarily

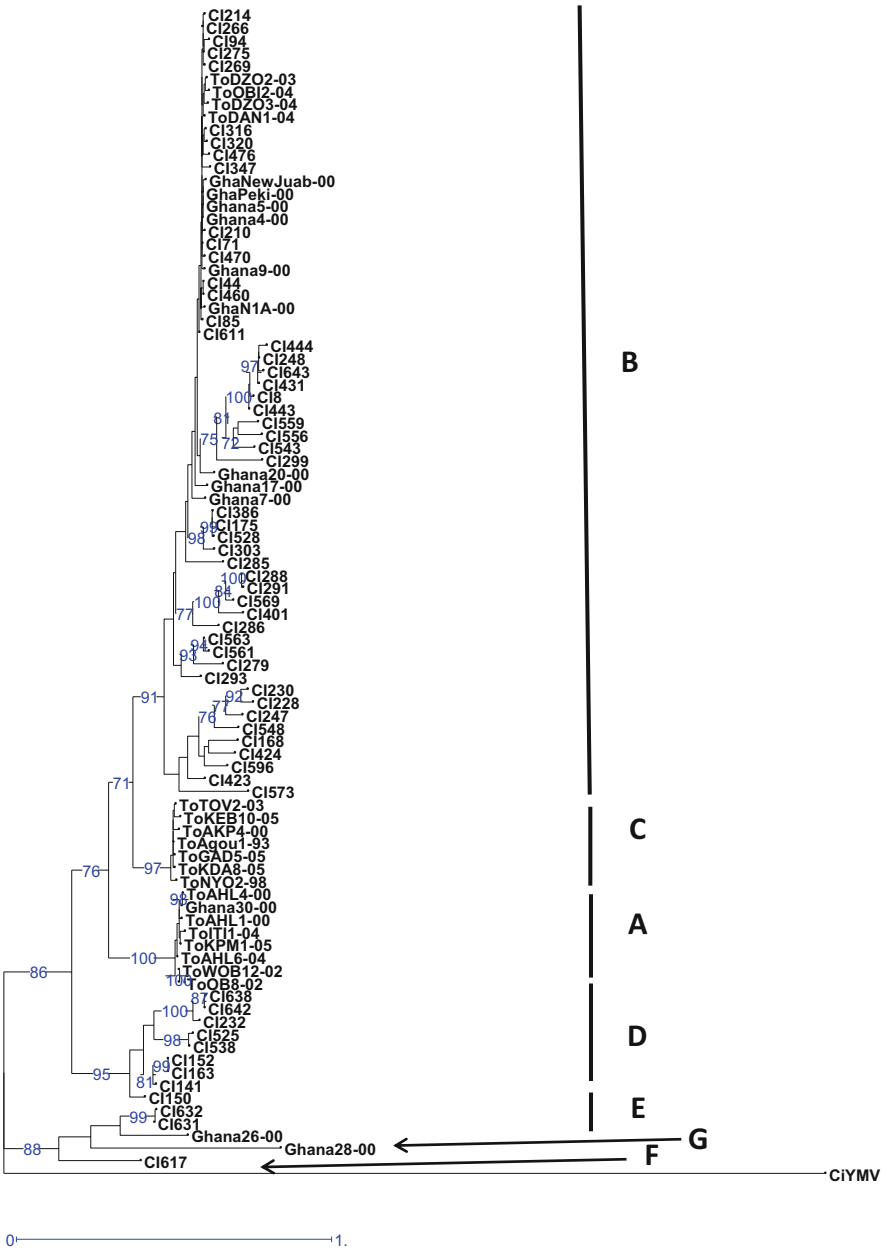

Fig. 10.6 Maximum likelihood phylogeny of CSSV sequences established by alignment of a 534 bp fragment of the first part of ORF3. Bootstrap values of 500 replicates are given above nodes when higher than 70 %. CSSV sequences representative of groups A, B, C, D, E, F, and G discovered in Togo, Côte d'Ivoire, and Ghana were used in comparison along with the Citrus mosaic virus sequence (CiYMV) (AF347695) used as the out-group. The names of sequences include the abbreviation of the country (CI for Côte d'Ivoire, Gha for Ghana, To for Togo), the abbreviation of the locality name or a sampling number, and the year of sampling (1993–2010 coded as 93–10). The names of sequences from Togo and Ghana are followed by their GenBank accession number

closely related at the DNA sequence level. CSSV populations have now been analyzed molecularly in the main West African countries (Côte d'Ivoire, Ghana, Nigeria, and Togo) (Oro et al. 2012; Kouakou et al. 2012). To date, this analysis has aligned the viral isolates into at least six structural groups according to the diversity in the first part of ORF3 (Fig. 10.6) and the 20 % threshold of nucleotide divergence with different well-structured subgroups for the B–C group. However, according to ICTV (International Committee on Taxonomy of Viruses) recommendations taking into account the nucleotide diversity in the RTase region, we could describe five different species, A, B–C, D, E, and G (data not shown). From these analyses, we can conclude that the CSSD is most likely due to a complex of viral species, reminiscent of the banana streak disease caused by banana streak virus (BSV), another badnavirus. Full sequences have been obtained and published for only three species of CSSV (A, B–C, D) (Muller and Sackey 2005; Kouakou et al. 2012) and have now been recognized as different viral species by ICTV.

As has been observed for most viruses studied, badnavirus populations contain recombinant viruses. This was illustrated for the first time for CSSV with the sequencing of isolate Wobe12 (species A) by Muller and Sackey (2005). Wobe12 possesses an ORF1 close to ORF1 (95.6 % nucleotide identity) of isolate Agou 1 (subgroup C of the B-C species) but shares only 75 % nucleotide identity with the complete sequence of Agou1. Recombinations allow the emergence of new viral isolates and increase the variability of viral populations beyond what is observed by studying a single region of the genome. The sites of recombination in the genome and their frequency should be extensively investigated in the future to have a better understanding of viral evolution during an epidemic.

10.6 Control Measures of the Disease

As a disease that does not persist in the soil and does not spread quickly or far in any considerable amount, the term "crowd disease" has been introduced by Vanderplank (1948), and the possible control measures have been described (Thresh et al. 1988).

Eradication Procedures

The official eradication campaign launched in Ghana in 1946 has been the most ambitious and costliest ever undertaken against a plant disease (Thresh et al. 1988). Replanting with healthy young trees is an integral part of the program. The initial eradication procedure was to remove only trees with symptoms. The adjoining trees within 30 paces were then inspected monthly and treated as necessary. Better results could have been obtained by removing all adjacent symptomless contacting trees or by even more drastic procedures of eliminating latent and missed

infections. However, this was not possible until 1957 because of the difficulty persuading farmers of the need to destroy seemingly healthy trees. The level of activity of the eradication campaigns has fluctuated greatly with serious interruptions and discontinuities as discussed in previous reviews (Owusu 1983; Thresh and Owusu 1986; Thresh et al. 1988), but surveys and treatments have continued on behalf of farmers, who have either received payments for trees removed or had trees replaced at the government's expense. Some innovations have been introduced, such as block plantings in the 1950s (Owusu 1983) and the "plant as you cut scheme" in the 1970s under which the government cut, replanted, and maintained the farms for some time before returning them to their owners.

There are many reasons to explain the difficulties in obtaining satisfactory results from the eradication measures. First of all, such measures require an extremely large amount of man power. The traditional practices of planting in Ghana are to establish small farms with irregular boundaries or an elongate shape, with very closely spaced trees planted to the very boundary of the available land. Additionally, seeds or seedlings used to replace dead or dying trees are sometimes planted adjacent to the older infected trees. All these practices favor the potential rapid reinfection of the treated plots (Thresh et al. 1988).

Cutting out infected trees has been used only to a limited extent outside Ghana. It was used for a time in Nigeria in the 1950s when drastic measures were adopted to treat outbreaks found outside the two worst affected "areas of mass infection." Treatments were then abandoned in all areas, and since then the policy has been to "live with" CSSD. This has been possible because CSSD in Nigeria is relatively mild and seldom kills trees, especially if mirids are being controlled.

To complete the eradication procedure and avoid reinfection of the treated areas, other methods should be adopted. The most effective is to clear whole areas and replant in large compact blocks because of the relatively small proportion of trees in the vulnerable peripheral areas. An isolation distance of 10 m or planting barriers or mixed cropping with banana, plantain, citrus, coffee, or other perennials that are immune to CSSV has also been recommended (Thresh et al. 1988).

Mealybug Control

CSSV is transmitted from infected trees to healthy trees by several species of cacao-infesting mealybug vectors and by no other means. This suggests that virus spread could be prevented or at least decreased by effective vector control. Considerable attention has been given to this possibility, but formidable difficulties have been encountered. The potential for using contact insecticides is limited because of the waxy secretions produced by mealybugs and by the carton tents constructed by some of the main vector species. This problem would be overcome by using a suitable systemic insecticide, but the difficulty is to identify one that is not prohibitively expensive and that can be used safely and without causing an undesirable

taint to or residues in the cacao beans produced by treated trees (Thresh and Owusu 1986).

Another possible approach to vector control is to do so indirectly by using insecticides to disrupt populations of attendant ants. This possibility was tried in Ghana in the 1950s, but the experiments were abandoned following undesirable side effects on the cacao fauna and the emergence of damaging insect pests that were previously unimportant.

Biological control of mealybugs by introducing or augmenting populations of natural enemies has also been tried and found to be unsuccessful. This is because mealybug vectors are indigenous, and populations are generally low and already under a high degree of natural predation (Thresh and Owusu 1986).

Mild Strain Protection

The use of mild strains in cross-protection has also been investigated as a means of biological control. Posnette and Todd (1955) successfully protected against the virulent New Juaben isolate in Ghana by previous infection of trees with mild strains of the virus. Despite this favorable result, however, many researchers still believe that cross-protection control should be sparingly used as a means of CSSD control because of the danger of the possibility of mild strains of a virus mutating into virulent ones especially if widely disseminated in millions of plants (Olunloyo 2001). Additionally, only a closely related mild strain is able to protect against another strain, as has been demonstrated for the *Citrus tristeza virus* (Folimonova 2013). It seems now that there are so many different CSSV isolates that it would be impossible to protect the cacao trees against them all.

Genetic Resistance

Until the 1950s, cacao plantings in West Africa were established almost exclusively with seed or seedlings of the uniform and very susceptible West African Amelonado variety. Attempts to control the disease through breeding started with the introduction of the Upper Amazon varieties (Posnette and Todd 1951), and some of the progenies have been particularly important like the "Series II" hybrids obtained by crossing selected Upper Amazon parents with either Amelonado or locally selected Trinitario type (Thresh and Owusu 1986). Today, immune varieties have not been identified despite all the studies conducted in the search for tolerant and resistant cacao trees (Adegbola 1971; Thresh and Owusu 1986). But, Upper Amazon × Upper Amazon hybrids with good agronomic characteristics have been demonstrated more resistant to infection than other genotypes being grown. Moreover, the ability of these hybrids to withstand infection was demonstrated in long-term field trials (Legg and Lockwood 1981). Even so, these hybrids have not been

produced in large quantities for distribution on a large scale. Benefits could be obtained by pursuing this approach so that hybrids with useful levels of resistance are used for all new plantings in CSSV-affected areas of West Africa.

Strict Indexing of Material for Export

The International Cocoa Quarantine Centre at Reading, UK (ICQC, R) handles all international movements of cacao breeding material. It is consequently in charge of the indexing procedure for cacao accessions used for budwood samples that are to be transferred from one country to another country. Buds are taken from imported budwood samples and grafted onto seedlings to raise new clonal plants at Reading. After 2 years of growth in the Quarantine Centre, the new plants are indexed on West African Amelonado (WAA) seedlings to determine whether the plants harbored *cacao swollen shoot virus* (CSSV). The new flush leaves formed on the WAA indicator plants are observed for virus-like symptoms.

10.7 Origin of the Disease/Current Reemergence

As this disease appeared soon after the introduction of cacao to West Africa and has been recorded in only some of the cacao-growing areas of West Africa, it is assumed to have originated in the wild vegetation of the secondary forests of West Africa. The presence of naturally infected wild hosts, the widespread potential susceptibility in indigenous species within the order Malvales, and the apparent uniqueness of the CSSV provided strong support for the idea that the virus was native to West Africa.

Indeed, some indigenous plants of the same family or of families closely related to *Theobroma cacao*, from the order of the Malvales, serve as a natural reservoir for the CSSV. In fact, the disease can be experimentally transmitted from *Ceiba pentandra*, *Cola chlamydantha*, *Cola gigantea*, and *Adansonia digitata* to cacao seedlings by mealybugs that are vectors of CSSV (Posnette et al. 1950). However, judging from the rates of transmission observed in these experiments, the virus concentration usually falls to a low level after the alternate hosts have been infected for a few months. The known wild hosts of cacao viruses are apparently tolerant of the virus, but not all plant species that harbor the virus show symptoms of the disease (Posnette 1981; Legg and Bonney 1967). The tendency is for symptoms in wild host species to occur only when newly infected. New shoots arising from a coppiced stump often show a recurrence of symptoms, presumably associated with a higher virus load (Posnette et al. 1950).

The high incidence of CSSV infection in *Cola chlamydantha* around the Wiawso District of the Western province of Ghana, and elsewhere, and the occurrence of infected *C. chlamydantha* specimens where CSSD is unknown strongly suggests

that viral infection of *Cola chlamydantha* antedates that of cacao (Todd 1951). The role of *Cola chlamydantha* as indigenous primary host of CSSV seems clear in its geographical range: Ivory Coast, the Western Province of Ghana, and Southern Nigeria. Outside this area, in Northern Nigeria, Central and Eastern provinces of Ghana, *Ceiba pentandra* and *Cola gigantea* are commonly found on cacao farms and are favored host species of the vectors and could equally act as primary hosts. A *Ceiba pentandra* tree was surprisingly observed by Attafuah et al. (1963) in the center of the first described outbreak of CSSD in Sierra Leone.

The species *Ceiba pentandra* is regularly described as originating in South America. However, the fact that the *Ceiba pentandra* is widespread in West Africa and was found naturally infected by CSSV in Ghana by Posnette et al. (1950) suggests the probable origin of this species was in both West Africa and in South America. Most of these experiments on the host range of CSSV and the positive transmissions from potential indigenous hosts to cacao trees have been conducted at the Cocoa Research Institute of Ghana (CRIG) formerly WACRI.

Indigenous plant species may even be new sources of inoculum. However, the low percentage of highly infected indigenous hosts and the differences in size between the cacao and the alternate host suggest transmission by this route is infrequent. Obviously, even the infrequent transmission of CSSV from alternate host to cacao was sufficient to initiate severe epidemics in cacao. Now that the disease is widespread on cacao in West Africa, the role of indigenous hosts is likely less prominent in the creation of new foci of the disease. Regardless, wild alternate hosts have the potential to contribute to the emergence of new aggressive virus species/isolates by new host shifts to cacao. This is of special concern since selective pressures in alternate host plants may induce a differential evolution of the virus as compared to its evolution in cacao.

The high variability observed within CSSV populations compared to its very short evolutionary history on cacao trees further suggests the existence of many parallel emergences, more likely by host shifts from different native hosts to cacao trees in the various countries of West Africa (unpublished results). Additionally, the geographic distribution of the CSSV diversity through the different countries of West Africa (Fig. 10.7) suggests the emergence of some CSSV isolates with a restricted dispersal in only one region unlike other isolates. For example, group B isolates are widespread in all the four countries analyzed. Four species of CSSV have been detected in Côte d'Ivoire so far (B-C, D, E, and F) with a very restricted dispersion of the E and F species. Only two CSSV species are present in Togo, subgroup C of the B-C species in the Kloto region and subgroup B and species A in the Litimé area (Wawa region). CSSV was identified much earlier in Ghana than any other country, and the number of species detected in Ghana is much higher. This is presumably due to the earlier introduction and cultivation of cacao in Ghana and its quicker adoption and larger total land devoted to its production. We detected four different species in the subsampling of the CSSV CRIG collection in Tafo, but recent sampling in infected plots located in several regions of Ghana suggests there may be more than seven species present (unpublished results, Abrokwah et al.).

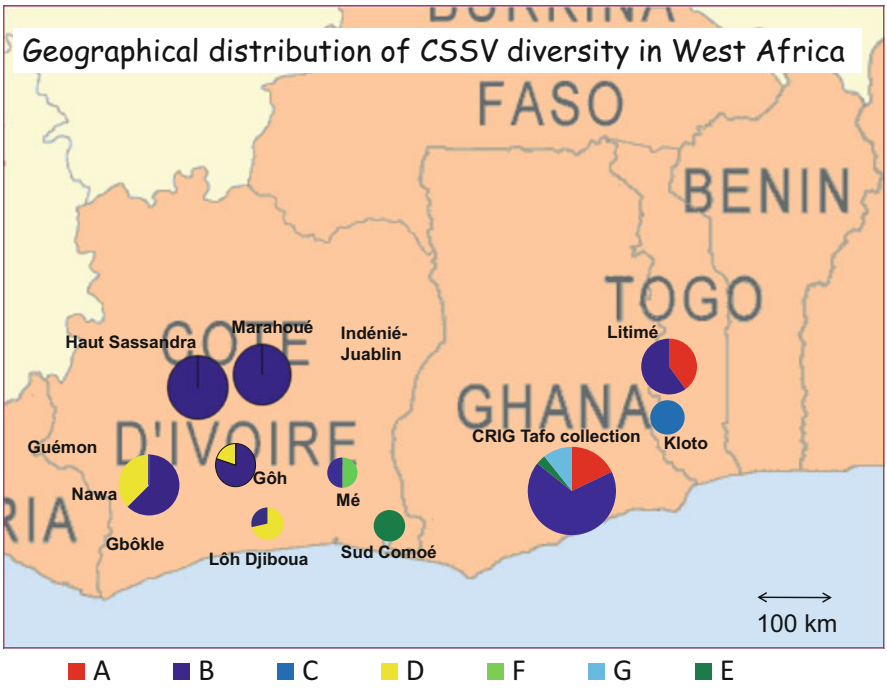

Fig. 10.7 Spatial distribution of CSSV groups of isolates in the different countries of West Africa

Different hypotheses have been developed to explain the diffusion of the disease from Ghana where it first emerged. In particular, the more widespread and very homogenous subgroup B (species B-C) can be detected in the main West African countries (Ghana, Ivory Coast, and Togo) where cacao is grown. Actually, the B subgroup has also been detected in Nigeria (Obok and Wetten, unpublished results). With subgroup B, it is possible to argue the diffusion of the disease occurred due to the movement of infected scions of Ghanaian hybrids as opposed to the parallel emergence of the same virus from the same alternative host plant species in very distant areas.

The increase in exchanges in cacao germplasm between different areas of a country, or between different countries, as a consequence of climate and environmental changes or political crisis, might explain the concomitant reemergence of the disease in the four West African countries. In particular, the migration of farmers from old infected areas, because of soil depletion or because of war in their countries, may have contributed to the spread of the disease by carrying with them contaminated germplasm to new areas.

It is also likely that the cutting of forest trees (deforestation) that harbored mealybugs in their canopies may have facilitated the transfer of CSSV vectors onto cacao trees. The continuing deforestation of Southern Ghana and Côte d'Ivoire and increased sun exposure appear to be associated with changes in insect fauna

compositions and populations. There has been a decline in populations of what was formerly the predominant vector (*Formicococcus njalensis*) and an increase in more mobile species (Thresh and Owusu 1986; Bigger 1981). Although not well studied, climate change may also be impacting the movement and evolution of mealybug populations.

10.8 List of Important Points for Future Research

To develop a polyvalent and early CSSV diagnostic that is faster than the indexing procedure by grafting onto susceptible stocks. The versatility of the diagnosis requires that the variability of the virus be exhaustively studied.

To set up a rigorous screening of cacao trees resistant to the virus, which will take into account the genetic diversity of the virus, as the variability of the virus has yet to be taken into account in the various programs to select resistant cacao trees. Knowledge about the stability of the different diversity groups over time will confirm the strategy of taking one or two isolates that are representative of each group to screen cacao trees for CSSV resistance.

Additional research is also required on alternative hosts of CSSV to confirm the use as barrier crops of nonhost CSSV plants.

New initiatives to gain additional information on the socioeconomic as well as agronomic factors involved successful replanting and such aspects as the degree of isolation required, the choice of barrier crop, and the amount of third-party support and intervention needed to locate and treat sources of infection and to carry out routine surveys.

Acknowledgment I wish to thank Mike Thresh for the numerous interesting discussions that help me approaching the problem of CSSV in its entirety. I thank Colin Campbell who helped me to write the tribute to Mike Thresh as an introduction of this chapter. I am grateful to Komlan Wegbe, Ekemini Obok, Francis Abrokwah and Henry Dzahini-Obiatey for giving to me some unpublished information.

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Part V
Pathogens of Local Concern and Potential to Spread

Chapter 11
Fruit and Canopy Pathogens of Unknown Potential Risk

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Abstract The worldwide threat to cacao production from the major pests and diseases continues to overshadow other problems of local or sporadic importance. Since the introduction of cacao (*Theobroma cacao*, L.) from its center of diversity in the Amazon forest into other regions of the world, several diseases, often regarded as minor pests, affects production of the crop in various cacao-producing regions and countries. These diseases may not be globally important, but may have very great local impact on production wherever they are found. Among these minor diseases are pink diseases caused by *Erythricium salmonicolor*, charcoal pod rot, caused by *Lasiodiplodia theobromae*, warty pod, a disease of unknown etiology, mealy pod caused by *Trachysphaera fructigena*, thread blight caused by *Marasmius* species, brown root rot caused by *Phellinus noxious*, and anthracnose of cacao caused by *Colletotrichum* species. Most often, the symptoms of cacao pod diseases are similar, and this often confuses farmers and extension personnel, resulting in either the overestimation or underestimation of these diseases in the field. This chapter discusses the situation and outlook, taxonomy, distribution, symptoms, modes of spread, and impact of these diseases on cacao. It also discusses control measures available for these diseases and future prospects for their management.

11.1 Pioneers in Cacao Plant Pathology Research: Dr. Job Thomas Dakwa

The time has come to acknowledge the contribution of one of the pioneers in cacao plant pathology. His name was Dr. Job Thomas Dakwa, but "Thomas" from biblical parlance was never a doubter or a double-minded person. Dr. J. T. Dakwa or J. T. as

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Fig. 11.1 Pioneers in cacao plant pathology research—Dr. Job Thomas Dakwa

he was popularly called was rather a man of strong conviction, who never compromised on his principles (Fig. 11.1). Anyone who has worked on cacao diseases and followed the International Cocoa Research Conferences around the world will certainly recognize the name, J. T. Dakwa. Dr. J. T. Dakwa was born at Begoro in 1936 as the third of six children and died in 2011. J. T. was an exceptionally brilliant student and a legendary mathematician. Thus, it took J. T. four, instead of five, years to complete his higher school education at Abuakwa State College. J. T. Dakwa completed his B.Sc. Degree program in Zoology, Botany, and Physics at the University of Ghana, Legon, in 1963, after which he was employed at the then West African Cocoa Research Institute (WACRI) now Cocoa Research Institute of Ghana (CRIG). However, after a few months, he left for a Master's Degree Program at the University of Ghana, Legon. Following the completion of the Master's Degree, J. T. was employed at CRIG in July, 1965. In 1967, he won a scholarship to pursue a Ph.D. study at the Imperial College of London, UK. Upon his return from the UK, Dr. Dakwa immersed himself in researching the pathology of the cacao tree and finding solutions to the diseases of this major cash crop of Ghana. He was particularly interested in assessing the effects of weather on the rates and patterns of disease development and researching the epidemiology of cacao black pod disease caused by *Phytophthora palmivora*. For many years cacao researchers had noted the morphological differences among *Phytophthora* isolates causing disease in *Theobroma cacao*, as well as differences in the geographical distribution, and aggressiveness. J. T. Dakwa was among the team of researchers who met at the Cocoa *Phytophthora* Workshop in Rothamsted Experimental Station in Harpenden, UK, in 1976 to elucidate the controversy of many morphological forms (variants) of *P. palmivora*, which resulted in the redesignation of the MF3 as *P. megakarya*. Dakwa was the first scientist to recognize the presence of *P. megakarya* as a black pod pathogen on cacao in Ghana and initiated pragmatic control measures of the disease (Dakwa 1987). This finding changed the focus of black pod disease research in Ghana. Dakwa's presence and contribution to the many International cacao conferences, seminars, and workshops were most notable. Not only did he work on *Phytophthora* diseases of cacao, but he also researched and published in Plant Disease Reporter and

Turrialba on mealy pod and *Colletotrichum* on cacao (Dakwa 1972a, 1974, 1976a, 1984, 1988, Dakwa and Danquah 1978) and on coffee rust caused by *Hemeleia vastatrix*. J. T. Dakwa also coauthored with A. Asare-Nyako the chapter on Black pod disease of cacao: the disease on roots. In: *Phytophthor*a Diseases of Cocoa Edited by P.H. Gregory. Apart from his busy work schedule, J. T. found time to pursue other professional interests. He was a member of the Ghana Science Association, The British Mycological Society, The Association of Applied Biologists, the Federation of British Plant Pathologists, and the International Society of Plant Pathologists (ISPP). He published proficiently in the journals of these associations. Further to his research work, J. T. Dakwa was a great teacher, and as a part-time lecturer at the Crop Science Department of the University of Ghana, Legon, in 1971–1972 and again in 1989–1990, he unearthed and nurtured the hidden talents of his students. J. T. Dakwa bridged the gap between scientific research and application of research findings in the field by visiting and advising farmers on pre- and post-harvest diseases of vegetables, root crops, fruits, and plantain both at the University of Ghana Research Station at Kade and at the Akomadan area. J. T. Dakwa was not only a bookworm and an accomplished researcher who made a deep impression on younger scientists but also an avid sportsman, playing both football and hockey. J. T. Dakwa played his role as an astute researcher, made major contribution to cacao mycology in particular and cacao plant pathology in general, and must be eulogized for his contribution to our understandings of cacao diseases.

11.2 Pink Disease (*Erythricium salmonicolor*) (Berk. & Broome) Burdsall

Situation and Outlook

Pink disease caused by *Erythricium salmonicolor* (Berk. & Broome) Burdsall (syn. *Corticium salmonicolor* Berk. & Broome) (syn. *Phanerochaete salmonicolor* Berk. & Broome, Jűlich) has been known in cacao for many years (Stockdale 1909), but has only been studied in detail as a pathogen of rubber (Holliday 1980a) and as the causal agent for canker in *Eucalyptus* spp. (Old and Davidson 2000). The common name of pink disease in French is '*malaidie rose*'; in Spanish and in Portuguese it is '*malrosado'*. Taxonomically, Erythricium belongs to the family, Phanerochaetaceae; order, Polyporales; class, Agaricomycetes and Phylum, Basidiomycota.

Erythricium salmonicolor (Berk. & Broome) Burdsall is not only a commonly distributed fungus, but it is also virtually omnivorous, having been found on many plant species and over 141 genera (Smith 1985). The host plants of economic importance of the pathogen include rubber, tea, coffee, cacao, grapefruit, orange, nutmeg, mango, apple, coca, and kola. In New Guinea and Malaysia, pink disease is associated with cover crops and shade trees such as pigeon pea (*Cajanus cajan*) and *Crotalaria*, *Tephrosia*, *Leucaena*, and *Gliricidia* (Wood and Lass 1985). The

pathogen has been reported in cacao in Brazil, Columbia, Ghana, Nigeria, Malaysia, Papua New Guinea, Peru, Western Samoa, and Trinidad. *Erythricium salmonicolor* was first reported in Ghana by Wharton (1962a) as a minor disease, but a current survey of cacao diseases in Ghana indicates that pink disease is spreading in the country and emerging as an important disease of cacao (Akrofi et al. 2014).

Symptoms

Pink disease of cacao is characterized initially by dieback of infected branches with the brown dead leaves within the otherwise green canopy of infected trees. The dead leaves remain attached to the declining trees for several weeks. The disease appears as a coating of brown to pink incrusted hyphae (fruiting bodies) with yellowing and browning of leaves and complete defoliation on severely affected branches and twigs. A closer observation of an infected branch reveals a coating of drab paint on the branch. Irrespective of age, affected branches manifest typical pinkish to orange coloration (Fig. 11.2a) and dieback symptoms, desiccated flowers (Fig. 11.2b), and mummified fruits (Fig. 11.2c) often attached to declining trees for several weeks. On the bark, a sparse white mycelium (web) is seen which is easily overlooked when the bark is wet. The affected bark of infected trees is usually dry with several longitudinal cracks (Fig. 11.2d). Although tree dieback occurs throughout the year, symptoms are more prevalent during the dry season, especially for trees on farms with little or no shade.

Four distinct growth forms of pink disease were identified on the bark of infected cacao trees in Ghana (Akrofi et al. 2014). These are cobweb, pink to salmon-colored incrustation, creamy pustules and orange fruiting bodies. The cobweb stage appears as a layer of light white to pink-colored vegetative mycelia on the surface of the bark (Fig. 11.2e), and it is followed by the formation of pink to salmon-colored pustules on any part of the infected branch (Fig. 11.2f). The third growth stage consists of creamy pustules which are more conspicuous on the underside of the infected branch (Fig. 11.2g); the creamy pustules develop into orange fruiting bodies on the dying infected stems, branches, and twigs (Fig. 11.2h). Also, Akrofi et al. (2014) observed variations in the colony morphology of *E. salmonicolor* isolated from the cobweb (Fig. 11.2i, j) and pink-colored pustules (Fig. 11.2k, l) after different periods of incubation at 28 ± 2 °C. On agar medium, the colonies from cobweb growth appeared cottony-white with thread-like mycelia at the advancing margins (Fig. 11.2i) similar to that reported by Mordue and Gibson (1976) but turned creamy with age (Fig. 11.2j). In contrast, colonies from pink pustules were initially pink (Fig. 11.2k) but turned creamy (Fig. 11.2l) within one week.

All four growth forms may be found together on the diseased bark at the same time, but the most conspicuous and distinctive are the salmon-pink encrustations formed by hyphal fruiting bodies on branches and stems of the tree, causing twig and branch injuries, stem canker, and eventually host plant death. Affected

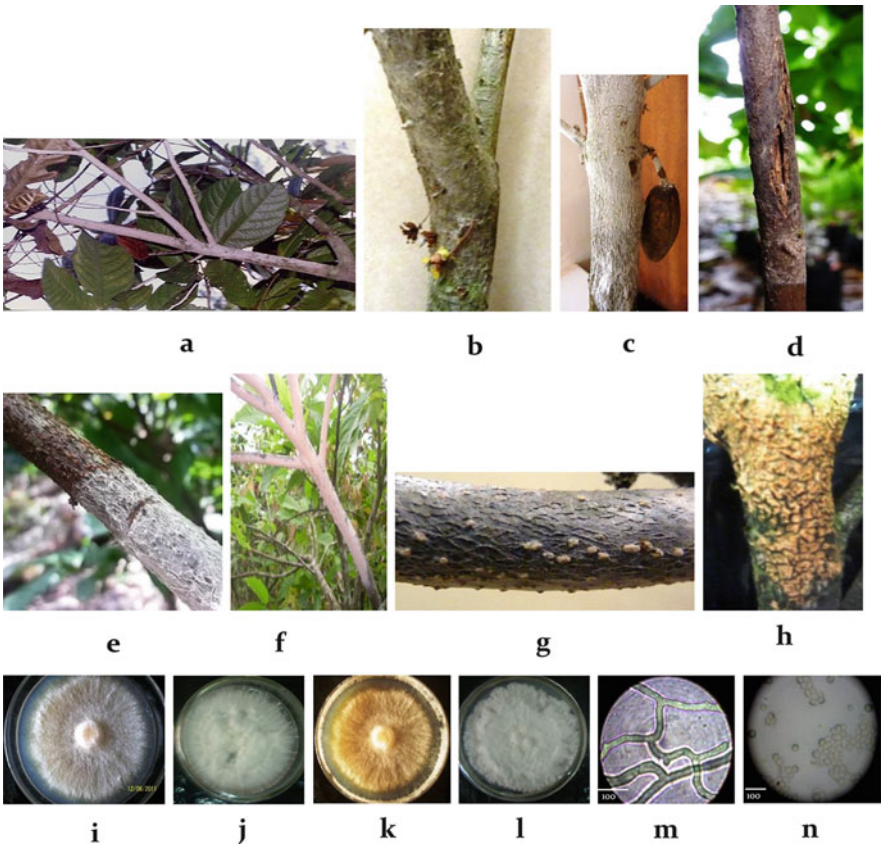

Fig. 11.2 Symptoms of pink disease on *Theobroma cacao*, L. *Top panel*: infection on trunk or branch showing (**a**) *pinkish* colouration of branch, (**b**) desiccated flowers on trunk, (**c**) mummified pod on trunk, and (**d**) severe longitudinal cracks on bark of declining infected trunk. *Middle panel*: development stages of *E. salmonicolor* on cacao showing (**e**) vegetative mycelia mat, (**f**) *pink* to *salmon-coloured* pustules, (**g**) creamy pustules, and (**h**) *orange-colored* pustules on a branch. *Lower panel*: colony morphology on Basidiomycetes Selective medium at 28 ± 2 °C from cobweb growth stage of *E. salmonicolor* (**i**) 7 days after inoculation, (**j**) 28 days after inoculation, (**k**) colony morphology from *pink*-colored pustules stage 3 days after inoculation, (**l**) 7 days after inoculation, (**m**) microscopic examination showing hyaline non-septate hyphae, and (**n**) microscopic examination showing irregularly shaped hyaline, unicellular, ellipsoid spores from pustules on naturally infected branches

branches become defoliated and die. The hyphal system is monomitic, hyaline, smooth, distinct with sparse branches usually at a fairly wide angle. There are no clamp connections evident (Fig 11.2m). The fungus produces two types of spores depending on the life cycle stage. One spore type (the basidiospore) is produced from a basidioma, the characteristic pink/white or pink/orange crust that develops mostly on the underside of the branch. Although it can develop to encircle the entire stem it can sometimes reach 2 m in length. The crust is initially smooth, but it

cracks and becomes paler as it ages. Basidiospores are broadly ellipsoidal with prominent apiculus, thin-walled, smooth, not amyloid, dextrinoid or cyanophilous, 10–13 × 6–9 μm. The other spore type (the conidia) is produced from orange/red blisters (pustules) scattered over the bark surface. The conidia are oblong to irregular in shape, thin-walled, catenulate, and hyaline (Fig. 11.2n). The spores measure 10–24 μm × 8–12 μm (Opoku et al. 2001).

Survival of E. salmonicolor

Erythricium salmonicolor survives dry periods in latent cankers (Luz 1983). Formation and release of basidiospores are dependent on regular heavy rainfall and the release usually starts 20–80 min after the start of rain. More spores are produced when rainfall is light and of shorter duration (Luz et al. 1985) and spore release can continue for up to 14 h after rainfall (Schneider-Christians et al. 1986). The spores must settle on moist brown bark for successful germination and penetration (Luz and Ram 1980). Basidiospores germinate and penetrate through undamaged bark tissues (Verma and Munjal 1983). In water, germination of basidiospores starts 60–90 min after release and 100 % germination occurs after 210 min (Schneider-Christians et al. 1986). Conidia remain viable for around 20 days under dry conditions, but high humidity is required for their germination.

Disease Spread

High humidity, heavy shade, and precipitation are important factors in the spread of the disease (Cook 1913; Briton-Jones 1934). The agents of spore dissemination are wind (Almeida and Luz 1986), rain splash (Schneider-Christians et al. 1983), red ants, and other insects (Briton-Jones 1934). In 1999, pink disease was first found on an experimental plot at Bunso in the Eastern region of Ghana and thereafter in farmers' fields 400 km away in the Western region. This discontinuous distribution of the disease on cacao farms in Ghana suggests that human activities may be involved in the spread of the disease.

Disease Impact

Pink disease can cause significant losses, ranging from the loss of individual branches to death of the whole tree if the main stem or several branches are affected. The pathogen usually causes girdling cankers which disrupts the physiological processes leading to defoliation and death of the distal parts of the tree. This ultimately results in reduction in photosynthetic production and subsequent

reduction in cacao yields (Akrofi et al. 2014). Flowers as well as pods are infected and pod numbers in both the canopy and on the trunk decrease as the severity of the disease increases. Disease incidence of about 80 % or more in cacao was reported in Western Samoa (Schneider-Christians et al. 1983) and the disease was also reported to severely affect 2–6 year-old plants, causing death of the whole tree (Brown and Friend 1973; Holliday 1980a). In Ghana, cacao yield losses due to the disease on experimental plots ranged between 60 and 100 % (Opoku et al. 2001).

Disease Control

The wide host range and several agents of spore dissemination of *E. salmonicolor* make local exclusion and eradication difficult as cross-infection can occur readily. Effective cultural control can be achieved through ensuring good drainage, shade reduction, and removal of susceptible forest and shade trees (Wood and Lass 1985). The disease can also be controlled by frequent pruning and burning of infected parts, supplemented with fungicide treatment (Opoku et al. 2001). However, this practice is effective only if the disease can be recognized early. Fungicides reported to show activity against *E. salmonicolor* include copper formulations such as Bordeaux mixture, copper oxychloride, copper carbonate (Ram et al. 1982; Kueh 1986; Thankamma 1989; Thankamma et al. 1986); tridemorph paints in an ammoniated latex base (Edathil and Jacob 1983); triadimefon granules (Villarraga 1987); chlorothalonil paints in a latex/bitumen base (Anon 1985); and fenpropimorph (Luz and Figueiredo 1982). In Ghana, cultural control, involving pruning of infected branches supplemented with 3–4 weekly spraying of copper hydroxide or copper–metalaxyl mixtures, was effective for the control of pink disease (Opoku et al. 2001; Akrofi et al. 2014). Early detection of pink disease enhances control.

11.3 Warty Pod

Warty pod of cacao, a disease of unknown etiology, is currently common on cacao farms in West Africa. The symptoms of the disease on cacao were described by Wharton (1962b), but the causal pathogen has not been identified. The symptoms include several protrusions of different ages, different sizes, and different shapes on young green pods, previously described as pyramid-shaped protrusions by Lass (1985). These protrusions appear like the early symptoms of frosty pod rot. The protrusions increase in size with age of the pod, turn yellow, and then into dark-brown lesions on ripening of the pod. The lesion, on the ripened pod, unlike that of Phytophthora infection is soft when pressed with the thumb and also without spores as with Phytophthora lesions, distinguishing warty pod infection from that of Phytophthora pod rot. Beans from ripened warty infected cacao pods are good and fermentable. Lass (1985) reported that *L. theobromae* can be isolated from

dark-brown lesions of warty pods. However, in our laboratory at the Cocoa Research Institute of Ghana, *L. theobromae* and *Fusarium* species were frequently isolated from the dark-brown warty pod lesions. These fungi could not induce the disease when inoculated onto pods, suggesting that they colonize the soft lesions and may not be responsible for causing warty pods. Damage is often localized, confined to pods on a few trees or several pods on the same tree. Currently, losses from the disease are usually unimportant to warrant any control measures.

11.4 Lasiodiplodia Pod Rot (*Lasiodiplodia theobromae*)

Situation and Outlook

Lasiodiplodia pod rot caused by *Lasiodiplodia theobromae* (syn. *Botryodiplodia theobromae* (Pat.) Griff. and Maubl) is the asexual state of the fungus *Botryosphaeria rhodina* (Berk and M. A. Curtis) Arx. *Lasiodiplodia theobromae* is also called "charcoal pod rot" or "diplodia pod rot" and in the West Indies "brown pod rot." The common name of Lasiodiplodia pod rot in French is "pourriture noire," in Spanish it is "podredumbre de carbon," and in Portuguese "podridão-carvão." In French and in Portuguese, brown pod rot refers to Phytophthora pod rot. It is therefore important to attach the name of the causal pathogen to the disease to avoid this confusion. Taxonomically, *Lasiodiplodia* belongs to the family, Botryosphaeriaceae; order, Botryosphaeriales; class, Dothideomycetes; and subclass, Ascomycota. The Botryosphaeriaceae are cosmopolitan and have a wide host range estimated to be more than 280 species ranging from monocotyledonous, to dicotyledenous, to gymnosperms (Domsch et al. 2007; Sutton 1980).

Lasiodiplodia species are common, especially on tropical and subtropical woody trees where they cause a variety of diseases. The pathogen relies on pruning wounds, earlier infection by other pathogens, and other stresses on the host for them to become opportunistic pathogens, causing branch and stem canker, gummosis, shoot blights, stem end rot, fruit rot, and/or dieback of many woody plants (Abdollahzadeh et al. 2010; Adesemoye et al. 2013; Slippers et al. 2005). *Lasiodiplodia theobromae* causes dieback and gummosis in mango trees and end rot of mango fruits in Sindh, Pakistan (Khanzada et al. 2004a, b); black branch and dieback disease of cashew in Brazil (Cardoso et al. 2002); collar rot of peanut in Virginia and North Carolina (Phillips and Porter 1998); gummosis of *Jatropha podagrica* in China (Fu et al. 2007), and collar and root rot of physic nut in India (Latha et al. 2009). *Lasiodiplodia* also causes tuber rot of yam, crown rot of banana, and charcoal pod rot of cacao (Sangeetha et al. 2011; Rossel et al. 2008; Opoku et al. 2007a, b; Khanzada et al. 2004a, b; Jiskani 2002).

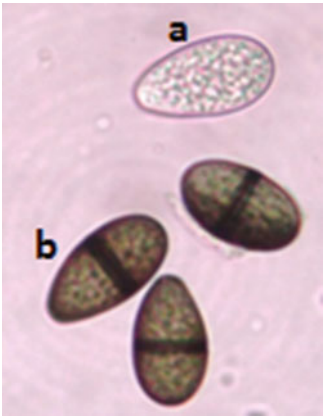

Fig. 11.3 Young unicellular, hyaline, granulose, ellipsoid thick-walled conidia of *L. theobromae* (**a**) and mature uni-septate conidia of *L. theobromae* (**b**)

Lasiodiplodia. theobromae was first reported on cacao as a minor charcoal pod rot disease in Cameroon in 1895, but since the late 1980s, serious dieback disease affecting cacao trees and attributed to *L. theobromae* has been reported (Mbenoun et al. 2007). Dieback disease associated with *Lasiodiplodia* had earlier been detected in cacao in Ghana in 1908 (Dungeon 1910). The pathogen is reported to be present in about 70 % of cacao farms surveyed and was associated with yield losses of about 80 % in Nigeria (Onyenka et al. 2005). Also in Ondo state of Nigeria, four different species of Botryosphaeriaceae were isolated from trunks, branches, and pods of cacao and these species were found to have some interaction with *Phytophthora* on the crop (Jaiyeola et al. 2014).

Symptoms

The pathogen is found all year round on both young and mature wounded cacao pods. On the pod, the disease starts as a brown lesion which turns black. The lesions subsequently produce a lot of black conidia so that diseased pods become covered with black sooty charcoal-like powder (Fig. 11.3c) in marked contrast to the white sporangia of Phytophthora pod rot and white conidia of *Trachysphaera fructigena* (mealy pod) (Fig. 11.3d). Also, the conidia of *Lasiodiplodia* are easily blown by wind in contrast to sporangia of *Phythophthora*. As a wound pathogen, infections are common on wounded pods, particularly, those pods eaten by rodents and birds.

Lasiodiplodia and species of *Fusarium* have been identified as inducing dieback disease and causing substantial yield loss in most cacao-producing countries in West Africa. The dieback is initiated by systemic growth of causal fungal pathogens via Mirid feeding wounds (lesions) to the stem, where cankers develop, causing tree death in more severe cases (Adu-Acheampong 2009). Irrespective of age, affected cacao trees manifest typical dieback symptoms. Leaves on the outer twigs turn yellow and the damage may then extend along the whole branch,

reaching the main trunk, eventually resulting in tree death. The twigs and branches of diseased trees show internal discoloration with brown streaks in the vascular tissue. White or yellowish exudates from infected trunks (gummosis) have also been reported (Adu-Acheampong 2009). Although sudden widespread wilting and death may occur, affected trees more typically decline over several months, during which time flushes of new growth may develop at the collar of declining trees. Desiccated leaves and mummified fruits remain attached to declining trees for several weeks. Although tree mortality occurs throughout the year, symptoms are more severe during the dry season, especially for trees with little or no shade (Mbenoun et al. 2007).

According to Sutton (1980), the genus is based on *Lasiodiplodia theobromae*. The colonies are grayish sepia to mouse gray to black, fluffy with abundant aerial mycelium; reverse side is fuscous black to black. The pycnidia are simple or compound, often aggregated, stromati, ostiolate, frequently setose, up to 5 mm wide. The conidiophores are hyaline, simple, sometimes septate, rarely branched, and arising from the inner cell lining of the pycnidial cavity. The conidia of *L. theobromae* are initially unicellular, hyaline, granulose, subovoid to ellipsoid-oblong, thick-walled, base truncated; mature conidia is uni-septate, cinnamon to fawn, often longitudinally striate (Fig. 11.3). The conidia measure 23.6–28.2 μm × 13.0–15.4 μm; they have length/breadth ratio of 1.9 and paraphyses length of 55 μm and width, 4 μm (Alves et al. 2008). The main features that distinguish this genus from other closely related genera are the presence of pycnidial paraphyses and longitudinal striations on mature conidia.

Disease Control

The *Botryosphaeriaceae* are difficult to control (Jaiyeola et al. 2014) because their spores could persist in soil and leaf litter in the orchard (Adesemoye et al. 2014; McDonald and Eskalen 2011) and also because of their wide host range. Losses due to charcoal pod rot on cacao are generally not high enough to justify control measures. However, proper farm sanitation by total removal of infected parts, pruning of infected branches, treatment of wounds, and the use of shade trees that do not attract rodents reduce the incidence of the disease.

11.5 Trachysphaera Pod Rot (Mealy Pod)

Situation and Outlook

Trachysphaera pod rot (mealy pod) is caused by *Trachysphaera fructigena*, an Oomycete of the class Pythiaceae. Closely related to *Phytophthora*, symptoms

produced by *T. fructigena* are similar to that of *Phytophthora*. *Trachysphaera fructigena* causes fruit rot of coffee, banana, avocado, and cacao. Mealy pod is of limited distribution and has only been recorded in West and Central Africa (Holliday 1980b). In French, the disease is commonly called "pourriture farineuse des cabosses." Due to its limited distribution, mealy pod has neither Portuguese nor Spanish common names.

Symptoms

The fungus is essentially a wound parasite (Holliday 1980b) and it is often associated with bird or rodent-damaged pods. These rodents may be rats or squirrels. *Trachysphaera fructigena* infects wounded cacao pod tissue to cause brown spreading lesions. On the surface of the lesions, a dense conidial mass, at first white and then becoming pinkish brown, giving a mealy appearance is produced. The conidia mass feels coarse when rubbed between the fingers due to echinulations on the conidia walls. The initial white conidial mass can easily be blown away by wind, but ants play some role in dissemination of the conidia. The symptoms of mealy pod are shown in Fig. 11.4d.

Pod losses in cacao due to *Trachysphaera* are generally low but Dakwa (1972b) found evidence of higher incidence of the disease in Ghana between July and November, which coincides with the rainy season.

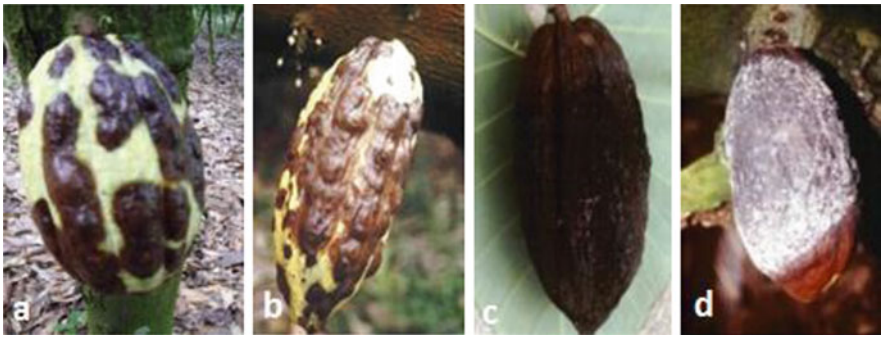

Fig. 11.4 Symptoms of minor cacao pod diseases in Ghana (A. Y. Akrofi). Ripe cacao pod showing (**a**) *dark* lesions, (**b**) *brown* lesions of protrusions typical of warty pods, (**c**) *black* sooty charcoal-like powder typical of charcoal pod rot, and (**d**) *white* powdery spores on surface of cacao pod typical of mealy pod infection

Disease Control

On cacao, infection by *T. fructigena* is an insignificant component of pod diseases and seldom requires targeted control. However, sanitation and avoiding damage to host tissues reduce the incidence of mealy pod. Furthermore, copper fungicide sprays have been reported to be effective on infections on cacao (Dakwa 1976b).

11.6 Thread Blight Diseases of Cacao

Situation and Outlook

Thread blight disease (Fig. 11.5) derives its name from mycelial threads (rhizomorphic strands) of the fungal pathogen that are seen growing on branches, twigs, and leaves of affected plants causing leaf blight. The disease affects several

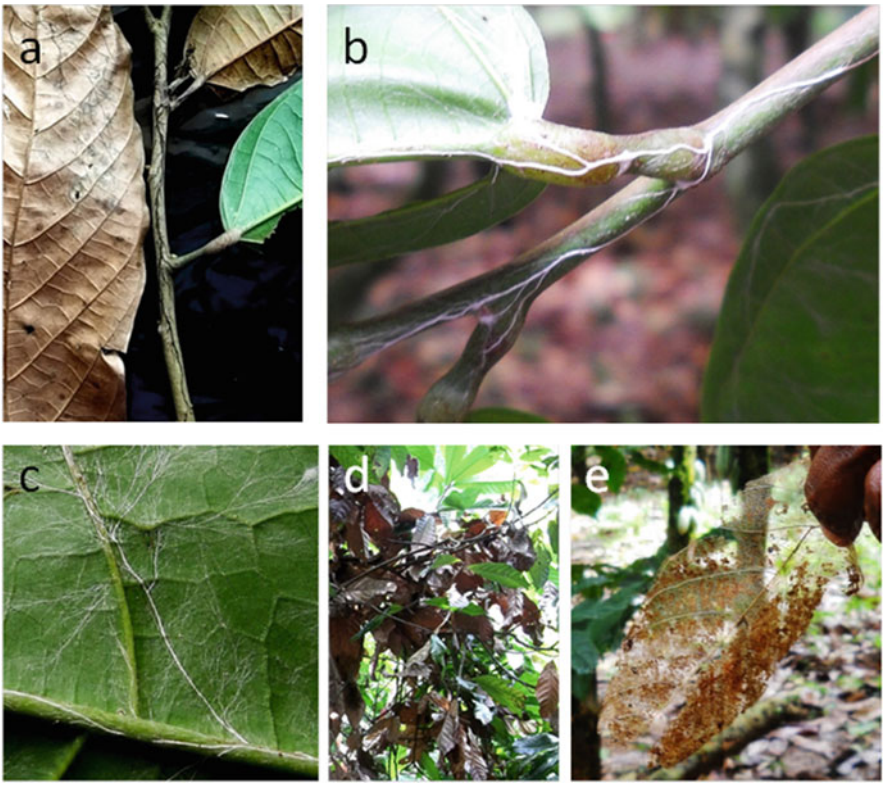

Fig. 11.5 Thread blight of cacao: (**a**) black thread, (**b**) white thread on branch, (**c**) white thread blight on cacao leaf, (**d**) cacao canopy affected by white thread blight, and (**e**) cacao leaf after being parasitized by *Marasmiellus scandens* leaving only vascular tissues

tropical and semitropical tree crops worldwide. In the Amazon where the cacao tree originated, thread blight has been reported on 45 native plant species and several others (Lourd and Alves 1987; Gasparotto and Silva 1999). Economic importance of this disease has been reported on some cultivated tree crops other than cacao. It is increasingly emerging as serious foliar disease in many cacao-growing regions. On most continents, the causal fungus is believed to have spread from the native hosts to introduced plants. This suggests that most thread blight fungi are native to the regions where they occur making their genetic distinctions difficult and controversial. Traditionally, thread blight fungi form marcescent basidiocarps (i.e., reviving after drying out) with tough or persistent pilei and stipes. They belong to the basidiomycete taxonomic class in the order Agaricales. Several morphological forms have been reported on cacao and most have been identified as distinct pathogens. Dennis and Reid (1957) reported that the white thread blight (Fig. 11.5b, c, d, e) on cacao is caused by *Marasmiellus scandens* which is the only preferred name, while *Marasmius scandens* (Massee) and *Marasmius byssicolor* (Petch) are used as synonyms. However, Opoku et al. (2007a, b) described *Marasmius byssicola* as the cause of the black thread blight (Fig. 11.5a) on cacao in Ghana. In the Americas, Barros (1981) reported brown thread blight in some parts of Colombia caused by *Koleroga noxia* (previously *Pellicularia Koleroga*).

Thread blight disease is widespread on cacao in Ghana and was identified in 78 % of farms visited during a recent survey in cacao-growing areas. The disease was severe in areas of heavy rainfall and poor farm management. Farms with high sanitation standards recorded lower incidence reflecting the importance of farm maintenance practice. The main damage from the disease is death of the leaves and accompanying defoliation and dieback of branches of the affected trees, which impacts the photosynthetic capacity of the trees. In a survey of 24-year-old mixed hybrid cacao trees, pod yields were reduced between 46 and 84 %. Another type of thread blight disease which is rarely seen is a fungus–plant cohabitation in which an epiphytic fungus, *Marasmius equicrinis*, is involved in what is commonly called "horse hair blight". Horse hair blight is of little economic importance in cacao cultivation.

Signs and Symptoms

The disease is identified through the appearance of white, cream, yellowish, dark-brown, or black network of the fungal mycelial threads growing on the lower surfaces over branches and leaves of affected plants. The fungi always grow toward the leaves branching off from the petioles. They can affect both adaxial and abaxial surfaces of young and old cacao leaves. The mycelia threads may grow along the main leaf vein for a distance before spreading out over the leaf blade into almost invisible fineness. The fine strands initiate light-brown necrosis which spreads within 21 days to other parts of the leaf. Leaf development stops when necrosis starts, becomes dry, and can detach from the branch. However, desiccated leaves usually remain hanging for a long time from mycelia of the fungus that grew over

the petiole from the branch (Fig. 11.5d). At an advanced stage, the desiccated leaves appear papery with the lamina disappearing leaving the veins (Fig. 11.5e).

Horse hair blight on the other hand is recognized through the tangles of shiny black mycelia threads of the fungus which do not adhere closely to the host plant along their entire length. They only attached at certain points along their mycelia threads by small dark-brown pads. After the natural death of leaves, they are held to the tree by the long free fungal threads which tangled them. The fungus obtains nourishment from the dead leaves, twigs, and older stem barks it entangles, but not from living tissues.

Spread

At certain stages of the mycelia growth, the fungus forms hyphal clumps on leaf edges or along the main veins. The clumps are irregularly concave bodies 1–3 mm high and 2–8 mm wide. These are aggregates of hyphae strands which serve as survival structures of the fungi on leaves but rarely found on branches. They absorb moisture readily under high humid conditions and appear sticky, adhere to healthy plants, and start new infections. The fungus mostly survives dry periods as mycelial threads on infected leaves, twigs, or branches. Dead leaves with mycelia are the main sources of inoculum and are spread by wind, rain, insects, nesting birds, and human activities. The primary infection is generally through direct contact with affected plant parts. The contact must remain unbroken for at least 16 h under high humidity (80–100 %) for the fungus to spread and infect. The fungus grows from the point of infection, mostly on the branches, extending to the leaves. In the initial stage of the disease development, the host plant manages to outgrow the infection after a period by growing new leaves to replace the defoliated ones. This makes it difficult to detect the initial damage caused by the disease and the effect only noticed when the plant succumbs to the pathogen at later stage. This makes the disease control difficult.

Spread of the disease in cacao farms is not contiguous but more prevalent in closely spaced or heavily shaded areas with overhanging branches. The disease may reach epidemic proportion in such conditions when warm temperatures and high humidity prevail. Under severe attack, some of the trees become massively defoliated resulting in broken canopies. Usually, serious damage is not observed on affected trees within the first year of infection. However, killing back in the canopy and loss of tree vigor are observed in subsequent years when control measures are not initiated. These result in gradual loss of production as severity of the disease increases.

Disease Management

Various management strategies have been used to control thread blight disease including the use of fungicides. Thread blight, once established, is difficult to

control with fungicides. Applying preventive fungicide sprays to trees prior to infection may also be an uneconomical way of protecting the plant from the disease. The disease can be managed effectively through pruning of infected brunches, twigs, and leaves. Thread blight fungi are mostly spread from infected plants through direct contact. Therefore, their control requires suppression of the initial infection to enhance reduction of subsequent infections. Pruning can be done immediately after the disease is noticed and the pruned parts burnt outside the farm to avoid reinfection of healthy trees. Pruning of infected twigs and branches promotes better penetration of sunlight and air. It is also recommended to undertake regular monitoring to detect any further reinfection of the healthy twigs because significant inhibition of disease severity can be achieved during the initial stages of disease development. However, in the case of severe infection, pruning may be combined with the application of 3 % Nordox (75 % copper (I) oxide) and 5 % Metalm (12 % metalaxyl and 60 % copper (I) oxide) which has proved effective against the mycelia growth of the pathogen in Ghana (Amoako-Atta et al. 2014). The role of alternative hosts and insects (black ants) in the spread of the disease in cacao farms is currently under investigations.

11.7 Anthracnose of Cacao

Situation and Outlook

Colletotrichum spp. are some of the most commonly occurring pathogens and endophytes and have a wide range of plant host. Anthracnose of cacao is caused by species within the *C. gloeosporioides* complex. This species complex is composed of 22 pathogenic and nonpathogenic species and one subspecies (Weir et al. 2012). Several of these species have been isolated from infected cacao tissue, including *C. siamense* (James et al. 2014) and *C. theobromicola* (Rojas et al. 2010). Additionally, *C. tropicale* has been isolated as an endophyte from cacao (Rojas et al. 2010) and has been tested in laboratory, greenhouse, and field studies as a potential biological control agent by itself or in a consortium (Arnold et al. 2003; Herre et al. 2007; Mejía et al. 2008). *Colletotrichum* species have long been known to have a long latent stage prior to causing disease (Baker et al. 1940).

Anthracnose of cacao is a cacao disease with worldwide distribution (Wood and Lass 2001). However, while occurring in South and Central America, the basidiomycete caused diseases of frosty pod rot and witches' broom are far more prevalent and cause more losses throughout the cacao-growing countries. The exception to this is South American regions growing the highly susceptible, yet highly desired "Porecelana," clones as these are highly susceptible to the disease. Anthracnose is of increasing importance on the Asian continent and is a major problem in India (Peter and Chandramohanan 2011).

Signs and Symptoms

C. theobromicola and other species in the *C. gloeosporioides* species complex cause anthracnose on young shoots, leaves, and stems. Tissues with the most sun exposure, such as that at the crown of the tree (Phillips-Mora and Rolando Cerda 2009), have stronger manifestation of anthracnose symptoms. Young leaves and new shoots are the primary tissue attacked by the fungus. Some of the most severe impacts of anthracnose of cacao are foliar symptoms. Typical symptoms on young leaves are brown necrotic lesions surrounded by a chlorotic yellow halo. These individualized lesions expand and coalesce to cause large blighted areas on the leaves. During heavy infections periods, this blight can lead to complete defoliation followed by branch dieback. After branch dieback, false brooms can form on branches where the dieback triggers growth of new shoots (Phillips-Mora and Rolando Cerda 2009). While leaf spots 0ccur on older leaves, they do not have a severe impact on tree health. They do serve as a source of inoculum for secondary infections. On cacao pods, lesions begin as dark sunken spots and lesions can eventually coalesce (Phillips-Mora and Rolando Cerda 2009). These infections rarely damage the beans contained in the pods, especially when infections occur in older pods. While not a major cause of bean loss, *Colletotrichum* spp. can form mycelia bearing spores on infected pods, leading to further infections (Rojas et al. 2010).

Spread

As with anthracnose on most plant species, spore production and subsequent disease spread are favored by the humid conditions found in most cacao-growing regions (Phillips-Mora and Rolando Cerda 2009). Anthracnose can spread via rain splash from soil or infected tissue, wind, insects, or tools (Phillips-Mora and Rolando Cerda 2009). Insect wounding of leaves increases the spread of the disease in the rainy season (Phillips-Mora and Rolando Cerda 2009). Under favorable conditions and due to the multiple mechanisms of disease spread, *Colletotrichum* infections can readily spread through susceptible plantations (Wood and Lass 2001).

Disease Management

Phytosanitation is listed as one of the top means to reduce anthracnose in mature trees (Wood and Lass 2001). Removal of dead wood, infected pods, and infected seedlings limits inoculum and thus spread, reducing the level of disease to manageable levels (Wood and Lass 2001). Under plantations and especially under plantlets in a nursery, mulching can avoid rain splash and reduces infection (Phillips-Mora

and Rolando Cerda 2009). A combination of a good phytosanitation program with a well-timed fungicide program can lead to relatively good levels of disease control.

Colletotrichum and Biological Control

Despite the ability of some species to cause disease, endophytic *Colletotrichum* spp. have been shown to have the ability to reduce cacao disease incidence and activate plant resistance (Arnold et al. 2003; Mejía et al. 2008; Mejia et al. 2014). One such species that has been a large focus of work is endophytic *C. tropicale* isolated from cacao in Panama (Rojas et al. 2010). Initial work demonstrated that *C. tropicale* in a consortium of other cacao fungal endophytes could reduce foliar cacao infection by *Phytophthora palmivora* (Arnold et al. 2003). In larger field level studies, application of this *C. tropicale* isolate reduced the severity of black pod rot in the field (Mejía et al. 2008). While *C. tropicale* reduced sporulation on *Moniliophthora roreri*-infected pods, it did not reduce incidence of the disease during the experimental period (Mejía et al. 2008). Work by Rojas et al. (2010) demonstrated that these endophytic cacao isolates in Central America were phylogenetically distinct from pathogenic isolates. More recently, Mejia et al. (2014) conducted microarray studies to compare gene expression differences between endophyte-free cacao leaves and those colonized by *C. tropicale*. *C. tropicale* induced the expression of hundreds of genes in cacao, including many defense genes (Mejia et al. 2014). At a physiological level, *C. tropicale* reduces photosynthetic rates and increased the content of lignin and cellulose, major component of plant cell walls (Mejia et al. 2014). The findings of this study suggested induction of resistance genes and alterations of host physiology as the mechanisms used to manage disease in the field (Mejía et al. 2008).

Overall, while there are cacao-growing regions that are heavily impacted by anthracnose, there is limited current literature on the disease. Much of the current work on cacao and *Colletotrichum* spp. is focusing on utilizing the species as a biological control agent. While this work has greatly increased our understanding of fungal endophytes in cacao, there is still a need for studies on latent infections of cacao and the many other nearby species that grow around cacao to ensure that this would be a safe product for development.

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Chapter 12
Ceratocystis Wilt Pathogens: History and Biology—Highlighting *C. cacaofunesta*, the Causal Agent of Wilt Disease of Cacao

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Abstract *Ceratocystis* is a genus of ascomycete fungi that includes aggressive pathogens of economically important plants worldwide. This fungus is the causal agent of Ceratocystis wilt disease and canker disease, which often kills the plant causing major losses in agricultural production. In the last two decades, emerging diseases related to *Ceratocystis* infections have been greatly increased. Ceratocystis wilt of cacao is caused by *C. cacaofunesta,* one of the three well-established host-specific pathogens in the genus. Ceratocystis wilt of cacao has caused sporadic epidemics in the Americas, but its importance is often underestimated. Furthermore, the disease represents a serious threat to the world's cacao production due to the risk of pathogen spread. Silvicultural practices in cacao agroforests, the marketing of seeds, and cacao grafting in association with a minimal knowledge of the biology of the pathogen effectively contributed to this threat. This chapter explores the controversial taxonomic and evolutionary history of the genus *Ceratocystis* as well as the biology of *C. cacaofunesta*.

12.1 Introduction

Ceratocystis pathogens are major economic problem on a wide variety of more than 40 economically important agricultural crop plants worldwide, including *Theobroma cacao*. This genus of fungi is easily disseminated by human activities and shows increased rates of dissemination due to the ease with which infected plants

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can contaminate crop management systems. Like the other ascomycota, *Ceratocystis* is a group of confusing taxonomy due to its conserved morphology and the surprisingly broad host and geographic range. In the last few decades, the use of molecular tools has resolved many of the complexities of the *Ceratocystis* species, as well as the movement of these pathogens worldwide with the emergence of new host-specialized lineages. However, their taxonomic status still requires careful consideration. Species identification continues to be a challenge for the group and the development of new methods for this purpose is essential.

In the recent years, the recognition of its economic importance has resulted in intense scientific interest for the *Ceratocystis* group, which has led to an increase in publications concerning this genus and a high demand for new species descriptions. The association of Ceratocystis wilt disease with the ecological interaction of these fungi and bark beetles has also been the focus of many recent studies.

C. cacaofunesta is the causal agent of Ceratocystis wilt of cacao and is one of the few species of this genus with well-established species status and host specificity and representing a significant risk to cacao production. The risk is especially high in the Americas, where it was first reported followed by its rapid spread throughout the continent. In this chapter, we organized information concerning the biology and pathogenic features of all *Ceratocystis* stricto sensu species, but with a special focus on *C. cacaofunesta*.

12.2 Impact of Ceratocystis Wilt Disease on Agriculture

The genus *Ceratocystis sensu stricto* Ellis & Halst. includes many aggressive plant pathogens that cause wilt, canker stain diseases, and tissue rot on a wide variety of perennial and economically important agronomic crop plants worldwide (Kile 1993). Specially, *C. fimbriata* has had recent sporadic epidemics and is particularly important as the causal agent of coffee canker disease in *Coffea* plantations in Colombia and Venezuela (Pontis 1951). Coffee canker disease has become one of the most important threats to coffee production in Colombia, causing losses equivalent to US$174 per hectare and decreasing the coffee tree density from 1000 trees/ha to 950 trees/ha, on average (Castro-Caicedo 1998). Also in Colombia, Ceratocystis wilt disease in citrus has caused significant losses, estimated between 1 and 10 % of the total production (Borja et al. 1995). In Brazil, *C. fimbriata* isolates vary greatly in their aggressiveness to different hosts. However, they do not have a strong host specialization (Harrington et al. 2011). *C. fimbriata* causes lethal, wilt-type diseases or cankers in important plantation or orchard crops, such as *Gmelina arborea*, rubber tree (*Hevea brasiliensis*), inhame (*Colocasia esculenta*), and figs (*Ficus carica*) (Ferreira et al. 2010; Harrington et al. 2011). Brazilian isolates of *C. fimbriata* have caused important losses in *Mangifera* plantations (Rossetto and Ribeiro 1990; Ribeiro et al. 1995). However, in Brazil one of the major economic hosts of *C. fimbriata* is *Eucalyptus*. Ceratocystis wilt disease of eucalyptus was described for the first time in 1997 in Bahia causing mortality of 40 % in clonal

plantations (Ferreira et al. 1999; Ferreira 2000), and in a short period of time, the disease spread to other Brazilian States (Ferreira et al. 2006). Genetic analyses of isolates of *C. fimbriata* suggest that Brazilian genotypes have now been moved to other regions of South America, Africa, and Asia in infected eucalyptus cuttings (Roux et al. 2000; Barnes et al. 2003; Van Wyk et al. 2006b, 2012; Ferreira et al. 2010, 2011; Harrington et al. 2011).

Ceratocystis wilt diseases have also caused important economic losses in Europe and Asia. *C. platani* causes canker stain of plane trees (*Platanus orientalis*) and is a major threat to European plantations (Engelbrecht et al. 2004). It has killed hybrid plane trees in Italy since the 1930s (Engelbrecht et al. 2004) and *C. platani* has spread to France, Spain, Switzerland, and Greece (Panconesi 1999; Tsopelas and Angelopoulos 2004). Actions to remove the susceptible plane tree hybrids in Europe have been estimated at a cost of 200 million euros (RCE 2014). In Asia, Ceratocystis wilt disease has caused significant losses on *Punica* plantations in India (Somasekhara 1999) and *Ipomoea* production in China and Japan (Clark and Moyer 1988).

Oak Wilt disease, caused by *C. fagacearum*, has received much research attention because the epidemics originate in forest ecosystems in the United States (Juzwik et al. 2008). However, the latest taxonomic study of this group showed that this pathogen no longer belongs to the *Ceratocystis* strict genus (De Beer et al. 2014).

Ceratocystis wilt disease of *Theobroma cacao* was reported for the first time in Ecuador in 1918 (Rorer 1918). The lack of references about the disease suggests that it was confined to Ecuador until the 1950s, when reports arose of the disease in neighboring countries (Fig. 12.1). Also during the 1950s, Ecuador saw an increase in the disease particularly on the cacao trees type "criollo" with loses totaling 65,000 trees (Desrosiers 1957; Delgado and Suárez 2003). In Colombia, in 1956, Ceratocystis wilt of cacao affected more than 5000 trees (Arbelaez 1957). In the same year in Venezuela, the disease destroyed over 20 % of existing plants, estimated to be about 120,000 cacao trees (Malaguti 1956). In the late 1950s, Trinidad and Tobago reported the death of 2153 trees infected with the disease (Iton 1960).

Since the 1930s, Brazil has occupied a prominent position among the largest cacao producers in the world (Sauer 1993). Specifically, the state of Bahia was responsible for about 75 % of the national cacao production. In 1989, *Moniliophthora perniciosa*, the causal agent of witches' broom disease was introduced in Bahia drastically affecting the Brazilian role in the world cacao market (Pereira et al. 1989). Different strategies were followed in an attempt to control witches' broom disease. Breeding studies of cacao led to the identification of the Theobahia variety, which showed high productivity and resistance to witches' broom disease (Monteiro et al. 1995). Local producers replaced the cacao production clones with Theobahia grafts (Souza and Dias 2001). However, the hybrid showed a high susceptibility to Ceratocystis wilt of cacao (Silva and Luz 2000). In 1997, this disease was found for the first time in Bahia in seedlings propagated for grafting (Bezerra 1997). According to official data, no farms were lost due to Ceratocystis

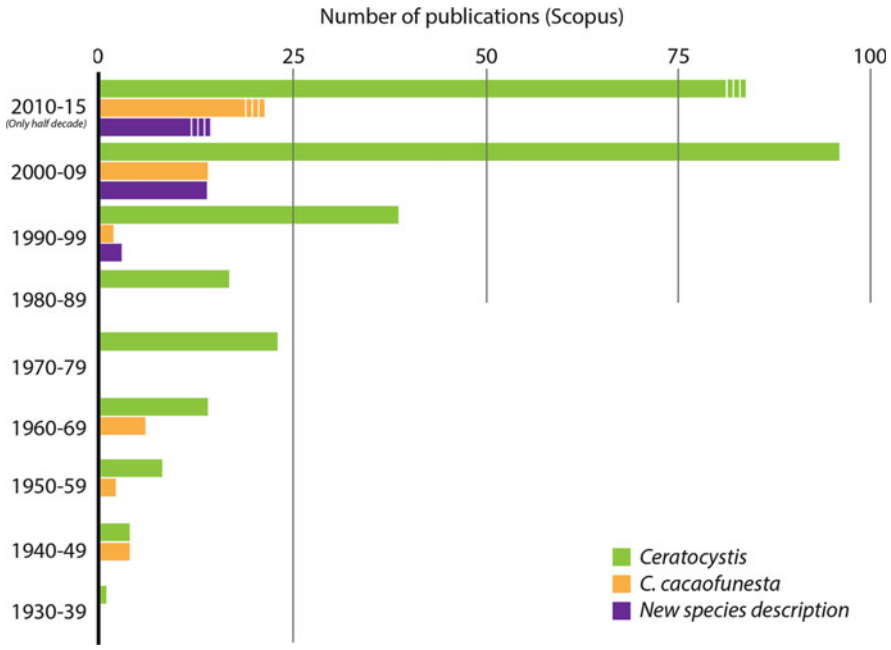


Fig. 12.1 Increase in *Ceratocystis* scientific publications. Data were obtained by searching "*Ceratocystis*" in the title, abstract, and keywords of the Scopus scientific bibliography databank. The searches were further filtered using "*cacaofunesta*" and "*sp. nov.*" It is noteworthy that the most recent data referenced in the last 5 years already comprised more than 80 % of the total number of publications from the previous decade

wilt of cacao, although the disease affected 24 % of the plantations in the region (Ram et al. 2004; Silva and Luz 2000). Figure 12.1 shows impact of Ceratocystis wilt of cacao on scientific research based on the number of publications and how the disease in Bahia changes the research focus after the disease was verified in the 1950s. Estimates suggest that millions of cacao trees have been killed due to Ceratocystis wilt of cacao although no quantifiable figures exist (Dand 2011).

Ceratocystis wilt of cacao is reemerging as an important disease because agricultural practices (like intensive pruning) facilitate the spread of the fungus into cuttings and seeds of cacao (Engelbrecht et al. 2007a). This is a serious threat to the producing countries, especially to African countries (Côte d'Ivoire and Ghana) which are the top cacao producers in the world (ICCO 2013). Recently, Mbenoun reported the isolation of two *Ceratocystis* spp. (*C. ethacetica* and *C. paradoxa*) in *Theobroma cacao* (Mbenoun 2014; Mbenoun et al. 2014a). Also, in Colombia, new *Ceratocystis* species were identified (*C. colombiana* and *C. papillata*) in coffee, cacao, and citrus trees (Van Wyk et al. 2010b).

The increase in the identification of new disease caused by *Ceratocystis* species found in cacao mirrors emerging *Ceratocystis* spp. diseases worldwide. Figure 12.1 shows that in the last 5 years there are already an approximately equal number of

references describing new species of *Ceratocystis* compared to all of the reported in the 2000s. In Brazil, cupuassu (*Theobroma grandiflora*) was recently described as a new host of *C. fimbriata* (Oliveira et al. 2013a). Pakistan, which is a major producer and exporter of mango (Al Adawi et al. 2013b), has reported large production losses of mango resulting from a Ceratocystis wilt disease caused by *C. manginecans* (Kazmi et al. 2005; Al Adawi et al. 2006). Ceratocystis wilt caused by *C. manginecans* has also devastated the mango industry in Oman (Al Adawi et al. 2006, 2013a; Van Wyk et al. 2007b). Recently, in Indonesia two new diseases of *Acacia mangium* were described and found to be associated with *C. manginecans* and *C. acaiivora* (Tarigan et al. 2011). Furthermore, in Australia, two new species of *Ceratocystis* were described on Eucalyptus (Nkuekam et al. 2012). Africa was the last continent where Ceratocystis wilt disease was officially described (Morris et al. 1993; Wingfield et al. 1996), however, the number of *Ceratocystis* pathogens identified in this region increased suddenly in the last year (Mbenoun 2014; Mbenoun et al. 2014a; Nkuekam et al. 2008, 2013; Heath et al. 2009; Van Wyk et al. 2012).

The data suggest that major new threat to agriculture lies in the emergence of new *Ceratocystis* species and adaptation to new hosts and new geographic regions. In 2003, Baker and coworkers described 31 host species that are infected by *Ceratocystis* species and today the number of hosts has increased to 42 (CABI 2015). This alarming situation has attracted the attention of the scientific community, resulting in intense research of Ceratocystis wilt diseases (Fig. 12.1).

12.3 Taxonomy

Taxonomic Status of the Genus Ceratocystis

The taxonomic history of the genus *Ceratocystis* is complex and has been subject of considerable study (De Beer et al. 2014; Harrington 2000; Wingfield et al. 1993). During the course of more than one century, it has been intertwined with several other genera like *Ophiostoma*, *Leptographium*, and *Raffaelea*; consequently the genus has been subjected to many name changes (Wingfield et al. 1993; De Beer et al. 2013).

Ceratocystis was originally described in 1890 by Halsted as the causal agent of black root rot of sweet potatoes (*Ipomoea batatas* Lam.). However, the complete mycological description of the pathogen was given by Halsted and Fairchild (1891). The specimen deposited by Halsted in 1891 was named *Ceratocystis fimbriata*, which is also the type species of this genus. This specimen was described as a parasitic fungus with a black perithecia and globose bases with long necks (sexual fruiting bodies) and sticky ascospore masses at their tip (Halsted 1890; Upadhyay 1981; De Beer et al. 2014). These morphological features are similar to features for species of the genus *Ophiostoma,* consequently *Ceratocystis* was once included in

this genus (De Hoog and Scheffer 1984). Like *Ceratocystis*, *Ophiostoma* also has an association with ambrosia beetle species and includes some plant pathogens like *O. ulmi* (Buisman), which causes an important vascular wilt disease of conifers (Harrington 2013; Brasier 1987). In the past, this similarity in morphology, ecology, and their association with insects for these two genera, resulted in confusion and they have constantly been treated collectively (De Beer et al. 2014).

A summary for the name change and synonym for species of *Ceratocystis* is provided here: The list illustrates the confusion and overlapping nomenclature for this genus.

Ceratocystis (ceratos=horn and cyst=pouch or sac)
Ceratocystis fimbriata, Ellis & Halst., In: Halsted, New Jersey Agric. Coll. Exp. Sta. Bull. 76:14. 1890.
=***Sphaeronaema fimbriatum*** (Ellis & Halsted) Sacc, Syll Fung. 10:125. 1892.
=***Ceratostomella fimbriata*** (Ellis & Halsted). Elliott, Phytopathology 13:56. 1923
=***Rostrella coffeae*** **Zimmermann**. Meded. Lds. Pl. Tuin, Batavia. 37:32. 1900.
=***Ophiostoma fimbriatum*** (Ellis & Halsted) Nannf, Svenska Skogsv Fören. Tidskr. 32:408. 1934.
=***Endoconidiophora fimbriata*** (Ellis & Halsted) R.W. Davidson, J. Agric. Res. 50:800. 1935.
=***Ophiostoma variosporum.*** Von Arx, Antonie van Leeuwenhoek. 1952.
=***Ceratocystis moniliformis f. coffea*** (Zimm.) C. Moreau, Bull. Sc. Minist. France Outre-Mer 5:424. 1954.

Type Species: Ceratocystis fimbriata *(Ellis & Halsted)*

The close relationship between species of *Ceratocystis* and *Ophiostoma* has given rise to the general name "ophiostomatoid fungi," a collective reference including the later described genus *Ceratocystiopsis* (Hausner et al. 1993a; Spatafora and Blackwell 1994). *Ceratocystis sensu lato* has also been used collectively for all the ophiostomatoid fungi (Wingfield et al. 1993). These genera have been subjected to many taxonomic studies designed to demarcate the ophiostomatoid fungi (De Beer et al. 2013).

Different data including morphological, ecological, and phylogenetic analysis based on DNA sequencing have shown clear evidence that these three genera are distinct and phylogenetically unrelated (De Hoog and Scheffer 1984; Wingfield et al. 1993; De Beer et al. 2014). *Ophiostoma* and *Ceratocystis* were separated based on their different anamorph states (De Hoog and Scheffer 1984). The asexual states of *Ceratocystis* species were previously classified in the genus *Chalara* and more recently in *Thielaviopsis* (Paulin-Mahady et al. 2002). In contrast, *Ophiostoma* have anamorphs in *Leptographium* Lagerberg and Melin (Minter et al. 1983). Species of *Ceratocystis sensu stricto* can also be distinguished from other ophiostomatoid fungi by not having cellulose and rhamnose in their cell walls

(De Hoog and Scheffer 1984; Marín Montoya and Wingfield 2006) and being sensitive to the antibiotic cycloheximide (Harrington 1981). Furthermore, members of *Ceratocystis* do not have obligate associations with ambrosia beetles, in contrast to the more specialized ecological relationship between species of *Ophiostoma* and ambrosia beetles (Harrington 2013). Most species of *Ceratocystis* produce fruity volatile metabolites that attract a wide range of insects (Goheen and Hansen 1993; Harrington 2013). Several authors have proposed that the morphological similarity between Ophiostoma fungi and *Ceratocystis* has resulted from convergent evolution of these structures, in response to their similar niches and especially to the adaptation of insect dispersal (Spatafora and Blackwell 1994).

The first study in this group using molecular tools was published by Hausner et al. (1993b), presenting phylogenetic support for the separation of ophiostomatoid fungi using the large subunit ribosomal RNA gene. Afterward, studies based on analysis of partial sequences from the small subunit ribosomal (rDNA) and β-tubulin genes have revealed that not only do *Ceratocystis* and *Ophiostoma* represent two distinct genera, but they also fall into two separate orders (Spatafora and Blackwell 1994). *Ceratocystis* was accommodated into the order Microascales, family Ceratocystidaceae, while *Ophiostoma* and *Ceratocystiopsis* now resides in a separate order, the Ophiostomatales in the Sordariomycetidae subclass, which is phylogenetically distinct from *Ceratocystis* (Spatafora and Blackwell 1994; De Beer et al. 2014).

Complexes of Species Within Ceratocystis

Despite the effort that has been made to delimit the genus *Ceratocystis* from other *Ophiostoma* fungi, additional work is required to investigate and define the species within this genus. Much confusion still exists regarding the taxonomy of *Ceratocystis* species. Taxonomic studies have shown high genetic diversity in this group leading to propose the use of species complexes within the genus (Webster and Butler 1967a; Harrington et al. 1998; Wingfield et al. 1996; Witthuhn et al. 1998; Harrington 2000). Traditional biological species concepts are often inappropriate for fungi, especially in species complexes like this, with high rates of asexual reproduction and several morphologically similar species. Therefore, Harrington and Rizzo (1999) proposed a species concept for fungi based on diagnostic phenotypic characters within monophyletic lineages of organisms, as populations or lineages with unique phenotypic characters, such as morphology and host specialization.

Recent phylogenetic studies using rDNA sequence data (Wingfield et al. 1994, 1996; Witthuhn et al. 1998; Barnes et al. 2003), mating-type genes (Witthuhn et al. 2000; Harrington et al. 2002), host specialization, isozyme variation (Engelbrecht and Harrington 2005; Johnson et al. 2005), phylogenetic analysis of the rRNA ITS regions (highly variable internal transcribed spacers) (Van Wyk et al. 2007a, 2009, 2010a; Harrington et al. 2011), and microsatellite DNA regions

(Barnes et al. 2001) have reinforced the hypothesis that *Ceratocystis* species, which have been contemplated as a single species, actually represent complexes of cryptic species (Marín Montoya and Wingfield 2006). In 2000, Harrington analyzed rDNA-ITS sequences, partial sequence of MAT-2 mating-type genes, and interfertility tests to isolate groups of *Ceratocystis*. The results suggested that the genus *Ceratocystis* is formed by a complex of species mainly defined by geographical origin and host-associated lineages (Harrington 2000; Engelbrecht and Harrington 2005). *Ceratocystis* species were separated into a North American complex, Latin American complex, and an Asian complex. In the Latin America complex, two specific host species were clearly recognized, *C. cacaofunesta* (Engelbrecht and Harrington 2005) and *C. platani* (Engelbrecht and Harrington 2005), which appear to have adapted to infect *Theobroma cacao* and *Platanus occidentalis* (sycamore), respectively (Engelbrecht and Harrington 2005; Harrington et al. 2014). This hypothesis has been supported using microsatellite and minisatellite markers, which grouped the host-specialized isolates in separate clades in accordance with their respective host and origins (Barnes et al. 2003). It has become evident that some of these species, in fact, represent a complex of several host-specialized forms and cryptic taxa, which suggests that host specialization may be an important factor in recent speciation events within the genus (Harrington et al. 2014).

Afterward, Wingfield et al. (2013) delimited five different taxonomic groups into the genus *Ceratocystis* supported by phylogenetic and ecological analyses. These included species of the *C. fimbriata* complex, *C. coerulescens* complex (Munch) Bakshi, *C. moniliformis* complex (Hedcock) Moreu, as well as the *Thielaviopsis* and *Ambrosiella* complexes (Wingfield et al. 2013; De Beer et al. 2014). Each of these groups includes several morphologically cryptic species (Engelbrecht and Harrington 2005; Mbenoun et al. 2014b). For example, species in the *C. fimbriata* complex include important plant pathogens like *C. albifundus*, a virulent pathogen of *Acacia mearnsii* in Africa (Roux and Wingfield 2013); *C. cacaofunesta*, a pathogen of cacao in South America (Engelbrecht et al. 2007a); *C. platani*, an invasive pathogen of Platanus trees in Europe (Ocasio-Morales et al. 2007); and *C. manginecans* that has devastated mangos (*Mangifera indica*) in the Middle East (Van Wyk et al. 2007b; Tarigan et al. 2011).

The total number of species described in this genus is not clear due to its complicated taxonomic history with other fungi. However, in 2013, Seifert and coworkers listed 68 species in this complex (Mbenoun et al. 2014b; De Beer et al. 2014). In fact, during the last decade, there has been an increase in the number of species described for this genus (Van Wyk et al. 2007a).

The most recent work published by De Beer et al. (2014), using multiple gene regions (60S, LSU, MCM7) as well as morphological and ecological data for 79 species, residing in *Ceratocystis sensu lato* including the *C. fimbriata, C. coerulescens*, and *C. moniliformis* complexes, defined these species complexes into discrete genera. The new genera are ***Ceratocystis*** Ellis & Halst (previously *C. fimbriata* complex), ***Chalaropsis*** Peyronel (previously *Thielaviopsis* complex) ***Endoconidiophora*** Munch (previously *C. coerulescens* complex part 1), ***Thielaviopsis*** Went (previously *C. paradoxa* complex), and ***Ambrosiella*** Brader

ex Arx & Hennebert (previously Ambrosiella), with two taxonomic novelties ***Davidsoniella gen. nov,*** Z.W. de Beer, T.A. Duong, & M.J. Wingf, 2014 (previously *C. coerulescens* complex part 2) and ***Huntiella gen. nov,*** Z.W. de Beer, T.A. Duong, & M.J. Wingf, 2014 (previously *C. moniliformis*) (De Beer et al. 2014; Fourie et al. 2015). In this study all species in the *C. fimbriata* complex remained in the *Ceratocystis* genus because the genus is typified by the type species *C. fimbriata*. The genus currently includes 32 species (see Fig. 12.2) and many of them are important plant pathogens of angiosperm trees or root crops (Kile 1993; Engelbrecht and Harrington 2005; Van Wyk et al. 2013; Roux and Wingfield 2013; De Beer et al. 2014). Some previous *Ceratocystis* species continue in an uncertain taxonomic position as *C. adiposa*, *C. radicicola*, and important pathogens like *C. fagacearum* and *C. paradoxa*. Those species remained outside the three major phylogenetic groups defined by Wingfield et al. (2013). However, for now, these species still remain in the *Ceratocystis* genus until more data and additional species discoveries can define their position (Wingfield et al. 2013; De Beer et al. 2014).

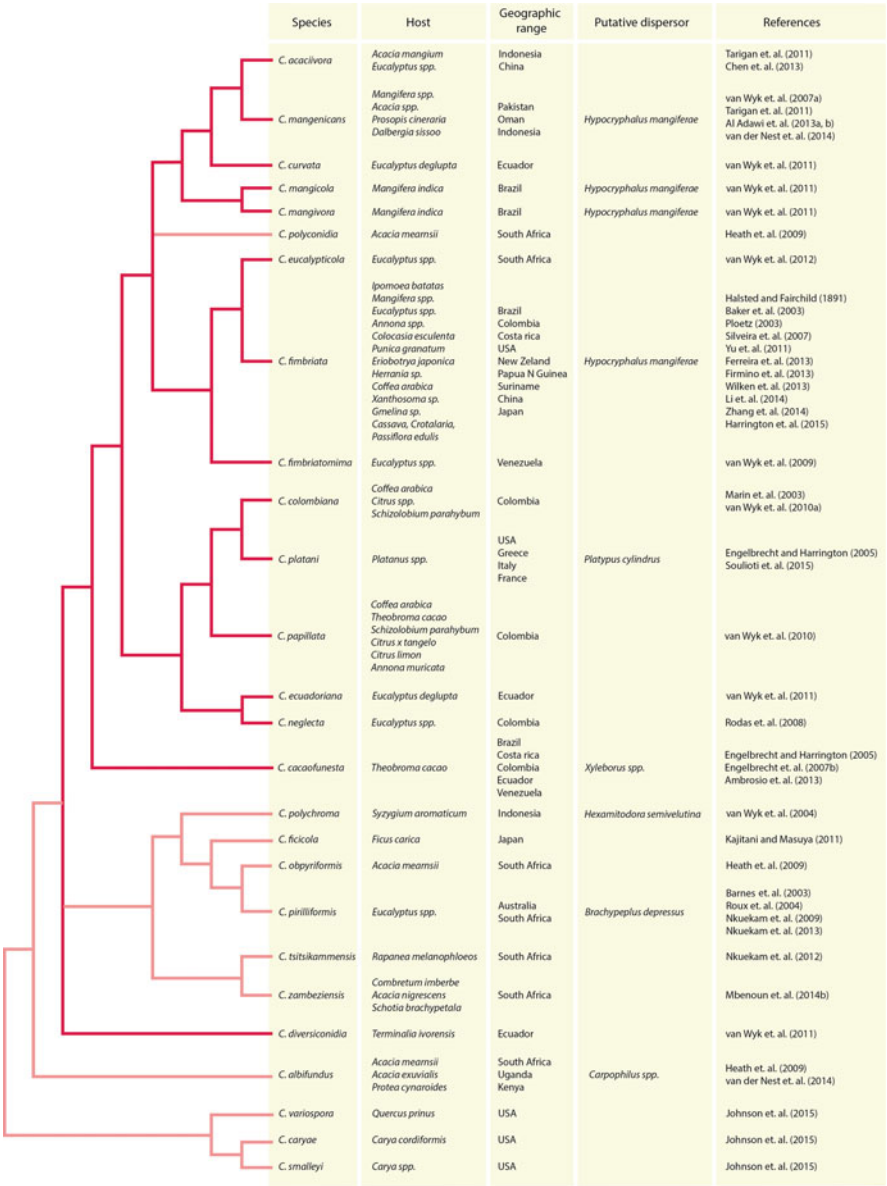
As previously mentioned, significant progress has been made to define and delimit *Ceratocystis* at the genus and species level. Many studies and the development of novel taxonomic tools, especially the ones used to characterize DNA sequence variation, will be required to clarify the internal structure for the genus. In some cases, species boundaries for *Ceratocystis* are very clear, for example in the cases of the tree pathogens *C. platani*, *C. cacaofunesta*, and *C. albifundus* (Wingfield et al. 1996; Engelbrecht and Harrington 2005), while in others, the distinction at the species level is still being debated (Fourie et al. 2015; Harrington et al. 2014). It is important to employ new methodologies and studies that can help to delineate the taxonomy and systematic nature of this important genus. Future studies and new data on whole genome sequences for *Ceratocystis* species could clarify the taxonomy of this group. At this moment, the complete genome is available for only some species like *C. fimbriata*, *C. moniliformis*, and *C. manginecans* (Fourie et al. 2015).

Ceratocystis cacaofunesta *the Causal Agent of Ceratocystis Wilt of Cacao as a Newly Described Species*

Scientific name: *Ceratocystis cacaofunesta* Engelbrecht and Harrington 2005. Mycologia 97:64. 2005.

Synonyms:

=*Ceratocystis fimbriata* (Ellis & Halsted 1890)
=*Ceratostomella fimbriata* (Ellis & Halsted 1890)



Species	Host	Geographic range	Putative dispersor	References
C. acacivora	*Acacia mangium* *Eucalyptus spp.*	Indonesia China		Tarigan et. al. (2011) Chen et. al. (2013)
C. mangenicans	*Mangifera spp.* *Acacia spp.* *Prosopis cineraria* *Dalbergia sissoo*	Pakistan Oman Indonesia	*Hypocryphalus mangiferae*	van Wyk et. al. (2007a) Tarigan et. al. (2011) Al Adawi et. al. (2013a, b) van der Nest et. al. (2014)
C. curvata	*Eucalyptus deglupta*	Ecuador		van Wyk et. al. (2011)
C. mangicola	*Mangifera indica*	Brazil	*Hypocryphalus mangiferae*	van Wyk et. al. (2011)
C. mangivora	*Mangifera indica*	Brazil	*Hypocryphalus mangiferae*	van Wyk et. al. (2011)
C. polyconidia	*Acacia mearnsii*	South Africa		Heath et. al. (2009)
C. eucalypticola	*Eucalyptus spp.*	South Africa		van Wyk et. al. (2012)
C. fimbriata	*Ipomoea batatas* *Mangifera spp.* *Eucalyptus spp.* *Annona spp.* *Colocasia esculenta* *Punica granatum* *Eriobotrya japonica* *Herrania sp.* *Coffea arabica* *Xanthosoma sp.* *Gmelina sp.* *Cassava, Crotalaria,* *Passiflora edulis*	Brazil Colombia Costa rica USA New Zeland Papua N Guinea Suriname China Japan	*Hypocryphalus mangiferae*	Halsted and Fairchild (1891) Baker et. al. (2003) Ploetz (2003) Silveira et. al. (2007) Yu et. al. (2011) Ferreira et. al. (2013) Firmino et. al. (2013) Wilken et. al. (2013) Li et. al. (2014) Zhang et. al. (2014) Harrington et. al. (2015)
C. fimbriatomima	*Eucalyptus spp.*	Venezuela		van Wyk et. al. (2009)
C. colombiana	*Coffea arabica* *Citrus spp.* *Schizolobium parahybum*	Colombia		Marin et. al. (2003) van Wyk et. al. (2010a)
C. platani	*Platanus spp.*	USA Greece Italy France	*Platypus cylindrus*	Engelbrecht and Harrington (2005) Soulioti et. al. (2015)
C. papillata	*Coffea arabica* *Theobroma cacao* *Schizolobium parahybum* *Citrus x tangelo* *Citrus limon* *Annona muricata*	Colombia		van Wyk et. al. (2010)
C. ecuadoriana	*Eucalyptus deglupta*	Ecuador		van Wyk et. al. (2011)
C. neglecta	*Eucalyptus spp.*	Colombia		Rodas et. al. (2008)
C. cacaofunesta	*Theobroma cacao*	Brazil Costa rica Colombia Ecuador Venezuela	*Xyleborus spp.*	Engelbrecht and Harrington (2005) Engelbrecht et. al. (2007b) Ambrosio et. al. (2013)
C. polychroma	*Syzygium aromaticum*	Indonesia	*Hexarmitodora semivelutina*	van Wyk et. al. (2004)
C. ficicola	*Ficus carica*	Japan		Kajitani and Masuya (2011)
C. obpyriformis	*Acacia mearnsii*	South Africa		Heath et. al. (2009)
C. pirilliformis	*Eucalyptus spp.*	Australia South Africa	*Brachypeplus depressus*	Barnes et. al. (2003) Roux et. al. (2004) Nkuekam et. al. (2009) Nkuekam et. al. (2013)
C. tsitsikammensis	*Rapanea melanophloeos*	South Africa		Nkuekam et. al. (2012)
C. zambeziensis	*Combretum imberbe* *Acacia nigrescens* *Schotia brachypetala*	South Africa		Mbenoun et. al. (2014b)
C. diversiconidia	*Terminalia ivorensis*	Ecuador		van Wyk et. al. (2011)
C. albifundus	*Acacia mearnsii* *Acacia exuvialis* *Protea cynaroides*	South Africa Uganda Kenya	*Carpophilus spp.*	Heath et. al. (2009) van der Nest et. al. (2014)
C. variospora	*Quercus prinus*	USA		Johnson et. al. (2015)
C. caryae	*Carya cordiformis*	USA		Johnson et. al. (2015)
C. smalleyi	*Carya spp.*	USA		Johnson et. al. (2015)

Fig. 12.2 Host species, geographic range, putative insect dispersers for species maintained in *Ceratocystis* genus after systematic revision of De Beer et al. (2014). The phylogenetic hypothesis shown on the *left* is composed of branches with strong statistical support (*dark red*) obtained by Fourie et al. (2015) and branches with medium support (*light red*) obtained by De Beer et al. (2014)

Common names: Ceratocystis wilt, cacao canker, Ceratostomella disease, *Xyleborus–Ceratostomella* complex, cacao wilt, Ceratocystis canker, mort subite (French), mal del machete (Spanish), necrosis del cacao, la llaga macana

del tronco del cacao, murcha de Ceratocystis em cacaueiro, mal do facão (Portuguese),

Taxonomic position

Kingdom: Fungi; Phylum: Ascomycota; Class: Sordariomycetes; Order: Microascales; Family: Ceratocystidaceae; Genus: *Ceratocystis*

The causal agent of Ceratocystis wilt of cacao, *Ceratocystis cacaofunesta,* was described in 2005 by Engelbrecht and Harrington as a new species within the Latin American clade of the *C. fimbriata* species complex. They differentiated *C. cacaofunesta* from other strains of the *C. fimbriata* complex by its specificity to infect *Theobroma cacao*, as well as phylogenetic data (ITS-rDNA sequences), intersterility (sexual incompatibility) tests, and some morphological differences like the rare production of doliform (barrel-shaped) conidia and minor differences in size of perithecial bases and ascospores (Engelbrecht and Harrington 2005; Engelbrecht et al. 2007b). Engelbrecht and Harrington employed a species concept defined by Harrington and Rizzo in 1999 (see complexes of species within *Ceratocystis above*). In previous studies, Baker and coworkers (2003) showed host specialization in a "pull" of lineages in reciprocal inoculation experiments on cacao (*Theobroma cacao*), sweet potato (*Ipomoea batatas*), and sycamore (*Platanus* spp.) which form three distinct ITS genotypes (Baker et al. 2003). In this work, besides *C. cacaofunesta,* the other identified species within the *C. fimbriata* species complex was *C. platani* (Harrington et al. 2014). Subsequent studies with microsatellite alleles (Steimel et al. 2004) and mating studies confirm the species boundaries between *C. fimbriata*, *C. platani*, and *C. cacaofunesta* (Engelbrecht and Harrington 2005; Ferreira et al. 2010).

C. cacaofunesta is a specialized pathogen on a single host, causing Ceratocystis wilt of cacao (Engelbrecht and Harrington 2005; Engelbrecht et al. 2007a, b; Ploetz 2007; Harrington et al. 2011; Ambrosio et al. 2013). The pathogen may be indigenous to the Americas, because this disease has been reported only in Central and South America (Harrington 2000; Baker et al. 2003; Engelbrecht and Harrington 2005; Engelbrecht et al. 2007a; Ferreira et al. 2010), where it is commonly referred as "Mal del machete" from the association of infections with contaminated machete wounds. The first report of this disease occurred in Ecuador in 1918, and initially the causal agent of this disease was identified as *C. fimbriata* (Rorer 1918). At that time, it was considered an unimportant disease because the variety of cacao prevalent in Ecuador was resistant to Ceratocystis wilt (Wood and Lass 2001). Then in 1957, Desrosiers (1957) reported a more virulent form of the fungus at the Pichilinque Research Station in Ecuador on the Criollo cacao varieties, which was less severe on Trinitario and Forastero cacao cultivars (Wood and Lass 2001). Thenceforth this disease has been reported in many countries of South America; Colombia, Brazil, Guyana, Peru, Trinidad and Tobago, and Venezuela; in Central America; Costa Rica, Guatemala, and México and in the Caribbean island of Haiti; where it has affected a significant number of cacao trees (Arbelaez 1957; Thorold 1975; Pontis 1951; Soria-Vasco and Salazar 1965; Engelbrecht et al. 2007a). Actually, this is one of the most important "emerging" diseases of cacao in Central and South America (Engelbrecht et al. 2007a; Ploetz 2007).

12.4 Life History

Morphology

The *Ceratocystis fimbriata sensu stricto* complex includes several morphologically cryptic species (Baker et al. 2003; Harrington 2000) and means that morphological variations in the genus are subtle, almost indistinguishable while its members are unable to interbreed (Engelbrecht and Harrington 2005). The main morphological differences between species lie in some aspects associated with reproductive structures (Engelbrecht and Harrington 2005). Although *C. fimbriata* is the type species of the complex, in this section we will describe the morphology of *Ceratocystis cacaofunesta,* the pathogen of *Theobroma cacao*.

Ceratocystis cacaofunesta (= *Ceratocystis* spp.) forms a filamentous tallus. The vegetative mycelium consists of septate, branched, and smooth-walled hyphae (Engelbrecht et al. 2004). In culture, initially the mycelium is hyaline and then it gradually becomes darker (Baker et al. 2003; Engelbrecht and Harrington 2005). This is because of the accumulation of melanin pigment in hyphal walls (Harris and Taber 1973). The fungus exhibits the three reproductive modes described in filamentous ascomycetes: vegetative propagation and asexual and sexual reproduction (Mbenoun 2014). The following paragraph describes the morphology of the reproductive structures of *C. cacaofunesta*.

Asexual spores (conidia) are produced during the anamorphic state of the fungus by conidiophores through ring wall building (Minter et al. 1983) (Fig. 12.3g). Those endoconidiophores develop from branches of the vegetative hyphae and arising scattered or in clusters, up to 40–295 mm long, including the basal cells and 2.0–8.0 mm wide at the base. They are 0–12 septate with phialide at the top (Engelbrecht and Harrington 2005). The most common phialide is lageniform, hyaline to light brown with 12–85 mm long, 2.0–9.0 mm wide in the middle and 2.0–6.5 mm wide at the tip (Engelbrecht and Harrington 2005). Also, wide-mouthed phialides with tones ranging from hyaline to light brown can be observed, albeit rarely, borne near to the base of perithecia. *C. cacaofunesta* mainly produces two types of conidia: (1) cylindrical endoconidia which are the most abundant spore forms produced by the fungus and (2) aleurioconidia which are produced by specialized conidiophores. Endoconidia are unicellular, hyaline to light brown, smooth, mainly cylindrical with truncated ends, straight, and biguttulate. Their dimensions range from 2.5 to 5.0 mm (Engelbrecht and Harrington 2005). Endoconidia are exuded through a cylindrical collarette on the phialide borne in chains of variable lengths; aleurioconidia are oval, thick walled and pigmented in olive-brown tones to allow for long-term survival. A third type of spores, the doliform conidia, could be produced by *C. cacaofunesta* but is rarely produced (Engelbrecht and Harrington 2005). Doliform conidia are produced from wide-mouthed phialides, often remaining in chains, and hyaline to light brown (Engelbrecht and Harrington 2005). Asexual and vegetative reproductions give rise to progeny that are genotypically identical to their parents.

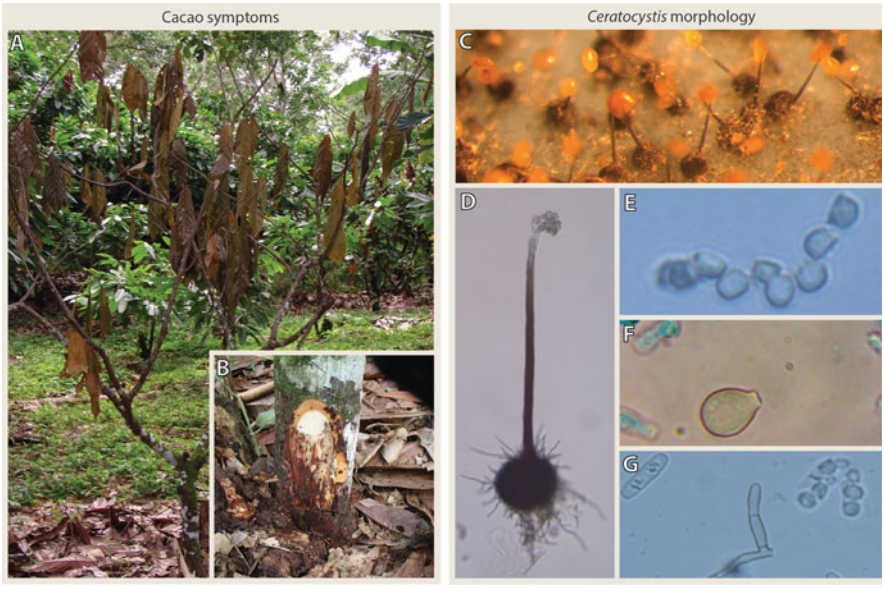


Fig. 12.3 Symptomatology of Ceratocystis wilt of cacao and morphological features of the causal agent *C. cacaofunesta*. (**a**) A dying cacao tree showing the typical symptoms: wilt and necrotic leaves, particularly in this disease the wilted leaves remain attached to the branches for several weeks after the tree dies. (**b**) Vertical cross section of cacao tree infected with *C. cacaofunesta* showing the dying regions of parenchyma in dark shadows. (**c**) Ascomata of *C. cacaofunesta* on Malt-Agar. Dark-brown perithecia with a globose base immersed in the substrate and orange sticky ascospore masses emerging from their necks. (**d**) Perithecium with masses of ascospores emerging from the ostiolar hyphae. (**e**) Hat-shaped hyaline ascospores. (**f**) Brown and globose aleurioconidia. (**g**) Mycelium and cylindrical endoconia of *C. cacaofunesta*

The teleomorphic state (sexual) of *C. cacaofunesta* is observed after 48 h of growth in cultured media with the beginning of the perithecium's rostrum formation (Santos et al. 2013). Sexual spores (ascospores) are produced in large numbers within asci (meiosporangia) which are organized inside the perithecium (ascocarp) (Fig. 12.3e). Ascospores are hyaline, single-celled, and are characteristically hat-shaped (Engelbrecht and Harrington 2005) (Fig. 12.3f). Perithecia can be produced superficially or embedded in the substrate. The perithecium is globose (95–305 μm wide and 100–275 μm tall) and dark brown to black. It has a long (310–1010 μm), straight and at the base (12–25 μm wide at the tip) neck which also is dark brown to black (Engelbrecht and Harrington 2005). The ostiolar hypha is divergent, light brown to hyaline, nonseptate, smooth-walled and ranging 30–125 mm long with an ostiole at the end. Each ascus consists of groups of eight haploid spores. When the ascospores are fully mature, the asci walls disintegrate and the spores are enveloped in a gelatinous mass (Engelbrecht and Harrington 2005; Chong 1961). Within the perithecium hydrostatic pressure is generated pulling the mucilaginous matrix up the perithecium neck and out of the ostiole.

Spores accumulate in a white to yellowish droplet at the tip of the perithecial neck (Hunt 1956; Upadhyay 1981) (Fig. 12.3d). It has been proposed that this is an adaptation to insect dispersal because this allows the spores to adhere to the insect exoskeletons (Malloch and Blackwell 1993). The ascospores typically germinate within 20–25 h on water agar with one or several germ tubes emerging from the top of the spore, thus establishing haploid mycelia (Santos et al. 2013).

Life Cycle

The life cycle of *C. cacaofunesta* will be explored here as a representation of all *Ceratocystis* species (Fig. 12.4). This is because the overall cycle is common throughout the genus, only varying in some minor aspects of the interaction with different plant hosts.

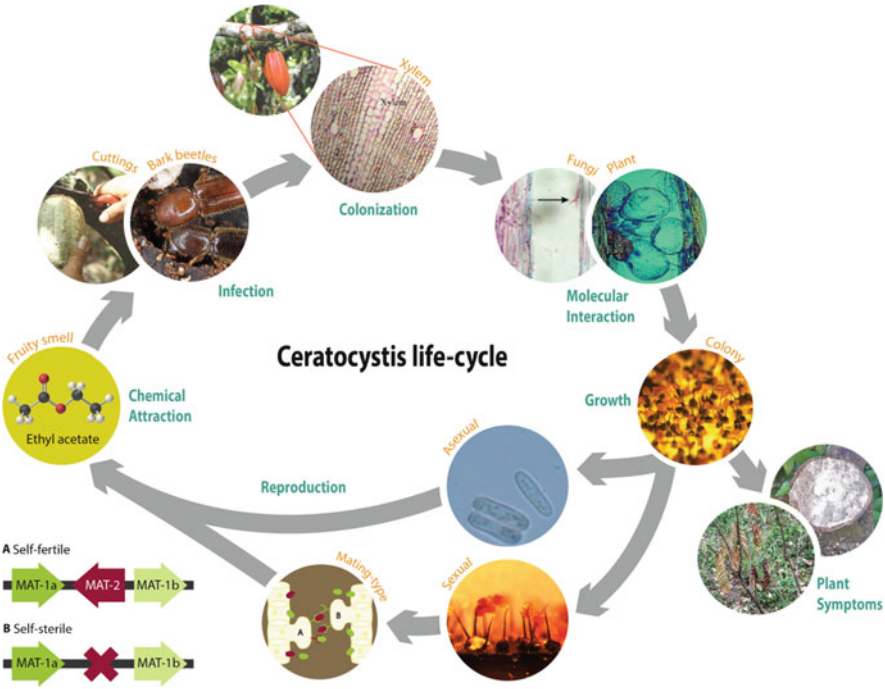


Fig. 12.4 *Ceratocystis* life cycle represented by *C. cacaofunesta* cycle on cacao hosts. Each stage is discussed in detail in the text

Infection

Since *Ceratocystis* species are primarily xylem pathogens, wounds to the xylem are the main infection pathway for most species of the genus (Harrington 2000). However, *Ceratocystis* also causes root infections via soil-borne aleuroconidia through root anastomoses between neighboring trees (Rossetto and Ribeiro 1990; Harrington 2009). This infection strategy has been well described for isolates of *C. fimbriata* infecting mango, eucalyptus, sweet potato and for isolates of *C. platani* (Engelbrecht and Harrington 2005; CABI 2015; Harrington 2000; Johnson et al. 2005).

C. cacaofunesta enters cacao tissue passively through wounds on the stem, caused by either insects (natural causes), often bark beetles (Goitía and Rosales 2001; Mazón et al. 2013), or infected cutting tools (Malaguti 1956; Saunders 1965; Santos et al. 2013). The fungus infects the xylem parenchyma cells reaching the xylem vessels in the stem (CABI 2015). *C. cacaofunesta* moves through the host into the secondary xylem (Malaguti 1952). In cacao, roots of infected trees by *C. cacaofunesta* often show necrosis suggesting that it is also a soil-borne pathogen (Engelbrecht et al. 2004, 2007a).

Recently, Araujo et al. (2014) showed that isolates of *C. fimbriata* obtained in Brazil are able to colonize all stem tissues of mango. Firmino et al. (2013) showed for the first time that *C. fimbriata* can infect fruit of *Passiflora edulis* (Firmino et al. 2013). The fact that *Ceratocystis* pathogens are able to infect other plant tissues, beyond the xylem vessels, has led to suggest that *Ceratocystis* are non-true vascular pathogens (Viégas 1960; Araujo et al. 2014).

Germination and Colonization

Since *C. cacaofunesta* (like all *Ceratocystis* species) is delivered directly to the targeted host tissue, it does not form plant penetration structures like appressoria or haustoria. Once inside the plant tissue, chlamydospores (aleurioconidia) germinate, probably triggered by exudates of the host plant (Ioannou et al. 1977). Spores of true vascular wilt pathogens, like *Verticillium* and *Fusarium*, germinate and grow well in in vitro xylem fluids (Kessler 1966). Amino acids and sugars contained in the xylem have been associated to wilt disease resistance (Booth 1969; Singh et al. 1971). However, studies of the nutritional conditions for the *Ceratocystis* fungi in the xylem are missing. In the natural host, fungal colonization is restricted to a small area around the wound site (Johnson et al. 2005). On susceptible host, *Ceratocystis* fungi colonize extensively the pith parenchyma in a radial direction in the stem tissues of plants (Clérivet et al. 2000).

Histopathological studies of *C. cacaofunesta—T. cacao* in resistant and susceptible seedlings showed that fungal spores grow well in both genotypes and produce perithecia (Santos et al. 2013). However, different patterns of fungal colonization

were observed. Tissues from the resistant genotype showed minimal fungal colonization, high amounts of non-germinated conidia and few perithecia without masses of spores, whereas, the susceptible genotype was highly colonized. In addition, in the susceptible (CCN 51) clone, the fungus produced numerous perithecia, releasing ascospores at 4 h after inoculation (Santos et al. 2013). The authors observed atypical smaller conidia protruding from the pits during the early stages of colonization and suggested that this is the reason for the rapid distribution of the fungi (Santos et al. 2013).

Fungi–Plant Interactions

A common mechanism of tree's resistance to pathogens is based on the ability to restrict them to a few cells (Duchesne et al. 1992). Host resistance in response to wilt pathogens infection includes preformed and induced mechanisms (Duchesne et al. 1992). The host-induced reaction includes the production of gel, gum, and tyloses which operates as occluding structures (Duchesne et al. 1992). These structures serve to limit the colonization of the vascular tissue by the pathogen (Duchesne et al. 1992). Ouellette and Rioux (1992) showed that this response is effective in blocking the advance of the pathogen if it is produced in advance of fungal ingress or during the colonization process. On the other hand, the accumulation of these compounds in xylem vessels leads to the obstruction of water and nutrient transport (Duchesne et al. 1992). Consequently, the branches wilt and die, compromising the whole tree (Clérivet et al. 2000). In addition, host responses include the deposition of phenolic compounds in the parenchyma cells adjacent to the xylem vessels in an attempt to impede the xylem vessel colonization by the wilt pathogens (Shi et al. 1992; Hall et al. 2011).

The cacao responses, both resistant and susceptible, to *C. cacaofunesta* infection included (1) discoloration of primary walls of infected xylem vessels and the surrounding parenchyma cells; (2) mobilization of polyphenolic in parenchyma cells; (3) translocation and accumulation of starch in the xylem; and (4) production of gums and tyloses. The main difference between genotypes was the intensities of those responses being more pronounced in the susceptible varieties (Santos et al. 2013).

C. cacaofunesta is a necrotrophic fungus. Necrotrophs are species that extract nutrients from dead cells killed prior to or during colonization (Stone 2001). Typically, diverse phytotoxic compounds are deployed to induce cell necrosis and cause leakage of nutrients (Dickman and de Figueiredo 2013). Phytotoxins have been implicated in a number of diseases caused by *Ceratocystis* spp. Ceratoplatanin (CP) is the phytotoxin best characterized in *Ceratocystis*. Ceratoplatanin occurs in filamentous fungi performing different functions like allergen in humans, elicitor of plant defenses, and inducer plant resistant (Pan and Cole 1995; Hall et al. 1999; Rementeria et al. 2005; Seidl et al. 2006; Zaparoli et al. 2009; de O Barsottini et al. 2013; Baccelli et al. 2014). The first member of

this family functionally described was a protein of *Ceratocystis platani* (Pazzagli et al. 1999). CP was found in both the fungal culture supernatants and the cell wall of the fungus. The proposed role for CP located in the cell wall is, similar to hydrophobins, to allow the attachment of hyphae to hydrophobic surfaces during the formation of aerial structures (Pazzagli et al. 1999). However, the monomers act as a toxin or elicitor inducing necrosis in tobacco cells (Pazzagli et al. 1999, 2001, 2004; Boddi et al. 2004; Scala et al. 2004). Certainly, other phytotoxic compounds may be produced by the fungus and an analysis of fungal genome sequences could elucidate this point. Wilt symptoms in cacao infected by *C. cacaofunesta* could be due to toxins in combination with vessel occlusion (Santos et al. 2013).

Plant Symptoms

Ceratocystis pathogens cause diverse diseases such as root and tuber rot, canker stain diseases, and vascular wilt (Kile 1993; CABI 2015). Infected trees may die over a period of months or years (Juzwik et al. 2011). Within *Ceratocystis*, there are many different genetic strains that vary in their aggressiveness and resistance host (Harrington et al. 2011). Thus, a plant can be infected with a mild strain and exhibits moderate symptoms or a plant can be infected with a severe strain and be killed suddenly. Either way, often these diseases are fatal for the plant. Other typical symptoms of cacao wilt disease is the black- brown discoloration of the vascular tissue, this symptom is particularly visible after the plant has shown advanced stages of wilt (Santos et al. 2013). The internal infected woody tissue shows a brown red or purplish color decreasing in intensity toward the healthy tissue (Iton and Conway 1961; Wood and Lass 2001).

The classical symptoms of the Ceratocystis wilt of cacao are leaf chlorosis and wilt within 2–4 weeks after the infection (Fig. 12.3c). Leaves remain adhered to the plant for weeks even after the death of the plant (Silva et al. 2004; Delgado and Suárez 2003; Chong 1961). Internally, there occurs vascular browning due the trapping of the conidia at the parenchyma cells surrounding the xylem vessels (Santos et al. 2013) (Fig. 12.3a, b). Host defense responses are the key to generating a containment barrier against the pathogen, as mentioned above. If the fungus is trapped at this time, the pathogen may be contained. It suggests that the restricted vessel occlusion could be a mechanism to contain the pathogen's spread in resistant genotypes (Talboys 1972). However, if conidia are released into xylem sap and disseminated, then a large number of vessels can be colonized and blocked concomitantly leading to the inability of the plant to transport the water resulting in wilt (Talboys 1972). In addition, phenolic compound deposition and necrosis of the vascular parenchyma cells contribute to wilt symptoms (Santos et al. 2013).

Santos et al. (2013) described the kinetics of the Ceratocystis wilt of cacao seedlings. Resistant and susceptible plants at 6 months of age were inoculated with a *C. cacaofunesta* spore suspension. Macroscopic symptoms of leaf chlorosis and

wilt were evident in the susceptible seedlings 7 days after inoculation. After 15 days, most of these plants were completely wilted (Santos et al. 2013).

Reproduction

Vegetative propagation occurs when an unspecialized mycelia section is detached from the main body of the thallus and forms an independent colony or mycelia mass. Asexual reproduction originates from numerous mitospores (conidia) which are sections of modified hyphae.

An early key step in sexual reproduction is mate type recognition (Ni et al. 2011). Following mating, cells undergo cell-cell fusion resulting in a dikaryotic state that prepares the cells for nuclear fusion and meiosis (Ni et al. 2011). Fungi have evolved systems to detect mating partners via mating type-specific peptide pheromones and receptors (Ni et al. 2011). The mating in ascomycetes is determined by a bipolar system consisting of two idiomorphics forms (MAT-1 and Mat-2) of the single locus (MAT-1). In heterothallic fungi, haploid hypha can't undergo sexual reproduction until it meets another compatible partner. The compatibility consists of the interaction of hyphae with different but complementary alleles at the mating locus. However, isolates of homothallic fungi are able to complete their sexual cycle independently, through self-fertile interactions with a single individual capable of sexual reproduction (Ni et al. 2011).

C. cacaofunesta, as other species, belonging to *Ceratocystis sensu stricto*, has a bipolar mating-type system (Harrington and McNew 1997). However, most of those fungi are homothallic by a unidirectional mating-type switching mechanism (Harrington and McNew 1997; Witthuhn et al. 2000). This switching of mating type occurs only in Mat-2 strains and it is irreversible. Harrington and McNew (1997) suggested that MAT-2 isolates may delete the MAT-2 gene and express MAT-1, being self-sterile. Therefore, selfings give rise to a mixture of both self-fertile (MAT-2) and self-sterile (MAT-1) offspring. Recently, Wilken et al. (2014) through genome analysis showed that the MAT-1 locus of *C. fimbriata* comprises three genes (MAT-1-1, MAT1-2, and MAT2-1) with their respective domains similar to other ascomycota MAT-genes, including the presence of introns in conserved positions. However, the MAT locus of *C. fimbriata* exhibits an atypical organization with gene rearrangements in the position of individual genes. Also, self-fertile isolates become self-sterile through the complete deletion of the entire MAT2 gene (3581 bp) and its flanking regions (1911 bp) from the MAT locus (Wilken et al. 2014).

MAT-1 and MAT-2 progenies of *Ceratocystis* species segregate in variable ratios ranging from 1:1 to 9:1 during a selfing event (Webster and Butler 1967a). However, several studies have reported that MAT-1 strains are less abundant that MAT-2 isolates in nature and grow slower (Wilken et al. 2014; Harrington and McNew 1997; Olson and Martin 1949). Harrington and McNew (1997) suggested that the decreased fitness of self-sterile offspring is a pleiotropic character

associated with the loss of MAT-2. Since MAT-2 encodes a transcription factor, its deletion could affect the expression of genes under its control. Another explanation is the occurrence of chromosome rearrangements (Harrington and McNew 1997). Therefore, the most common reproduction in *Ceratocystis* is selfing (MAT-2), limiting the genetic diversity of the group (Harrington et al. 1998). Far from being a disadvantage, this feature could facilitate rapid speciation by interruption of the gene flow. Thus, introduced populations could persist in an essentially clonal manner (Engelbrecht et al. 2007b). In this regard, the self-fertile to self-sterile switch could be a mechanism of the fungus to avoid the accumulation of deleterious mutations linked to selfing and clonal reproduction throughout obligate outcrossing (Gioti et al. 2012).

Production of Volatiles from Species of Ceratocystis

Many species of the genus *Ceratocystis* have demonstrated the ability to produce a wide variety of fruity aromas. These compounds are important in the life cycle of these fungi, because it attracts the insect vectors (Iton 1966; Engelbrecht et al. 2004). In his classification of the genus *Ceratocystis*, Hunt (1956) mentions different types of aromas produced by different species suggesting that volatile metabolic products might be used as taxonomic markers. In 1983, Sprecher and Hanssen identified several acyclic monoterpene alcohols, aldehydes, and acetates as metabolic products from cultures of *C. fimbriata*. Since then, many other monoterpenes and sesquiterpenes compounds have been reported in other *Ceratocystis* species (Lanza and Palmer 1977; Sprecher and Hanssen 1983). Furthermore, from these volatiles, various others terpenes with fruity or floral aromas have been identified and are being used for large-scale production of bioflavors (Krings and Berger 1998; Vandamme and Soetaert 2002). Various analytical techniques have been applied to the identification of these volatile compounds, including gas chromatographic (GC) and gas chromatography-mass spectrometry (GC-MSD) (Christen et al. 1997; Sanchez et al. 2002). Volatile compounds produced by species of *Ceratocystis* are thought to be important in the attraction of insects, but some of these compounds are phytotoxic and may have a role in host–pathogen interactions (Tabachnik and DeVay 1980; Engelbrecht et al. 2004).

12.5 Evolutionary Biology

Ceratocystis spp. includes fungi that have primarily been shaped by the evolutionary forces resulting from their interaction with the plant hosts and with insect dispersers. Additionally, human activities may have complicated attempts to understand the pathosystem evolution by increasing their rates of evolution, creating new niches by introducing the pathogens to new areas and new hosts, and exerting

additional selection pressures (Ennos and McConnell 1995). In the next sections we will review the current knowledge of the ecology and evolution of *Ceratocystis* at the species and population levels of biological organization.

Genetic Diversity

C. fimbriata (Halsted and Fairchild 1891), the first described *Ceratocystis* species, was considered to have a broad geographic host range until the first proposition that the species actually consists of a complex of species (Webster and Butler 1967a, b). Only more recently, DNA-based techniques have made it possible to easily distinguish independently evolving lineages that might otherwise have been assigned to *C. fimbriata*. In the last year, many other fungal species initially described as members of the *Ceratocystis* genus were proposed to actually be separated into six different genera of the same family (De Beer et al. 2014). As a result, much of the diversity initially attributed to one species, *C. fimbriata,* is actually structured among several species.

Among the Brazilian group of *Ceratocystis fimbriata*, two host specific lineages were described as different species based on molecular phylogenies and intersterility tests: *C. cacaofunesta* and *C. platani* (Engelbrecht and Harrington 2005). Another four lineages from *Gmelina*, inhame, fig, and *Eucalyptus* were separated along host lines by analysis of microsatellite markers (Ferreira et al. 2010) and in the phylogenetic analysis of MAT1-2 gene loci but they are interfertile (Harrington et al. 2011). Mango isolates of *C. fimbriata* showed a wider molecular variation and did not group as a clade in the phylogeny of Brazilian lineages, having a lineage grouping with different lineages from other hosts and being interfertile with them all (Harrington et al. 2011).

Within *Ceratocystis* species, gene diversity is relatively low, around 0.2 for Nei's diversity for clone-corrected populations, but consistent for putative native populations of the fungus such as *C. albifundus* in South Africa (Roux et al. 2001; Barnes et al. 2005), *C. platani* and *C. cacaofunesta* in South America (Engelbrecht et al. 2004, 2007b), *C. fimbriata* populations on mango in Brazil (São Paulo and Rio de Janeiro) and on Eucalyptus planted in naturally infested soil, also in Brazil (Minas Gerais and Bahia) (Ferreira et al. 2010). Lower levels of genetic diversity and even clonal structures of *Ceratocystis* groups characterize populations that have undergone recent bottlenecks. These bottlenecks occur mainly in the form of founder effects of recently colonized areas/hosts resulting from human activity with one or a few pathogen variants. This was the case for *C. pirilliformis* and *C. eucalyptcola* (= *C. fimbriata*) from *Eucalyptus* in South Africa (Nkuekam et al. 2009; Van Wyk et al. 2012), for *C. fimbriata* from mango in the Brazilian state of Mato Grosso do Sul, for *Eucalyptus* in the Brazilian state of Bahia (Ferreira et al. 2010), for pomegranate wilt caused *by Ceratocystis fimbriata*, taro and lowquat in China (Huang et al. 2003), and for *C. mangenicans* from mango in Oman and Pakistan (Al Adawi et al. 2013b).

Among *C. cacaofunesta* populations, the gene diversity values for the Rondonian (Brazil) and Ecuadorian populations of *C. cacaofunesta* are similar to those of native populations of other homothallic *Ceratocystis* species. Populations from Costa Rica, Colombia, and Bahia (Brazil) showed lower gene diversity in nuclear and mitochondrial markers suggesting that these three populations on cacao are the result of introductions (Engelbrecht et al. 2007b). The Rondonian population is within the Upper Amazon center of diversity for *T. cacao* (Cheesman 1944; Bastos and Evans 1978; N'goran et al. 2000) and is possibly the source of the introduced populations in Bahia (Brazil), Costa Rica, and Colombia (Engelbrecht et al. 2007b). The Ecuadorian population may be native on other species of *Theobroma* and shows a higher genetic divergence from other populations not only based on molecular markers but also in the difficulty in obtaining hybrid progeny from strain pairings (Baker et al. 2003). Actually *C. cacaofunesta* have a restricted distribution, and is present only in the central and South America regions. However, potentially this pathogen could be moved to others regions with the major f risk being in areas like Africa and Asia, where the majority of cacao is produced.

The relatively low levels of overall gene diversity in *Ceratocystis* populations may be related to three important characteristics leading to limited outcrossing (Yu et al. 2011; Harrington et al. 2015). First, members of the *C. fimbriata* complex are homothallic due to unidirectional mating-type switching (Witthuhn et al. 2000). Second, their natural insect dispersers have a limited flight range (Harrington 2013). Third, the long-term dispersal is usually associated with human transport of infected plant tissues usually resulting in the introduction of only one or a few genetic variants of the pathogen (Ferreira et al. 2011, 2013).

Geographic Range and Dispersal

Nowadays, the *Ceratocystis* genus has a worldwide distribution (Fig. 12.5). There is a higher density of species and occurrences in the South American tropics suggest that this region is the ancestral range of *Ceratocystis* species. The diversity patterns described from genetic markers such as DNA fingerprints and microsatellite markers, mitochondrial sequences, and RFLP haplotypes can help to distinguish indigenous populations from introduced ones. There is strong evidence that members of *Ceratocystis* Latin American clade colonized other hosts and continents in vegetative cuttings taken from asymptomatic hosts (Engelbrecht et al. 2007b; Ferreira et al. 2010; Harrington et al. 2015), especially Eucalyptus cuttings from Brazil that were introduced into China as early as 1984 (Chen et al. 2013; Harrington et al. 2015). Among clones of Eucalyptus spp. that were widely planted in South China in the 1990s, were *E. urophylla* × *E. grandis* hybrids from Brazil and *E. urophylla* clones from Indonesia, where the ITS5 haplotype of *C. fimbriata* was identified in Eucalyptus and *A. mangium* (Harrington et al. 2014).

Ceratocystis-infected Eucalyptus was also introduced into South Africa probably from a Latin American lineage of *C. fimbriata* (Van Wyk et al. 2006b) where it

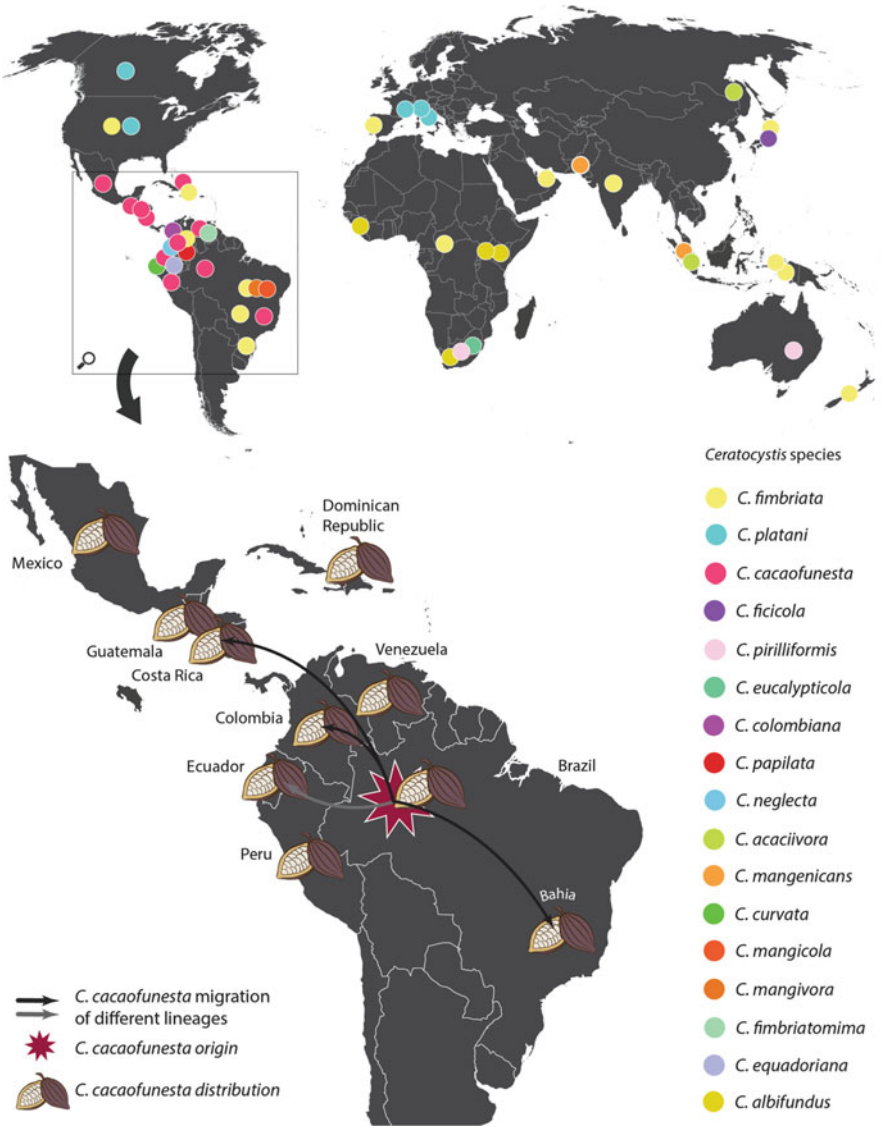


Fig. 12.5 Map of geographic distribution for *Ceratocystis*. Migration of *C. cacaofunesta* in Central and South America

diverged for reduced outcrossing and low diversity resulting from a founder effect and was recently proposed to be a new species, *C. eucalyptcolla* (Van Wyk et al. 2012). Another African lineage of *Ceratocystis* isolated in natural savanna trees, described as *C. thulamelensis*, is more closely related to South America lineages of *C. fimbriata* than to other South African lineages suggesting it was also introduced (Mbenoun et al. 2014b).

Ceratocystis platani is probably native to the southwest region of North America, but has spread throughout the Italian peninsula and too many areas of France (Ferrari and Pichenot 1976), Switzerland, and Spain (Ruperez and Muñoz 1980; Matasci and Gessler 1997). In 2003, the disease was first observed in plantings of *Platanus orientalis* in Greece, causing widespread mortality (Tsopelas and Angelopoulos 2004). Since then, *C. platani* has spread into the native range of *P. orientalis* in southern and north-western Greece causing massive epidemics and extensive ecosystem degradation in natural stands of *P. orientalis* (Ocasio-Morales et al. 2007; Tsopelas et al. 2011).

Insect–Pathogen Interactions

One of the reasons *Ceratocystis* was first considered an Ophiostomatoid fungus is the presence of previously described adaptations to insect dispersal, including the long-necked perithecium with passive discharge of adhesive ascospores from evanescent asci. However, those adaptations are considered a product of convergent evolution, i.e., evolving independently (Malloch and Blackwell 1993). Among *Ceratocystis s. l.* the ecological interactions with insects are mainly opportunistic and highly variable (Harrington 2009). Species initially attributed to the *C. fimbriata* complex are now known to have close associations, mainly with casual insects (Juzwik et al. 1998) mediated by the production of fruity aromas. These aromas are attractants for fungal-feeding insects (Lin and Phelan 1992; Kile 1993). Fungal-feeding insects such as drosophilid flies (Diptera) and nitidulid and scotilid beetles (Coleoptera) have been frequently associated with mycelial mats of *Ceratocystis,* but *Ceratocystis sensu stricto* usually do not have specific insect vectors (Kile 1993).

Nitidulid beetle transmission of *C. fimbriata* has been demonstrated with the *Populus* (Hinds 1972), *Platanus* (Crone 1963) and *Prunus* (Moller and DeVay 1968) isolates of the fungus. *Ceratocystis fagacearum*, the causal agent of Oak Wilt disease in North America has a known ecological relationship with nitidulid beetles. Many species of nitidulids inhabit *C. fagacearum* mats, but *Carpophilus sayi* and *Colopterus truncatus* appear to be particularly important vectors in the Upper Midwest and suggests that *C. fagacearum* may have coevolved with these vectors (Juzwik et al. 2004; Ambourn et al. 2005).

Some *Ceratocystis sensu lato* fungi, currently placed in the related genus *Ambrosiella* (De Beer et al. 2014), show an obligate relationship with Ambrosia beetles (Coleoptera: Scotilinae) (Engelbrecht and Harrington 2005). Ambrosia beetles cultivate ambrosiella fungi for feeding benefiting the fungi with a specialized transport to new host plants. Many of the ambrosia beetles have special spore-carrying sacs, called mycangia, and the fungal symbionts are transported in these sacs (Beaver 1989). Although ambrosia beetles are not common vectors of *Ceratocystis sensu stricto*, there are two descriptions suggesting that ambrosia beetles can vector *Ceratocystis*. The beetle *Hypocryphalus mangiferae,* largely associated with

infected mango trees in Pakistan and Oman (Masood et al. 2008), showed evidence that the beetle contributes to the transport *C. mangenicans* and xylem infect and spread (Masood et al. 2010). *Platypus cylindrus,* a previously described ambrosian beetle (Baker 1963), is developing a secondary mutualistic relationship with *C. platani* that infects *Platanus* spp. trees and was introduced in Europe (Soulioti et al. 2015).

Ambrosia beetle frass is also an important form of dispersal for the Latin American *C. platani* and *C. cacaofunesta,* which may be soil borne (Harrington 2009). Ambrosia beetles attack trees previously colonized by the pathogens (Ocasio-Morales et al. 2007) and the sawdust and fungal propagules expelled from the trees as the adult beetles clean their tunnels may be dispersed by wind or rain splash for relatively short distances (Iton and Conway 1961). On cacao, *Xyleborus* spp. (*Scolytidae*) selectively attack trees infected with *C. fimbriata* (former *C. cacaofunesta*), especially preferring trees with deteriorated bark (Saunders 1965). The adult female beetles bore into the tree perpendicular to the bark, usually at the base of the trunk (Iton and Conway 1961). Branching tunnels in which eggs are laid form horizontal planar galleries. *Ceratocystis* and other fungi may live within these galleries (Iton and Conway 1961).

Speciation Patterns

While lineages of *C. fimbriata* were deeply studied and host-specific lineages were described as new species, the hypothesis of host specialization in *Ceratocystis* has gained strength (Baker et al. 2003; Engelbrecht and Harrington 2005; Van Wyk et al. 2007a; Harrington 2009). Harrington (2000) used rDNA-ITS and MAT-2 mating-type gene sequences in a phylogenetic analysis that revealed three geographic clades within *C. fimbriata*, centered in Asia, North America, and Latin America. Within each geographic clade were several host-associated lineages that were considered possible cryptic species. Baker et al. (2003) identified two strongly supported lineages delineated by the ITS phylogenetic analysis that contained only isolates from cacao (*Theobroma cacao*) and sycamore (*Platanus* spp.). These two monophyletic lineages also showed intersterility, although not total, with each other and all Latin American lineages from different hosts. These lineages were later described as the species *C. cacaofunesta* and *C. platani*, with the aid of slightly divergent morphological characteristics (Engelbrecht and Harrington 2005).

In Indonesia, *Ceratocystis* spp. were first noted when *C. fimbriata* was reported in 1900 on *Coffea arabica* L. on the island of Java (Zimmerman 1900). Subsequently, various species of *Ceratocystis* have been reported from other hosts on many Indonesian islands. Examples include *C. fimbriata* from *Hevea brasiliensis* Müll.Arg in Sumatra, Kalimantan and Java (Leefmans 1934; Tayler and Stephens 1929; Wright 1925); *C. polychroma* M. Van Wyk, M.J. Wingf., and E.C.Y. Liew from *Syzygium aromaticum* (L.) Merrill and Perry in Sulawesi (Van Wyk et al. 2004) and *C. tribiliformis* M. Van Wyk and M.J. Wingf. from *Pinus merkusii*

Jungh. and de Vriese in Sumatra (Van Wyk et al. 2006a). In Japan, *C. ficicola* is phylogenetically more related to *Ceratocystis* species infecting Eucalyptus than to other lineages of *C. fimbriata* infecting fig hosts (Kajitani and Masuya 2011). The same pattern appears in *C. fimbriata* isolates in Colombia that are phylogenetically more related to *C. fimbriata* infecting other hosts in different countries (Marin et al. 2003). All of this evidence strongly suggests that host specialization may be an important force driving speciation in this genus.

Ecological speciation from host specializations appears to be a major route for the emergence of fungal plant diseases and some life-history traits of fungal plant pathogens can facilitate rapid ecological divergence (Giraud et al. 2010). *Ceratocystis* fits all the traits (Giraud et al. 2010). First, the strong disruptive selection imposed by different hosts requires rapid adaptation pathways. Although *Ceratocystis*, as many other fungal pathogens, shows a relatively low genetic diversity, it overcomes this limitation by the strategy of producing a large number of spores, increasing both the possibility of survival on a new host and increasing levels of adaptive variation created by mutation. Second, a high frequency of mating within a host occurs in *Ceratocystis* as a result of selfing and low dispersal distances by insects (Harrington 2009), creating pleiotropy between host adaptation and assortative mating. Third, the high frequency of asexual reproduction with rare events of sexual recombination (Witthuhn et al. 2000) prevents gene-flow among divergent lineages. Fourth, a low number of genes underlying the specificity of host–pathogen interactions are primarily evidenced by the comparison of *C. cacaofunesta* and *C. fimbriata* genomes that show a similarity of 98 % in gene composition (unpublished data).

Although many *Ceratocystis* species are concordant with the hypothesis of host specialization, worldwide migrations often induced by human activities opened the possibility of many allopatric speciations not necessarily associated with host-shifts. For example, *C. fimbriata* rapidly diversified in Indonesia with and without host-shifts. Among those diversifications, species of Eucalyptus infecting *Ceratocystis* in Indonesia have a single common ancestor with other geographic areas (Van Wyk et al. 2012) but diverged and have been described as different species worldwide: *C. atrox* (Van Wyk et al. 2007b) and *C. corymbiicola* (Nkuekam et al. 2012) from Australia, *C. pirilliformis* (Barnes et al. 2003) from Australia and South Africa, *C. neglecta* (Rodas et al. 2008) from Colombia, *C. fimbriatomima* (Van Wyk et al. 2008) from Venezuela, *C. zombamontana* (Heath et al. 2009) from Malawi, and *C. eucalypticola* from South Africa (Van Wyk et al. 2012). All of these species from Eucalyptus can be distinguished from each other based on phylogenetic inference and they have some morphological features that can be used to recognize them.

Harrington et al. (2011) also showed evidence that refutes the idea of host specialization for Latin American lineages of *C. fimbriata*. The Brazilian isolates appeared previously to be closely related to each other and to *C. fimbriata sensu stricto* based on the phylogenetic analysis of MAT1-2 sequences, which separated *Gmelina* isolates, inhame isolates, fig isolates, and Eucalyptus isolates from Bahia into groups (Engelbrecht et al. 2007b). If this genetic structure was being produced

by host specialization, one could expect isolates of one host to be particularly aggressive on cultivated hosts from the same family or genus of their native hosts. Harrington et al. (2011) studied the aggressiveness of isolates to other native and cultivated hosts and found that the variation in aggressiveness among isolates from a single host is as large as among hosts group of species. The explanation proposed is that the pathogen is not specialized to any particular host. Instead, an individual isolate has the potential for a wide range of aggressiveness to various hosts due to numerous quantitative genetic traits (Pariaud et al. 2009; Harrington et al. 2011).

12.6 Diagnosis and Control Strategies

Detection and Identification

In cacao and generally others *Ceratocystis* host trees like *Mangifera*, *Acacia* spp., *Prunus*, etc.; the principal sign of this disease is a wound caused by contaminated pruning tools and insect attacks. Another visible sign is that the leaves on a plant suddenly wilt, and the wilted leaves remain attached to the branches for several weeks after the tree dies (Engelbrecht et al. 2007a; Harrington 2000; Santos et al. 2013).

In plant material like stems and trunks, reproductive structures of the pathogen *C. cacaofunesta* are only visible at a very late stage of the infection process on mature trees. Santos and coworkers (2013) noted the absence of hyphae and conidia in inoculated susceptible and resistant cacao seedlings prior to wilting (Santos et al. 2013). In other species of *Ceratocystis* these structures can be detected by direct observation (naked eye) and usually recognized by their distinctive perithecia. Reproductive structures are difficult to observe in infected cacao trees. However, it is easy to obtain them, from parts of infected plants in a humid environment at temperature around 25 °C. The procedure usually results in ascomata production in a few days. In cases where there are no perithecia productions, pure culture of the fungus is required for reliable identification (Engelbrecht and Harrington 2005). Species of *Ceratocystis* can be isolated on potato dextrose agar, carrot agar, or malt extract agar (Harrington 2000). A carrot assay is a selective medium and has been used successfully for isolating *Ceratocystis*. The assay consists of placing a small piece of infected plant material between two fresh carrot disks that have been sterilized and incubate the plates at 25–27 °C in high humidity during 4–10 days. In this period, the production of fungal perithecia is initiated (Moller and DeVay 1968; Engelbrecht and Harrington 2005). Then, the ascospore masses from the perithecia are formed on the carrot disks are transferred to malt extract agar media for purification and subsequently storage in sterile water at room temperature (Castellani 1939) or in a cool chamber. It has been reported that the fungus can also be isolated from the body of ambrosia beetles using the

carrot assay (Goitía and Rosales 2001). *C. cacaofunesta* grows rapidly in malt extract agar. After approximately 24 h, a hyaline mycelium appears, and overtime the pathogen produces characteristic dark-brown perithecia (see above: morphology).

C. cacaofunesta has been recently differentiated from other strains of the *C. fimbriata*. Unfortunately, we don't have yet a diagnostic protocol for its detection and identification as well as for its differentiation from other related fungal species. However, it is recommended to use the identification of the pathogen based on its culture and morphological characteristics reported by Engelbrecht and Harrington (2005). Molecular or serological diagnostic techniques have not been available, but there are microsatellite markers developed by Steimel et al. (2004) which had been used in population studies (Engelbrecht et al. 2007b) with intersterile populations and show unique microsatellite markers (Engelbrecht et al. 2007b). *C. cacaofunesta* can be confused with other species of *Ceratocystis*, principally with *Ceratocystis moniliformis,* but can be differentiated because the latter species is only weakly pathogenic (CABI 2001). *C. moniliformis* grows much more quickly on agar medium in laboratory conditions. In addition, its morphological characters are different: the perithecial bases of *C. moniliformis* have spine-like ornamentations and fungus fails to produce aleurioconidia (Hunt 1956; Harrington 2013).

Control Strategies

There is a wide variety of circumstances that hinder the implementation of controls throughout the multiple host ranges of *Ceratocystis* spp. However, most of the controls are expensive and may be harmful to the environment (Juzwik et al. 2011). Thus, prevention is a recommended approach for cacao farmers. Nevertheless, if the plantation is already infected or there is an imminent risk of infection it's recommended to implement mitigation measures (exclusion and eradication). Control measures like chemical and biological control and host resistant genotypes all target and interrupt the principal stages of the infection cycle of the fungus (Juzwik et al. 2011).

Avoidance Wound avoidance is the key to management diseases caused by *Ceratocystis* spp., including Ceratocystis wilt of cacao. Since it is a vascular pathogen, the recommended preventive measures are as follows: (1) Ensuring the health of new planting or grafting material since they can carry the pathogen from the nursery; (2) avoid causing wounds on trees; (3) prevent field personnel from climbing the trees during harvest or pruning during collection; (4) disinfect tools with any of the following products [sodium hypochlorite 5 %; formalin; benomyl (Benlate), thiabendazole (Mertect), and carbendazim (Bavistin and/or Derosal)], in doses of 4 cc or 1 ml/l of water; (5) pruning should be done preferably in the dry season and protecting wounds immediately after cutting with potassium permanganate 1 % or one of the above products; (6) a healing paste (pitch) can be applied to

large wounds and should be monitored periodically to ensure proper healing; and (7) periodical monitoring of the trees should be conducted, in order to detect early infections, so that subsequent sampling and treatment can be conducted.

Exclusion Nowadays, chemical treatments are used in packing materials to help control the spread of wood-boring insects. Thus, it could prevent the spread of *Ceratocystis* in insect droppings before reaching the final destination (FAO 2009). After pruning, when the wood is still moist is when the formation of mycelium begins beneath the bark (Gibbs and French 1980). Apparently, those conditions favor the fungal growth but it does not survive for long periods in the stem wood (Merek and Fergus 1954). A good practice recommendation is to clean the trunk with water and 8-hydroxyquinoline sulfate solution. Take care with the cutting implements and climbing ropes when they are used to avoid mechanical transmission. Finally, it is important to mention that some of the diseases caused by *Ceratocystis* spp. are categorized as an A1 quarantine pest by principal phytosanitary protection organizations around the world: the European and Mediterranean Plant Protection Organization (EPPO), Inter-African Phytosanitary Council (IAPSC), and the North American Plant Protection Organization (NAPPO).

Eradication The eradication of Ceratocystis wilt of cacao during local epidemics has been possible by following good sanitation practices (Harrington 2013). However, when an exotic disease epidemic arrives, it can be a difficult task to complete the eradication even in a limited area. This is because the high prevalence of the pathogen on non-host plants. Nevertheless, when the pathogen is quickly recognized, Ceratocystis wilt of cacao can be easily eliminated. All symptomatic trees, recently killed tree as well as neighboring trees must be removed in order to reduce disease prevalence and prevent the spread (Harrington 2013). After removal of all diseased trees, it is recommended to treat the diseased stumps with ammate, aresenite, or 2,4,5-trichloroethane-rophenoxyacetic acid (2,4,5-T). This treatment reduces the survival time of *Ceratocystis* (Merek and Fergus 1954). Additionally, farmers should take into account that by cutting diseased trees, there is a risk that the conidia will move to adjacent trees through root grafts. Therefore, root grafts between trees must be severed before felling the diseased trees.

The wood of the diseased trees can be utilized by the wood industry, burned, or buried, as long as the bark is removed and the wood is dry (French and Juzwik 1999). The tools and equipment that will be used to process the diseased trees must be disinfected with alcohol, sodium hypochlorite, or other chemicals. Treatments to the infected area should include the spraying of glyphosate or other herbicides to kill the root systems of infected plants and to further prevent the spread by root grafts (Harrington 2013).

Diseased trees should not be cut on windy days and their movement in and out of the affected area should be limited to minimize or prevent the spread of fungal infected particles such as sawdust. Additionally, the contaminated soil must be removed and disinfected. Physical and chemical methods are recommended, such as water vapor or by using products like metam sodium, dazomet, and

dicloropropen (Yuce et al. 2011). McCracken and Burkhardt (1979) reported that felling and removing from the area of diseased trees was not needed because *Ceratocystis* spp. had a short survival period. However, more recent studies have shown that *Ceratocystis* fungi can survive for long periods as aleurioconidia in sapwood. Therefore, the authors suggest removing the debris before the insects enter the timber and spread contaminated frass (Harrington 2013).

Protection Tree protection against *Ceratocystis* spp. involves fungicide injections, wound dressings, and trenching or other barriers. However, fungicide treatment can be problematic because vascular infected tissue is hard to be reached. In addition, the treatment may not be efficient when providing a single sustained dose. The high cost of the treatment and the tree injury caused during the injection process must be balanced with the likelihood that the treatment will reduce the risk of infection. Moreover, preventive treatments may only delay the onset of the disease. Fungicide treatment is not useful when the plant is already infected, as it does not stop the advance of the pathogen (Blaedow et al. 2010). Another disadvantage of triazols is the limited movement of the compound into the root system and short retention in the tree.

Walter et al. (1952) recognized that wound dressings commonly had sawdust with viable *C. platani* inoculum, which could survive for months. Fungicidal wound dressings have been recommended for the protection of pruning or other wounds from infection by *C. variospora* (DeVay et al. 1968) and other *Ceratocystis* spp. Latex paint is the recommended treatment because of its effectiveness and its lack of toxicity to the tree (French and Juzwik 1999; Camilli et al. 2007). Wound dressings also can inhibit the growth of *Ceratocystis* (Davis and Peterson 1973).

Reductions in root graft transmission by the use of root-free zones have been practiced for many years and can be highly effective in reducing losses caused by *Ceratocystis* (Bretz 1951; Gehring 1995; Cummings-Carlson et al. 2010; Juzwik et al. 2011). Generally, a trench is made to delimit the infected trees from healthy trees. The placement of the trench can be calculated based on the probability of disease spread (Bruhn et al. 1991). Generally, this protective practice involves two barriers, primary barrier using at least one ring of healthy trees beyond the symptomatic trees and a secondary barrier placed inside the first one that helps to protect the ring of healthy trees.

Host–Plant Resistance Plant resistant to *Ceratocystis* pathogens has been used successfully in *Mangifera* (Ribeiro et al. 1995; Rossetto et al. 1997), *Ipomoea* (Martin 1954), and *Crotalaria* (Ribeiro et al. 1995). In *Coffea,* three genotypes (MEG 639-601; MEG 639-617, and MEG 639-704) were selected from breeding programs (Castillo 1982; Castro-Caicedo and Cortina-Guerrero 2009; Castro-Caicedo et al. 2013). *Citrus* species have been shown to vary in their susceptibility to Colombian strains of *C. fimbriata* (Paez and Castano 2001). In contrast to other *Ceratocystis*, soil-borne populations of *C. fimbriata,* in Brazil this fungus can be quite diverse, and individual isolates vary greatly in aggressiveness to Eucalyptus and other hosts (Harrington et al. 2011). Four highly resistant clones of Eucalyptus

(Clones C15, C16, C17, and C18) are available to manage this disease (Zauza et al. 2004). Subsequently, Rosado et al. (2010) studied Ceratocystis wilt resistance by stem inoculations of *E. grandis* and *E. urophylla* genotypes and estimated the heritability and gains of selection in families derived from controlled interspecific crosses. As a result, a high degree of genetic control and low allelic dominance of the trait were found and five QTLs for Ceratocystis wilt resistance on Eucalyptus were mapped using microsatellite genetic maps for the interspecific, full-sibling family DGxUGL [(*E. grandis x E. dunnii* Maiden) x (*E. urophylla x E. globulus*)]. However, in both *E. grandis* and *E. urophylla*, there are clones that range from highly resistant to highly susceptible and the crosses of these two species show a high degree of heritability of resistance (Rosado et al. 2010). In cacao, most authors agree with the use of resistant genotypes as the more efficient strategy against *C. cacaofunesta* (Table 12.1). This is because the results are more stable and beneficial to the environment (Silva et al. 2004; Delgado 2003; Engelbrecht and Harrington 2005; Alarcon 1994; Guerrero 1975; Chong 1961). Desrosiers (1956) began finding differentiation between cacao varieties based on their susceptibility to infection with *Ceratostomella fimbriata*. During 1980s, international breeding programs for resistance to *Ceratocystis* were developed (Gardella et al. 1982; Ocampo et al. 1982) and resistant genotypes were identified under local conditions (Mata 1991; Delgado 2003). A classical method, developed by Delgado and Echandi (1965), has been employed to assess resistant genotypes. Basically, propagule suspensions are applied to branch sections to evaluate perithecial development. Ceratocystis wilt of cacao has gained great importance because much of the germplasm that has been selected for that is resistant to witches' broom and has proven to be susceptible to *Ceratocystis* (Engelbrecht et al. 2007a). Silva et al. (2004) reported the susceptibility of the clone IMC-67 to a *C. cacaofunesta*

Table 12.1 Different responses of cacao clones to *C. cacaofunesta* infection

Host response	Clones	References
Resistant	CEPEC 2002 (VB 1151), TSH 1188, PS 13:19 and CEPEC 2007 (VB 681)	CEPLAC collection; Oliveira et al. (2009)
Intermediate resistant	TSAN 792, PH 15, CP 44, CP 46, and ICS 1	Oliveira et al. (2009)
Susceptible	CCN 10, CA 1.4, SJ 02, CCN 51, and PH 16	Oliveira et al. (2009)
Resistant to both colonization and reproduction of the fungus	AMA-10, EBC-122, EBC-138, EBC-142, EBC-148, and LCT-EEN 73	Delgado (2003)
Resistant for avoid the pathogen reproduction	AMA-4, AMA-14, AMA-9, CUR-3, LCT-EEN 57, and LCT-EEN 382	Delgado (2003)
Resistant	POUND-12, EET-399 and EET-400	Delgado and Suárez (2003)
Resistant to Ecuadorian isolate, susceptible to Brazilian strain	IMC-47 and IMC-67	Delgado and Suárez (2003)

Brazilian isolated, which is concerning since this is one of the primary resistant clones used for rootstock to combat this disease.

Chemical Control Fungicides were first used with some success to treat tapping panels of *Hevea* (Chee 1970), in *Ipomoea* fields (Martin 1971) and in postharvest dips of *Ipomoea* roots (Daines 1971; Yang et al. 2000). Also, in *Ficus*, fungicides were used to control Ceratocystis wilt disease (Hirota et al. 1984). Stems of *Platanus* species have been injected with fungicides resulting in protection of the plant against *C. fimbriata* (Causin et al. 1995; Minervini et al. 2001). In cacao, because Ceratocystis wilt disease kills the plant, fungicides have the potential to prevent crop losses, but this is normally not economically feasible (Ferreira et al. 2013). Thus, sanitation is more effective for control of this disease. For example, disinfecting machetes and pruning tools between plants may help control the disease in *Platanus* (Walter et al. 1952 *Prunus* (Teviotdale and Harper 1991) and cacao and a 10 % solution of formalin is recommended to disinfect tools (FEDECACAO 2007). Moreover, all cacao cuttings from regions with Ceratocystis wilt disease should be grafted and grown under strict quarantine in a greenhouse before field release to ensure that they are free from *C. cacaofunesta* (Engelbrecht et al. 2007a).

Biological Control Several isolates of the genus *Trichoderma* have been tested in in vitro assays to evaluate the antagonistic potential to cacao pathogens such as *Moniliophthora perniciosa*, *Phytophthora palmivora*, and *Ceratocystis cacaofunesta*. Only *T. harzianum* and *T. pseudokoningii* have shown potential biocontrol in vivo suggesting that they could be used as control agents (Oliveira et al. 2013b). Moreover, the effectiveness of two kinds of trap for collecting scolytids, bottle traps with windows (situated at 1 m and 3 m above ground level) and funnel traps, was tested to control the scolytid wood-boring beetles. Funnel traps collected significantly more individuals than bottle traps. The scolytid diversity collected by each kind of trap was dissimilar, having only 60 % of species in common. These scolytids, especially *Xyleborus* spp., are among the most harmful cacao pests in America, because of their mutualistic association with *Ceratocystis cacaofunesta* (Mazón et al. 2013).

Phytosanitary Risk

Because most forms of the species are easily transmitted in cuttings, unrestricted movement of cuttings or other propagative material is potentially dangerous. It is likely that *Ceratocystis* has been spread to new countries or regions through cuttings of *Populus*, *Theobroma*, *Eucalyptus*, and *Syngonium* and on storage roots of *Ipomoea*. Circumstantial evidence points to packing materials as the source of the plane tree pathogen in southern Europe. *Ceratocystis* is known to survive up to 5 years in wood, probably in aleurioconidia form. *Ceratocystis fimbriata* is listed among the highest risk pathogens that could be imported into the United States on eucalyptus logs and chips from South America (Kliejunas et al. 2001). The

Platanus form (*C. fimbriata* f. *platani*) is listed as an EPPO A2 quarantine pest (OEPP/EPPO 1986).

12.7 Summary

The genus *Ceratocystis sensu stricto* Ellis & Halst. includes many aggressive plant pathogens causing wilt, canker stain diseases, and tissue rot on a wide variety of perennial and economically important agronomic crop worldwide.

In recent years, the recognition of the economic importance of *Ceratocystis* has resulted in an intense scientific interest, as evidenced by an increase in publications.

The major threat to agriculture from *Ceratocystis* is the emergence of new fungal species and their appearance on new hosts and in new geographical areas.

Ceratocystis wilt of cacao is reemerging as an important disease and represents a serious threat to major cacao producers in the world.

The taxonomic history of the genus *Ceratocystis* is complex and has been intertwined with several other genera like *Ophiostoma*, *Leptographium*, and *Raffaelea*. Consequently, the genus has been subjected to many name changes.

The genus *Ceratocystis fimbriata* was placed into the order Microascales, family Ceratocystidaceae. This delimitation has made it possible to study the genus independently and discover new cryptic species.

The genus *Ceratocystis* accommodates many important fungi that include many plant pathogens. Recent studies showed high genetic diversity in this group and have resulted in the proposal to establish species complexes within the genus.

The traditional biological species concepts is inappropriate for this group with high rates of asexual reproduction and several morphological similarities; the similarity in these group can be explained by convergent evolution linked to an association with the dispersal insects.

Species in *Ceratocystis* has been recently defined based on unique morphological characteristics, by mating studies, intersterility tests, host ranges, and phylogenetic inference base on DNA sequence data. However, additional research and continued investigation are required to clearly define all of the species within this genus.

The causal agent of Ceratocystis wilt of cacao is a newly described species, *Ceratocystis cacaofunesta* (formerly *C. fimbriata)*, that causes a serious disease of *Theobroma cacao* in Central and South America.

Ceratocystis spp. exhibits the three reproductive modes described in filamentous ascomycetes: vegetative propagation, sexual, and asexual reproduction.

C. cacaofunesta is homothallic by a unidirectional mating-type switching mechanism.

The classical symptoms of the Ceratocystis wilt of cacao are chlorosis of the leaves and wilt within 2–4 weeks after the infection. Leaves remain attached to the plant for weeks even after the death of the plant.

The cacao disease responses, both resistant and susceptible, to *C. cacaofunesta* infection included (1) discoloration of primary walls of infected xylem vessels and

the surrounding parenchyma cells; (2) mobilization of polyphenolic in parenchyma cells; (3) translocation and accumulation of starch in the xylem; and (4) production of gums and tyloses.

Wilt symptoms of cacao infected by *C. cacaofunesta* could be due to toxin activity combined with vessel occlusion.

Ceratocystis species maintain low levels of genetic diversity within populations, because the main reproduction method is asexual and its current dispersal mechanism is highly associated with human transport of clonal isolates.

A strong association with bark beetles for dispersal was reported in many *Ceratocystis* species. It is *currently c*onsidered an inefficient disperser due to the large-scale human-related migrations; however, the past evolution of *Ceratocystis* populations and species might be *highly shape*d by this ecological association. Further studies are necessary to establish the current role of bark beetles and *Ceratocystis* infections.

Wound avoidance is the key to the management of *Ceratocystis* spp. diseases.

The eradication of Ceratocystis wilt has been possible by following good sanitation practices when epidemics caused by *Ceratocystis* are local.

Clean nursery stocks could be produced if care is taken to make the collections of cuttings from trees free of diseases. Follow up monitoring of nurseries stocks should be done using molecular tools.

Treating trees with fungicides to prevent Ceratocystis wilt can be problematic because of the difficulty of treating internal vascular tissues and in providing a single sustained dose.

In cacao, resistant genotypes are the most efficient strategy against *C. cacaofunesta,* because it is more stable and beneficial to the environment.

Because Ceratocystis wilt of cacao kills the plant, fungicides have the potential to prevent crop losses, but in most cases are not economically feasible.

Biological control has the potential to control against Ceratocystis wilt of cacao, but requires field validation.

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Chapter 13
Armillaria Root Rot of *Theobroma cacao*

Jolanda Roux and Martin P.A. Coetzee

Abstract Armillaria root rot of *Theobroma cacao* was reported for the first time more than a century ago. The pathogen causes a root rot of these trees, resulting in tree death. Mortality commonly occurs in distinct disease centers, often around stumps of dead native trees removed for the establishment of cacao orchards. The cause of Armillaria root rot was, and often continues to be, ascribed to *Armillaria mellea*. Advances in fungal taxonomy have, however, shown that *A. mellea* is restricted to eastern Asia, Europe, and North America and that the cause of Armillaria root rot of cacao, in for example Africa, represents a distinct species. Management of Armillaria root rot is often unsuccessful due to the persistence of fungal inoculum in roots, stumps, and soil. Some success has been achieved in managing the disease in other crops, but due to the relatively low acknowledged incidence of Armillaria root rot in cacao plantations, limited experience in controlling the disease exists for this crop. Care should be taken, however, as the impact of root and wood rot pathogens such as *Armillaria* species results not only in tree death but also in reduced tree vigor and yield. The availability of genomes for both *T. cacao* and *Armillaria* species provides valuable opportunities for future endeavors to reduce the impact of this disease in cacao plantations.

13.1 Introduction

Armillaria species (Basidiomycota, Agaricales, and Physalacriaceae) are well-known root rot pathogens of many plant species, including both woody and herbaceous plants globally (Raabe 1962; Watling et al. 1982; Wargo and Shaw 1985; Guillaumin and Legrand 2013). They have been described as aggressive

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killers of healthy trees, secondary pathogens of stressed trees, and saprophytic decayers of dead trees (Wargo and Shaw 1985), with more than 500 species of woody plants recorded as hosts (Morrison et al. 1991). Depending on the environment and hosts, infection by *Armillaria* species may have a significant impact both in natural forests, such as in the natural deciduous forests of Australia (Kile 1980; Shearer et al. 1997), and in agricultural and plantation setups (Shaw and Kile 1991).

Armillaria root rot of *Theobroma cacao* has been known since the early 1900s in West Africa (Gravier 1907, in Thorold 1975; Dade 1927) and from the Americas (Averna-Sacca 1920; Rombouts 1937). It is, however, not considered a serious problem when compared to diseases caused by, for example, *Phytophthora* and *Moniliophthora*. In a recent symposium paper by Ploetz (2007), Armillaria root rot was not even mentioned. Recent studies in Cameroon, however, suggest that Armillaria root rot may be of more importance than currently acknowledged. In at least two cases where tree death was ascribed to another pathogen, investigation of the problems revealed Armillaria root rot as the cause of death (Roux et al. 2012).

In this chapter, we summarize the small amount of literature available pertaining to Armillaria root rot of *T. cacao*. We also provide a summary of some of the information available regarding *Armillaria* species in general, the epidemiology of the diseases they cause, and the management strategies used to reduce their impact.

13.2 History and Impact of Armillaria Root Rot

The first report of Armillaria root rot of *T. cacao* is unclear with Fox, in Popoola (2004), reporting that it was apparently first reported by Eady (1922), on cacao in Togo. Thorold (1975), however, lists Gravier (1907) as already having recorded *Armillariella mellea* (*Armillaria mellea*) from these plants in Sao Tomé and Noack also reported it from cacao in Cameroon in 1907.

In Ghana, *A. mellea* (a species name that was generally applied to all fungi causing Armillaria root rot in Africa) was classified as an injurious pest soon after its discovery as the cause of tree death, and failure to control it was punishable by law (Popoola 2004).

> "*A. mellea* was, therefore, classified as an injurious pest in the Injurious Pest Ordinance of 1924 (Anon 1924). Failure on the part of a farmer, who fails to maintain good sanitary condition and apply the 'prescribed treatment' to cases of attack was considered an offence punishable by fines or imprisonment. This was to show the importance of the menace of the disease on the economy' of Ghana."—From Popoola (2004)

The impact of Armillaria root rot on different crops varies, but infection by the causal fungus eventually results in tree death. Because *Armillaria* species may take several years to kill an infected host (Raabe 1966, 1979), the true impact on crop production is likely underestimated. Extensive studies in vineyards in the USA have shown that infection by *A. mellea* significantly decreases yield and growth, lowers root absorption of macronutrients, and results in delayed ripening of fruit (Baumgartner 2004; Baumgartner and Warnock 2006). Losses of up to 30 % have

been reported from cassava farms and from forestry plantations in Zimbabwe as a result of Armillaria root rot of these plants (Mwenje et al. 1998). In many regions the disease has led to the abandonment of orchards as control measures are often too expensive and/or ineffective.

13.3 Taxonomy and Nomenclature of *Armillaria*

The type species of the genus *Armillaria*, *Armillaria mellea* (Vahl: Fr.) Karsten, was first described in 1790 by Vahl as *Agaricus melleus*. It was later transferred to the genus *Armillaria* by Kummer in 1871. Other genus names previously assigned to *Armillaria* species include *Armillariella*, *Clitocybe*, and *Lepiota*. Until the 1970s, all species of *Armillaria* with an annulus were included in *A. mellea* (Watling et al. 1982; Guillaumin and Legrand 2013).

The genus *Armillaria* contains more than 51 morphological species, as well as unique biological and phylogenetic species (Coetzee et al. 2011). Originally, as was the case with other fungi, only the morphological species concept (MSC) was applied to identify *Armillaria* species. However, identification based on basidiocarp morphology has several limitations, of which the lack of basidiocarp production is but one. Basidiocarps are ephemeral and irregularly produced and thus not always available for identification. When succeeding in inducing basidiocarp production in culture, their morphology often does not correlate with those generated in the field. A significant constraint, as with the MSC in other fungi, is the lack of resolution in morphological features as the basidiocarp morphology of many species is nearly identical (e.g., Bérubé and Dessureault 1989). Several additional phenotypic characters were introduced to assist with the identification of *Armillaria* species. These include response to temperature (Mohammed et al. 1994; Rishbeth 1986), response to phenolic acids and terpenes (e.g., Rishbeth 1986), isozyme and protein profiles (e.g., Coetzee et al. 2009; Morrison et al. 1985; Mwenje and Ride 1996; Mwenje et al. 2006), and their reaction to mono- and polyclonal antibodies (e.g., Fox and Hahne 1989; Lung-Escarmant et al. 1985).

The biological species concept (BSC) has been used, in addition to morphology, for several decades to identify *Armillaria* species. Using mating tests it was shown that *A. mellea* sensu lato represented a species complex (Korhonen 1978; Anderson and Ullrich 1979). The BSC, however, has a number of constraints, both technically and genetically. The mating interaction between two strains is often ambiguous with no clear demarcation line between the isolates indicating mating incompatibility. Partial interfertility has been observed between certain morphologically defined species (e.g., Bérubé et al. 1996). Tester strains become unsuitable for mating test when they aged (Harrington et al. 1992). Although species in *Armillaria* can be differentiated based on the BSC, applying the concept in taxonomy and diagnostics should be done with caution (Korhonen 1978, 1983; Harrington et al. 1992; Banik and Burdsall 1998; Morrison et al. 1985; Bérubé et al. 1996).

Currently, all identifications of *Armillaria* species routinely include DNA-based techniques, especially DNA sequencing. Previously, DNA–DNA hybridization (Jahnke et al. 1987), amplified fragment length polymorphisms (AFLPs) (Pérez-Sierra et al. 2004; Wingfield et al. 2009), and restriction fragment length polymorphisms (RFLPs) of genomic DNA or amplified genomic regions (e.g., Anderson et al. 1987; Smith and Anderson 1989; Harrington and Wingfield 1995; Coetzee et al. 2000a, b; Chillali et al. 1997, 1998) were also employed in conjunction with other species concepts. DNA sequencing and phylogenetic analyses of the ribosomal DNA (rDNA) operon intergenic spacer I (IGS-1), rDNA internally transcribed spacer regions (ITS 1 and ITS 2, including the 5.8S gene), and a portion of the translation elongation factor 1-α (TEF1-α) gene are most commonly used (Anderson and Stasovski 1992; Coetzee et al. 2000b, 2001a, 2005; Maphosa et al. 2006). The phylogenetic species concept (PSC) has allowed for the identification of distinct taxonomic groupings that could not be identified using the MSC or BSC.

The taxonomy of *Armillaria* in Africa, and other continents, has been problematic, with many currently known species previously being assigned to *A. mellea* sensu lato, or assigned as subspecies. Recent and ongoing research has, however, shown that the fungus causing disease of plantation and native forest trees in Africa is not *A. mellea* (Coetzee et al. 2000a; Maphosa et al. 2006; Mwenje et al. 2006). The majority of cases of Armillaria root rot of agricultural and horticultural crops in Africa seem to be caused by native species of *Armillaria*. Although *A. mellea* has been reported from South Africa, it was shown that the fungus was introduced into the country from Europe (Coetzee et al. 2001b).

Apart from a single, ongoing study to identify the cause of Armillaria root rot in Cameroonian cacao plantations, the identities of the species causing root rot of cacao remain uncertain. Isolates of *Armillaria* are obtained from dying and dead *T. cacao* trees in two regions in Cameroon group within the *A. fuscipes* sensu lato clade (Roux et al. 2012). This clade, however, includes many subclades and may constitute several distinct species (Coetzee et al. 2005).

13.4 Distribution and Host Range

Armillaria species have a global distribution and attack more than 500 plant species (Raabe 1962; Shaw and Kile 1991; Kile et al. 1994). The majority of studies on these fungi, however, are from North America (e.g., Bliss 1951; Shaw and Roth 1976; Anderson and Ullrich 1979; Morrison 2004; Ross-Davis et al. 2012; Elías-Román et al. 2013), Europe (e.g., Hintikka 1974; Termorshuizen and Arnolds 1987, 1994; Morrison 2004; Keča and Solheim 2011; Tsykun et al. 2013), and Australia and New Zealand (e.g., Shaw et al. 1981; Kile and Watling 1988; Hood 1989; Shearer et al. 1998; Coetzee et al. 2001a; Dunne et al. 2002; Morrison 2004). More recently in-depth studies of the species diversity and distribution of *Armillaria*

Table 13.1 Reports of Armillaria root rot from *Theobroma cacao*

Country	Reference
Brazil	Rombouts (1937), Averna-Sacca (1920), Shaw and Kile (1991)
Cameroon	Noack (1907), Grimaldi (1954) in Thorold (1975), Shaw and Kile (1991)
China	Beijing Forestry University (1983) in Shaw and Kile (1991)
Colombia	Averna-Sacca (1920), Shaw and Kile (1991)
Congo (Belgian)	Dade (1927)
Democratic Republic of Congo (Zaire)	Steyaert (1948) in Thorold (1975), Shaw and Kile (1991)
Dominican Republic	Ciferri (1961) in Thorold (1975)
Equatorial Guinea (Fernando Po)	Nosti (1953) in Thorold (1975)
Gabon	Mohammed (1992)
Ghana	Noack (1907), Dade (1927), Shaw and Kile (1991)
Ivory Coast	Noack (1907), Mallamaire (1935) in Thorold (1975), Shaw and Kile (1991)
Madagascar	Orian (1954) in Thorold (1975), Shaw and Kile (1991)
Mexico	Limon (1945), Shaw and Kile (1991)
Nigeria	Bailey (1966) in Thorold (1975), Shaw and Kile (1991), Popoola (2004)
Papua New Guinea	Dumbleton (1954) in Thorold (1975), Shaw and Kile (1991)
Principe	Shaw and Kile (1991)
Sao Tome	Gravier (1907) in Thorold (1975), Rishbeth (1980), Shaw and Kile (1991), Mohammed (1992)
Sierra Leone	Noack (1907), Deighton (1936)
Togo	Eady (1922) in Popoola (2004), Shaw and Kile (1991)
Uganda	Noack (1907), Dade (1927), Shaw and Kile (1991)

species have emerged from Africa, Asia, and South America (Matsushita and Suzuki 2005; Qin et al. 2007; Pildain et al. 2010; Coetzee et al. 2000a, 2001a, b, 2005; Mohammed 1992; Mwenje and Ride 1996; Maphosa et al. 2006).

Reports of *Armillaria* from *T. cacao* date back to the early 1900s (Table 13.1). Little to no research on the disease in cacao plantations has, however, been published in the last few decades.

13.5 Biology of the Pathogen

The vegetative state of *Armillaria* species, which is the most abundant state, is diploid (2n) with mycelia lacking clamp connections, rather than dikaryotic (two different haploid nuclei sharing a mycelium) as is the general nuclear state for other Basidiomycota (Motta 1969; Korhonen and Hintikka 1974). The majority of the species have a tetrapolar heterothallic mating system (Baumgartner et al. 2011);

thus mating will occur only between hyphae with different alleles at two mating type loci. A few species are homothallic, such as the African form of *A. mellea* (Guillaumin et al. 1994), producing diploid mycelium from a basidiospore and therefore being able to propagate in an asexual manner. Mating is controlled by a bifactorial (having two mating type loci) mating system for all species, except for *A. heimii*, which has a unifactorial (having one mating type locus) mating system (Ullrich and Anderson 1978; Abomo-Ndongo et al. 1997).

Heterothallic *Armillaria* species produce basidiomes that give rise to sexual basidiospores that are haploid. In compatible matings, the haploid hyphal cells at the mating interaction zone will briefly become dikaryotic, before fusion of nuclei to produce clampless secondary mycelia with one diploid nucleus per cell (Anderson and Ullrich 1982). A brief, second dikaryotic stage occurs during fruiting to produce haploid basidiospores. These spores will germinate to form haploid, uninucleate, primary mycelia.

Homothallic species of *Armillaria* exhibit a type of homoheteromixis or secondary homothallism (Abomo-Ndongo et al. 1997; Ota et al. 1998). In this system, four haploid nuclei are produced in the basidium after meiotic division of the diploid nucleus. The haploid nuclei fuse into pairs after which they migrate to two of the four basidiospores. This is followed by mitosis after which one of the nuclei might migrate back to the basidium, leaving the basidiospore with one diploid nucleus. Basidiomes of homothallic strains yield a mixture of uninucleate, binucleate, and anucleate basidiospores (Abomo-Ndongo et al. 1997; Ota et al. 1998). Fertile basidiospores can subsequently germinate to produce diploid colonies.

Colonies produced by *Armillaria* species survive as facultative necrotrophs that can colonize living roots, kill the root tissue, and then utilize the dead material as a source of nutrition (Rishbeth 1985; Baumgartner et al. 2011). In the parasitic phase, *Armillaria* species colonize the cambium of living roots, killing the cambium. In the saprophytic phase, they utilize the dead tissue, with mycelium often persisting on residual roots and stumps for many years.

13.6 Characteristic Symptoms and Progress of Infection

Infection of trees by *Armillaria* species are mostly only recognized when tree canopies wilt and die. However, other signs may be present at a much earlier stage in the disease and include canopy chlorosis, crown thinning, dwarfed leaves and die-back, and cracking (Fig. 13.1a) and bleeding at the bases of infected trees. These symptoms may be associated with excessive fruit production, unseasonal fruiting, and/or the production of fruits that are smaller than usual.

When cutting into the bases and root collars of infected trees, the presence of white (when fresh) to cream (older) mycelial mats between the bark and wood, associated with dead/dying wood, will be observed (Fig. 13.1b). These mycelial mats may also be found in the wood (Fig. 13.1c, d) and roots of trees (Fig. 13.1f).

Fig. 13.1 Symptoms and signs of Armillaria infection of *Theobroma cacao*. (**a**) Cracking of bark at the base of an infected tree, (**b**) bark and cambium lesion, together with white fungal/mycelium fan after removal of bark, (**c**) cracking of the xylem and mycelial fans in xylem of infected tree, (**d**) white mycelial mat and gum production in the stem, (**e**) basidiomes (mushrooms) of a typical *Armillaria* sp. in Cameroon found on a native tree species, (**f**) white mycelial mats in an infected and dead *T. cacao* root, and (**g**) typical *Armillaria* sp. rhizomorphs on the base of an infected tree

They have a distinct mushroom smell, are robust, and can be peeled off from the tree. Infected tap roots and stems may split as a result of infection (Fig. 13.1c). The latter feature has been pointed out as a peculiarity of Armillaria root disease in the tropics and it was for this reason that the disease has also been known as "collar crack disease" and "splitkanker" (Dade 1927; Gadd, CH in Popoola 2004).

Armillaria species cause white rot of wood, in which both lignin and polysaccharides are degraded. Rotting wood is often fibrous in texture, spongy, and wet. Black zone lines are often seen in the decaying wood. These lines are composed of thickened, dark fungal cells that may play a role in the protection of the *Armillaria* sp. from unfavorable conditions and/or competition from other fungi in the rotting wood (Worrall 1994). Infection by some species of *Armillaria* may result in luminescence, producing a faint glow from the wood under low light.

Basidiomes (mushrooms, Fig. 13.1e) and rhizomorphs (Fig. 13.1g) may or may not be present on infected trees. The formation of these structures is dependent on specific environmental factors and their production is, therefore, erratic. Finding basidiomes is especially difficult as they are soft and ephemeral, lasting only a few days. They can be found at the bases of infected trees, the root collar, or growing out from the roots. Basidiomes are fleshy, mostly honey brown in color, but can vary from yellow to brown or olivaceous depending on the species and environment. They have tufts of dark hair and whitish gills, and the majority (*A. tabescens* and *A. ectypa* do not) have a ring (annulus), resulting from a partial veil, on their stipes. *Armillaria* species produce white to cream-colored spore prints (Watling et al. 1982; Guillaumin and Legrand 2013).

Rhizomorphs, resembling root or thread-like structures, are produced by most species and can be found under the bark, on the roots, and in the soil surrounding the infected tree. When cultivated, all species of *Armillaria* produce these structures on growth medium. The rhizomorphs may be monopodially or dichotomously branched, cylindrical or flattened, reddish brown to black (e.g., Bérubé and Dessureault 1988, 1989; Morrison 2004). When they are actively growing they may have a cream-colored tip. The inner tissue is whitish mycelium, which may become tan with age. They have been shown to translocate nutrients over several meters (Lamour et al. 2007).

Vegetative spread of *Armillaria* species results in characteristic disease centers with the oldest mortality at the center as the pathogen progressively grows from tree to tree. These centers often develop around dead native trees in the plantation. Disease centers can continue to grow over many years, killing trees of different ages (Shaw and Kile 1991).

Disease progress in terms of mortality increased in grape vineyards over a 2-year period from ~5–11 % and increased in terms of symptomatic plants from 1 to 8 % (Baumgartner and Rizzo 2002). Symptoms often only become visible shortly before tree death (Rhoads 1956; Edgar et al. 1976; Hintikka 1974; Baumgartner and Rizzo 2002). In *Pinus* plantations in the USA disease increased from less than 5 % to more than 20 % in 7 years (Bruhn et al. 1994). Foliar symptom development and tree death are higher in *Pinus* species when the root collar is infected than when roots

are infected (Shaw 1980). Aboveground symptoms typically do not appear until 50–75 % of roots have been colonized (Marsh 1952; Baumgartner and Rizzo 2002).

13.7 Epidemiology

Armillaria root rot is often a problem in areas where native forests/bush were cleared to establish plantations/orchards (Baumgartner 2004; Shaw and Kile 1991). The mycelium survives saprophytically in residual woody roots after cutting of trees and can remain buried in the soil to serve as inoculum for infection of the next crop (Redfern and Filip 1991). After its host plant has died it can survive as a saprophytic white rotter. Depending on environmental conditions, stump size, and other factors, *Armillaria* species may survive for decades in stumps.

Armillaria species may disperse using two routes, namely through airborne spores or vegetatively through the growth of the pathogen between trees via root contact or via the growth of rhizomorphs (Prospero et al. 2006). Vegetative growth most commonly leads to the successful spread of the fungus and is considered the more important dispersal mechanism of the two.

Basidiomes (mushrooms) of *Armillaria* species produce sexual basidiospores that are airborne. These may land on exposed stump surfaces or wounds that expose the wood of trees, leading to infection. From the stumps the pathogen may then infect living trees by growing down into the roots of the stumps and via root contact infect healthy trees. Basidiomes can remain dormant for months and survive relatively harsh environmental conditions (Shaw 1981). Their exact role and importance in initiating new infection centers, however, remain an enigma. Attempts to demonstrate infection by basidiospores in nature have generally been unsuccessful. Indirect evidence from population structure studies (Legrand et al. 1996; Dettman and van der Kamp 2001; Szewczyk et al. 2014), however, clearly indicates that establishment from basidiospores does happen.

Vegetative dispersal of *Armillaria* species may take place in one of two ways. Mycelium may be transferred between trees where diseased roots come into contact with healthy roots or via rhizomorphs that grow through the soil. Rhizomorphs may grow along root surfaces, under the bark of dead trees, or for several meters through the soil to infect new hosts (Garrett 1956, 1960; Prospero et al. 2006).

Armillaria species may cause disease on both healthy and stressed trees, depending on the species and age (Prospero et al. 2004; Baumgartner et al. 2011). Factors that may cause suitable stress to trees include biotic and abiotic factors such as drought, soil compaction, defoliation, and other diseases. Several studies have indicated a higher level of disease incidence and development under wet conditions (Wargo 1984; Rizzo et al. 1998; Baumgartner and Rizzo 2002).

13.8 Diversity of the Pathogen

Armillaria species include some of the world's largest asexual clones. For example, a single clone of *A. gallica,* in Michigan, has reached a size of 15 hectares (ha) with an estimated age of more than 1500 years (Smith et al. 1992). In contrast, genetic diversity may be high in small areas or between populations.

Population structures of *Armillaria* species have been studied in multiple ways, including culture characteristics, mating-type alleles, and isozymes. These techniques have been used to infer mycelial types, clones, and genotypes (Worrall 1994). Initially, and most commonly, pairings between diploid isolates were used to determine somatic compatibility between isolates (Adams 1974; Kile 1983; Rizzo and Harrington 1992). Isolates that are incompatible belong to different clones, while those that are compatible represent a single clone. More recently, molecular markers have been developed to study the population and genetic diversity of *Armillaria* species (Saville et al. 1996; Langrell et al. 2001; Brazee et al. 2012; Baumgartner et al. 2010). In recent years, microsatellites, or simple sequence repeats, have mostly been used to infer mating structure, genetic diversity, and gene flow among populations of *Armillaria* species (Prospero et al. 2008; Baumgartner et al. 2010; Xing et al. 2014). Some of these markers have proven effective for multiple species of *Armillaria* (Prospero et al. 2010).

Population studies in *Armillaria* have been used to investigate the establishment of new infection centers, the factors contributing to the establishment and growth of these infections, as well as the generation of diversity in intraspecific interactions. The presence of multiple somatic incompatibility groups among different trees, for example, suggests infection by basidiospores, rather than vegetative growth (Shaw and Roth 1976). Microsatellite markers have revealed that *Armillaria* populations are panmictic, with evidence of gene flow between populations separated by 1000–3000 km. This is speculated to be achieved through basidiospore dispersal (Prospero et al. 2008; Baumgartner et al. 2010).

13.9 Control

Although Armillaria root rot of trees is one of the oldest tree diseases known (e.g., Hartig 1874), disease management efforts are often ineffective. The fact that mycelium of *Armillaria* species can survive saprophytically in roots in the soil makes it especially difficult to manage Armillaria root rot, particularly in woody systems. A number of management options have, however, been attempted and research is ongoing to optimize some of these.

Considerable research has focused on the prevention of infection of agronomic crops by *Armillaria* species. Techniques employed include avoidance of sites with a history of Armillaria root rot, soil fumigation, manual removal of infected plant material, soil inoculations with antagonistic fungi or bacteria, trenching, and root

collar excavation. Avoidance of sites with *Armillaria* infections can, however, be difficult, particularly where no records exist of *Armillaria* incidence, or where natural forest is cleared for cacao plantations.

Methylbromide and carbon disulfide have been most commonly used to fumigate soil and infected stumps before plantation establishment (Bliss 1951; Adaskaveg et al. 1999). Both compounds are reported to kill mycelium in partially decayed roots up to 1 m deep in the soil (Munnecke et al. 1973, 1981; Ohr et al. 1973; Baumgartner 2004). Several other chemicals, including systemic fungicides, have also been tested. These include methyl isothiocyanate, metam-sodium, chloropicrin, and sodium tetrathiocarbonate (Adaskaveg et al. 1999). However, fumigants do not provide long-term control (Adaskaveg et al. 1999) and should form part of an integrated management system.

Manual removal of roots, stumps, and other woody material from plantations/orchards after deep tilling was first suggested by Hartig in 1874 and remains one of the main recommendations to reduce the incidence of Armillaria root rot globally (Bliss 1951; Baumgartner and Rizzo 2002). Trenching is often applied with inoculum removal, to prevent spread through infected tree roots and rhizomorphs from infection centers (Bliss 1951). This is done by digging a trench up to 1 m deep, lining it with plastic, and backfilling the trench. This is only successful if the pathogen is limited in its occurrence in the area and the trench is dug well beyond the last affected trees.

Root collar excavation can be used both pre- and post-planting and relies on the reduction of plant tissue moisture. This is achieved through permanent removal of soil from the base of a tree's trunk resulting in the lowering of moisture in the root collar. The reduction in moisture reduces the chance of *Armillaria* infection and spread. It may even lead to the receding of mycelial fans from the root collar, before it has had the opportunity to infect underlying vascular tissue, thereby improving the function of vascular tissue at the base of the trunk (Baumgartner 2004). This technique does not provide a cure for Armillaria root rot, but based on reports from hosts such as *Pinus* species buys time, through reducing tree death, and provides opportunity for the establishment of new plants (Shaw 1980; Baumgartner 2004). Root collar excavations are of limited value once *Armillaria* has girdled or killed substantial portions of the tree's trunk.

Studies to investigate the potential of soil inoculation with antagonistic fungi and bacteria have received considerable interest in agriculture, including the control of Armillaria root rot. Biological control using, for example, *Trichoderma* species to infect mycelium weakened by sublethal doses of fumigants has been applied with some success (Garrett 1957; Munnecke et al. 1973, 1981). However, it is difficult to achieve necessary concentrations of biological control agents at depths more than 0.3 m (Otieno et al. 2003). Furthermore, it has been suggested that *Trichoderma*-based products are not effective against *Armillaria* as they cannot penetrate into the wood and/or through the black zone lines caused by *Armillaria* infection (Downer 2004). Similarly, the use of antagonistic bacteria (e.g., *Bacillus subtilis*) via drip-irrigation systems (Baumgartner and Warnock 2006) has been tested experimentally. Although these treatments show great potential under laboratory conditions,

their efficacy under natural conditions with multiple factors impacting on these systems has resulted in less encouraging results. Baumgartner (2000) also showed that mycorrhizal fungi do not protect tree roots from infection by *Armillaria* species. This is because they infect root hairs and young roots, whereas *Armillaria* species infects woody tissue.

Therapeutic treatments are available for *Armillaria* infections of grape vines and other fruit trees in the USA. However, effectiveness of these treatments depends on the damage and level of infection (Baumgartner and Rizzo 2002). Chemical control, apart from soil and stump fumigation before plantation establishment, is not widely used to manage Armillaria root rot. As with other diseases, registration issues and cost are often inhibitive. Where chemical control has been attempted, it has not been successful in curing existing infections of trees or has at best been inconsistent. Several fungicides have been tested as protectants, eradicants, and curatives (Hagle and Shaw 1991). Therapeutic injections of propiconazole into almond trees in the USA were successful in reducing mortality of infected trees during two growing seasons compared to untreated trees (Adaskaveg et al. 1999).

13.10 Breeding for Resistance and Future Research

Armillaria root rot of *T. cacao* has not yet had a serious impact on this crop. As a result, no known breeding programs have been established to select for genetic material tolerant/resistant to the disease. In other crops, e.g., grapevine (Baumgartner and Rizzo 2006), stone fruit (Guillaumin et al. 1989; Wilkins et al. 2002), and walnut (Reil 1997), resistant root stocks have been developed against Armillaria root rot.

The potential exists for selective breeding based on genetic resistance traits of *T. cacao* against Armillaria root rot. Both the *T. cacao* (Argout et al. 2011) and *A. mellea* genomes (Collins et al. 2013) have been sequenced, and the genome sequencing of other *Armillaria* species is also in the pipeline. Investigating these genomes will provide significant opportunities for understanding the molecular interaction between *Armillaria* species and *T. cacao*. The information gained from these studies can be implemented in future breeding programs.

13.11 The Way Forward

Many instances of cacao death caused by Armillaria root rot may be misidentified due to inaccurate diagnostics. It is, therefore, important that cacao growers and researchers are trained regularly in disease and pest recognition. Care should be taken to ensure accurate diagnostics so as to implement the most accurate disease management strategies.

The identification of *Armillaria* species associated with Armillaria root rot should receive attention. In many cases the cause of Armillaria root rot is still routinely referred to as being *A. mellea*, which is inaccurate and causes confusion with regard to quarantine and disease management.

Strict quarantine, restricting the movement of microbes and insects between countries and continents, should continue and should include bans on the movement of any soil or growth media or any rooted plants where the possibility existed of infection by *Armillaria* or other root pathogens.

The availability of the cacao genome and increasing numbers of *Armillaria* species genomes will allow for more accurate resistance breeding in the future.

13.12 Conclusions/Summary

Although Armillaria root rot of *T. cacao* has not received much attention in the past few decades, its presence and possible impact on tree health should not be ignored. As is evident from the multitude of other tree crops, the impact of *Armillaria* species can be severe, either as primary pathogens or in combination with other biotic and abiotic factors. Its impact, especially in regions where *T. cacao* is intercropped with native trees in cleared natural areas, is most likely underestimated, as has been seen recently in Cameroon. Management of Armillaria root rot disease is currently complex, labor intensive, and expensive. The existence of genome sequences of the causal agents and other modern technologies, as well as extensive experiences from other crop systems, however, provides excellent opportunities for future management of the disease in cacao plantations.

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Chapter 14
Root-Infecting Fungi Attacking *Theobroma cacao*

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Abstract In this chapter, we consider some of the "root-infecting" pathogens of *Theobroma cacao*. Despite sometimes being described as minor pathogens of the crop, they can cause serious losses locally. We have chosen to examine four pathogens—*Verticillium dahliae* (Verticillium wilt or sudden death), *Rigidoporus microporus* (white root disease), *Rosellinia* spp. (black rot), and *Phellinus noxious* (brown root rot). These are all soil-borne, root-infecting fungi, but whilst the basidiomycetes and *Rosellinia* (ascomycete) are unspecialized, opportunistic root rot pathogens whose pathogenicity is part of a saprophytic lifestyle, *Verticillium* is a highly specialized pathogen adapted for colonization of the xylem system. Yet, despite differences in their pathogenicity, there are some important practical similarities with regard to their management. These pathogens are extremely difficult to manage given their soil-borne nature, colonization of alternative hosts and/or colonization of woody debris, and the perennial nature of the cacao host plant. Currently, cultural methods are often used as the main management approaches with chemical control being considered too costly or ineffective. Development of breeding programs against the unspecialized pathogens is one area of research that should be expanded; successful breeding programs have been undertaken for *Verticillium* in Brazil. Biological control is another area where further work is needed for all these pathogens both with regard to the direct application of biological control agents (BCAs) themselves and on the interaction with BCAs and various soil amendments to enhance naturally occurring antagonists. There may be differences in approach here based on modes of pathogenicity, but inevitably, we should be aiming at an integrated approach for these pathogens.

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14.1 *Verticillium dahliae*: Verticillium Wilt

Introduction

Verticillium spp. are soil-borne fungi with worldwide distributions. They cause vascular disease resulting in severe yield losses in many crops in temperate and subtropical regions, yet are generally less destructive in the tropics (Subbarao et al. 1995). *V. dahliae* is more destructive in warmer climates, compared to *V. albo-atrum* which is more problematic in crops of higher latitudes with humid climates. This is linked to their temperature requirements with *V. dahliae* favoring temperatures of 25–28 °C, while *V. albo-atrum* has a lower temperature range (20–25 °C). Globally, *Verticillium* spp. colonize a wide range of plant genera and species. *V. dahliae* infects members of the *Malvaceae* such as cacao and cotton, *Solanaceae*, *Asteraceae*, *Convolvulaceae*, *Papilionaceae*, *Labiatae*, and *Chenopodiaceae* (Resende et al. 1994; Domsch et al. 2007).

Verticillium wilt of cacao, caused by *V. dahliae*, is a serious problem in the States of Bahia and Espírito Santo in Brazil (Resende et al. 1995). This disease is more common in areas subject to droughts, causing annual plant mortality of up to 10 % in unshaded cacao-growing areas (Almeida et al. 1989). Verticillium wilt of cacao has also been reported in Uganda inducing losses of up 30 % (Emechebe et al. 1971) and of the four principal diseases affecting cacao in that country, *V. dahliae* was considered to cause the greatest crop damage (Matovu 1973). This is one reason why cacao has not become a significant crop in Uganda (Leakey 1965; Resende et al. 1995; Sekamate and Okwakol 2007). More recently, interest in cacao production in Uganda is increasing. Uganda exported 19,439 tons of the commodity worth US$46.6 million in 2013 compared to 17,935 tons worth US$43 million during 2012 and the Ugandan government plans to distribute further cacao seedlings to encourage farmers to grow the crop (Nabwiiso 2015). It will be interesting to see if Verticillium wilt will become a problem to this new enterprise. *V. dahliae* has also been found on cacao in Colombia (Granada 1989; Resende et al. 1995).

Taxonomy

Two species of *Verticillium*, *V. dahliae* Klebahn and *V. albo-atrum* Reinke & Berthold, are very similar and prior to the 1970s, some workers, particularly in North America, considered *Verticillium dahliae* to be synonymous with *V. albo-atrum*. Current thinking regards *V. dahliae* as a highly variable and probably mutable species. The fungus originally had a much more restricted range that has become greatly extended as a result of human activity (CPC 2015). *V. dahliae* can apparently respond locally to selection pressure imposed by vegetation and cropping patterns, and this microevolution is imposed on a base population whose character may be more or less variable and reflects the origins of the parent

population. Evidence of this occurring has been reported in *Verticillium dahliae* isolates from cacao in Uganda with cross-infection of isolates from cotton and cacao (Resende et al. 1994). Verticillium wilt of cotton is widespread in Uganda and the country is perhaps unique in growing cacao in the same ecological zone as cotton (Resende et al. 1994). There is much to be learned about intraspecific variation in *V. dahliae* and the structures and affinities of populations throughout the extensive range of this species.

Taxonomically, *V. dahliae* is separated from *V. albo-atrum* mainly by the presence of microsclerotia as resting structures and these withstand adverse environmental conditions up to 13 years (Schnathorst 1981). The distinctness of the two species is now widely accepted and has been confirmed by molecular evidence (Typas 2000). When freshly isolated, the two species can be readily distinguished on the basis of morphological and other characteristics (Mace et al. 1981; Heale 2000). However, loss of pigment and ability to produce conidia in culture make identification of old isolates more difficult. Colonies are moderately fast growing, white at first, with little to moderate aerial mycelium and a regular margin. Colonies turn black from the center after a week or so as a result of microsclerotia production. *Verticillium dahliae* is the anamorphic form of an ascomycete, belonging to the family Plectospharellaceae, Class Sordariomycetes. The vegetative mycelium of *V. dahliae* is hyaline, usually branched, septate, and multinucleate. Conidia are ellipsoidal to ovoid, hyaline, mostly one celled, and 2.5–6 × 1.5–3.0 μm in diameter, produced at the tips of narrow, pointed, conidiogenous cells subtended in whorls (2–3 per node) on more-or-less erect, hyaline, verticillate conidiophores (Gómez-Alpízar 2001; Fradin and Thomma 2006). Conidia are produced in succession to form moist spore balls at the tips of conidiogenous cells, giving characteristic appearance to conidiophores in culture. Microsclerotia are commonly observed in *V. dahlia*, but rarely in *V. albo-atrum*. Microsclerotia are dark brown to black, torulose, almost globose, and of irregular shape and size (50–200 × 15–100 μm). In culture, white sectors may appear as a result of partial loss of the ability to produce microsclerotia, or pigmentation may be lost entirely.

Symptoms

Symptoms of Verticillium wilt in cacao are similar to symptoms seen in many crops attacked by *Verticillium* spp. These include epinasty (downward growth of petioles), yellowing, necrosis, and wilting or abscission of leaves, followed by stunting or death of the plant (Resende et al. 1996a). The disease is often referred to as sudden death. Generally with *Verticillium* diseases, wilting starts from the tip of an infected leaf, and the symptoms start in the oldest shoots as invasion is acropetal, i.e., from the base to the apex (Fradin and Thomma 2006). However, Cooper et al. (2000) reported the opposite to acropetal progression in inoculated cacao plants in the glasshouse with increased symptoms in upper leaves which corresponded to increased colonization. These authors attributed this to vessel

Fig. 14.1 Aerial symptoms of Verticillium wilt on cacao in the field (DRC) showing chlorosis and dessication of the leaves. *Source*: Eric Boa, University of Aberdeen

anatomy with increased interconnections within and between vascular bundles higher up the stem. In the field, infected cacao plants generally exhibit sudden wilting and subsequent necrosis of leaves and flushes (Fig. 14.1). In cacao-growing areas of NE Brazil, it is common to observe diseased trees with or without leaves, but no attention has been given to the pattern of defoliation caused by isolates from distinct trees (Resende 1994). These defoliating and non-defoliating types of symptom development occur on other hosts too. *V. dahliae* pathotypes have been described as defoliating or non-defoliating Schnathorst and Mathre (1966), but other authors (Ashworth 1983) have suggested a continuum of symptoms related to the relative aggressiveness amongst strains of *V. dahliae*, rather than the occurrence of distinct pathotypes.

Wilting symptoms in *Verticillium*-infected plants are thought to be due to the formation of vascular occlusions resulting in water stress and defoliation and may also involve growth regulator imbalances. Talboys (1968) suggested that defoliation was related to the level of water stress, while Tzeng and DeVay (1985) and Resende et al. (1996a) demonstrated enhanced production of ethylene from cotton and cacao plants, respectively, infected with defoliating isolates compared to those infected with non-defoliating isolates. In cacao stem sections, a brown discoloration of the vascular tissues is observed accompanied by tyloses and deposition of gels and gums seen internally in the vessels. In the field, brown streaks or bands are seen within the sapwood of infected trees (Fig. 14.2). Recovery is also seen in the field and is a feature of Verticillium wilt of apricot, cacao, cherry, olive, and peach as well as various ornamental and shade trees and bears significance for management

Fig. 14.2 Internal symptoms of Verticillium wilt of cacao (DRC) showing blackening of the infected vessels in the wood. *Source*: Eric Boa, University of Aberdeen

of the disease (Jiménez-Díaz et al. 2012). Symptom levels depend mainly on the concentration of inoculum, pathotype of *Verticillium*, plant variety and stage of plant development, temperature, soil moisture, and nutrition, especially potassium content (Resende 1994). Symptoms intensify when the evapotranspiration rates are high, as well as under drought. The area of NE Brazil where Verticillium wilt is observed is prone to droughts.

Biology

V. dahliae can survive for many years in soil as microsclerotia, either free or embedded in plant debris, which are stimulated to germinate in response to root exudates (Mace et al. 1981; Mol 1995). Microsclerotia can germinate multiple times with hyphae growing toward roots of potential hosts within an estimated sphere of influence of 300 μm or less (Huisman 1982). Upon reaching the root, the fungus colonizes the root surface, often at or near root tips, or following root hairs to the root surface. Hyphae grow between epidermal cells (Klosterman et al. 2009)

and then proceed (inter and intracellularly) through the cortex and into the xylem (Bowers et al. 1996). Various host responses may occur in tissues as a defensive response to infection (Mueller and Morgham 1993; Resende 1994; Bowers et al. 1996). Once in the xylem, the pathogen spreads by mycelial growth as well as by production of conidia which are transported in the transpiration stream, rapidly becoming systemic in susceptible crops. The major disease effects are believed to result from occlusion of vessels and the production of toxins (Mace et al. 1981). Resende et al. (1995) reported systematic colonization of inoculated susceptible cacao seedlings while in more resistant cultivars, production of tyloses, gels, and gums in association with accumulation of various antifungal compounds restricted colonization. The host–pathogen interaction in cacao has been extensively studied by Resende and coworkers with identification of four phytoalexins including arjunolic acid and elemental sulfur (Resende et al. 1996b; Cooper et al. 1996).

During tissue necrosis or plant senescence, *V. dahliae* enters a saprophytic stage with the stems and roots of the plant becoming colonized in addition to the vascular tissue. Large numbers of microsclerotia are formed (Mol 1995) which can act as inoculum for subsequent plantings. Microsclerotia survive well over a range of soil moisture and temperature conditions, but rapidly lose viability in wet, warm soil (Green 1980). Both bacteria and fungi attack and degrade the microsclerotia in soil (Tjamos 2000).

V. dahliae is considered as a monocyclic disease, i.e., only one disease cycle and a single inoculum production occur during one growing season. Consequently, inoculum levels of *V. dahliae* (microsclerotia per gram of soil) in the soil at planting time play a critical role in the development of disease on many crops (Xiao et al. 1998; Xiao and Subbarao 1998). In contrast, *V. albo-atrum* may produce conidia on infected plant tissues above ground. These conidia become airborne and contribute to spread of the disease. Therefore, the diseases caused by *V. albo-atrum* can sometimes be polycyclic (Fradin and Thomma 2006). However, Resende et al. (1994) reported the asymptomatic colonization of dicotyledonous weeds by *V. dahliae* from cacao fields. This indicates an alternative mechanism of survival but could also indicate inoculum levels present in cacao fields have been underestimated. Such asymptomatic colonization could also serve as a source of transmission to other localities and regions. It has been suggested that much of the national and international movement of *V. dahliae* (in other hosts) has probably occurred with contaminated planting material and on weed seeds (Evans 1971). In cotton, the pathogen is found principally as microsclerotia associated with the lint, but also internally at up to 10 microsclerotia/seed (Sackston 1983) and infected cotton seeds are considered to be the primary route for introduction into new geographical areas (Ma and Shezeng 2000).

Management

Management of diseases caused by *V. dahliae* is difficult because of the long survival of the pathogen in soil and in plant debris (via microsclerotia) and in alternative hosts including many weed species. Transmission of the pathogen can occur through a number of agencies including infected plant material, contaminated soil, and in irrigation water.

An integrated approach for the management of many Verticillium wilts has been advocated for a number of crops and this approach includes: (1) site selection to avoid planting in high-risk soils; (2) use of disease-free planting material; (3) reduction or elimination of inoculum in soil; (4) protection of healthy planting material from infection; (5) use of resistant cultivars and rootstocks; (6) cultural practices; (7) soil solarization; and (8) organic or biological soil amendments. Such an ecologically based approach aims to minimize damage caused by the pathogen through the combined use of all available management measures, either simultaneously or sequentially, and through actions taken both prior to and after establishing the crop. However, an added problem for management of *Verticillium* wilt in cacao is that, being a tree crop, some of these management options are irrelevant. Site selection, away from planting in high-risk soils, is one option for new cacao plantings and for existing plantings. Pereira et al. (2008) suggested management of the disease through the use of genetic resistance and associated cultural measures, such as the use of healthy seedlings, removal of infected crop residues, balanced fertilization, irrigation, and proper application of systemic fungicides. Several cacao cultivars such as Pound 7 and SIC 2 have been shown to be more resistant to the disease than other cultivars (Resende et al. 1995). These authors reported the use of a stem puncture technique as compared to the more usual soil drench method. The stem puncture technique is able to differentiate resistance and susceptibility more rapidly, using less inoculum and has potential as a large-scale screening method for breeding programs against this pathogen (Resende et al. 1995). Breeding for resistance against Verticillium wilts remains the most logical approach to management of these diseases (Klosterman et al. 2009) and this includes cacao.

Cavalcanti and Resende (2005) have claimed that induced resistance represents a promising strategy as part of an integrated management approach of *Verticillium* wilt of cacao because it is possible to use susceptible cultivars with good agronomic characteristics. Progress has been made in this area through the use of the *S*-methyl ester of benzo-(1,2,3)-thiadiazole-7-carbothioic acid (acibenzolar-*S*-methyl, ASM). However, the efficacy of acibenzolar-*S*-methyl depends on the time of application and the dosage and more experimentation is needed.

With regard to the use of systemic fungicides for Verticillium wilt of cacao, this is uneconomic on a field scale and impractical due to application constraints and because the fungus survives in plant debris or in soil for prolonged periods of time. Crop rotation has been advocated in managing Verticillium wilt diseases in many crops (Butterfield et al. 1978), but there is also considerable disagreement as to its

overall effectiveness (Huisman and Ashworth 1976). Removal of infected crop debris at harvest has also been advocated in some cropping systems (Mol et al. 1995) but also deemed ineffective in others. These highly variable results are likely due to the differences in strains or pathogen pre-planting populations between locations, differences in soils and/or soil microflora, or varying success in the exclusion of weeds from rotation crops.

Pre-plant soil disinfection using a range of biocidal chemicals has been used worldwide for many decades in order to manage Verticillium wilts in high-value crop production but with increasing concerns about the environmental impact of these chemicals, other soil treatments have also been attempted. Solarization, where plastic sheets are applied to soils allowing them to heat the soil with solar energy, is one alternative and this has been used successfully to manage wilt in a number of crops including perennials, prior to planting or even in established orchards (Ashworth and Gaona 1982; Tjamos et al. 1991; Stapleton et al. 1993). This could be attempted in cacao.

The addition of organic soil amendments, from plant and animal sources, can be effective in reducing disease in some cropping systems (Lazarovits et al. 2000). Organic soil amendments reduce the survival of microsclerotia through increasing the populations of antagonistic soil microflora. The effectiveness of this approach is subject to variations in soil types, locations, and/or cropping systems (Lazarovits et al. 2000). Specific biocontrol agents are being developed that may be useful against *V. dahliae* (Tjamos 2000). Biocontrol agents, both fungal and bacterial, that can be applied to seed and roots of planting stock are being studied. Specific examples of fungi with activity against wilt include *Heteroconium chaetospira* (Narisawa et al. 2000) and *Talaromyces flavus* (Fravel et al. 1995; Madi et al. 1997). Plant growth promoting rhizobacteria such as *Pseudomonas* and other genera obtained from the rhizosphere can also be effective (Berg and Ballin 1994). This is an area where more research can be undertaken in the context of Verticillium wilt of cacao.

For many crops, *V. dahliae* is easily transmitted in vegetative planting stocks serving as a means of introducing highly aggressive strains and as a direct inoculum source. With regard to cacao, efforts should be made to prevent the entry of the pathogen into other cacao-producing regions in West Africa and Asia through infected material. It is necessary to restrict the movement of germplasm into areas where the disease does not occur and to collect branches for bud grafting from areas free of pathogen. Resende et al. (2010) advocated that if the plant material is from affected areas, then it must be placed in quarantine for observation and analyses since the fungus can remain dormant inside the plant tissue. Vascular discoloration symptoms are often observed. The absence of the pathogen must be confirmed through direct plating on alcohol agar medium before being dispatched (Freitas and Mendes 2005).

14.2 *Rosellinia*: Black Rot Disease

Introduction

Rosellinia species have commonly been recorded in both temperate and tropical regions (Petrini 1993). Only a few species are known to occur as root pathogens and only two, *Rosellinia bunodes* (Berk. et Br.) Sacc. and *R. pepo* Pat., are considered as actual root-infecting pathogens of cacao. A third species, *R. paraguayensis* Starb., has only been recorded on cacao in Grenada (Waterston 1941). Both *R. bunodes* (see also http://www.cabi.org/isc/datasheet/47857) and *R. pepo* occur in tropical areas in Central and South America, West Africa, the West Indies, and Asia (Waterston 1941; Saccas 1956; Sivanesan and Holliday 1972; Castro et al. 2013) and can cause root rot in a large and diverse number of economically important crops like banana, cacao, coffee, tea, citrus, rubber, avocado, and various important timber species (Aranzazu 1996; Booth and Holliday 1972; Saccas 1956; Sivanesan and Holliday 1972; Waterston 1941).

Black root rot or Rosellinia root rot is an important cacao disease in some parts of Latin America and the Caribbean (Aranzazu et al. 1999; Cadavid 1995; Merchán 1989a, 1989b; Waterston 1941). Its first mention in this region was probably that of Barber (1893, cited by Nowell 1916). Since then, both pathogens have been reported in an increasing number of countries. According to Aranzazu et al. (1999), in some areas of Colombia over 50 % of cacao-producing farms are affected by *Rosellinia*. In one estate, within 2 years between 0.3 and 4.4 % of cacao trees were lost to *Rosellinia* spp. Seven years after planting, less than half of the original population had survived in those disease foci (Cadavid 1995). In the Biguá-Peruíbe region, São Paulo, Brazil, up to 20 % of 3-year-old cacao trees died (Feitosa and Pimentel 1991). In CATIE's cacao germplasm bank in Cabiria (Costa Rica), 45 clones, some of them unique, were lost to a complex of *Ceratocystis cacaofunesta* and *Rosellinia* between 1997 and 2001, which is equivalent to 1.4 % per year (Mendoza et al. 2003).

Taxonomy

The genus *Rosellinia* belongs to the subdivision Ascomycotina; class Euascomycetes; subclass Pyrenomycetes; order Sphaeriales, syn. Xylariales and was erected by De Notaris in 1844 with *R. aquila* (Fr.:Fr.) De Not. as the type species. *Rosellinia bunodes* and *R. pepo* were first described around the turn of the nineteenth century. *R. bunodes* was first described as *Sphaeria bunodes* Berk. et Br. in 1873 and as *R. bunodes* (Berk et Br.) Sacc. in 1882 (Saccas 1956; Sivanesan and Holliday 1972). The fungus, *R. pepo* Pat., was described in 1908 (Booth and Holliday 1972). Generally, *Rosellinia* spp. possess smooth perithecia with dark, one-celled ascospores. Size and shape of ascospores and perithecia are useful

Fig. 14.3 *Rosellinia* sp. on cacao roots. *Source*: G. M. ten Hoopen

parameters for species identification. However, a problem in identifying *Rosellinia* species may arise as it can be difficult to obtain the teleomorph. Therefore, identification is often based on vegetative structures alone (Castro et al. 2013).

Symptoms

Rosellinia root rots are often characterized by patches that extend in a circular pattern due to the way in which the pathogen infects neighboring plants. *Rosellinia* spp. are thought to spread through direct root contacts between plants (Aranzazu et al. 1999) and it is unclear what role ascospores or sclerotia play in the epidemiology. *R. pepo* occurs on roots as grayish cobweb-like strands (Fig. 14.3) that become black and coalesce into a woolly mass. Beneath the bark, white, star-like fans can be observed. *Rosellinia bunodes* on roots shows firmly attached black

branching strands that may thicken into irregular knots. *Rosellinia bunodes* may extend well above the soil surface in humid conditions (Sivanesan and Holliday 1972). In cacao, the first symptoms include yellowing and drying of the leaves, defoliation, drying of tree branches, and finally the bush or tree dies. Immature fruits tend to ripen prematurely, remain empty of beans, and, when not harvested, turn black and dry out (Merchán 1989a, 1993; Mendoza 2000). In cacao in the Americas, it seems that *Rosellinia* and *Ceratocystis cacaofunesta* act together as they are often found together. Aranzazu et al. (1999) mentioned that cacao trees attacked by *Rosellinia* are subsequently attacked by *Ceratocystis*. However, as *R. bunodes* and *R. pepo* are considered opportunistic pathogens (Waterston 1941), ten Hoopen and Krauss (2006) believed that *Rosellinia* acts as a secondary invader after infection with *C. cacaofunesta*. *Ceratocystis cacaofunesta* causes a relatively rapid death in cacao. Leaves wilt and dry on the tree where they remain suspended for considerable time. In contrast, if *Rosellinia* is the primary cause of death, chlorotic leaves are shed gradually, usually before they are fully necrotic. In either case, *Rosellinia* may be observed on the roots and base of the dying tree. At this point, a transversal cut should give diagnostic certainty: *C. cacaofunesta* causes a vascular discoloration, which is absent, in trees killed by *Rosellinia*.

Biology

In Colombia, where Rosellinia root rot is particularly serious, *Rosellinia* is consistently associated with fairly acidic soils rich in O.M. (Aranzazu et al. 1999; Ruiz and Leguizamón 1996; Stell 1929; Waterston 1941) and a high soil moisture level (López and Fernández 1966). However, Waterston (1941) and Cadavid (1995) did not find any relationship between acidity and disease occurrence in Colombia and the West Indies, respectively. Furthermore, Waterston (1941) and López and Fernández (1966) suggested that disease development is favored by soils with low phosphate contents, although no supporting experimental data were presented. Work by Mendoza et al. (2003) confirmed that organic matter and pH are important factors in disease development and management and organic matter probably more important than pH. Phosphate levels seemingly do not affect *Rosellinia* (Mendoza et al. 2003).

Management

Although *Rosellinia* is considered a facultative and weak pathogen (Aranzazu 1996; Waterston 1941), management of the disease, once it erupts, is difficult. Cultural, chemical, genetic, and biological control measures have been examined, although none of these measures has been explored in detail. As early as in 1908, Stockdale (1908) described cultural methods to manage the disease. Nowell (1916)

expanded these methods and to date there are some measures available to farmers. Cultural management measures consist generally of three steps. The first step is to isolate the diseased tree(s)/area(s), using trenches and/or root pruning (Aranzazu 1997; Aranzazu et al. 1999; Nowell 1916). The second step consists of the removal and burning of coarse woody debris (CWD) in the form of the infected trees, including the infected root system and CWD lying on the ground (Aranzazu et al. 1999; Nowell 1916; Waterston 1941). The third and final step is the application of lime to increase the pH and facilitate the decomposition of organic material (Aranzazu et al. 1999; Nowell 1916). Various authors have promoted the use of lime to manage Rosellinia root rot, as acid soils are said to favor the development of the disease (Aranzazu et al. 1999; Herrera and Grillo 1989; Nowell 1916). Solarization of diseased areas might also help in eradicating the pathogen (Aranzazu et al. 1999; Nowell 1916; ten Hoopen and Krauss 2006; Sztejnberg et al. 1987).

The use of fungicides to manage *Rosellinia* has proven relatively ineffective and uneconomical (Aranzazu et al. 1999; Bautista and Rivera 1997; Cubillos 1988) and has even been reported to accelerate disease development by killing the native, antagonistic microflora (Aranzazu et al. 1999). In coffee, only one fungicide out of five tested, tolclofos methyl (Rizolex), was successful in managing *Rosellinia* over a 3-year period but only when applied at a dose of 72 kg ha^{-1} (15 g per planting hole, 4800 plants ha^{-1}) twice a year. Studies by Cubillos (1988) and Merchán (1989b), using different fungicides to manage *Rosellinia* in cacao, showed no disease control. Since numerous fungicides were inhibitory in vitro (Achicanoy 1989), delivery to the target zone is likely to have been one of the limiting factors. Due to the high costs of the fungicides, their relative ineffectiveness, and the damage to the environment, alternative methods to manage *Rosellinia* in cacao are necessary.

Merchán (1990) mentioned that cacao clones IMC67, Pound7, and UF613 might be tolerant to *Rosellinia pepo*. García et al. (2005) could not confirm tolerance of these clones when seedlings derived from open-pollinated seeds were incubated with a mixture of three *Rosellinia* isolates. Merchán (1993) evaluated 13 clones, 17 simple hybrids, and 29 double hybrids of cacao, but none showed any resistance to *R. pepo*. However, no methodology or exact data were presented nor did he report which clones and hybrids were tested.

Sztejnberg and Jabareen (1985, 1986) investigated resistance of persimmon rootstock to the anamorph (*Dematophora necatrix*). Resistant persimmon seedlings excreted phenols in soils infested with *R. necatrix* isolated from apples. The phenols inhibited the growth of the apple isolate but not a strain isolated from persimmon. Phenolic compounds extracted from roots of pecan (*Carya illinoinensis*), persimmon, passion fruit, and an apple cultivar, all resistant to *R. necatrix*, inhibited fungal growth, whereas those from peach (*Prunus persica*), almond (*Prunus* sp.), and another apple cultivar, all susceptible, did not (Sztejnberg et al. 1983). This potential resistant mechanism should be investigated further as it could be indicative of general resistance to root pathogens.

Biological control of *Rosellinia* using fungal antagonists has received some attention (see also Chap. 17) yet remains an area which should be explored in

more detail. For further information regarding soil-borne *Rosellinia*, see ten Hoopen and Krauss (2006).

14.3 *Rigidoporus microporus:* White Root Disease

Introduction

Rigidoporus microporus causes white root rot disease on tropical crops, such as cacao, cassava, tea, and rubber (*Hevea brasiliensis*). White root disease of cacao is found globally wherever cacao is cultivated. Locally, it can be severe on cacao, but most fundamental research has been conducted on rubber.

Taxonomy

Ridley (1904) identified the causal pathogen of white root disease of rubber as *Fomes semitostus* but since then various other synonyms have been used. Nomenclature and various other identifications were detailed by Napper (1932) and Fox (1961). *Fomes lignosus* (Klotzsch) Bres. was used in many publications until Pegler and Waterston (1968a) adopted the name of *Rigidoporus lignosus* (Klotzsch) Imazeki. *Rigidoporus microporus* (Sw.) Overeem (1924) is currently the preferred name. The fungus is in the Family Meripilaceae, order Polyporales.

According to Pegler and Waterston (1968a):

Basidiomata are annual, rarely perennial, sessile with broad basal attachments, often imbricate, occasionally resupinate. Pileus: 3–10 × 4–22 × 0.3–1.5 cm, applanate, dimidiate, thin, upper surface reddish brown with a bright yellow margin, later fading to a uniform wood-brown color, velutinate then glabrescent, concentrically zonate-sulcate; margin thin, decurrent. Context up to 1 cm thick, white, round to angular, 5–9 mm, 50–140 µm diameter, dissepiments 15–40 µm thick; tubes occasionally stratified, up to 6 mm long, reddish brown. Basidiospores: 3.5–4.5 × 3.5–4 (average 4.2 × 3.7) µm, subglobose, hyaline, smooth, thin-walled with few contents. Basidia: short clavate, four-spored. Cystidia: absent, hyphal system monomitic, non-agglutinated. Generative hyphae: 2–7 µm diameter, hyaline or with a pale brownish tint, wall thin to slightly thickened, freely branching, simple septate. Radial growth in culture 5–5.5 cm in 7 days at 25 °C on malt agar, mat white becoming brownish with marginal rhizomorphic strand.

Fig. 14.4 Mycelial strands of *Rigidoporus* on the base of diseased cacao collar in Indonesia 2007 (*Source*: Julie Flood, CABI)

Symptoms

Cacao leaves lose their luster and green color. Leaves appear leathery and curve downward. Leaves change in color from dark green to yellowish brown and finally coppery brown. The discoloration of the foliage may start on one branch, but spreads to the whole canopy. Infected trees may flower and fruit out of season. Eventually, the leaves drop and branches die back leading to tree death. Large vacant spaces are left in fields as the disease progresses. A network of firmly attached rhizomorphs forms on roots. As they grow the ends of rhizomorphs form whitish, mycelial fans which can be seen on the collars of infected trees (Fig. 14.4). Rhizomorphs are firmly attached to infected roots, branching or ramifying into a network as they grow along. Typically they are white and flat at the growing end, but as they mature, they become rounded and darker in color and can acquire the color of the soil. Roots are soft and watery with a creamy color when severely infected. Characteristic, bracket-shaped fruiting bodies form on tree collars.

Biology

The fungus is a rhizomorphic root-infecting fungus with an ectotrophic growth habit (Pegler and Waterston 1968a). The rhizomorphs extend ahead of the root rot and spread the disease to the tree collar (Fig. 14.4) and to other roots of the infected tree. The pathogen spreads from diseased trees to the roots of adjacent healthy trees through root contact and via rhizomorphs. Infected trees are killed, thus forming vacant patches in plantations.

The fungus has been recorded in Asia, Africa, Oceania, Australasia, and the tropical Americas. Losses can be significant locally. At replanting time, when cacao

trees have been cleared from the land, remnants of infected roots, timber, and stumps that are left in the soil provide inoculum sources which initiate new disease points in replanting. Young plants are killed relatively quickly, but older trees with more extensive root systems will spread the fungus to adjacent trees through root contact. The size of the inoculum determines its viability and success in initiation of infection (John 1966).

Once established, the pathogen perpetuates itself from existing infected plants. Fruiting bodies of the fungus are produced under humid conditions during rainy seasons, especially on stumps and at the base of trees covered with weeds or creeping legumes. Fruiting bodies are often seen in many tiers, their upper surface being orange yellow and lower surface orange, red, or brownish. When cut across, an upper white layer and a lower reddish brown layer can be distinguished (Rubber Research Institute of Malaysia 1974a).

The fungus produces abundant basidiospores: estimated at 10,000–100,000 spores per cm^2 of hymenium per day for many days (Fox 1971). The spores are disseminated aerially and infect wounds and cut surfaces of stumps. However, due to the difficulty in initiating infection in artificial inoculations, it is presumed that basidiospores are less significant in disease spread (Fox 1971). Spores germinate readily in water and on growth medium (John 1965), but the success of spore inoculations is low. However, John (1965) did succeed in inoculating cut surfaces of stumps when soil was used to cover the inoculated surfaces. Observations of infection of isolated stumps that could only be attributed to aerial spores prompted the suggestion to cover cut surfaces of stumps with creosote to prevent spore infection (John 1964; Rubber Research Institute of Malaysia 1974b), though this practice has since been questioned (Fox 1977). Once infection is established, the fungus spreads by the growth of the rhizomorphs. Following removal of the stand for replanting, the fungus survives in the infected roots and other debris that remains in the soil.

Most of the basidiospores are released at night (John 1965), and the relationship of spore liberation to temperature and humidity was described by Hilton (1960). The disease is serious in moist soils (Peries and Liyanage 1985); the effect of soil moisture content and temperature on fungal growth was described by Fox (1971). The optimum temperature for growth of the fungus is 28 °C and optimum pH is 8.0 (Fox 1971, 1977; Peries and Liyanage 1985).

White root disease was ranked by Peries and Liyanage (1985) as the second most important disease of rubber, while Allen and Cronin (1994) ranked it the most important in the main rubber-growing countries. The disease can be locally important in cacao, e.g., in cacao blocks in Indonesian estates (Flood 2007). It is most important within the first 5 years after establishment of a plantation and was responsible for the destruction of several hectares of cacao in experimental blocks at Bah Lias Estate, North Sumatra, in 2007. Both established trees and newly planted experimental clones were killed (Flood 2007). Losses to white root disease vary from country to country and from field to field and are greatly influenced by moisture level. Soepena (1993) reported many cases of the disease in 3-year-old rubber plantings with 30 % incidence in Indonesian rubber estates; the incidence

was greater (40–60 %) in smallholdings. Such areas would have to be replanted early (before 20 years old) due to stand loss. A similar trend of additional white root disease in cacao in smallholder blocks is also likely because of the difficulty in managing the disease.

Management

Management of the disease requires early detection and early detection is a major problem because visible foliar symptoms are normally only observed on plants with advanced disease. Rhizomorphs advance ahead of the disease: the disease may be detected by observing the presence of rhizomorphs at the base of the tree after removal of the soil. The process is referred to as collar inspection, but tree to tree inspection is costly; most workers in estates look for leaf symptoms with conformation by collar inspection. Inevitably this is too late. Fernando et al. (2012) reported the successful use of mulching to detect the fungal growth. In artificially inoculated polybag rubber plants, the pathogen was detected earlier in mulched plants compared with non-mulched plants. The practicality of this approach on a plantation level is doubtful as mulching will also be costly in terms of labor, and the authors stated that they do need to demonstrate its applicability under natural field conditions. However, any improvement in detection could link with chemical disease management being more efficient. Fernando et al. (2012) also reported that further investigation was necessary to assess the pathogenicity behind the rapid upward movement of the pathogen; further study of the host–pathogen interaction was needed.

Once the disease is confirmed all efforts should be made to prevent it from spreading to neighboring trees (irrespective of host). White root disease management should begin early, normally about 1 year after planting, and be continued thereafter.

Cultural practices form a major component of white root disease management, especially if trying to reduce inoculum levels. When replanting rubber areas the methods used when clearing old trees will determine the resulting inoculum. Complete mechanical clearing including uprooting the trees and collecting and disposing of roots results in the lowest incidence of root disease after replanting (Newsam 1963). This approach would be similar with cacao on estates, but this procedure would be too expensive for smallholders, who may simply fell and clear the trees with chainsaws, possibly applying aboricides to root decay (Lim and Abdul Aziz 1981). Undoubtedly, the reason why there is such a high incidence of white root disease in smallholder blocks is due to the cost of land preparation (removal of inoculum) at replanting. Painting stumps may prevent colonization of the stumps via basidiospores (John 1965), but the practice has been questioned (Fox 1977). Planting of creeping legume covers promotes faster decay of inoculum and creates a disease suppressive environment (Fox 1965, 1977). Similar field orientated work should also be conducted for cacao.

Root injury should be avoided to prevent infection by the spores. If possible, infected trees including their roots should be removed. If it is not possible to remove infected trees, they should be surrounded by trenches to prevent root contact with healthy trees (Rubber Research Institute of Malaysia 1974b).

The use of chemicals to manage white root disease in rubber is a common practice in Malaysia, Indonesia, and Thailand. Formerly, collar protectant dressings (i.e., formulations of fungicide in bituminous compounds) were painted onto exposed parts of lateral and tap roots, as well as tree collars after surgically removing all infected tissues (Fox 1966). Quintozene may be used as a fungicide (Fox 1966). Applying the collar protectant dressing involves digging and is labor intensive and, hence, costly. It is thus being replaced by drenching aqueous solutions of fungicides in a shallow trench dug immediately around the base of the tree. In Malaysia, triadimefon and propiconazole were first used (Tan and Ismail 1992). Since then, hexaconazole has been found effective. In Africa, tridemorph was first used in the drenching treatment (Canh 1986). Later, cyproconazole and triadimenol were also found effective (Gohet et al. 1991). Soil drenching of fungicide as a method of managing the pathogen (on rubber) is more suitable for younger trees as it is less or even not effective on larger and more mature trees. It is also not effective on trees with advanced disease, i.e., trees with obvious foliage symptoms (Tan and Ismail 1992).

Amending the soil with sulfur is practiced in Malaysia and Indonesia. Sulfur is nontoxic to the pathogen in laboratory tests. The effectiveness of sulfur amendments is attributed to a reduction in pH resulting in the buildup of fungal antagonists, especially species of *Trichoderma* and *Penicillium* (Peries and Liyanage 1983; Hashim and Azaldin 1985). For rubber, sulfur is applied to the soil in the planting hole at the time of planting. There is considerable interest in the use of microorganisms, especially *Trichoderma* species, in rubber. *T. koningii* is being marketed in Indonesia for the management of white root disease (Soepena 1993) and can be integrated with soil sulfur amendments. This approach should be attempted with cacao.

More recently, attempts have been made to develop a screen for resistance against *Rigidoporu*s in cacao. Azmi (2005, 2006) reported attempts to develop a screen for resistance in cacao against white root rot. Using 10-day-old cacao seedlings, tap roots were inserted into colonized rubber wood blocks (RWB) and covered in soil. ICS 60 and PA300 had significantly less infection. Further research (Nuraziawati et al. 2008) reported that several cacao clones had some degree of resistance to the pathogen; PA300 remained the most resistant under the conditions tested. Planting more resistant germplasm would be useful to both estates and smallholders alike and such clones should be tested in the field as part of an integrated approach to the management of white root rot.

14.4 *Phellinus noxius*: Brown Root Disease

Introduction

Phellinus noxius (Corner) G. Cunn causes brown root disease of several economically important hosts including cacao. The pathogen was first described in Singapore as *Fomes noxius* by Corner (1932), but was reclassified as *Phellinus noxius* by Cunningham (1965). The disease is called brown root rot disease or "pourriture brune des racines" in French, "pudrición castana de las raices" in Spanish, and "podridão marrom-das-raizes" in Portuguese.

Taxonomy

Phellinus noxius is a member of the family Hymenochaetaceae, order Aphyllophorales, and phylum Basidiomycota of the kingdom Fungi. The pathogen occurs throughout most tropical/subtropical areas being found in Africa, Asia, Australia and Oceania, Central America, and the Caribbean. The pathogen has a wide host range, spanning over 100 genera in both monocotyledons and dicotyledons. Some of the most significant hosts are mahogany, teak, rubber, oil palm, tea, coffee, and, of course, cacao. The disease has been reported on cacao in Nigeria, Ghana, Sri Lanka, Malaysia, Papua New Guinea, and Samoa (Lass 1986).

According to Pegler and Waterston (1968b):

The fruiting bodies, sporophores, or basidioma are perennial, solitary or imbricate, sessile with a broad basal attachment, commonly resupinate. Pileus: 5–13 × 6–25 × 2–4 cm, applanate, dimidiate or appressed-reflexed; upper surface deep reddish brown to umbrinous, soon blackening, at first tomentose, glabrescent, sometimes with narrow concentric zonation, developing a thick crust; margin white then concolorous, obtuse. Context up to 1 cm thick, golden brown, blackening with KOH, silky-zonate fibrous, woody. Pore surface grayish-brown to umbrinous; pores irregular, polygonal, 6–8/mm, 75–175 μm diameter, dissepiments 25–100 μm thick, brittle and lacerate; tubes stratified, developing 2–5 layers, 1–4 mm to each layer, darker than context, carbonaceous. Basidiospores: c. 4 × 3 μm, ovoid to broadly ellipsoid, hyaline, with a smooth, slightly thickened wall, and irregular guttulate contents. Basidia: 12–16 × 4–5 μm, short clavate, 4-spored. Setae absent. Setal hyphae present both in the context and the dissepiment trama. Context setal hyphae radially arranged, up to 600 × 4–13 μm, unbranched or rarely branching, with a thick dark chestnut brown wall and capillary lumen; apex acute to obtuse, occasionally nodulose. Tramal setal hyphae diverging to project into the tube cavity, 55–100 × 9–18 μm, with a thick dark chestnut-brown wall (2.5–7.5 μm thick) and a broad obtuse apex. Hyphal system dimitic with generative and skeletal hyphae, non-agglutinated in the context, but strongly agglutinated in the dissepiments. Generative hyphae: 1–6.5 μm diameter, hyaline or brownish, wall thin to somewhat

Fig. 14.5 Typical *P. noxius* symptoms of hard brittle encrustation of soil on the trunk of a cacao tree. Soil particles held in place by exudates from the brown rhizomorphs (*Source*: Andrews Akrofi, CRIG)

thickening, freely branching, and simple septate. Skeletal hyphae: 5–9 μm diameter, unbranched, of unlimited growth, with a thick reddish brown wall (up to 2.5 μm thick) and continuous lumen, and nonseptate.

According to Bartz (2007):

When formed, the pileus may appear as a flattened half circle, with a dark reddish brown upper surface that darkens with age. The upper surface appears hairy at first, but gradually losing the woolly appearance with age and developing a thick crust. The margin is white and blunt or rounded at the apex.

Symptoms

The symptoms of infection by *Phellinus noxius* are similar regardless of host. The disease begins in the roots and spreads to the collar colonizing both lateral and tap roots. The entire root system can be girdled before the development of foliar symptoms. The fungus has an ectotrophic extension habit. A sleeve is formed by basidiomata growing along the root surface slightly ahead of root tissue colonization. The fungal sleeve becomes encrusted with soil particles as it ages. Early during infection the fungal mycelium is golden, and the infected wood is brown.

Extensive root damage has usually occurred before above ground symptoms are observed. The most characteristic symptom of the disease is the hard brittle encrustation of soil around the root, held by exudates from the brown rhizomorphs (Fig. 14.5). Encrustation consists of brown mycelium with firmly embedded soil and small stones. Encrustation may be visible above ground as the fungus moves towards the collar of the tree. In the diseased wood, dark lines are visible due to the presence of hyphae. As the wood decay proceeds it becomes light, dry and friable, and honeycombed.

The rhizomorphs develop into a complete fungal sheath over the root surface and eventually turn black. *P. noxius* colonizes and degrades both cortex and lignified xylem tissues. Infected root tissues turn brown in the center and then turn white and soft with brown lines forming a network throughout. The development of above ground mycelia mats or fruiting bodies is irregular and is not correlated with the degree of colonization observed below ground. The trunk diameter may be reduced at the collar, a trait easily spotted in the field. In Ghana, as in Papua New Guinea, the symptoms produced consist of brown crust of mycelium on the lower trunk up to a height of 1 m.

Foliar symptoms consist of wilting of leaves, followed by chlorosis and then browning and defoliation. On infected cacao, the symptoms include yellowing of leaves followed by drying up of leaves, defoliation, and dieback. Infected trees with highly decayed root systems lose support and may be blown over in strong winds. The progression of symptoms may be slow or the tree may suddenly wilt over a few days. The large sporophores are hard, purplish-brown brackets, with yellowish-white margins and concentric blackish zones near the edges. They form above ground on trunk encrustations but are rarely seen.

Aboveground symptoms are similar to those seen with other root rot fungi (such as *Rigidoporus*) and collar rot fungi (such as *Phytophthora palmivora* of cacao); pathogen identification cannot be made using symptomatology alone. The soil-encrusted mycelium sleeve or sheath (Fig. 14.5) is unique to *P. noxius* and is used to rapidly distinguish it from other pathogens in the field. Infections can be traced to roots of other trees by following the encrustations.

Biology

For isolation of the pathogen, a "stick trapping technique" is used. This typically involves insertion of shoots of a woody host plant into the soil at the collar region of a potentially infected tree. The shoot is examined for the presence of mycelium after 3 weeks. Soil diluted in water or infected plant tissue cut into small cubes can be plated on a selective medium to obtain a pure culture (Chang 1995). The colony morphology of *Phellinus noxius* varies with the medium. On malt agar, colonies show increase in the white and brown plaques while on potato dextrose agar, colonies are initially white, becoming brown with irregular lines or patches of darker tissue with age. Although clamp connections are produced, they are not commonly observed in culture. Arthrospores and trichocysts are produced abundantly in culture but are not observed in the field (Ann et al. 2002). The appearance of *P. noxious* is aptly described by Ann et al. (2002). Perennial basidiocarps are formed on trunks of infected trees or in culture on sawdust medium. They can be single or overlapping.

Due to its wide host range and geographical distribution, the economic impact of *P. noxius* is variable. For example, the disease losses can vary from insignificant to 60 % in rubber tree plantations after 21 years (Nandris et al. 1987). Once present in a field, the disease can cause severe losses if not managed properly, as a result of it

spreading between trees by root contacts. *Phellinus noxius* is one of several basidiomycetes causing heart rots of *Acacia mangium* in South-East Asia (See et al. 1996) and root rot of hoop pine (*Araucaria cunninghamii*) and avocado (*Persea americana*) in New South Wales and Queensland (Bolland 1984; Dann et al. 2009). Though *P. noxius* has been reported as a disease of cacao, it tends to be locally damaging and its impact on cacao production has not been studied.

Infected plant debris is the most common source of inoculum in new plantations. Seedlings can be infected in the nursery and can serve as the initial source of inoculum when transplanted into the field. Brown root rot commonly spreads by root to root contact, although basidiospores are also a potential source of inoculum. Infections generally start from infected tree stumps, and once established, the fungus can persist for up to 60 years in infested soil. The fungus colonizes the root system and moves to the collar. Basidiocarps may form on the trunk of the host. Basidiospores can infect newly cut tree stumps, but this process is not thought important to the development of epidemics.

Management

As with all these root-infecting fungi affecting cacao, the management depends on early detection and inevitably; as with other fungi discussed here, detection of the pathogen before the appearance of typical visual symptoms (wilting and chlorosis) is very difficult and time-consuming. Also when visual symptoms are observed, the disease is often too advanced for prophylactic treatment. Methods of detection include scraping away the soil around the collar and the main roots and looking for the distinctive mycelial sleeve or sheath, or baiting out the pathogen (Nandris et al. 1987). The only practical method for identifying the pathogen in a plantation is to look for the mycelial encrustations on the roots of dead or dying trees.

Thus, much of the management of brown rot depends on routine inspection and removal of infected trees. Although digging trenches around infected trees and locating the infection front by digging along infected roots has been recommended, these practices are of limited practical value on a large scale.

When clearing land, the establishment of ground cover is recommended to accelerate the decay of infected root fragments that might serve as inoculum for the crop being planted. Spore infection can be prevented by chemically poisoning the stumps with compounds/herbicides which themselves are not toxic to *Phellinus noxius* (Anon 1976). Spores require a freshly cut surface and cannot infect a dead surface. The fungus can remain viable in infected root fragments for up to 4 years. The disease can be spread by the movement of infected root fragments in soil, and soil should not be moved from affected areas. Planting affected areas with non-susceptible annual crops can accelerate the breakdown of infected root fragments. Infested areas should not be replanted with susceptible trees for several years. This recommendation is often ignored in the replanting of old plantations,

primarily due to the perceived economic benefits, but the earlier replanting, in fact, may result in the pathogen rapidly destroying the new plants.

Various fungicides, including triadimefon, prochloraz, and mepronil, have been found to have activity against the *Phellinus noxius* (Lim et al. 1990; Mappes and Hiepko 1984), but they are not economically viable as routine field treatments. Although soil fumigants can be effective, they are not used on a large scale due to the cost and associated health risks (Chang and Chang 1999). *Trichoderma* species compete well against *Phellinus noxius* and can prevent spore infection of freshly cut stumps. However, the methods used to precolonize stumps with *Trichoderma* species are complex compared to simply poisoning the stumps and, therefore rarely used. Since it is possible to use biocontrol in the rhizosphere with good results (Lim and Teh 1990; Jacob et al. 1991; Kothandaraman et al. 1991), this is an area that should be more intensely studied. More research on host resistance is also needed, since variation in susceptibility has been observed among different host species or cultivars within a species.

14.5 Discussion

In this chapter, we consider some of the soil-borne, root-infecting fungi that attack cacao. Often these pathogens are referred to as minor pathogens of the crop, but they can be locally very severe, e.g., Verticillium wilt is reported to cause an annual plant mortality of up to 10 % in unshaded cacao-growing areas in Brazil (Almeida et al. 1989) and was responsible for losses up to 30 % in Uganda (Emechebe et al. 1971). Many consider that the disease was responsible for the failure for the cacao industry to expand in Uganda. Likewise, *Rosellinia* is a significant constraint to cacao production in Colombia with over 50 % losses on one estate (Cadavid 1995) and in Brazil, 20 % of 3-year-old cacao trees died (Feitosa and Pimentel 1991). A further consequence can be the loss of unique germplasm such as in CATIE's cacao germplasm bank in Cabiria in Costa Rica. Forty-five clones succumbed to a complex of *Ceratocystis cacaofunesta* and *Rosellinia* between 1997 and 2001 (Mendoza et al. 2003) or the loss of germplasm following *Rigidoporus* attack in experimental breeding trials in Bah Lias Research Station (Flood 2007).

Although the fungi examined here are soil-borne, root-infecting fungi, and have some similarities, there are also notable differences. The basidiomycete pathogens (*R. microporus* and *P. noxious*) and the ascomycete pathogens (*R. bunodes* and *R. pepo*) are unspecialized, root rot pathogens whose pathogenicity is part of a saprophytic lifestyle, while *Verticillium* is a highly specialized pathogen adapted for colonization of the xylem system. However, such specialization comes at a cost in terms of competitive saprophytic ability. In keeping with their saprophytic ability, the other pathogens can actively grow through the soil (basidiomycetes via rhizomorphs) seeking out new hosts and are able to colonize any woody debris in the soil; spread can also occur via root to root contact. *Verticillium* does not

actively grow through the soil. The fungus remains in the soil or in plant debris in the form of resistant resting structures (microsclerotia). These germinate only in response to exudates from host plants in close proximity so active growth in the soil is over very short distances as the pathogen is susceptible to antagonistic interactions from other soil microbiota. An additional survival strategy developed by *Verticillium dahliae* strains affecting cacao is that of asymptomatic colonization of alternative hosts especially weed species that grow in the cacao plantations in Brazil (Resende et al. 1994). The other pathogens discussed here have extremely broad host ranges in keeping with their unspecialized mode of pathogenicity and degradation of woody materials.

Nevertheless, despite these differences, there are some important practical similarities with regard to their management. These pathogens are extremely difficult to manage given their soil-borne nature, colonization of alternative hosts, or colonization of woody debris—maintaining inoculum that is difficult to target. Additional problems occur with cacao being a perennial crop grown in a plantation situation or in smallholder blocks, each with its own difficulties of implementing management. Chemical use is therefore generally limited because it is extremely costly for a large estate or in a smallholder situation where access to finance and knowledge are limiting factors. The perennial nature of the crop indicates reapplication of chemicals (further costs) and translocation of systemic fungicides within a tree crop can be inefficient. Pesticide residues can also be problematic given environmental and consumer concerns about residues. Soil drenching as practiced in rubber estates (Tan and Ismail 1992) for white root disease may not be practical for cacao in terms of costs, application, and residues. Cultural methods are important to management for all these diseases and often are the only management option available. The reduction of inoculum through clean clearing of the sites at replanting (to remove debris), trenching to isolate the infected trees, or clearing potential weed hosts are elements of cultural methods. Further approaches such as the appropriateness of soil solarization methodologies as has been done for other crops (Tjamos et al. 1991) could be investigated and tried on an experimental scale. Ten Hoopen and Krauss (2006) suggested its use for areas affected by *Rosellinia*.

One area of investigation that would be extremely useful to both improved cultural and chemical management is that of early detection. By the time aerial symptoms are seen, it is too late for anything to be done for the individual tree and that tree will already be a source of infection for others. Improving early detection could allow for rapid chemical inputs for example where the individual tree is of importance, e.g., in a germplasm collection or early detection could allow for further cultural methods to try to prevent further spread from an initial focus. The development of early detection methods for these pathogens is a research gap that needs to be addressed.

Another research gap is the development of screens for resistance with subsequent development of breeding programs for some of these pathogens. The exception to this is *Verticillium* where some extensive research has already been undertaken to develop rapid screening methods and breeding programs in Brazil (Resende et al. 1995). Several cacao cultivars such as Pound 7 and SIC 2 have been

shown to be more resistant to the disease. Pound 7 was one clone that showed less susceptibility to *Rosellinia* too (Merchán 1990), although García et al. (2005) could not confirm this so more research is needed. Some preliminary work has been done with *Rigidoporus* to develop a screening system using rubber wood blocks (Azmi (2005, 2006) and this approach could be expanded for other basidiomycete pathogens.

Biological control is another area of management that should be investigated further. This could include direct use of antagonistic organisms such as *Trichoderma* as used against *Rigidoporus* (Soepena 1993) or *Rosellinia* (Mendoza et al. 2003) or biological control agents (BCAs) could be combined with various soil amendments such as sulfur that encourages the buildup of native soil antagonists (Peries and Liyanage 1983; Hashim and Azaldin 1985). The use of lime has been advocated for *Rosellinia* management to aid in the decomposition of debris and increasing the pH (Aranzazu et al. 1999). Soil amendments have been shown to be effective in reducing Verticillium wilt in some cropping situations (Lazarovits et al. 2000). The mechanism primarily being used is antagonists targeting the survival of microsclerotia in soil, but this approach seems to have had highly variable results and success was limited to certain localities, soils, etc. (Lazarovits et al. 2000). This is one area where more comprehensive research could be undertaken in the context of Verticillium wilt of cacao.

Why are these approaches not being investigated more widely? Why is there not more research conducted on integration of these methods? One reason for such approaches not being tried and adopted will be lack of funding for research on these pathogens. In a climate of restricted funding for research as is the case in many cacao-producing countries, prioritization will be given to the diseases that cause the most significant losses and have a potentially global impact; the diseases discussed in this chapter are not considered so important even though they can be locally severe. The cost of initiating long-term and expensive breeding programs can be so high that many developing countries would choose to spend their limited research funds on a pathogen that has a larger impact. Nevertheless, any knowledge of susceptibility/resistance of varieties and clones or use of integrated approaches would be useful for growers where these pathogens are known to be serious constraints. Smallholders would particularly benefit. They may have a small area of land and once there is a problem with these soil-borne pathogens, it is easier to replant with a less susceptible variety than to consider expensive and physically difficult management such as uprooting, trenching, etc. Such farmers may well feel their only option is to move to an alternative crop and grow something else which is perceived to be easier to manage, e.g., oil palm.

With regard to the future, some of these diseases could undoubtedly become more problematic. Verticillium wilt is currently particularly important in NE Brazil where the crop is grown at the very edge of its geographic range, but with predicted climatic change, more intermittent rains, and unseasonal droughts, this disease could become more serious over a wider area. In Africa too, e.g., in Uganda, with the proposed planting of more cacao, Verticillium wilt could again become severe and raised awareness of its potential threat needs to be disseminated. Currently,

some of these pathogens have restricted geographical distributions, e.g., *Phellinus noxius* has not been detected in South America, a region where cacao is extensively grown. The hyphae and fruiting bodies of the fungus may be borne externally or internally on roots, stems, bulbs, tubers, corms, rhizomes of susceptible hosts, and this poses risk of introduction into uninfected cacao-growing regions. *Rosellinia* spp. attacking cacao are found in cacao-producing countries on all three continents, but it seems that its economic impact remains confined to the Americas (ten Hoopen and Krauss 2006). Why this is so remains unclear, yet introduction of infected soil or plant material from areas where *Rosellinia* is capable of attacking cacao into areas where this disease is, for now, absent could pose an important risk. Also, these fungi do have very broad ranges and introduction could come from other timber or fruit trees so any alerts should include other potential hosts other than cacao. Verticillium wilt has been reported in the Americas and in Africa, but although *Verticillium dahliae* has a global distribution, Verticillium wilt of cacao has not yet been reported in cacao in Asia. This may be because the disease has not been identified in Asian cacao-producing countries or it has been misidentified.

One way of raising awareness of the importance of these diseases globally is through the publication of information such as in this chapter but direct targeting of producers would also be beneficial.

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Part VI
Non-microbial Maladies of Cacao

Chapter 15
Cherelle Wilt of Cacao: A Physiological Condition

Rachel L. Melnick

Abstract Cacao beans are produced in pods (fruits) of the trees of *Theobroma cacao* L. Cacao pods are produced after pollination of the small cauliflorous flowers covering the branches and trunk of the tree. Despite abundant flowering, only 0.5–5 % of cacao flowers become pollinated. Pollinated flowers then develop into immature pods, commonly known as cherelles. Despite abundant flowers and pod set of trees, few cherelles develop into mature pods. Up to 75 % of cherelles are lost to a thinning condition known as cherelle wilt. Additionally, cherelles can be lost at an early stage to insect, stramenopile, and fungal pests. This chapter will discuss the physiological changes in young fruit that cause cherelle wilt, as well as the physiological changes induced in pods caused by cherelle wilt. Lastly, the chapter will discuss whether there is a clear link between cherelle wilt and frosty pod rot caused by *Moniliophthora roreri*.

15.1 Growth of Cacao Pods and Flowers

Cacao beans are seeds of *Theobroma cacao* L. that are produced in pods (fruits). Cacao has perfect flowers that attach to floral cushions by a long, thin pedicel (Wood and Lass 2001). Cacao flowers are cauliflorous flowers, meaning that flower cushions arise from the old wood of trunks and branches (Wood and Lass 2001), as seen in Fig. 15.1.

The heaviest flowering and pod set occurs on the oldest wood of the trunk (Brooks and Guard 1952). Despite abundant flowering, only 0.5–5 % of cacao flowers set fruit and the unpollinated flowers abscise within 32 h of anthesis (Aneja et al. 1999). Abscission is hormone mediated, as ABA levels increase drastically prior to abscission, and is essential for senescence, while ethylene plays a supportive role (Aneja et al. 1999).

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Fig. 15.1 Floral cushions on the old wood of cacao trees in Pichilingue, Ecuador

Flowers are pollinated by small flying aphids, midges, and thrips (Wood and Lass 2001). Exogenous environmental factors that impact tree growth and development also influence flowering in cacao. The switch from low to high humidity and low to high soil moisture, such as seen at the onset of the rainy season in South America, triggers the floral flushing (Sale 1970). Trees that had pods constantly artificially removed had increased flowering intensity (Valle et al. 1990). However, hand-pollinated trees had greater cherelle wilt than naturally pollinated trees, despite having the same number of mature pods, suggesting an attempt by the tree to regulate pod production. It should be noted that flower production requires extensive energy from the tree as seen by the increased energy costs put toward flowering measured in higher flowering clones when compared to clones that do not produce a high level of flowers (Hasenstein and Zavada 2001; Valle et al. 1990).

After successful pollination, the initial signs of fruit development are seen at 6–8 days (Hasenstein and Zavada 2001). These immature pods are commonly known as cherelles. Only a small number of cherelles develop into mature pods, as many are lost to pests and other conditions. Up to 75 % of cherelles are lost to a physiological condition known as cherelle wilt (Ashiru 1971; Wood and Lass 2001). In some fields and under certain conditions, specific trees can have 100 % cherelle wilt (Fig. 15.2).

In some plantations, a combination of pests and cherelle wilt can result in major pod losses. An unmanaged plantation in Costa Rica was reported to have only 18 % healthy pods at the end of the growing season due to these losses (Porras and Gonzalez 1984). Despite the large losses of pods to cherelle wilt, there is far less research conducted on wilting than there has been on the diseases that infect cacao

Fig. 15.2 A young cacao tree in intercropped fields with plantain showing 100 % pod loss. Trees such as this one are an exception

pods. Additionally, there has been speculation that not all pods lost at an early stage are due to thinning, but instead that some of the pod losses may be due to biotic factors such as diseases.

15.2 Cherelle Wilt: Symptoms of Physiological Thinning

One aspect of cherelle wilt that is fairly well understood occurs in pods that form from successful pollinations of incompatible flowers. However, we still do not fully understand the exact processes that induce cherelle wilt when pollen incompatibility is not involved. There has been significant work on the physiological changes to cherelles that bring about wilting. The processes and physical manifestation of the conditions are outlined in Fig. 15.3. The initial symptoms of cherelle wilt are loss of water balance in the pod that causes a loss of turgor pressure in the pods (Thrower 1960). The earliest observable change that occurs during cherelle wilt is occlusion of xylem vessels by a mucilage-like substance in the cherelle peduncle (Nichols 1961). These occlusions block growth and development by limiting water movement into the pods. During the very early stages of cherelle wilt, pods appear to lose turgor pressure, after which the cherelle yellows over a week (Thrower 1960). The yellowing often starts at the pistil end of the pod and then spreads to the entire pod (Thrower 1960). After yellowing, the pods turn blackish brown.

At the end of wilting, acceleration of loculus growth causes differential swelling of the pod (Nichols 1964). As this occurs, there is also enlargement of pericarp cells

Fig. 15.3 Proposed model for timing of cherelle wilt in cacao

and existing vascular bundles (Nichols 1964). This is associated with the pods becoming blackish brown. While existing vascular bundles increase in size, there is an overall cessation in the formation of vascular bundles in the outer tissues of the inner pericarp and lignification of the middle pericarp (Nichols 1964), leading to mummification of the cherelle. Mummified cherelles remain attached to the tree.

Cherelle wilt occurs in two stages or flushes that McKelvie (1956) referred to as first wilt and second wilt. The peak period for the initial stage of cherelle wilt begins at 50 days after pollination and ends when the cell walls develop in the seed endosperm (McKelvie 1956). Seventy days after pollination is the peak of the second stage of cherelle wilt (McKelvie 1956). Second stage wilt declines as pod metabolism increases, after which the pod is no longer susceptible to wilt (McKelvie 1956). Pods that wilt during the second stage of wilt typically have smaller stalks and larger embryos than pods that are developing normally (McKelvie 1956).

The number of pods maintained on a cacao tree appears to be related to the number of flowers that are successfully pollinated. Over three years, fallen flowers were assessed weekly, and cherelle wilt was assessed biweekly in mature cacao fields (Valle et al. 1990). Hand-pollinated trees had an increased number of pods, but also an increased level of cherelle wilt when compared to naturally pollinated trees, causing hand and naturally pollinated pods to have the same amount of mature pods (Valle et al. 1990). Through this 3-year study, the researchers concluded that cherelle wilt was a means for the cacao tree to adjust the number of pods to a number that the tree could support (Valle et al. 1990).

Pollen compatibility also plays a role in the loss of pods due to cherelle wilt. The incompatibility response in *T. cacao* flowers occurs in two stages modulated by phytohormones. The two stages that impact fruit set are pollination and gamete fusion (Cope 1962). According to Baker et al. (1997), an early release of abscisic acid (ABA) occurs at pollination during the pollen–stigma interaction. The hormonal content of the cacao flower likely plays a role in modulating self-incompatibility (Hasenstein and Zavada 2001). This release of ABA occurs during

the self-incompatibility reaction and is likely required for later floral abscission. The ABA release is absent in unpollinated flowers. The increasing levels of ethylene are not balanced by increasing auxin, resulting in the typical abscission seen in unpollinated flowers (Baker et al. 1997). The second stage occurs after the pollen tube contacts the ovule. This contact alters the levels of auxins and ethylene that fully determines whether the flower will become a fruit or abscise from the tree (Baker et al. 1997). For flowers, abscission is most likely to occur when the levels of both ethylene and ABA are high, which is the common condition in self-incompatible flowers (Baker et al. 1997). Abscission is overcome when increasing auxin levels balance the increasing ethylene levels, allowing fruit set to occur (Baker et al. 1997). Fruit set occurring on self-incompatible trees has been seen by other authors (Lanaud et al. 1987); however, these pods are often aborted by the tree (Bos et al. 2007). Certain self-incompatible clones have been shown to have low pod set under open pollination, but have an increased number of seeds in the mature pods (Falque et al. 1996).

15.3 Cherelle Wilt: Potential Causes of Induction of Cherelle Wilt

During the early stages of cherelle wilt research; there was a focus on determining whether cherelle wilt was related to the development of the pod or the physiological state of the tree itself. It is likely that there is an association between cherelle wilt and the growth of other cacao tree tissues. Several studies showed that there was an increase in cherelle wilt during or immediately following periods of leaf flushing (Alvim 1954; Humphries 1944). This association between cherelle wilt and leaf flushing suggested that the tree was not able to produce enough energy to support both the increased leaf formation and pod development. There also might be a relationship between the root/shoot balance of the tree and the likelihood of individual cherelles wilting. Cherelles on branches are more likely to develop cherelle wilt than those on the main trunk of the tree (Uthaiah and Sulladmath 1981), suggesting a relationship between wilt and sugar and water transport. Studies in Trinidad found that pods on thinner branches located at a greater distance from the roots were far more likely to wilt than pods on large branches or the trunk (Humphries 1943). Cherelle wilt was more prevalent on very young fruit trees and decreased as the trees matured and produced more flushes (Humphries 1944). The authors believed that this was due to young trees not having enough nutrient reserve to support the development of pod, even though the increased number of leaves increased photosynthetic capacity and the nutritional status of the tree (Humphries 1944).

The association of fruit thinning in temperate fruit trees, such as apple, has long been associated with a hormone deficiency, thus leading others to speculate that hormone deficiency also plays a role in cherelle wilt in cacao. It has also long been

thought that a lack of hormone production in the endosperm could cause a decrease of water and sugar transport in pods, inducing the wilt condition (McKelvie 1956). This line of thought has caused several scientists to look at application of phytohormones to the trees as a means to reduce cherelle wilt. Application of Ethephon, an ethylene-producing substance, to the pedicels of young fruit induced wilt, while application of gibberellic acid prevented wilt in cherelles that were 10–50 days old and treated with Ethephon (Orchard et al. 1981). Application of gibberellic acid inhibitor chlorocholine chloride induced formation of more flowers and increased fruit set on the trees, but cherelle wilt specifically was not addressed in this study (Santoso and Purwanto 2013). Studies with Amazonian cacao in India found that an application of benzyladenine with indole acetic acid or gibberellic acid singularly reduced cherelle wilt in trees (Uthaiah and Sulladmath 1981). Despite decades of this and similar research on the utilization of phytohormone applications to trees to reduce cherelle wilt, no specific phytohormonal treatment was found to be effective across different clones and countries. Treatment, environmental conditions, and tree clone all have a larger influence on cherelle wilt than the application of the plant growth regulators.

The attempt to utilize hormones to reduce cherelle wilt arose from research demonstrating that hormone levels and ratios determine both flower abscission and cherelle wilt. As previously stated, flower abscission occurs when the ethylene and ABA levels are high and is overcome when increasing auxin levels balance the increasing ethylene levels (Baker et al. 1997). Uthaiah and Sullabmath (1980) found lower levels of cytokinins in wilting pods and detected no cytokinins in normal pods after the end of the susceptibility period to cherelle wilt. Despite the previous work on the impact of hormones on cherelle wilt, the exact role of hormones in inducing cherelle wilt remains unknown. This research has also not resulted in a treatment that is feasible and economical to reduce wilt. However, knowledge of the role of hormones in wilt could be used to improve cacao breeding efforts. The sequencing of the *T. cacao* genome (Argout et al. 2010) and utilization of molecular techniques such as real-time quantitative polymerase chain reaction (RT-qPCR) and RNA sequencing (RNA-seq) have been used to study cacao processes (in particular its reaction to pathogens) and could provide insight into the biochemical pathways and individual genes involved in inducing cherelle wilt. This knowledge could then be translated to assist in selection of lines that are less likely to wilt or are more resilient to poor environmental conditions.

15.4 Molecular Physiology of Cherelle Wilt

While the role of hormones in cherelle wilt is not fully understood, the levels of metabolites and associated altered gene expression have been investigated. The changes in the metabolite concentrations in the pods following the initiation of cherelle wilt are those of a senescing plant tissue. Xylem occlusion and cession of vascular bundle formation (Nichols 1961, 1964) have a large impact on the key

transport carbohydrates, sucrose, fructose, and myo-inositol in wilting cherelles, which are reduced in wilting pods (Melnick et al. 2013b). By-products of the glyoxal pathway can be induced under starvation conditions and were elevated during cherelle wilt (Melnick et al. 2013b). GABA, an amino acid which accumulates during periods of tree stress, increased in wilting cherelles, suggesting that the xylem occlusions are inducing osmotic stress (Melnick et al. 2013b). Asparagine is the most abundant amino acid detected in wilting cherelles (Melnick et al. 2013b). Asparagine levels can increase during senescence when sucrose is limited (Buchanan-Wollaston 1997), which would occur in wilting pods (Nichols 1964).

Expression of cacao genes encoding proteins commonly associated with the senescence and the osmotic stress responses were induced during cherelle wilt. Energy reallocation appears to be altered in the pods as seen by the altered expression of a putative neutral invertase which hydrolyzes sucrose (Sturm 1999). With decreased sucrose moving into pods, existing stores would need to be reallocated. Cherelle wilt also alters expression of genes encoding proteins associated with the biosynthesis and function of jasmonic acid and ethylene. Jasmonic acid and ethylene are phytohormones associated with senescence in cacao (Melnick et al. 2013b).

Similar hormones might be involved in pollination, flower abscission, and cherelle wilt. The expression of cacao genes putatively associated with the interacting pathways of polyamine and ethylene biosynthesis was altered during wilt. These data draw correlations with earlier findings that both pollination and fruit set alter ethylene levels of the flowers and pods (Baker et al. 1997). Genes putatively encoding *S*-adenosylmethionine synthetase, an enzyme involved in the biosynthesis of the ethylene precursor *S*-adenosylmethionine, are induced during cherelle wilt (Melnick et al. 2013b). *S*-adenosylmethionine synthetase and ethylene are related to ripening and may lead to the pod yellowing seen during wilt, although this needs to be tested further.

As during flower abscission, ABA levels are also altered during cherelle wilt. Cherelle wilt induced the expression of cacao genes involved in ABA signaling and reduced the expression of genes related to ABA biosynthesis (Melnick et al. 2013b). Altered expression of ABA-associated genes suggests that a disruption in ABA homeostasis occurs and points toward altered ABA action in response to wilt. Cytokinin biosynthesis and action also appear to be altered during cherelle wilt. The expression of putative cytokinin oxidase/dehydrogenases was induced during wilt (Melnick et al. 2013b), suggesting that the tree is regulating the levels of cytokinins in the pods. It should be noted that CKXs irreversibly inactivate cytokinins to reduce overall levels during seed development (Schmülling et al. 2003), which aligns with the studies of Uthaiah and Sullabmath (1980) which demonstrated that cytokinins are not detected in normal pods after the end of the susceptibility period to cherelle wilt.

Aspects of the physiology and molecular biology associated with the initial induction of cherelle wilt likely represent the largest gap in our knowledge on the condition. To better assess the level of hormones in the pods, time series studies across a range of self-compatible and self-incompatible clones need to be

conducted to determine which hormones are involved and how they interact to modulate cherelle wilt. Coupling this with RNA-seq data would strengthen the understanding at a genetic level. An increased understanding of this process and the pathways involved could improve breeding strategies for this orphan crop, by providing a cherelle wilt suppressing selection criteria that could result in higher yielding trees.

15.5 Impact of Environment on Cherelle Wilt

Environmental conditions have a large impact on cherelle wilt. Excess rain and extreme temperatures increase the incidence of cherelle wilt (Naundorf and Vellamil 1949). Fruit arising from flower cushions bearing fewer flowers are less likely to succumb to cherelle wilt (Naundorf and Vellamil 1949), suggesting that the tree may manage the cost of fruit production not only at a pod level, but also at the flower production level.

One of the environmental factors that impacts pod set and cherelle wilt is shade. There is increasing controversy over whether sun or shade grown cacao results in better yields. With increased planting of cacao in larger shade-free plantations, the impact of shade on cherelle wilt can be important for production. Shade management is also essential for managing cacao diseases, as reducing the level of humidity in the under canopy can reduce the spread and sporulation of stramenopile and fungal pathogens. Many of the experiments on the impact of shade on cherelle wilt conclude that the impacts of shade on soil fertility and the resulting nutritional level of the tree had a bigger impact on wilt than the shade itself. In experiments on the impact of shade on flowering and pod set in Ghana, trees that were fertilized and under light shade produced more pods than unfertilized trees under high shade or no shade (Asomaning et al. 1971), supporting that the robust growth of fertilized trees reduced wilt regardless of shade. In work with hand-pollinated pods in Central Sulawesi Indonesia, shade in different agroforestry systems did not impact cherelle wilt (Bos et al. 2007). Again, nutritional levels of the tree impacted cherelle wilt, as nitrogen-fixing legumes were more common under forest trees than under shade trees (Bos et al. 2007). It should be noted that reduction of shade increased leaf production, which is likely what causes the associated increase in cacao yield (Wood and Lass 2001). As previously stated, increased photosynthates provide additional nutrients to support pod development. Cacao trees grown under shade must also compete with the shade trees for nutrients in the soil, resulting in the reduced yield found under heavy shade (Wood and Lass 2001). Overall, the impact of shade on cherelle wilt is not a shade response, but instead is a response to the overall soil health and nutritional levels of the trees.

15.6 Impact of Biological Control Agents of Cherelle Wilt

While there has been a wide range of research utilizing biological control agents to manage cacao pod diseases, a few of these studies assessed whether the biological control agent also affected cherelle wilt. Much of the biological control work against cacao diseases has focused on utilizing native fungal mycoparasites and endophytes as potential biological control agents. In most of these studies, researchers categorically state that they do not measure cherelle wilt incidence in the studies, but also often state that it is difficult to differentiate between cherelle lost to wilt from those lost to disease at this early developmental stage. There are a few studies that are exceptions. Fungal mycoparasites were unable to reduce cherelle wilt in studies conducted by Krauss and Soberanis (2001) in Peru and Deberdt et al. (2008) in Africa. In these studies, the inability to reduce cherelle wilt by the mycoparasites appears to be related to their inability to colonize pods and cushions, as their mode of action was direct antagonism of the pathogens.

Additional studies were conducted by Melnick et al. (2013a) in Ecuador to determine if endophytic *Bacillus* spp. from cacao could reduce black pod rot and frosty pods rot, but also measured the number of pods lost to cherelle wilt. Pods were internally colonized by endophytic *Bacillus* spp. at one day after inoculation due to application with the surfactant Silwet L-77 (Melnick et al. 2013a). Silwet L-77 reduced the surface tension of the solution, allowing for substomatal infiltration into the stomata that carried bacteria directly into the treated tissues. The *Bacillus* spp. had no impact on wilt for the clone "CCN-51,", but there was a significant reduction in cherelle wilt of "Nacional" pods. Application of *Bacillus pumilus* (isolate ET) reduced the incidence of cherelle wilt at two months after inoculation (Melnick et al. 2013a). The reduction of cherelle wilt could have been due to the ability of *Bacillus* spp. to produce phytohormones (Idris et al. 2007) and alter osmotic and plant defense pathways (Ongena and Jacques 2008). The increase in young pods did not increase the number of healthy pods at the end of the experiment due to an associated increase in frosty pod rot. Further research is needed to clarify the potential of *Bacillus* spp. to reduce cherelle wilt.

Assessment of whether the applied biological control agents impact cherelle wilt at the field level should be considered a standard practice when carrying out biological control studies. The reason for this is twofold. First, there is a large amount of anecdotal data, only some of which is mentioned in publications, that discusses the ability of biocontrol agents to reduce the levels of minor pathogens in cacao. For example, when applied to the aerial portion of the tree, *Trichoderma asperellum* PR11 was found to be a more consistent colonist of the cacao floral cushions than the bark or soil, although it had no impact on the incidence of cherelle wilt (Deberdt et al. 2008). Also, mycoparasitic *Trichoderma stromaticum* alternated with copper fungicide increased flowering and reduced the amount of witches' broom infection on cacao (http://www.dropdata.org/cocoa/cocoa_biological.htm), but the impact on cherelle wilt was not fully characterized. The standing hypothesis is that biocontrol agents clean the canopy, especially the flower cushions, of minor

pathogens and the healthier cushions flower more and set more fruit. Yet, researchers have not sufficiently confirmed this accepted dogma through follow-up experiments. This research is needed to ascertain the ability of the specific biocontrol isolates to clean flower cushions or to reduce cherelle wilt or that this phenomenon is consistent across clones and environments. It should be noted that losses of cherelles to minor pathogens might be confused with losses due to physiological thinning processes, a topic that is discussed in later sections.

The second reason that biological control agents should be tested against cherelle wilt is that agents are more likely to be commercialized and adopted by growers if they have more than one benefit to production and yield. There are significant costs in application of biological control agents in cacao, through labor, purchasing of backpack sprayers with specialized nozzles, and buying the actual product itself. For small-scale farmers who might not have the means to apply synthetic pesticide, the capital involved in investing in biological control agents is a potential barrier to the adoption of this management strategy. A biological control agent that could increase yield in the absence of the pathogen by promoting flowering and reducing cherelle wilt could largely improve the economic outcomes for farmers applying the product, thus making the investment in the product more likely by increasing the likelihood for a return on the investment.

15.7 Pests of Cherelles

Biotic factors, such as pests and diseases, have been speculated to cause cherelle wilt beyond physiological thinning (Nichols 1965). True bug damage to pods is thought to cause up to 10 % loss of cherelles due to abortion of fruit development, yet mature fruit mortality was not impacted (Yede et al. 2012). While not cited as a cause of cherelle wilt, *Colletotrichum theobromicola* (formerly known as *C. gloeosporioides*) is known to cause fruit rot of cherelles (Peter and Chandramohanan 2011). *C. theobromicola* infection of cherelles is minor when compared to damage caused to leaves and branches (Peter and Chandramohanan 2011). *Phytophthora palmivora*, causal agent of black pod rot, also infects cherelles in some situations, but Peter and Chandramohanan (2011) did not see any losses of cherelles from *P. palmivora* in their study. The symptoms of *Phytophthora* infection can be distinguished from cherelle wilt because small brown lesions that coalesce are typical of black pod rot as opposed to the yellowing that becomes brownish black, which is typical of cherelle wilt (Thrower 1960). Minor pathogens could be causing a portion of the pods to be lost to cherelle wilt, but many articles have stated that it is difficult to determine if cherelles are lost to pests or to wilt. Although the processes and symptoms of wilt and disease would seem to be distinguishable early in these processes, after cherelles completely necrose and become colonized by secondary microbes, determining the original causal agent and process are indeed difficult. As a result, the true number of cherelles lost to minor pathogens remains unknown.

The fungal pathogen *Moniliophthora perniciosa* can infect the meristematic tissues of flowers and floral cushions. The infection alters the physiology and gene expression resulting in the development of parthenocarpic pods or conversion of flower cushion tissues into vegetative brooms (Melnick et al. 2012). Despite the ability of the pathogen to infect flower cushions, there has been no clear link between this pathogen and cherelle wilt. Suarez-Capello (1977) demonstrated that while floral cushions can become infected with *M. perniciosa*, *M. perniciosa* did not grow into the flower. The pathogen itself produces phytohormones (Kilaru et al. 2007) and can alter the expression of genes putatively involved in hormone function and biosynthesis, resulting in parthenocarpic pods (Melnick et al. 2012). Fortunately, parthenocarpic pods are very easily distinguished from wilting pods by visual inspection.

Of all cacao pathogens, only *Moniliophthora roreri*, causal agent of frosty pod rot, exclusively infects pods. It has been speculated that *Moniliophthora roreri* might cause cherelle wilt, as the early stages of the diseases resemble those of cherelle wilt. Cacao pods are most susceptible to *M. roreri* at 0–3 months after pollination, which overlaps with the susceptibility to cherelle wilt. The earliest symptom of *M. roreri* infection of cherelles is deformation of pods in the form of localized swellings (Aime and Phillips-Mora 2005), as seen in Fig. 15.4.

M. roreri also leads to premature ripening of cacao pods, meaning that typical green cherelles can become yellow or have areas of the pods that become yellow

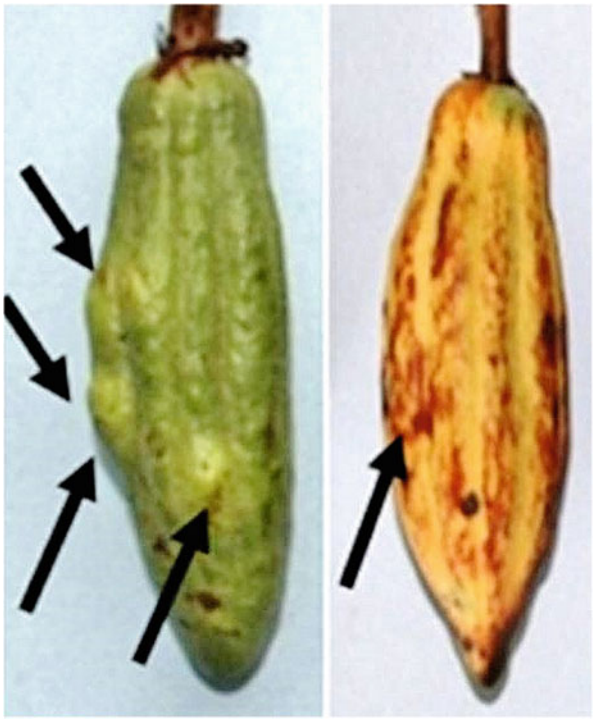

Fig. 15.4 Cacao pods infected with *M. roreri*. Localized swelling of the young cherelles is indicated by the *arrows*

(Aime and Phillips-Mora 2005), as seen in Fig. 15.4. As previously mentioned, yellowing is also the first visible symptom of cherelle wilt. At this early stage, it is very difficult to differentiate pods that are infected with *M. roreri* from those undergoing cherelle , wilt through visual inspection (Leach et al. 2002; Krauss et al. 2010). The localized swellings, while common, do not always occur after *M. roreri* infection. This overlapping symptomatology and period of susceptibility further support the speculation that *M. roreri* infection could result in cherelle wilt. Coupling this information with the sporulation of saprophytic organisms that invade mummified cherelles, it is understandable that some have thought that early invasion by *M. roreri* might be a significant contributor to cherelle wilt. This hypothesis will be discussed in the following section.

15.8 Cherelle Wilt and Frosty Pod, Is There a Relationship

One issue that has been speculated on for many years is whether *M. roreri* affects or causes cherelle wilt. As mentioned previously, both cherelle wilt and the early symptoms of frosty pod rot cause premature ripening in pods. This is very evident in clones that are green when immature and ripen to a yellow, as yellow immature pods are evident on trees. Malformation, when it occurred, was most often visible as swelling (Fig. 15.4) and is always indicative of *M. roreri* infection and not cherelle wilt (Melnick et al. 2013b).

Melnick et al. (2013b) visualized *M. roreri* hyphae in young infected cherelles. The hyphae isolated from yellow and deformed areas resembled hyphae found in older pods at the biotrophic stages of the *M. roreri* lifecycle. Hyphae isolated from cherelles undergoing wilt (without visible malformations) were thin and did not resemble *M. roreri* (Melnick et al. 2013b). Molecular analysis for the presence of *M. roreri* through RT-qPCR confirmed that *M. roreri* was present in senescing malformed cherelles but was not detected in wilting cherelles (Melnick et al. 2013b). It is interesting to note that *M. roreri* was generally unable to complete its lifecycle on infected senescing cherelles, as *M. roreri* sporophores and meiospores were not seen by Melnick et al. (2013b). This was confirmed by the lack of sporulation on obviously wilted cherelles in field experiments in Costa Rica (Phillips-Mora, CATIE, personal communication). Pods at the second stage of wilt susceptibility can become infected with *M. roreri* and sometimes sporulate early, but do not resemble those lost only to cherelle wilt (Bailey, USDA-ARS, personal communication). In trials with hand-pollinated, flowers, 3-month-old pods occasionally senesced early (within 30 days after infection with *M. roreri*) and the fungus sporulated on the pods (Bailey, USDA-ARS, personal communication).

Researchers looking at *M. roreri* spread in Mexico were unable to isolate *M. roreri* from wilted cherelles, despite plating the necrotic tissue onto potato dextrose agar and incubating them in the humid conditions that would induce sporulation (de la Cruz et al. 2011). While cherelle wilt does not seem to be caused by *M. roreri*, the unique physiology of cherelles results in a cessation of frosty pod disease progression in infected cherelles that wilt. The pod senescence process

associated with physiological wilt, made obvious by the shift from green color to yellow in *M. roreri*-infected cherelles, had little impact on the expression of *M. roreri* genes putatively encoding proteins associated with fungal metabolism, growth, parthenogenesis, and detoxification processes (Melnick et al. 2013b). Expression of these genes in *M. roreri* is altered during the biotrophic to saprotrophic shift, which occurs during typical frosty pod rot disease development in pods that reach mature sizes. It is possible that the premature yellowing in *M. roreri*-infected cherelles was due to overly intensive invasion of the fungus stressing the pods. Another possibility is that the wilting of *M. roreri*-infected cherelles is due to the convergence of two separate processes: the infection of a cherelle that was destined to wilt and cessation of pod development due to infection. The sparse distribution of mycelium seen in yellow deformed cherelles further supported the idea that the early stages of wilt had little effect on the development of *M. roreri*. A proliferation of thin saprophytic *M. roreri* mycelia was not commonly observed in infected senescing cherelles until the cherelles had become necrotic, which coincided with a large shift in the expression of *M. roreri* genes. It is suggested that high disease incidence of *M. roreri* can stress the tree and reallocate resources, causing a loss of young pods that mimic cherelle wilt (Wilberth, personal communication, CABI). Also, infection of young cherelles might mimic cherelle wilt if caught before malformations develop (Wilberth, personal communication, CABI). It would seem that the *M. roreri*'s shift from biotroph to saprotroph was a consequence of the cherelle necrosis rather than a cause (Melnick et al. 2013b).

Once cherelles infected with *M. roreri* begin to senesce, necrosis-inducing factors produced by *M. roreri* may influence the resulting symptoms. The shift from cherelles that are yellowing to necrosis (or browning) in *M. roreri*-infected pods was associated with an induction of the *M. roreri* necrosis and ethylene-producing protein gene *MrNep2* (Melnick et al. 2013b). Necrosis and ethylene-producing proteins are commonly produced by many microbes, including plant pathogens. The action of these proteins is plant cell death. In addition to becoming necrotic, the fungus rots the infected cherelles, leading to the cherelle becoming softer and losing turgor pressure. Lignin and pectin-degrading associated genes are induced in the fungus during the shift to necrosis (Melnick et al. 2013b). Lignin and pectin provide structure to plant cell walls; therefore, their degradation would lead to the softening of the pods seen during the rotting stage of the disease.

The premature tissue senescence in aborting *M. roreri*-infected cherelles stresses the *M. roreri* within the pods, resulting in altered expression of fungal genes. These responses included the induction of genes related to sequester of toxic plant metabolites, such as *MrYOR1*, a putative ABC transporter, *MrNAHG*, a putative salicylate hydrolase, and *MrP450b*, a putative cytochrome P450 (Melnick et al. 2013b). The stressful environment found in wilting cherelles could explain the failure to observe meiospores on necrotic and mummified *M. roreri*-infected pods (Melnick et al. 2013b). The environment of the wilting cherelles appears to truncate the disease cycle of frosty pod, altering the lifecycle of *M. roreri* by

limiting sporulation. Disruption of the *M. roreri* lifecycle would prevent infected cherelles from being a major source of inoculum in the field.

Despite this research supporting the conclusion that *M. roreri* is not a main cause of cherelle wilt, few detailed studies have looked at the loss of cherelles to minor pathogens such as *Colletotrichum* spp. While the focus of the work in the Americas and Africa is on diseases, insect pests are larger problems for production in Asia. Bos et al. (2007) found that 2 % of the pods lost to cherelle wilt were fed on by insects prior to wilt. Again, this is an under-researched topic so our understanding of the impact of insects on cherelle wilt or whether insects can initiate cherelle wilt is limited. Overall, scientists are making greater efforts to assess the impact of pest management treatments on cherelle wilt instead of not measuring or removing these pods from the study. The only way to understand what causes the loss of young pods is to thoroughly quantify the losses and attempt to determine the cause.

15.9 Conclusions

Despite the unknowns, we do know that there are massive changes in the physiology of the pods that eliminate the movement of water and nutrients into developing cherelles. This shift in physiology is associated with a massive reprogramming of cherelle gene expression including the induction of numerous stress-responsive genes along with the induction of genes putatively associated with the biosynthesis of JA, ethylene, and polyamines. The review of the literature fully supports that cherelle wilt is a physiological condition related to the compatibility of the clones and natural thinning. Cherelle wilt is impacted by clone, tree age, tree nutrition, and the environment (in particular soil moisture and nutrition). Despite the speculation that disease can cause cherelle wilt, there is little understanding of the role of minor pathogens and no clear link between *Moniliophthora* spp. and cherelle wilt. The induction of cherelle wilt is related to the hormone levels in the pods, but the biochemical pathways critical to the wilt process remain undefined. An increased understanding of these processes could not only be used to increase yields by improving management practices reducing wilt, but also be used in breeding programs to develop more robust trees capable of supporting a larger number of pods.

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Chapter 16
Epiphytic and Parasitic Plants Associated with Cacao

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Abstract Cacao plantations are generally considered to be one of the most biologically rich agricultural systems and are assumed to be an important substitute habitat for various biota. This biota includes major and minor pathogens, some of which have been already discussed in various chapters of this book and some of which are epiphytes and some parasites. This chapter discusses the situation and outlook, and control of epiphytes and parasitic plants on cacao.

16.1 Epiphytes

Situation and Outlook

An epiphyte (Fig. 16.1) is a plant that grows upon another plant (such as a tree), or sometimes upon another object (as a building), merely for physical support and not for nutrients. The term epiphyte derives from the Greek epi (meaning "upon") and phyton (meaning "plant"). Epiphytes grow on plants called phorophytes (phor = carry). The relationship is usually one of phoresis [an interaction in which a larger organism (the phorophyte) carries a smaller one (the phoront), but the larger organism is not harmed] or one of commensalism (Hopkins 1999). Being autotrophs, epiphytic plants use photosynthesis to produce required energy. True epiphytes, also termed holoepiphytes, never root in soil; they complete their entire life cycle anchored to a host plant. True epiphytes are also known as aerophytes (air plants) because they have no attachment to the ground or other obvious nutrient source. Where nonaquatic, they derive their moisture and nutrients from the air, rain, and, sometimes, from debris accumulating around them instead of from the structure that anchors them. Roots that may develop are primarily for attachment, and specialized structures (e.g., cups and scales) may be used to collect or hold moisture.

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Fig. 16.1 Epiphytes on cacao. (**a**) *Microgramma owariensis*, (**b**) *Asplenium africanum*, and (**c**) *Platycerium stemaria* on cacao in Ghana (Pictures by K. Acheampong)

It is estimated that there are about 29,000 epiphytic species, representing between 8 and 10 % of all known vascular plant species and comprising 25–50 % of plant species richness in tropical forests (Gentry and Dodson 1987; Hietz et al. 2006; Nieder et al. 2001). Epiphytism is reported to exist in 83 vascular plant families. A report on nonvascular epiphyte infestations of cacao in tropical agroforestry systems indicates that their diversity on cacao trees is comparable to that of rainforest trees (Anderson and Gradstein 2005; Rice and Greenberg, 2000), but species richness was usually lower, and individual species were found at lower heights on the trunks. The report from cacao plantations in western Ecuador documented 112 species (51 bryophytes and 61 lichens). The vast majority of the species are widespread neotropical or pantropical species; one species, *Spruceanthus theobromae* (Spruce) Gradst., is endemic to cacao plantations of western Ecuador. A survey of epiphyte diversity on cacao plantations in Ghana showed that the most commonly found nonvascular epiphytes in the country were mosses and lichens and, to a less frequent extent, liverworts. Fourteen different vascular epiphytes were also found on cacao farms in Ghana. The common vascular epiphytes were *Bulbophyllum* spp., *Platycerium stemaria*, *Microgramma owariensis*, *Tridactyle* spp., *Diaphananthe bidens*, *Angraecum birimense*, and *Asplenium africanum*. Others which were less frequently observed on the farms included *Rhipsalis cassutha*, *Chasmanthera dependens*, *Drynaria laurentii*, *Aerangis biloba*, *Chamaengis vesicata,* and *Rangaeris rhipsalisocia*. Mosses and lichens were essentially found on all fields surveyed, but their severity on cacao trees varied between the regions.

Influence of Epiphyte Infestations on Cacao Production

In natural forest environments, epiphytes are considered to be a significant component of biodiversity, but in many tree crop plantations in the wet and humid tropics,

including cacao and citrus, they may be sufficiently detrimental to be regarded as pests. For instance, Mabbett (2013) observed that thick layers of lichen growing on the surfaces of cacao, coffee, and citrus leaves can significantly reduce photosynthesis. There is an additional risk of leaf stomata being blocked, with corresponding inhibition of gaseous exchange, if the epiphytic layer occurs on the abaxial (lower) surface of the leaf.

Some climbing epiphytes, including *Bulbophyllum* spp., interfere with the supply of light available to host plants, whilst mosses and lichens potentially support the growth of parasitic organisms (Thorold 1952; Thorold 1975; Robe and Griffiths 1992; Benzing 1998). Epiphytes may cause the death and peeling away of the epidermal layers of the trunk and branches of host plants (Adenikinju and Akinfenwa 1991). Moreover, Adenikinju and Akinfenwa (1991) reported that bryophytes and lichens obstructed the development of the cauliflorous flowers of cacao, causing losses in the final cacao bean yield. Although there have been divergent views on the benefits of epiphyte control in cacao farms as preserving biodiversity, epiphytes are generally believed to have long-term detrimental effects on cacao trees (Becker and Wurzel 1987; Zinsmeister and Mues 1988; Adenikinju and Akinfenwa 1991; Agbeniyi and Adenikinju 2001; Thorold 1952; Thorold 1975; Dormon et al. 2004; Sporn et al. 2007).

Epiphyte Control in Cacao Farms

Different methods, including herbivores and application of assorted solutions (Myers and Heck 2013; Littler and Littler 1999; Acheampong et al. 2013), have been employed to achieve epiphyte control in varied situations. In cacao plantations in Ghana, different concentrations of sodium chloride (NaCl) solution controlled different epiphytes. *Bulbophyllum* sp. and mosses were controlled by 2 % NaCl, *A. africanum* and *P. stemaria* by 4 % NaCl solution, and *A. birrimense* by 6 % NaCl solution (Acheampong and Ofori Frimpong 2004). Furthermore, Acheampong et al. (2013) showed that one-time spraying with cacao pulp juice fermented for 5 days, at the rate of 20 ml/m^2, killed 50 % of lichens, liverworts, mosses, *Platycerium stemaria,* and *Bulbophyllum* spp. in 27, 23, 19, 25, and 29 days, respectively. The fermented juice was also better for control of the nonvascular epiphytes than one-time spraying with 4 % aqueous NaCl at the same rate. The juice was, however, not effective against the vascular epiphytes, *Platycerium stemaria* and *Bulbophyllum* spp. The fermented cacao pulp juice caused only slight injuries (apparent loss of chlorophyll and necrotic spots) along the leaf margins in *Platycerium stemaria*. In *Bulbophyllum* spp., injury (leaf chlorosis) was confined to the older leaves after application of the fermented pulp juice. Sprayable vinegar and copper sulfate have also been used to control epiphyte infestations in Ecuador and Nigeria (Wood 1985; Anderson and Gradstein 2005; Olaiya and Agbeniyi 2003). While it may be more feasible to control some of the epiphytes that infest cacao plantations by such methods, we recommend managing epiphyte infestations

by cultural means such as manual removal of vascular epiphytes and the provision of adequate shade (15–18 recommended shade trees per hectare), so as to reduce humidity.

16.2 Parasitic Plants

Situation and Outlook

Parasitic plants, a group to which mistletoes (Fig. 16.2) belong, are common on branches of trees and shrubs. Originally, all mistletoes were grouped within the family Loranthaceae. However, the genera *Viscum* and *Phoradendron* have been recently classified under Santalaceae (Angiosperm Phylogeny Group 2003). Mistletoes have long been recorded as causing damage to cacao in Columbia (Anon 1980), Costa Rica (Kuijt 1969), Trinidad (Briton-Jones 1934), and most severely in Ghana (Room 1971; Phillips 1977; Amoako-Attah et al. 2014). In Ghana, Appiah and Owusu (1997) reported that six of the 15 different species of mistletoe are found on cacao. These include *Tapinanthus bangwensis* (Engl. and Krause) Danser, *T. truncatus* (Engl.) Danser, *T. buntingii* (Sprague) Danser, and *Tapinanthus bangwensis* (Engl.) van Tiegh. Opoku and Baah (2010) also reported that about 14 % of cacao trees in Ghana are infested with mistletoes and more than 70 % of these are attributed to *T. bangwensis*. The parasite is also found on a wide range of other trees, most of which are associated with cacao farms as intercropped or shade trees. Field inspections of cacao farms in Ghana revealed that mistletoes do not attack monocotyledonous plants and were seldom found on Anacardiaceae (mango and cashew) and Fabaceae (*Gliricidia sepium*) families, suggesting that plants in

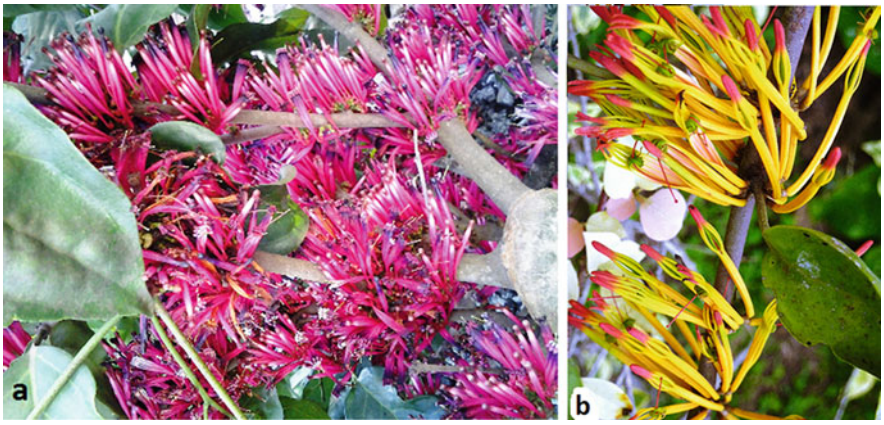

Fig. 16.2 The two most common mistletoe types on cacao in Ghana. (**a**) *T. bangwensis* showing *green* broadly ovate or ovate-lanceolate leaves and profuse *red* tubular flowers arising in clusters in the leaf axil and (**b**) *Phragmanthera incana* showing ovate-lanceolate leaves and *yellow* flowers

different families might have some physical, anatomical, and/or biochemical properties that inhibit the parasites' germination, growth, and/or establishment (Amoako-Attah et al. 2014).

Life Cycle of T. bangwensis

The life cycle of *Tapinanthus bangwensis* (Engl. and Krause) Danser has been aptly described (Lass 1985; Amoako-Attah et al. 2014). In Ghana, *T. bangwensis* is reported to flower twice a year, bearing bright red flowers which are pollinated by sunbirds and bees (Lass 1985). The bright red berries need a month to ripen and are eaten by various species of birds, which serve as disseminating agents. *Tapinanthus bangwensis* can germinate on a wide range of substrates including soil, glass, stone, wood, paper, cloth, and agar media (Phillips 1991). This demonstrates that germination of *T. bangwensis* in nature or culture depends only on nutritive reserves of the embryo and not on the substrate, as long as free water is present (Wood and Laas 1985; Boussim et al. 1993; Engone et al. 2005). Amoako-Attah et al. (2014) showed that *T. bangwensis* germination, holdfast and haustorium development, and leaf emergence occurred on artificial media and thus were not dependent on contact with a living host. Germination was usually within 24 h and this may be an adaptation to overcome harsh environmental conditions associated with the tropical climates where it occurs. However, mistletoe was exacting in terms of light and temperature required for development (Ellenberg 1996). In the laboratory, Amoako-Attah et al. (2014) found that seeds of *T. bangwensis* growing under continuous darkness died before one week. Rapid seedling development was enhanced by temperatures ranging between 25 and 30 °C and by longer photoperiods of 16–24 h. However, leaf emergence was faster at 25 °C than at 30 °C and development was better stimulated in 16 h light than in continuous light (24 h).

Mistletoe Control

The maintenance of overhead shade on cacao farms to prevent mistletoe germination is a useful measure. The overhead shade may reduce exposure of cacao trees to mistletoe seeds dropped by birds; however, too much shade results in a humid environment and encourages the development and spread of fungal diseases. The number of recommended shade trees on a cacao farm is between 15 and 18 trees per hectare (Opoku-Ameyaw et al. 2010). Mistletoe has a wide host range and most of the shade trees on cacao farms, including fruit trees such as avocado (*Persea americana*) and *Citrus sinensis*, are infected by the parasite. Nevertheless, monocotyledonous plants are not infected by mistletoes, and plants within the Anacardiaceae (mango and cashew) and Fabaceae (*Gliricidia sepium*) families are seldom infected by mistletoes (Amoako-Attah et al. 2014). Such plants may

be recommended to provide shade on cacao farms. In controlling mistletoes, physical pruning of infected branches, about 10 cm toward the trunk of the host with long-handled pruning knives, is recommended (Lass 1985). However, this practice has some limitations. Pruning at flowering peaks, when mistletoes are most visible to farmers, leads to reinfestation (Appiah and Owusu 1997). However, resprouting of cortex-embedded haustorial strands in pruned branches has been reported (Dzerefos et al. 1998; Opoku and Baah 2010). Furthermore, pruners and other tools used to remove mistletoes can aid in seed dispersal (Phillips 1977) and, consequently, abet spread of the parasite.

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Part VII
Disease Management

Chapter 17
Biological Control of Cacao Diseases

G.M. ten Hoopen and Ulrike Krauss

Abstract This chapter discusses the advances in biological control of cacao diseases over the last 15 years. Most attention has been focused on biological control of frosty pod rot (*Moniliophthora roreri*), witches' broom (*Moniliophthora perniciosa*) and black pod disease (*Phytophthora* spp.). Research on biocontrol of other diseases in the cacao phyllosphere or rhizosphere is scarce or in its infancy. There is, however, a steady increase in information regarding the factors influencing and the mechanisms underlying biological control of cacao diseases as well as practical aspects such as inoculum production, formulation and application. There has been a clear shift away from inundative approaches using epiphytic BCAs towards more classical biocontrol approaches using bacterial and fungal endophytes as well as vesicular arbuscular mycorrhiza. These have the advantage that they can permanently establish themselves in the cacao tree. Moreover, besides direct competition for space and nutrients, antibiosis and mycoparasitism, through induced resistance and growth promotion, endophytes have a larger arsenal of mechanisms through which they can help protect their host. Endophytic BCAs could thus provide more effective and sustainable disease control. Recent advances in our understanding of the mechanisms through which endophytic biocontrol agents can reduce pest and disease impact provide possibilities for innovative disease control strategies, including combination therapies together with natural or chemical products. Continued work on production, formulation and application is also necessary in order for biocontrol to become economically interesting. However, biological control will not become a stand-alone solution for disease control but should become part of integrated pest management strategies, with cultural management as a central and reinforcing pillar.

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17.1 Introduction

Cacao diseases are the single most important cause of yield loss in cacao production. It is estimated that over 40 % of all cacao production is lost annually to just five diseases: frosty pod rot (FPR), caused by *Moniliophthora roreri* (Chaps. 3 and 4); witches' broom disease (WB), caused by *Moniliophthora perniciosa* (Chaps. 5 and 6); black pod disease (BPD), caused by *Phytophthora* spp. (Chaps. 7 and 8); vascular streak dieback (VSD), caused by *Ceratobasidium theobromae* (Chap. 9); and cacao swollen shoot disease (CSSV), caused by the cacao swollen shoot virus (Chap. 10) (Flood et al. 2004). Additional losses are inflicted by other diseases such as Ceratocystis wilt, caused by *Ceratocystis cacaofunesta* (Chap. 12), and Rosellinia root rot and numerous other root rotting pathogens (Chap. 14) as well as by insect and vertebrate pests. There are many other known pathogens of cacao, yet their potential for causing widespread disease is poorly understood (Chap. 11).

Although genetic control, or disease resistance of the host plant, will probably offer the best option for economic and sustainable control of cacao diseases in the long run, to date, no total resistance against any of the major diseases exists (Phillips-Mora et al. 2005, 2012; Guest 2007; Schnell et al. 2007; Efombagn et al. 2013; Chap. 18). Subsequently, disease management relies heavily on cultural and chemical management practices.

Cultural control is the backbone for all control measures of cacao pests and diseases. Cultural control is the deliberate alteration of the production system, either the cropping system itself or specific crop production practices, to reduce pest populations or avoid pest injury to crops. Cultural control in cacao involves among others, shade management, weeding, cacao pruning and phytosanitation, e. g. the removal of diseased pods. In some cases, as, for example, with frosty pod and witches' broom, phytosanitary practices, i.e. the removal of diseased pods and brooms, are currently the principal means available to smallholder farmers to manage these diseases. However, the effectiveness of phytosanitation depends partly on the disease to be managed. Although proven to be fairly effective against *M. roreri* and *M. perniciosa* (Soberanis et al. 1999; Leach et al. 2002; Medeiros et al. 2010), it is less effective against black pod rot caused by *Phytophthora megakarya* (Ndoumbe-Nkeng et al. 2004). Phytosanitation is, however, labour intensive and expensive. In the case of witches' broom, it is one of the most expensive management practices (Pomella et al. 2007) and thus not often or not regularly practised by farmers (Ndoumbe-Nkeng et al. 2004; Opoku et al. 2000; Medeiros et al. 2010). Another example where cultural control has been the prime management measure is the case of CSSV. For decades, the "cutting out method," the removal of infected and neighbouring cacao trees, has been implemented in order to remove sources of inoculum, however with variable results (Ameyaw et al. 2014).

Chemical control, although fairly effective against some cacao diseases, e.g. black pod disease in Africa (Deberdt et al. 2008; Gockowski et al. 2010), has major negative externalities such as human health problems, pollution, reductions in the

populations of beneficial organisms, the emergence of secondary diseases or pathogen resistance to pesticides (Avelino et al. 2011 and references therein). In other cases, chemical control of cacao diseases is just not very effective. The fast expansion of cacao pods, longitudinal growth can be over 3 mm per day (ten Hoopen et al. 2012 and unpublished results), has been identified as a possible limiting factor for chemical disease control (Evans et al. 1977). Bateman et al. (2005a) evaluated modern fungicides, such as triazoles and oxathiins, together with copper for frosty pod control. Copper hydroxide at 1500 g a i. ha^{-1} (grams of active ingredient per hectare) showed the most consistent yet incomplete improvement in healthy pod yield. However, applications under Costa Rican conditions became cost-effective only when farm gate prices reached \$1.25 kg^{-1}. A systemic fungicide, the oxathiin flutolanil, improved total pod production at 300 g a i. ha^{-1} but gave proportionally less control at the height of the production season. Lastly, for example, in the case of organic cacao production, chemical control is simply not allowed, although limited use of copper compounds may still be permitted. Therefore, and even though chemical control will remain, at least for now, a principal control strategy, it is clear that due to the numerous problems associated with chemical disease control, other options have to be explored. One of these options is biological control of cacao diseases. Biological control can help conserve the stability and diversity of agricultural communities, reduce synthetic inputs and help farmers to adapt to a rapidly changing world where agricultural intensification, land-use and climate change could increase the risk for devastating large-scale disease epidemics (Crowder and Harwood 2014).

Biological Control of Plant Diseases

Biological control is defined here as the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be (Eilenberg et al. 2001). The mechanisms through which organisms can exert disease control are many and diverse. Four key mechanisms are involved sometimes together: parasitism, antibiosis, competition for resources and induced resistance.

Biological control can be roughly divided into three somewhat overlapping categories: inundative, classical and conservation biological control (see also Box 17.1). Inoculation biological control is considered by some as a fourth category (Box 17.1). Inundative biocontrol involves regular inundation by or augmentation of native or endemic natural enemies from the release site (Evans 1999). Inundated systems tend to revert to their original state (ten Hoopen et al. 2003; Krauss et al. 2006) rendering biocontrol limited in efficacy and durability. Inoculation biological control differs from inundative control in that it expects the released organism to control the target after multiplication. Similar to inundative control, repeated introductions of the biological control agent are necessary. In both cases, some of the risks associated with classical biocontrol are less likely to occur. Classical

biological control is normally self-perpetuating and self-regulating but comes with all the risk of the introduction of an exotic species (Box 17.1). Classical biocontrol was originally developed for the control of exotic insect pests and weeds and research on its use for the control of pathogens, such as *M. roreri*, and *M. perniciosa* is still in its infancy. Recent progress in transferring the classical biocontrol approach towards the management of cacao diseases is cutting-edge research.

Conservation biological control (Coll 2009) has also been gaining interest in recent times. The aim of conservation biological control is to stimulate resident populations of biocontrol agents using benign interventions that do not harm microorganisms (Everett et al. 2005). In all four of the before-mentioned biocontrol approaches, there is an aspect of human intervention, the application of biocontrol agents or interventions, that stimulates resident populations of biocontrol agents. Yet macro-organisms (including plants) maintain a natural relationship with many microbes living in (endophytes) or on (epiphytes) the plant's tissues. Certain of these microbes (e.g. epiphytic and endophytic bacteria and fungi, arbuscular mycorrhizae, nitrogen-fixing bacteria) can provide at least some protection from disease attacks (e.g. Arnold and Herre 2003; Herre et al. 2007; Tchameni et al. 2011). This biological control service/activity (Coll 2009) is one of the environmental (regulation) services (Millennium Ecosystem Assessment 2005) provided naturally by microbes. However, the extent to which these naturally occurring control agents reduce disease impact remains largely unknown. It is only relatively recently that recognition of the essential role certain of these microbial symbionts may play in their host's ecology and evolutionary success (Mejía et al. 2014) has led to research on exploiting these relationships for disease control (Backman and Sikora 2008).

Box 17.1: Biological Control (after Eilenberg et al. 2001)

Biological control or biocontrol is defined as: "The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be."

Biocontrol can be roughly divided in three more or less overlapping categories: (I) inundative, (II) classical and (III) conservation biological control. Eilenberg et al. (2001) also proposed a fourth category, inoculation biocontrol.

(I) Inundative biocontrol can be defined as "the use of living organisms to control pests when control is achieved exclusively by the released organisms themselves."

(II) Classical biological control is defined as "the intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control."

(continued)

Box 17.1 (continued)

(III) Conservation biological control is a pest management approach based on manipulation of agroecosystems to promote pest suppression by naturally occurring predators, parasitoids and pathogens (Barbosa 1999; Coll 2009).

(IV) Inoculation biological control is defined as "the intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not permanently." It differs from inundative control in that it expects the released organism to control the target after multiplication.

There is increasing attention on biological control using nonpathogenic endophytic symbionts (Mejía et al. 2014). Carroll's (1986) definition that endophytes are organisms that cause asymptomatic infections within plant tissue excluded pathogenic and mycorrhizal fungi. Petrini (1991) proposed that the definition should be expanded: "to include all organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to their host." This would account for those endophytic organisms that have a more or less lengthy epiphytic phase and also for latent pathogens that may live symptomless in their hosts for some time in their life. The microbial consortia of endophytes effectively impact plant health in nature (Backman and Sikora 2008). Although endophytes do not produce visible effects on their hosts, they can have pervasive genetic and phenotypic effects which can result in increased pathogen resistance (Mejía et al. 2014). Like pathogens, these beneficial endophytic organisms are confronted with the innate immune system of the plant. Their colonization success essentially depends on the evolution of strategies for defence evasion. This modulation of plant defence responses probably aids in establishing the balance between the host and endophyte and ultimately can result in enhanced defensive capacity of the plant (Hermosa et al. 2013; Pozo et al. 2013; Mejía et al. 2014).

The potential nontarget effects of biological control agents (BCAs) include competitive displacement, allergenicity, toxicity and pathogenicity. An additional factor to consider is the potential of the BCA to reproduce and spread to other nontarget environments. Since some BCAs are hypovirulent forms of plant pathogens, or close relatives of fungal pathogens (Freeman and Rodriguez 1993; Mejía et al. 2008), there is a possibility of gene transfer. This could result in a loss of control abilities and even a gain of virulence in the BCA (Brimner and Boland 2003). Gene transfer could also alter the genetic composition of native populations of the BCA.

Biological Control of Cacao Diseases

Of the five most important cacao diseases, those that have received most attention with respect to the development of biological control options are BPD, FPR and WB, all three diseases that primarily attack cacao pods. Considerably less attention has been given to the biological control of CSSV and VSD. With regard to other diseases, only a few have received some attention, as, for example, Rosellinia root rot. A thorough research of the literature and the Internet[1] leads us to believe that many of the "minor" pathogens have not been systematically researched for biological control options. Most of the research on biological control of cacao diseases has been effectuated in the Americas where black pod, frosty pod, and witches' broom occur, oftentimes side by side (e.g. Bateman et al. 2005a; Hidalgo et al. 2003; Krauss and Soberanis 2001a, b, 2002; Loguercio et al. 2009a, b; Mejía et al. 2008). Biological control of black pod disease has received some attention in the Americas (e.g. Krauss et al. 2006; Hanada et al. 2010) but less so than in Africa, where *P. megakarya*, the most aggressive (Nyassé et al. 1999; Appiah et al. 2004) of all *Phytophthora* spp. attacking cacao, is present (e.g. Deberdt et al. 2008; Tondje et al. 2007). To our knowledge, Asian research on biological control of cacao diseases is just starting as evidenced by two 2015 publications, one on the possibilities for biocontrol of VSD (Rosmana et al. 2015) and one on the use of *Trichoderma* for black pod control (Sriwati et al. 2015).

Many of the first studies on biocontrol of cacao diseases focused on inundative approaches using locally isolated fungal antagonists, generally belonging to the genera *Trichoderma* and *Clonostachys*, anamorphs of the ascomycete genera *Hypocrea* and *Bionectria*, respectively. Although field trials in Peru showed promising results for the control of FPR (Krauss and Soberanis 2001a, b, 2002), trials in Panama and Costa Rica were more variable (Hidalgo et al. 2003; Krauss et al. 2003, 2006; Bateman et al. 2005a). Similarly, in Cameroon field trials, using a locally soil isolated *Trichoderma asperellum* (Samuels et al. 1999) initially also provided promising results (Tondje et al. 2007; Deberdt et al. 2008). Follow-up trials, however, again showed considerable variability (ten Hoopen et al. 2010a). This variability in results is a common feature in biological control, and the mechanisms responsible should be better understood.

As mentioned previously, classical biocontrol of pathogens has received relatively little attention but has now become one of the main areas of interest in cacao biocontrol research. Evans et al. (2003a) collected supposedly co-evolved mycoparasitic fungi for frosty pod control from *T. gileri*, the purported original forest host for *M. roreri*, in western Ecuador. Many of these fungi belonged to the genera *Clonostachys* and *Trichoderma*. Subsequent field screens showed that

[1] Search algorithm on Google, Google Scholar and DuckDuckGo was Theobroma (biocontrol OR biological control) (*Fusarium* OR *Trachysphaera* OR *Botryodiplodia* OR *Macrophoma* OR *Geotrichum* OR *Calonectria* OR virus OR Phytoplasm OR bacteria or viral OR *Corticum* OR *Colletotrichum* OR *Phellinus* OR *Rigidoporus* or *Armillaria* OR *Pseudomonas*).

certain of these co-evolved biocontrol agents could survive for periods up to 12 weeks in/on cacao pods and could partly control sporulation of *M. roreri* (Evans et al. 2003a). Arnold et al. (2003) showed that endophytic colonization of cacao leaves reduced their susceptibility to *P. palmivora*. These studies started a new direction in the research for biological control of cacao diseases, focusing on the use of endophytes originating from the cacao tree itself and/or several closely related species. This also led to the description of several new endophytic *Trichoderma* species, such as *T. stromaticum* (Samuels et al. 2000), *T. koningiopsis* (Samuels et al. 2006a), *T. theobromicola* and *T. paucisporum* (Samuels et al. 2006b), *T. martiale* (Hanada et al. 2008) and *T. evansii* (Samuels and Ismaiel 2009), many with antagonistic capacities towards cacao pathogens. Moreover, these studies also put into evidence that many other well-known species of the genus *Trichoderma*, originally thought to be primarily a soilborne genus, have the capacity to form symbiotic relationships with cacao such as *T. hamatum*, *T. harzianum* and *T. asperellum* (Bailey et al. 2009; Evans et al. 2003a; Harman et al. 2004; Rosmana et al. 2015). Recent advances in this field show the intricate relationship between cacao and its microbial symbionts and are starting to elucidate the mechanisms through which these symbionts can contribute to the protection of the plant against cacao pests and diseases.

The objective of this chapter is to provide a general overview of the current knowledge and recent advances in the area of biological control of cacao diseases over the last 15 years and to discuss possible ways forward to make biological control an integral part of cacao management strategies. This chapter is not intended to provide an overview of all organisms being isolated and tested for their potential to control cacao pathogens in vitro but will focus primarily on the results of in vivo and field experiments. It will discuss some of the mechanisms involved and discuss factors that might contribute to its success, or lack thereof, under field conditions. Special attention will be given to the use of endophytic organisms, as it seems that these constitute a very promising avenue for sustainable control of cacao diseases. Even though not normally classed as biological control, the use of plant extracts or other environmentally friendly products has also been advocated for pest and disease control. In this chapter, attention will also be given to some of the advances in this area for the control of cacao pathogens.

Finally, the chapter will present a brief overview of the successes and failures obtained in the biological control of specific cacao diseases before discussing future avenues to explore.

17.2 Phyllosphere Biological Control

Most studies on the biological control of cacao diseases have focused on the control of diseases that attack cacao pods, specifically black pod disease, frosty pod rot and witches' broom disease. Besides the fact that these three diseases are among the most important, the clearly evident presence of diseased pods, which are the source

of the cacao beans that provide revenue to the farmers, may have contributed to this bias.

Cacao pods are located in the phyllosphere. The phyllosphere is a term probably first coined by Last (1955) and Ruinen (1956) and is often used to describe the total above-ground portions of plants as habitat for microorganisms (Last 1955; Ruinen 1956). Some authors employ the term phylloplane. However, its two-dimensional connotation does not do justice to the three dimensions that characterize the phyllosphere (Leveau 2006). The phyllosphere community is composed of epiphytes on the surface and endophytes in the internal tissue of above-ground plant parts. Both epi-and endophytes have been used as biocontrol agents against cacao diseases. Epiphytic fungal biocontrol agents have primarily been used in inundative biocontrol, whereas several co-evolved endophytes have been used in more classical biocontrol approaches. For convenience, the use of endophytes from outside the geographical region of origin of cacao for the control of indigenous diseases in Africa and Asia such as VSD, CSSV or black pod caused by *P. megakarya*, so-called new encounter diseases (NEDs) (Guest and Keane 2007; Samuels et al. 2012), is also considered under the heading of classical biocontrol although it does not completely fit the description as given in Box 17.1.

Inundative Control

Several studies have used inundative biocontrol approaches using locally isolated epiphytic, or in some cases soilborne, BCAs (e.g. Krauss and Soberanis 2001a, b, 2002; Hidalgo et al. 2003; Mendoza-Garcia et al. 2003; Bateman et al. 2005a; Tondje et al. 2007; Deberdt et al. 2008; Mbarga et al. 2014). The use of these BCAs relied primarily on exploiting their capacity to compete for space and nutrients and to parasitize a pathogen or their capacity for producing antibiotic substances that can limit pathogen growth (antibiosis). A large variety of fungal and bacterial epiphytes is present on aerial cacao parts (ten Hoopen et al. 2003; Urdaneta and Delgado 2007). Some of these epiphytes are antagonists of pathogens. For instance, numerous epiphytic fungal parasites of the genera *Clonostachys* and *Fusarium* and to a lesser extent *Trichoderma*, potential agents for biocontrol of BPD (*P. palmivora*) and FPR, were detected on cacao flowers and pods in Costa Rica (ten Hoopen et al. 2003). ten Hoopen et al. (2003) also found that there were no differences in the frequency of occurrence between the three main cacao cultivars, ‘Criollo’, ‘Forastero’ and ‘Trinitario’, nor between clones within these groups, nor between four distinct FPR susceptibility classes of segregating F1 hybrids. These results suggest that mycoparasite abundance and genetic disease resistance are independent phenomena and should lead to additive effects if employed simultaneously in an integrated disease management programme. Furthermore, the common practice of growing cacao in cultivar mixtures to encourage cross-pollination poses no obstacle to the establishment of mycoparasite inoculum for the purpose of biocontrol.

Inundative biocontrol of FPR using native epiphytic fungi was successful in Peru. Native *Clonostachys rosea* reduced FPR by some 15–25 % under field conditions. Mixtures of local antagonists controlled three cacao pod diseases (FPR, WB and BPD) simultaneously, leading to yield increases of up to 16.7 % (Krauss and Soberanis 2001a, 2002). However, the inundative mycofungicide approach proved less promising in Costa Rica (Hidalgo et al. 2003; Krauss et al. 2003). Even biocontrol isolates proven to be effective in the above-mentioned inundative trials in Peru fell short of expectations in both Costa Rica (Bateman et al. 2005a) and Panama (Krauss et al. 2006), despite apparently similar agro-ecological conditions. Since in Central America both the crop and the pathogen are removed from their centre of origin, classical biocontrol of FPR, using co-evolved biocontrol agents, may be more applicable. None of the introduced Peruvian antagonists could be viewed as co-evolved (Krauss and Soberanis 2001a, b; Krauss et al. 2002).

In a 3-year field trial in Cameroon, inundative biocontrol of BPD, caused by *P. megakarya*, with a local, soil-isolated *T. asperellum* (isolate PR11) reduced black pod incidence by 23–24 % compared with a water control treatment (Tondje et al. 2007; Deberdt et al. 2008). However, follow-up trials under different agro-ecological conditions provided more variable results (ten Hoopen et al. 2010a). This difference in results is partly explained by the climatological differences between trial sites, the work by Deberdt et al. (2008) being done in an agro-ecological zone generally drier, and thus less conducive to disease development, than the study area of ten Hoopen et al. (2010a). Since *P. megakarya* is considered to be native to Africa, it can be considered a new encounter disease (NED). *P. megakarya* probably jumped hosts somewhere in the south-western forest region of Cameroon (near Mount Cameroon) (Mfegue 2012). Holmes et al. (2003) isolated *P. megakarya* in south-western Cameroon from a fruit of *Irvingia* and speculated that *Irvingia* could be a native forest host. However, given that the fruit was collected from the forest soil, this precludes any definite conclusions since infection could have occurred after the fruit had fallen. Thus, there is a lack of knowledge as to the native host(s) of *P. megakarya* (Mfegue 2012), and the search for co-evolved biocontrol agents of this particular pathogen remains therefore in suspense.

One of the reasons often evocated to explain the variability in biocontrol trials between years and sites is that epiphytic BCAs are subjected to numerous stresses. These can be biotic, e.g. competition with existing flora and fauna (e.g. de Souza et al. 2008), or abiotic, e.g. humidity and temperature (e.g. Loguercio et al. 2009b). Both types can hinder successful establishment, germination and growth on the pod surface and thus reduce biocontrol efficacy (Crozier et al. 2015).

Some of the means proposed to compensate for the biotic and abiotic stresses encountered in the phyllosphere or even the rhizosphere of cacao has been the use of specific formulations favouring germination and growth of the BCA and/or biocontrol mixtures. The idea behind the latter is that under changing environmental conditions, different mycoparasites are capable of exercising control. Using mycoparasite mixtures has also been proposed when simultaneous control of several cacao diseases is sought. In several South American countries, FPR, WB

and BPD are present. The use of mycoparasite mixtures as tested in Peru for the simultaneous control of these three diseases showed that mixtures were consistently better than single strains (Krauss and Soberanis 2001a, b, 2002). However, control efficacy varied for the different diseases. The latter is partly explained by the biology of the pathogens to be controlled. Firstly, fungal pathogens such as *M. roreri* and *M. perniciosa* have cell walls mainly constituted of chitin, whereas the oomycete *Phytophthora* has cell walls mainly constituted of cellulose. Thus for simultaneous control, BCAs should be capable of metabolizing both chitin and cellulose. Secondly, both *Moniliophthora* spp. exhibit a biotrophic phase in which they germinate, after arriving on the cacao pod surface, and subsequently penetrate the host tissue. The biotrophic phase is followed by a necrotrophic phase within the brooms and/or cacao pods during which the cacao pod is destroyed from the inside out. Contrary to *Moniliophthora* spp., *Phytophthora* spp., after stomatal entry or direct penetration, directly start to necrose pod tissues and destroy the pod from the outside inwards. In other words, an epiphytic BCA has probably a larger window of opportunity to control *Phytophthora* than it does for *Moniliophthora* spp. Therefore, one argument in favour of the use of endophytic BCAs is that they would probably have a larger window of opportunity to control *Moniliophthora* spp. Moreover, endophytes are less exposed to changing environmental conditions (Backman and Sikora 2008) and, when infecting cacao tissues, trigger molecular responses associated with stress and plant defence (Bailey et al. 2006; Mejía et al. 2014) that could result in induced systemic resistance.

Classical Biocontrol

In the early 2000s, work by, e.g. Arnold et al. (2003) and Evans et al. (2003a) started a classical biocontrol approach of cacao diseases by using endophytes isolated from *T. cacao* and closely related tree species in their region of origin. The use of endophytes now seems to be the most promising way forward, and much of the recent cacao biocontrol literature focuses on using endophytes.

Recent papers have shown that the phyllosphere of cacao and closely related *Theobroma* and *Herrania* spp. are very diverse with respect to the diversity of endophytes, fungal and bacterial, present (e.g. Arnold et al. 2003; Rubini et al. 2005; Crozier et al. 2006; Thomas et al. 2008; Melnick et al. 2011). Most of these studies have been carried out in Latin America, the region of origin of *Theobroma* and *Herrania* species. Yet there is a growing interest in studying the endophytic community of cacao on other continents for their potential in controlling (indigenous) diseases. In Cameroon, for example, a study is ongoing which looks at the diversity of cacao fungal endophytes and their potential in protecting the plant against either black pod disease caused by *P. megakarya* or the cacao pest *Sahlbergella singularis* (D. Begoude, personal communication). Recent studies from Indonesia showed the presence of endophytic *T. virens*, *T. longibrachiatum* and *T. asperellum* in cacao (Sriwati et al. 2011, 2015; Rosmana et al. 2015) whereas

Amin et al. (2014) studied the endophytic community in cacao clones from different VSD susceptibility classes. It seems, however, that endophytic diversity is decreased in countries where cacao is not native (Schmidt et al. 2010). Interestingly, the search for endophytic co-evolved antagonists in the centre of origin of certain pathogens, such as the new encounter diseases CSSV, VSD and BP caused by *P. megakarya*, has not yet received the attention it deserves. The reason for this seems simple, as for most NEDs native hosts remain largely unidentified (Guest and Keane 2007; Mfegue 2012; see also the corresponding chapters in this book).

It is important to note though that the difference between epiphytes and endophytes is sometimes blurry. *Clonostachys* spp., common as epiphytes on cacao pods (Krauss and Soberanis 2001a, b, 2002; ten Hoopen et al. 2003), have also been found as endophytes (Evans et al. 2003a). *T. asperellum*, in Cameroon a common soil-based antagonist of *P. megakarya*, has been found as an endophyte in Brazil (Hanada et al. 2009) and recently in Sulawesi (Rosmana et al. 2015; Sriwati et al. 2015). *T. stromaticum* isolates belonging to genetic group II are generally better endophytic colonizers (de Souza et al. 2008) than isolates from genetic group I. It seems that for certain species of biocontrol agents, the capability to colonize plant host tissue is isolate dependent.

Somewhat similar to ten Hoopen et al. (2003), Rubini et al. (2005) found that the number of cultivable endophytic fungi, using PDA medium, recovered from three cacao tree categories, resistant to *M. perniciosa*, healthy or symptomatic (i.e. diseased), did not differ between categories. Yet the frequency in which endophytes with the capacity to inhibit *M. perniciosa* were found decreased from healthy (19 %), to resistant (16.1 %) to symptomatic plants (14.3 %). Interestingly, only 43 out of the 256 endophytic fungi tested in vitro against *M. perniciosa* showed inhibitory qualities and only one isolate, identified as *Gliocladium catenulatum*, was able to substantially (70.8 %) reduce witches' broom disease symptoms (Rubini et al. 2005). These data seem to indicate that cacao genetic make-up and endophyte abundance are also independent phenomena. However, more research is necessary to determine the interactions between genetic disease resistance of cacao and the microbial assemblages living in or on the cacao tree.

Numerous other foliar endophytic fungal (FEF) species isolated from *T. cacao*, *T. gileri* and *T. grandiflorum* show potential as biological control agents and have in several cases been shown to reduce leaf and pod damage caused by *T. cacao* pathogens and herbivores (Arnold et al. 2003; Evans et al. 2003a; Rubini et al. 2005; Bailey et al. 2006; Mejía et al. 2008; Bae et al. 2009; Hanada et al. 2010; Krauss et al. 2010). They also include many novel species from genera well known to be antagonists of cacao pathogens, such as *Trichoderma* and *Clonostachys* spp. (Hanada et al. 2008; Samuels et al. 2006a, b). For many of these endophytic *Trichoderma* isolates, besides direct mycoparasitism, competition, niche exclusion, antibiosis and/or induced systemic resistance have been demonstrated to be operational to varying degrees (Bailey et al. 2006, 2008; de Souza et al. 2008; Krauss et al. 2010; Samuels et al. 2006b; Thomas et al. 2008), and they can even promote plant growth or reduce/delay drought stress (Harman et al. 2004; Bae et al. 2009; Bailey et al. 2011).

Evans et al. (2003a) collected numerous candidates for FPR control from *T. gileri* in western Ecuador, most notably *Clonostachys* and *Trichoderma* spp. Subsequent field screens in Costa Rica demonstrated that *Trichoderma ovalisporum* (Holmes et al. 2004) strain TK1 could be re-isolated from cacao pods 10 weeks after inoculation and suggested an adverse effect on sporulation by *M. roreri* (Evans et al. 2003b; Holmes et al. 2006), which rendered it a candidate for full-scale field trials. Soon *T. ovalisporum* (TK-1) yielded respectable results, in field trials in both Ecuador and Costa Rica (Holmes et al. 2006; Krauss et al. 2010). A recent study by Crozier et al. (2015) showed that the efficacy of this endophyte for FPR control could be further increased by formulating it using a corn-oil-based formulation, yielding 30.7 % healthy pods over a 2-year period compared to 18.5 % healthy pods for plots treated with copper oxychloride. *T. ovalisporum* TK1, as many of the other *Trichoderma* spp. used in biocontrol of cacao diseases, is not only an endophyte but also a mycoparasite.

Another *Trichoderma* species, *T. stromaticum* (Samuels et al. 2000), is probably a co-evolved antagonist of *M. perniciosa* and is now being used as a semicommercial product, with promising results for the management of witches' broom in Brazil (Loguercio et al. 2009a, b; de Souza et al. 2008). Since this species has been isolated in the native range of *T. cacao*, it also goes to show why conservation of the biodiversity of host plants and their associated fungi in their natural habitat (in situ conservation; see e.g. Krauss (2004) and Avelino et al. (2011)) is of such great importance. This is especially true for NEDs for which native hosts and co-evolved antagonists still have to be identified.

Essential for the success of endophytes in biological control is their establishment in/on the plant tissues. Under natural conditions, as for most tropical tree species, leaves are flushed in a largely endophyte-free condition (Arnold and Herre 2003; Arnold et al. 2003). Spores deposited on the leaf surface can directly penetrate the leaf (Herre et al. 2007), and previously endophyte-free leaves become saturated with endophytes. It is not yet clear how these fungi can subsist sometimes for several years within host leaves (Herre et al. 2007). In the case of classical biocontrol, the objective is to inoculate the cacao plant or parts thereof with selected BCAs. In a study by Bailey et al. (2008), which evaluated different cacao seedling inoculation methods using several endophytic *Trichoderma* species, it was shown that general aggressiveness of the isolate may be more critical to the isolate's ability to colonize woody tissues than the source and method of the original isolation. Moreover, there were marked differences in colonization efficiency due to inoculation method.

It has been clearly demonstrated that *Trichoderma* species are capable of colonizing above-ground plant tissues, including the bark, the xylem, the apical meristem and to a lesser extent the leaves (Evans et al. 2003a; Bailey et al. 2006, 2008, 2009). Yet the impact of black pod, frosty pod and to a lesser extent witches' broom is at the pod level. Colonization of the pod, either directly, after the application of an endophytic BCA to the pod surface, or indirectly, from within endophyte-colonized plants themselves, is a necessity when the BCA acts through

mechanisms such as mycoparasitism, competition for space and nutrients, etc. that ask for a certain proximity between pathogen and BCA.

Trichoderma species can form endophytic associations within *Theobroma cacao* trichomes (Bailey et al. 2009). It has been hypothesized that the relatively high density of trichomes on young cacao pods compared with mature pods explains the differential susceptibility of the different developmental stages of cacao pods (Flores et al. 1994). If trichomes facilitate the infection of cacao pods, they may also facilitate the establishment of certain epiphytic and/or endophytic BCAs and thus influence biocontrol efficacy. As the density of trichomes is dependent on pod age, biocontrol applications at the early stages of development could be more effective than at later stages.

How exactly these endophytic organisms protect the cacao plant against pathogen attacks is as yet poorly understood, but recent advances are starting to elucidate the various mechanisms through which these endophytes can exercise disease control. Besides mycoparasitism, endophytic fungi can also produce antifungal compounds (Aneja et al. 2005; Talontsi et al. 2013; Sriwati et al. 2015) which in some cases can be found within the cacao plant after colonization by the specific antifungal compound producing endophyte (Herre et al. 2007). Yet increased host resistance can also be obtained by the induction of increased expression of host genes which are effective in enhancing disease resistance (Bailey et al. 2006; Mejía et al. 2014; see also Sect. 17.5).

These recent scientific advances demonstrate that the cacao tree consists of both host tissues and multiple microbial symbionts (e.g. foliar fungal or bacterial endophytes, arbuscular mycorrhizae, nitrogen fixing bacteria, etc.) that play essential roles in their host's ecological and evolutionary success (Mejía et al. 2014). Studies of their separate and combined effects on host genetic and phenotypic expression as well as any other direct or indirect effects they may have on host plant defences are needed in order to exploit the full potential of these symbionts for the control of pests and diseases.

Bacteria for Biocontrol of Cacao Diseases

The use of bacteria, especially *Bacillus* and fluorescent *Pseudomonas* spp., to control cacao diseases has started to receive attention, although less so than the use of fungal antagonists. An inundative control approach with local bacteria was tested in field assays in Coast Rica. So-called effective microorganisms (EM) from various sources were enriched by anaerobic fermentation in mixed culture. As a result, the exact composition of each batch is likely to vary and is generally not known. Incipient results from short field tests (less than one season) looked promising (Najár and Thomas 2001; Okumoto et al. 2002). However, the lack of a follow-up publication suggests that initial hopes were not confirmed in subsequent trials.

A study by Melnick et al. (2008) showed that bacteria of the genus *Bacillus* were capable of colonizing cacao leaves, primarily as epiphytes but also as endophytes. Their presence led to a significant decrease in disease severity when the leaves were challenged with *P. capsici*. Moreover, *Phytophthora* suppression by *Bacillus cereus* isolate BT8 only occurred in non-colonized leaves that had developed after bacterial applications and persisted in older leaves of BT8 colonized plants. According to Melnick et al. (2008), these results are strongly indicative of systemically induced disease resistance, and there is strong indication that no direct effects (e.g. antagonism) were involved. Although disease suppression was sustainable (>68–70 days) after a single application, Melnick et al. (2008) believe that reapplications would be necessary in order to maintain disease resistance yet likely less frequent than conventional fungicide applications. In a follow-up study on the use of endospore-forming bacteria for the control of cacao diseases, Melnick et al. (2011) found that cacao can support the coexistence of multiple endospore-forming bacterial species from different genera. Certain of these bacteria exhibited antagonism against three main cacao pathogens, *Phytophthora capsici*, *M. roreri* and *M. perniciosa*. Leite et al. (2013) showed that the bacterial endophytes *Bacillus subtilis* and *Enterobacter cloacae* isolated from cacao could both systematically colonize and promote growth of cacao seedlings. Falcäo et al. (2014) not only confirmed the growth-enhancing capacities of *B. subtilis* but also that it increased grafting success rates and that it showed antagonistic effects against *M. perniciosa*. Actinomycetes isolated from the surface of cacao pods were able to completely inhibit basidiospore germination of *M. perniciosa* under laboratory conditions (Macagnan et al. 2006). Chitinolytic bacteria belonging to the genera *Enterobacter* and *Bacillus* have also been used to reduce leaf anthracnose of cacao caused by *Colletotrichum gloeosporioides* (Suryanto et al. 2014).

Many *Bacillus* spp. are chitinolytic (Melnick et al. 2011) and could possibly directly antagonize fungal pathogens such as *M roreri* and *M. perniciosa*, yet chitinolytic *Bacillus* spp. will probably be less effective against Phytophthora pod rot given that *Phytophthora* has cell walls primarily composed of cellulose. Actinomycetes capable of inhibiting basidiospore germination of *M. perniciosa* in the lab were less successful in the field, with inhibition of *M. perniciosa* ranging from 6 to 21 %, yet had no effect on black pod rot caused by *P. palmivora* (Macagnan et al. 2006).

Fluorescent *Pseudomonas* species have proven to be antagonistic against *Phytophthora infestans* and *P. capsici* (Tran et al. 2007, 2008). Akrofi et al. (2012) thus hypothesized that fluorescent pseudomonas could also be useful for the control of cacao black pod disease. Effectively, they found that cacao harbours *Pseudomonas* strains capable of, at least *in vitro*, inhibiting mycelial growth, causing lysis of zoospores from both *P. palmivora* and *P. megakarya*. Other bacteria, such as rhizobacteria, have also proven to be able to antagonize *P. palmivora* and reduce lesion size on detached pods (Koranteng and Awuah 2011).

The use of bacteria for disease control in cacao is still in its infancy. Yet given the interesting results presented in the few available papers, it certainly seems to

merit further attention. The use of bacteria in biological control of cacao diseases might provide additional benefits related to reduced production costs and/or longer shelf life of the biocontrol product (Yánez-Mendizábal et al. 2012; Schisler et al. 2004). Another intriguing possibility to explore would be the use of bacterial and fungal BCA mixtures.

17.3 Rhizosphere Biological Control

Biological control of soilborne cacao diseases is surprisingly poorly investigated. One soilborne disease of cacao that has received some attention is caused by two species of *Rosellinia*, *R. bunodes* and *R. pepo*. Inundative applications with fungal antagonists isolated from soil, either as single strain or in strain mixtures, showed that in pot bioassays, mixtures of antagonists exhibited more control than single strains and mixtures were effective over a wider range of soil conditions (Mendoza-Garcia et al. 2003).

Many *Phytophthora* species have a soilborne phase in their natural life cycles (Ristaino and Gumpertz 2000) even though disease expression often occurs on aerial plant parts. Primary inoculum of *P. megakarya* is located in the soil (Ristaino and Gumpertz 2000; Mfegue 2012), and with the onset of the rainy season, through rain splash, cacao pods become infected, and from, there the repeated cycles of pod infection and sporulation are at the origin of losses that can reach up to 80 % when no control measures are in place. Thus, if this soilborne inoculum could be eliminated or prevented from reaching cacao pods, losses from black pod could possibly be reduced. Efforts to confine the pathogen in the soil by adding coffee parchment mulch (7.5 cm) were compared with single treatments of Terraseal WL60 (2,800 l ha^{-1}) DuPont soil fungicide No. 1823 (9 kg ha^{-1}) and malachite green (17 kg ha^{-1}). None of these treatments gave any black pod control (Gorentz 1974 and references therein). Efforts to control soilborne inoculum of *Phytophthora* using soil applications of fungicides provided 50 % control of black pod in some cases (Gorentz 1974 and references therein). However, in these trials, the lower portion of cacao trees was also sprayed, which could confound the results. One of the problems with soil-oriented fungicide applications is how to assure that the fungicide arrives at the biological target. Soil absorption of the fungicide can limit its movement, whereas roots and other soil organisms can create canals for fungicide transport. Porosity and surface tension also influence water and thus fungicide movement (e.g. Bailey and White 1970; Krauss 1994; Krauss and Deacon 1994).

The use of a biological control agent, a living organism, in this case a *T. asperellum* (PR11) isolated in Cameroon from the soil environment, has the benefit that the organism is adapted to the environment it is applied to and could actively grow in it. Moreover, several studies have demonstrated that *Trichoderma* spp. are chemotrophic towards other fungi or induce chemotropism towards themselves (Chet et al. 1981; Sharma 2011). Efforts to control soilborne inoculum of *Phytophthora ramorum* and another straminopilous species, *Pythium myriotylum*,

with *T. asperellum* PR11 have proven successful (Mbarga et al. 2012; Widmer 2014[2]). A study by Ndoungue Djeumekop et al. (2012) showed that repeated inundative soil applications of *T. asperellum* PR11 were capable of significantly reducing the amount of soil inoculum of *P. megakarya* compared with a control and fungicide (Ridomil Gold WP66, a.i. CuO 600 g kg^{-1} and Metalaxyl-M 60 g kg^{-1}) treatment. Moreover, in a 2-year field study, soil applications with *Trichoderma* PR11 actually reduced the number and percentage of rotten pods compared to a water-only control treatment but was worse than the fungicide treatment (Ndoungue et al., unpublished results). It is hypothesized that this difference in results, a larger reduction in soil inoculum yet less control of pod rot by *T. asperellum* PR11 compared with the fungicide, is due to the uptake of the fungicide by the cacao tree which would infer additional control of *P. megakarya*.

Research on the biological control of soilborne cacao diseases remains scarce, even though in some areas, soilborne pathogens can create substantial production losses (e.g. Cadavid 1995). Moreover, none of these studies have started studying persistence of applied BCAs in the soil or differentiated between inundative and classical biological control approaches. Somewhat more research has been done on the beneficial effects of vesicular arbuscular mycorrhiza (VAM) which in some cases can also provide disease control and which, due to the establishment of a more permanent symbiotic relationship between the plant and mycorrhiza, could possibly be a more sustainable and cost-effective means of fighting soilborne cacao diseases.

Vesicular Arbuscular Mycorrhiza

Microbial organisms living in the rhizosphere at the root–soil interface are known to be able to influence plant growth, health and soil quality. Rhizosphere microorganisms, primarily bacteria and fungi, can establish a variety of relationships, symbiotic and/or saprophytic with detrimental or beneficial effects. Some of the mutualistic microorganisms are capable of entering the root system of host plants and adopt a (semi)-endophytic lifestyle which is beneficial for both the host (plant) and the microorganism. Among these endophytic organisms, we find mycorrhizal fungi (Pozo et al. 2013). Most of the major plant families form arbuscular mycorrhizal (AM) associations. The AM symbiosis increases plant growth, enhances available soil nutrient uptake and increases plant resistance to abiotic (drought, salinity, heavy metal toxicity) and biotic stresses (pathogen attacks) (Pozo et al. 2013 and references therein).

Similar to endophytes in aboveground plant parts, mycorrhiza, when colonizing the host plant, can either directly compete with soilborne pathogens for space and nutrients or can modulate plant defence responses which may in turn result in enhanced resistance against pest and diseases. What is interesting is that this

[2] Widmer's (2014) *Trichoderma asperellum* isolate 02-64 corresponds with isolate PR11.

activated defence is often also expressed in aboveground plant tissues as an induced systemic resistance effect (Graham 2001; Pozo and Azcón-Aguilar 2007; Van Wees et al. 2008). Moreover, colonization by AM fungi can alter the microbial community surrounding the roots with either beneficial or detrimental effects for soilborne pathogen control (e.g. Ravnskov et al. 2006; Wamberg et al. 2003). More recent findings indicate that a primary mechanism of pathogen control occurs through the ability of AM fungi to influence plant gene expression (Pozo et al. 2013) similar to what occurs when, e.g. a foliar endophyte, *Colletotrichum tropicale*, infects cacao leaves (Mejía et al. 2014).

Thus, the activity and diversity of mycorrhizal fungi influence ecosystem functioning. Therefore, managing AM fungi (native populations or introduced) can be a sustainable strategy in agriculture as it can help reduce the use of chemicals and energy in agriculture, minimizing agricultural degradation (Jeffries and Barea 2012).

However, it is recognized that different species of AM fungi can differentially affect plant physiology and growth of their host. In some cases, a given AMF species can even produce a net loss in growth compared with other AMF species or non-AMF controls. Similarly, a given AMF may affect hosts differently. Finally, hosts can produce different effects on the growth and spore production of AMF species. Foliar endophytes seem to have similar effects (Mejía et al. 2014).

Relatively little information exists about the role of AMF in cacao-based systems (Chulan and Ragu 1986; Cuenca et al. 1990: Chulan and Martin 1992). There are a few recent papers describing the diversity and temporal changes in AM fungi in cacao production systems (e.g. Soumaila et al. 2012; Snoeck et al. 2010). Both Iritié et al. (2012) and Snoeck et al. (2010) in Ivory Coast and Cameroon, respectively, found that the genus *Glomus* was generally most abundant. Both studies also found *Acaulospora* and *Gigaspora* spp., whereas in Cameroon, two other genera were identified, *Scutellospora* and *Archaospora*. It is surprising though that very little attention has been given to the role of these mycorrhizae in the control of cacao diseases. In a paper by Herre et al. (2007), it was shown that *T. cacao* seedlings inoculated with common AMF showed dramatically reduced damage in the leaves due to *P. palmivora*. In this case, since AMF are confined to the roots, there was no possibility of a direct physical interaction between AMF and *P. palmivora*. Besides the possibility that AMF-derived products were translocated to the leaf, the anti-pathogen effects observed in the leaves were probably due to an indirect effect (Herre et al. 2007).

A study by Tchameni et al. (2011) showed the beneficial effects for cacao seedlings of soil inoculation with either *Trichoderma asperellum* PR11 or a mixture of two AMF (*Gigaspora margarita* and *Acaulospora tuberculata*) or a combination of all three. In all cases, cacao seedling growth (plant height and root and shoot weights) was increased compared with non-inoculated control seedlings. Moreover, increased resistance to *P. megakarya* was observed in the leaves of these seedlings. However, there were important differences between treatments. In general, the combination treatment AMF/*T. asperellum* was somewhat less effective than either *T. asperellum* or the two AMF applied separately. Dual inoculation with *T.*

asperellum and the AMF significantly reduced the level of root colonization by AMF. Based on their results, the authors concluded that for the successful use of combination treatments of AMF and saprophytic fungi, such as *Trichoderma* spp., it is necessary to study the functional compatibility between AMF, antagonist and host, not only in terms of plant nutrient and growth responses but also in terms of biocontrol activity. In a follow-up study on the mechanisms behind the observed increased resistance to *P. megakarya*, Tchameni et al. (2012) studied the effects of *G. margarita* and *Glomus mosseae* on amino acid synthesis and reduction in cacao seedlings. They observed a positive correlation between mycorrhizal colonization and the level of amino acids present in seedling leaves and a negative correlation between amino acid levels and disease severity when detached leaves of the cacao seedlings plants were challenged with *P. megakarya*. These amino acids could serve as precursors for the synthesis of proteins involved in plant defence mechanisms.

Mycorrhiza could possibly play an important role in the control of cacao diseases, both in the rhizosphere through direct interaction with the pathogen and in the rhizo- and phyllosphere through mechanisms of induced resistance. However, for now, their potential remains largely unexplored. Moreover, belowground and aboveground interactions between plants, endosymbionts and pathogens may influence each other (Van Der Putten et al. 2001). Omacini et al. (2006), for example, showed that an endophyte-infected grass species, *Lolium multiflorum*, had lower levels of mycorrhizal colonization. However, the presence of endophyte-infected plants caused an increase in mycorrhizal colonization in non-endophyte-infected conspecific neighbours when endophyte-infected and noninfected plants were grown together (Omacini et al. 2006). Exploring these relationships in cacao and their potential for increasing biocontrol efficacy remains to be done.

17.4 Factors Influencing Biocontrol Efficacy

In order for biological control to be effective, it is necessary, besides having an effective biocontrol agent, that the biocontrol agent is capable of germination, growth and, in the case of endophytes, penetration of the cacao tissue in order to become established.

The best strategy for obtaining promising antagonists for an inundative biocontrol approach is to isolate native microbes from healthy plants and/or plant parts of the same species that is to be protected. Disease pressure should be as high as possible and agro-ecological conditions comparable to where subsequent biocontrol is expected. The argument in favour of this approach is good adaptation of the agent to local agro-ecological conditions. In order to achieve good control, these agents typically have to be applied in relatively large quantities and repeatedly throughout the season, which is expensive.

In the case of classical biocontrol, co-evolved antagonists should be isolated from the native host in its geographical region of origin. Such has been done for co-

evolved antagonists of FPR and WB. Yet since for certain diseases, e.g. BPD caused by *P. megakarya* and VSD in Asia, the native hosts remain unknown, this has not been possible for all diseases attacking cacao.

Cacao is often grown in spatially and structurally complex and species-diverse agroforestry systems which can have an effect on biocontrol efficacy. Combining different plant species in agroecosystems can enhance ecological pest and disease regulation mechanisms. Resource availability and microclimatic variation affect processes related to pest and pathogen life cycles. Although these mechanisms are supported both by empirical research and epidemiological models, their relative importance in a real complex agroecosystem is still poorly understood (Gidoin et al. 2014).

Structural optimization of agroforestry systems is complex. Trees intercept solar radiation, affecting resource availability for plant parasites, influence wind and rain effects and promote wetness in the plantation, favouring propagule viability and infection (Avelino et al. 2011; Loguercio et al. 2009b; Gidoin et al. 2014; Poppenborg and Hölscher 2009; Ngo Bieng et al. 2013). The impact of shade trees is variable between seasons and years and can differentially affect pathogens, BCAs and crop plants. Phytophthora pod rot is increased by intermediate shade levels, whereas stem canker caused by the same pathogen is increased in fields exposed to full sunlight due to water stress (Schroth et al. 2000 and references therein). Moreover, there are edge effects due to spatial arrangements. Evans (1981a), for example, noted that cacao grown in unshaded blocks without windbreaks demonstrated increased turbulence, especially at plantation edges, and that these currents favoured inoculum movement of *Moniliophthora perniciosa*, with a resulting increase in both pod and flower infections. Antagonistic effects of shade also exist. Work by Monteith and Butler (1979) and Butler (1980) showed that wind speed plays an important role in the duration of condensed water on cacao pods and pod wetness duration affects black pod rot. Increasing wind speed in the cacao canopy by reducing shade tree density and even by making occasional openings in the cacao canopy will reduce pod wetness duration, which in turn reduces the occurrence of black pod rot. Yet balanced against this will be the changing pattern of rainfall. Without a shade canopy, direct rain hitting the pod surface and the soil may increase intensity and velocity of raindrop splash, which, when combined with increased wind velocity, could result in increased long-range spore dispersal (Evans 1998). Notwithstanding the diverse effects of shade, biocontrol of pests and diseases affecting plant aerial organs seems to be improved by it (e.g. Gidoin et al. 2014).

Although mixed biocontrol inocula (*Clonostachys* spp. and *Trichoderma* spp.) were able to reduce incidences of frosty pod rot, witches' broom and black pod rot (*P. palmivora*) in shaded and non-shaded cacao in Peru, the reduced disease loss attributed to the control only resulted in a net economic return in shaded plantations (Krauss and Soberanis 2001b). Different isolates of *T. stromaticum*, antagonist of *M. perniciosa*, have also been reported to respond differently to microclimate variation in terms of sporulation and antagonism at different cacao canopy levels (Loguercio et al. 2009b). Litter and pruning residues of shade trees may also play an

important role in soil pest and disease control through the improvement of the level of soil microbial activity, particularly that of antagonists (Wardle et al. 1995; Altieri 1999). In Papua New Guinea, leaf litter mulch has been reported to reduce the survival of *P. palmivora* under cacao trees by accelerating substrate decomposition and by stimulating the activity of antagonistic and hyperparasitic microbes (Konam and Guest 2002).

The cacao production system thus influences, through changes in (micro)climatic conditions and natural microbial assemblages, the efficacy of biocontrol treatments. The use of endophytes could partially sidestep some of the side effects of abiotic and biotic factors on the biological control agent (Backman and Sikora 2008).

(Micro)climate

Germination, growth and when applicable host tissue colonization have to occur in an environment prone to drying out and often with little available nutrients (Crozier et al. 2015). Temperature and relative humidity have been shown to affect both growth and biocontrol efficacy of *Trichoderma* species on aerial plant parts (Hannusch and Boland 1996a, b; Dik et al. 1999). Fluctuations in relative humidity, however, appear to have the greater effect on the biocontrol activity of *Trichoderma* species. In bioassays as well as glasshouse trials for the control of grey mould of cucumber caused by *Botrytis cinerea*, relative humidity affected efficacy of the *Trichoderma* biological control agents more than temperature (Elad et al. 1993; Dik et al. 1999), and this is thought to be related to conidial germination for which a period of wetness is required (Hjeljord and Tronsmo 1998). Furthermore, moisture affects nutrient availability, through solute transport and the presence of water films (Hjeljord and Tronsmo 1998). Hence, elucidating the effects of meteorological factors may provide a better understanding of field inconsistencies in biocontrol. Unravelling these effects may thus provide a starting point towards developing recommendations for the timing of BCA applications (Loguercio et al. 2009b).

Loguercio et al. (2009b) showed that sporulation of *T. stromaticum* isolates on brooms caused by *M. perniciosa* was consistently preceded by a specific set of environmental factors: reduced night-to-day temperature fluctuations, RH, broom moisture, zero wind and shading. They also observed that *T. stromaticum* isolates behaved differently along different tree heights in terms of sporulation biocontrol activity. The five tested isolates belong to two different genetic groups, and these results suggested isolate-specific responses to weather variation.

Begoude et al. (2007) in a study to evaluate in vitro the influence of environmental parameters, water activity a_w, temperature and pH, on the radial growth rate of four *Trichoderma asperellum* strains antagonistic to *P. megakarya* found that a_w was a crucial environmental factor. Under certain conditions, low a_w could prevent growth of *T. asperellum* strains. When these same strains were applied to cacao in the field, high levels of wash-off due to rain and fast desiccation of the conidia on

cacao pods limited their biocontrol efficacy (Deberdt et al. 2008). Most *Trichoderma* are thermally mesophilic and do not tolerate high osmotic potentials caused by drying conditions in the environment. This is particularly relevant when the pathogen is more adapted to water stress than the BCA, as e.g. in the case of *M. perniciosa* and its BCA *T. stromaticum* (Loguercio et al. 2009b).

The knowledge of weather effects on specific BCAs, combined with their specific biological requirements, can be used to improve biocontrol formulations. Understanding differential sensitivities of BCAs to meteorological factors could help to develop BCAs for characteristic crop microclimates, for example, full-sun and shade-grown cacao, and could help prepare for the foreseen changes in climate due to global warming (Loguercio et al. 2009b).

Microbial Communities

The presence of epiphytic and endophytic microbial communities could also hamper biocontrol efficacy. ten Hoopen et al. (2003), Krauss et al. (2006) and ten Hoopen (2007) showed that inundative applications of epiphytic BCAs were only capable of altering the microbial community composition on pod surfaces for limited periods of time, underlining the need for repeated applications to ensure that the selected BCA can exert some kind of disease control. De Souza et al. (2008) showed that endophytic colonization of sterile cacao seedlings was more successful than colonization of non-sterile seedlings, likely due to competition between the applied BCA and microbial communities already present in the non-sterile seedlings. Thus, competition between the many endophytic and endophytic microbes found on cacao trees will likely limit the ability of single BCAs to completely colonize a cacao tree (Bailey et al. 2006).

Some of the disappointing field results, after having obtained encouraging results in laboratory and greenhouse trials, which is often the case in biocontrol, can be linked back to the use of sterile seedlings and/or sterile soils during the process of BCA screening. Since in these trials BCAs do not have to compete with already present microbial communities, results of trials might be skewed in favour of good colonizers. In the field, however, good competitive qualities are equally important.

Spatial Arrangement

Preserving natural forest around agroforestry plots can be instrumental in natural biocontrol of plant diseases and is particularly common in systems with perennial plants and high microbial diversity (Allen et al. 1999). Natural forest surrounding agroforestry plots can lead to a reduction of disease by hosting naturally occurring biocontrol agents and by luring virus vectors away from the crop (Krauss 2004).

According to Thurston (1998), fragmented landscapes with a large interface between planted and spontaneous flora facilitate the immigration of biocontrol agents and reduce losses due to disease.

Endophyte transmission in woody perennials, such as cacao, is usually horizontal via water-dispersed spores (Carroll 1988; Arnold et al. 2003; Hanada et al. 2010), and infection depends strongly on the surrounding flora (Rodrigues et al. 1995; Arnold and Herre 2003). Arnold and Herre (2003) also emphasized the importance of closed (vs open), forest canopy on enhancing the rate of initial endophyte colonization of seedlings. However, according to Herre et al. (2007), these experiments confounded intact or open canopy cover with intact or absent leaf litter, respectively, endophyte accumulation being much more rapid in the presence of leaf litter. The end result of these interactions can be an extraordinarily high degree of endophyte diversity in tropical woody perennials (Arnold et al. 2000).

Similarly, wind patterns can have a dramatic effect: nonpathogenic epiphytes on orange leaves significantly increased when grown downwind from plant species other than citrus. Epiphyte populations were highest on the windward side of the orchard and declined with distance from the other plant species (Lindow and Andersen 1996). Such movement can be manipulated by the spatial arrangement of agroforestry species, e.g. wind breaks.

Biocontrol agents employed for the control of cacao diseases tend to be suspended and applied in water. Typically water-dispersed microorganisms can become wind-borne in the form of aerosols when the water droplets in which they are suspended evaporate (Fitt et al. 1989). Aerosol transport is more important in microbial spread on the landscape scale than rain splash because fine mists remain suspended in the air for longer periods of time and are thus subjected to more cumulative wind. Aerosols are particularly important in the upward movement of propagules. Fogs and aerosols are also more susceptible to interception by windbreaks than truly wind-dispersed microbes (Krauss 2004), which can limit their spread but also maintain applied inoculum in place.

According to Boa (2000), agroforesters should aim for a high degree of plant structural complexity (habitat diversity) and functional complexity (trophic links and nutrient cycling), in order to achieve the more complex and stable diversity which is required to reduce the risk from pests and diseases than merely to reduce the risk of crop failure. Thus, the surrounding plant biodiversity as well as their spatial arrangement may be pivotal in maintaining a high functional diversity of potentially useful plant-associated microorganism. Given the increasing interest in co-evolved pathogens, endophytes and antagonists for biocontrol of cacao diseases, conservation of host plant biodiversity in its natural habitat and research into the plant/microbe interactions are gaining importance with likely applications in agriculture and biotechnology (Krauss 2004).

17.5 Mechanisms of Biocontrol

Mycoparasitism, Antibiosis and Niche Exclusion

Mechanisms through which BCAs can control pathogens have been the object of a large number of studies (e.g. Harman and Kubicek 1998; Harman et al. 2004; Benitez et al. 2004; Hermosa et al. 2013). Numerous BCAs have the capability to directly mycoparasitize other fungi, including many cacao pathogens such as *M. perniciosa*, *M. roreri*, or *Phytophthora* spp. (Krauss and Soberanis 2001a; Tondje et al. 2007; de Souza et al. 2008; Sriwati et al. 2015). Competition for space and nutrients as well as antibiosis have also been described as control mechanisms of numerous BCAs. For example, *T. stromaticum*, *T. paucisporum* and *T. theobromicola*, isolated from the cacao canopy (some endophytically), as well as other *Trichoderma* spp. isolated from the soil (e.g. *T. virens*, *T. asperellum*, *T. harzianum*) produce a large number of antifungal compounds that can inhibit *M. perniciosa*, *M. roreri* or *Phytophthora* spp. (e.g. Sriwati et al. 2015; de Marco and Felix 2002; Aneja et al. 2005; Tondje et al. 2007; Pakora 2013). Nonanoic acid is a compound produced by the endophyte *T. harzianum* which has strong in vitro inhibitory effects against *M. roreri* and *P. perniciosa* (Aneja et al. 2005). Seedlings inoculated with this endophyte possess this chemical, whereas uninoculated seedlings do not (Herre et al. 2007). These are general non-specific mechanisms that directly affect pathogens. For more details, we refer to, e.g. Harman and Kubicek (1998), Harman et al. (2004), Pakora (2013) and references therein. Unfortunately, much of this mechanistic research is carried out on nutrient-rich laboratory media that are not representative of what is likely to happen on or in cacao pods and should thus be interpreted with caution.

Indirect control mechanisms include plant growth stimulation, increased stress tolerance and induced resistance which make the plant better able to cope with the effects of pathogen attacks. Plant growth is induced in certain *Trichoderma*–plant interactions (see Harman et al. 2004; de Souza et al. 2008; Hermosa et al. 2013). In the case of cacao, *T. stromaticum* and *T. asperellum* seem to stimulate the formation of active flower cushions (Tondje et al. 2007; Medeiros et al. 2010). However, how is yet unclear. An endophytic *T. hamatum* isolate (DIS219b) is capable of enhancing cacao seedling growth and augmenting their tolerance to drought stress (Bae et al. 2009; Bailey et al. 2011). Tchameni et al. (2011, 2012) showed the positive effects of a *T. asperellum* and mycorrhizal fungi on cacao growth and resistance to *P. megakarya*, linking increased resistance to increased amino acid synthesis.

Again these mechanisms are non-specific and may occur in many BCA–plant interactions (Harman et al. 2004; Druzhinina et al. 2011; Hermosa et al. 2013). Yet it is these mechanisms that are currently arousing a lot of interest in the scientific community working on the biological control of cacao diseases and important advances in our understanding of the how of these mechanisms come from their work.

Growth Enhancement, Stress Tolerance and Induced Resistance

As mentioned before, pathogens and beneficial endophytic organisms are confronted with the innate immune systems of the plant. Colonization success essentially depends on the evolution of strategies for immune evasion (Pozo et al. 2013; Hermosa et al. 2013). Besides their primary innate immune response to pathogen attacks, plants can activate a second line of defence, referred to as induced resistance. Induced resistance is defined here as the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or abiotic agents (inducing agents) (Kloepper et al. 1992). This type of resistance often acts systematically throughout the plant and can be effective against a broad spectrum of attackers. Induced resistance can be broadly divided into two types: systemic acquired resistance (SAR) and induced systemic resistance (ISR).

SAR is commonly induced by certain chemicals and biotrophic and avirulent forms of pathogens (Durrant and Dong 2004; Hermosa et al. 2013). This mechanism involves the accumulation of salicylic acid (SA) and pathogenesis-related proteins (PRs) (Ryals et al. 1996). ISR is induced by pathogens with a necrotrophic lifestyle and that are commonly deterred by jasmonates. A third type of induced resistance is rhizobacteria–ISR although the process through which rhizobacteria induce resistance is quite different from pathogen–ISR and SA and PR proteins are not involved (Van Loon et al. 1998; Harman et al. 2004; Hermosa et al. 2013). *Trichoderma* can induce resistance and PR proteins seem to be involved [chitinase, peroxidase, glucanase and cellulase (Yedidia et al. 1999, 2000)]. There is considerable crosstalk between these mechanisms which often results in reciprocal antagonism.

The induction of resistance in plants by *Trichoderma* spp., as a way to manage pathogen attacks, has received less attention than, for example, the responses induced by rhizobacteria. According to Harman et al. (2004), this may be because much research focused on direct effects such as antibiosis and mycoparasitism. However, recent studies have started to elucidate the mechanisms through which endophytic *Trichoderma* spp. can contribute to plant growth and defence against abiotic and biotic stresses.

In a study by Bailey et al. (2006), it was shown that during the colonization of cacao seedlings by four endophytic isolates of *Trichoderma*, isolate-specific gene expression induction patterns occurred. Several of the cacao expressed sequence tags (ESTs) induced due to colonization share homology with genes reported to function in plant responses to environmental stresses, including plant symbiont and plant pathogen interactions. Interestingly, one of these ESTs, P31, seems closely related to the major intrinsic protein family (MIP), of which some are active in selective water transport. The repression of MIP gene expression may reduce water permeability and encourage water conservation during droughts. Enhanced drought tolerance has been observed in endophyte-colonized cacao (Bae et al. 2009; Bailey et al. 2011) and has been demonstrated with root-colonizing *T. harzianum* isolate T-

22 in maize (Harman et al. 2004). Bailey et al. (2006) also found altered fungal gene expression during seedling colonization by *Trichoderma*. Some of the identified fungal ESTs seem to be of importance in the establishment of endophytic associations. Several other preferentially expressed genes seem to influence the biocontrol capacity of the *Trichoderma* isolates. The patterns of altered gene expression in cacao and *Trichoderma* suggest a communication system involving genetic crosstalk, resulting in an endophytic association that seems to be able to prime the plant for subsequent pathogen attacks (Bailey et al. 2006). The advantage of primed plants is that they do not express costly defence responses until recognition of a potential intruder.

Mejía et al. (2014) showed that *Colletotrichum tropicale*, routinely the dominant species found in foliar endophytic fungal communities that establish in healthy cacao leaves (Rojas et al. 2010), can exert profound influences on host gene expression related to changes in many aspects of the hosts' physiology, metabolism, anatomy and, most interestingly, resistance to pathogens. In the case of *C. tropicale*, enhanced resistance to pathogen damage resulted through up-regulated increased host gene expression and was not due to any direct endophyte–pathogen interaction or through any chemical compound produced by *C. tropicale*. Yet the endophytic colonization also entails costs for the host, e.g. reduced photosynthetic capacity, although these may be offset when pathogen and herbivore pressures increase (Mejía et al. 2014). For more information on the mechanisms through which *Trichoderma* spp. and plant interact, we refer to Harman et al. (2004), Hermosa et al. (2013) and references therein.

Based on the before-mentioned, it seems that endophytes can employ a wider range of mechanisms that can reduce the impact of cacao diseases compared with epiphytes. Moreover, since endophytes could become permanently established in the interior of the plant, they potentially have a larger opportunity to exercise biocontrol activity. This strongly argues in favour of using endophytic BCAs for control of cacao diseases.

17.6 Plant and Microbial Extracts, Natural Products

Although not considered biological control, since no living organisms are employed, the use of plant and microbial extracts as well as other natural products is discussed here since, similar to biological control, these are considered environmentally friendly with supposedly minimal negative externalities.

Very little work on the use of plant extracts to control cacao diseases has been done. In a study by Sonwa et al. (2002), it was shown that farmers in Cameroon use plant extracts together with either insecticides or fungicides since they believe that these plant extracts increase effectiveness of the pesticides. Coulibaly et al. (2002) identified extracts from several plant species that are commonly being used either alone or mixed with other plant extracts and/or fungicides. Among them are *Cannabis sativa*, *Erythrophleum ivorense*, *Guibourtia tessmannii* and *Nicotiana*

tabacum. Extracts are collected, among other tissues, from bark and roots, and according to farmers, their effectiveness depends on the number of species used, diversified species mixtures supposedly being more effective. Unfortunately, in neither of these cases, verification of the efficacy of these plant extracts to control pests and diseases has been undertaken.

As far as we are aware, the only field experimentation using a plant extract, in this case oil extracted from neem seeds, is that of Pohe and Agneroh (2013). They demonstrated that neem seed oil at doses of 10–15 l ha^{-1} seems to be as efficient as the recommended fungicide for control of black pod due to *P. megakarya* in Ivory Coast. Similar work is currently being conducted in Cameroon.

Widmer and Laurent (2006) showed that rosemary and lavender extracts reduced germination of *P. megakarya*, *P. capsici* and *P. palmivora*, with *P. megakarya* being most sensitive to these plant extracts. The active compounds were caffeic acid and rosmarinic acid or some simple derivative thereof. Synthetic caffeic and rosmarinic acid completely inhibited zoospore germination of these three species at doses of 3 and 6 g l^{-1}. In contrast, these acids did not inhibit conidial germination of *T. asperellum* spores, and thus these extracts could provide an alternative to chemical fungicides in reducing damage by cacao black pod disease and could possibly be used in combination with a biological control agent. Ambang et al. (2010) studied the effect of yellow oleander (*Thevetia peruviana*) seed extracts on in vitro growth of *P. megakarya*. Their findings demonstrated that crude extracts could have fungicidal as well as fungistatic effects depending on the dose applied and the *P. megakarya* isolate being tested. Glycerol, and particularly its in vivo derivative glycerol-3-phosphate, plays an important role in plant defensive responses (Zhang et al. 2014). Glycerol is an environmentally friendly, non-toxic, edible and biodegradable sugar alcohol. When applied to leaves, it can stimulate short-term plant growth and has been recognized as being capable of inducing systemic acquired resistance. Under laboratory conditions, exogenous application to cacao leaves decreased lesion formation by *P. capsici* significantly (Zhang et al. 2014).

Although there are some promising results, the use of plant or other environmentally friendly extracts or products for the control of cacao diseases has to date not received sufficient attention. None of the proposed products, except neem seed oil, has been tested under field conditions. If plant extracts or other environmentally products are to be integrated in an IPM scheme, more research will be necessary. Furthermore, these extracts will face all the challenges (variability, lack of rainfastness, systemic action or spread over growing pod, etc.) that limit the cost-effectiveness of chemical fungicides to a similar or even greater extent.

17.7 Production, Formulation and Application of Biopesticides

Contrary to fungicides, biocontrol agents are capable of actively colonizing, epiphytically or endophytically, plant tissues in the rhizosphere and phyllosphere. The rapid increase in pod surface area at certain stages of pod development (ten Hoopen et al. 2012), considered to be a constraint for effective chemical control, should not influence control if BCAs are capable of establishing themselves and actively grow on or in the expanding plant tissues.

Three key aspects have a major influence on the efficacy of a BCA: inoculum quality, which is tightly linked with production methods, formulation and delivery.

Inoculum Production

As mentioned previously, Najár and Thomas (2001) produced effective microorganisms (EM) in an anaerobic liquid culture in the presence of molasses, alcohol and/or vinegar, and as with all enrichment cultures, the outcome is variable, with limited control by the researcher.

The production of many fungal biocontrol candidates has often been achieved by using nutrient-enriched, aerobic solid-state fermentation. *Trichoderma* spp. and other endophytes are generally produced on rice or Biodac®, a granular cellulose complex (e.g. Krauss et al. 2010; Mejía et al. 2008). The former can be extracted with a MycoHarvester[3] to yield a relatively stable powder consisting of dry spores with similar size. The mass production of *C. rosea* was markedly improved by a two-step liquid-/solid-state fermentation: Guata, an inert polyester fibre, was coated with a nutrient solution and inoculated with a starter culture from a liquid culture (Krauss et al. 2002). From these low-weight fibrous mats, spores can be extracted by washing directly in the field (Krauss et al. 2006) or at the research station for overnight refrigerated storage (Hidalgo et al. 2003).

Formulation

Formulations can facilitate the attachment of conidia to plant surfaces (Carver and Gurr 2006 and references therein) and promote germination and subsequent colonization of plant tissues. Bateman and Alves (2000) recommend oil-based formulations for conidial biological control formulations because of their greater ability to adhere to the substrate. Moreover, such formulations slow down the desiccation

[3] (http://www.dropdata.net/mycoharvester/).

process under fluctuating environmental conditions. Formulation trials have considered stickers, oil adjuvants (Hidalgo et al. 2003; Krauss et al. 2010; Mbarga et al. 2014; Crozier et al. 2015) and nutrient amendments (Krauss et al. 2006; Hanada et al. 2009; Mbarga et al. 2014; Crozier et al. 2015). Nutrient sources e.g., are known to promote germination and growth of *Trichoderma* (Crozier et al. 2015).

Stickers were somewhat beneficial in combination with systemic agents (chemical as well as microbial, i.e. endophytes) for FPR control (Krauss et al. 2010). However, BCAs interacted with additives, and it is not yet possible to draw firm conclusions. Tondje et al. (2007) used a cassava flour formulation of *T. asperellum* which reduced black pod disease in Cameroon compared with a water only control (Deberdt et al. 2008) although not always consistently (unpublished results). However, the independent effects of cassava flour or the *Trichoderma* spores were not determined. Sucrose (2 %) and soybean oil (2 %) in formulations with *T. martiale* did not improve *P. palmivora* control (Hanada et al. 2009). Molasses amendments to biocontrol mixtures in Panama improved yield but did not alter FPR incidence. The colonization of pods by mycoparasitic BCAs was also unaltered by molasses, leaving the question of operational mechanism for yield enhancement unanswered (Krauss et al. 2006). A reason for the somewhat mitigated results of nutrient additions to formulations could be that these could also favour the pathogen. Macagnan et al. (2006) even hypothesized that their formulations were responsible for reducing the effectiveness of applied actinomycetes for *M. perniciosa* control. However, results so far do not seem to confirm these ideas. Crozier et al. (2015) did not observe an increase in disease for any of the formulations used. Krauss et al. (2006) even found that molasses-only treatments reduced disease losses and increased yield compared with an absolute control. It was hypothesized that molasses increased the efficacy of native antagonists.

As far as we are aware, until recently, invert or reverse emulsions (of the water in oil type), are the only type of oil-based formulations of *Trichoderma* spp. conidia to have been used so far. Recent work from Costa Rica, where a nutrient-amended invert corn oil emulsion was used to formulate endophytic *Trichoderma* spp. for FPR control, has shown promising results over two full cacao production seasons (Crozier et al. 2015). The formulation containing the endophytic *T. ovalisporum* TK1 recorded a record high of 30.7 % healthy pods (Crozier et al. 2015).

Recent trials in Cameroon with nutrient-amended oil formulations for *T. asperellum* PR11 showed that a soy-bean oil formulation with 4 % glucose, increased shelf life of the conidia and when applied to pods in the field demonstrated significant protection against *P. megakarya* when evaluated under laboratory conditions (Mbarga et al. 2014). This oil formulation offers an important advantage compared with reverse emulsions: it is better adapted to side lever knapsack (SLK) sprayers, which are the sprayers most commonly used by small-scale farmers (Bateman et al. 2005a).

Delivery and Timing

Delivery of BCAs to their biological target has two major components, the application method being used and timing of applications.

A study by Hidalgo et al. (2003) compared motorized mistblowers fitted with rotary atomisers and hydraulic sprayers fitted with D2-45 hollow cone nozzles for the control of FPR using BCAs and a copper-based fungicide. Their overall finding was that directional hydraulic sprays were somewhat superior to the motorized mistblower technique. A follow-up study showed that best yields were achieved with sprays that maximize deposits on pods (Bateman et al. 2005a). Both trials, however, showed that agents interacted with sprayers (Bateman et al. 2005a; Hidalgo et al. 2003).

For the targeted delivery of BCAs to cacao pods, the only plant parts infected by *M. roreri*, nozzle configuration of hydraulic sprayers had the largest impact. Using tracer solutions as a model, the combinations DC31-25 and D1.5-25 (both at 200 kPa) that produce spray angles of approximately 25° and 35°, respectively, achieved substantially better deposits on pods per volume emitted than a D2-45 (at 300 kPa) that gave a 40° cone of spray which is more commonly used by cacao farmers (Bateman 2004). It is important to know that formulation can also affect droplet deposition (Crozier et al. 2015), and additional research is needed to better understand the interactions between BCAs, their formulation and application. In the case of cacao root diseases, formulation and application methods of BCAs are still unsatisfactory and very poorly investigated (ten Hoopen and Krauss 2006).

Correct timing of applications is equally or even more important than application means. Correct timing of applications will maximize their benefits and reduce costs (Bateman et al. 2005b). In the case of inundative biocontrol, repeated applications are a necessity. Krauss and Soberanis (2002) found that an adjusted BCA-application regime of three applications, one at the onset of flowering and an additional two applications 8 and 10 weeks later, was superior to three applications in regular intervals and was comparable with five monthly and ten biweekly applications for both percentage and number of healthy pods.

In the case of classical biological control, the idea is that the BCA becomes permanently established and provides long-term disease control. Establishment can be the bottleneck with this approach. Endophytes, theoretically, could permanently establish themselves in cacao trees and through, e.g. induced resistance confer permanent disease control. However, if control relies on/or includes mechanisms such as direct competition, antibiosis and mycoparasitism, the endophyte has to be in close proximity to the pathogen. If endophytes become permanently established in the trunk and braches, they could directly interact with pathogens such as CSSV and/or VSD. Yet in the case of FPR, BPD and WB, which for a large part attack cacao pods, the endophyte, in order to be close to the pathogen, should be able to colonize newly formed pods. If so, one or a few applications of the BCA at the seedling stage or later on in the field will suffice to confer resistance to the tree. If endophytes already present in the tree are not capable or are poor colonizers of

newly formed pods, repeated pod-directed applications, at least once yearly, will be necessary to manage diseases. A single application of TK1 conidia in water or with the sticker NP-7 showed initially 80–100 % pod colonization, yet these dropped to 20–50 % after 3 weeks and 10–20 % after 7 weeks (Krauss et al. 2010). Hanada et al. (2008, 2009) observed that after a single application of *T. martiale*, spore viability decreased 70 % within ten days, but pod colonization remained near 90 % for up to 40 days after which control decreased. These results indeed seem to indicate that in the case of pod pathogens, repeated applications of endophytes are a necessity.

In the case of endophytes, inoculation of cacao seedlings has shown that colonization success is dependent upon time and opportunity, species and even species isolate used as well as inoculation methods (e.g. Bailey et al. 2008; Rosmana et al. 2015). The observed failures of certain *Trichoderma* isolates to colonize growing points will limit subsequent colonization of the growing plant and over time minimize the presence of the endophytic *Trichoderma* and thus the capacity to exercise disease control (Bailey et al. 2006).

Adverse Effects

Biological control agents are perceived to be less harmful than synthetic fungicides, having fewer nontarget and environmental effects, to be efficient against fungicide-resistant pathogens and to be at lower risk of resistance development. Moreover, they can be used in farming systems where the use of synthetic fungicides is restricted (Brimner and Boland 2003). Although interactions between BCAs and pathogens are generally beneficial from a disease management point of view, the organisms involved may have detrimental impacts on other organisms in the systems where they are applied. The potential nontarget effects of BCAs include competitive displacement, allergenicity, toxicity and pathogenicity (Cook et al. 1996). Competitive displacement occurs when a BCA expels or replaces native nontarget species through competition for space or nutrients. Allergenicity may occur in humans and animals that develop sensitivity to spores or formulations of the BCAs. The release of antibiotics or alkaloids, which may aid in disease suppression, may have toxic effects on nontarget species. The potential of a BCA to infect or destroy organisms other than the target pest or disease is of most concern from a risk management perspective (Brimner and Boland 2003). As mentioned before, certain BCAs and endophytes are related to pathogens or hypovirulent pathogens (Carroll 1988) which could present a risk concerning the possibility of gene transfer.

There are numerous examples of nontarget effects for the use of biocontrol fungi (see, e.g. Brimner and Boland 2003). However, in cacao, examples are scarce. One of the possible adverse effects of BCA applications is competitive displacement. Inundative applications of four bacteria on cacao did not significantly shift the microbial community of either bacilli or total eubacteria (Melnick et al. 2011).

These findings are in line with the work of ten Hoopen (2007) who found that applications of an epiphytic antagonist (*Clonostachys byssicola*) did not seem to significantly alter the microbial population structure on the surface of treated pods. Moreover, as mentioned before, ten Hoopen et al. (2003) and Krauss et al. (2006) demonstrated that even though inundative applications of epiphytic BCAs are capable of altering the microbial community composition on pod surfaces, these quickly return to their original state.

However, simultaneous inoculation of soil with *T. asperellum* PR11 and two VAM did not always positively benefit cacao seedlings compared with plants inoculated with either *T. asperellum* or VAM only, suggesting some kind of negative interaction between the two. It has to be noted though that the ability to impede mycorrhizal colonization of plant roots may be dependent on the order of inoculation. In one case, when plants were inoculated with the mycorrhiza 2 weeks before BCA inoculation, no apparent effect on colonization was observed (Brimner and Boland 2003).

In contrast to chemical plant disease control, there has never been a report of resistance developing to biocontrol. This statement assumes that genetically modified plants with a gene from a BCA no longer constitute biological control. Moreover, GMO plants that constitutively express the Bt toxin have triggered the development of Bt-resistant insects (Bagla 2010).

For a more general overview of the potential risks of biological control, we refer to Hokkanen and Lynch (1995) and Brimner and Boland (2003).

17.8 Biological Control of *Phytophthora* spp.

Black pod rot is caused by several *Phytophthora* species. The species concerned are *Phytophthora palmivora*, which has a global distribution; *P. megakarya*, which is restricted to West and Central Africa and is now the single most important cacao pathogen in this region (Mfegue 2012; Opoku et al. 2000; Chap. 8); and *P. tropicalis* (Aragaki and Uchida 2001) and *P. capsici*, common in Central and South America (Guest 2007). It has to be noted, however, that the exact relationship between *P. tropicalis* and *P. capsici* has not yet been clarified and possibly all isolates previously considered *P. capsici* may in fact be *P. tropicalis* (see also Chap. 7). Several other species have been described to cause black pod, such as *P. hevea*, *P. citrophthora*, *P. megasperma*, and *P. katsurae*, yet these are of less importance (Chap. 7).

Biological control of black pod has probably received most attention in Cameroon, where *P. megakarya* can cause substantial pod loss when no control measures are in place (e.g. Nyassé et al. 1999; Ndoumbe-Nkeng et al. 2004; Mfegue 2012). Black pod biocontrol is also starting to attract interest in other African countries as evidenced by BCA screening efforts in Ivory Coast (Kebe et al. 2009; Mpika et al. 2009), Ghana (Akrofi et al. 2012; Koranteng and Awuah 2011) and Nigeria (Adedeji et al. 2008; Adebola and Amadi 2010, 2012). However, field assays

have been primarily reported upon in Cameroon (Tondje et al. 2007; Deberdt et al. 2008; ten Hoopen et al. 2010a) and Nigeria (Agbeniyi et al. 2014). Nonetheless, black pod remains a worldwide problem and efforts to control it using BCAs have also been undertaken in North, Central and South America (Krauss et al. 2006; Hanada et al. 2009; Cuervo-Parra et al. 2014) and have started to rouse interest in Asia (Sriwati et al. 2015).

In Cameroon, through a USDA-funded project, research towards the development of biological control options of cacao black pod disease started at the end of the 1990s and continues until this day. Early work focused on the use of *Geniculosporium* species, commonly found as endophytes of tree species, and the form genus for the anamorphs of several Hypoxylon species (Tondje et al. 2006, and references therein). Tondje et al. (2006) found that all *Geniculosporium* strains tested exhibited some degree of antagonism against *P. megakarya*, although none of the strains could completely prevent cacao pod husks from infection by *P. megakarya*. Subsequently, attention shifted towards the use of *Trichoderma* spp. because of successes elsewhere, its well-known abilities as a biocontrol agent and the findings that cacao harbours endophytic *Trichodermas*.

Tondje et al. (2007) described the isolation, identification and screening process of several *T. asperellum* isolates with great potential for biocontrol of black pod in Cameroon and reported that *T. asperellum* exhibited mycoparasitic activities on *P. capsici*, *P. citrophthora*, and *P. palmivora*. Furthermore, culture filtrates of the *Trichoderma* isolate showed substantial laminarinase and cellulose activities, the two enzymes that may adversely affect the cell walls of *Phytophthora* (Tondje et al. 2007). Deberdt et al. (2008) showed that one of these isolates, PR11 formulated as wettable powder using cassava flour as carrier, performed well under field conditions reducing black pod incidence by about 33 % the first year of field trials and by almost 66 % the following year. A carry-over effect was noticed during the third year of observations. Subsequent trials however, showed more variable results (ten Hoopen et al. 2010a and unpublished data). It was hypothesized that the formulation being used was partly to blame, due to a lack of germination of the *T. asperellum* spores. A recent study by Mbarga et al. (2014) focused on the development of a novel oil-based formulation to increase control efficacy and to reduce variability in control results. Their results seemed to indicate that the formulation has a twofold effect. The constituents of the formulation seem to create a protective barrier around the cacao pod, partly inhibiting infection by *Phytophthora*, and seem to protect the *Trichoderma* spores against microclimatic variability and to improve germination.

In subsequent field trials, results effectively demonstrated that a blanco formulation (without PR11) as well as a pure spore suspension could reduce black pod rot incidence. Yet what was most interesting was that the complete formulation was as good as the copper fungicide control treatment, which showed the synergistic effects between *T. asperellum* PR11 and the formulation (G. M. ten Hoopen, personal communication).

In Latin America, where black pod is oftentimes found in combination with frosty pod rot and/or witches' broom, black pod disease is often of secondary

concern (Hidalgo et al. 2003; Krauss et al. 2010) yet not always (Krauss et al. 2006). However, biological control efforts towards controlling WB and/or FPR can also have an effect on black pod disease. The effect of three endophytic fungi, *Colletotrichum gloeosporioides*, *C. rosea* and *Botryosphaeria ribis*, on pod loss due to *M. roreri* and *Phytophthora* species was assessed in Panama. The result showed a significant decline in losses due to Phytophthora pod rot from treatment with *C. gloeosporioides* and reduced incidence of sporulating lesions by *M. roreri* after treatment with *C. rosea* (Mejía et al. 2008).

Hanada et al. (2009), using the endophytic *Trichoderma martiale* strain ALF247, demonstrated its capacity to reduce black pod disease severity under field conditions. This effect was more pronounced with increasing concentration of *T. martiale* spores but was not affected by the composition of the conidial suspension. However, even at the highest concentration (5×10^7 conidia ml^{-1}), disease symptoms were still prevalent on inoculated pods. Together with the fact that the number of remaining viable conidia on the pod surface decreased sharply after only 5–10 days, although biocontrol effects could be observed up to 30 days, this suggests that repeated applications are a necessity. Moreover, this trial did not reflect actual farmer field conditions, and the results should thus be interpreted cautiously.

17.9 Biological Control of *Moniliophthora roreri*

Frosty pod rot (FPR) of cacao is caused by the hemibiotrophic basidiomycete *Moniliophthora roreri* that results in yield losses of around 70–80 % in most areas within a few years of pathogen establishment and can lead to complete crop failure (Krauss et al. 2003; Chaps. 3 and 4). Spores of *M. roreri* germinate and penetrate the pod surface soon after landing there and establish a systemic pod infection, which can be latent for 2 months (Evans 1981b). During the essentially non-symptomatic phase, the seed chamber, where the actual cacao beans normally mature, is completely destroyed. Only then does the pathogen emerge to the pod surface, where it causes brown lesions, followed by rapid and prolific sporulation within 1 week (Leach et al. 2002). The fact that much of the pathogen development and damage takes place deep inside an intact pod poses significant challenges to FPR management by any agent that requires spatial proximity for effective control. In consideration of the long latency period of *M. roreri* (Chap. 3), this section only considers research with a field phase of duration not less than 4 months. This criterion eliminated a substantial number of papers on microbial and plant extracts tested in vitro.

Cultural control has proven to be the central pillar of any integrated control strategy for FPR in afflicted countries. Disease-resistant germplasm (Phillips-Mora et al. 2005, 2012; Schnell et al. 2007) and chemical and biological agents (Bateman et al. 2005a, undated; Hidalgo et al. 2003; Krauss and Soberanis 2001b, 2002; Krauss et al. 2003, 2010; Torres de la Cruz et al. 2013) are being developed as supplementary management options. Biological control of *Moniliophthora* spp.

efforts can be divided into two fundamental biocontrol strategies: classical and inundative biocontrol.

Inundative approaches have been used in Peru, Panama and Costa Rica with varying results (Krauss and Soberanis 2001a, b, 2002; Hidalgo et al. 2003; Bateman et al. 2005a; Krauss et al. 2006). In Peru native *C. rosea* reduced FPR by some 15–25 % under field conditions. Mixtures of local antagonists controlled three cacao pod diseases (FPR, witches' broom and black pod) simultaneous, leading to yield increases of up to 16.7 % (Krauss and Soberanis 2001a, 2002). Increasing application frequency improved control of FPR and WB yet concomitantly increases costs (Krauss and Soberanis 2002). However, biocontrol was still most economical compared with cultural or chemical control alone. Fertilization increased the percentage of healthy pods as well as yield yet reduced the benefit–cost ratio. No interaction between biocontrol and fertilization was observed, which means both techniques can be applied independently of each other (Krauss and Soberanis 2002). Field trials in Costa Rica using a single strain and a mixture of locally isolated BCAs did not significantly reduce disease incidence (Hidalgo et al. 2003). Follow-up trials in Costa Rica using the Peruvian BC isolates once again did not significantly reduce FPR incidence (Bateman et al. 2005a). Trials in Panama, using mixtures of local BCAs and one Peruvian *T. asperellum*, strain Tr-4, did show significant reductions in the number of sporulating pods, yet with regard to disease incidence, data were less conclusive (Krauss et al. 2006).

Due to the unsatisfactory results with the inundative approach and based on positive results from two trials (Evans et al. 2003b; Holmes et al. 2006), attention shifted towards the use of the endophytic *T. ovalisporum* TK1. In 2003, a 3-year full-scale field trial was set up in Costa Rica. TK1 was applied either in water, with a sticker or adjuvant, or in sequential combinations with fungicides (Krauss et al. 2010). In the final year of trials, TK1 improved healthy pod yields (absolute and relative). This was the first of the Costa Rican La Lola trials (Hidalgo et al. 2003; Bateman et al. 2005a) in which the BCA achieved a significant yield improvement. Since there were indications that the formulations used had an effect on biocontrol efficacy, follow-up trials focusing on formulation improvement were prioritized. Crozier et al. (2015) effectively showed that averaged over 2 years *T. ovalisporum* TK1 formulated in an invert 50 % v/v corn oil, 2.5 % v/v lecithin and 1 % w/v potato dextrose broth increased yield to 30.7 % healthy pods compared with 9.7 % in the water control.

Mejía et al. (2008) used cacao endophytes, isolated from leaves of cacao in a native Panamanian forest. While no FPR reduction was recorded in a 7-month trial with single spray inoculations, for one isolate of *C. rosea*, sporulation of *M. roreri* was significantly reduced. For the reasons outlined above, one would expect the inoculative biocontrol approach to work best if isolates that have co-evolved with the pathogen are used, which was not the case here.

17.10 Biological Control of *Moniliophthora perniciosa*

One of the most well-known cases of biological control of a cacao disease is the case of witches' broom (*Moniliophthora perniciosa*, Chaps. 5 and 6). Various articles have been published on the potential use of biocontrol fungi against witches' broom disease (Costa and Bezerra 1994; Costa et al. 1996; Bastos 1988, 1996a, b, 2012; Krauss and Soberanis 2001b; Holmes et al. 2004; Rubini et al. 2005; Pomella et al. 2007; de Souza et al. 2008; Loguercio et al. 2009a, b). Several of these articles focus on *T. stromaticum* which is an endophyte of cacao, cupuassu (*T. grandiflorum*) and *Herrania* spp. (de Souza et al. 2006). This species is an effective mycoparasite of *M. perniciosa* (de Souza et al. 2008). Dead brooms and old infected pods are colonized by *T. stromaticum* and cause a reduction in basidiocarp production and consequently inoculum levels due to accelerated decomposition of dead infected tissues (see de Souza et al. 2008). *Trichoderma stromaticum* has been used on an experimental basis as of 1999 (de Souza et al. 2008) as part of the integrated management of cacao.

Two genetic groups (groups I and II) exist within *T. stromaticum* with different capabilities in controlling witches' broom and colonizing *T. cacao*. Only isolates of group II have been found endophytically in cacao and closely related species in nature (de Souza et al. 2006). In vivo and field trials showed that *T. stromaticum* is capable of colonizing cacao trees and seedlings endophytically yet does not seem to induce plant growth or resistance to *M. perniciosa* (de Souza et al. 2006). In the field, tree colonization was observed in trunk samples 30 days after spraying 300 ml per tree of spore suspensions containing 10^7 spores ml^{-1}. Yet after 120 days, only *T. stromaticum* isolates belonging to genetic group II were recovered (de Souza et al. 2008). This again shows that colonization capacity is isolate specific and that great care has to be taken when extrapolating observations on the capabilities of biocontrol agents. Bailey et al. (2008) found that *T. stromaticum* isolate DIS 185c was an inefficient colonizer of cacao.

In a 3-year field trial, Medeiros et al. (2010) showed that alternating treatments with copper hydroxide and *T. stromaticum* reduced vegetative broom formation and yielded consistently higher number of pods per tree by increasing the number of pod-forming flower cushions. Yet *T. stromaticum* alone did not seem to protect the pods from witches' broom disease (Medeiros et al. 2010). This combination treatment fungicide/BCA also significantly reduced witches' broom incidence on pods. Estimated dry bean yield was also highest for the combination treatment. Alternating copper applications with biocontrol sprays without the use of frequent broom removal reduced disease incidence by 50–60 %, increased total pod yield per tree, estimated bean yield per ha by 50–100 % and considerably increased, by 100–300 %, estimated healthy bean yield equivalents. However, Medeiros et al. (2010) point out that this disease management strategy should go hand in hand with fertilizer applications to assure the increased yield. Especially in abandoned or neglected cacao plantations, disease management strategies that go hand in hand

with fertilizer application seem to be the way to assure economically viable pod yields (Krauss and Soberanis 2001b; Medeiros et al. 2010).

17.11 Biological Control of Other Cacao Diseases

Little to nothing has been done on biological control of most other cacao diseases presented in this book. A thorough search of the literature only provided a few references regarding biological control of Rosellinia root rot, CSSV and VSD and one on control of cacao leaf anthracnose caused by *Colletotrichum gloeosporioides* using chitinolytic bacteria.

Rosellinia *spp.*

Rosellinia root rot of cacao is a soilborne disease caused by two species, *Rosellinia pepo* and *R. bunodes*. Unfortunately, only a few studies have focused on biological control of *Rosellinia* spp. in cacao. Among the fungal control genera investigated, *Trichoderma* species have received most of the attention for their biocontrol potential against *R. bunodes* (Ruiz and Leguizamón 1996; Castro 1995).

Mendoza-Garcia et al. (2003) showed that mixtures of *Clonostachys* and *Trichoderma* can be effective against *R. bunodes* in the greenhouse. However, the effectiveness depended on soil pH and the amount of soil organic matter. The authors concluded that in order to obtain efficient control, application of mixed BCAs should be accompanied by liming to increase the soil pH and by the removal of organic material. Similar observations regarding organic matter content and biocontrol with *Trichoderma* spp. were made in coffee (Ruiz and Leguizamón 1996).

Similar work as done by ten Hoopen et al. (2003) showed that mycoparasite populations in the rhizosphere of cacao are independent of the germplasm, which means that genetic resistance and biological control can have additive effects (Argyle et al. 2003).

Even though the few studies that focused on biological control of Rosellinia root disease in cacao showed promising results, verification of the potential of these BCAs under field conditions remains to be done. To date we are only aware of one field trial, in which *Trichoderma koningii* was applied to control *R. pepo* in a cacao plantation. This study did not, however, show any disease control. Curiously, control in vitro by *T. koningii* was proportional to the amount of the antagonist applied, whereas in the field, the lowest tree mortality was obtained with the lowest application dose and highest tree mortality with the highest application dose of *T. koningii* (Merchán 1993). For a more complete overview of biological control of *Rosellinia* spp., we refer to ten Hoopen and Krauss (2006).

Vascular Streak Dieback

To date there are no effective control measures for VSD. Yet given that *Ceratobasidium theobromae*, the causal agent, is a systemic pathogen and infection hyphae are located within the xylem vessels of stems and leaves, the use of endophytic biocontrol agents might provide some level of protection to the disease. The first article, and thus far the only one, looking at the use of endophytic *Trichoderma* strains for the control of VSD was published in 2015 (Rosmana et al. 2015). They showed that cacao seedlings inoculated though roots with four different *Trichoderma asperellum* isolates from pods and/or sapwood could resist artificial leaf inoculation with *C. theobromae*. Interestingly, the authors were not able to recover *T. asperellum* from the leaves even though the roots were colonized, which would indicate that these isolates stimulated resistance in above-ground plant parts. This agrees with yet unpublished findings of Vanhove et al., who used *T. asperellum* and three *Enterobacter* spp., which had been selected for their ability to induce systemic resistance in horticultural crops in temperate areas, to inoculate cacao roots by watering the seedlings. After 8 months, the number of VSD-affected leaves was significantly reduced.

Epiphytic application of the commercial *Trichoderma harzianum* T-22 to leaves of mature cacao in a plantation in Malaysia with naturally high VSD disease pressure led to a reduced number of leaves exhibiting VSD symptoms compared with the control after 3 months (Vanhove et al. unpublished). When plants previously infected with *C. theobromae* were inoculated with *T. asperellum* through connections at the site of shoot grafts, VSD symptoms were significantly reduced (Rosmana et al. 2015). In this case, the mode of action seemed to be through direct mycoparasitism or antibiosis. These encouraging results once again demonstrate the potential of biological control for cacao diseases. Currently, the most promising of the four *T. asperellum* strains is being tested under field conditions in Sulawesi (Rosmana et al. 2015).

Cacao Swollen Shoot Virus Disease

This disease, contrary to most other diseases discussed in the chapter, which are fungal or straminipilous in origin, is caused by a virus. CSSV is a *Badnavirus* and is semi-persistently transmitted to cacao trees through the feeding actions of at least 13 mealybug insect species (Hughes and Ollenu 1994; Ameyaw et al. 2014). This disease is principally controlled through the cutting out method, the complete removal of infected and neighbouring trees. Biological efforts for control of this disease have been limited in scope and have largely focused on direct and indirect vector control.

Direct vector control, using the entomopathogenous fungus *Aspergillus parasiticus* (Speare), capable of killing the mealybug *Planococcoides njalensis*

(Laing) under laboratory conditions, was ineffective under field conditions (Asante and Ackonor 1996). The use of parasitoids or native and exogenous predatory insects has had very little success and was unable to substantially decrease mealybug populations (Asante and Ackonor 1996; Ackonnor 1997).

Since mealybugs can spread the disease radially when being carried from one tree to an adjacent tree by attendant ants (*Crematogaster* and *Camponotus* spp.) (Hughes and Ollenu 1994), control of attendant ants could possibly reduce disease impact. Strickland (1951), when looking at one of the most important mealybug vectors of swollen shoot virus *P. njalensis*, found that it was almost invariably attended in the field by Crematogasterine ant species and found that *Crematogaster* density was closely correlated with mealybug density. Strickland (1951) also found that *Oecophylla* was strongly negatively correlated with *Crematogaster* spp. and thus proposed that specific control efforts to reduce mealybugs by killing attendant ant species (*Crematogaster*, *Camponotus*) should be non-lethal to *Oecophylla* and other large predatory ants common on cacao. Unfortunately, we are unaware if anyone has put these recommendations to the test.

Another approach to protect cacao from CSSV has been reviewed by Hughes and Ollenu (1994). They discussed the use of attenuated forms of the virus. Mild strains of severe viruses may, when inoculated to susceptible plants, prevent infection by closely related severe strains. Unfortunately, results have been mitigated. Yet mild strain protection of cacao against CSSV remains an intriguing possibility, especially since it seems that CSSV infection of cacao trees might infer some protection against black pod rot caused by *Phytophthora* spp. Nyadanu et al. (2012) found that cacao leaf discs from virus-infected plants displayed higher levels of resistance to *Phytophthora* spp. than leaf discs from healthy plants.

In conclusion, biological control of CSSV has not had the attention certain other diseases have received. Moreover, most biocontrol efforts have had little to no impact on CSSV disease. However, given the current interest and promising results of biocontrol of cacao diseases using endosymbionts, it would be worthwhile to see whether endophytic biocontrol agents could play a role in reducing the impact of CSSV disease.

17.12 The Nagoya Protocol: Implications for Biocontrol in Cacao-Producing Countries

The future of biological control and thus also biocontrol of cacao diseases depends largely on access to reliable biological control agents. Within the biocontrol R&D community, the Nagoya Protocol has created concerns in that it could hamper access to classical biocontrol candidates and the development of economical biocontrol agents. The Nagoya Protocol is a legally binding multilateral environmental agreement (MEA) under the Convention on Biological Diversity (CBD) on "Access to Genetic Resources and the Fair and Equitable Sharing of Benefits

Arising from their Utilization". The protocol aims to create an enabling framework for mutually beneficial access to the genetic resources of countries subscribing to the protocol.

The International Organisation for Biological Control (IOBC) established a commission on Access and Benefit Sharing (ABS) and Biological Control in 2008. The resulting report (Cock et al. 2009) was mostly concerned with parasitoids and herbivores as biocontrol agents of invertebrate pest and weeds, respectively, in staple crops. Nowadays, there is increasing appreciation that a legal and regulatory vacuum is no longer desirable and legally regulated ABS will ultimately benefit all parties. Furthermore, benefits for the use of microbial diversity need not be monetary but could take many different forms, such as recognition of intellectual property, co-authorship, access to value added products (improved formulations), etc. The latter is especially important since classical biocontrol is not designed to achieve corporate revenue. It aims for cost-effective control of an introduced pest over a wide area, frequently in several countries (Cock et al. 2009) and the beneficiaries, typically farmers, may have their problems reduced without necessarily actively using BCAs. To date low-income countries have contributed slightly more BCAs than they have received (Cock et al. 2009).

In contrast, agents for augmentative or inundative control are often developed by public organizations and/or private companies with no protection of the organisms or intellectual property rights (Klapwijk 2011). The history of commercial mass production and sale of natural enemies spans less than 50 years and is (still) limited to a relatively small number of companies worldwide, most of which are located in developed countries (Schloen et al. 2011), with an estimated market at end-user level in 2008 of US$100–135 million and profit margins around 3–5 %. Commercial strain *Trichoderma harzianum* T-22 has been patented in the USA, and freeze-dried samples are being sold by the American Type Culture Collection (ATCC) for US$354 for commercial purposes and for US$295 if non-for-profit. The formulated biocontrol product, RootShield, with 1.15 % active ingredient, is sold for around US$200 kg^{-1}. The strain was tested against Rosellinia root rot in cacao in Costa Rica (Mendoza-Garcia et al. 2003) and VSD in Malaysia (Vanhove et al. unpublished).

Cacao-producing countries tend to be net exporters of biodiversity. Understandably, these states wish to safeguard their natural resources against unilateral exploitation and biopiracy. However, they are often unable to add value through costly R&D, steps typically carried out in more developed countries. Thus, there is an urgent need to assist the Parties to the Nagoya Protocol in its implementation, in order to provide legal clarity on access facilitation, benefit-sharing obligations and compliance measures. Contrary to initial fears, the mechanisms and model agreements provided under the ongoing ABS capacity-building initiative can accelerate and facilitate setting up complex multinational collaborations, in which the provider country and the foreign investors feel their respective interests are sufficiently protected, and both will receive their mutually agreed fair share of any subsequent benefit in countries party to the agreement, whether monetary or not. This MEA streamlines procedures, thereby reducing administrative bottlenecks and potential

delays. ABS agreements involve not only CBD/Nagoya Protocol Parties but also private companies. Thus incentives may be needed to encourage benefit sharing. Negotiations should also consider that genetic resources that are originally accessed for one purpose may subsequently enter different value chains. The long-term vision of these contracts necessitates provisions for extending commitments to third party or successor users, such as new/restructured companies, additional sectors and/or countries. However, if legislative drafters do not consider BCAs, their development and use may become a challenging process for international researchers, national collaborators, practitioners and tertiary beneficiaries.

17.13 Conclusion and Perspectives

The recent advances in biological control of cacao diseases give rise to optimism, yet a lot remains to be done. Increasing evidence exists that especially *Trichoderma* species have significant potential for direct biocontrol of cacao diseases in aboveground plant parts (e.g. Krauss et al. 2010; Mbarga et al. 2014; Crozier et al. 2015) and can have profound effects on the expression of host genes which can increase plant resistance to biotic, e.g. pests and diseases, and abiotic stresses, e.g. drought tolerance (e.g. Bailey et al. 2006; Bae et al. 2009; Mejía et al. 2014). The shift away from inundative BC to a more classical approach using endophytic organisms seems to be justified in light of recent advances (e.g. Bailey et al. 2006; Mejía et al. 2014; Crozier et al. 2015; Rosmana et al. 2015) as endophytes seem to have a wider range of mechanisms for control and control might be more durable. It has to be noted however that exciting as these new developments are regarding the interactions between endophytes and their host plants, the fact remains that under natural conditions, cacao and its microbial symbionts are still overcome by pathogens and substantial disease losses occur. An important question is: to what degree are plant properties, in this case disease resistance, due to endophytic fungi (Herre et al. 2007)? If fungal effects are small, then viewing plants as just plants is fine; if fungal effects are large (as data seems to suggest), then we need to reconsider how to study plant "characteristics" (Herre et al. 2007). Thus, future research should also look at what influences endophyte community composition within a plant (in space and time).

It is clear that there are a large number of potential biocontrol candidates out there. Unfortunately a lot of the biocontrol research of cacao has remained at the laboratory and nursery stage. Numerous studies have already provided evidence of the capacities of many fungal and bacterial BCAs to control diseases. The subsequent steps towards full-scale field trials are oftentimes lacking, and promising results from the laboratory and nursery stages are not translated into likewise field results. Moreover, trials in experimental fields are not representative of farmer fields, and trials under farmer field conditions often show very variable results.

The lack of clear and economically interesting results is also evidenced by the fact that biological control of cacao diseases is not yet an integral part of cacao crop

management strategies. As for now, the only biocontrol product being used on a large-scale seems to be Tricovab (*T. stromaticum*) in Brazil, mostly because the Brazilian government's Executive Commission for Cacao Farm Planning (CEPLAC) [4] is producing and distributing this biofungicide.

Even though there is potentially a huge market for biological control products for cacao diseases, the lack of consistency and lengthy and complicated registration processes may be inhibitory for industry to develop such products. Many *Trichoderma* may instead be marketed as plant inoculants or plant-strengthening agents, which for the respective manufacturers circumvents the time and expenses necessary for product registration as plant protection products (Hermosa et al. 2013).

Classical Biocontrol for Co-evolved Versus New Encounter Diseases

In the case of co-evolved pathogens of cacao, such as *M. roreri* and *M. perniciosa*, a large number of co-evolved microbial antagonists have been identified (e.g. Arnold et al. 2003; Rubini et al. 2005; Crozier et al. 2006; Thomas et al. 2008; Melnick et al. 2011). In the case of new encounter diseases such as VSD, CSSV and black pod due to *P. megakarya*, this is not yet the case. Moreover, since endophytic diversity in cacao seems to be less in countries where cacao is not native (Schmidt et al. 2010), the search for co-evolved antagonists in the centre of origin of these diseases would seem a priority. Unfortunately, such efforts are seriously hampered by the lack of information on indigenous hosts, and more efforts should be dedicated to their identification. Finally, seedling or graft inoculation with promising co-evolved endophytes, fungal (including mycorrhiza) and/or bacterial, either singly or in mixtures, could then provide a basic level of disease protection, particularly in locations outside the region of origin of cacao.

Improving Biocontrol Efficacy

Theobroma cacao does not just host one single endophytic species yet harbours whole communities of fungal and bacterial endophytes (Melnick et al. 2011). The presence of these endophytes can reduce disease susceptibility (Arnold et al. 2003; Herre et al. 2007; Hanada et al. 2010). However, the capability of endophytes to colonize different plant parts seems to be isolate dependent (e.g. Bailey et al. 2006; de Souza et al. 2008). Moreover, Herre et al. (2007) indicated that colonization of

[4] See http://www.freshfruitportal.com/2013/06/05/brazil-to-launch-biofungicide-against-cacao-disease/?country=others (last consulted on 11 June 2015).

cacao by fungal endophytes is not homogenous in space and time. Fluctuations of endophytic communities occur in nature in response to season (climate) temperature (Melnick et al. 2008 and refs therein) and the presence or absence of pathogens. Therefore it would be interesting, similar to work done on epiphytic mycoparasite mixtures, to study in more detail the potential of endophyte mixtures to control cacao pathogens. Yet the use of multiple organisms in a consortium that will coexist in the internal plant tissues is just beginning to be undertaken (Backman and Sikora 2008).

Single antagonists are unlikely to be active in all environments in which they are applied, whereas mixed inocula will have a wider range of environmental adaptations such as different pH values, soil organic matter, phosphorus and moisture content and could preferentially colonize different plant parts. While mixed fungal BCAs have proven advantageous for the simultaneous control of several cacao diseases, they did not consistently add value to FPR control alone, although increased consistency of performance is one of the hypothesized benefits. Some mycoparasite isolates have been reported to discriminate between individual *M. roreri* host strains (Samuels et al. 2006a, b), a phenomenon that can be overcome by appropriate mixtures (Krauss and Soberanis 2001a). The design of compatible, mixed inocula with increased resilience to agro-climatic variations is in its early stages of development (ten Hoopen et al. 2010b; Krauss et al. 2013) but certainly a step in the right direction. This approach may be best suited for areas of mixed infection by several pathogens and/or high agro-ecological fluctuations.

Intriguing possibilities also exist for the development of mixed bacterial and fungal inocula, including VAM, or combination treatments of natural or chemical fungicides and BCAs. Tchameni et al. (2011) showed that dual inoculation with *T. asperellum* and two AMF could be beneficial to cacao seedlings, showing the interest of using a mix of potentially beneficial organisms to reduce pathogen impact, although *T. asperellum* PR 11 did seem to have a negative impact on root colonization by AMF. Similarly, Lutz et al. (2004) studied the signalling between bacterial and fungal biocontrol agents in a mixture and found that negative as well as positive effects on the expression of key biocontrol genes occurred. Many *Trichoderma* are relatively tolerant of copper hydroxide, a commonly used fungicide for the control of cacao diseases. Combination treatments of fungicides with BCAs have been tested with various results (e.g. Krauss et al. 2010; Medeiros et al. 2010; ten Hoopen et al. 2010a; Agbeniyi et al. 2014). Similar to certain fungicides, plant extracts capable of inhibiting germination of *Phytophthora* spp. are compatible with *T. asperellum* (Widmer and Laurent (2006)).

An important consideration to take into account is the disease to be controlled. Whereas endophytic colonization of trunks and/or branches might confer durable control for vascular diseases such as VSD or CSSVD, diseases that primarily attack cacao pods, such as FPR, BPD and WB, might require repeated applications, at least once a year. If so, continued work on formulation, to stimulate adherence, germination and pod colonization, and application, delivery methods and timing is still necessary, especially considering recent advances (Mbarga et al. 2014; Crozier et al. 2015) that have shown the positive impacts of improved formulations.

Other questions to ask: what are the mechanisms used by endophytes to influence the outcome of host–pathogen interactions, are they localized or systemic (all plant tissues), and are they short lasting, long lasting, or permanent? Answers are coming (Bailey et al. 2006; Hermosa et al. 2013; Mejía et al. 2014), albeit slowly, to some of these questions and allow for a better understanding of the processes governing plant disease resistance. However, one major question comes up: how to utilize this knowledge for the development of biocontrol products that will be economically interesting for producers and users.

Adverse Effects

For now very little knowledge is available on the long-term effects of the use of selected endophytic BCAs on naturally occurring endophytic microbial communities. Work by ten Hoopen et al. (2003) and Rubini et al. (2005) seems to suggest that cacao genetic make-up and epi- and endophytic diversity and abundance are independent phenomena although this has to be confirmed by more detailed studies. However, if so, biological control and breeding for disease resistance would be complementary components in IPM schemes. The use of co-evolved BCAs from the region of origin of cacao (South America) for control of cacao diseases elsewhere, Africa and or Asia, for example, has not been considered (yet). If this option is to be explored, careful risk analyses will be a prerequisite. However, given the promising results using locally isolated endophytic BCAs for control of VSD and BPD in Indonesia (Rosmana et al. 2015; Sriwati et al. 2015), it seems we could avoid, for now, such an approach. Moreover, the search for co-evolved antagonists of NEDs should provide additional alternatives. Nonetheless, if FPR and/or WB reach Africa, such a classical biocontrol approach could certainly be of worth.

Other Diseases

As evidenced in this book, cacao is subject to attacks by a large number of diverse pathogens. Numerous of these pathogens have not (yet) been subjected to biological control efforts. Based on the biology and epidemiology of the pathogen under consideration, specific biological management options could be explored. For example, in the case of root pathogens, the use of soilborne *Trichoderma* isolates together with VAM and soil amendments could be an option. In the case of VSD, the use of epiphytic and endophytic microbes to reduce leaf infection as well as endophytes to protect against vascular colonization should be explored (Guest and Keane 2007). Similar to other basidiocarp-producing cacao pathogens, such as WB, biological control could also be focused against the basidiocarp stage.

In all cases, the search for local endophytic BCAs, if possibly co-evolved with the target pathogen, should be prioritized. Strict and careful screening processes

should be developed allowing for the selection of biocontrol candidates that are not only screened for their functional capacity with their host but also with regard to other biocontrol agents.

Integrated Pest Management

Biocontrol is capable of reducing disease incidence but not of eliminating a pathogen completely. As a result, biocontrol is best suited for integrated disease management programmes where it can supplement other control strategies such as cultural and genetic control. Cultural measures can be further enhanced by integrating cacao into a disease-resilient agroforestry system (AFS) (Ratnadass et al. 2012). A diversified AFS is not only a good risk avoidance strategy but also a tool to stabilize the dynamics of trophic processes and to optimize shade cover, temperature and aeration for a healthy cacao crop and to create conducive conditions for the establishment of applied biocontrol agents as well as the activity of naturally present antagonists (Krauss 2004; ten Hoopen et al. 2003). The quality and quantity of biodiversity, as well as its spatial arrangement within the landscape, are also of importance (Schroth et al. 2000; Ngo Bieng et al. 2013; Gidoin et al. 2014). Genetic disease resistance and BCA abundance can be exploited simultaneously to create additive affects. Similarly, combination treatments using several BCAs, either with fungicides and/or natural products, should improve disease management (e.g. ten Hoopen et al. 2010a; Krauss et al. 2010). With cultural management as a central pillar, biological control will hopefully soon become an integral part of IPM of cacao diseases.

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Chapter 18
Breeding for Disease Resistance in Cacao

Osman A. Gutiérrez, Alina S. Campbell, and Wilbert Phillips-Mora

Abstract Cacao production must increase in order to meet the projected rise in the demand for chocolate. Approximately one-third of global production is lost annually to diseases and insects. Four diseases account for the greatest losses worldwide: black pod, caused by four *Phytophthora* spp.; witches' broom, caused by *Moniliophthora perniciosa*; cacao swollen shoot virus, caused by a member of the genus *Badnavirus*; and frosty pod, caused by *Moniliophthora roreri*. At the present time, only 30 % of material currently under cultivation is of improved varieties, therefore, there is an urgent need for the development of new, high-yielding, disease-resistant varieties. Sustainable production increases could be achieved if improved varieties were used by the farmers. Cacao breeding was started in Trinidad in the 1930s by F. J. Pound and within a few decades cacao research centers had been established in all the major cacao producing areas worldwide including West Africa and Southeast Asia. Pound and other researchers have made several expeditions to the Amazon to collect wild cacao germplasm. In addition to using the germplasm collected from the wild and farmers' fields to find new sources of resistance genes, researchers have developed breeding programs that cross and select cacao genotypes in order to accumulate desirable genes for resistance, as well as good horticultural and quality traits. Recently, numerous molecular tools, including the genome sequences of two varieties of cacao, have been developed and/or made available to accelerate the breeding process. International private/public collaborations are in progress to identify candidate resistance genes, map these in the sequenced genomes, and develop molecular markers associated with these genes. Researchers will use these markers in genomics-assisted breeding programs to screen young cacao plants and select those with desirable traits.

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18.1 Introduction

Chocolate is produced from the seeds of cacao (*Theobroma cacao* L.), a tree native to the Amazon basin (Motamayor and Lanaud 2002). The genus *Theobroma* is assigned to the subfamily Sterculiaceae within the family Malvaceae (Whitlock and Baum 1999). The 22 species comprising the *Theobroma* genus are small, understory, lowland, rainforest trees whose native ranges stretch from Southern Mexico to the Amazon basin (Purseglove 1968). In addition to *T. cacao*, three other species (*T. bicolor*, *T. grandiflorum*, and *T. angustifolium*) are cultivated (Cuatrecasas 1964; Silva et al. 2004). However, *T. cacao* is the only tropical fruit tree species within the Malvaceae that produces fruit with significant economic value. Its seeds contain large amounts of fat and are the source of cocoa solids and cocoa butter. Cacao ranks fourth in the league of tropical crops, behind palm oil, rubber, and bananas. At $12 billion of traded value annually, cacao ranks far behind palm oil products which are valued at $60 billion and natural rubber which is valued at around $29 billion. Due to rising demand and price per metric ton (MT), cacao is now comparable to bananas, which have an annual traded value of $15 billion (Hardman 2014).

When Columbus arrived in the Americas for the first time, cacao was being cultivated and consumed by the Aztecs. It had previously been used by the Mayas and is believed to have been taken to Mexico and Central America over 2000 years ago (Hurst et al. 2002; Motamayor and Lanaud 2002). The first record of *T. cacao* outside the Americas was in 1670 when a single plant was introduced from Mexico to the Philippines. Most of the cacao grown in the Philippines until the late nineteenth century is believed to have originated from this plant (Blanco 1837). Cacao was introduced to West Africa in 1822, where it was cultivated in São Tomé and Príncipe (Bartley 2005). Cacao is currently grown in over 50 countries throughout the tropics (Knight 2000), primarily within 20° north and 20° south of the equator (Minifie 1989). Over 80 % of cacao is produced by smallholder farmers, providing an important source of income in many rural communities (Hasnah et al. 2011).

As the primary regions for chocolate manufacturing, Europe and the United States are the main importers of post-processing cocoa products. However, from 2008 to 2011, China grew from the 12th to 9th largest importer of cocoa paste and from the 15th to 9th largest importer of cocoa powder and cake (WCF 2015). A combination of the increase in demand from the chocolate industry in countries with emerging economies like Brazil, Russia, India, China, and South Africa and an unreliable production system has raised the possibility of a supply deficit of cacao beans in the future. Confectionery manufacturers are concerned because there could be a shortage of one million MT of cacao projected by 2020 (Hardman 2014). Production will need to be increased in order to meet the anticipated increase in the demand for chocolate (Ploetz 2007b; WCF 2015); however, cacao yields are affected by numerous pests and diseases.

18.2 Disease Problems in Cacao

Approximately one-third of global cacao production is lost annually due to diseases and insects (Lass 2004). Disease limits cacao production in many tropical areas around the world and has led to production areas being abandoned (Enríquez et al. 1982; Krauss and Soberanis 2001). Although details of individual diseases can be found in other chapters within this book, a brief introduction is provided below. The major cacao diseases are caused by oomycetes, fungi, and viruses, while mirids and the cacao pod borer are the most damaging insects. Most varieties currently grown are highly susceptible to diseases (Adejumo 2005; Phillips-Mora and Wilkinson 2007). The hot and humid conditions that are ideal for cacao production are also ideal for disease development. Although cacao suffers from many different diseases, four diseases account for the greatest losses worldwide: black pod, caused by four *Phytophthora* spp.; witches' broom, caused by *Moniliophthora perniciosa*; cacao swollen shoot, caused by a member of the genus *Badnavirus*; and frosty pod, caused by *Moniliophthora roreri* (Cilas and Despréaux 2004; Fulton 1989; Ploetz 2007b).

Because of its worldwide distribution and severe impact on pod yield and quality, black pod (Chaps. 7 and 8) is considered the most damaging disease of cacao with estimated annual losses of 20–25 % (Evans 2007). It is caused by *Phytophthora palmivora*, *P. megakarya*, *P. citrophthora*, and *P. capsici* (Bowers et al. 2001), of which the first two are the most significant (Adomako 2007; Guest 2007). *P. palmivora* is found in all cacao-producing regions, while the more aggressive species, *P. megakarya*, is only present in West Africa (Guest 2007). However, *P. megakarya* has the potential to cause significantly greater reductions in cacao production in West Africa if it continues to spread at the current rate (Gregory 1974).

Witches' broom (Chaps. 5 and 6) is the second most important disease of cacao in terms of lost annual production, with losses up to 90 % occurring in the most severe cases (Dias and Resende 2001; Ploetz 2007b). Witches' broom disease occurs in all cacao-growing regions of South America and in some Caribbean islands (End et al. 2014). It is present in Panama, but not in other Central American countries (Phillips-Mora and Cerda Bustillos 2009). The pathogen that causes witches' broom (*M. perniciosa*) is in the same genus as *M. roreri* which causes frosty pod (a.k.a. monilia or moniliasis), another significant disease of cacao. Within 10 years of the discovery of witches' broom in the state of Bahia, Brazil in 1989, production had fallen from 400,000 tons/yr to 130,000 tons/yr (Bowers et al. 2001; Meinhardt et al. 2008).

Cacao swollen shoot virus (CSSV, Chap. 10) is transmitted by mealybugs and causes an important disease that impacts cacao production in West Africa. It was first identified in Ghana (Posnette 1940; Stevens 1936), where it has caused enormous economic damage since the 1930s, but it was only restricted to small areas in Togo and Côte d'Ivoire until recently. Currently, outbreaks in the main producing areas in Côte d'Ivoire, Ghana, and Togo are causing serious problems.

Other affected countries are Nigeria, Cameroon, Liberia, Sierra Leone, Benin, and Sri Lanka (End et al. 2014). CSSV is not found in Central and South America; it is indigenous to West Africa, where it is found in forest trees such as *Ceiba pentandra* and appears to be nonpathogenic.

Although frosty pod rot (Chaps. 3 and 4) causes less damage to the cacao industry overall than black pod or witches broom, it has the potential to become a much more serious threat (Phillips-Mora and Wilkinson 2007). Damage is currently limited by the absence of *M. roreri* in major cacao-producing countries. It is restricted to cacao-producing regions of the Americas, excluding Brazil and the Caribbean countries (Evans 2007; Phillips-Mora and Wilkinson 2007). In places where frosty pod occurs alongside black pod and witches' broom, frosty pod causes the highest yield losses. It frequently causes over 80 % crop losses (Phillips-Mora and Wilkinson 2007).

Ceratocystis wilt (a.k.a. mal de machete or mal de choroní, Chap. 12) is another disease affecting production in the Americas (Wood and Lass 1985). It is caused by *Ceratocystis cacaofunesta*, a vascular wilt fungal pathogen transmitted by boring insects in the *Xyleborus* genus (Ambrosio et al. 2013). This disease is very important in the Caribbean and in Central and South America where it has been reported to kill 50 % of the trees in some cacao plantations (Iton 1966).

Vascular streak dieback (VSD, Chap. 9) is caused by the basidiomycete *Ceratobasidium theobromae* (P.H.B. Talbot & Keane) Samuels & Keane (Syn. *Oncobasidium theobromae*), a near-obligate parasite of cacao (End et al. 2014). It was first reported in the 1960s in Papua New Guinea where it caused substantial losses of trees. The disease has spread and is now found throughout Southeast Asia (Guest and Keane 2007) and is the most serious disease affecting cacao production in the continent (Varghese et al. 1987).

In addition, there are numerous diseases that cause relatively minor losses globally but can cause severe losses locally. Some diseases of local or emerging importance include Rosellinia disease (caused by *Rosellinia pepo*, *R. bunodes*, and *R. paraguayensis*, Chap. 14), Verticillium wilt (caused by *Verticillium dahlia* and *V. albo-atrum*, Chap. 14), pink disease (caused by *Erythricium salmonicolor*, Chap. 11), and anthracnose (caused by *Colletotrichum gloeosporioides*, Chap. 11) (End et al. 2014; Rojas et al. 2010).

Most cacao is grown in a low-input system, and minimal chemical control is used (Hebbar 2007). In addition to being cultivated in areas where environmental conditions are favorable for disease development, the perennial nature of cacao makes disease management particularly challenging because susceptible host tissue is present over a longer period of time (Ploetz 2007a). Control measures used on annual crops are not always effective on perennial crops.

18.3 Principles of Plant Pathology and Host–Microbe Interactions

Cacao is native to the Amazon but is currently cultivated in tropical regions worldwide. This has brought it in contact with numerous microbes and insects with which it did not coevolve. A lack of coevolution between plants and pathogens can result in scenarios where, in the new range, hosts have little or no resistance and pathogens are therefore much more virulent than if they had evolved together (Stukenbrock and McDonald 2008). These conditions are ideal for the development of serious epidemics and have been seen with cacao diseases such as CSSV, vascular streak dieback, Ceratocystis wilt, and *P. megakarya*-caused black pod (Ploetz 2007b). However, other serious diseases, such as witches' broom, are caused by organisms that have evolved with *T. cacao*.

The goal of plant disease management is to minimize the damage caused by plant diseases (Maloy 2005). Complete control is not necessary since damage only needs to be kept below economically significant thresholds (managed). In 1929, H.H. Whetzel outlined four plant disease control principles: exclusion, eradication, protection, and immunization. These were expanded by the US National Academy of Sciences in 1968 and are known as the six traditional principles of plant disease control (Maloy 2005). They are (1) avoidance of infection by selecting environments or seasons where inoculum is absent, (2) exclusion and/or (3) eradication of the pathogen from an area, (4) protection of plants from infection by using chemicals or barriers, (5) use of disease resistance in crops, and (6) therapy of infected plants (Arneson 2001). The goal of the first three is a pathogen free environment, at least during the period of time when the host plant is susceptible. The last three focus on the plant host.

Plants regularly interact with numerous potentially damaging organisms such as microbial pathogens, nematodes, and insects, but most of these interactions do not result in disease or crop damage (Parker et al. 2000). A pathogen that is highly virulent on one plant species can be nonpathogenic on many others. Resistance of all members of a plant species to a particular parasite or pathogen is known as non-host resistance (Jones and Takemoto 2004; Mysore and Ryu 2004; Thordal-Christensen 2003). These incompatible host–microbe interactions occur when (1) the plant is unable to support the biological requirements of the potential pathogen, (2) preformed plant defenses prevent pathogen infection and colonization, or (3) effective plant defenses are induced following plant recognition of the pathogen.

In contrast, compatible host–microbe interactions are those where pathogens successfully infect a host and disease develops. Compatible interactions occur when preformed defenses are insufficient to prevent infection, the plant does not detect the pathogen, or activated defenses are ineffective (Hammond-Kosack and Jones 1996). Some pathogen species have different subgroups, or types, that cause both compatible and incompatible reactions on a host plant and may have varying levels of virulence. These subgroups are referred to as races (Keen 1990).

Even if the pathogen and host are compatible, they must come in contact in order for disease to occur. In some cases, the lack of disease on particular genotypes can be attributed to disease escape. In such cases the host, pathogen, and conducive environment do not exist simultaneously for a long enough period for infection and/or disease to occur (Agrios 2005; Russell 1978). Disease escape is seen in cacao clones that have a different, or reduced, period of pod production (Surujdeo-Maharaj et al. 2003). For areas with long dry seasons, disease escape is an effective way to reduce yield loss to witches' broom (Purdy and Schmidt 1996). Appropriately timed hand-pollination could ensure that pods mature when inoculum pressure is lowest (Edwards 1978; Evans et al. 1977). In Ecuador, the wide range of disease intensities seen between trees in a mixed planting was attributed to differences in pod susceptibility to infection and the timing of their peak production (Evans et al. 1977). Pods that develop and mature during the dry season avoid heavy infection by *M. perniciosa* and *M. roreri* in Ecuador (Edwards 1978). Trees that primarily produce pods outside of the window during which disease pressure is the greatest experience less disease and lower yield loss (Evans et al. 1977).

18.4 Defense Responses in Plants

Preformed Defense Responses

Defense responses can largely be categorized as preformed or induced. Preformed compounds (antimicrobial secondary metabolites, i.e. phytoanticipins) and many structural defenses (wax layers and rigid cell walls) are present regardless of whether an attack has taken place, while induced defenses develop in response to an attack (Mysore and Ryu 2004; Nürnberger et al. 2004; Six and Wingfield 2011). The physical resistance provided by structural defenses such as cell walls and lignin, deters insects and protects against active penetration by fungi. Waxy cuticles and trichomes inhibit spore germination by preventing water accumulation on the leaf surface (Freeman and Beattie 2008). Some plants may also have fungitoxic leaf exudates that prevent germination or inhibit growth following germination. Plants with resistance due to preformed structural defenses may develop disease if a pathogen enters through a wound or following artificial inoculation (Agrios 2005).

The preformed structural barriers in cacao include the higher concentration of epicuticular wax found on fruits of the *Phytophthora*-tolerant clones PA 150 and SCA 6, in relation to the more susceptible clone SIC 2 (Sena-Gomes et al. 1995). The low stomatal frequency (Sena-Gomes and Rocha 1995) and greater cuticle thickness (Sena-Gomes and Machado 1994) of SCA 6 appear to be more important for its resistance to certain pathogens than the size of its stomata.

Preformed compounds within plant cells provide a second layer of defense in situations where structural defenses were bypassed or ineffective. Antifungal compounds and plant proteins that inhibit pathogen colonization are present at low

levels inside cells prior to infection (Walton 2001). Alkaloids, such as caffeine and nicotine, are toxic to microbes and decrease palatability for insects (Freeman and Beattie 2008). Spence (1961) theorized that the resistance of some cacao genotypes to *P. palmivora* was due to the presence of certain partially identified polyphenolic substances in the epicarp that inhibited zoospore germination and germ tube formation. Constitutive phenols have also been associated with resistance to *C. fimbriata* in some cacao genotypes (Capriles de Reyes and Reyes 1968).

Induced Defense Responses

Plants lack immune systems with mobile defense cells, such as those present in animals. Instead, they rely on the immunity of each individual cell and systemic signals originating from infection sites. Most living plant cells are able to detect invading pathogens. This can occur at the cell surface, where specific receptors detect molecules present in microbes, such as flagellin, or when gene products in the plant cell recognize specific disease-causing effector molecules produced by the pathogen (Freeman and Beattie 2008; Spoel and Dong 2012). The induced defense responses are triggered following the detection of a pathogen.

Effector recognition leads to an oxidative burst, ion channel gating, or protein kinase cascades, which then activate cellular changes, such as cell wall reinforcement, or changes in gene expression that further increase defense responses (Agrios 2005). Toxic chemicals and pathogen-degrading enzymes are produced following pathogen detection. Phytoalexins are antimicrobial compounds produced in healthy cells in response to materials diffusing from adjacent damaged cells (Walton 2001), resistance occurs when they reach concentrations sufficient to restrict pathogen development (Freeman and Beattie 2008). Cacao genotypes resistant to the fungal pathogen, *Verticillium dahlia*, were found to produce four phytoalexins in response to infection; two phenolics, a triterpenoid, and elemental sulfur (Resende et al. 1996). Sulfur accumulation was restricted to tissues and structures most likely to come in direct contact with xylem-invading pathogens and persisted for over 60 days (Resende et al. 1996).

The hypersensitive response (HR) is an inducible defense response characterized by rapid cell death around an infection site. It is associated with incompatible interactions between plants and microbes and has been observed in response to fungi, bacteria, nematodes, and viruses (Hammond-Kosack and Jones 1996; Holub et al. 1994). Inoculating an organism that is pathogenic on one plant species, into another, non-host, plant species can elicit a HR (Mysore and Ryu 2004). HR ranges from the death of a single cell, to expanding necrotic lesions accompanied by limited pathogen colonization (Holub et al. 1994). It is affected by environmental conditions and has been found to be diminished under high humidity (Hammond-Kosack and Jones 1996; Klement 1982). By limiting access to water and nutrients, the HR can limit the growth and spread of obligate biotrophic pathogens (ones that only subsist on living host tissue), or hemi-biotrophic pathogens (subsist on living

and dead host tissue at different stages in their lives) (Glazebrook 2005; Hammond-Kosack and Jones 1996). The role of HR in preventing colonization by pathogens that kill host tissue during colonization (necrotrophs) is less clear because these can obtain nutrients from dead plant cells (Glazebrook 2005). However, the pathogen may be adversely affected by toxic substances released from the cell as it dies (Osbourn 1996). Baker and Holliday (1957) observed a hypersensitive response in Trinitario cacao clones and in progeny of a highly witches' broom-resistant Amazon Forastero. Resende and Bezerra (1996) observed a HR in cacao following artificial inoculation with a strain of *M. perniciosa* that had been isolated from *Solanum stipulaceum*.

Plants have also evolved inducible systemic defense responses such as systemic acquired resistance (SAR), where the activation of local defenses induces a higher overall resistance state throughout the plant, protecting it from a wide range of pathogens (Spoel and Dong 2012). In spite of plants' lack of mobile immune cells such as those found in animals, plants have the capability to recognize a pathogen and turn on signaling pathways which lead to the activation of defense mechanisms in distant cells (Spoel and Dong 2012). Signaling molecules, such as salicylic and jasmonic acids, travel systemically throughout the plant activating defense responses, such as callose deposition between cell walls, to make them more resistant to insect feeding and pathogen entry (Freeman and Beattie 2008).

Salicylic acid appears to induce resistance to *P. palmivora* in cacao (Aguilar et al. 2000). In the clones ICS 1 and IMC 67, lesion number and size on leaves infected with *P. palmivora* decreased significantly following the application of salicylic acid to the plant, seed, or soil (Okey and Sreenivasan 1996).

18.5 Gene-for-Gene Interactions

The recognition of the pathogen by the host plant that results in disease resistance is often controlled by single complementary genes in the plant and the pathogen and is therefore known as gene-for-gene resistance (Keen 1990). This concept, originally proposed by Flor (1947), states that a resistance (R) gene in the plant matches an effector-coding, avirulence (AVR) gene in the pathogen (Spoel and Dong 2012). Recognition sets off a cascade of defense responses that prevent the pathogen from colonizing and result in an incompatible interaction (Keen 1990). R gene-mediated resistance is usually accompanied by an oxidative burst, that is, rapid production of reactive oxygen species (ROS) and the activation of a salicylic acid-dependent signaling pathway (Glazebrook 2005). The HR is also typically triggered by a gene-for-gene interaction.

Plant R genes encode proteins that allow plants to recognize pathogen AVR gene products and activate downstream defenses (Dangl and Jones 2001). Each of these genes tends to be dominantly inherited, and if either partner lacks a functional allele, recognition and resistance do not occur and disease develops (Keen 1990). However, a plant may have numerous resistance genes that protect against a

particular pathogen species. Thus, for a pathogen race to escape detection, it must carry nonfunctional alleles for all of the avirulence genes corresponding to the R genes carried by the plant (Keen 1990). Functionally, races are defined by the collection of avirulence genes that they contain (Keen 1990). Gene-for-gene interactions are common among host plants and biotrophic pathogens, those which garner nutrients from living plant cells (Oliver and Ipcho 2004). The major pathogens affecting cacao, such as *P. palmivora*, *M. roreri*, and *M. perniciosa*, are hemibiotrophs, meaning they behave as biotrophs during part of their life cycle (Evans et al. 1978; Marelli et al. 2009; Oliver and Ipcho 2004; St. Leger and Screen 2001).

R genes controlling a plant's resistance to bacterial, fungal, and viral pathogens have been found (Staskawicz et al. 1995). Although these organisms infect and colonize the plant in distinct ways, the structural similarity of R proteins associated with each organism indicates that they share recognition and signal transduction mechanisms (Hammond-Kosack and Parker 2003; Staskawicz et al. 1995) and may involve common downstream defense pathways (Feys and Parker 2000).

Most R proteins have putative protein-interaction/recognition domains such as leucine-rich repeats (LRR) and leucine zippers (LZ), and signaling functions such as a kinase domain or nucleotide-binding site (NBS) (Feys and Parker 2000). LRR domains contribute to specificity in pathogen recognition (Dangl and Jones 2001). Most R genes in plants are grouped into five categories based on the presence and arrangement of functional domains: LRR, LZ, NBS, transmembrane (TM), coiled coil (CC), toll/interleukin-1 receptor (TIR), and serine-threonine kinase (STK) (Martin et al. 2003; van Ooijen et al. 2007). Although resistance gene homologues are common in the cacao genome (Kuhn et al. 2003), functional links to resistance for these resistance genes, as in gene-for-gene interactions, have not been established.

18.6 Quantifying Resistance

Measuring individual components of pathogen, host, or disease development, can help determine the mechanisms leading to disease resistance. These components can include rates of colonization, germination, and sporulation, as well as the time required for each. Different components of resistance can be evaluated for each disease (Dias and Resende 2001) and in most cases are adapted to the specific disease being studied. The ability to identify significant sources of resistance to a disease can be severely limited if tools/measures giving consistent, quantifiable results are not available. In the case of black pod, the percentage of infected pods, incubation period of the pathogen, lesion size, and sporulation have been studied (Phillips-Mora and Castillo 1999). Lesion size is the primary characteristic used to assess resistance to black pod, and can be determined based on lesion diameter (Phillips-Mora and Castillo 1999) (Fig. 18.1), or lesion area calculated using digital image analysis software. When multiple *Phytophthora* species are present in a

Rating scale to assess resistance to *Phytophthora palmivora*
(following paper disc inoculation)

Disease severity scale

Symbol	Reaction	Scale
HR	Highly resistant	0.0 - 2.0
R	Resistant	2.1 - 4.0
MR	Moderately resistant	4.1 - 6.0
MS	Moderately susceptible	6.1 - 8.0
S	Susceptible	8.1 - 10.0
HS	Highly susceptible	> 10.0

Adapted from figure created by Adriana Arciniegas
CATIE Cacao Breeding Program

Fig. 18.1 Lesion diameter-based rating scale used to assess resistance to *Phytophthora palmivora* (following paper disc inoculation)

country, selection should be done based on the more aggressive and widespread isolate. In Trinidad and Peru, selections based on resistance to the predominant *P. palmivora* provided effective resistance against *P. capsici* which is also present in the country (Iwaro et al. 1996; Rios-Ruiz 1989). Frosty pod screening studies rely primarily on the extent of internal severity (IS) (Fig. 18.2); this best defines the pathogen's ability to damage the commercial product (Phillips-Mora and Galindo 1988; Suárez-Capello 1999) and appears to be the most indicative of resistance (Phillips-Mora 1999). IS is highly significant and positively correlated with external severity (ES) (Fig. 18.3). Therefore, there is an implicit consideration of ES even when only IS is measured (Phillips-Mora and Galindo 1988). For witches' broom, trees are mainly evaluated based on broom characteristics such as the number, size, and type. The resistance of vegetative parts to infection with *M. perniciosa* has been considered important since the beginning of the evaluation of resistance; however, the resistance in cherelles, fruits, or flower cushions are also evaluated as resistance parameters (Andebrhan et al. 1998; Fonseca et al. 1985; Paim et al. 2006; Silva et al. 2014; Thevenin et al. 2005).

Tolerance is the ability of a susceptible plant to maintain adequate yield in spite of being infected (Caldwell et al. 1958). Tolerant genotypes experience less yield loss relative to disease severity/pathogen development than other genotypes (Schafer 1971). Tolerance differs from resistance in that tolerant plants may have the same level of infection as susceptible plants, but without incurring as much

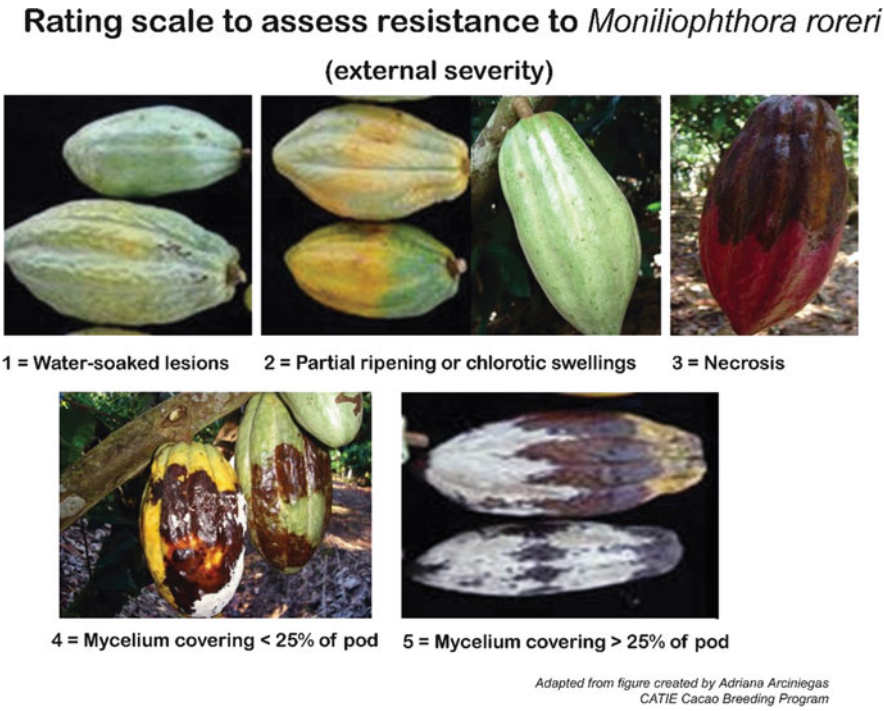

Fig. 18.2 Rating scale to assess resistance to *Moniliophthora roreri* (external severity)

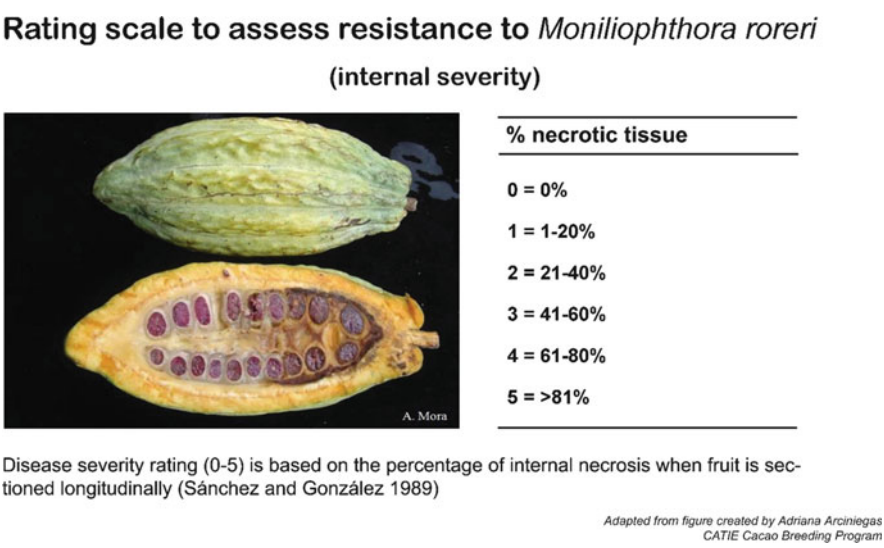

Fig. 18.3 Rating scale to assess resistance to *Moniliophthora roreri* (internal severity)

damage (Simons 1969). However, there is no standard resistance terminology and terms such as resistance and tolerance are defined differently by different individuals. A broad concept of resistance is used in this chapter, and it is not restricted to direct resistance of the plant to the pathogen (incompatible interactions).

18.7 Qualitative vs. Quantitative Resistance and Resistance Durability

Vertical vs. Horizontal Resistance

To describe different reactions that resistant plants have to disease, van der Plank (1963, 1966) divided resistance into two categories, vertical and horizontal, based on the number of genes involved and their mode of action. He described vertical (also called qualitative) resistance as controlled by one to a few host genes, each having a large effect on disease development. Qualitative resistance is typically effective against a subset of the pathogen population, completely controlling races carrying an AVR gene that encodes a gene product recognized by the plant host. Qualitative resistance has no major effect on disease caused by races of the pathogen not carrying that specific AVR gene. Horizontal (also called quantitative) resistance acts against the entire pathogen population to a limited level and results from the combined effect of several genes. Quantitative resistance can stem from any aspect of plant biology, physiology, or morphology that impedes pathogen colonization and sporulation and includes resistance due to disease escape. Quantitative resistance in perennial crops is valuable against nonlethal diseases but less useful against those that result in the death of the plant.

For example, the continuous range of levels of resistance to the development of black pod in some studies (Iwaro et al. 1996; Ndoumbé et al. 2001) indicates that it is controlled by several genes, each exerting a small effect. According to van der Vossen (1997), black pod resistance is incomplete and of a quantitative nature. Although the type of resistance responses seen with most cacao diseases is quantitative, results from an inoculation study with *M. perniciosa* suggest that the witches' broom resistance observed in SCA 6 is controlled by a single major recessive gene (Meinhardt et al. 2008).

Resistance Durability

The plant immune system is commonly explained using the four phase "zigzag" model described by Jones and Dangl (2006). Pathogen-associated molecular pattern (PAMP) molecules present in microbes, such as peptidoglycan in bacterial cell walls and flagellin, are detected by plant receptors in the first phase. This triggers

plant defense responses that initiate PAMP-triggered immunity (PTI). In the second phase, pathogens deliver effector molecules that disrupt PTI, resulting in effector-triggered susceptibility (ETS). In the third phase, a pathogen effector is recognized by a protein encoded by a plant resistance gene. This results in effector-triggered immunity (ETI). In the last phase, selection pressure has selected for pathogen isolates that do not produce the previously recognized effector. This prevents the pathogen from triggering ETI. The plant, however, is also under selection pressure, favoring plants with receptors that identify other pathogen effectors. This dynamic is comparable to an evolutionary arms race between plants and potentially pathogenic organisms.

Induced defenses can be general responses, effective against a range of microbes, or they can be specific to certain pathogens and/or races resulting from co-evolution between host and pathogen (Spoel and Dong 2012). The zigzag model describes an ongoing coevolution whereby some individuals within a plant species are resistant to some individuals within a pathogen species (necessitating the race classification) (Jones and Dangl 2006). This model explains some of the factors controlling the durability of resistance genes incorporated by plant breeders.

Durability refers to the amount of time between the introduction of a resistant genotype and the adaptation of the pathogen, allowing it to overcome the host's resistance. Resistance durability depends on the biology of the pathogen (mutation rate, number of generations, reproductive strategy, dispersal) (McDonald and Linde 2002) and the biology of the host (annual vs. perennial, number of genes controlling the interaction, etc.). Pathogens are under selection pressure to alter or delete the gene encoding the effector being recognized. In general, genetically variable pathogens that propagate sexually and have large, rapidly multiplying populations are able to overcome the plant's resistance more quickly (McDonald and Linde 2002). Durable resistance lasts many seasons or indefinitely.

Qualitative resistance is considered less durable than quantitative resistance because it is controlled by one, or a few, genes and is often race-specific (Van der Plank 1966). Race-specific resistance does not protect against an entire pathogen species, and a small proportion of the pathogen population may still be able to infect and cause disease on resistant host genotypes. In these cases, durability is quickly lost as this pathogen subset expands and occupies the niche left by incompatible strains of the pathogen (McDonald and Linde 2002). In other cases, mutations are required for the pathogen to be able to infect a resistant genotype. Qualitative resistance often involves an interaction between a single resistance gene in the plant host, and a single avirulence gene in the pathogen. This means that a mutation in a single gene could allow pathogens to bypass recognition and overcome this kind of resistance (Lo Iacono et al. 2013). In addition, the large effect that qualitative resistance has on the pathogen, because pathogen multiplication/survival is greatly affected, puts high selection pressure on the population. Quantitative resistance puts less selection pressure on the pathogen because it involves many genes, each one responsible for a small part of the overall resistance. Many mutations would be required before the quantitative resistance was overcome by the pathogen.

Several factors affect the expression of inherited resistance in cacao, and clones may not be equally resistant in different geographic locations, particularly when it is controlled by minor effect genes (i.e. quantitative resistance). Before selecting clones for use in further crosses or commercial production, they should be tested in different locations in order to determine how stable their resistance is under a wide range of conditions. Factors such as nutrition, temperature, and light intensity can affect resistance in the field (Ward 1889). Also, pathogen species are genetically diverse and populations differ between cacao production areas, so resistance that is effective in one area may not be effective or stable in other areas (Bowers et al. 2001).

The durability of any resistance source is determined by the inability of the pathogen to adapt over time. There are few reports of resistance durability or stability in cacao (Rios-Ruiz 2001). In the clone SCA 6, resistance to witches' broom has been durable in Trinidad (Laker et al. 1988a, b), but not in Ecuador (Bartley 1977) where another strain of *M. perniciosa* is prevalent (Wheeler and Mepsted 1988), nor in Peru where the tree experiences somewhat high rates of infection (Rios-Ruiz 1989). Pathogen isolates from Ecuador induced more severe symptoms on several cacao clones than isolates from Trinidad, particularly those that had SCA 6 as a parent (Wheeler and Mepsted 1988). *M. perniciosa* can be divided into two groups based on their ability to produce symptoms on SCA 6 (Wheeler and Mepsted 1988). Pathotype A, which occurs in Ecuador, Bolivia, and Colombia is more pathogenic than pathotype B which is present in Trinidad and Brazil (Wheeler and Mepsted 1988). The resistance of the clone IMC 67 to Ceratocystis wilt appears to have regional stability (Rios-Ruiz 2001). To determine the stability of resistance to *M. roreri*, Phillips-Mora et al. (2005) inoculated several clones with seven isolates of the pathogen, belonging to four pathogen groups. The clone ICS 95, which had shown resistance to the pathogen in Colombia (Arguello-Castellanos 1997), Costa Rica (Phillips-Mora 1999), and Peru (Evans et al. 1998), was highly resistant to all isolates tested (Phillips-Mora et al. 2005), indicating it may be possible to breed clones with broad resistance to *M. roreri*. It is unclear, however, whether the resistance to *M. roreri* will be durable.

18.8 Genetic Diversity and Cacao Breeding Programs

World cacao production more than doubled between 1984 (1.5 million tons) and 2006 (3.8 million tons) (Eskes 2011) mainly due to a substantial increase in the area under production. Since continued expansion of the area under cultivation is unsustainable, future production increases should come from increases in productivity. This could be achieved by using high-yielding and disease-resistant planting material.

Breeding for disease resistance is one of the six traditional principles of plant disease control (Anonymous 1968). Host resistance is the ability of the host to hinder the growth and/or development of the pathogen (Robinson 1969). It usually

functions by impeding the multiplication of pathogen, which then reduces the infection rate (Parlevliet 1979). Cacao breeding involves germplasm collection and characterization, genetic studies, and the creation of new clones for distribution to farmers.

Very limited numbers of superior *T. cacao* clones are available for farmers, only 30 % of trees currently being cultivated are selected varieties, and most of these are progeny from biparental crosses (full-sib families) (Bhattacharjee and Kumar 2007). Productivity and disease resistance are the primary traits targeted by cacao breeders. Cacao genotypes with at least some level of resistance to black pod (Efombagn et al. 2007; Iwaro et al. 2006; Tan and Tan 1990; Thevenin et al. 2012), witches' broom (de Albuquerque et al. 2010; Lima et al. 2013; Santos et al. 2005; Surujdeo-Maharaj et al. 2003, 2004), and frosty pod (Phillips-Mora et al. 2005) have been identified. However, long generation times make breeding tree crops particularly expensive and time-intensive (Irizarry and Rivera 1998) and it can take decades for new cacao clones to become commercially available.

Some serious diseases, such as witches' broom and black pod, are caused by organisms that have evolved with *T. cacao*. Resistance to these can often be found in a crop's center of origin (Leppik 1970; Simmonds and Smart 1999). Indeed, genotypes from the upper Amazon, considered the center of origin of cacao, presented greater frequency of characteristics associated with *Phytophthora* resistance than was found in widely cultivated genotypes from Mexico, Central America, the Caribbean, and Bahia, Brazil (SIAL and EEG) (Pires et al. 2000). These collections also contained a greater proportion of witches' broom-resistant genotypes, mainly from Peru (SCA 6 and SCA 12) and from Acre, Brazil (CSul and RB) (Pires et al. 2000).

It can be more difficult to find germplasm resistant to new-encounter diseases, where pathogens emerged following the introduction/cultivation of a crop in a new location or the introduction of the pathogen to new locations (Anderson et al. 2004). Cacao has been particularly susceptible to new-encounter diseases, which include CSSV and VSD (Evans 2007; Lenne and Wood 1991). Pathogens affecting indigenous hosts quickly adapted to cacao after it was introduced and cultivated on a large scale (Evans 2007). For example, CSSV affects *Cola chlamydantha*, a common West African forest tree, and other species to a lesser extent (Posnette 1981). The original host of *Ceratobasidium theobromae* in Southeast Asia and Melanesia, where VSD first appeared on cacao, has not been identified (Keane 1997). Although it can be more difficult to find resistance to new-encounter diseases (Lenne and Wood 1991), genotypes were found with resistance to both CSSV and VSD and have been used as parents in the breeding programs in Ghana and Papua New Guinea (Legg and Lockwood 1981; Tan and Tan 1988). Results from inoculation studies in Ghana indicate that resistance to CSSV is not confined to a single group of cacao (Adomako et al. 2006).

Yield is a major objective in any breeding program and has been an aim of cacao improvement programs since the 1930s, when the first program was established in Trinidad. It is usually measured by counting the number of pods or weighing the freshly harvested seeds. The number of pods can be converted into yield by using

the pod index, which is the amount of pods necessary to produce 1 kg of dry cacao. The three main yield components are the number of pods per tree or area, the number of beans per pod, and the weight of 100 individual beans (also known as seed index). However, productivity is highly influenced by environmental factors, and seed production per pod can vary substantially even within the same tree (Bartley 2005). On a global scale and due to their great relevance, productivity and disease resistance have received the most attention from breeders, followed by sexual compatibility and quality (Lopes et al. 2011). Despite early work on disease resistance, yield remained the primary focus of cacao breeding programs until the early 1980s (Andebrhan et al. 1998).

Germplasm Resources

There is significant genetic variability in the cacao germplasm collected from the Upper Amazonian region, which is considered its center of diversity (Lo Iacono et al. 2013; Motamayor et al. 2008). Much of the disease-resistant germplasm used in cacao breeding programs was collected in the Amazon and along its tributaries. These collection expeditions found significant morphological differences among geographically separated populations (Allen and Lass 1983; Evans et al. 1977) along different river tributaries (Bartley 2005).

Many expeditions have been carried out throughout Latin America to collect *T. cacao* germplasm for breeding and conservation, but labeling errors, as well as the lack of knowledge concerning its relationship to cultivated material, has limited its use in breeding programs (Motamayor et al. 2008). Until recently, cacao germplasm had been classified into three major morphological/geographic groups: Criollo, Forastero, and Trinitario (Motamayor et al. 2008). After analyzing 1241 accessions with 106 Simple Sequence Repeat (SSR) markers, Motamayor et al. (2008) proposed a new classification that divided germplasm into 10 major groups: Marañon, Curaray, Criollo, Iquitos, Nanay, Contamana, Amelonado, Purús, Nacional, and Guiana. This new classification, which may be expanded in the future, reflects the genetic diversity available to breeders more accurately than previous classifications. Now the genetic relationships among new and old germplasm resources can be easily established using molecular tools.

Cultivated populations and secondary centers of diversity also contain substantial genetic diversity and can be valuable sources of germplasm for breeding programs (Bartley 2005; Lenne and Wood 1991). Farmers in Cameroon identified trees with potential resistance to *P. megakarya* in fields under high disease pressure (Efombagn et al. 2007). Although these farmer selections were less resistant than some accessions from local germplasm collections, they are still considered valuable additions to the breeding program (Efombagn et al. 2007). A search for trees resistant to *M. perniciosa* on plantations in Bahia, Brazil, also gave promising

results (Pinto and Pires 1998) and the genetic diversity among farmer selections is also being considered in other cacao-producing areas.

Ultimately, the future of the world cacao economy depends on the availability of genetic diversity and the sustainable use of this broad genetic base to breed improved varieties (CacaoNet 2012). Cacao genetic diversity is an essential element in the development of new and improved varieties to achieve a more sustainable and cost-effective means of cacao production, thus contributing to the economies of cacao producing countries. Over the past 100 years, cacao germplasm has been collected from the wild, farmers' fields, and genetic improvement programs. This material is currently grown in germplasm collections, living gene banks controlled by research institutes in the respective countries where they are located (Turnbull et al. 2010). In addition to safeguarding genetic diversity, several of these centers also characterize the material, recording morphological and agronomical characteristics, as well as evaluating disease resistance, and plant and seed chemistry.

There are many national collections in the Americas, the center of origin of the crop, and in cacao-producing countries in Africa and Southeast Asia. Over 40 collections maintain more than 24,000 accessions of cacao (CacaoNet 2012). The largest collections in Africa are maintained by the Centre National de la Recherche Agronomique of Ivory Coast (CNRA) and by the Cocoa Research Institute of Ghana (CRIG) (Eskes and Efron 2006). Brazil, Colombia, Ecuador, French Guiana, Mexico, Venezuela, and Bolivia have collections with local genotypes (Dias and Resende 2001). Brazil has focused on cacao genetic conservation and has carried out numerous collecting trips in the Amazon (Almeida et al. 1987). Over 90 % of the accessions in the germplasm collection in Belem, Brazil are from the wild. It is one of the most extensive collections of wild cacao, established with material CEPLAC scientists collected from the Brazilian Amazon (Almeida et al. 1987).

Two international collections are managed by the Cocoa Research Unit of the University of the West Indies (CRU/UWI), Trinidad and Tobago, and the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), Costa Rica (CacaoNet 2012). These two institutes have entered into agreements with the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) to maintain global collections of cacao genetic resources for long-term conservation and to make this germplasm freely available to any qualified institution or individual (CacaoNet 2012). Trinidad's conserved cacao accessions include genotypes from the Upper Amazon collections made by F. J. Pound in the early 1900s with the specific goal of locating material resistant to witches' broom disease (Turnbull et al. 2010).

Most collections have some degree of duplication, internally and with other collections (CacaoNet 2012). At the same time, only a few have a strategic safety duplication of their unique materials at a different site to guard against natural disasters (e.g., CEPLAC and CATIE's Genebanks). Misidentification of trees within collections, which can be as high as 30 %, is also a significant problem in every collection (Motilal and Butler 2003; Motilal et al. 2013).

The International Cocoa Germplasm Database (ICGD) is the primary source of information on available germplasm, including origin, morphological and agronomic characteristics, susceptibility to pests and diseases, and the location where it is being held (Motilal and Butler 2003). It was set up in 1990 to organize data from cacao accessions and make them publicly available. The database is continually being updated, with photographs and data of new and existing genotypes. ICGD contains information on approximately 13,800 genetically distinct accessions which are held in over 40 major genebanks and quarantine stations around the world (www.icgd.rdg.ac.uk). This database provides a single point of reference, and can be used by breeders to search for material with particular traits. Systematically screening genebank accessions and uploading the resulting information into the database would greatly benefit breeding programs.

Breeding Strategies

Most cacao trees currently in the field are the product of mass selections carried out by farmers over generations and are derived from few individuals (Bartley 2005; Hunter 1990). Although these farmer selections produce morphologically similar trees with increased homozygosity, these populations are still heterogeneous and are composed of largely heterozygous individuals (Bartley 2005).

Scientists worldwide are looking for ways to accelerate the production of cacao trees that can resist emerging pests and diseases, tolerate droughts, meet manufacturer's needs, and produce higher yields (CacaoNet 2012). In addition to finding sources of resistance, breeding disease-resistant plants involves crossing and selecting genotypes in order to accumulate desirable genes for resistance and agronomic traits (Keane 2012).

The first step in assessing the potential role of a genotype in a breeding program is to determine the characteristics it possesses (Bartley 2005). Phenotype is the observable properties of an organism and is controlled by genetic makeup and the environment. Once desirable characteristics/phenotypes have been identified, breeders must determine how the loci controlling these traits are inherited. Inheritance dynamics in cacao are known for a few traits such as coloring, compatibility, and seed size that have been researched in detail (Bartley 2005). Breeding programs in other allogamous crops like maize have generated many families through inbreeding and hybridization, and segregation of many traits have been analyzed to determine their inheritance. It can take many years of observation to build an adequate phenotypic basis for analyzing a trait's inheritance, especially in cacao due to the number of years it takes for plants to produce fruit.

Cacao is grown commercially using either clones or seedlings, and different breeding strategies are used for each. In much of West Africa, seedlings from biparental crosses (full-sib families) are used to establish commercial farms. Many farmers plant seeds from their best-performing trees, however, the resulting progeny often do not breed true and may have low productivity or be very

susceptible to diseases (Bowers et al. 2001). Since cacao is primarily an open-pollinated species, trees with desirable traits often produce offspring lacking these traits (Bowers et al. 2001).

Crosses between genetically distinct genotypes can produce progeny that are more productive and resistant to disease than the parents, a process known as heterosis (Shull and Gowen 1952). Heterosis, also known as hybrid vigor, was the basis of cacao breeding between the 1950s and 1990s (Bhattacharjee and Kumar 2007). To breeders of maize, and other crops, the term hybrid describes the offspring of two homozygous (inbred) parents (Ordas et al. 2012). In cacao, however, hybrid refers to the offspring of a cross regardless of the parents' level of homozygosity.

At CRIG in Ghana, Upper Amazon parents are crossed with Trinitario parents and the full-sib families are evaluated for productivity, resistance to CSSV, and resistance to black pod. Once good families have been identified the parental clones are placed in seed gardens. Most of the Upper Amazon parents are self-incompatible, so seed production is by natural pollination in biclonal gardens, and the seed is distributed to farmers as a hybrid family. Unfortunately, many of the original Upper Amazon clones used as parents in the 1940s and 1950s are still being used in West African programs, without having undergone any improvement through recurrent selection (Wood and Lass 1985). Average yields in West Africa are still around 300–400 kg/ha, while in Asia and the Americas, each hectare produces 500 kg and 500–600 kg, respectively. Within a continent, yield varies by country and the type of cacao being cultivated (WCF 2015).

In the Caribbean, South Asia, and parts of South and Central America, cacao is usually propagated using clones. Malaysia and Indonesia successfully implemented cloning for commercial cacao production in the 1980s (Wood and Lass 1985). The breeding program in Trinidad has concentrated on the development of new clones using single tree selection for low pod index, resistance to black pod, and resistance to witches' broom (Iwaro and Butler 2000; Surujdeo-Maharaj et al. 2004). Since clones are genetically identical, they maintain the traits of the original genotype such as disease resistance and productivity. Scions can be grafted directly onto fruit-bearing trees to replace existing canopies, as has been done with witches' broom-susceptible canopies in Brazil. This makes use of the existing root system, and farms can be commercially profitable within a couple of years (Dias 2001).

Most cacao breeding programs have only made modest gains in the genetic improvement of cacao. One of the main reasons for this is the lack of standardized production procedures and the fact that many of the programs are severely underfunded and not able to conduct proper phenotypic evaluation. Misidentification of parents has also been a significant problem, and many of the full-sib families evaluated from select parents have significant pollen contamination (Schnell et al. 2005). Disease-resistant germplasm has been identified in Colombia (Arguello-Castellanos 1997), Ecuador (Delgado et al. 1960; Suárez-Capello 1999), Peru (Arévalo et al. 1999), and Costa Rica (Phillips-Mora 1999), but is relatively uncommon.

As in other perennial crops, genetic improvement in cacao is very slow; a single selection cycle frequently takes more than a decade and it is often necessary to complete two or more cycles before being able to release a new variety (Phillips-Mora et al. 2012). Even for annual crops it has been estimated that the development of a new variety requires 10–20 years of work (Briggs and Knowles 1977). Many cacao breeding programs stop after the first generation, they cross accessions from germplasm collections, and the best hybrids, or full-sib families, are recommended for planting (Bhattacharjee and Kumar 2007). Other programs have a more structured approach and make crosses according to factorial, diallel, or other, mating designs (Ndoumbé et al. 2001). Structured breeding uses mating designs with parents that have complementary traits of interest (e.g. resistance to different diseases); well-designed trials minimize the number of years needed to develop a new cultivar.

Diallel Crosses

Lines being evaluated may be crossed in a diallel design, where genotypes are crossed in pairwise combinations. In a complete diallel, each line is crossed with all others, but a partial or incomplete diallel cross can be used instead, if the number of progeny is too large. Diallel crosses are widely used to estimate the general combining ability (GCA) and specific combining ability (SCA) of genotypes in regards to quantitative traits (Bos and Caligari 2008; Griffing 1956). Parameters such as GCA and SCA provide information on the characteristics of the gene (s) controlling trait expression, and they help breeders choose parents that are more likely to produce high-performing offspring (Machikowa et al. 2011).

The GCA is the average performance of a genotype and it is related to narrow sense heritability and can be used to estimate additive gene action (Falconer and Mackay 1996). GCA depends on how closely related the parents are (Bos and Caligari 2008). SCA is the difference between expected performance based on GCA of the parents and the actual performance of a parent in a specific cross (Machikowa et al. 2011; Sprague and Tatum 1942). SCA can be used to estimate nonadditive gene actions (Falconer and Mackay 1996).

Diallel analyses have been used in many crops, including cacao, to determine genetic parameters of quantitative traits in cases where parents differ significantly (Arunga et al. 2010; Rivaie 2013). Larger populations are needed when parents have low levels of heterozygosity (Dickinson and Jinks 1956). In cacao, crosses are done by isolating flowers with an artificial barrier and hand-pollinating them with pollen from the selected genotype (Rivaie 2013). This prevents pollen contamination from nearby trees not selected for the cross.

Recurrent Selection

Cacao breeding is currently based on recurrent selection (RS) from populations with a wide genetic base (Baudouin et al. 1997). Breeders can use molecular markers to select the most genetically diverse parents, which increases the chances of accumulating resistance genes from different sources, and hopefully with different modes of action (Baudouin et al. 1997). Genotypes resistant to witches' broom disease have been crossed with the goal of accumulating different resistance genes within a plant, thereby increasing its durability (Lopes et al. 2011).

Recurrent selection is a cyclical approach, where the best performing progeny in a population are crossed, the resulting population is evaluated, and the cycle is repeated. The goal is to increase the frequency of desired alleles in each successive population, improving its overall performance, and the performance of the best individuals within it (Gallais 1978). RS is effective for combining several traits, and ideally, the improved population will retain its genetic variability (Baudouin et al. 1997).

Reciprocal recurrent selection (RRS) is a variation of recurrent selection where lines A and B are crossed in a reciprocal manner. This means that A females are crossed with B males, and A males are crossed with B females, generating two full-sib families AB and BA (Kojima and Kelleher 1963). Both populations are evaluated in a single cycle.

The Ivory Coast began an RRS approach in 1990, using two genetic groups differing in several target traits. One group is composed of Upper Amazon selections that have shown good combining ability for early yield and resistance to black pod. The other group is composed of genetically similar lower Amazon and Trinitario selections, which have shown high yield potential, self-compatibility, and flavor (Pokou et al. 2009). The goal is to develop high-yielding plants that are more resistant to disease (Pokou et al. 2009). Twelve of the 55 parental clones included in the program had good levels of resistance to BP (Lachenaud et al. 2000).

Cacao Breeding Programs

Organized cacao breeding began in Trinidad in the 1930s, when the geneticist, Dr. F. J. Pound was given the task of improving Trinidad's cacao population in terms of productivity and resistance to witches' broom (Monteiro et al. 2009). Pound selected trees around the island based on yield components. These clones, a total of 108, are known as Imperial College Selections (ICS) (Pound 1934, 1935, 1936). Some of them like ICS 95 are still planted in many commercial fields around the world and have also been used as parents in cacao breeding programs. The cultivar CCN 51 has ICS 95 as one of its progenitors (Boza et al. 2014). Also an outbreak of witches' broom in the country prompted studies into disease resistance

and expeditions into the Amazon in search of resistant material (Pound 1938, 1943). Around 2,000 genotypes were derived from the material (pods) collected in these trips, including SCA 6 and SCA 12, two of the most frequently used resistant sources in breeding programs worldwide (Monteiro et al. 2009). Although they were initially described as being immune to witches' broom, they were reclassified as resistant after developing mild disease symptoms (Rios-Ruiz 2001).

Breeders in Trinidad developed the Trinidad Select Hybrids (TSH) using consecutive cycles of crosses and recurrent selection for over 60 years. The initial goals were high-yielding plants resistant to *Ceratocystis* and *M. perniciosa* (Maharaj et al. 2011). Clones were selected for commercial distribution at several points during the breeding program: early selections (TSH 728, 730, 919, 973, 1076, and 1095), mid selections (TSH 1102, 1104, 1188, and 1220), and recent selections (TSH 1313, 1315, 1330, 1344, 1347, 1350, 1352, 1362, 1364, and 1380) (Maharaj et al. 2011). These clones appear to maintain their disease resistance in the germplasm collection and commercial farms (Laker et al. 1988a). The potential yield of the Trinidad Select Hybrids (TSH) is 2,000 kg/ha while the average yield of unimproved seedlings is approximately 300 kg/ha.

In Brazil, the planting material used by farmers was derived from the early introductions of "Cacau Comun" for over 200 years after the introduction of cacao. After the 1940s, plant selections were made in plantations by researchers working at institutions such as the Cacao Institute of Bahia (ICB), Agronomic Institute of East (IPEAL) and Experimental Station of Goytacazes (EEG) (Lopes et al. 2011). With the foundation of the Executive Commission of the Cacao Plan (CEPLAC), the focus changed to the development of interclonal hybrids (full-sib families) between germplasm of different origins (Dias and Kageyama 1995). Upon the arrival of witches' broom in 1989 and Ceratocystis wilt (*Ceratocystis cacaofunesta*) in 1990, breeding for resistance became an important goal in Brazil and the breeding programs currently concentrate on the development of resistant clones (Lopes et al. 2011). Black pod resistance is a priority, and since the arrival of *M. roreri* to Bolivia, researchers in Brazil have begun to evaluate their clones for frosty pod rot resistance by conducting field trials in neighboring countries such as Peru.

A breeding program at the Tropical Agricultural Research and Higher Education Center (CATIE) in Costa Rica has been developing high-yielding cacao varieties with tolerance to frosty pod rot and black pod, and with superior quality profiles (Phillips-Mora 2010). Although frosty pod rot is currently confined to 13 countries in the Americas, its potential for dispersal to other countries and continents is high, placing the global chocolate industry at risk. To facilitate their breeding program, CATIE developed artificial inoculation methods for evaluating the response of material in their genebank to frosty pod (Phillips-Mora and Galindo 1988; Sánchez et al. 1987) and black pod (Phillips-Mora and Galindo 1989). After evaluating nearly 800 clones to frosty pod rot at CATIE, it was concluded that resistance is an uncommon trait since only 10 % of the material showed resistant (2 % loss) or moderately resistant (8 % loss) responses (Phillips-Mora et al. 2012). For black pod the situation has been more favorable, with nearly a third of the clones showing

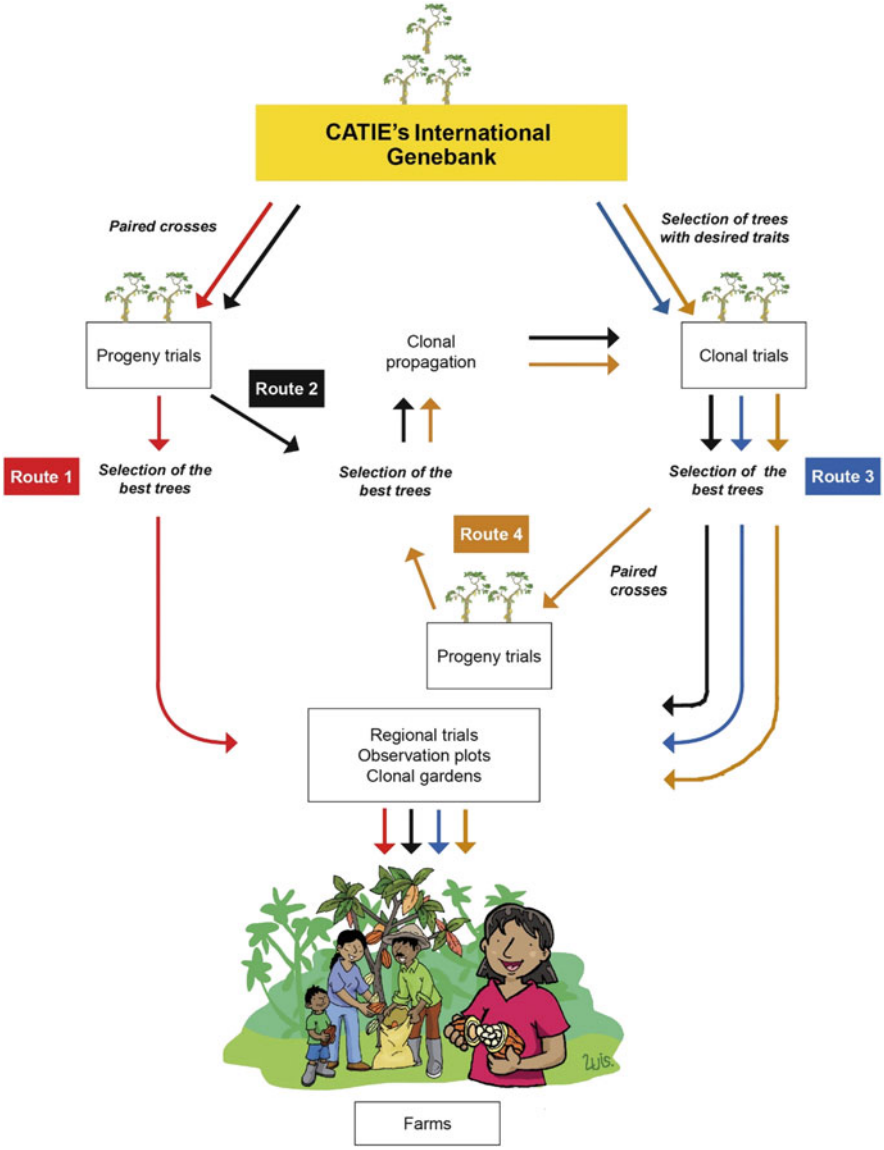

Fig. 18.4 Strategies for breeding cacao

high levels of resistance. In 2007, the program released four superior clones that are currently available to more than 10,000 farm families in Central America and Mexico (Fig. 18.4). Clone CATIE-R6 has an outstanding combination of traits: high resistance to frosty pod and black pod, high yield, and a fine flavor profile, receiving an award at the 2009 Salon du Chocolat, in Paris.

Peru is best known for the germplasm collected by Pound in the 1930s. The Peruvian breeding began in the 1950s and focused on high-yielding material, but emphasis had shifted to witches' broom resistance by the following decade. Unfortunately, many clones were lost during the 1980s due to problems stemming from political instability (Arévalo et al. 1999). In the 1990s, selections were made on abandoned farms under heavy witches' broom or frosty pod rot pressure. Further testing showed some of these Huallaga and Ucayali collections to be resistant to *M. perniciosa* (Arévalo et al. 1999). Although nearly all Peruvian accessions are highly susceptible to *M. roreri*, reduced sporulation occurs on ICS 95 and some clones from the Uycali region (Rios-Ruiz 2001).

Ecuador is known for its high quality cacao. They are the top producers of beans used in fine flavor chocolates worldwide and in 2013 surpassed Brazil as the leading cacao producer in the Americas (Gill 2014). The first scientific inquiry into frosty pod rot was carried out in Ecuador by J. Rorer, a plant pathologist who visited the country from 1917 to 1925. This work led to the first identification of the pathogen, *M. roreri*, and to the first report of witches' broom in Ecuador (Rorer 1926). Very little research was done on cacao diseases after this, until the 1940s, when the Tropical Experimental Station was established at Pichilingue (Evans et al. 1977). The cacao breeding program initially focused on resistance to witches' broom and frosty pod but was expanded to include Ceratocystis wilt once it was discovered that widely planted Trinitario clones were highly susceptible to it (Evans et al. 1977). In the early 1960s, Homero Castro developed the highly productive and disease-tolerant clone, CCN 51, which is used in 90 % of all new plantings in Ecuador. CCN 51 is used as a parent in many cacao breeding programs worldwide due to its high general and specific combining abilities (Boza et al. 2014).

Cacao was first introduced to Africa in the late 1800s (Wood and Lass 1985). In the late 1930s, a cacao breeding program emphasizing resistance to CSSV began at the Central Cacao Research Station, which later became the West African Cacao Research Institute (WACRI) (Wood and Lass 1985; Fowler 1948). Amazon materials were introduced in the 1940s and distributed as unselected bulk varieties to farmers in Nigeria, Ghana, and some areas of Western Cameroon (Frison and Eskes 1999). The introduced Amazon germplasm was more disease resistant, particularly to CSSV. WACRI was dissolved in 1962, following the independence of Ghana and Nigeria and was reestablished as the Cocoa Research Institute of Ghana (CRIG). This center is currently responsible for cacao breeding research in the country. The Centre Nacional de Recherche Agronomique (CNRA) in Cote d'Ivoire has been focusing on black pod resistance for the past few decades and relies on direct involvement of farmers in selecting resistant genotypes on their farms (Pokou et al. 2008). Farmers in areas with high disease pressure have selected trees for inclusion in the breeding program.

In South Asia, the programs at the Indonesian Coffee and Cocoa Research Institute (ICCRI), Papua New Guinea Cocoa and Coconut Institute (CCI) and at the Malaysian Cocoa Board (MCB) have concentrated on the development of clones with resistance to cocoa pod borer, VSD, and black pod, and a number of productive new clones have been selected in these programs.

In Indonesia, cacao was introduced in the early 1900s and production came mainly from Criollo types that later became known as the Java Criollo. Due to their susceptibility to *Phytophthora* canker, new clones called DR were developed but they only performed well in East Java. In the 1970s, a breeding program was started by the research Institute of Sumatra Planters (RISPA) and they selected 130 clones (Wood and Lass 1985). Currently, the Indonesian Coffee and Cocoa Research Institute (ICCRI) maintains an active cacao breeding program concentrated in the deployment of high-yielding clones with resistance to VSD and black pod.

During the 1940s, Upper Amazon material from the WACRI's 1944 introductions in combination with several Trinitario accessions were introduced into Malaysia. Some of these accessions showed resistance to VSD. In the early 1950s, Amelonado pods were introduced from Ghana and distributed to stations across the country. However, this material was susceptible to VSD and did not perform well (Wood and Lass 1985).

Cacao of Trinitario type was introduced to Papua New Guinea in the early 1900s. After years of selections at Keravat, New Britain, several clones known as the "Kerava" clones were released in the 1950s (Wood and Lass 1985). Later, in the 1980s, SG 1 and SG 2, interclonal hybrids (full-sib families) obtained from crosses between the two racial groups, Trinitario and Upper Amazon Forastero, were developed and released by PNG CCRI (Efron et al. 2005). Presently, their breeding program concentrates on the development of clones with resistance to VSD as well as black pod, since these are major factors limiting cacao production in the country.

In 1955, the U.S. Department of Agriculture initiated a program for the quarantine and distribution of cacao cultivars as a service to the cacao research centers located in the tropics (Fisher et al. 1960; Soderholm and Vasquez 1985). This activity was divided between two locations, with the Miami station serving as the quarantine facility and the Mayaguez station maintaining the germplasm. This activity was partially supported by the American Cocoa Research Institute and continued until 1992 when Hurricane Andrew destroyed the quarantine facilities and cacao collections at Miami. Cacao breeding programs around the world also suffered during the 1990s as a result of low cacao prices. Some programs had to reduce their activities, while others stopped completely due to lack of funds (Eskes 2011). Another quarantine facility, known as the International Cocoa Quarantine Centre, is located at the University of Reading, in the UK and facilitates the transfer of germplasm free of pests and diseases to Africa, Asia, and Australia/Oceania. This center is financially supported by the University of Reading, the UK chocolate and cocoa industries, and the USDA-ARS.

In 1998, the USDA-ARS restarted the cacao breeding program based at the Subtropical Horticultural Research Station in Miami. The primary goal of the program is to develop a biotechnology-based approach to improve *T. cacao* for increased disease resistance, especially to witches' broom, frosty pod, and black pod. The USDA-ARS and Mars Inc. are currently coordinating a joint international cacao genetics project that feeds information into national and regional breeding

programs to increase the efficiency of developing superior varieties for cacao farmers worldwide. This collaboration uses marker-assisted selection (MAS) to reduce the time and resources needed to develop improved clones.

18.9 Genomics-Assisted Breeding

Cacao improvement programs depend on the availability of substantial genetic diversity together with an understanding of how best to use it. Powerful new technologies, such as molecular genetics, genomics, proteomics, and eco-geographical remote-sensing techniques, have increased the value of these genetic resources (CacaoNet 2012). The sequencing of two different cacao genomes, the Belizean Criollo genotype B97-61/B2 (Argout et al. 2011) and a Costa Rican Forastero genotype, Matina 1-6 (Motamayor et al. 2013) has substantially increased the amount of resources available for the development of new molecular markers that can help in the development of new cacao varieties. These new tools represent promising steps in advancing breeders' ability to deliver improved trees to farmers, and along with advances in informatics, have markedly increased the capacity to use, analyze, and communicate related data and information.

Over 600 microsatellite markers have been developed and mapped by Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) and USDA-Miami (Brown et al. 2005; Lanaud et al. 1999, 2009; Pugh et al. 2004). Microsatellite alleles differing in length have been assayed by Polyacrylamide Gel Electrophoresis (PAGE) as well as Capillary Array Electrophoresis (CAE). Unfortunately, SSR marker results from different laboratories are difficult to compare due to variation in allele sizes from platform to platform, which complicates the sharing of genotype data from different research groups (Livingstone et al. 2011).

Recently, single nucleotide polymorphism (SNPs) markers have been developed and used in genetic research in humans, animals, and several crop systems (Altshuler et al. 2000; Ha et al. 2007; Page et al. 2002). SNP markers are PCR-based markers, biallelic, unambiguous, and can be screened by a variety of non-electrophoretic means. In contrast to SSRs, they are platform-independent and their results are easily repeatable. In addition, SNPs are found more frequently than SSRs in the coding regions of genes (Rafalski 2002). In cacao, SNPs have been developed and used for comparative genomic studies, consensus genetics maps, marker-assisted breeding, and determining off-types in clonal collections (Allegre et al. 2012; Ji et al. 2013; Kuhn et al. 2012; Livingstone et al. 2011, 2012; Takrama et al. 2012, 2014). More recently, Livingstone et al. (2015) released a 6K SNP array that represents an additional tool for use in genomics-assisted breeding projects.

18.10 Status of Breeding for Disease Resistance

Black Pod

Resistance to black pod was found to be inherited in a quantitative manner, with narrow sense and broad sense heritability estimated to be 0.33 and 0.51, respectively (Iwaro and Butler 2000). Thevenin et al. (2012) assessed the resistance of 186 accessions belonging to the Guiana group (Motamayor et al. 2008) in relation to the Guianan strain (GY 27) of *P. palmivora* and established that the Guiana genetic cluster is an important source of resistance to *P. palmivora*. Fifty nine of these clones were as resistant to *P. palmivora* as SCA 6, which is used as the resistant control. In addition, resistance to *P. megakarya* was found in wild material collected in French Guiana (Paulin et al. 2008). A significant amount of work has been done to locate QTLs for resistance to black pod. So far, 65 QTLs have been reported in linkage groups (LG) LG1, LG2, LG4, LG5, LG8, and LG10 (Brown et al. 2007; Lanaud et al. 2009); however, only 13 of them are considered consensus QTLs and they are located on LG1, LG2, LG4, and LG5. Resistance has been evaluated using percent pod rot in the field and leaf disk inoculation; however, a weak correlation often exists between the two methods of measuring resistance (Risterucci et al. 2000). Different QTLs were detected with each method, indicating perhaps different mechanisms of resistance or low repeatability of the assay.

Frosty Pod

The last 30 years of research at CATIE have resulted in identification of frosty pod tolerant clones with distinct genetic and/or geographic origins. Studies in Costa Rica (Enríquez and Soria 1999) indicated that resistance to *P. palmivora* and *M. roreri* is controlled by several genes. However, while *P. palmivora* resistance appears to be dominant, resistance to *M. roreri* is likely a recessive trait among the germplasm sources being used (Phillips-Mora and Castillo 1999). This explains the high proportion of black pod-resistant progeny resulting from a cross between clones that are resistant and susceptible to *P. palmivora*. A number of cultivars have been identified at CATIE that have resistance to *M. roreri* including ICS 43, UF 273, UF 712, and EET 75. The inheritance of resistance was studied in the progeny of these clones and results indicate that it is polygenic (Phillips-Mora and Galindo 1988). Five QTLs for frosty pod resistance have been reported to be associated with LG2, LG7, and LG8 (Brown et al. 2007; Cervantes-Martinez et al. 2006). Resistant clones are being crossed progressively to obtain varieties with increased levels of resistance, thus exploiting the predominantly additive character that this trait has in cacao (Cervantes-Martinez et al. 2006).

Witches' Broom

Inoculation methods have been developed to evaluate witches' broom resistance in the field (Sreenivasan 1995), in the greenhouse (Frias et al. 1995), and in the laboratory (Fonseca and Wheeler 1990). Host resistance can include several mechanisms, including the resistance of the canopy, cushions, flowers, and pods to infection by *M. perniciosa* (Pires et al. 1999). An agar block method designed for large-scale screening of seedlings can predict canopy resistance, but the relationship between canopy and pod resistance needs additional study (Surujdeo-Maharaj et al. 2003).

The most widely used sources of resistance to witches' broom are SCA 6 and SCA 12, two of the clones collected by Pound during one of his expeditions to the Amazon in the 1930s (Pound 1938). These clones and their progeny are resistant to witches' broom in Brazil and Trinidad, but not in Ecuador. There appear to be high levels of pre- and post-penetration resistance to *M. perniciosa* in SCA 6, and intermediate levels in its progeny, such as the series TSH and TSA (Pires et al. 1999). Also, additional sources of resistance to witches' broom have been identified in the clones CAB 208 and CAB 214, which are unrelated to SCA 6, the predominant source of resistance used in breeding programs (de Albuquerque et al. 2010).

Several QTLs associated with witches' broom resistance have been discovered. Using an F_2 population obtained from a cross of SCA 6 (resistant) by ICS 1 (susceptible), a QTL that accounts for 35 % of the variation with a dominant effect was reported by Queiroz et al. (2003). Later, Brown et al. (2005), utilizing a similar population, reported two QTLs with dominant effects. One major QTL is located on LG9 and a minor one on LG1. Subsequently, Faleiro et al. (2006), using the same population as Queiroz et al. (2003), identified that a major QTL is also associated with the SSR marker TcCIR 35 as previously reported by Brown et al. (2005). Lanaud et al. (2009) used a QTL meta-analysis approach to demonstrate the importance of a LG9 region associated with resistance to witches' broom and black pod.

CEPLAC is producing and distributing clones TSH 1188, TSH 565, TSH 516 (from Trinidad), CEPEC 42 (from Brazil), and EET 397 (from Ecuador) for renovating cacao farms by grafting these clones onto existing rootstocks to replace the witches' broom susceptible canopies (Ferreira 1997; Pinto and Pires 1998). They are productive and have demonstrated high levels of resistance in southern Bahia, but SCA 6 is the only source of witches' broom resistance in these materials (Pinto and Pires 1998).

Cuttings of resistant plant material are available, from Biofabrica, a Brazilian government supported nursery (http://www.biofabricadecacau.com.br). Resistant germplasm has been planted, or grafted onto existing rootstocks, on approximately 150,000 of the 500,000 ha under production in Bahia (Hebbar 2007). The seed-propagated "Theobahia" variety is moderately resistant (Ferreira 1997) and has also been widely distributed in Bahia (Monteiro et al. 1995).

Ceratocystis Wilt

Soria and Salazar (1965) evaluated 143 clones of the IICA germplasm collection as well as 239 trees that were the progeny of crosses between a resistant parent and a susceptible one and between two susceptible parents for resistance to Ceratocystis wilt. They concluded that the clones SPA 9, IMC 67, Pound 12, and PA 121 were resistant. Very few resistant progeny were observed among hybrids resulting from the cross of a resistant and a susceptible parent, however, resistant individuals were found among the progeny of crosses between susceptible parents. They proposed that resistance was controlled by up to three independent recessive gene pairs or a pair of recessive genes and one or more modifiers. Later Gardella et al. (1982), using the clones SPA 9, IMC 67, Pound 12, UF 613, ICS 1, and ICS 45, as well as their progenies, found that resistance to *Ceratocystis* in the clones SPA 9 and IMC 67 was controlled by a single dominant gene. The cultivar IMC 67 is often used as a rootstock as it is homozygous for the resistance-conferring allele, which acts in a dominant manner. Open-pollinated seedlings of this cultivar contain at least one copy of the allele and are resistant. The resistant alleles in IMC 67 were derived from Pound 18, one of the original seedlings collected by J.F. Pound in the headwaters of the Amazon, and the IMC (Iquitos Mixed Calabacillos) are seedlings of Pound 18. Up to this moment, this resistant gene has not been associated with any molecular marker and its location is unknown. Silva et al. (2012) evaluated seedlings of open-pollinated progenies of 20 diverse cacao clones that have shown resistance to witches' broom or resistance to Ceratocystis wilt. They found that the progeny of clones FCB 01, CSG 70, BOBA 01, VB 902, TSH 1188, VB 1151, PS 1319, and MAC 01 were the most resistant to the diseases. Also Santos et al. (2012), using 143 F_2 individuals originated from the selfing of hybrid-clone TSH 516 (SCA 6 $\times$ ICS 1), found that the resistance was quantitatively inherited and involved two QTLs located on LG3 and LG9. Most recently, Marelli et al. (2014) evaluated 266 plants obtained from a cross of a *Ceratocystis*-resistant genotype TSH 1188 and the susceptible CCN 51. Using SNP markers and simple interval mapping, they identified a 1.2 Mbp region of chromosome 6 associated with resistance. Using association mapping methodology, they also identified markers on chromosome 6.

Cacao Swollen Shoot Virus

Posnette and Todd (1955) observed that seedlings of the Upper Amazon parentage were less susceptible to CSSV and that the Nanay, Iquitos, Scavina, and inter-Nanay crosses were resistant to the virus. Also, resistant mutants have been produced in cacao by subjecting scion wood of the Upper Amazon clone T85/799 to gamma rays, and screening budded plants derived from this for resistance (Adu-Ampomah et al. 1996). Mutant clones obtained by this procedure were screened

with a high concentration of the severe strain 1A of CSSV and showed resistance. In Ghana, Padi et al. (2013) evaluated the level of resistance of a gamma radiation mutant, mvT85, that was derived from clone T85/799, as well as old and new cacao clones using the CSSV strain 1A. They also used SNP markers to genotype the clone T85/799 and the mutant mvT85. They concluded that there were no differences between the SNP profiles of the two, and there was an absence of segregation for resistance to CCSV in the full-sib and backcross mvT85-derived populations. Furthermore, they did not observe any differences in the level of CCSV resistance between mvT85 and T85/799. They also concluded that the older clones were more susceptible to CCSV and a longer period of 8 months was the most adequate for estimating the level of resistance among clones. In addition, they stated that clones obtained from crosses using Catongo, RB 49, and C-SUL 7 (Lower Amazon origin genotypes) demonstrated more resistance to CSSV. Recently, Ofori et al. (2015) studied the association between total phenolic content (TPC) and CSSV resistance. They inoculated 53 cacao families with the severe strain 1A of CSSV and evaluated disease severity after the first, second, third, and seventh flushes. Results indicated that even though they observed significant differences for disease severity, the broad and narrow sense heritability estimates were low. The correlation between TCP and disease severity was also low. They concluded that additive effects were involved in the inheritance of disease severity and that the clones GU 239/H, GU 290/H, and GU 225/V that presented positive GCA effects for CSSV resistance could be used as parents in the development of CSSV-resistant progenies.

Vascular Streak Dieback

Genetic variation for resistance to VSD exists, and it is durable and quantitatively inherited (Tan and Tan 1988). In crosses between Trinitario female parents and Amazonian male parents, all disease ratings had a significant GCA, while only the parents of dead plants were found to have significant SCA. Gene effects for resistance are additive, indicating that selection for progeny resistant to VSD is effective (Tan and Tan 1988).

In Indonesia, sources of resistance to VSD have been identified and tested in participatory plant breeding programs. Out of 49 clones that were tested only eight clones, including DRC 15, KA2 106, and a local Sulawesi selection, VSD2Ldg, were identified as resistant to VSD (McMahon et al. 2010). A PCR-based protocol has been developed to identify *C. theobromae* in cacao tissue, enabling early detection of the pathogen in plants (Samuels et al. 2012).

Breeding for VSD resistance has been a major objective of the Coconut and Cacao Institute (CCI) breeding program in Papua New Guinea, and genetic resistance has limited losses in commercial plantings. Upper Amazon clones and seedlings in the heterogeneous, partly outbreeding Trinitario population were screened, and promising individuals were crossed to produce the resistant hybrids that are now widely grown throughout the country (Guest and Keane 2007).

Recently, Epaina (2014) studied VSD and black pod resistance using a population derived from the cross between two Trinitario cacao genotypes: K82 and KA2-101. The former is resistant to black pod but susceptible to VSD, while the latter is susceptible to BP but resistant to VSD. He genotyped this population with SSR and SNP markers and used QTL mapping methodology to identify 10 QTLs associated with VSD resistance located on LGs 1,2, 3, 4, 5, 8, and 9.

Recommendations

Worldwide cacao losses due to diseases are very high; therefore, the development of high-yielding, disease-resistant varieties is a priority for cacao breeders. Mislabeling of accessions, low exchange of germplasm among breeders, differences in screening methodologies, and incomplete characterization of the pathotypes of fungi and or viruses used for screening have slowed the development of resistant varieties. Also, the lack of collaborations among private and public research institutions has limited cacao breeding progress.

To determine the stability of resistance while taking into account pathogen variability among locations, clones need to be evaluated in different countries. Misidentification can be minimized by using only plants that have been previously genotyped with molecular markers. The standardization of screening protocols used by researchers is also important. This includes artificial inoculation techniques for fungi, oomycetes, and viruses as well as measures of susceptibility and resistance. Standardization is expected to reduce some of the variation seen between experiments conducted in different countries on the same clones. In addition, the development of diagnostic tools to detect CSSV infection is paramount. Up to the present time, the available diagnostic tools that have been developed are not capable of detecting all the CSSV strains present across countries and within different regions of the same country. This situation has contributed to the spread of CSSV-infected material and slowed the development of resistant germplasm because breeders cannot determine the level of infection that is present in a plant that is not showing CSSV symptoms.

With the sequencing of the two different cacao genotypes, the Belizean Criollo B97-61/B2 and the Forastero genotype, Matina 1-6, an immense amount of molecular tools have become available. Thousands of SNP markers have been identified and are being used by breeders in the development of new cacao clones.

To date, several population-specific QTLs associated with resistance to BP, WB, and VSD have been discovered across the cacao genome, and have been mapped to known common regions in chromosomes 4, 8, and 9. This may indicate the presence of gene clusters associated with disease resistance. In addition, the development of methodologies such as genome-wide association studies and genomic selection could be used in the screening of accessions available in germplasm collections, as well as in farmers' fields, in order to discover new sources of genes for resistance against fungi and viruses, as well as associated with good

horticultural and quality traits. These novel genes could then be incorporated into ongoing breeding programs.

There is currently a need for an increase in cacao production, however, this cannot be achieved without greater collaboration among cacao researchers around the world. Since the early 2000s, cacao-breeder working groups have been created in Africa and Asia and most recently in the Americas. These organizations are essential because they allow the development of joint projects such as performance trials, exchange of germplasm, as well as the sharing of scientific information among breeders. These associations also allow increased communication between the public and the private sectors regarding their respective needs.

Following these recommendations will enable breeders to develop improved varieties in a shorter amount of time.

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Glossary of Terms

Abiotic Physical rather than biological; not derived from living organisms/relating to or resulting from physical phenomena.

Etiology The science of the causes of disease, especially the nature of the causal agent.

AFLP Amplified fragment length polymorphism.

Alleles Variants of a gene, occupying the same relative position on a chromosome and being separated during meiosis.

Alternative host A plant other than the main host on which the pathogen can survive.

Anamorph An asexual reproductive stage in a fungal life cycle.

Anastomosis The physical connect and mixing of two different fungal mycelia.

Antagonistic A biological structure or chemical agent that interferes with the physiological action of another.

Anthracnose A plant disease typically caused by *Colletotrichum*, characterized by having limited necrosis and black sunken lesions.

Applanate Flattened.

Appressed Closely flattened down.

Arriba Cacao from the Guayas Basin of Ecuador, derived from the indigenous Nacional variety.

Arthrospore A specialized uninucleate cell functioning as a spore and derived from the disarticulation of cells of a formerly vegetative branch.

Ascomycota The largest fungal phylum for which the ascus is the diagnostic character.

Ascospore A spore produced in an ascus by "free cell formation."

Asexual Not having or involving sexual union of gametes.

Atypical Different from what is common or typical.

Basidiocarp The multicellular structure of a basidiomycete fungus on which the spore-producing hymenium produced, a mushroom.

Basidioma A basidium-producing organ or fruit body.

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Basidiomycota Phylum in the kingdom Fungi, producing basidiospores in the sexual phase; include, for example, mushrooms, bracket fungi, earth stars, jelly fungi.

Basidiospore Reproductive spores from basidiomycete fungi.

Basidium (pl. basidia) Cell produced by Basidiomycotina on which (generally four) basiodiospores form.

Bifactorial mating system Each haploid nucleus has two mating-type loci; different alleles at each locus are needed for a sexual compatible interaction between hyphae from individuals of the same species.

Biocontrol (Biological Control) The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be.

Biological control Control of pathogens or pests using biological agents.

Biological species concepts In the chapter regarding *Ceratocystis* we utilize the phylogenetic species concept defining a species as the smallest aggregation of populations with a common lineage that share unique, diagnosable phenotypic and genetics characters.

Biotic Relating to or resulting from living organisms.

Biotroph An organism that acquires nutrients from a host without the death of the host tissues.

Biotrophic Obtaining nutrients from living host cells.

Bottleneck A sharp reduction in the size of a population due to environmental events or human activities that can change gene frequencies by sampling errors acting in a similar way to genetic drift.

Budwood Twigs with vegetative buds used for clonal propagation by grafting or budding.

Cabruca system Cultivation of cacao under thinned primary rain forest, as practiced in the Bahia region of Brazil.

Cacao Spanish corruption of the Nahuatl name for the tree (*Theobroma cacao*) and its product. In this text cacao is used specifically for the tree and its parts (i.e., cacao pod) and not for products produced from the tree (i.e., cocoa butter).

Caducous Separating easily or before the expected time.

Catongo Cacao variety possessing the anthocyanin inhibitor gene (shoots and beans lacking the purple coloration).

Cheilocystidium A sterile, distinctively shaped body occurring at the edge of an agaric basidioma (mushroom).

Chemotropic response Directed or oriented growth or movement in response to a chemical stimulus. The movement is either towards (positive) or away (negative) from the chemical stimulus.

Cherelle Immature pod of cacao, typically less than 2 months old.

Chitinolytic Being able to break down chitin.

Chlamydospore A thick-walled asexual resting spore that develops from a portion of a hyphal cell. The chlamydospore is a resistant propagule of indeterminate resting period.

Chlorosis Symptoms caused by the loss of chlorophyll, with characteristic paling, yellowing, or patchiness of leaf color.

Chromosome A unit package of DNA and proteins that is carried as hereditary material.

Chupon Vegetative offshoot of the stem allowing for the continual vertical growth of the plant.

Clade A group of organisms that consists of a common ancestor and all its lineal descendants, and represents a single branch on the tree of life.

Cladogram A diagram depicting possible branching lineages. Cladograms consist of clades and the branching points that differentiate clades and members are called nodes.

Clamp connection A hyphal outgrowth which at cell division and septum formation connects the resulting two cells; diagnostic of Basidiomycota.

Clavate Club-like.

Clonal lineage Organisms that share a common ancestor and have the same or highly similar DNA sequence.

Coenocytic mycelium Hyphal strands that have no dividing walls (aseptate) with a multinucleate cytoplasm.

Coevolution Cases where two (or more) species reciprocally affect each other's evolution. For example, an evolutionary change in the morphology of a plant might affect the morphology of its pathogen, which in turn might affect the evolution of the plant, which might affect the evolution of the pathogen. . .and so on.

Colony pattern A group of hyphae (mycelium) derived from single or multiple propagules that has a macroscopic recognizable shape or design as a whole during growth.

Compatibility type Versions of the same fungal species that can undergo sexual reproduction; mating types that are genetically similar but physiologically different and needing each other for sexual reproduction.

Complex of species A group of closely related species very similar in appearance, such that boundaries between them are often unclear.

Concolorous Of one color.

Conidia Sometimes termed asexual chlamydospores, or chlamydoconidia are asexual, non-motile spores of a fungus.

Conidiogenous Producing conidia.

Conserved region Stretch of DNA in which the sequence of nucleotides is unaltered.

Conspecific Belonging or referring to the same species.

Convergent evolution The independent evolution of similar features in species of different lineages, creating analogous characteristics that have similar form or function, but that were not present in the last common ancestor of those groups.

Correlated Concerning the relationship between two variables.

Corticioid Flattened, smooth (of basidiocarps).

Cyst A walled resting structure from single or multiple cells.

Cystidium Sterile body, frequently of a distinctive shape occurring at the surface of a basidioma, particularly the hymenium, from which it frequently projects.

Decurrent Of lamellae, running down the stipe.

Diatoms Algal form of plankton with silicaeous walls occurring singly or in small clusters.

Dieback Disease form characterized by death of shoot or branch, beginning at or near the apex and spreading back to older tissues.

Dikaryon (Dikaryotic) Fungus in which the cells have two genetically distinct haploid nuclei.

Dimidiate Shield-like; appearing to lack one half or having one half very much smaller than the other.

Diploid Having twice the haploid content of the genome.

Dissepiment A partition, e.g., between pore of a polypore.

Dolipore septum Septum with pores located in thickened region characteristic of some Basidiomycotina species.

Ectotrophic Growing outside the root or between the cells.

Effective microorganisms (EM) Beneficial organism used as an amendment.

Ellipsoid An object that is elliptic in outline; oval with tapered ends and widest in center.

Endophyte Organism inhabiting internal plant tissues without causing apparent harm.

Endospore An asexual spore that is formed within a cell.

Eukaryotic An organism whose cells contain hereditary material enveloped in a membrane.

Evolutionary forces The forces of evolution are processes that change gene frequencies in populations and they include genetic drift, mutation, migration, and natural selection.

Extragenic Not under the control of genes.

Fixation index A comparative measure ranging from 0 to 1 of genetic variability within and among populations that indicates the ability of the populations to interbreed freely (zero value) or non-breeding (unity value) from not having any shared genetic diversity.

Flower cushions Compact inflorescences formed on woody tissue of suitable physiological age.

Forastero Original used to refer to a cacao variety foreign to or not native in the region of cultivation (mainly in Mesoamerica) but is now utilized to denote the cacao germplasm (both wild populations and cultivated forms) from South America other than Criollo and Trinitario.

Founder effect The loss of genetic variation that occurs when a new population is established by a very small number of individuals from a larger population, through sampling errors acting in a similar way to genetic drift.

Gametangia Hyphal structures acting as male (antheridia) or female (oogonia) reproductive structures.

GenBank An online repository of nucleic, protein, and organismal information.

Gene diversity The diversity of genetic forms of one or many genes in populations or species accounting for number and frequency of forms.

Gene flow Also called migration—any movement of individuals, and/or the genetic material they carry, from one population to another.

Generative hyphae Are branched, thin-walled, septate, and commonly with clamp connections.

Genetic drift Change in gene frequency of an allele in a population over time.

Genome Complete set of DNA containing all the genes for an organism.

Germ tube The germinating hypha from a spore.

Germplasm The living genetic resources such as seeds or tissue that is maintained for the purpose of animal and plant breeding, preservation, and other research uses.

Glabrescent Becoming smooth.

Haploid Having a complete but single unpaired set of genetic information.

Haustoria Are fungal mycelia structures that penetrate the host's tissue and draws nutrients from it.

Haustorium An outgrowth produced by some parasitic organisms that penetrates the host cell and acts as an absorptive organ.

He (expected heterozygosity) Theoretical maximum fractional number of mixed genic states of total genes of an individual or fraction of individuals in a population who show a mixed genic state at a particular gene.

Hemibiotroph A pathogen that lives on living tissues without killing those tissues for part of its life cycle but ultimately changes and kills and infects those tissues.

Heterokaryotic A condition where all of the nuclei in a multinucleate cell are genetically different

Heterothallic Mating system with compatible mating-type genes of a single type being present in a genome; hypha of the compatible mating type is therefore needed for sexual production.

Holobasidiate Metabasidium undivided.

Holocarpic The whole thallus (entire body) can become segmented into spores.

Homogenous Uniform composition; having the same type.

Homoheteromixis (secondary homothallism), in the basidiomycetes, a system whereby the basidiospore contains dikaryotic or diploid nuclei, resulting in dikaryotic or diploid hyphae upon germination that are phenotypically similar to homothallic hyphae instead of haploid hyphae as is the case with heterothallism.

Homokaryotic A condition where all of the nuclei in a multinucleate cell are genetically identical.

Homologs A gene related to a second gene because they are derived from a common ancestral DNA sequence.

Homothallic Mating system with mating-type genes of both types present in a genome; an individual is therefore self-fertile, thus able to reproduce without a partner of the opposite mating type, also referred to as selfing.

Hormone A chemical substance produced in one area and usually affecting metabolic activities elsewhere.

Hyaline Smooth, translucent.

Hymenium Spore-bearing layer of a fruit body.

Hyperplasia Overproduction of cells or tissues in reaction to a disease-producing agent.

Hypertrophy Increased size of cells or tissues in reaction to a disease-producing agent.

Hyphae The tubular structures that constitute the mycelium.

ICGT (International Cocoa Genebank Trinidad) A universal collection of cacao genetic resources that is the largest in the public domain. The collection is curated by the Cocoa Research Centre of The University of the West Indies and is located in central Trinidad in the country of Trinidad and Tobago in the Caribbean.

Incubation period The time taken between infection by a pathogen and development of observable symptoms in the host.

Induced resistance A plant defense state associated with an enhanced ability to resist pathogen attack by stronger activation of cellular defense responses.

Induced Systemic resistance (ISR) Induced resistance potentiated by plant growth-promoting rhizobacteria which does not involve the accumulation of pathogenesis-related proteins or salicylic acid but instead relies on pathways regulated by jasmonate and ethylene.

Integrated Pest Management (IPM) The attempt to prevent pathogens, insects, and weeds from causing economic crop losses by using a variety of management methods that are cost-effective and cause the least damage to the environment.

Interfertility tests *Ceratocystis* species is used with a diagnostic species to identify biological species and identify barriers to gene flow between species.

Internal resistance Host-induced resistance after pathogen ingress.

Internal transcribed spacer region or ITS A locus or region in the DNA that is found between the genes for Small Subunit Ribosomal RNA (SrRNA) and the Large Subunit Ribosomal RNA (LrRNA). It is used in taxonomy and molecular phylogeny as a universal DNA barcode marker for fungi because of a high degree of variation.

Interspecific hybridization The sexual mating between two distinct species.

Irregular guttulate contents Having one of more oil-like drops (guttules) inside.

Isolate (1) verb: To separate a microorganism from host or substrate and establish it in pure culture; (2) noun: a single spore or pure culture and the subcultures derived from it.

Isozyme (or isoenzyme) Proteins that perform the same enzymatic function but with different amino acid sequence.

Isozyme analysis Characterization of types of isozymes; genetic diversity studies based on frequency of isozymes.

ITS (internal transcribed spacer) Noncoding DNA sequence located between the small and large subunit ribosomal RNA genes.

ITS polymorphism Difference in DNA sequence of the ITS region.

Koch's postulates Four criteria which establish whether a pathogenic organism causes a particular disease.

Limoniform Shaped like a lemon.

Mating types Molecular mechanisms that regulate compatibility in fungi; genotypic diversity is generated through sexual recombination; this process is controlled in some *Ceratocystis* species by two alleles, MAT-1 and MAT-2, at the same mating-type locus.

Meiosis Specialized cell division prior to sexual reproduction that produces gametes with half of the initial chromosomal content.

Mesophilic Growing best at moderate temperatures.

Microsclerotium A small sclerotium or resting structure.

Mitochondrion An organelle in eukaryotic cells that is bounded with a double membrane.

Mitotic recombination Type of genetic rearrangement occurring in somatic cells.

Monilioid hyphae Shortened hyphae sometimes forming reproductive bodies.

Monokaryon (Monokaryotic) Fungus in which the cells have genetically identical haploid nuclei.

Monomitic Having hyphae of one kind (generative hyphae) as opposed to dimitric, having two kinds of hyphae (generative and skeletal) and trimetric, having three kinds of hyphae (generative, skeletal, and binding).

Mt Cox I Mitochondrially encoded cytochrome C oxidase I; one of three mitochondrial subunits of the respiratory complex IV enzyme.

MtDNA restriction fragment length polymorphism Genetic difference arising from differences in DNA sequence of mitochondrial DNA detected by the presence of restriction enzyme sites.

Mutation A permanent change of the nucleotide sequence of the genome of an organism.

Mycelium The mass of hyphae that forms the vegetative body of a fungus.

Mycobiota The total fungal inventory of an area.

Mycolaminaran A β-1,3; β-1,6 polysaccharide of D-glucose units.

Mycoparasitism Parasitism by a fungus of another fungus (one fungus living on another).

Mycorrhiza The association of a fungus with the roots of a vascular plant in a symbiotic to mildly parasitic relationship.

Mycotoxin A toxin or toxic metabolite derived from a fungus.

Necrosis The condition of premature death of plants cells.

Necrotroph Plant pathogen that kills the host cells and then utilizes the cells for feeding.

Necrotrophic Obtaining nutrients from dead host cells.

Niche The role and position a species has in its environment; how it meets its needs for food and shelter, how it survives, and how it reproduces. A species' niche includes all of its interactions with the biotic and abiotic factors of its environment.

Nodulose (of spores). Having broad based, blunt, wart-like excrescences.

Non-agglutinated Not fixed together.
Nuclear polymorphism Genetic difference in DNA sequence of the genetic material of the nucleus.
Nursery A place where young plants or trees are raised.
Obovoid Egg-shaped with a narrow end at the base.
Obpyriform Pear-shaped with a narrow end at the base.
Obturbinate A conical shape having a wider base at the bottom.
Occluded pedicel Closed off with walls; often used for the lumina of hyphae.
Oomycete Filamentous algae or fungi that are typically aquatic but with some terrestrial members; all oomycetes have a saprophytic or pathogenic life cycle.
Ophiostomatoid fungi Antique group that includes species of *Ceratocystis*, *Ophiostoma*, and *Ceratocystiopsis*.
Ovoid Shaped like an egg.
Palindromic Having the same sequence forward or backward.
Panmictic Random mating among members of a population without preference for certain traits.
Papilla Nipple-shaped protuberance.
Parasexual recombination An asexual form of genetic recombination that does not involve meiosis or the fusion of gametes.
Parthenocarpic fruits Fruits induced without fertilization and therefore seedless.
Pathogen A microorganism that can cause disease.
Pathotype A subdivision of a species distinguished by common characters of pathogenicity.
Pedicel Stalk of a single structure.
Peduncle Stalk of a compound structure.
Perithecia A flask-shaped sexual fruiting body produced by *Ceratocystis* species.
Perithecial base Basal segment in certain ascomycetous fungi, a flask-like case containing the spore sacs.
Perithecium Subglobose or flask-like structure which contains ascospores with an ostiole (opening) at end of the neck (associated with Ascomycetes).
Persistent Existing for an undetermined and longer period of time than expected.
Petiole Stalk of leaf.
Phenological Relationship of climatic conditions with periodic biological events.
Phenotypic characters Recognizable trait, feature, or property of an organism.
Phyllosphere The total aboveground portions of plants as habitat for microorganisms.
Phylogenetics The study of the evolutionary history, development, and relationships among groups of organisms (e.g., species or populations).
Pileus The hymenium supporting part of the basidioma on non-resupinate Agaricomycetes; the cap of a fruiting body.
Plasma membrane The cell membrane or plasmalemma that surrounds the protoplasm.
Pleomorphic Fungi having multiple independent forms.
Polyene antibiotics Antimicrobial compounds that contain many carbon–carbon double bonds.

Polysaccharide Extremely long chain containing repeat units of carbohydrate molecules.

Post-penetration resistance Host defense to pathogen after the external protective layers of the host have been breached; similar to internal resistance.

Pre-breeding Preliminary breeding to obtain suitable parent material that would be used in the final crosses to generate desirable varieties.

Primers Short stretches of nucleotide bases that will bind to a particular DNA region and allow the initiation of DNA polymerization.

Pulvinus A prominent swelling at the base of the petiole of a cacao leaf.

Pyriform Pear-shaped.

QTLs A quantitative trait locus (QTL) is a section of DNA (the locus) that correlates with variation in a phenotype (the quantitative trait).

Quarantine pest A pest of potential economic importance to a growing region that is not yet present, or present but not widely distributed and is officially controlled.

RAPD Randomly amplified polymorphic DNA.

Refractario trees Cacao cultivars from western Ecuador selected for "resistance" to pod diseases, based on escape from infection rather than genetic resistance.

Reniform Kidney-shaped.

Repetitive sequence Stretch of bases in DNA that is present as a set, multiple times with or without interruptions.

Restriction digest DNA after being subjected to enzymes that cut at specific base sequences in DNA.

Resupinate (of a basidioma or fruit body) Being flat on the substrate with the hymenium on the outer side.

RFLP Restriction fragment length polymorphism.

Rhizobacteria Root-colonizing bacteria.

Rhizomorphs Shoestring like aggregated hyphae, enabling fungi to move over a distance and often able to penetrate a host.

Rhizosphere The total belowground portions of plants and the soil immediately surrounding them as habitats for microorganisms.

Saprotroph An organism feeding on dead tissue, not forming part of a living host.

Sclerotium Firm, compact masses of hyphae, with or without addition of host tissue or soil, normally having no spores in or on it, acts a resting structure.

Septum A partition.

Sequence An ordered stretch of DNA.

Serological diagnostic Identification of foreign proteins by a process that matches with an antibody protein.

Somatic incompatibility Vegetative interaction between hyphae; incompatible reaction leads to cell death at the zone where hyphae from two incompatible strains are encountered.

Speciation The evolutionary process by which new biological species arise from an ancestor species.

Spherical Ball-shaped.

Sporangial stalk Filament that holds the sporangium.
Sporangium A structure in which spores are formed.
Sporophore Fruiting body.
Stellate Star-shaped.
Sterigmata Extensions (generally four) of the cell wall of the basidium on which basidiospores develop.
Strain A described/characterized purified isolate.
Striate Showing stripes or streaks.
Subspherical Nearly but not fully spherical.
Sulcate Grooved.
Synonym Another name for a species or group, especially a later name not currently employed for the taxon.
Synonymy Having the same name.
Systemic Acquired Resistance (SAR) A "whole-plant" resistance response that occurs following an earlier exposure to virulent, avirulent, or nonpathogenic microbes or to elicitor chemicals and which involves coordinated accumulation of pathogenesis-related proteins (and transcripts) and salicylic acid throughout the plant.
Systemic (of a pesticide) entering the plant and passing through the tissues or (of a pesticide) absorbed and circulated by a plant or other organism.
Taxonomic status The current standing valid/accepted name for a taxon by the 18th International Botanical Congress.
Teleomorph Sexual reproductive stage of a fungal life cycle.
Tetrapolar See bifactorial mating-type system.
Thallus A plant body that is not differentiated into roots, stems, and leaves and lacks a true vascular system.
Tinsel flagellum Threadlike projection, with multiple longitudinal rows of finer projections, that is capable of causing motion.
Tomentose Covered in soft matted hairs (downy).
Torulose Cylindrical but having swellings at intervals.
Trama The layer of hyphae in the central part of a lamella of an agaric.
Transcriptome An organism's complete set of RNA used to identify expressed genes.
Triazol Refers to any of the heterocyclic compounds with molecular formula C2H3N3, having a five-membered ring of two carbon atoms and three nitrogen atoms.
Trichome A small hair or other outgrowth from the epidermis of a plant, typically unicellular and glandular.
Trinitario Phenotypes derived from cacao populations in Trinidad.
Turbinate Shaped like a top; an inverted conical shape.
Tylose An outgrowth of xylem parenchyma cells which occlude xylem vessels acting as a resistance mechanism.
Umbrinous Umber in color.
Unidirectional mating-type switching A sexual reproduction mechanism described in a few fungal genera. The switching of mating type is irreversible

and results in the production of both self-fertile and self-sterile isolates by a homothallic species.

Vegetative incompatibility See Somatic incompatibility.

Velutinate Thickly covered in delicate hairs; velvet-like.

Verrucose Having a rough or warty surface.

Verticillate Having parts arranged in whorls.

Vesicle Any membrane-bound globule within the cytoplasm.

Whiplash flagellum Threadlike smooth projection capable of causing motion.

Wilt disease A disease characterized by chlorosis and wilting of the plant tissue due to the impairment of water supply as a result of the xylem infection by fungal pathogens.

Zonate Having concentric lines often forming alternating pale and darker zones near the margins.

Zoospore A motile asexual spore with one or more flagella and without a cell wall.

Sources for Glossary Terms

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