The Pennsylvania State University

The Graduate School

Department of Plant Pathology

SOLANUM LYCOPERSICUM AS A MODEL SYSTEM TO STUDY PATHOGENICITY MECHANISMS OF *MONILIOPHTHORA PERNICIOSA*, THE CAUSAL AGENT OF WITCHES' BROOM DISEASE OF *THEOBROMA CACAO*

A Dissertation in

Plant Pathology

by

Jean-Philippe Marelli

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2008

The dissertation of Jean-Philippe Marelli was reviewed and approved* by the following:

Mark J. Guiltinan
Professor of Horticulture
Dissertation Co-advisor
Co-Chair of Committee

Seogchan Kang
Associate Professor of Plant Pathology
Dissertation Co-advisor
Co-Chair of Committee

Paul A. Backman
Professor of Plant Pathology.

Paula McSteen
Assistant professor of Biology

Wenda Bauchspies
Assistant professor of Science, Technology and Society

Dr. Barbara Christ
Professor of Plant Pathology
Head of the Department of Plant Pathology

*Signatures are on file in the Graduate School

In memory of my father, Paul Marelli, to whom I owe this accomplishment and much more...

ABSTRACT

Theobroma cacao produces cocoa beans, an essential ingredient for the production of chocolate. This research focuses on enhancing disease resistance in *Theobroma cacao* via two approaches: The first approach targeted improved understanding of the pathogenicity mechanisms of *Moniliophthora perniciosa*, the causal agent of witches' broom disease because of its economical impact in South America and the potential threat to unaffected areas in other continents. The second approach involved identifying tomato varieties that differentially interact with *M. perniciosa* to study mechanisms of pathogenesis and resistance toward the goal of making tomato as a surrogate model for cacao witches' broom. Certain strains of *M. perniciosa* can infect wild and cultivated solanaceous plants, including *Solanum lycopersicum* (tomato), which allowed the advantage and convenience of working with an annual plant and make use of the numerous genetic and genomic resources available from tomato plants. The primary goal in this thesis was to elucidate cellular and physiological mechanisms underlying the establishment of fungal biotrophy. The research strongly suggests that during the biotrophic stage, the fungus causes cell multiplication and cell enlargement leading to swelling of stem and petioles. Consistent with these cellular changes, using an auxin reporter system (DR5-GUS) transgenically introduced to a susceptible tomato plant, an overproduction of auxin was found at the sites of infection. To discover new sources of resistance against *M. perniciosa* in tomato, *S. habrochaites*, a wild relative of *S. lycopersicon*, and near isogenic lines (NILs) derived from a cross between them were screened. *Solanum habrochaites* can block the establishment of biotrophy. One hundred tomato NILs containing different chromosomal sections of the genome of *S. habrochaites* in a *S. lycopersicon* background were the source for identifying genome regions of *S. habrochaites* that contribute to the observed resistance against *M. perniciosa*. A strong QTL for disease severity ($P<0.05$), shoot diameter ($P<0.001$), and shoot fresh and dry weight ($P<0.0001$) was detected on the short arm of chromosome one. This genomic region contains a cluster of genes for resistance against the fungal biotrophic pathogen *Cladosporium fulvum*. Implications of this thesis for improving cacao breeding strategies will be discussed.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

It would be impossible to summarize in a dissertation all the knowledge acquired during a PhD degree for that it is not only scientific, technical and academic knowledge, but also knowledge about one self, others, and life in general. I do think that so far, this degree has been my toughest experience, where not only I have been prepared to be a good researcher, but also a humble man in front of the many storms of life, which can, at times be cumbersome. Therefore, I would like to thank all those people, who not only made my carrier possible here at Penn State, but also those who helped me during those hard times.

In the Guiltinan lab, I would like to thank Mark Guiltinan, for his guidance and effort to attune my scientific thinking. Special thanks go to Siela Maximova, who not only helped me with the admission process at Penn State but also was always available for advice in designing and analyzing experiments. Many thanks go to the lab technicians, Ann Young and Sharon Pishak, without whom this research would have required many more years. Special thanks go to the post-docs, Joe Verica and Marna Yandeau-Nelson, for editing my manuscripts and helping me with lab protocols.

In the Kang lab, I would like to thank Seogchan Kang, for his patience, advice and understanding all along my PhD. Many times it is good to have a model, someone you admire for their hard work and their tenacity. For me that person was Dr. Sook-Yang Park, one of the post-docs, who helped me numerous times in my experimental work. Many thanks also go to Dr Lee for his help establishing hydroponic systems for tomato.

In Brazil, I met people, who inspired me, for their brilliant ideas, the hours spent talking about science. One of those persons was Dr. Karina Gramacho, from the CEPLAC institute, who either personally or via e-mail, never stopped inspiring this work.

In France, I would like to thank Dr. Vincent Pétiard, from the Nestlé Company, who proposed an excellent idea for Chapter 3 of this dissertation and provided the financial and experimental back-up to make it come true. At the same time, I would like to thank Dr. Bernard Masseret, also from Nestlé, who is an excellent person to work with, and also became a good friend.

In the department of Plant Pathology, I specially would like to thank Dr Barbara Christ, whose support and care for the students make her, in my opinion, an excellent department head, and who was there when I needed help. Also many thanks go to the building coordinator, Teresa Shirk, for her help getting growth chamber space, and her service to make sure all the equipment was working properly.

On campus I also met people who listened to me, and gave me confidence in myself. Those persons were Wenda Bauchspies, Fred Gildow, and Victoria Crain.

For the completion of this thesis, I would like to specially thank all my committee members, Mark Guiltinan, Seogchan Kang, Wenda Bauchspies, Paul Backman, and Paula McSteen for the many hours spent at committee meetings giving their input and ideas to improve the quality of this dissertation. Also, special thanks go to Mr. Roger Dudik, who not only did an excellent job with the final corrections, but also accompanied me until the day of the defense with his good humor and support.

I would like to thank the funding agents for this research, the World Cocoa Foundation (ex ACRI), the department of Plant Pathology at Penn State, and the Nestlé Company.

Finally, all along my PhD degree, I met those who became my best friends, and who in one way or another were an essential part of this success. I would specially like to thank, Andres Diaz, Alma Rodriguez Estrada, Leila Rodriguez, Luz Marina Cardozo and Jacob Easley.

Chapter 1

Lessons from the past: intersections of science, economy and politics in plant disease epidemics

Abstract

Bahia was one of the largest cacao growing areas in the world before the introduction of the witches' broom disease in 1989. Recently, accusations of an intentional introduction of the pathogen by a Brazilian leftist political party have resurrected this episode. The first section of this chapter tackles this question by reviewing the origins of witches' broom and the spread patterns throughout South America during the 20th century. This review revealed a significant relationship linking how the social, political and economic contexts have influenced the spread of this disease. The second and third sections study two additional tropical diseases (fusarium wilt of bananas and coffee rust) and use them to study how human activities in general affect the appearance and spread of plant diseases. This leads to raising the question of whether or not the "disease triangle", a common concept in Plant Pathology, reflects non-pathological factors such as politics, economy and culture.

Introduction

The confluence of interest in Plant Pathology and intellectual interest in Social Sciences created curiosity of the role human's social and cultural activities play in the development of disease pandemics which are often overlooked in plant pathology alone. Most books and treatises of plant pathology mention the influence of humans as a "vector" of disease by spreading the pathogen through infectious material (Agrios 2005; Boroadbent 1960). However, references mentioning the role of socio-economical and cultural contexts in the spreading of plant disease and the strategies adopted by communities to control an epidemic are rare, if not absent, in discussions of plant pathology.

The traditional view to understand the different factors influencing the development of a plant disease was typified by the pioneer plant pathologist Gäumann, who recognized that for an epidemic to occur, an accumulation of susceptible individuals, the presence of an aggressive pathogen, and the optimal climatic conditions for the development of the pathogen are necessary (Gäumann 1950). This concept, represented by a triangle, has each of the three sides representing one of the three factors. If one side of the triangle has a reduced magnitude, the area of the triangle decreases as well. Therefore, the disease triangle is not only an illustrative concept, but also a tool to quantify the effectiveness of a disease management strategy (Sanders 2000). Scholars have suggested that other factors influencing plant disease such as time, humans, animals and insects should be added to the triangle as additional factors (Agrios 2005). Increasing the number of factors adds one or more vertex to the triangle to create a

disease tetrahedron or other polygon (Francl 2001). Agrios proposed to add a line extending from the base to the upper vertex, adding a fifth factor to the tetrahedron to represent time. Although extensive literature considered the influence of time and vectors, the influence of humans as a major social, cultural and economic factor is lacking or diminished (Agrios 2005).

Francl recognized that human activities such as planting practices, genetic improvement through breeding and genetic manipulation can have an influence on plant disease epidemics (Francl 2001). However, he argued that:

> Humans constitute a part of the pathosystem environment in the sense that we are external to the host-parasite interaction. Thus, regardless of our dominant influence, a view devoting a dimension to ourselves may be considered anthropocentric'.

However, ignoring the human influence denies the human role as part of the system and is even more anthropocentric, considering that influences on the disease triangle include complex cultural, economic and social factors that affect its shape and the extent to which the epidemic develops. For example, the scientific knowledge surrounding the study of a particular disease, the political environment and the economical context in which the location affected is evolving, are all factors that need to be considered in understanding the disease pandemic. By thinking of all these "other" factors that surround a plant pathogen epidemic leads to considering science , technology and society (STS) studies to answer questions about the relationship of scientific knowledge and human activities that could allow characterization of some of those aspects. The central dogma of STS is that "science and technology", or technosciences, are social and cultural phenomena (Bauchspies *et al.* 2006). STS scholars focus on the

context which generates scientific knowledge and how the context shapes the final outcome (Bauchspies 2000). In the current case, the focus is on how the consideration of a particular disease, the approaches to control it, and the social and cultural context in which it develops shapes its effect on the natural environment. This, in fact, asks the question, “How does society at a certain time of history affect plant pathogens and the science of plant pathology?”

The rationale for this paper is that the social activities of practitioners shape the science of plant pathology as much as in any other sciences. In addition, the knowledge generated on plant pathogen taxonomy, biology, epidemiology and control strategies is a social construct and evolves within the cultural, political and economic context at the time of the epidemic. Therefore, it is possible to link the three biological factors that generate the development of a plant disease (host, pathogen, and environment) with the social factors that shape society (culture, politics and economy) in a new model that will incorporate all of those influences.

This paper, describes a disease that is an appropriate case to illustrate the concept. The mystery and intricate relationship with human culture of this disease begins with its name: the witches’ broom disease of *Theobroma cacao*. This disease has caused considerable economic damage in South America since the late eighteen hundreds and even today is a primary limitation to cacao production. Through a description of its history, highlights of the social, political and economical factors that catalyzed the various epidemics that severely affected the cacao economy in South America and the Caribbean become evident. Also, in order to generalize the concept to other cases, a comparison and contrast of the witches’ broom epidemic with two other tropical diseases

shows these other two evolved in a very similar environment as did the witches' broom. These other diseases are the rust of the coffee shrub, and the Panama disease of the banana tree. Finally, after having shown how humans can affect plant disease epidemics, a discussion of the integration of these factors into the disease triangle demonstrates a way to better enhance understanding of plant pathogens.

Tropical plant diseases that changed history

Cacao witches' broom: chronicle of a disease foretold

Theobroma cacao is an under storey tree, native to the Amazon forest. Genetic evidence suggests that indigenous people acquired it to be cultivated in Meso America (including Mexico – North America) where it became a major economic crop for the Mayans and the Aztecs (Motamayor et al. 2002). During the Classic, Post-Classic and Colonial Periods, cacao beans represented a type of currency, used for trade (Muhs *et al.* 1985). Also, the Aztecs confectioned a drink, called "Xocolatl" in Nauhatl, the Aztec language, which served as an offering to the gods, and the elite used it as a medicinal potion (Dillinger et al. 2000).

After the Spanish conquest by Hernando Cortés in 1520, cacao consumption by the Spaniards became common in the colonies mostly among women who drank a modified version of Xocolatl by adding sugar, cinnamon, ginger, hazelnut and many other ingredients not native to the Americas (Dillinger et al. 2000; Jamieson 2001). Due to increased consumer demand, the Spanish pushed for intensive Mexican cacao

cultivation which until then had been carried out traditionally by Mayans. Between the sixteenth and the seventeenth century the main centers of production changed considerably. In Mexico, the rapid intensification of cacao cultivation and the loss of farm labor due to infectious diseases brought by the Spaniards, caused a considerable decline in cacao cultivation (Jamieson 2001). New centers of production founded in Suriname, Ecuador and Brazil by European entrepreneurs, supplied cacao beans for the increasing demand in the New World. As opposed to the traditional Mexican small holdings , large plantations, where cacao was grown intensively characterized the new producing countries (Jamieson 2001).

The witches' broom disease of cacao caused by a fungal pathogen, M*oniliophthora perniciosa*, has expanded almost in parallel with the increasing acreage devoted to cacao in South America (Fig. 1-1).

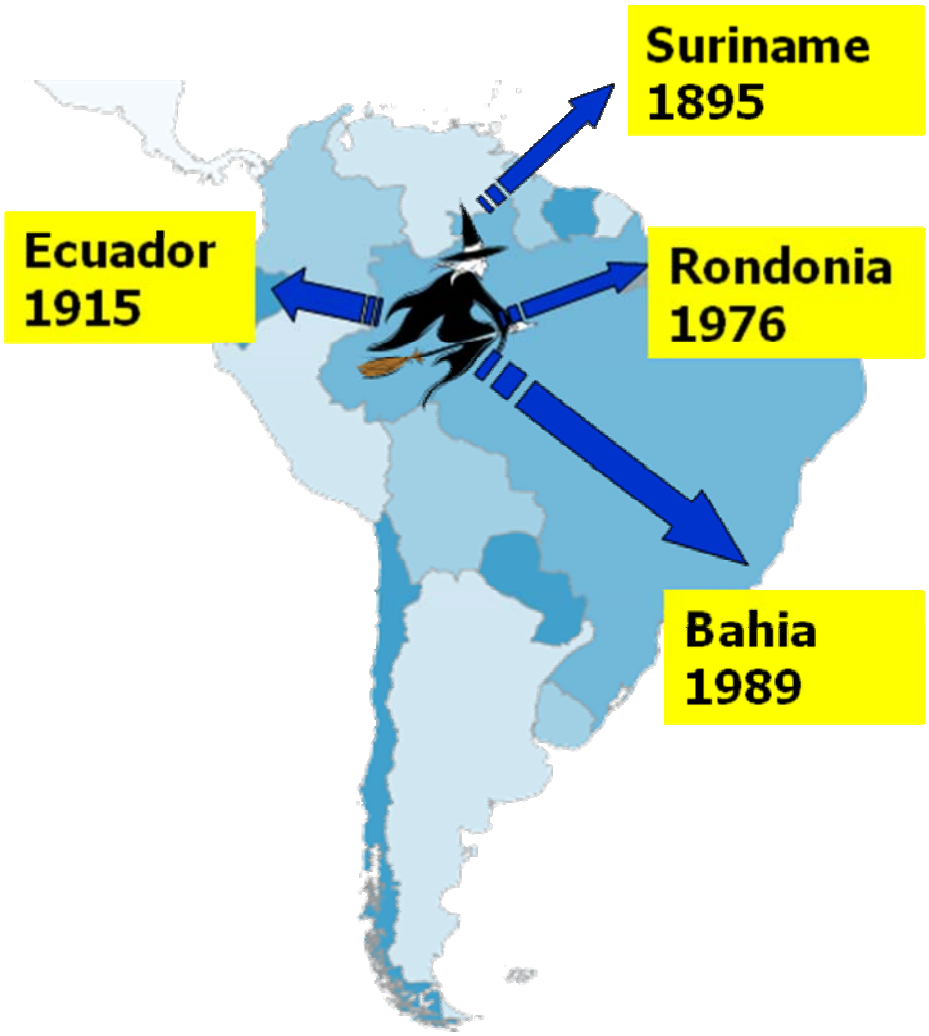


Fig. 1-1: Main epidemic events of the witches' broom disease in South America during the 19th and 20th centuries.

The fungus first caused economic damage in Suriname (Fig 1-1), where an epidemic, recorded in 1895, caused losses approaching 50% of the annual yield (Rorer 1913). The disease was first called *Krulotten* in Dutch, because of its resemblance to a hook in the diseased branch. In 1901, Dutch mycologist Friedrich A.F.C. Went, who spent several years collecting material in Suriname and studying it in Utrecht, The Netherlands, conducted the first detailed study of the disease (Stahel 1919). His

conclusions were that the fungus, which had been previously named *Exoascus theobromae,* was present in infected tissues, but the spores could not be observed. He also confirmed that the same fungus was present in infected fruits where he found the same type of hyphae as in the branches (Rorer 1913). A few years later, two other mycologists, Van Host, director of agriculture in Dutch Guyana and his assistant Drost, completed a second set of studies and isolated the fungus that they renamed *Colletotrichum luxificum*, since they could find fructifications of this fungus growing on the surface of the pods. However they were unable to reproduce the disease by infecting cacao plants with spores of *C. luxificum* in the greenhouse (Bancroft 1910). In 1913, James Birch Rorer , sent to Suriname by the Board of Agriculture of Trinidad and Tobago, over concerns on the spread of witches' broom to these islands, expressed serious doubts that the identity of the fungus was *C. luxifixum*, since he found that the morphology of the fungus growing inside the infected tissues and the one isolated from the pod surfaces differed (Rorer 1913). Two years later, Gerold Stahel, a German mycologist working in Suriname extended on J.B Rorer's observations, and, by controlled inoculation experiments, discovered that the actual causal agent of the *Krulotten* was a basidiomycete producing fruiting bodies (Fig. 1-2) at the surface of dead branches. He renamed the basidiomycete *Marasmius perniciosus* nov. spec. (Stahel 1915). Despite all the identification work done in Suriname, the disease could not be contained and ruined most cacao growers. Singer renamed it *Crinipellis perniciosa* based on morphological characteristics (Singer 1942). A few years ago, Aime & Philips-Mora analyzed the fungus classification once again based on genomic sequence information and renamed it *Moniliophthora perniciosa* (Aime and Phillips-Mora 2005).

Fig. 1-2: Fruiting bodies of *Moniliphthora perniciosa* (the witches' broom pathogen). The spores are produced under the pink mushroom caps. (Marelli 2008)

In Ecuador, the cacao plantations first established plantings of local garden varieties (Criollos) well adapted to the local environmental conditions. The planted areas expanded during the sixteenth and seventeenth centuries and Ecuador became the main supplier of cacao for New Spain, reaching 6,300 tons per year by the 1820s (Jamieson 2001). Because of the high prices for cacao, a new social class of cacao growers emerged called the "cacao millionaires". The profitability of cacao increased as a result of smuggling activities, strictly forbidden by the crown, between Ecuador and the other colonies in Latin America (Mahony 1996). In order to increase productivity, new varieties of cacao were introduced in Ecuador from various locations (Venezuela,

Trinidad). Those exotic varieties, known to be resistant to witches' broom (present at low levels in Ecuador), were high yielding varieties (Wood and Lass 1985). However, the vegetative cycle of this exotic material was very different under the Ecuadorian conditions, and as opposed to the local Ecuadorian trees that had a constant flushing and flowering cycle along the year, the exotic cacao only produced new flushes and flowers at the beginning of the rainy season, coinciding with the high levels of spores of *M. perniciosa* in the air. This significantly increased the infestation of trees in Ecuador, and by the 1920's the disease became epidemic (Evans *et al.* 1977). The combination of witches' broom epidemics and another devastating disease called frosty pod led to the decline of Ecuadorian cacao production reduced from 50,000 tons to 15,000 tons in a period of 16 years (Stell 1933).

During this epidemic period, a few farmers applied the idea of breeding for resistant varieties, which had been mentioned during the Suriname epidemic but never put in practice. One of them, Don Carlos Seminario, a cacao grower and researcher from the Balao district, noticed in 1923 that many trees were able to withstand the disease (Stell 1933). Based on these observations, Don Carlos Seminario, planted seeds from the largest pods of those resistant trees and placed them under infected plant material to expose them to the spores of the fungus. With this method, he was able to select the most resistant trees. By 1933, he had around one million of these trees in his fields. Nevertheless, most cacao millionaires lacked encouragement to replant cacao due to the low market prices after the crisis of the stock exchange in 1929, and many of them abandoned their cocoa farms to invest in banana and coffee crops that were more profitable at that time (Stell 1933).

In Brazil, cacao is endemic to the Amazon region. The witches' broom disease was present but was not economically damaging until the 1970s. Cacao was not extensively grown as a cultivated crop until pioneer settlers from the southern and northeastern part of the country , encouraged by the government, colonized the rainforest (particularly in Rondonia) and established cacao fields for a cash crop (Laker and Silva 1990). As a consequence of the increased cacao acreage, *M. perniciosa* became a considerable problem and caused an outbreak in the Amazon region no later than 1976 (Wheeler 1987). When witches' broom became epidemic, many growers, inexperienced in cacao cultivation, became overwhelmed by the disease. Many of them either cut down their plantations to grow a different crop or abandoned the infected fields, thereby contributing to the rapid spread of the disease. The witches' broom epidemic, the aging plantations and the competition from Bahia, the other cacao producing region of Brazil, accelerated the decline of cacao cultivation in the Brazilian Amazon (Keithan 1939).

In Bahia, in the northeastern part of Brazil, *Theobroma cacao* had been introduced in 1736 by a French planter, Louis Frederic Warneaux (Keithan 1939), but the crop only became economically important in 1835, due to increasing demand for cacao beans in North America and Europe, the availability of cheap labor from recently freed slaves, and the adaptability of the crop to the growing conditions in Bahia (Keithan 1939). During the 18th century, the major land owners in southern Bahia were the "coronels"- rich growers initially founders of the national guard, from which they derived a military rank – who cultivated cacao for export and dominated the area, controlled by a total oligarchy (Pang 1979). The supremacy of the "coronels" best illustrated in the novels of Jorge Amado, involved high crime between clans , human abuse of field

workers, and extreme political control over the region (Amado 1912). One of the most powerful coronels in Bahia was Antônio Pessoa da Costa e Silva who also was a state senator of Bahia and defended cacao interests during the entire duration of Brazilian's first republic (Pang 1979). The maintenance of the "coronels" political power in Brazil's rural areas depended mainly on the independence of regions like Bahia from the central power and the importance of their agricultural production to the local economies (Nunes Leal 1977). When the effect of the economic crash of 1929 was felt on coffee and cacao prices (Fig 1-3), the relative importance of cacao in the states's economy shifted towards industry and services, which also led to the end of the "coronels" influence (Pang 1979). The cacao farms, subsequently managed by the sons of the "coronels", who represented a higher educated class, mostly living in Europe and the big Brazilian metropolies (São Paolo and Rio), had little interest in the management and production of the plantations in Bahia (Mahony 1996).

In 1936, Brazil was the second world cacao producer, 134,172 tons, just behind Ivory Coast (Fig 1-3) (Keithan 1939). A period of sharp decline in cacao prices followed, and many cacao growers could hardly recover their production costs (Fig 1-3). In addition, the competition from Ivory Coast, with its low quality cacao produced in large quantities, created difficulty for Bahia to compete. In 1957, a support institution, CEPLAC (Comissão Executiva do Plano de Recuperação da Lavoura Cacaueira), created to recover the cacao industry in decline, adopted roles of improving the cacao germplasm through breeding programs, offering extension services to the growers, and providing research on alternative crops for cacao plantations (Anonymous 1981). This organization also served to establish quarantine regulations to avoid the introduction of cacao pests

and diseases, including the most feared witches' broom that had already had deleterious effects in the Amazon (Silva 1987). During the 30 years that followed the creation of CEPLAC, the cacao production of Bahia climbed from 190,000 t to 300,000 tons, placing Brazil as the second world exporter just below Ivory Coast (Fig. 1-3).

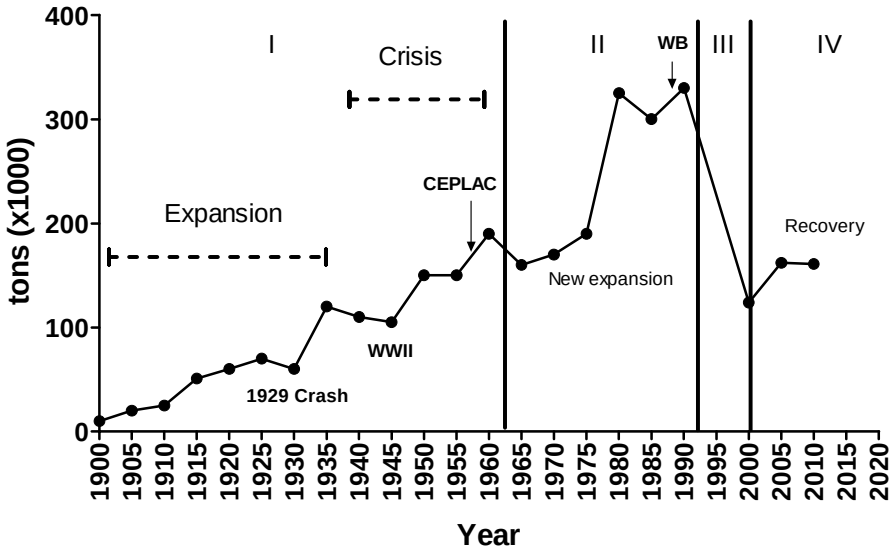


Fig. 1-3: Analysis of the cacao production in Bahia, Brazil. WWII: World War II. CEPLAC: Cacao National Recovery Commission. WB: Witches' broom epidemic. Based on Ruf et al (Ruf 2002) , ICCO annual report (ICCO 2007).

On May 30 1989, the witches' broom dise**a**se appeared in Bahia (Anonymous 1989). CEPLAC declared an emergency alert and eradication of diseased trees began immediately. A buffer zone of 134 ha (0.02% of cacao cultivated area in Bahia) was established around the only known epidemic focus by spraying with fungicides, killing the trees with herbicides and finally burning them along with the alternative known host species of *M. perniciosa* in that area (Pereira *et al.* 1996). CEPLAC personnel knew that they had to move quickly because of the dense planting of cacao in the area and the

known susceptibility of the type of cacao planted in Bahia (var. commun). However, they faced several socio-political pressures during the operation. First, CEPLAC felt that if not successful the institution would suffer from severe criticism from farmers that were already discontent with the directions CEPLAC was taking on cacao matters (Gareez 1985). Second, because of the harsh measures taken to eliminate infected trees, many farmers objected collaboration and they ignored the catastrophic effects that the disease could have if unrestrained (Pereira, Almoida, and Santos 1996). A second focus was discovered days later, which had not been reported by the farmers. The new area affected was so extensive that no eradication was possible at that point and the disease spread rapidly to all the cacao plantations in Bahia.

Because the second focus locale was overlooked, many rumors circulated about how the disease arrived to Bahia and how it was allowed to spread. The first speculation came from CEPLAC itself who filed a lawsuit after the disease started to spread, after workers had discovered infected material hanging on the trees of the first farms that were affected (Gramacho, personal communication). This accusation made world news after an article appeared in the *New Scientist* in 1991 stating suspicion of an act of bio-terrorism against the right wing political power (the descendants of the coronels), orchestred by the leftist party, the "Partido dos Trabalhadores" (PT, workers party) (Homewood 1991). More, recently, a journalist from the Brazilian magazine *Veja*, Policarpo Junior, published the confession of Luis Enrique Franco Timoteo, accusing himself, the actual director of CEPLAC, Gustavo Moura and five other technicians of CEPLAC of having organized a voluntary introduction of the disease on Bahia in 1989. The accusations stated that in 1987, members of the PT working for CEPLAC, including Mr. Moura,

organized the introduction in order to reduce the political power of the land owners whose main resource was cacao (Junior 2007). They were supposedly bringing the infected material from Rondonia (Amazon) from were Timoteo originated. He claimed that he was selected by the group because of his knowledge of the area and made several trips from 1987 to 1989 carrying infected cacao shoots in rice bags in order to bypass the quarantine controls. According to him, the material was then dispersed along route BR101 by CEPLAC workers by hanging the infected branches under the trees (Fig. 1-4).

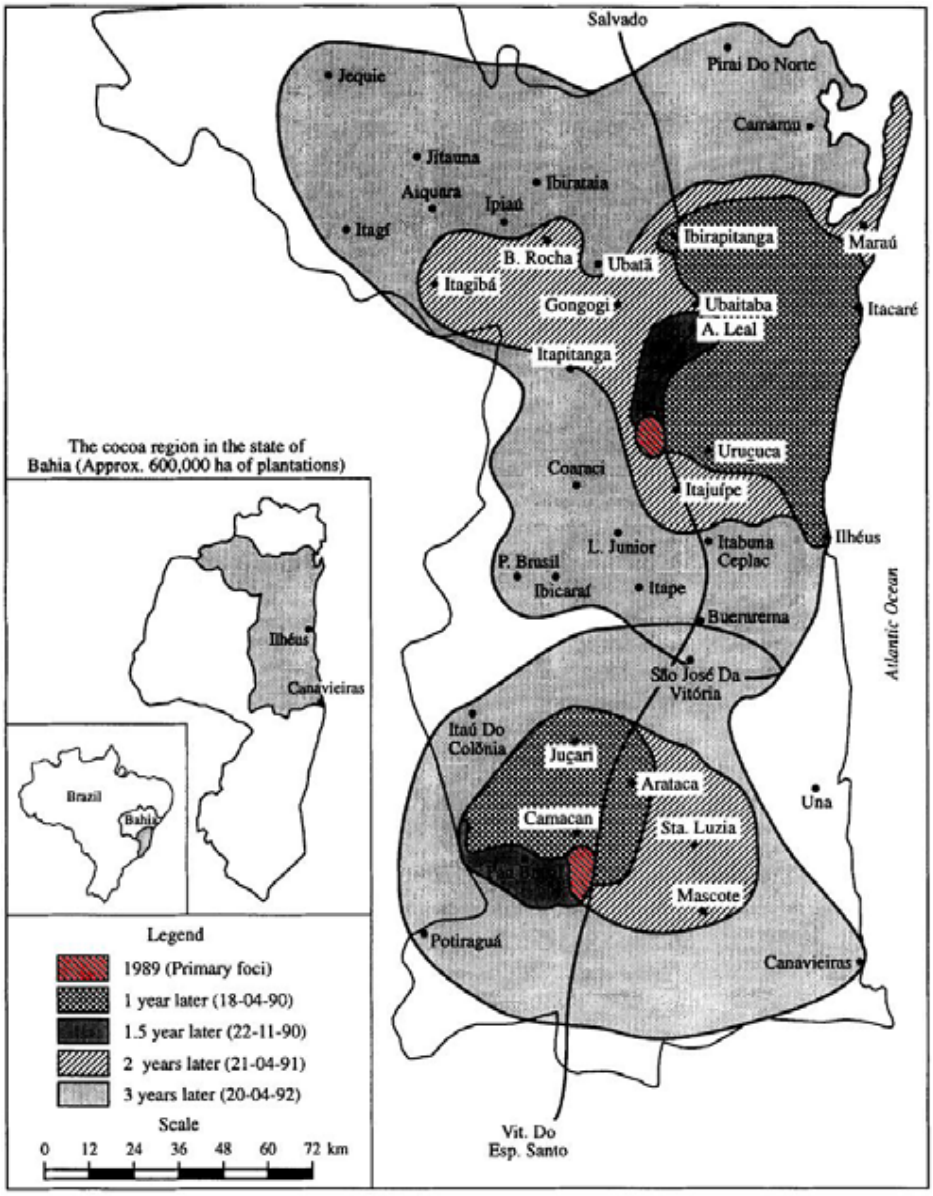


Fig. 1-4: Geographical spread of witches' broom in the growing area of Bahia, Brazil, originating from the first sites of introduction of Uruçuca and Camacan. The line going across the cacao growing area is the road BR101 (Pereira, Almoida, and Santos 1996)

The first targets were the plantations (Uruçuca and Camacan) belonging to the most powerful growers. After the article appeared on June 20 2007, the cacao growers requested a trial to clarify the accusations of Timoteo. After almost six months of

hearings, the jury decided to close the case and to call for a “non-lieu” stating that insufficient evidence existed to support the accusation of Ricardo Moura and the four other CEPLAC agents. They agreed, nonetheless, that the introduction of the witches’ broom in Bahia was a deliberate criminal intent.

Many scientists have since then given their opinions about the case. Dr Gonçalo Guimaraes Pereira, from the UNICAMP University of São Paolo, declared, during a meeting in Washington, DC in November 2007, that he found only 2 races of *M. perniciosa* present in Bahia, both of Amazonian origin (Rincones et al. 2006). According to him, this would represent enough evidence that *M. perniciosa* originated from the Amazon, but refutes the hypothesis that the introduction was criminal in the way Timoteo described it. Dr. Pereira declared that because the Amazon is considered to be a center of high diversity of the fungus, if the pathogen had been introduced repeatedly over a two year period, more than two races of the pathogen would be present in Bahia now. This explanation has since been rejected by other scientists based on the fact that no causational relation exists between the presence of only 2 races of the pathogen in Bahia and the Timoteo’s plan of introduction. Many environmental and genetic factors determine the capacity of a strain of *M. perniciosa* to sporulate (Anderbrhan 1987). The explanation for only two types of strains could be that those strains were the ones best adapted to the environmental conditions of Bahia and were the most virulent.

Another scientific argument on which scientists in Bahia and around the world have commented is the distribution of the epidemic foci and their linear spread from two focal points, 117 km apart. Normally, in a natural setting, the foci of the disease would have been situated west of the cacao growing area and in a random fashion (Fig 1-4). The

unnatural distribution of the foci shows clearly that they were in fact located along route BR101, and that the two farms first affected (Uruçuca and Camacan) were some of the most important of the area, very close to what was described by Timoteo (Fig 1-4).

The dependence of the cacao economy on world market prices was a circumstantial factor that certainly influenced the disease spread and was so detrimental to the affected countries. Both in Ecuador and Brazil, the disease appeared when cacao prices were in decline because of over-production and competition from West Africa. This resulted in a lack of motivation to save the crop by the growers, who abandoned or sold their estates instead of seeking solutions to control the disease.

Witches' broom had a catastrophic effect on cacao production in South America. Evident from this historical review is that besides the intriguing life cycle of the pathogen, the mystery of witches' broom appearance contributed to the difficult transfer of scientific and technical knowledge to the rapidly growing cacao farms in South America during the twentieth century. The capitalistic nature of the cacao market, inducing price fluctuations, and the political climate in the producing countries also influenced the epidemic dynamics.

The case of witches' broom contains several avenues of evidence indicating how humans can contribute directly through their culture, politic and economic systems to the spread of a disease epidemic. The legend of witches' broom is still alive today, through the unsolved question of whether it was voluntarily introduced based on political interests or by accident. This entangles the story even more with human history and the importance of both mystery and science will remain.

In the following section, two selected supplemental examples that occurred with other agricultural commodities in tropical regions lend support to demonstrate that this is not an isolated case in the science of Plant Pathology. Although limited to certain regions of the world, these examples allow establishing a common pattern in the interaction between the science of Plant Pathology and society in contexts that are comparable. First is a description of the case of the devastating Panama disease of bananas, caused by the fungus *Fusarium oxysporum*, f. sp. *cubense*, and second is a description of a disease of coffee, that changed history (the coffee rust disease caused by *Hemileia vastatrix*).

Fusarium wilt epidemic of bananas and US domination in Latin America

Banana cultivation as a local commodity began in the Caribbean as a result of importation by the Portuguese from the Canary Islands in 1516 (Inibap 2005). Bananas are a rich source of carbohydrates and calories, and thus became omnipresent in the daily diets of the Caribbean people. The crop progressed to Central America where it became part of the markets established by the end of the nineteenth century (Soluri 2000). In Honduras 1000 small scale farmers (also called "poquiteros") cultivated at least 10,300 hectares of bananas over seven municipalities in 1899. Their cultivation practices involved slash and burn of the land, planting and weeding, and incurred no major pest or disease problems (Soluri 2000). Most of the export crop in Honduras originated from harbors on Honduras's Atlantic coast and arrived in the United States, primarily at New Orleans, New York and Boston.

The major drawback for large scale banana export was its perishable nature. But its nutritional properties combined with its relatively low price, quickly made bananas a commodity (Soluri 2005). From the harbors, bananas had to be shipped in steamboats and many shipments perished while being transported to the United States (Bucheli 2004) [1]. In 1885, Lorenzo Dow Baker and Andrew Preston understood that a modern shipment system was necessary to create a profitable banana trade. They created the Boston Fruit Company and bought a fleet, (the Great White Fleet) of ships conditioned only for banana transportation (Bucheli 2004).

At the same time, Henry Meiggs, an American contractor in railroad construction started to work with his nephew, Minor C. Keith in Costa Rica. Building railroads through the jungle was extremely risky for non-natives because of the numerous diseases, especially malaria, yellow fever and hookworm (Tucker 2000). When M.C. Keith took over the project, he brought a supplementary labor force composed of inmates from the jails of New Orleans and Italian immigrants from Louisiana. Because of the harsh environment, many died during the railroad construction (Bucheli 2004). When M.C Keith did not receive the promised payments from the Costa Rican government, he planted bananas along the railroads to feed the workers. When he finished the railroad, he realized that transporting passengers had limited profit; however, if he transported a commodity like his readily available bananas to the harbors, he could easily increase profits. He insured his success by coordinating the shipments to the US with the Tropical Trading and Transport Company. This extended his production areas to South America,

[1] For example, 114 US import companies tried to develop a banana trade between 1870 and 1899, but because of the difficulty of importing a fresh product, only 22 companies had survived by 1899

by creating other plantations in the region of Magdalena in Colombia (Bucheli 2004). In 1899, Minor C. Keith and the Boston Fruit Company dominated the banana market in South America. The same year, the broker company used by Minor C. Keith threatened him with bankruptcy. His only solution for the survival of his company was to merge with the Boston Fruit Company. On March 30, 1899, the two parties created what was going to be the most powerful American corporation in Latin America of the century, the United Fruit Company (UFC).

The activities of the UFC involved the extension of their plantations by creating new railroads and also builing infrastructure for the workers in the banana plantations. The area of banana growing comprised the low wetlands of Central America from the north coast of Honduras to the Chiriqui lagoon along the coast of Panama. During the Spanish occupation, those low lands remained unexploited because the "Ladinos" (descendants of Spanish settlers in Central America) had no interest in the lowland rainforest due to diseases and "bizarre inhabitants" (Tucker 2000). When the Central American nations obtained their independence in the 1820s, the newly formed governments viewed North American investments as a way to dominate the hostile rainforest environment. Therefore, by building railroads and exploiting the land, the United Fruit Company became a source of technological advance and a "necessity for economic growth" by the local governments (Soluri 2000).

The interest of local governments in foreign investments helped the company during the expansion of its cultivated lands. First, because most of the land for banana plantations was unclaimed, the UFC could easily become a landowner as it progressed through the jungle. Second, in areas of conflict between small growers and the company,

the government always favored the UFC. Third, the company controlled all the different steps of the production from planting to shipping and distribution of bananas in the US markets which led to a “vertical integration” of the company (Bucheli 2004). The immense economical power that the company acquired allowed the UFC to dominate the banana trade during much of the 20th century.

In the 1920s a problem emerged in the plantations. Some banana trees were wilting without reason, and apparently a plague was propagating rapidly between the plantations (Fig. 1-5). The appearance of this disease, later called Panama disease of bananas, first reported in ´Bocas de Toro`, Panama, became one of the most difficult challenges for the banana industry in the 20th century.

Fig. 1-5: Banana plants presenting the typical wilting symptoms caused Panama disease. (Courtesy of Scot Nelson).

The causal agent of the banana wilt was officially recognized as being *Fusarium oxysporum* f.sp *cubense* (FOC). The UFC hired scientists from Europe and the United States to study the pathogen and define control measures. The strategies put in place to control *Fusarium* wilt arose from three major developments.

First, in 1916, a soil scientist, Samuel Prescott, hired by the UFC to perform an extensive survey of the soils in the banana plantations in Central America. His report stated that the fungus was present in every single plantation but to different degrees. His

findings allowed him to underlay an ecological hypothesis “that the fungus was an opportunistic agent that took advantage in poor, exhausted soils” giving more importance to the nature of the soil than to the pathogen itself (Marquardt 2001). His hypothesis proved wrong for most soils, and no real correlation existed between soil fitness and disease resistance. However, he did establish an empirically weak correlation between soil acidity and resistance by showing that naturally acidic soils were less conductive to disease spread and would prevent the banana trees to become diseased. This became a criterion for the company’s selection of the best soils where new plantations would be established. In fact, relocation was the only “control measure” that the company used to escape the disease. The average life expectancy of a banana plantation was normally between fifteen to twenty years. When infected with FOC, the plantation was only productive for two to five years. After the FOC pandemic, new areas had to be found that were free of the disease. Or, in other words, unexploited forest land was cleared and planted with young banana trees, starting again an endless cycle of deforestation crop production, and abandonment.

The second major development was made by Claude Wadlaw, a Scottish plant pathologist, part of a team of agronomists who came to Honduras to study the disease. In his opinion, the company was performing an “exploitation of the native fertility of virgin soil with a minimum amount of detailed treatment” and that “once the vitality bequeathed by the region’s former giant forests became depleted, disease arrived and the pioneer industry moved on” (Marquardt 2001). This view of low agronomically sound cultivation of bananas led the company to change radically its system of production. One of the arguments made by Wadlaw was that if banana production were modernized, this would

lead to a better management of the soil and improved disease management[2]. After his study, the company implemented most of his ideas by using deeper, better maintained drainage systems, active soil improvement through tillage, liming (to reduce acidity) [3] and fertilization. Moreover, this change corresponded also to the separation of conception and execution in the banana plantation as it was seen in other industries (Marquardt 2001). The West Indians were not responsible for the cultivation practices anymore and were replaced by agricultural technicians, mostly American or Europeans.

The third strategy adopted by the UFC was never really adopted until the 1960s. It consisted of research of resistant cultivars of banana[4]. As a result, the company invested considerable amounts of money to collect *Musa* species from around the world to look for other sources of resistance (Marquardt 2001). M. Vining Dunlap, who was head of the project reported two "Cavendish" varieties, "Valery" and "Lacatan", that were very productive and resistant to FOC. (Marquardt 2001). However, the company's whole export system was based on the trade variety "Gros Michel" that could be collected in the field and shipped directly. This was possible because the skin of "Gros Michel" was hard enough to resist the transportation and still be undamaged when sold in stores. If "Valery" or " Lacatan" were shipped in the same way they would be damaged and scratched making them unmarketable. The company considered switching to the resistant bananas to be unprofitable at this time. Instead they decided to search for more

---

[2] Before 1928, most of the production system was developed based on the indigenous knowledge of the West Indians where bananas have been cultivated since the 16th century.

[3] A less acidic soil would provide a better growth environment for the plant, but could also promote fungal spore survival

[4] The domesticated banana is parthenocarpic (sterile) and its breeding proved to be extremely difficult to accomplish.

land and modernize the cultivation of “Gros Michel”, the only banana that could be sold to the demanding American consumers.

Ultimately, the company scientists believed that soil degradation caused the disease. This appeared reasonable at the time because the banana plantations were clearly consuming the soil nutrients of the alluvial lowlands of Central America. Moreover, the low technological process, mainly by West Indian contract workers, was replaced after the 1930 by highly mechanized equipment and chemical fertilizers, which improved the soil. A genetic fix (that would target specifically the pathogen) was found in the resistant “Cavendish cultivars” but it was not given as much importance. Most of the infrastructure was designed exclusively to export “Gros Michel” bananas, which didn’t require any special handling for shipping as opposed to the resistant “Cavendish” that were easily damaged by handling. Not until the 1960s, did companies and scientists recognized that replacing the “Gros Michel” by the “Cavendish” variety was the only efficient method to combat the disease.

The agro-ecological landscape of banana plantations, as well as the spread of the epidemic of FOC in the Americas, was molded by the company’s strategies to escape the Panama disease instead of finding a solution to eradicate the fungus. The unawareness of the responsible agent (the Fusarium fungus) prevented the company to find solutions that could target the fungus, such as the use of fungicides, or resistant varieties. Although resistant varieties were known, the economic power of the UFC combined with the availability of virgin rain forest and their influence in local governments made adopting the ‘escape’ strategy easier rather than changing the entire export system, a requirement to be able to change the type of banana exported. The latter would have demanded an

expensive change in infrastructure in direct conflict with the mandate American and European consumers who demanded bananas to be as perfect as possible (Soluri 2005).

Nowadays, all the bananas cultivated are of the "Cavendish" variety. Recently, emergence of new race of FOC (race II), aggressive on "Cavendish" had made world news. If this new form of the pathogen arrives in the banana growing area, it could have a devastating effect on the crop and diminish considerably banana production (Ploetz 2005).

Coffee rust: when a local disease becomes a global problem

The coffee plant, evergreen Rubiaceae (genus *Coffea*), originated in Ethiopia and the Arabs cultivated coffee in Yemen during the sixteenth century. The pleasant and stimulating effect of caffeine led to a transition from the medicinal properties of coffee to a ritual drink in coffee-houses and sites of social and philosophical exchange (Jamieson 2001). Until the late seventeenth century, coffee was only produced by the Arabs who monopolized the production by preventing any plant from leaving Yemeni territory. When coffee became a popular drink in Europe, its market value increased, and the European powers became interested into cultivating it in their colonies (Clearance-Smith and Topik 2003). Unlike cacao, coffee was not brought to Europe by the colonists, but by European visitors to the middle-east (Jamieson 2001). The Dutch were the first Europeans to plant coffee in their colonies in Java, Indonesia, and Ceylon in the 1690s. When Ceylon became a British colony in 1850, coffee plantations developed rapidly on the island that was governed by Sir Edward Barnes. Most of the growers were European

entrepreneurs who hired locals for the hard work of coffee cultivation which was labor intensive only at the annual harvest (Clearance-Smith and Topik 2003). By the end of the nineteenth century, the island of Ceylon was primarily covered with coffee plantations that had replaced the natural flora of its hillsides. This rapid development in the coffee industry enabled building roads that facilitated penetration into Ceylon's "hill country". In 1869, coffee plantations covered 90,000 acres and generated exports worth twenty millions of dollars per annum (Topik and Wells 1998)

In 1869, Rev. M. J. Berkeley, a respected mycologist at Kew Gardens, received from Ceylon a leaf sample that was infected with a fungus (Berkeley 1809). He described the fungus as growing on the underside of the leaves and "with its spawn system within the leaf tissue" and named it *Hemileia vastatrix*. Berkeley suggested the fungus would be difficult to eradicate and that an application of sulfur powder was urgently needed in Ceylon to control the disease. Despite his recommendations, the Celanese government took no special measure against the coffee rust disease. The reason for the inaction was that many farmers believed that their crops were suffering from a physiological disorder and did not believe that a microbe could cause the defoliation observed on the affected plants (Ayres 2005). Ignoring the recommendations made by Berkeley in 1809, led to a reduction in the average annual yield per acre by 57.5 % between 1881 and 1885 (Ayres 2005).

In response to the epidemic devastating the coffee plantations, the Celanese government finally reacted and formed an inquiry commission to request trained mycologists from Kew Gardens to work on the disease. The first scientist sent to Ceylon in 1879 was Daniel Morris. His hypothesis, based upon his observations, was that the

fungus created an external web with structures that allowed it to feed on the leaves. The web theory explained why the fungus resided on the leaf surface under humid conditions and penetrated leaves when unfavorable dry conditions prevailed [5]. Henry Marshall Ward, Daniel Morris' successor, arrived in Ceylon the following year but arrived too late to eradicate the disease. A disciple of the renowned Anton de Bary, he tried to prove, using the scientific method, that the fungus observed at the surface of the diseased coffee trees was actually the cause and not the consequence of the problem. At the time, the theory of spontaneous generation was still widely accepted and pathogens were not believed to be the causal agents of disease (Caponi 2002). Anton de Bary, in 1841, was the first mycologist to prove that pathogens were the causal agents of disease by providing experimental evidence that the cause of the potato late blight was a microorganism, later called *Phytophthora infestans*. Following his teachers steps, Ward conducted an experiment in which he placed microscope slides in place of the coffee leaves in order to observe the numerous types of spores deposited on a leaves by natural means. By doing this, he observed that most spores that germinated formed a web similar to the one previously reported by Morris. However, he noticed that this web formed from another fungi and not *Hemileia*. Ward described the biology of *Hemileia* as very close to the other known rust fungi of wheat, sunflowers, and corn[6] . Ward also discovered that the germinating spores were at their most vulnerable stage, and recommended the application of a fungicide on the leaves as a protectant before the

[5] During dry conditions, the infected leaf became dry and fell from the tree causing defoliation.

[6] The spores (urediospores) germinated under wet conditions on the leaves, and during dry periods the fungus emerged from the leaf epidermis to expose its fructifications. The spores liberated from those fructifications were wind-blown and deposited on neighboring shrubs

spores arrived (Fig. 1-6). He suggested that monoculture of a susceptible genotype of coffee to the rust disease was the main reason why the coffee rust had caused so much damage and spread so rapidly in Ceylon (Large 2003; Schumann 1991). Unfortunately, at the end of his studies, he was unable to offer a remedy to the farmers and was despised by the coffee industry. However, his contributions to the science of plant pathology placed him among the most eminent scientists of the field (Carefoot 1967).

Fig. 1-6: Sporulating lesions of *Hemileia vastatrix,* the coffee rust pathogen.(Marelli 2008).

The decline of the Celanese industry in 1880 triggered the development of coffee production in South America where coffee had been introduced from Asia by the Portuguese in the early eighteenth century. In addition, the Dutch and the French empires contributed to this transfer by collecting coffee seedlings and exhibiting them in their botanical gardens in Europe (Jamieson 2001). The main producing area in South America began in Southern Brazil where the main workforce was of slaves (Roseberry 1991). The

slave-holding landlords established the first coffee *fazendas* (slave estates) near the Paraiba river valley in Rio de Janeiro's back country (Fig. 1-7) .

Fig. 1-7: Brazilian stamps describing the size and labor required in a typical coffee plantation in Brazil at the beginning of the 20th century

The climatic conditions were perfect for coffee cultivation. However, traditional cultural practices which involved slash and burn of the forest and tilling planting on the steep slopes provoked rapid erosion of the soil. This translated into a decline in productivity which was offset by the availability of new virgin territories in the vast Brazilian rainforest. Most of the coffee growing by Brazilian landowners was accomplished without any agronomic knowledge of the crop, mainly because growers were not trained for crop production and were not involved in the cultivation of the crop (Topik and Wells 1998). This lack of knowledge contributed to the lack of productivity

and the degradation of the environment (Tucker 2000). Coffee growing also developed later in Central America, the Caribbean, and Colombia, where, the first coffee settlers had only small farms and did not use slave labor on their plantations (Roseberry 1991). These differences had important consequences in the agro-ecological coffee systems in Brazil and the rest of South America (Tucker 2000). On the one hand the Brazilian coffee production, having the support of slavery, could be done by penetrating the forest and replacing it by coffee trees planted in monoculture, and was oriented towards export. On the other hand, in Colombia, where the cultivation of coffee only began in 1870, the cultivation of coffee occurred on the Andean slopes, where farmers had a more isolated method of production, and because they could not use slave labor , the size of their plantations was smaller than in Brazil (Roseberry 1991). Despite these differences, the two countries became the main exporters of coffee.

Both countries knew that one of the greatest threats for their crops was coffee rust, the disease that devastated the crops in Ceylon earlier in the nineteenth century. Brazil and Colombia implemented strict quarantine measures that targeted not only farmers but also the general public by the transmission of television advertisements warning the general public against the dangers of importing plant material that could carry the rust. Despite these stringent quarantines by South American countries, the rust fungus arrived in Brazil in 1970. Coffee rust was first detected in Bahia, near the cocoa region by cocoa Plant Pathologist Arnaldo Medeiros during a Phytopathology meeting. Possible introduction possibilities included transfer on clothes and shoes from Africa, infected planting material on airplanes and winds. The monoculture and the low technological expertize of the famers favored rust spread in Brazil and the epidemic

could not be contained (Schumann 1991). From Brazil, rust spread to neighboring countries, Bolivia and Peru, and finally made its way to Central America (Nicaragua, El Salvador). In 1983, the disease arrived in Colombia, the world's second coffee producer.

Two methods were utilized to fight the pathogen. The first was the discovery of genetic resistance in hybrids *C. arabica* and *C. canephora* in East Timor. Those hybrids had a similar quality to that of the original Arabica, but were resistant to various strains of the fungus (Kushalappa and Eskes 1989). At the same time, the 1960s also corresponded to the commercialization of copper fungicides and dithiocarbamate fungicides that are protectant chemicals (Kushalappa and Eskes 1989). The main problem with coffee remains that most of the trees planted in South America come from a single cutting coming from a tree growing in the conservatory of King Louis XIV since 1713 and most of the genetic diversity from this center of origin (Ethiopia) has been lost.

One of the most significant environmental consequences of the coffee rust epidemic was an increased use of fungicides that became required for growing coffee in South America. Many growers who could not afford the use of fungicides had to cease coffee production and turn to a more profitable crop. The heavy use of fungicides also had effects on soil ecology (Fulton 1984).

Both genetic resistance -discovered through plant breeding- and fungicides, products of the "Green Revolution" allowed the growers to continue producing coffee. Nevertheless, the rust epidemic caused an economical disaster and an ecological havoc in Latin America.

Conclusion: A multidimensional disease triangle

This paper shows that human activities: Culture, politics and economics do have an influence on the initiation and spread of a plant disease epidemic. These factors represent additional dimensions to the traditional disease triangle mentioned earlier. The three categories (cultures, politics and economics) can be symbolized in a second triangle facing the disease triangle and linked by a z-axis to make a "triangular disease prism" (Fig. 1-8). This links a quantifiable concept (the interaction of host, pathogen and environment) with a more qualitative representation of human intervention in disease epidemics (culture, politics and economics). The problem resides now in how to study this new side of the prism. More interdisciplinary research, combining plant pathology with Science Technology and Society (STS) studies, will be the best avenue studying this new "multi-dimensional" disease prism.

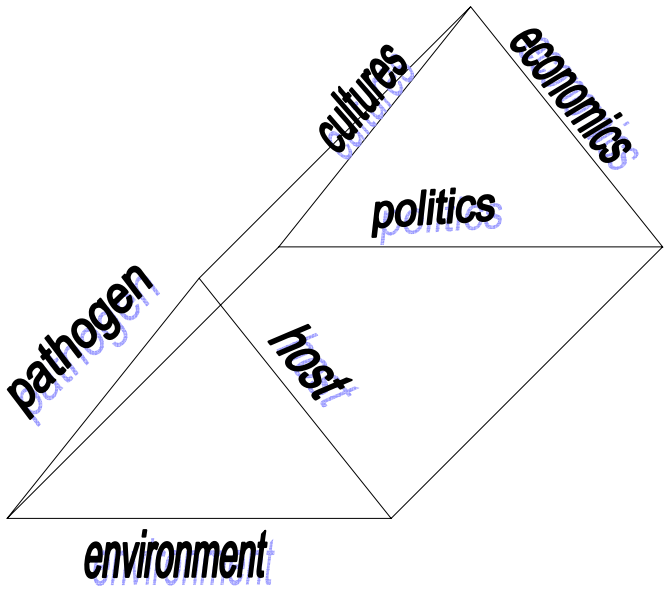


Fig. 1-8: The Multidimensional disease triangle

References

Agrios, G. N. 2005. *Plant Pathology*. Fifth ed. Burlington, MA: Elsevier Academic Press.

Aime, M. C., and W. Phillips-Mora. 2005. The causal agents of witches' broom and frosty pod rot of cacao (chocolate, *Theobroma cacao*) form a new lineage of Marasmiaceae. *Mycologia* 97 (5):1012-1022.

Amado, J. 1912. *Cacau*. 53 ed. Rio de Janeiro: ABDR.

Anderbrhan, T. 1987. Studies on the epidemiology and control of witches'broom disease of cacao in the Brazilean Amazon. Paper read at International Cocoa Research Conference, May 1987, at Santo Domingo, Dominican Republic.

Anonymous. 1981. Brazil's Big Cocoa Push: Growth Is a Gamble. *Financial Times of London world business weekly* 4 (34):44.

Anonymous. 1989. Vassourada. *O estado de Sao Paolo*, May 30 1989.

Ayres, P. G. 2005. *Harry Marshall Ward and the fungal thread of death*. St Paul, Minnesota: American Phytopathological Society press.

Bancroft, K. 1910. Colletotrichum luxifucum, Van Hall and Drost. In *A handbook of the fungus diseases of West Indian plants*. London: Published privately by the author.

Bauchspies, W. K. 2000. Cultural Re-constructions of an Adoptive Child: Science. *Cultural Dynamics* 12 (2):237-260.

Bauchspies, W. K., J. Croissant, and S. Restivo. 2006. *Science, technology and society: a sociological approach*. First ed. New York: Backwell publishing.

Berkeley, M. J. 1809. *The gardener chronicle and agricultural gazette*, November 6 1809, 1157.

Boroadbent, L. 1960. Dispersal of inoculum by insects and other animals, including man. In *Plant Pathology*, edited by J. G. Horsefall and A. E. Dimond. New York: Academic Press.

Bucheli, M. 2004. *Bananas and business : the United Fruit Company in Colombia, 1899-2000*. New York: New York University Press.

Caponi, S. 2002. La generación espontánea y la preocupación higienista por la diseminación de los gérmenes. *História, Ciências, Saúde — Manguinhos* 9 (3):591-608.

Carefoot, G. R. 1967. *Famine on the wind : man's battle against plant disease*. New York: Rand McNally Press.

Clearance-Smith, W. G., and S. Topik. 2003. *The global coffee economy in Africa, Asia and Latin America, 1500-1989*. New York: Cambridge University Press.

Dillinger, T. L., P. Barriga, S. Escarcega, M. Jimenez, D. S. Lowe, and L. E. Grivetti. 2000. Food of the Gods: Cure for Humanity ? A Cultural History of the Medicinal and Ritual Use of Chocolate. *Journal of Nutrition*:2057S-2072S.

Evans, H. C., D. F. Edwards, and M. Rodriguez. 1977. Research on Cacao Diseases in Ecuador: Past and Present. *PNAS* 23 (1):68-80.

Francl, L. J. 2001. *The disease tirangle: a plant pathological paradigm revisited.* Available from http://www.apsnet.org/education/InstructorCommunication/Teaching/Articles/Francl/.

Fulton, R. H. 1984. *Coffee rust in the Americas*. St. Paul, Minnesotta: American Phytopathological Society Press.

Gareez, A. N. R. 1985. *CEPLAC de Corpo e Alma*. Ilheus, Bahia: CEPLAC.

Gäumann, E. A. 1950. *Principles of plant infection : a text-book of general plant pathology for biologists, agriculturists, forsters and plant breeders*. Edited by W. B. Brierley. London C. Lockwood.

Homewood, B. 1991. Fungus threat to Brazil's 300 million cocoa trees. *New Scientist*:16.

ICCO. 2007. International Cocoa Organization Annual Report 2005/2006: ICCO.

Inibap. 2005. *No end to the banana*. Available from http://www.inibap.org/exhibit/exhibit_en.htm.

Jamieson, R. W. 2001. The essence of commodification: caffeine dependencies in the early modern world. *Journal of Social History* 35 (2):269-294.

Junior, P. 2007. Bioterrismo na Bahia. *Veja*.

Keithan, E. 1939. Cacao industry of Brazil. *Economic Geography* 15 (2):195-204.

Kushalappa, A. C., and A. B. Eskes. 1989. *Coffee rust : epidemiology, resistance, and management*. Boca Raton, Florida: CRC Press.

Laker, H. A., and Silva. 1990. Witches' Broom Disease of Cocoa in the State of Rondonia, Brazil: Historical Perspectives and Present Situation. *Cocoa Growers Bulletin*:45 - 57.

Large, E. C. 2003. *The advance of the fungi*. St. Paul, Minnesota: The American Phytopathological Society.

Mahony, M. A. 1996. The world cacao made: Society, politics, and history in southern Bahia, Brazil, 1822-1919. Ph.D., Yale University, United States -- Connecticut.

Marquardt, S. 2001. "Green Havoc": Panama Disease, Environmental Change, and Labor Process in the Central American Banana Industry. *The American Historical Review* 106 (1):49-88.

Motamayor, J. C., A. M. Risterucci, P. A. Lopez, C. F. Ortiz, A. Moreno, and C. Lanaud. 2002. Cacao domestication I: the origin of the cacao cultivated by the Mayas. *Heredity* 89:380-386.

Muhs, D. R., R. R. Kautz, and J. J. MacKinnon. 1985. Soils and the location of cacao orchards at a Maya site in western Belize. *Journal of Archeological Science* 12:121-137.

Nunes Leal, V. 1977. Local Government autonomy and coronelismo. In *Coronelismo: The municipality and representative governement in Brazil*
edited by M. Deas, C. T. Smith and J. Street. New York: Cambridge University Press.

Pang, E.-S. 1979. Coronelismo: an oligarchic approach to Brazilian politics. In *Bahia in the first Brazilian republic*. Gainesville: University of Florida Press.

Pang, E.-S. 1979. The quest for "Order and Progress", 1889-1904. In *Bahia in the first Brazilian republic*. Gainesville: University of Florida press.

Pereira, J. L., L. C. C. d. Almoida, and S. M. Santos. 1996. Witches' broom disease of cocoa in Bahia: attempts at eradication and containment. *Crop Protection* 15 (8):743-752.

Ploetz, R. 2005. *Panama disease: an old nemesis rears its ugly head. Part 2. The Cavendish era and beyond*. The American Phytopathological Society. Available from http://www.apsnet.org/online/feature/panama.

Rincones, J., G. D. Mazotti, G. W. Grifith, A. Pomela, A. Figueira, G. hhA. Leal, M. V. Queiroz, J. F. Pereira, R. A. Azevedo, G. A. G. Pereira, and L. W. Meinhardt. 2006. Genetic variability and chromosome-length polymorphisms of the witches' broom pathogen *Crinipellis perniciosa* from various parts in South America. *Mycological Research* 110:821-832.

Rorer, J. B. 1913. The Suriname Witch-broom Disease of Cacao. *Board of Agriculture, Trinidad and Tobago* Circular No. 10.

Roseberry, W. 1991. La Falta de Brazos: Land and Labor in the Coffee Economies of Nineteenth-Century Latin America. *Theory and Society* 20 (3):351-381.

Ruf, F. 2002. "Ilheus, terre du cacao et de l'argent". In *Production de cacao et replantation à Bahia (Brésil)*, edited by F. Ruf, M. Forget and A. Gasparetto. Montpellier: CIRAD.

Sanders. *Turfgrass diseases: a triangular view of diagnosis and management* Penn State College of Agriculture Cooperative Extension. Available from http://pubs.cas.psu.edu/PubAuthor.asp?varAuthor=Sanders&Submit2=Go.

Schumann, G. L. 1991. *Plant Diseases: Their Biology and Social Impact*. St Paul, Minnesota: Amercian Phytopathological Society Press.

Silva, P. 1987. Cacao enfermidades, inspeção e quarentena. *Boletim Tecnico*:3 - 13.

Singer, R. 1942. A monographic study of the genera "Crinipellis" and "Chaerocalathus". *Lilloa* 8:441-534.

Soluri, J. 2000. People, plants and pathogens: the eco-social dynamics of export banana production in Honduras, 1875-1950. *Hispanic American Historical Review* 80 (3):463-501.

Soluri, J. 2005. *Banana cultures. Agriculture, consumption, and environmental change in Honduras and the United States* First ed. Austin: University of Texas Press.

Stahel, G. 1915. Marasmius Perniciosus Nov Spec. *departement van Den Landbouw in Surinam* Bulletin No.33.

Stahel, G. 1919. Contribution to the knowledge of witchbroom disease. *Tropical Agriculture* 4 (6):167-176.

Stell, F. 1933. Report of Mr. F. Stell, Mycologist, Department of Agriculture, on his Visit to Ecuador to Study Witchbroom Disease (Marasmius perniciosus) of Cocoa.

Topik, S. C., and A. Wells. 1998. *The second conquest of Latin America : coffee, henequen, and oil during the export boom, 1850-1930*. Austin: University of Texas Press, Institute of Latin American Studies.

Tucker, R. P. 2000. *Insatiable appetite : the United States and the ecological degradation of the tropical world*. Berkeley: University of California Press.

Wheeler, B. E. J. 1987. Plant Pathology in a Developing World. *Plant Pathology*:430 - 437.

Wood, G. A. R., and R. A. Lass. 1985. *Cocoa*. Fourth ed. New York: Longman.

Chapter 2

Solanum lycopersicum as a model system to study pathogenicity mechanisms of *Moniliophthora perniciosa,* the causal agent of witches' broom disease of *Theobroma cacao*

Abstract

The C-biotype of *Moniliophthora perniciosa* is the causal agent of witches' broom disease of *Theobroma cacao* L. While this disease is of major economic importance, the pathogenicity mechanisms and plant responses underlying the disease are difficult to study given the cacao tree's long life cycle and the limited availability of genetic and genomic resources for this system. The S-biotype of *M. perniciosa* infects solanaceous hosts, particularly pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum).* These species are much more amenable for performing studies of mechanisms underpinning host-pathogen interactions as compared to cacao. A phylogenetic analysis performed in this study demonstrated that S-biotype strains clustered with C-biotype strains, indicating that these biotypes are not genetically distinct. A comparative analysis demonstrated that disease progression in tomato infected with the S biotype is similar to that described for cacao infected with the C- biotype. The major symptoms observed in both systems are swelling of the infected shoots and activation and proliferation of axillary meristems. Cellular changes observed in infected tissues correspond to an increase in cell size and numbers of xylem vessels and phloem parenchyma along the infected stem. Observations revealed that fungal colonization is biotrophic during the first phase of

infection, with appearance of calcium oxalate crystals in close association with hyphal growth. In summary, despite different host specificity, both biotypes of *M. perniciosa* exhibit similar disease-related characteristics, supporting that the tomato-*M. perniciosa* system can serve a surrogate model to increase understanding of witches' broom disease of cacao.

Key words: *Moniliophthora perniciosa*, *Theobroma cacao* L., *Solanum lycopersicum*, fungal pathogenicity, plant defense

Introduction

Moniliophthora (= *Crinipellis*) *perniciosa* (Stahel) Sing., is a basidiomycete fungus (Aime and Phillips-Mora 2005), closely related to the common button mushroom *Agaricus bisporus* (Purdy and Schmidt 1996). The fungus is a pathogen of *Theobroma cacao* L., the chocolate tree, and the disease's common name, witches' broom, is due to the appearance of dried and distorted shoots on the infected trees (Stahel 1919). *Moniliophthora perniciosa* is indigenous to the Amazon basin region in association with several plant species in the genus *Theobroma* (Malvaceae) and also in plants from families unrelated to the Malvaceae (Bastos *et al.* 1988). Multiple biotypes of *M. perniciosa* are defined based on host adaptation: C-biotype infecting *T. cacao* ; B-biotype found on members of the Bixaceae (Bastos and Anderbrhan 1986); L- biotype associated with members of the Bignoniaceae (Griffith and Hedger 1994); S-biotype associated with members of the Solanaceae (Bastos and Evans 1985); and H-biotype that can infect members of the Malpighiaceae (Resende *et al.* 2000).

Although cross infectivity between the different biotypes has not been clearly demonstrated, morphological and genetic evidence suggests that these biotypes belong to a single species (Arruda *et al.* 2005; Griffith *et al.* 2003).

In nature, basidiospores of *M. perniciosa*, are the only known infectious propagules, germinating on young cacao tissues such as apical and axillary meristems, young leaves, and developing flowers (Purdy and Schmidt 1996), and subsequently penetrating plant tissues directly or entering through stomata (Frias and Purdy 1991; Sreenivasan and Dabydeen 1989). The pathogen exhibits two phases in its disease cycle, the biotrophic phase and the necrotrophic phase. Germinated basidiospores form a swollen (5-20 µm) monokaryotic hyphae that grows intercellularly as a biotroph (Evans 1980) and causes symptoms that include shoot swelling, petiole swelling, and loss of apical dominance (Scarpari *et al.* 2005). The pathogen can also attack flower cushions (clusters of floral meristems and remnants of old flower tissues), resulting in the transformation of floral meristems into single or compound vegetative shoots (phyllody). Infected immature flowers lead to the formation of sterile abnormally shaped fruits, called “chirimollas” (Wheeler and Suarez 1993). Fruits can also be infected, resulting in necrotic lesions on the epidermal layers and a characteristic rotting of the mucilage and beans (Silva *et al.* 2002). The biotrophic phase usually lasts for about 60 days in artificially inoculated plants (Scarpari et al. 2005). In the necrotrophic phase of infection, from the apex downwards, the green broom becomes brown and dies within a week (Silva et al. 2002). This phase is associated with the appearance of intra-cellular hyphae, which is dikaryotic and exhibits clamp connections (Radzali *et al.* 1996). When the dried

broom is exposed to alternate periods of dry and wet weather, basidiocarps emerge to complete the life cycle (Purdy and Schmidt 1996).

A witches' broom epidemic recently occurred in 1989 in Bahia, Brazil, once the second most productive cacao-producing region in the world. In the subsequent ten years, cocoa bean production in this region has declined from 300,000 tons to 130,000 tons causing a loss of $235 million annually and socioeconomic hardship in the region (Bowers *et al.* 2001). The development of effective control measures against *M. perniciosa* is one of the major challenges in boosting cacao production in South America. Equally critical is avoiding the introduction of the pathogen to Africa and Asia where the disease has not yet been observed (Evans 2002). A better understanding of the physiological and genetic mechanisms underpinning the development of this disease is vital for protecting global cacao production from witches' broom. However, research on *M. perniciosa/T.cacao* interactions has been difficult because of cacao's slow and long life cycle, irreproducible infection efficiencies under laboratory conditions, very limited genetic/genomic resources, and the complexity of *M. perniciosa*'s disease cycle (Cronshaw and Evans 1978; Evans 1978).

To address this problem, this study aimed to develop a surrogate pathosystem based on cultivated solanaceous plants and the S-biotype of *M. perniciosa*. *Solanum lycopersicum* (tomato), in particular, has been extensively used as a model plant for basic biological research, offering numerous genetic and genomic tools and resources. These include high density molecular maps (Tanksley 1993), conserved ortholog set

(COS) markers (Fulton *et al.* 2002), near isogenic lines (Monforte and Tanksley 2000), microarrays (Alba *et al.* 2004) and mutant collections (Menda *et al.* 2004).

As the first step of this study, phylogenic relationships, colonization patterns, and the disease cycles of the S and C biotypes, were investigated. Cellular mechanisms underlying the development of the typical symptoms of the biotrophic phase (stem swelling and release of axillary buds) were also studied via morphometric analyses of the affected areas. Lastly, utilizing transgenic tomato plants harboring an auxin responsive reporter gene (DR5-GUS auxin responsive promoter), changes of this hormone occurring during infection, were monitored. In general, the two pathosystems exhibit similar characteristics in both fungal infectivity and disease symptoms displayed, supporting the suitability of tomato as a surrogate model plant for dissecting the molecular and cellular mechanisms regulating pathogenesis associated with witches' broom of cacao.

Materials and Methods

Moniliophthora perniciosa isolates

All isolates of *M. perniciosa* used in this study (Table 2.1) are maintained in a solution of MES 0.01 M and 16% glycerol in liquid nitrogen as part of the

Moniliophthora collection at the Pennsylvania State University (APHIS permit # 72545). Spores or mycelium were plated on Potato Dextrose Agar (Difco) and grown at 25 °C for 1 month for genomic DNA extraction.

Phylogenetic analysis

To determine the phylogenetic relationships between the S and C biotypes, nine isolates from the Penn State Collection and 18 isolates from the Genebank were analyzed (Table 2-1).

Isolate	Host	Original Material	Geographical Location	ITS Genebank accession Number.	Species
				EU047931	
73-63	*S.paniculatum*	Green broom	Camaca-Serra Boa, BA, Brazil		*M. perniciosa*
92-10-7	*S.rugorosum*	n/a[1]	Manaus, AM, Brazil	EU047930	*M. perniciosa*
1189G	*T.cacao*	Green Broom	Itanhem Sombra da Tarde, BA,Bazil	EU047932	*M. perniciosa*
1441G	*T.cacao*	Green Broom	CEPEC, BA, Brazil	EU047933	*M. perniciosa*
1480	*T.cacao*	Green Broom	n/a	EU047934	*M. perniciosa*
KG06	*S.paniculatum*	Green Broom	Vale do Jequitinhonha, BA, Brazil	EU047935	*M. perniciosa*
73-6-01	*S.paniculatum*	n/a	Itajuipe Almirante, BA,Brazil	EU047929	*M. perniciosa*
1188	*T.cacao*	n/a	n/a	EU047936	*M. perniciosa*
M.Gflorum	*T. grandiflorun*	n/a	n/a, Ecuador	EU047937	*M. roreri*
CANG/1	*T.cacao*	Basidioma in culture	Canavieiras, BA, Brazil	AY317131[G]	*M. perniciosa*
UB/21998	*T.cacao*	Basidioma in culture	Ilheus, BA,Brazil	AY317132[G]	*M. perniciosa*
BE12/18	*T.cacao*	Basidioma in culture	Belem, Para,Brazil	AY317133[G]	*M. perniciosa*
UB/2021	*T.cacao*	Basidioma in culture	Manaus, AM,Brazil	AY317134[G]	*M. perniciosa*
UB/2027	*T.cacao*	Basidioma in culture	Ouro Preto, RO,Brazil	AY317135[G]	*M. perniciosa*
UB/2041	*S.lycocarpum*	Basidiome/necrotic broom	Lavras, MG, Brazil	AY317136[G]	*M. perniciosa*
UB/2054	*T.grandiflorum*	Basidiome/necrotic broom	Manaus, AM, Brazil	AY317127[G]	*M. perniciosa*
FA617	*T. grandiflorun*	Basidiome/necrotic broom	Itajuipe Almirante,BA, Brazil	AY317129[G]	*M. perniciosa*
FA619	*S.paniculatum*	Basidiome/necrotic broom	Itajuipe Almirante,BA, Brazil	AY317130[G]	*M. perniciosa*
n/a	*T.grandiflorum*	Vegetative Dikaryotic Mycelium	n/a, Brazil	AY216468[G]	*M. perniciosa*
n/a	*T.subincantum*	Vegetative Dikaryotic Mycelium	na/na, Brazil	AY216469[G]	*M. perniciosa*
CP-37	*S. paniculatum*	Vegetative Dikaryotic Mycelium	n/a,BA, Brazil	AY216470[G]	*M. perniciosa*
n/a	*S. paniculatum*	Vegetative Dikaryotic Mycelium	n/a, Brazil	AY176316[G]	*M. perniciosa*
CP-83	*Herrania sp.*	Vegetative Dikaryotic Mycelium	n/a,BA,Brazil	AY216471[G]	*M. perniciosa*
CP-104	*C. frutescens*	Vegetative Dikaryotic Mycelium	n/a, Bahia, Brazil	AY216472[G]	*M. perniciosa*
RNBP1	*T.cacao*	Vegetative Dikaryotic Mycelium	Quillabamba,Peru	AF335590[G]	*M. perniciosa*
n/a	*n/a*	n/a	n/a	AY230255[G]	*M.roreri var. g ileri*
strainCBB-361	*n/a*	Vegetative Dikaryotic Mycelium	n/a	AY216476[G]	*Marasmius sp.*

[1]: not available

Genomic DNA was extracted from vegetative dikaryotic mycelium as previously described (Thomson and Henry 1993). The region spanning the internal transcribed spacers of ribosomal RNA encoding genes ITS1, 5.8S RNA, and ITS2 was amplified from each strain by PCR using the universal primer pair ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG). Amplification was verified on a 0.8% agarose gel, and PCR products were purified using Qiagen DNA purification columns (Qiagen cat # 28104). PCR products were sequenced using an ABI Hitachi 3730XL DNA analyzer (Applied Biosystems). Eighteen additional ITS sequences of *M. perniciosa*, generated in a previous study (Arruda et al. 2005), were obtained from Genebank. The resulting 27 ITS sequences (Table 2.1) were aligned using the Clustal W algorithm with MEGA 3.1 software (Kumar *et al.* 2004). A neighbor-joining tree was constructed with MEGA 3.1 with statistical support for branch topology tested by bootstrap analysis derived from 1000 replicates. To root the tree, isolate CBB-361 of *Marasmius* sp. (Arruda *et al*. 2005), was used as an outgroup.

Inoculum production

Basidiocarps were produced from S- and C-biotype strains using the bran-vermiculite *in vitro* method (Griffith and Hedger 1993; Niella *et al.* 1999). For the S-biotype, basidiocarps were produced from strain 73-6-01, which was collected from infected *Solanum paniculatum* in Itajuipe, Bahia (Brazil). An infected cacao plant in

Bahia was the source of strain 73SP16 (C-biotype). “Cookies” of the bran cultures were hung in a 40 gallon fish tank and maintained at 25° C with alternations between a 10-hour period at 100% humidity and a 14-hour period at 50% humidity. Water mist from an ultra-sonic humidifier supplied humidity. After 2 to 3 months, basidiocarps were harvested and the inoculum was collected by attaching the mushroom caps onto Petri-dish lids with petroleum jelly and placing them over flasks containing 5-10 ml of MES 0.01 M, 16% glycerol buffer. The resulting spore suspension was then frozen in liquid nitrogen until used.

Plant material and sterilization

Seeds of *S. lycopersicum* (cvs. New Yorker, NC841.73 and Micro-Tom), and *Capsicum annum* (cv. Tequila Sunrise) were surface-sterilized with 2% NaOCl (commercial bleach) for 20 min and washed 3 times with sterile distilled water for 2 min each time. Seeds were pre-germinated in the dark over moist sterile filter paper for 3-4 days at 25ºC. After germination, seeds were sown in 100 ml pots containing Scotts© ready-earth soil and grown at 25ºC with 16:8 hr light-dark periods at 60% humidity. Seedlings of cacao (cv. Comum) were sown and kept in a greenhouse under the same conditions. The plants were fertilized once a week with 1/10 Hoagland solution (160 ppm).

Plant infection

The method of (Surujdeo *et al.* 2003) was used to infect plants. Using a hemacytometer, inoculum concentration was adjusted to 6 x 10^5 spores per ml with a solution of 0.01M MES and 0.25 % agar. A 10 µl drop of inoculum was placed on the youngest emerging leaf using a micro-pipette. The plants were then placed in the dark for 48 h in a dew chamber (Percival # Model E-54U DL) at temperatures ranging from 25-27ºC and 100% humidity. Under these conditions small water droplets accumulated on the leaves. The rate of spore germination was evaluated before and after plant inoculation by plating 10µl inoculum on a water agar plate that was incubated under the same conditions as the inoculated plants. The plants were subsequently transferred to a growth chamber set at 25ºC with a relative humidity of 60% and a 16:8 hour light: dark period for 2-3 months. Images of infected plants were taken every week using a C-4000 zoom digital camera (Olympus) to record morphological changes.

Morphometric analysis

To investigate the cellular basis of stem swelling, the first emerging leaf of ten day-old tomato plants (NC841.73) was inoculated, and stem samples (five 1-cm long sections) from these plants, as well as from mock-inoculated plants were collected 8

weeks post inoculation. The middle of the hypocotyl (H), the cotyledonary node (C) and internodes 1, 2 and 4 were used as reference points. Stem sections were fixed in formaldehyde acetic alcohol (50% ethanol, 5% glacial acetic acid, 10% formalin, and 35% water) for 24 hrs at room temperature. Fixed samples were dehydrated by treating them with a series of ethanol solutions: 50% (1 hr), 70% (15 min), 95% (8.5 hrs), and 100% (1.5 hrs). Paraffin embedding was conducted using a Shandon Citadel 2000 paraffin processor (Thermo Scientific). Five to ten µm cross-sections were generated using a Leica 2000 paraffin microtome (Leica Microsystems) and placed onto positively charged slides (Thermo Scientific Shandon, NC9180670). Sections were cleared with xylene and the cell walls and fungal hyphae were stained with calcofluor (0.01%). Alternatively, sections were stained with Pianeze IIIB to specifically stain fungal hyphae and observations were conducted via brightfield microscopy as previously described (Gramacho 1999; Vaughan 1914). Fungal hyphae stained with Pianeze III B (Gramacho 1999) appeared pink when observed using difference interference contrast (DIC). A microscope connected to an AxioCam MRm digital camera (Zeiss) was used to collect the images. The presence of calcium oxalate crystals was observed under polarized light and by staining with silver nitrate as previously described (Kurrein *et al.* 1975).

To measure the total area of whole sections, mosaics of 10x images were taken using a Olympus BX60 microscope with a motorized stage, under UV light and taken with a Hamamatsu cooled digital camera (ORCA 100, Model #C4742-95). For precise cell measurements, an inverted Axiovert 200 Zeiss microscope (Zeiss) with UV filter was used.

Individual images were processed manually using a tablet PC to correct for image artifacts and measured using the "analyze particles" function on Image J V. 1.38X (Abramoff *et al.* 2004). The total area of each tissue type, cell size distribution, cell density (number of cells per unit area) and total number of cells (cell density X total area) were quantified and compared between infected and control plants. In total, this analysis involved six biological replicates for each of the five positions studied. Cell numbers between control and infected plants, and cell size distribution (logarithmic transformations) were compared using a Student t-test and a Mann-Whitney test respectively using the statistical package R (Ihaka and Gentleman 1996).

Genetic Transformation of tomato plants with the GUS gene under the control of the DR5 auxin inducible promoter

The DR5 construct is a synthetic promoter inducible by auxin and consists of 7 tandem repeats (TGTCTC) spaced by 5 bp (Hagen and Guilfoyle 2002). *Agrobacterium* mediated transformation (ATMT) of tomato (var. Micro-Tom) was performed at the University of California Davis Plant Transformation Facilities. Transgenic plants expressing GUS were grown and infected as described above. Six weeks after inoculation, plants were sectioned at the sites of swelling and stained for GUS activity as previously described (Jefferson *et al.* 1987).

Results

Symptomology in cacao, pepper, and tomato

In order to develop a surrogate model system for studying witches' broom of cacao, we first compared the symptomology of different biotypes on several plant species with that of cacao. As previously described (Silva et al. 2002), infected cacao plants exhibited the first symptom of infection about 30 days post inoculation (dpi), in the form of a slight swelling of the stems (Fig 2-1A). Activation of axillary meristems and swelling near the infection point was observed during later stages of infection during the biotrophic phase of the disease (Fig 2-1-B).

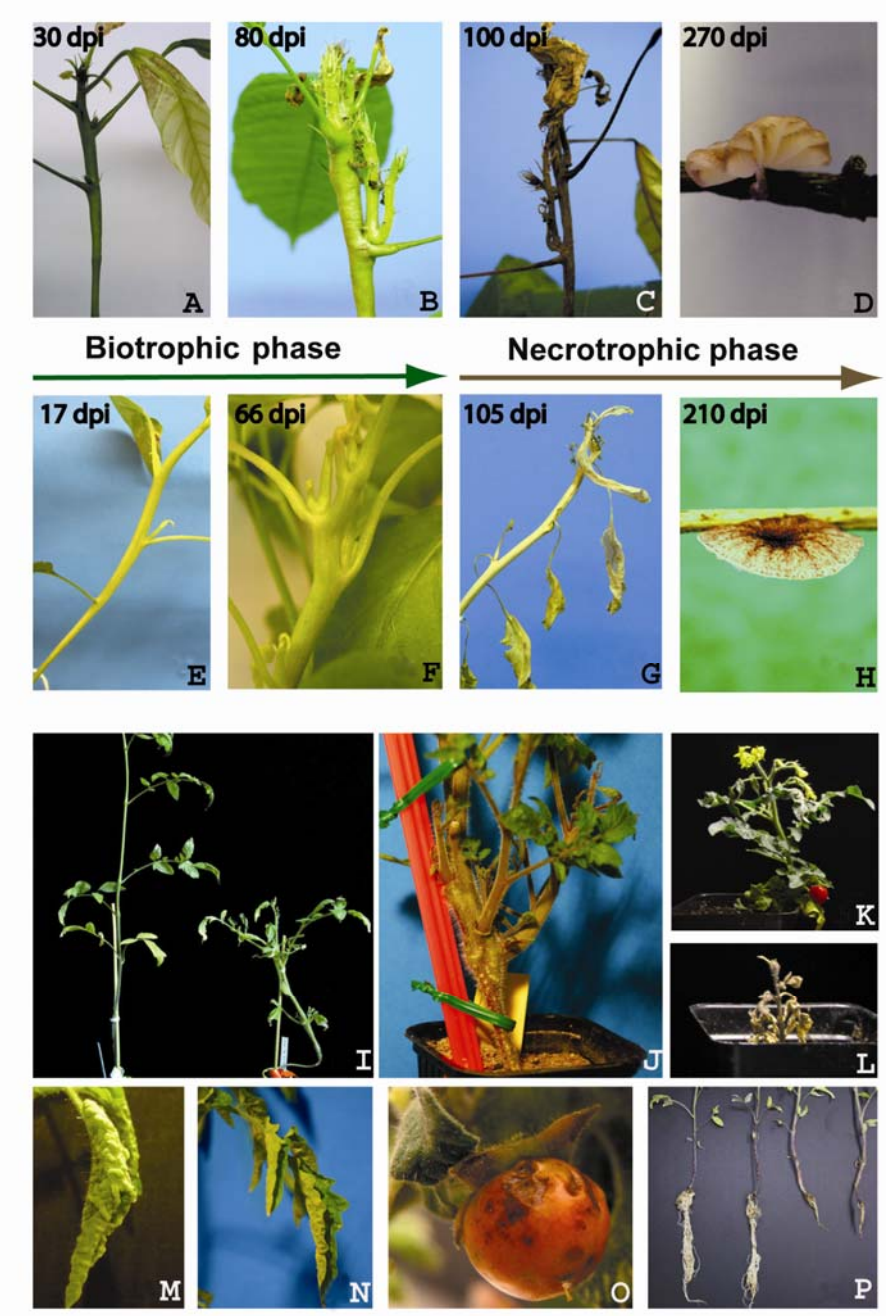


Fig. 2-1: Symptoms observed in plants (cacao and solanaceous hosts) infected with *M. perniciosa*. (A-H) Disease cycles of *M. perniciosa* in *T. cacao* and in *C. annuum*. (I-J). The biotrophic phase in *S. lycopersicon*; (I) left un-inoculated plant, right, inoculated plant that expresses swelling, activation of axillary buds and stunting. (J) Stem swelling and activation of meristems on an infected plant. (L) The necrotrophic phase in Micro-Tom compared to (K) a non-inoculated plant bearing fruits and flowers. (M) Blisters on leaves of an infected plant. (N) Leaf curling in an infected plant. (O) Necrosis of the pericarp on tomato fruits of Micro-tom. (P) Root atrophy in infected tomato plants.

The developmental switch from the biotrophic to the necrotrophic phase of infection occurred at about 100 dpi, causing a total necrosis of the shoot within 2-3 days (Fig 2-1C). Exposing the dried shoot to conditions conducive for sporulation caused basidiocarps to form on the dry branches within 270 dpi (Fig 2-1D).

The pepper variety, Tequila Sunrise, was used for verifying the pathogenicity of S-biotype strain 73-6-01 (Fig. 2-1E to 3-1H). Witches' broom symptoms (stem swelling and activation of the axillary meristems) were recorded at 17 dpi (Fig 2-1E). The biotrophic phase, as indicated by stem swelling and activation of axillary meristems, lasted up to 90 dpi in pepper (Fig 2-1F). Total necrosis of the infected shoot was observed at 105 dpi, but the progression was slower than in cacao (Fig 2-1G). When the dry shoot was exposed to sporulation conditions, basidiocarps appeared at 210 dpi (Fig 2-1H).

The same general progression of symptoms was also observed in tomato plants inoculated with S-biotype strain 73-6-01, with a few differences (Fig 2-1I-P). First, whereas the tomato variety Micro-Tom switched to the necrotrophic phase within one month (Fig 2-1K and Fig 2-1L), the tomato variety NC841.73 did not switch to the necrotrophic phase under the same growing conditions (Fig 2-1I and Fig 2-1J). Second, in addition to the expected stem swelling and activation of axillary meristems (Fig 2-1J), infected tomato plants developed additional symptoms not observed on cacao or pepper plants. About 15 dpi, plants developed blisters along leaf veins (Fig 2-1M). Inspection of these blisters under microscopy revealed that they consisted of an enlarged dedifferentiated group of cells resembling callus.

Additional symptoms included leaf curling (Fig 2-1N), fruit necrosis on Micro-Tom (2-1O), and hypertrophy of the root system (Fig 2-1P).

Phylogenetic analysis of strains of the C and S biotypes of M. perniciosa

To assess the genetic relatedness between the S and C biotypes of *M. perniciosa*, a phylogenetic analysis based on DNA sequences of the internal transcribed spacer (ITS) regions of ribosomal RNA encoding genes was performed (Fig 2-2).

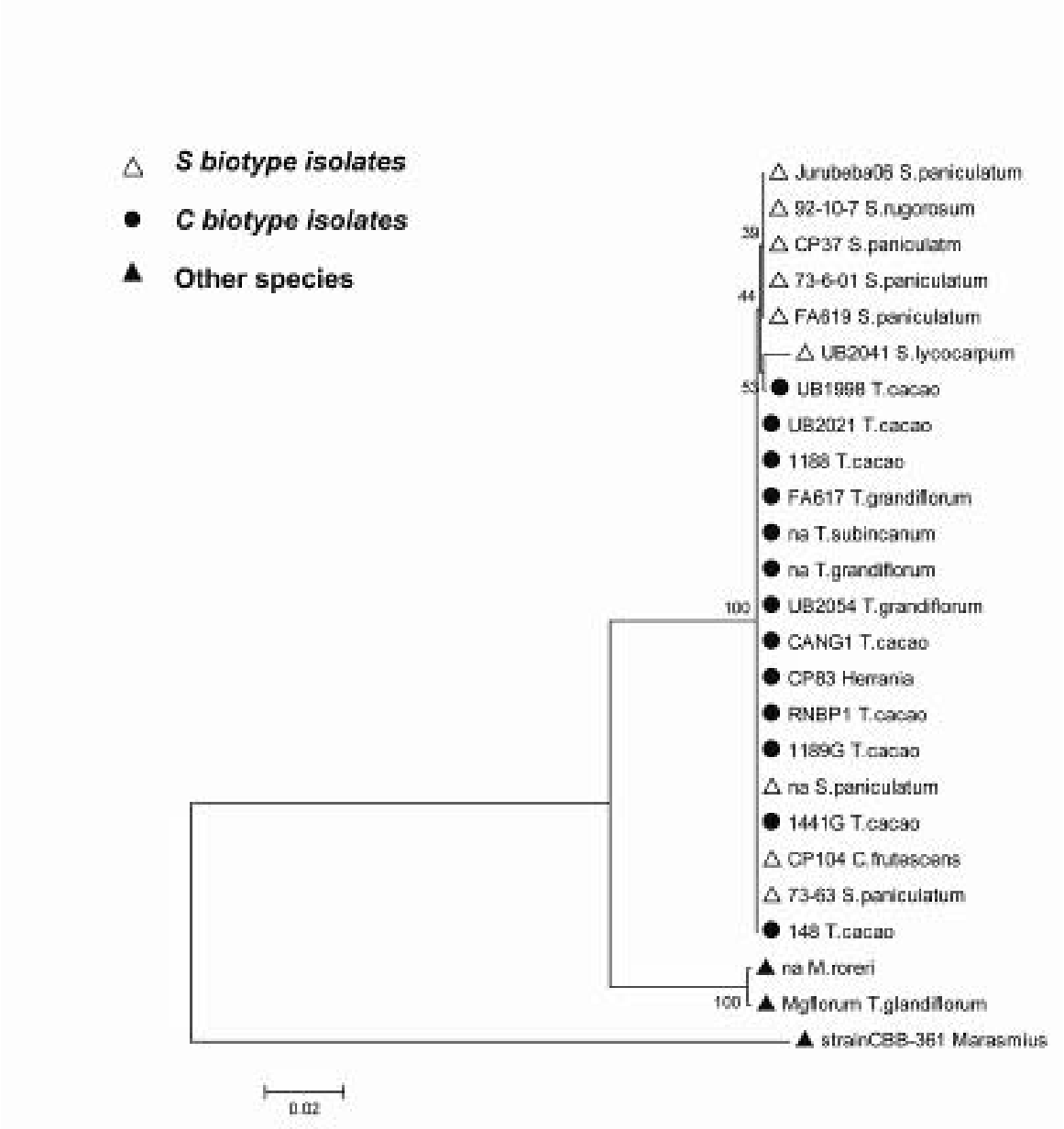


Fig. 2-2: Phylogenetic tree based on DNA sequences of the ITS regions (ITS1, 5.8S RNA, and ITS2) from isolates of *Moniliophthora* spp., *M. roreri* and *Marasmius* spp. (CBB361). The symbol preceding each strain name indicates the biotype (as indicated in the key on the left side of the picture). The numbers above the branches indicate the bootstrapping support. The tree was generated by neighbor joining analysis using MEGA 3.1, and statistical support for branch topology was tested by bootstrap analysis derived from 1000 replicates.

Since a previous report indicates that *M. roreri* is closely related to *M. perniciosa* (Aime and Phillips-Mora 2005), one *M. roreri* isolate collected from *Theobroma grandiflorum* (Mgflorum) and one *M. roreri* var. gileri type from *T.cacao* were included in the analysis. Additionally, strain CBB361of Marasmius spp. (both *M. perniciosa* and *M. roreri* belong to the Marasmiaceae) was used as the outgroup to root the tree.

The resulting phylogenetic tree consists of three well-resolved groups (Fig 2-2). The first group included all isolates of *M. perniciosa* collected from cacao, pepper, solanaceous hosts (*S. paniculatum*, *S. lycocarpum*, *S rugorosum*) and cacao relatives (*T. grandiflorum*, *T. subincanum* and Herrania). Some S-biotype isolates (Jurubeba06, 73-6-01, CP37 and 92-10-7) appeared to cluster, but the bootstrap support for this subgroup (39%, Fig. 2-2) was not strong. The second group contained two strains of *M. roreri* (na_M.roreri, and Mgflorum), supporting the proposed divergence from the first group. The third group was represented by (strain CBB361 of *Marasmius* spp. (the outgroup).

Cellular changes underlying symptoms in tomato plants colonized with M. perniciosa

The increase of stem area (swelling) could result from increased cell numbers, increased cell size, increase in inter-cellular spaces, or a combination of these cellular changes. To investigate the mechanisms underpinning stem swelling, cell numbers and tissue areas in cross sections of infected and control stems were compared (Fig 2-3). The total area of cortex, phloem, xylem and pith in control shoots was unquestionably higher

as seen on the calcofluor stained cross sections (Fig 2-3A and B). To understand in more detail the cellular changes caused by fungal colonization that occur during the biotrophic phase, the fold increase between infected and control areas of the different tissues (cortex, phloem, xylem, pith) were determined by quantitative analysis (Fig 2-3 C). The most significant difference between control and infected plants appeared at positions H and C (Fig 2-3C). At the hypocotyl position, the phloem and xylem areas where 14 and 10 times larger respectively in infected plants compared to controls. At the cotyledon position (C), the phloem and xylem areas were 7 and 5 times larger respectively, on infected plants compared to controls. On more distal positions of the plant shoot (1st, 2nd and 4th internode), this difference was attenuated for phloem and xylem. The cortex was between 2 and 5 times larger in infected plants compared to controls (Fig 2-3C). The pith was only one to two times larger in the infected tissues compared to the control tissues, except in the fourth internode where it became 5 times larger in infected tissues (Fig 2-3C).

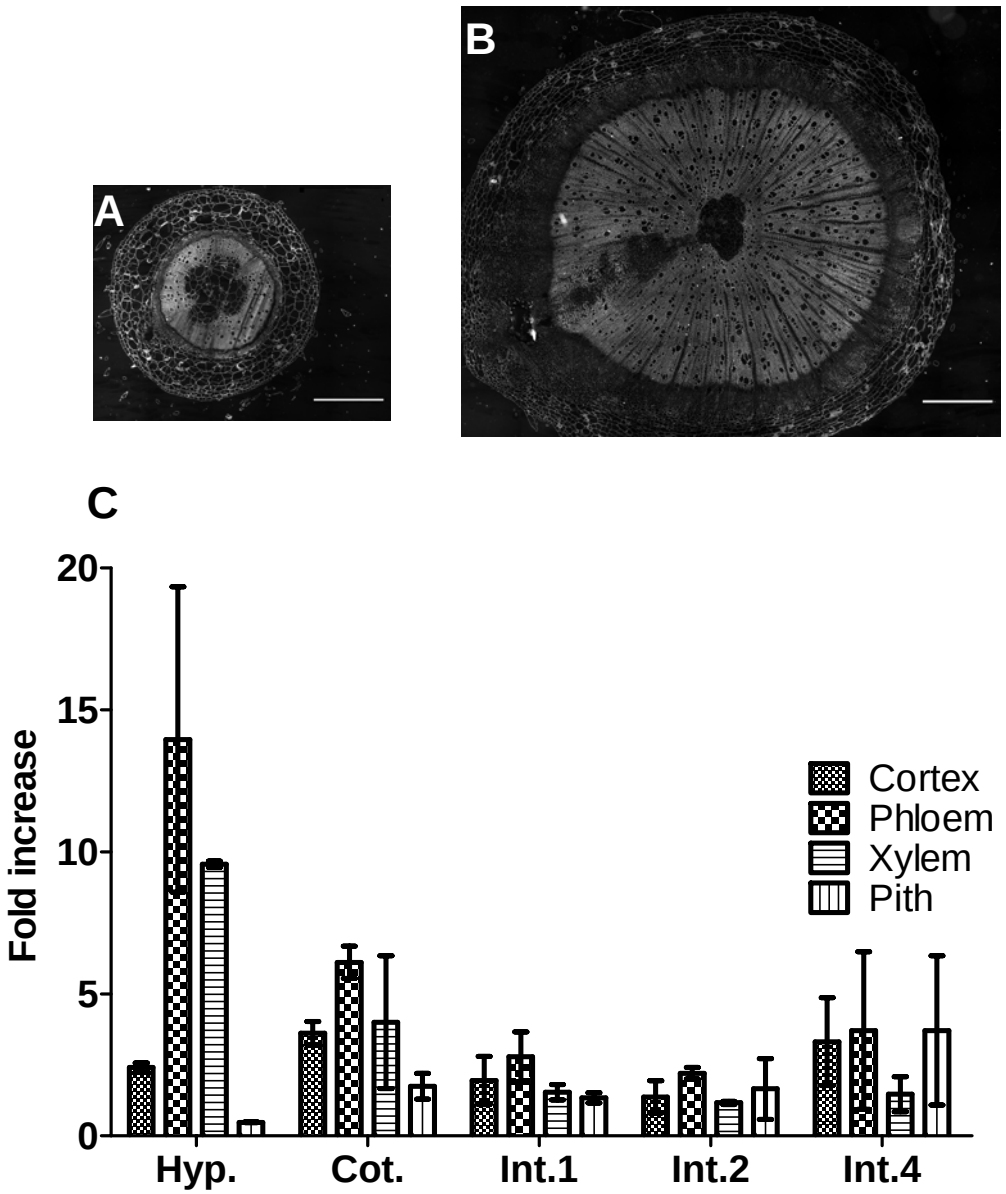


Fig. 2-3: Cellular changes induced by *M. perniciosa* infection in tomato. Photomicrographs of stem cross sections at the hypocotyl position in non-infected (A) and infected (B) plants (Bar=1000 µm) are shown. (C) The total fold increase in tissue area compared to the controls, measured at five different sections along the plant stem, Hypocotyls (Hyp.), Cotyledon node (Cot.), first internode ,(Int.1), second internode (Int.2) and fourth internode (Int.4) are shown.

A more detailed quantification was performed by counting the number of cells and the average cell size in the most affected tissues (phloem and xylem). In phloem, the total number of cells in infected plants was significantly higher than in the uninfected control plants at all positions (Fig 2-4C). At the hypocotyl position (H), 10 times as many cells appeared as in the control plants, whereas at the 4th internode, only a 1.5 fold increase occurred (Fig 2-4C). Because cell size in the tissues varied substantially, the cell size distributions between control and infected plants at five positions along the stem axis was compared. The distribution significantly shifted toward larger cells in the stems of infected plants compared to uninfected control plants (Fig 2-4D-H). Those differences were attenuated at the 4th internode, where the distributions, although still different, overlapped (Fig 2-4H).

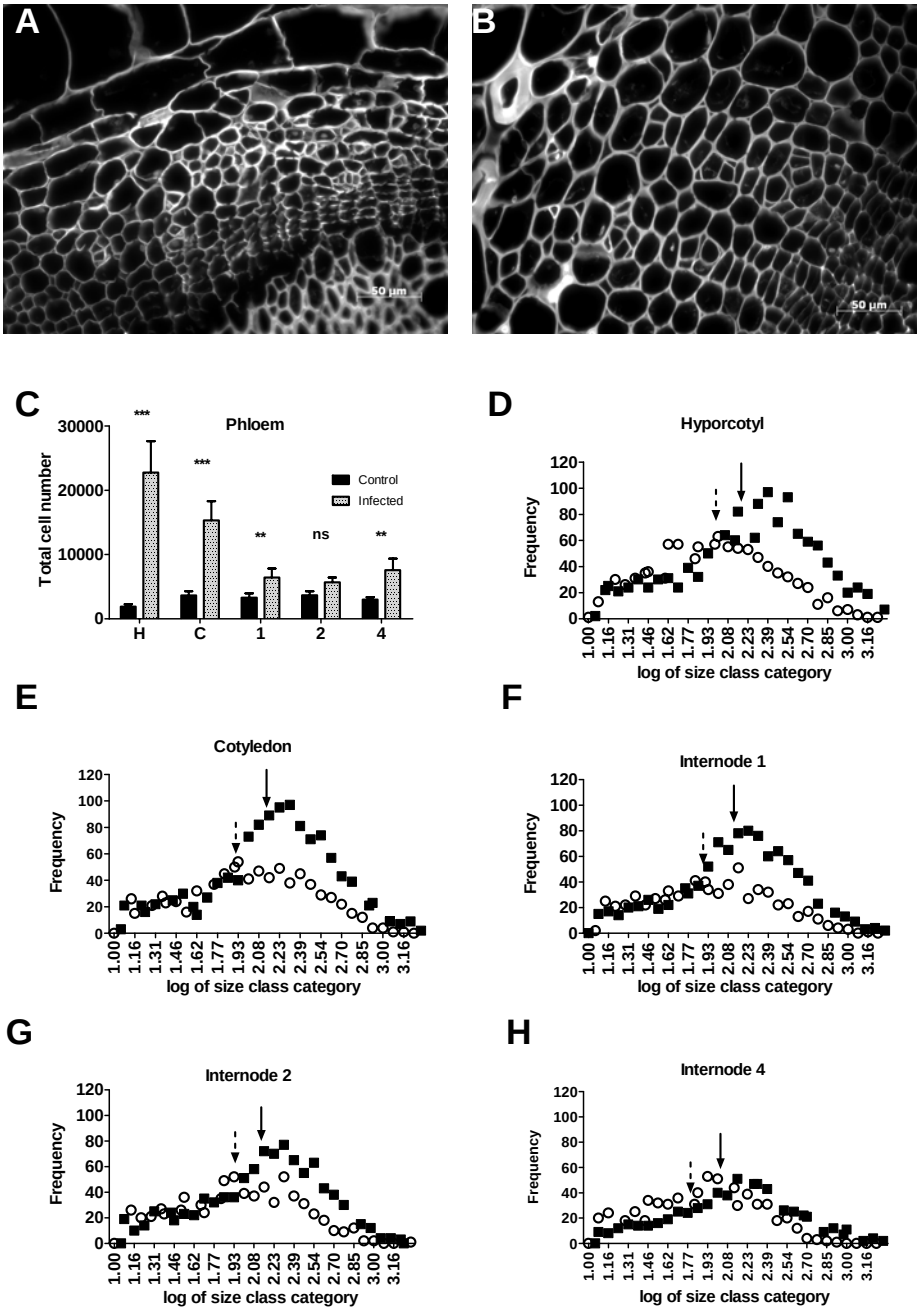


Fig. 2-4: Cellular changes induced by *C. perniciosa* infection on tomato phloem. Photomicrographs of phloem tissue sections in non-infected (A) and infected (B) plants measured at five different sections along the plant stem (C). Mean values were compared using a student t-test. Distributions of cell sizes using a logarithmic transformation (D-H). The distributions were compared using a Mann-Whitney test. The mean size values of the cell size for control (dashed arrows) and infected (solid arrows) are shown.

In the xylem, clear differences between infected and control plants also appeared (Fig 2-5). The total number of cells was significantly higher at four positions H, C, 1 and 2 (Fig 2-5 C). However, at position 4 no significant differences in cell numbers were observed (Fig 2-5C). Similar to the phloem, the cell size distribution also significantly shifted toward higher values in infected plants (Fig 2-5). However, the distributions of the control and infected plants overlap when measurements were made at the 4^{th} internode (Fig 2-5 H).

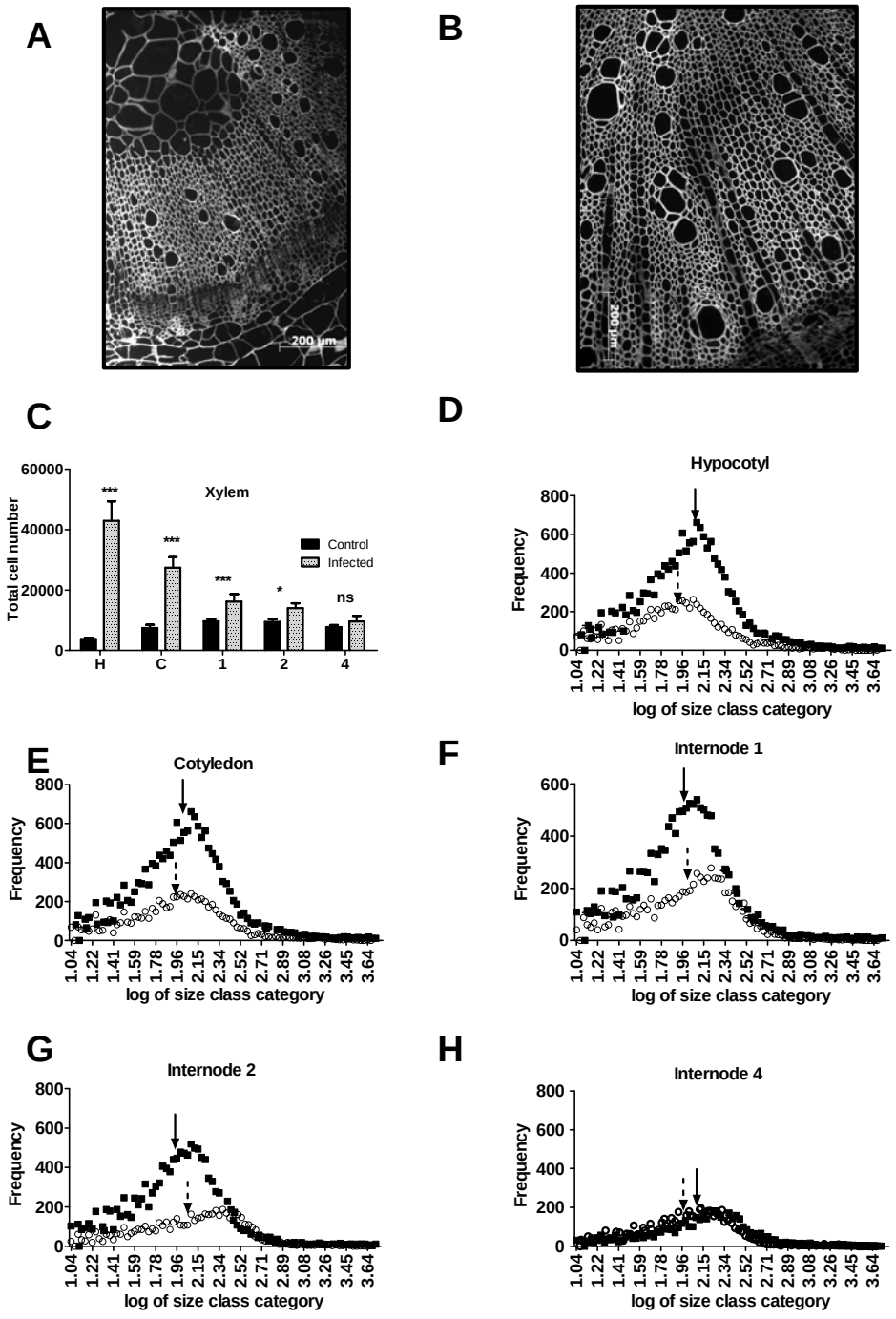


Fig. 2-5: Cellular changes induced by *C. perniciosa* infection on tomato xylem. Photomicrographs of phloem tissue sections in non-infected (A) and infected (B) plants. Quantification of total cell number measured at five different sections along the plant stem (C). Mean values were compared using a student t-test. Distributions of cell sizes using a logarithmic transformation (D-H). The distributions were compared using a Mann-Whitney test. The mean size values of the cell size for control (dashed arrows) and infected (solid arrows) are shown.

A generally accepted principle is that auxin is a key regulator of cell division and cell expansion in most plant tissues (Taiz and Zeiger 1998). Transgenic tomato plants, carrying an auxin responsive promoter-reporter gene (DR5-GUS), were used to investigate the possibility that the increase in cell numbers and sizes might be due to an increase in auxin level (Hagen and Guilfoyle 2002). In DR5 transgenic Micro-Tom tomato plants, a clear difference in the levels of GUS expression between infected and control plants appeared (Fig 2-6 A and B). Uninfected DR5-GUS control plants had a low expression of GUS near the primary xylem (Fig 2-6A) while infected plants exhibited a higher GUS staining in the primary xylem which correlated with the zone of stem swelling, increased cell enlargement and numbers as described earlier (Fig 2-6B). This increased staining was reproducible in at least 2 different transgenic lines.

Septate biotrophic hyphae grew intercellularly, predominantly associated with phloem parenchyma cells, but did not appear to colonize the plant extensively (Fig 6C). Intercellular crystals were often co-localized with hyphae in the apoplastic space (Fig 6C). Using the polarized light method and chemical staining with silver nitrate (Kurrein, Green, and Rowles 1975), these crystals were identified as calcium oxalate (CaC_2O_4) .

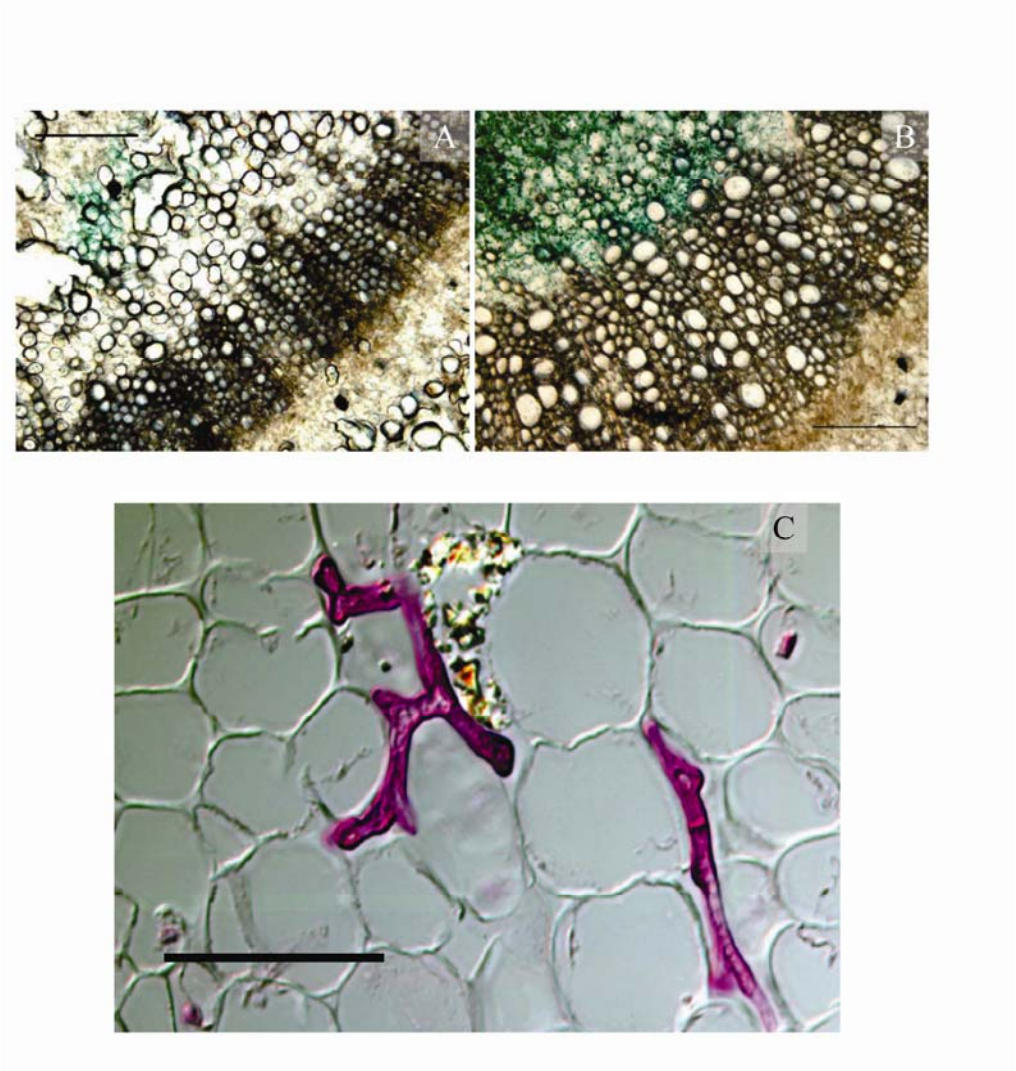


Fig. 2-6: Fungal colonization on infected tomato tissue and auxin localization. (A) Presence of GUS expression on control plant (B) GUS expression on infected plant. Plants were sectioned six weeks after inoculation and strained for GUS following the standard GUS staining protocol (Jefferson et al., 1987). Bar=100 µm (C) Biotrophic fungal hyphae growing in the phloem parenchyma cells near calcium oxalate clusters. Bar=50 µm.

Infected plants, exhibited a higher GUS staining in the primary xylem which correlated with the zone of stem swelling, increased cell enlargement and numbers as described earlier (Fig 2-6B). This increased staining was observed reproducible in at least 2 different transgenic lines.

Discussion

To validate the use of the tomato/ *M. perniciosa* model as a surrogate for studying witches' broom disease of cacao, the genetic relationship between isolates of the *M. perniciosa* C and S biotypes was investigated, and comparative morphological/cellular analyses between cacao and tomato during disease progression were conducted. Isolates of *M. perniciosa* from solanaceous hosts were found to belong to the same phylogenetic group as those collected from *T. cacao* or its relatives, *T. grandiflorum*, *T. subincatum*, and *Herrania* sp. A similar study, which used ITS sequences to compare isolates of the C, S, and H biotypes of *M. perniciosa*, reported that whereas the S- and C- biotypes grouped in the same phylogenetic clade, the H-biotype isolate collected from *H. acutifolia* formed a different species and was renamed to *Crinipellis brasiliensis* (based on the previous name, *Crinipellis perniciosa*) (Arruda et al. 2005). In another study involving isolates of the C and S biotypes, the numbers of chromosomes varied considerably among strains, but no correlation between karyotypes and host specificity could be established (Rincones *et al.* 2006). Based on AFLP and RAPD analyses, the S-biotype formed a distinct group from the C-biotype (Anderbrhan and Furtek 1994; Ploetz *et al.* 2005). However, in both studies only one or two isolates of the S-biotype were included. In contrast, in this study nine isolates of S-biotype strains collected from four different

solanaceous hosts were characterized. The clustering pattern between S- and C-biotype isolates suggests that these biotypes are not genetically distinct. Cross inoculations performed on cacao and tomato using both S and C biotypes, show that only the S-biotype can induce symptoms on tomato and only the C-biotype can induce symptoms on cacao (data not shown).

The symptoms observed on cacao and solanaceous showed very strong similarities (Fig 2-1). The tomato variety Micro-Tom could switch to the necrotrophic phase whereas this phase was never observed on NC841-73. This could indicate that the tomato variety NC841-73 does not express the components necessary for the switch to occur.

In infected tomato plants, *M. perniciosa* induced the enlargement of stem tissues, particularly in phloem and xylem regions (Fig 2-3). This is consistent with previous studies of cacao where xylem and phloem parenchyma tissues were significantly larger in infected plants (Orchard *et al.* 1994). Recent studies with susceptible and resistant cacao clones also showed that the radius of the xylem and phloem in the stems of susceptible plants was 50% larger than that of uninfected plants (Gramacho et al., personal communication). In tomato plants infected with *M. perniciosa*, the enlargement of the host's vascular tissue was associated with a higher cell number in the phloem and xylem of the infected tissues as compared to control tissues (Figs 2-4 and 2-5), suggesting that stem enlargement is caused by increased cell division in those tissues (hyperplasia).

The cells were larger in infected phloem and xylem tissues as shown by the significant upward shifts in the cell size distribution in the infected plants (Figs 2-4 and 2-5). This result also indicates an increase in cell sizes in the infected plants

(hypertrophy). To present knowledge, this is the first published account of the involvement of both hypertrophy and hyperplasia in the stem swelling caused by *M. perniciosa*. The major effect was observed in the hypocotyl and attenuated in the upper parts of the stem. Since inoculation occurred on the first emerging leaves, this data shows that the cellular alterations caused by the fungus located around the inoculation point, decrease as a function of distance above the site of infection. Isolation of the fungus from infected plants has shown that fungal colonization is limited to sites below and above the inoculation point and does not reach the apical meristem (data not shown). This could explain the observed decrease in the disease phenotype at positions near the apical meristem.

The increase in stem diameter due to cell enlargement and cell division suggested that plant hormones might be involved in symptom development. Auxins play important roles in plant development and cell enlargement but can also be involved in disease development in many plant-pathogen interactions (Jameson 2000). The best known system is crown gall formation caused by *Agrobacterium tumefaciens*, a pathogenic bacterium that induces overproduction of IAA (Indole Acetic Acid) in its hosts (Schwalm *et al.* 2003). Likewise, *Pseudomonas syringae* also produces auxins during infection (Glickmann *et al.* 1998). In *M. perniciosa*, the level of IAA oxydase, measured in liquid cultures, was high (Krupasagar and Sequeira 1969), suggesting that the fungus may be able to alter auxin concentrations by degrading it. Recently, the whole genome of *M. perniciosa* has been sequenced, in which genes for auxin biosynthesis were identified. This suggests that the fungus may also be able to synthesize the hormone (Gonçalo Guimaraes Pereira personal comunication). Moreover, auxins have been shown to induce

xylogenesis (formation of xylem tissue) (Taiz and Zeiger 1998), which is one of the most noticeable changes observed during infection by *M. perniciosa* (Fig 2-3). Other changes include the activation of axillary buds and the reduction of root biomass, which were also observed in tomato and pepper plants. This activation could be caused by the disruption of the natural auxin gradient, which regulates apical dominance in plants (Leyser 2003). The result from transgenic plants carrying GUS under the control of the DR5 inducible promoter suggests altered auxin levels in the infected plants. The question of whether the fungus produces auxins or induces auxin biosynthesis remains to be investigated.

In infected tomato tissues, *M. perniciosa* intercellular biotrophic hyphae were found associated with calcium oxalate crystals (Fig 2-6). Since production of these crystals occurred in the apoplastic space and not in specialized plant cells (idioblasts), and were often associated with fungal hyphae (Dutton and Evans 1996), these crystals likely originated from the fungus. Consistent with this observation, as compared to non-inoculated control plants, increased calcium oxalate crystals was observed in susceptible genotypes of cacao infected with *M. perniciosa* (Ceita *et al.* 2007). A sharp decrease in crystals was also observed during the rapid necrosis leading to the necrotrophic phase, suggesting a role of oxalate in programmed cell death (Ceita et al. 2007). The deposition of calcium oxalate is a noted phenomenon of other fungal species (Whitney and Arnott 1987). For example, during plant infection, the plant pathogen *Sclerotinia sclerotiorum* produces oxalic acid in millimolar quantities, acidifying the middle lamellae, sequestering calcium in the form of calcium oxalate and providing a favorable acid environment for enzymes to digest plant cells in a matter of hours (Hegedus and Rimmer 2005). Although in the case of *M. perniciosa* the biotrophic phase can last several

months, without degradation of plant cells, the production of oxalate at low levels and the sequestration of calcium in the form of calcium oxalate might aid fungal growth intercellularly. The reaction of oxalic acid with calcium creating calcium oxalate crystals could result in the weakening of the middle lamella, thus allowing the fungus to grow easily between cells.

The data presented here clearly demonstrates that the S-biotype of *M. perniciosa* is pathogenic to *S. lycopersicum* (tomato), and *Capsicum annuum* (pepper) and produces symptoms similar to those of the witches' broom disease, such as activation of axillary buds, cell enlargement and multiplication, oxalate crystal production and switch to a necrotrophic phase (Fig 2-1). This indicates that tomato plants are suitable as a surrogate model to study the witches' broom disease of cacao.

A close observation of the interaction between the S-biotype of *M. perniciosa* and the surrogate model tomato plants provided an insight into pathogenesis mechanisms. This insight is potentially useful for developing methods to control the disease in cacao. For example, tomato can potentially be used to test the efficacy of various control strategies such as biological control agents, transgenic expression of pathogenesis-related proteins (e.g. chitinase), and novel target-based chemicals. The feasibility of large scale experiments and the availability of various types of materials for genetic mapping in tomato plants is an advantage that can shorten the time necessary to elucidate the mechanisms of the *M. perniciosa* infection process. The information extracted from the infection biology of *M. perniciosa* in tomato plants could be very useful for understanding complex mechanisms for finding target-based control agents applicable to cacao in control programs.

References

Abramoff, M. D., P. J. Magelhaes, and S. J. Ram. 2004. Image Processing with ImageJ. *Biophotonics* 11 (7):36-42.

Aime, M. C., and W. Phillips-Mora. 2005. The causal agents of witches' broom and frosty pod rot of cacao (chocolate, *Theobroma cacao*) form a new lineage of Marasmiaceae. *Mycologia* 97 (5):1012-1022.

Alba, R., Z. J. Fei, P. Payton, Y. Liu, S. L. Moore, P. Debbie, J. Cohn, M. D'Ascenzo, J. S. Gordon, J. K. C. Rose, G. Martin, S. D. Tanksley, M. Bouzayen, M. M. Jahn, and J. Giovannoni. 2004. ESTs, cDNA microarrays, and gene expression profiling: tools for dissecting plant physiology and development. *Plant Journal* 39 (5):697-714.

Anderbrhan, T., and D. B. Furtek. 1994. Random amplified polymorphic DNA (RAPD) analysis of *Crinipellis perniciosa* isolates from different hosts. *Plant Pathology* 43:1020-1027.

Arruda, M. C. C., G. F. Sepulveda, R. N. G. Miller, M. A. S. V. Ferreira, D. V. R. Santiago, M. L. V. Resende, J. C. Dianese, and M. S. S. Felipe. 2005. *Crinipellis brasiliensis*, a new species based on morphological and molecular data. *Mycologia* 97 (6):1348-1361.

Bastos, C. N., and H. C. Evans. 1985. A new pathotype of *Crinipellis perniciosa* (Witches' broom disease) on solanaceous hosts. *Plant Pathology* 34:306-312.

Bastos, C. N., and T. Anderbrhan. 1986. Urucu (Bixa Orellana): a new host of witches'broom disease (*Crinipellis perniciosa*) of cocoa. *Fitopatologia Brasilera* 11:963-965.

Bastos, C. N., T. Anderbrhan, and L. C. Almeida. 1988. Morphological and pathological comparison of *Crinipellis perniciosa* isolates. *Fitopatologia Brasilera* 13 (3):2002-2006.

Bowers, J. H., B. A. Bailey, P. K. Hebbar, S.Sanogo, and R. D. Lumsden. 2001. *The impact of plant diseases on world chocolate production.* Plant Health Progress . Available from http://www.apsnet.org/online/feature/cacao/.

Ceita, G. O., J. N. A. Macedo, T. B. Santos , L. Alemanno, A. S. Gesteira, F. Micheli, A. C. Mariano, K. P. Gramacho, D. C. Silva, L. Meinhardt, P. Mazzafera, G. A. Guimaraes Pereira, and J. C. M. Cascardo. 2007. Involvement of calcium oxalate degradation during programmed cell death in *Theobroma cacao* tissues triggered by the hemibiotrophic fungus *Moniliophthora perniciosa*. *Plant Science* 173:106-117.

Cronshaw, D. K., and H. C. Evans. 1978. Witches' broom disease of cocoa (*Crinipellis perniciosa*) in Ecuador. II- Methods of infection. *Annals of Applied Biology* 89:193-200.

Dutton, M. V., and C. S. Evans. 1996. Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. *Canadian Journal of Microbiology* 42 (9):881-895.

Evans, H. 2002. Globalization and the threat from invasive, alien species. Paper read at Globalization of Agricultural Research, at Costa Rica.

Evans, H. C. 1978. Witches' broom disease of cocoa (Crinipellis perniciosa) in Ecuador. I- The fungus. *Annals of Applied Biology* 89:185-192.

Evans, H. C. 1980. Pleomorphism in *Crinipellis perniciosa*, causal agent of witches' broom disease of cocoa. *Transactions of the British Mycological Society* 74 (3):515-523.

Frias, G. A., and L. H. Purdy. 1991. Infection biology of *Crinipellis perniciosa* on vegetative flushes of cacao. *Plant Disease* 75:552-556.

Fulton, T. M., R. Ven der Hoeven, N. T. Eanneta, and S. D. Tanksley. 2002. Identification, analysis and utilization of conserved ortholog set markers for comparative genomics in higher plants. *The Plant Cell* 14:1457-1467.

Glickmann, E., L. Gardan, S. Jacquet, S. Hussain, M. Elasri, A. Petit, and Y. Dessaux. 1998. Auxin production is a common feature of most pathovars of Pseudomonas syringae. *Molecular Plant-Microbe Interactions* 11 (2):156-162.

Gramacho, K. P. 1999. Disease resistance and pathogenic variability in the fusiform rust-slash pine pathosystem. PhD thesis, Forest Pathology, University of Florida, Gainsville.

Griffith, G. W., and J. N. Hedger. 1993. A novel method for producing basidiocarps of the cocoa pathogen *Crinipellis perniciosa* using a bran-vermiculite medium. *Netherlands Journal of Plant Pathology* 99:227-230.

Griffith, G. W., and J. N. Hedger. 1994. Spatial distribution of mycelia of the liana (L-) biotype of the garic *Crinipellis perniciosa* (Stahel) Singer in tropical forest. *New Phytologist* 127:243-259.

Griffith, G. W., J. Nicholson, A. Negnninger, R. N. Birch, and J. N. Hedger. 2003. Witches' brooms and frosty pods: two major pathogens of cacao. *New Zealand Journal of Botany* 41:423-435.

Hagen, G., and T. Guilfoyle. 2002. Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Molecular Biology* 49:373-385.

Hegedus, D. D., and S. R. Rimmer. 2005. *Sclerotinia sclerotiorum*: When "to be or not to be" a pathogen? *FEMS Microbiology Letters* 251 (2):177-184.

Ihaka, R., and R. Gentleman. 1996. R: A language for data analysis and graphics. *Journal of Computational and Graphical Statistics* 5 (3):299-314.

Jameson, P. E. 2000. Cytokinins and auxins in plant-pathogen interactions – An overview. *Plant Growth Regulation* 32 (2-3):369-380.

Jefferson, R. A., T. A. Kavanagh, and M. W. Bevan. 1987. Gus Fusions - Beta-Glucuronidase as a Sensitive and Versatile Gene Fusion Marker in Higher-Plants. *Embo Journal* 6 (13):3901-3907.

Krupasagar, V., and L. Sequeira. 1969. Auxin destruction by *Marasmius perniciosus*. *American Journal of Botany* 56 (4):390-397.

Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* 5:150-163.

Kurrein, F., G. Green, and S. Rowles. 1975. Localized deposition of calcium oxalate around a pulmonary *Aspergillus niger* fungus ball. *American Journal of Clinical Pathology* 64:556-563.

Leyser, O. 2003. Regulation of shoot branching by auxin. *Trends in Plant Science* 8 (11):541-545.

Menda, N., Y. Semel, D. Peled, Y. Eshed, and D. Zamir. 2004. In silico screening of a saturated mutation library of tomato. *The Plant Journal* 38 (5):861-872.

Monforte, A. J., and S. D. Tanksley. 2000. Development of a set of near isogenic and backcross recombinant inbred lines containing most of the *Lycopersicon hirsitum* genome in a *L. esculentum* genetic background: A tool for gene mapping and gene discovery. *Genome* 43:803-813.

Niella, G. R., M. I. Resende, H. A. Castro, L. H. C. P. Silva, and J. A. Carvalho. 1999. Aperfeicoamento da metodologia de producao artificial de basidiocarpos de *Crinipellis perniciosa*. *Fitopatologia brasileria* 23:523-527.

Orchard, J., H. A. Collin, K. Hardwick, and S. Isaac. 1994. Changes in morphology and measurement of cytokinin levels during the development of witches' broom on cocoa. *Plant Pathology* 43:65-72.

Ploetz, R., R. J. Shnell, Z. Ying, Q. Zheng, C. T. Olano, J. C. Matamayor, and E. S. Johnson. 2005. Analysis of molecular diversity in *Crinipellis perniciosa* with AFLP markers. *European Journal of Plant Pathology* 111:317-326.

Purdy, L. H., and R. A. Schmidt. 1996. Status of cacao witches broom : biology, epidemiology, and management. *Annual Review of Phytopathology* 34:573-94.

Radzali, B. M., A. C. Hamish, S. Issac, and K. Hardwick. 1996. Effects of the fungus *Crinipellis perniciosa*, causal agent of witches' broom disease, on cell and tissue cultures of cocoa (*Theobroma cacao* L.). *Plant Pathology* 45:145-154.

Resende, M. L. V., B. A. N. Gutemberg, L. H. C. P. Silva, G. R. Niella, G. A. Carvalho, D. V. R. Santiago, and J. L. Bezerra. 2000. *Crinipellis perniciosa* proveniente de um

novo hospedeiro, *Heteropteys acutifolia*, é patogenico ao cacaueiro. *Fitopatol. bras* 25:88-91.

Rincones, J., G. D. Mazotti, G. W. Grifith, A. Pomela, A. Figueira, G. A. Leal, M. V. Queiroz, J. F. Pereira, R. A. Azevedo, G. A. G. Pereira, and L. W. Meinhardt. 2006. Genetic variability and chromosome-length polymorphisms of the witches' broom pathogen *Crinipellis perniciosa* from various parts in South America. *Mycological Research* 110:821-832.

Scarpari, L. M., L. W. Meinhardt, P. Mazzafera, A. W. V. Pomella, M. A. Schiavinato, J. C. M. Cascardo, and G. A. G. Pereira. 2005. Biochemical changes during the development of witches'broom: the most important disease of cocoa in Brazil caused by *Crinipellis perniciosa*. *Journal of Experimental Botany* 56 (413):865-877.

Schwalm, K., R. Aloni, M. Langhans, M. W. Heller, S. Stich, and C. I. Ullrich. 2003. Flavonoid-related regulation of auxin accumulation in *Agrobacterium tumefaciens*-induced plant tumors. *Planta* 218:163–178.

Silva, S. D. V. M., E. D. M. N. Luz, L. C. Almeida, K. P. Gramacho, and J. L. Bezerra. 2002. Redescicao da sintomatologia causada por *Crinipellis perniciosa* em cacaueiro. *Agrotropica* 14 (1):1-10.

Sreenivasan, T. N., and S. Dabydeen. 1989. Modes of Penetration of Young Cocoa Leaves by *Crinipellis perniciosa*. *Plant Disease* 73:478-481.

Stahel, G. 1919. Contribution to the knowledge of witchbroom disease. *Tropical Agriculture* 4 (6):167-176.

Surujdeo, S., P. Umaharan, D. R. Butler, and T. N. Sreenivasan. 2003. An optimized screening method for identifying levels of resistance to *Crinipellis perniciosa* in cocoa (*Theobroma cacao*). *Plant Pathology* 52:464-475.

Taiz, L., and E. Zeiger. 1998. *Plant Physiology*. second ed. Sunderland, Massachussets: Sinauer Associates, Inc.

Tanksley, S., S D. 1993. Mapping Polygenes. *Annual Review of Genetics* 27 (1):205-233.

Thomson, D., and R. J. Henry. 1993. Single step protocol for preparation of plant tissue for analysis by PCR. *Biotechniques* 19:394-397.

Vaughan, R. E. 1914. A method for the differential staining of fungus and host cells. *Annals of the Missouri Botanical Garden* 1:241-242.

Wheeler, B. E. J., and C. Suarez. 1993. The pathosystem. In *Disease Management in Cocoa*, edited by Chapman and Hall. New York.

Whitney, K. D., and H. J. Arnott. 1987. Calcium oxalate crystal morphology and development in *Agaricus bisporus*. *Mycologia* 79 (2):180-187.

Chapter 3

Discovery of new sources of resistance to the witches' broom pathogen of cacao in near isogenic lines of tomato

Abstract

Theobroma cacao, a plant cultivated in the tropics, produces cocoa beans sold in the international market for the production of chocolate. Because of economic impact in South America, witches' broom disease, caused by *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora, comb. nov.(=*Crinipellis perniciosa*) is among the diseases of foremost concern to the cacao industry. As an alternative cacao host, the tomato plant (*Solanum lycopersicum*), used in inoculation experiments, showed susceptibility to the strains of the S-biotype (strain 73-6-01) of *M. perniciosa*. Comparatively, the tomato's wild relative, *Solanum habrochaites,* was highly resistant to the disease when exposed to the same strain of *M. perniciosa*. One hundred near isogenic lines of tomato containing chromosomal sections of the genome of *S. habrochaites* in a *S. lycopersicon* background provided specimens to identify genome regions of *S. habrochaites* that could be responsible for the observed resistance to *M. perniciosa*. A strong QTL for resistance on the short arm of chromosome 1 was detected for disease severity ($P<0.05$), shoot diameter ($P<0.001$), and shoot fresh and dry weight ($P<0.0001$). That genomic area is known to contain a cluster of genes for resistance against the pathogen *Cladosporium fulvum*. This chapter discusses implications for improving cacao breeding strategies.

Introduction

Moniliophthora perniciosa (*Crinipellis perniciosa*) is a fungal pathogen of *Theobroma cacao.* By infecting shoots, flowers and pods of the cacao tree, the fungus has caused severe damage to cacao crops in South America since the late nineteenth century (Griffith et al. 2003). The disease cycle consists of a biotrophic phase, where monokaryotic fungal hyphae grow intercellularly, inducing cellular enlargement of host tissues (Chapter 2) and activation of axillary meristems. Ninety days after infection, the infected tissue necroses, the hyphae become dikaryotic, penetrate the cells, eventually producing spores in fruiting bodies (basidiocarps) (Purdy and Schmidt 1996). The infective propagules, the basidiospores, dispersed by rain aerosols, land on actively growing tissues such as leaf or floral meristems to initiate a new round of infection (Purdy and Schmidt 1996).

The main control strategies to combat this disease include cultural/sanitation controls (periodic removal of infected plant parts), biological control (use of *Trichoderma* spp., a hyperparasite of *M. perniciosa*), and genetic control (Pereira 2000).

The most practical control strategy to halt witches' broom infection is breeding for disease resistance. *T.cacao* varieties that are tolerant to *M. perniciosa* have been developed beginning in 1940's by Pound (Wood and Lass 1985). Recently, a major and a minor QTL for resistance against *M. perniciosa* have been found in an F2 population, a cross between resistant and susceptible parents of *T. cacao* in Brazil (Brown *et al.* 2005).

Due to the perennial nature of the cacao plant and the extended period required for fruit set (3 years), breeding for a new cacao variety requires as long as 15 years.

Moreover, the genetic pool available to breeders has been shown to be very narrow, limiting the possibilities of finding durable resistance (Motamayor *et al.* 2003). For these two reasons, finding a faster way to identify novel genes for resistance against *M. perniciosa* is imperative.

One characteristic of *M. perniciosa* is its ability to infect plants in various families. The C-biotype infects *Theobroma* spp and *Herrania* spp (Griffith and Hedger 1994). The L-biotype was found in association with lianas such as *Arrabidea verrucosa*, but is not pathogenic to these species. The B-biotype can infect members of the Bixaceae family. The S-biotype, was found to be associated with wild solanaceous species such as *Solanum rugorosum* (Bastos and Evans 1985; Griffith and Hedger 1994). Spores of *M. perniciosa* collected on *Solanum paniculatum* in the state of Bahia in Brazil after the witches' broom epidemic appeared to produce symptoms on cacao (Lopes *et al.* 2001). Using spores of the S-biotype, allowed reproducing the disease in cultivated tomato plants (see Chapter 2). Given all the genetic and genomic tools available for tomato this surrogate plant model is a useful tool for dissecting the genetic and molecular mechanisms underlying development of the witches' broom disease (Marelli *et al.* 2005).

Tomato's (*S. lycopersicum*) wild relatives, such as *Solanum habrochaites*, *Solanum pimpinellifolium*, and *Solanum pennellii*, contain a more diverse gene pool, useful for crop improvement (Tanksley 1993). When introgressed into an *S. lycopersicum* background, genes whose function was silent in the wild relative, sometime express a phenotype in cultivated tomato (Tanksley 1993). This highlights the use of introgression lines for genetic breeding using Near Isogenic Lines (NILs) of wild relatives as an important tool (Tanksley and McCouch 1997). In 2000, Monforte and

Tanskley obtained 99 Near Isogenic Lines (NILs) and backcross recombinant lines (BCRILs) by crossing *S. lycoperscion* cv E6203 and *S. habrochaites* cv LA1777 (Monforte and Tanksley 2000). For their study, these researchers selected single segments of introgressed LA1777 DNA in a *S. lycopersicum* background. They achieved this by successive back-crossing and selecting events leading to single homozygous insertions of small amounts of LA1777 genome into the cultivated tomato genome (Bernacchi *et al.* 1998). Genetic maps of NILs and BCRILs contained 95 molecular markers evenly distributed along the tomato genome and allowed for a precise localization of every introgression of LA1777 in the tomato genome (Monforte and Tanksley 2000). More recently, PCR-based markers added more information to the genetic map of the NILs and better segregated single and multiple insertions (Tripodi P, Tanksley SD, Grandillo S, unpublished data).

One advantage of using tomato as surrogate model for the witches' broom disease is the availability of genetic resources to study plant responses to pathogen attack (Chapter 2). Based on initial data showing a differential response between *S. lycopersicum* and *S. habrochaites* (*S. habrochaites* was immune to the disease over three inoculation experiments, data not shown), this chapter proposes to investigate the possibility of using the NILs developed between *S. lycopersicum* and *S. habrochaites* to map genetic resistance against *M. perniciosa* in tomato.

Materials and Methods

Plant material and growth

The plant population used corresponds to the *Solanum habrochaites* introgression lines derived from a cross between *S. lycopersicon* (M82) and *S. habrochaites* (LA1777) developed by Steven Tanksley and associates (Monforte and Tanksley 2000). A core set of 57 lines have a single segment of *S. habrochaites* introgressed into the *S. lycopersicon* background. The remaining 41 lines have additional multiple introgressions to increase genome coverage. In total, the population covers 85% of the genome of *S. habrochaites*. Steven Tanksley (Cornell University, Ithaca NY) provided seeds for the different lines.

The experiment completed at the Nestlé R&D center in Tours, France, used seeds germinated on June 6, 2007 and grown hydroponically in sand. Plant irrigation nutrition was by a Yamasaki's solution (Macronutrients: 0.354 g/l $Ca(NO_3)_2$.4H2O, 0.404 g/l KNO_3, 0.076 g/l $NH_4H_2PO_4$, and 0.24 g/l $MgSO_4.7H_2O$; Micronutrients: 0.016 g/l Fe-EDTA, 0.0012 g/l H_3BO_4, 0.00072 g/l $MnSO_4$, 4H2O, 0.00009 g/l, $ZnSO_4$, $7H_2O$, 0.00004 g/l $CuSO_4.5H_2O$, 0.0001 g/l $(NH_4)_2Mo_7O_{24}$,.$4H_2O$, and 0.00164 g/l NaCl). Plants were grown in a phytotron at 27 ºC, 80% of relative humidity and a 16:8 light: dark period and remained in those conditions for 10 days before inoculation with *M. perniciosa* basidiospores.

Inoculum production

The *M.p* strain 73-6-01 used in this study initially developed on *Solanum paniculatum* in Itajuipe, Bahia (Brazil) and proved *a posteriori* to be pathogenic on the parent TA496 of the tomato plant (data not shown). Production of basidiocarps of 73-6-01 used the bran-vermiculite *in vitro* method (Griffith and Hedger 1993; Niella et al. 1999) and was performed at The Pennsylvania State University under APHIS permit # 72545. After 2 to 3 months of alternate periods of wetness and dryness, basidiocarps were harvested and the inoculum collected. The procedure placed the mushroom caps on petroleum jelly coated Petri-dish lids positioned over flasks containing 5-10 ml of buffer solution made of MES 0.01 M and 16% glycerol. The resulting spore suspension was then concentrated, by centrifugation, at 4000 rpm. After evaluating the spore concentration and initial germination, aliquots of 1.5 ml, frozen in cryovials in liquid nitrogen, were shipped to France in a dry shipper (Taylor-Wharton, USA). The cryovials remained stored at Nestlé R&D centre's liquid nitrogen collection until the day of the experiment. Because of low seed germination of some of the plant lines, the inoculation had to be divided into two independent experiments. The first inoculation took place on June 21, 2007, and the second in July 10, 2007. Inoculation for both experiments occurred when plants reached an age of 10 days.

Plant inoculation

The inoculum was prepared by thawing the spores at room temperature. The percentage of viable spores was evaluated by germinating an aliquot of the spore

suspension for 5 hours at 25 ºC in the dark on 2% water agar plates. The initial spore suspension was diluted to 6 x 10^5 viable spores/ml in a 0.01 MES, 2% agar solution that had been previously sterilized and cooled to room temperature. The plants were inoculated by placing a 10 µL drop of spore suspension over the youngest emerging leaf of the tomato seedlings. Inoculated plants were kept in the dark, at 25 ºC, with 100% humidity and water condensing on the plants, for 48 hours.

Quantification of disease severity

Disease assessment occurred 6 weeks after infection, from August 1, 2007 to August 10, 2007. Disease assessment for the second experiment occurred on August 13, 2007. A new method to assess symptoms of *M.perniciosa* on tomato was developed: the symptoms scored for each plant were (1) leaf blisters (2) leaf yellowing, (3) stem and petiole swelling, and (4) purple color on the stem (probably due to anthocyanin production). Depending on the severity of each of the above symptoms, a visual grade, from 0 to 3, was recorded for each symptom. The number of active axillary buds (5) quantified the extent of apical dominance. The total score calculation occurred by adding the individual scores for every symptom observed and the total number of axillary buds (Severity score = 1+2+3+4+5).

Quantification of growth parameters

Evaluation of quantitative growth parameters occurred weekly. Shoot diameters at the hypocotyls (diam1) and at the third internodes (diam2) were measured using a digital caliper (VWR, cat# 62379-531). At the end of the experiment, fresh weight of shoots (SFW) was recorded. Shoot dry weights (SDW) were also obtained after placing the plants in an oven at 45 ºC for 48 hrs.

Statistical analysis

Mean values for each trait were calculated for the different Isogenic Lines and compared to the mean value of the inoculated susceptible control TA496 using a Student t-test.

For every parameter measured, variations that significantly differed were compared to the saturated genetic map of *S. habrochaites* introgression lines containing new genetic markers (Tipodi P, Tanksley SD, Grandillo S, unpublished data) and summarized in Table 3-1.

Results

Susceptibility of introgression lines to M. perniciosa

In order to search for genomic regions in the tomato genome conferring resistance to witches' broom infection, an initial screen was performed by infection replicates of the NILs collection grown in highly controlled growth chamber conditions. Because 13 out of 98 NILs did not germinate well, this was followed by a second screen. Due to variation in spore germination and plantlet survival, the data between the two experiments differs significantly, but reproducible trends were observed.

Table 3-1: Mean disease severity, mean diameter in mm at the first (diam1) and third (diam2) internodes, and mean shoot fresh (SFW), and dry weights (SDW) in mg, of the introgression lines and the control parents (S, R). Means of each IL were compared to the inoculated susceptible parent (TA496) by a Student t-test ($P<0.05$). Values in bold were significantly different from the inoculated control. The ILs highlighted in dark grey are the lines that showed a significant reduction in disease severity as well as a significant effect in all the quantitative parameters. The lines highlighted in light grey presented a significant effect in all, or 3 out of 4, of the quantitative parameters measured but did not achieve significant resistance.

IL	Chr.[a]	Exp.	N[b]	Disease severity	Diam 1 (mm)	Diam 2 (mm)	SFW[f (mg)]	SDW[g (mg)]
TA496	S[d]	1	8	7.37 (10.2)[c] ± 3.02 (8.28)[c]	4.81(2.12)[c] ± 1.63 (0.67)[c]	4.59 (2.88)[c] ± 0.87 (0.23)[c]	2745.62 (1263.4)[c] ± 757.30 (195.81)[c]	317 (16.2)[c] ± 120.47 (19.7)[c]
LA1777	R[e]	1	2	1 ± 1.41	2.22 ± 0.51	2.15 ± 0.33	212.00 ± 132.94	15.5 ± 10.61
05T1-539-32	3, 7	1	10	5.5 ± 2.92	3.63 ± 0.61	**2.88 ± 0.60**	**721.33 ± 453.45**	**80.33 ± 76.45**
05-T1-539-4	6, 7	1	10	9.55 ± 4.15	4.43 ± 0.88	4.18 ± 0.59	2369.56 ± 638.66	270.22 ± 91.01
05-T1561-20	2	1	9	7.62 ± 2.67	4.5 ± 0.96	2.89 ± 0.24	2026.87 ± 437.62	248.87 ± 105.91
05T1-563-5	5	1	9	6 ± 2.77	4.62 ± 0.87	4.1 ± 0.51	2156.57 ± 544.38	219.33 ± 108.02
05T1-703-17	10	1	9	6.44 ± 3.26	4.57 ± 0.66	5.14 ± 0.98	2579.12 ± 1066.33	275.5 ± 147.50
05-T1814-13	10	1	9	10 ± 4.36	4.99 ± 1.34	4.37 ± 0.86	2528.22 ± 872.67	301 ± 141.01
TA1105	2	1	3	8 ± 1.73	4.21 ± 2.54	4.88 ± 0.33	3144.33 ± 437.07	435.67 ± 124.13
TA1111	no	1	9	7.33 ± 1.01	3.89 ± 0.33	3.86 ± 0.51	2074.37 ± 823.68	240.87 ± 110.14
TA1116	1,2,3,5,6	1	10	8.4 ± 3.95	4.53 ± 0.80	4.65 ± 0.53	3150.00 ± 921.44	400.2 ± 139.03
TA1117 (het)	5	1	7	14 **± 4.07**	4.53 ± 0.56	4.17 ± 0.82	2732.86 ± 700.71	383.14 ± 149.19
TA1118	4, 5	1	7	6.57 ± 2	**3.39 ± 0.60**	**3.08 ± 0.83**	**743.00 ± 480.11**	**56.29 ± 57.57**
TA1119	1, 10	1	5	**11.2 ± 1**	5.89 ± 1.97	4.83 ± 0.47	2708.20 ± 717.10	283.4 ± 122.03
TA1121	12	1	6	4.8 ± 2.07	3.59 ± 0.98	3.43 ± 0.53	**510.60 ± 362.71**	**34.8 ± 34.78**
TA1127	no	1	10	10.5 ± 5.15	4.18 ± 0.71	4.28 ± 0.47	2063.90 ± 537.93	248.9 ± 84.03
TA1128	1	1	10	**2.5 ± 2.32**	**3.15 ± 0.57**	**3.38 ± 0.74**	**1257.78 ± 681.57**	**101.9 ± 85.86**
TA1133	4	1	4	10.25 ± 3.92	4.82 ± 0.34	4.88 ± 0.61	2725.25 ± 714.62	334 ± 67.92
TA1138	1, 4, 7	1	10	6.4 ± 0.74	**2.29 ± 0.49**	**2.33 ± 0.44**	**315.80 ± 214.60**	**25 ± 19.29**
TA1173 (het)	3	1	10	9.67 ± 2.69	4.83 ± 0.61	4.86 ± 0.36	2722.87 ± 879.26	322.44 ± 119.22
TA1223	1	1	4	6.75 ± 4.50	3.53 ± 0.93	3.94 ± 0.51	2100.25 ± 1075.62	230 ± 189.09
TA1225	1	1	9	8 ± 3.71	3.66 ± 0.51	4.08 ± 1.16	2399.67 ± 814.34	304 ± 141.23
TA1229	1, 7	1	10	5 ± 2.64	3.59 ± 0.60	4.09 ± 0.80	2394.90 ± 932.33	296.5 ± 173.66
TA1258	1	1	9	6.11 ± 2.45	3.43 ± 0.49	3.71 ± 0.62	2160.22 ± 439.33	199.89 ± 43.64
TA1262	2	1	5	9 ± 5.41	5.13 ± 1.01	5.02 ± 0.58	2885.40 ± 477.92	331.4 ± 65.92
TA1266	2	2	10	11 ± 7.5[b]	**3.45 ± 0.79[b]**	3.98 ± 0.59	1478.80 ± 323.61	31 ± 27.9
TA1271	no	1	7	6.86 ± 3.15	4.41 ± 0.82	4.54 ± 0.60	2345.14 ± 587.00	280.86 ± 87.19
TA1276	1, 3	1	5	6.8 ± 1.64	4.51 ± 0.38	5.08 ± 0.50	3350.80 ± 515.51	410.2 ± 109.85
TA1277	3, 12	1	5	10.8 ± 3.08	3.46 ± 0.57	**3.21 ± 0.70**	**996.80 ± 737.10**	**96.2 ± 72.38**
TA1280	4, 7	1	9	7.37 ± 2.62	4.5 ± 0.60	4.36 ± 0.28	2016.12 ± 723.32	200.37 ± 95.45
TA1281	4, 10	2	10	11.9 ± 6.76[b]	1.96 ± 0.33	2.49 ± 0.12	1283.60 ± 162.40	14.4 ± 13.22
TA1287	5	1	4	5.33 ± 1.53	**3.06 ± 0.06**	**2.66 ± 0.37**	**563.50 ± 144.96**	**38.5 ± 7.78**
TA1293	5	1	8	9.5 ± 1.77	4.63 ± 2.27	3.77 ± 0.59	1685.43 ± 671.88	**165.75 ± 108.63**
TA1303	7	2	10	7.7 ± 5.94[b]	**3.22 ± 0.41**	3.14 ± 0.56	**1885.25 ± 364.85**	**87.625 ± 47.90**
TA1304	3, 4, 7, 9, 1	1	8	8.62 ± 4.07	5.32 ± 0.34	5.7 ± 1.46	3310.87 ± 838.01	439.25 ± 114.16
TA1308	7	1	8	5.5 ± 2.51	**2.49 ± 0.77**	**2.5 ± 0.67**	**242.62 ± 142.44**	**42.42 ± 58.88**
TA1312	7	1	9	9.25 ± 3.46	5.13 ± 0.63	5.33 ± 0.59	3262.87 ± 504.74	394.87 ± 181.90
TA1314	8	2	10	9.2 ± 5.11[b]	2.4 ± 0.74	2.21 ± 1.20	1453.63 ± 317.36	34.5 ± 29.53
TA1315	no	1	9	10.25 ± 2.38	5.07 ± 1.07	4.26 ± 0.78	1976.25 ± 848.26	208.62 ± 119.04
TA1316	7, 8	1	10	**10.67 ± 3.31**	4.36 ± 0.81	4.45 ± 0.79	2844.22 ± 1928.74	432.67 ± 565.17
TA1317	8	1	10	**11.1 ± 3.31**	4.75 ± 0.62	4.8 ± 0.47	2812.80 ± 1152.66	406.4 ± 180.28
TA1318	8	1	3	13.33 ± 5.51	4.08 ± 0.47	4.41 ± 0.21	2738.67 ± 829.44	284.67 ± 113.30
TA1320	8	1	4	8.5 ± 3.20	4.68 ± 0.61	4.89 ± 0.64	2481.00 ± 649.23	313.25 ± 128.93
TA1321	2, 9	1	8	8.12 ± 3.56	4.81 ± 0.87	4.91 ± 1.01	2863.62 ± 863.44	340.12 ± 138.13
TA1324	4, 9	2	10	5.8 ± 2.61	2.35 ± 0.19	2.71 ± 0.62	**1601.80 ± 221.24**	**51.7 ± 30.42**
TA1325	7, 9	1	7	8.86 ± 2.82	3.97 ± 0.61	3.66 ± 0.79	2011.14 ± 832.80	243.57 ± 134.81
TA1326	9	2	10	5.4 ± 1.58	2.31 ± 0.18	2.49 ± 1.14	**1485.20 ± 126.30**	**46.2 ± 19.83**
TA1328	2	1	9	6.22 ± 3.48	5.55 ± 1.61	4.79 ± 0.50	3033.67 ± 996.64	412.78 ± 204.40
TA1330-???	9, 11	1	6	4.8 ± 0.89	**2.64 ± 0.49**	8.13 ± 0.31	**480.00 ± 238.31**	**30 ± 14.84**
TA1331	4, 9	2	10	11.1 ± 6.10	3.76 ± 0.39	0.58 ± 1.11	1451.33 ± 349.48	30.5 ± 13.44

IL	Chr.[a]	Exp.	N[b]	Disease severity	Diameter 1	Diameter 2	SFW[f]	SDW[g]
TA1332	1, 3, 9, 12	2	10	**4.3 ± 1.06**	2.97 ± 0.36	**3.46 ± 0.73**	**1799.00 ± 339.14**	**76.7 ± 46.66**
TA1337	1, 10	1	8	9.87 ± 3.76	5.6 ± 1.47	4.89 ± 0.57	2927.29 ± 565.37	380.62 ± 109.10
TA1338	no	1	9	7.62 ± 3.37	4.74 ± 0.74	4.91 ± 0.82	2219.00 ± 673.13	371.86 ± 157.26
TA1339	1, 12	1	10	**10.9 ± 3.41**	3.66 ± 0.62	4.32 ± 0.84	2745.40 ± 878.66	316.2 ± 115.49
TA1342	7	1	10	9.1 ± 3.14	4.16 ± 0.92	4.13 ± 0.75	2024.50 ± 859.36	232.7 ± 141.95
TA1343	11	1	10	6.9 ± 1.57	**3.13 ± 0.73**	**3 ± 0.71**	**761.20 ± 666.82**	**75.99 ± 76.80**
TA1344	7, 8	1	9	9.22 ± 2.69	4.73 ± 0.40	5.18 ± 0.48	2978.50 ± 415.32	363.77 ± 109.20
TA1349	6, 11	1	10	7.8 ± 2.95	4.39 ± 1.22	4.21 ± 0.47	2436.44 ± 574.45	280.2 ± 120.50
TA1350	8, 9, 12	1	9	7.44 ± 2.91	5.1 ± 0.91	5.31 ± 0.32	3334.33 ± 547.69	416.77 ± 149.27
TA1456	4	1	10	7.6 ± 3.17	4.58 ± 0.52	4.86 ± 0.55	2882.50 ± 723.76	342.2 ± 104.55
TA1459	4	1	9	8.33 ± 4.47	**2.83 ± 0.79**	**2.5 ± 0.55**	**556.11 ± 420.75**	**47.11 ± 39.86**
TA1467	4	1	7	9.57 ± 4.04	3.54 ± 0.59	**3.51 ± 0.78**	1610.85 ± 822.31	183 ± 132.07
TA1473	4	1	9	7.44 ± 2.96	3.48 ± 0.55	**3.79 ± 0.54**	**1566.89 ± 538.95**	170.22 ± 83.69
TA1474	4	1	9	8.77 ± 1.90	**3.43 ± 0.35**	**3.59 ± 0.23**	**1619.33 ± 242.99**	**177.22 ± 68.59**
TA1475	4	1	1	na[h] ± na	na ± na	na ± na	na ± na	na ± na
TA1476	4	1	7	8.28 ± 2.34	4.38 ± 1.51	4.56 ± 0.49	2566.43 ± 407.33	335.43 ± 115.93
TA1535	1, 2, 8, 12	1	4	6.25 ± 3.40	4.67 ± 1.01	6.55 ± 1.74	2658.00 ± 1732.12	294.95 ± 192.35
TA1536	1	1	10	**3.9 ± 3.35**	**3.26 ± 0.56**	**2.95 ± 0.29**	**924.55 ± 586.91**	**77.6 ± 59.59**
TA1537	2	1	8	**11.62 ± 4.49**	5.31 ± 0.79	5.42 ± 0.96	2952.14 ± 1528.13	342.25 ± 178.34
TA1538	1, 2, 3	1	10	6.7 ± 3.69	4.53 ± 0.84	4.94 ± 0.56	3278.70 ± 626.50	437.9 ± 133.72
TA1539	1, 3, 7, 6	1	7	9.67 ± 3.76	4.04 ± 0.66	3.44 ± 1.24	1607.83 ± 896.87	208.67 ± 131.49
TA1541	1, 3, 8	1	8	6.87 ± 1.04	**2.35 ± 0.38**	**1.88 ± 0.65**	**150.25 ± 42.59**	**15.73 ± 5.12**
TA1542	1, 4	1	1	na ± na	na ± na	na ± na	na ± na	na ± na
TA1543	5	1	4	6 ± 1.29	**3.02 ± 0.24**	**2.75 ± 0.48**	**722.00 ± 460.35**	**52 ± 52.13**
TA1544	2, 5, 7, 8	1	10	7.8 ± 3.51	3.7 ± 1.25	**3.37 ± 0.58**	**1098.60 ± 590.29**	**116.9 ± 66.25**
TA1545	6, 8, 10	1	6	9.67 ± 2.08	4.5 ± 1.50	3.78 ± 0.85	1896.00 ± 528.23	211 ± 58.62
TA1546	6, 10	1	10	8 ± 2.07	4.67 ± 0.95	4.59 ± 0.72	2300.30 ± 623.91	270.8 ± 112.03
TA1547	7, 9, 10, 12	1	10	7.5 ± 1.23	**2.96 ± 0.29**	2.67 ± 0.51	**518.90 ± 303.31**	**36.88 ± 28.21**
TA1548	8, 10	1	8	9.37 ± 4.68	5.28 ± 0.62	**5.78 ± 0.33**	3433.33 ± 696.02	325.12 ± 113.84
TA1550	10, 12	2	10	5.7[b] ± 4.9[b]	2.4 ± 0.22	4.79 ± 0.78	1386.33 ± 166.22	30.11111 ± 20.24
TA1551	10	1	3	6 ± 0.00	**2.25 ± 0.09**	**1.86 ± 0.48**	**81.33 ± 38.37**	**8.23 ± 5.41**
TA1552	1, 10, 12	1	4	8 ± 2.06	4.3 ± 0.21	4.6 ± 0.19	2272.25 ± 1542.42	430.33 ± 229.57
TA1553	1, 7, 12	1	9	7.67 ± 4.24	4.2 ± 0.47	4.55 ± 2.22	2145.33 ± 625.08	229.22 ± 95.65
TA1554	10, 11, 12	1	5	8.2 ± 3.51	3.72 ± 0.69	**3.35 ± 0.40**	**1075.40 ± 544.54**	**84.8 ± 56.46**
TA1555	8, 10, 11, 1	1	5	9.8 ± 5.10	6.35 ± 3.48	4.92 ± 0.56	2430.00 ± 1274.99	311.6 ± 183.35
TA1559	6	1	10	5.6 ± 2.11	4.27 ± 0.47	4.22 ± 0.70	2050.80 ± 800.78	261.2 ± 133.08
TA1562	4, 5, 8	1	8	7 ± 2.07	**2.94 ± 0.74**	**2.4 ± 0.50**	**152.67 ± 92.26**	**17.33 ± 16.32**
TA1631	no	1	7	10.57 ± 4.16	4.49 ± 0.70	4.06 ± 0.70	1878.29 ± 539.80	209.29 ± 130.86
TA1633	7, 10	1	10	11.6 ± 5.88	6.21 ± 2.22	5.28 ± 1.56	3096.50 ± 529.94	343.7 ± 82.98
TA1635	10	1	10	7.5 ± 3.80	4.52 ± 0.69	4.16 ± 0.93	2274.11 ± 730.55	230.7 ± 96.70
TA1638	1, 12	1	10	4.8 ± 1.48	**2.55 ± 0.47**	**2.39 ± 0.46**	**368.60 ± 323.72**	**30 ± 29.47**
TA1645	1, 8, 11, 12	1	7	8.43 ± 6.62	4.43 ± 1.16	4.82 ± 0.30	2295.29 ± 535.11	413.14 ± 168.15
TA1649	2, 3, 6	1	10	5.6 ± 3.43	4.16 ± 0.52	4.3 ± 0.46	2358.90 ± 454.11	281.2 ± 109.85
TA1652	3, 6, 7, 8, 1	1	6	7.5 ± 2.93	**2.9 ± 0.42**	**2.6 ± 0.72**	**463.67 ± 554.26**	**54.3 ± 70.62**
TA1656	1, 5, 6, 9, 11	1	9	6.37 ± 3.62	**3.07 ± 0.91**	**2.71 ± 0.55**	**647.75 ± 532.36**	**63.12 ± 65.23**
TA2608	1, 4, 5	1	10	6.78 ± 3.00	3.52 ± 0.83	**2.92 ± 0.78**	**828.44 ± 402.24**	**59.78 ± 40.20**
TA517	4	1	6	5.5 ± 2.28	3.03 ± 0.90	**2.99 ± 0.24**	**1163.00 ± 541.16**	**100.5 ± 62.50**
TA523	1	1	10	9 ± 4.19	3.94 ± 1.24	4.17 ± 0.78	2397.00 ± 1007.70	290.5 ± 151.24

[a] Chromosome numbers containing one or multiple *S. habrochaites* introgressions
[b] Number of plants inoculated with *M. perniciosa*
[c] Average value of control parents in the second experiment
[d] Suceptible parent, *Solanum lycopersicum* ,TA4969,
[e] Resistant parent, *Solanum habrochaites*, LA1777
[f] SWF : Shoot Fresh Weight
[g] SDW : Shoot Dry Weight

The mean values of disease severity, diam1, diam 2, SFW and SDW, presented in Table 3-1, indicate the lines that presented significant differences. The lines, highlighted in light grey, presented significant differences in growth parameters but not in disease severity. Among these the lines, the ones containing single introgressions were T1459 and TA1479 (chromosome 4); lines TA1287, TA1543 (chromosome 5); lines TA1303 and TA1308 (chromosome 7); line TA1551 (chromosome 10) and TA1308 (Chromosome 11). Only two lines, TA1128 and TA1536 that had overlapping introgressions on the short arm of chromosome 1 (Table3-1, Fig 3-1) presented significant disease reduction and a significant effect in all the quantitative parameters tested.

Pearson correlation coefficients revealed correlations between disease severity, diam1, diam2 and SFW/SDW. Significant correlations ($P<0.0001$) exist between disease severity and diam1 ($r=0.45$); S and diam2 ($r=0.34$); disease severity and SFW ($r=0.40$); disease severity and SDW ($r=0.41$). These correlations indicate that a strong QTL for resistance to *M. perniciosa* is located where all parameters are significantly affected. The remaining lines that presented significant effects (TA1118, TA1138, TA1541, TA1547, TA1562, TA1658, TA1652, and TA1656) had multiple introgressions of LA1777 in one single chromosome (Table 3-1).

Identification of QTL Mp1.1 on Chromosome 1

The most evident result found is on the IL TA1128 and TA1536. These lines, containing single homozygous introgressions at the extremity of chromosome 1, showed significant effects on disease severity and associated quantitative traits (Table 3-1). A detailed map shows that these introgressions are located between marker CT233 and TG58 on the tomato genetic map. The size of the concerned genomic region is approximately 20 cM. For clarity, the QTL identified for resistance to *M. perniciosa* is called **M.p1.1** (Fig 3-1).

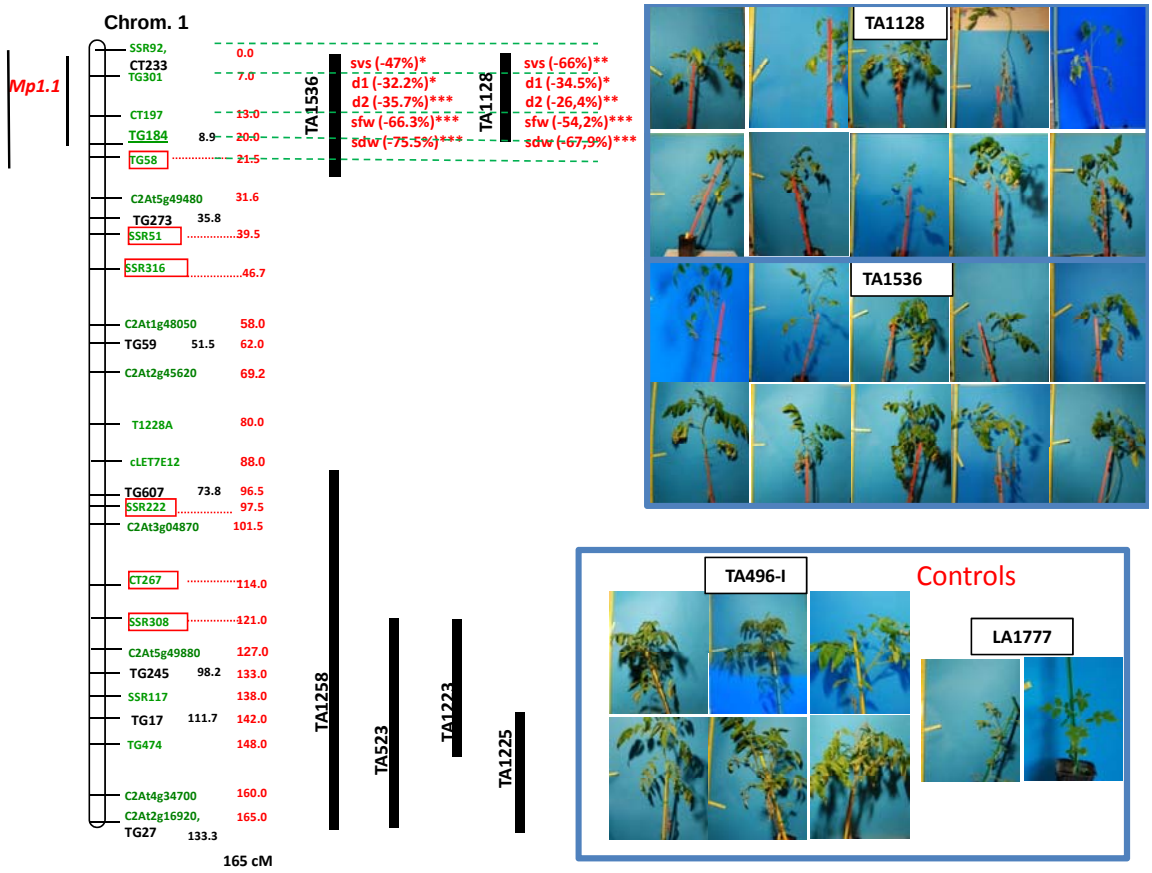


Fig. 3-1: Detailed chromosomal position, dimension, identity and phenotypic effect of ILs present in Chromosome 1. svs: disease severity; d1: diameter at first internode; d2: diameter at third internode; sfw: shoot fresh weight; sdw, shoot dry weight. Both NILs have low severity score, i.e. less blisters, less swelling, less curling and reduced branching compared to the control.

Discussion

Identity of the genomic region of Mp1.1

Previous studies have characterized the genomic region on the short arm of chromosome 1, containing Mp1.1 (Thomas *et al.* 1997; Yuan *et al.* 2002). This genomic region contains "gene galaxies" such as Northern lights and Milky Way that concentrate a high number of resistant genes homologous to the *Cladosporium fulvum* resistant genes Cf-9 (Parniske and Jones 1999). Those genes, called Hcr9, are present at high copy numbers in these regions and only a few of them have been fully characterized. Therefore, the interesting revelation is that the region that contains a putative QTL for resistance to *M. perniciosa* is located in these regions with a high density of resistance genes. Over the course of this experiment, observations revealed only the biotrophic phase of *M. perniciosa* (to observe the saprophytic phase required a longer observation time, which was not technically achievable) and leads to speculation that the genes for resistance act specifically in preventing the establishment of this phase on the host.

C. fulvum is also a biotrophic organism, and the resistance response is strictly a gene-for-gene interaction, extensively described in previous studies (Hammondkosack, Jones *et al.* 1994; Hammondkosack, Harrison *et al.* 1994). One possibility could be that a homolog to Cf-9 has the ability to recognize avirulent factors produced in the aposplast by *M. perniciosa*, thereby triggering the resistance response cascade. Already shown is

that homologs of the Cf genes can be effective against other pathogens such as *Venturia inequalis* in apple cultivation (Vinatzer *et al.* 2001).

Applications to cacao breeding

A better characterization of *Mp1.1* in tomato could lead to several applications for cacao breeding. A possible outcome could be mapping Conserved Ortholog Sequences (COS) between tomato and cacao in order to locate the COS that flank *Mp1.1* in tomato and then map it back via synteny in the cacao genome. To compare the tomato genome to that of other plant species such as *Arabidopsis*, the COS sequences have been useful (Fulton et al. 2002). The possibility of finding QTL in cacao through synteny with the tomato is an important advancement for cacao breeding and can significantly shorten the long and laborious work required to breed cacao. In addition, this investigative line makes possible comparing the QTLs found in tomato with other QTLs for disease resistance in tomato and cacao in order to verify the involvement of this QTL in disease resistance. Map-based cloning of the genes located within *Mp1.1* could also open opportunities for a transgenic approach in producing resistant cacao. Map-base cloning has been successful in tomato to clone disease resistance genes (Martin *et al.* 1991).

Confirmation of results

A small subset of plants of the NILs TA1536 and TA1128 were inoculated with spores of *M. perniciosa* and compared to the controls TA496 and LA1777 following the same protocol described in the Materials and Methods. Among the inoculated TA496 plants, the mean disease incidence (number of plants presenting witches' broom symptoms) was 33.3%. In the line TA1536 the disease incidence was 9% whereas in the line TA1128, none of the plants presented symptoms. Neither the mock inoculated controls nor the resistant parents showed symptoms. This result confirms that the lines TA1536 and TA1128 present a certain level of resistance to the witches' broom pathogen.

References

Bastos, C. N., and H. C. Evans. 1985. A new pathotype of *Crinipellis perniciosa* (Witches' broom disease) on solanaceous hosts. *Plant Pathology* 34:306-312.

Bernacchi, D., T. Beck-Bunn, D. Emmatty, Y. Eshed, S. Inai, J. Lopez, V. Petiard, H. Sayama, J. Uhlig, D. Zamir, and S. Tanksley. 1998. Advanced backcross QTL analysis of tomato. II. Evaluation of near-isogenic lines carrying single-donor introgressions for desirable wild QTL-alleles derived from *Lycopersicon hirsutum* and *L. pimpinellifolium*. *TAG Theoretical and Applied Genetics* 97 (1-2):170-180.

Brown, S. J., R. J. Schnell, J. C. Motamayor, U. Lopes, D. N. Kuhn, and J. W. Borrone. 2005. Resistance gene mapping for witches' broom disease in *Theobroma cacao* L. in an F2 population using SSR markers and candidate genes. *J. Amer. Soc. Hort. Sci.* 130 (3):366-373.

Fulton, T. M., R. Ven der Hoeven, N. T. Eanneta, and S. D. Tanksley. 2002. Identification, analysis and utilization of conserved ortholog set markers for comparative genomics in higher plants. *The Plant Cell* 14:1457-1467.

Griffith, G. W., and J. N. Hedger. 1993. A novel method for producing basidiocarps of the cocoa pathogen *Crinipellis perniciosa* using a bran-vermiculite medium. *Netherlands Journal of Plant Pathology* 99:227-230.

Griffith, G. W., and J. N. Hedger. 1994. The breeding biology of biotypes of witches' broom pathogen of cocoa, *Crinipellis perniciosa*. *Heredity* 72:278-289.

Griffith, G. W., J. Nicholson, A. Negnninger, R. N. Birch, and J. N. Hedger. 2003. Witches' brooms and frosty pods: two major pathogens of cacao. *New Zealand Journal of Botany* 41:423-435.

Hammondkosack, K. E., D. A. Jones, and J. D. G. Jones. 1994. Identification of 2 genes required in tomato for full Cf-9-dependent resistance to *Cladosporium-fulvum*. *Plant Cell* 6 (3):361-374.

Hammondkosack, K. E., K. Harrison, and J. D. G. Jones. 1994. Developmentally-regulated cell-death on expression of the fungal avirulence gene avr9 in tomato seedlings carrying the disease-resistance gene Cf-9. *Proceedings of the National Academy of Sciences of the United States of America* 91 (22):10445-10449.

Lopes, J. R. M., E. D. M. N. Luz, and J. L. Bezerra. 2001. Suscetibilidade do cupuacuzeiro e outras especied vegetais a isolados de *Crinipellis perniciosa* obtidos de quatro hospedeiros diferentes no sul da bahia. *Fitopatologia brasilera* 26 (3):601-605.

Marelli, J., S. Kang, S. Maximova, P. Backman, K. Gramacho, and M. Guiltinan. 2005. Histology and physiology of the interaction between Crinipellis perniciosa and Lycopersicon esculentum. *Phytopathology* 95 (6):S65.

Martin, G. B., J. G. K. Williams, and S. D. Tanksley. 1991. Rapid identification of markers linked to a Pseudomonas resistance gene in tomato by using random primers and near-isogenic lines. *Proceedings of the National Academy of Sciences of the United States of America* 88 (6):2336-2340.

Monforte, A. J., and S. D. Tanksley. 2000. Development of a set of near isogenic and backcross recombinant inbred lines containing most of the *Lycopersicon hirsitum* genome in a *L. esculentum* genetic background: A tool for gene mapping and gene discovery. *Genome* 43:803-813.

Motamayor, J. C., A. M. Risterucci, M. Heath, and C. Lanaud. 2003. Cacao domestication II: progenitor germplasm of the Trinitario cacao cultivar. *Heredity* 91 (3):322-330.

Niella, G. R., M. I. Resende, H. A. Castro, L. H. C. P. Silva, and J. A. Carvalho. 1999. Aperfeicoamento da metodologia de producao artificial de basidiocarpos de *Crinipellis perniciosa*. *Fitopatologia brasileria* 23:523-527.

Parniske, M., and J. D. G. Jones. 1999. Recombination between diverged clusters of the tomato Cf-9 plant disease resistance gene family. *Proceedings of the National Academy of Sciences of the United States of America* 96 (10):5850-5855.

Pereira, J. L. 2000. Management of Witches'broom Disease of Cocoa : A contemporary retrospective. Bahia, Brazil: Centro de Pesquisas do Cacau, CEPEC/CEPLAC.

Purdy, L. H., and R. A. Schmidt. 1996. Status of cacao witches broom : biology, epidemiology, and management. *Annual Review of Phytopathology* 34:573-94.

Tanksley, S., S D. 1993. Mapping Polygenes. *Annual Review of Genetics* 27 (1):205-233.

Tanksley, S., S D, and S. R. McCouch. 1997. Seed banks and molecular maps:unlocking genetic potential from the wild. *Science* 277:1063-1066.

Thomas, C. M., D. A. Jones, M. Parniske, K. Harrison, P. J. Balint-Kurti, K. Hatzixanthis, and J. D. G. Jones. 1997. Characterization of the tomato Cf-4 gene for resistance to Cladosporium fulvum identifies sequences that determine recognitional specificity in Cf-4 and Cf-9. *Plant Cell* 9 (12):2209-2224.

Vinatzer, B. A., A. Patocchi, L. Gianfranceschi, S. Tartarini, H. B. Zhang, C. Gessler, and S. Sansavini. 2001. Apple contains receptor-like genes homologous to the Cladosporium fulvum resistance gene family of tomato with a cluster of genes cosegregating with Vf apple scab resistance. *Mol. Plant-Microbe Interact.* 14 (4):508-515.

Wood, G. A. R., and R. A. Lass. 1985. *Cocoa*. Fourth ed. New York: Longman.

Yuan, Y., J. Haanstra, P. Lindhout, and G. Bonnema. 2002. The Cladosporium fulvum resistance gene Cf-ECP3 is part of the Orion cluster on the short arm of tomato Chromosome 1. *Molecular Breeding* 10 (1):45-50.

Chapter 4

Over-expression of a cacao class I chitinase gene in *Theobroma cacao* L. enhances resistance against the pathogen, *Colletotrichum gloeosporioides*

Maximova, S.N., J-P. Marelli, A. Young, S. Pishak, J.A. Verica and M.J. Guiltinan. 2006. Over-expression of a cacao class I chitinase gene in Theobroma cacao L. enhances resistance against the pathogen, Colletotrichum gloeosporioides. *Planta*. 224 (4): 740-749.

Introduction to published paper

Colletotrichum gloeosporioides (*Glomerella cingulata*) is an ascomycete pathogen that causes leaf and pod anthracnose on *Theobroma cacao* (Wood and Lass 1985) as well as in many other tropical crops (Agrios 2005). In *T. cacao*, the fungus attacks primarily young, soft leaves causing brown lesions surrounded by a characteristic clear yellow halo. When the lesions expand, they coalesce to form large blighted areas that can lead to defoliation. On pods, disease symptoms appear as dark sunken lesions that expand and agglomerate but rarely damage the beans. The infected cherelle (young cacao fruit) wilts and remains on the trunk as dead and mummified fruits (Mohanan *et al.* 1989). The primary source of inoculum comes from the acervuli forming under the leaf or fruit epidermis and releasing pink conidia. Cacao anthracnose is one of the major cacao problems in India (Mohanan et al. 1989) and has been reported to cause significant

damage to trees in Brazil and in Venezuela, where the variety "Porcelana" is very susceptible to this disease (Luis Cedeño, personal communication).

In addition to being a pathogen, *C. gloeosporioides* has also been identified as a fungal endophyte in *T. cacao* trees (Arnold *et al.* 2003). It was frequently isolated from *T. cacao* leaves, occurring in 71.9% of mature and old leaves per tree (Arnold et al. 2003). The fungus was also infiltrated to *T. cacao* seedlings where it remained asymptomatic and could be used to elicit resistance to *Phytophthora* spp., a major cacao pathogen in South America and Africa (Arnold et al. 2003). Therefore, *C. gloeosporioides* in *T.cacao* can behave as a fungal pathogen or as a beneficial endophyte.

For the current study, initially, isolation of the fungus was serendipitous. A cacao plant, previously inoculated with *M. perniciosa*, died in the growth chamber. To confirm the involvement of *M. perniciosa* in plant death, Koch postulates were performed[7]. A diseased root showing symptoms was used to isolate the causal agent. Morphological and genetic identification led to the isolation of *Colletotrichum gloeosporioides* from the diseased root. When applying conidia of *C. gloeosporioides* to young leaves, necrotic lesions appeared at the point of inoculation. Wounding with fine needles prior to inoculum deposition would initiate the formation of lesions that were quantifiable using a binocular microscope. From the leaf lesions, conidia of *C. gloeosporioides* were re-isolated, which completed the four Koch postulates[1]. Based on this discovery,

[7] Koch postulates are four criteria to demonstrate that a pathogen is the causal agent of a disease. The criteria are: (1) the pathogen must be found in diseased tissues but not in healthy tissues. (2) The pathogen must be isolated from diseased tissues and grown in pure culture. (3) The application of pathogen infective structures to a healthy plant must induce the same disease symptoms. (4) The pathogen must be re-isolated from the experimental host and be identical to the one in (2) (Koch 1983).

development of a leaf bioassay allowed testing for anti-fungal activity of transgenic cacao plants' over-expression of a chitinase gene and provided biological evidence of the efficacy of these transgenic *T. cacao* plants. My contribution to this paper was specifically to collect the data for Figs. 5 and 6, and partial writing of the paper in collaboration with the first author.

The paper appeared in *Planta* under the title: "Over-expression of a cacao class I chitinase gene in *Theobroma cacao* L. enhances resistance against the pathogen, *Colletotrichum gloeosporioides*" in 2006. It is reprinted in this dissertation from the original article by permission from the journal.

References

Agrios, G. N. 2005. *Plant Pathology*. Fifth ed. Burlington, MA: Elsevier Academic Press.

Arnold, A. E., L. C. Mejia, D. Kyllo, E. I. Rojas, L. Maynard, N. Robbins, and E. A. Herre. 2003. Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences* 100 (26):15649-15654.

Koch, R. 1983. *Journal of Hygene Infantile* 14:319-333.

Mohanan, R. C., K. M. Kaveriappa, and K. K. N. Nambiar. 1989. Epidemiological studies of *Colletotrichum gloeosprioides* disease of cocoa. *Annals of Applied Biology* 114:15-22.

Wood, G. A. R., and R. A. Lass. 1985. *Cocoa*. Fourth ed. New York: Longman.

Chapter 5

General Conclusions

As outlined in Chapter 1, the witches' broom disease has become a serious problem for cacao from the moment humans have started to grow the crop industrially. A recurrent cycle of (1) increased plantation area (2) economic prosperity (3) witches' broom epidemic and (4) economic crisis has been observed in Suriname, Ecuador, and finally Brazil. One of the highlights of this chapter is that this situation is not unique to witches' broom and cacao but has also been observed for fusarium blight of bananas and the coffee rust, to only cite a few. Many times disease epidemics not only appear because the three conditions of the disease triangle are met (a favorable environment, an aggressive pathogen, and a susceptible host) but also because of the local economic, political and cultural context. The role of scientists to prevent or cure a plant disease such as witches' broom is to understand all the factors that can trigger an epidemic, which are not only biological but also sociological.

The remaining chapters of this thesis deal mainly with the biological aspect of the witches 'broom pathogen, *Moniliophthora perniciosa.* Chapter 2 demonstrates that the tomato plant (*Solanum lycopersicum*) can be used as a model plant because of the parallels that exist between S-biotype strains of *M. perniciosa* infecting tomato and C-biotype strains of *M. perniciosa* infecting cacao. New findings on the mechanisms of infection such as the cell hypertrophy and hyperplasia in the swelling areas give

indications on how the pathogen increases the biomass of the plant during the biotrophic phase. The presence of auxin, revealed by the activation of the DR5 responsive promoter, shows that auxin is involved in the interaction. In addition, the presence of crystals of calcium oxalate present in the intercellular areas indicates that oxalic acid might be involved in *M. perniciosa* pathogenicity.

Chapter 3 is a direct application of the tomato model to accelerate the discovery of control strategies against *M. perniciosa.* By using Near Isogenic Lines of tomato created using a resistant wild relative of tomato, *Solanum habrochaites,* resistant to *M. perniciosa* and the susceptible *Solanum lycopersicum*, it was possible to look for the genomic region that confers resistance to *M. perniciosa*. The NILs have a small portion of the genome of the resistant parent in the background of the susceptible parent. After a screen of all the lines with *M. perniciosa*, that genomic region was found in the NILs TA1536 and TA1128, located on the short arm of Chromosome 1. This chromosomal region is known to be very rich in resistant genes and contains gene galaxies such as the "Milky Way' and the 'Northern Lights'. In particular this region contains the HCr9 genes (Homolog to Cf-9 resistance genes) which confer resistance against the foliar pathogen *Cladosporium fulvum,* also a biotrophic pathogen.

Chapter 4 takes another strategy to accelerate breeding for resistance using the transgenic approach. A cacao endochitinase gene isolated from the pods (fruits) was fused to the CaMV 35S constitutive promoter and introduced transgenically in the SCA6 genotype. The fungal pathogen, *Colletotrichum gloeosporioides,* isolated from cacao, served as experimental model to test the efficacy of the transgenic cacao plants to

stop fungal colonization. The plants that had the highest expression levels were also the ones able to limit the best fungal colonization and necrosis.

Several experiments could be done as a follow-up of this dissertation. In Chapter 2, the oxalic acid involvement in pathogenicity would be a key finding to understand how the pathogen enters the biotrophic phase and how it can switch to the saprophytic phase. Characterization of the production of oxalic acid in cultures of *M. perniciosa* by using pH indicators could be a starting point. Then, by performing random mutagenesis (EMS, or UV), oxalic acid deficient mutants could be generated in order to test the importance of oxalic acid in pathogenicity. Eventually, the optimization of genetic transformation system to produce gene knock-out transformants would be necessary.

In Chapter 3, the *Mp1.1* genomic region would need to be characterized by using sub-NILs in order to isolate a smaller genomic region containing the resistant gene(s). Because the short arm of chromosome 1 is very dense in resistance genes, cloning a resistance gene would be difficult. However, since the genetic markers surrounding that genomic region (CT233 and TG184) are known, screening several cacao varieties and relatives to detect those markers in the cacao genome could be feasible. Once characterized, those markers could be used for Marker Assisted Selection (MAS) in cacao breeding programs.

In Chapter 4, it is still necessary to test the transgenic cacao plants with commercially important pathogens such as *M. perniciosa* and *M. roreri*. This has been attempted several times at Penn State but the cacao genotype SCA6, used for genetic transformation was resistant to the strains of *M. perniciosa* used in the experiments. Two

new strains, Castenhal-I and APC3, acquired by the PSU collection, are pathogenic on SCA6 and could be used to test the susceptibility of the transgenic plants.

To illustrate some of the findings and observations made in this dissertation, a hypothetical model of the interaction between *M. perniciosa* and tomato is shown below (Fig. 5-1). In the first case scenario, the plant has a resistance gene, as in the resistant NILs found in chapter 3. Similarly to HCr9 resistant genes that function by recognizing an avirulence factor produced by *Cladosporium fulvum*, the resistant tomato lines could block the infection process by recognizing avirulence factors from *M. perniciosa* and trigger a hypersensitive response (HR). In the second case scenario, the tomato plant does not have a resistant gene, and therefore is unable to stop fungal colonization. The fungus induces hormone imbalance and overproduction of auxin causing cell division and enlargement in the vascular tissues. The presence of calcium oxalate crystals could be the result of oxalic acid production by the fungus to silence the plant defense responses.

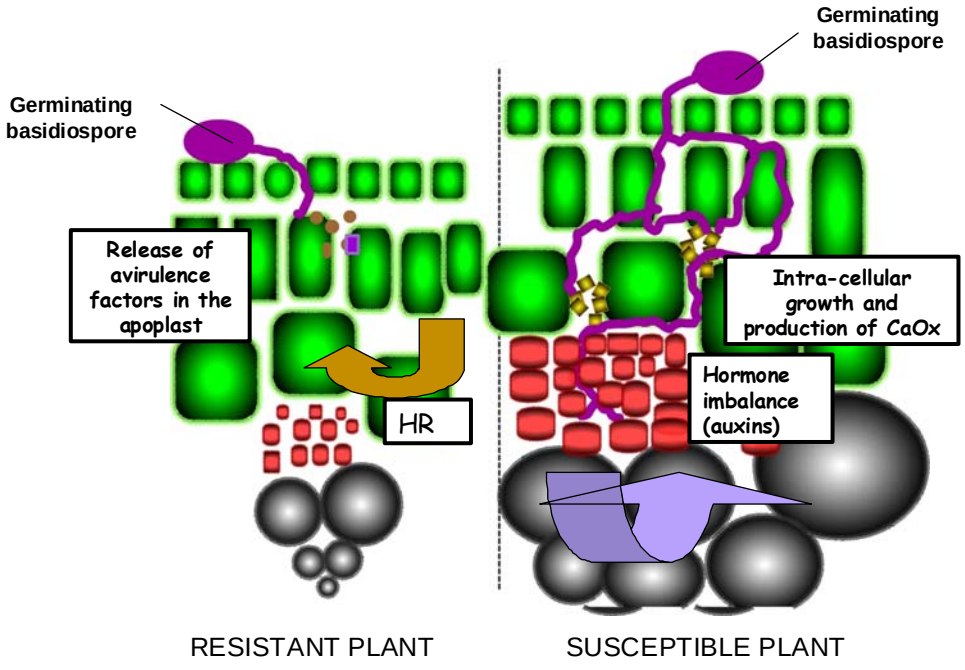


Fig. 5-1: Hypothetical model of the interaction between *M. perniciosa* and tomato

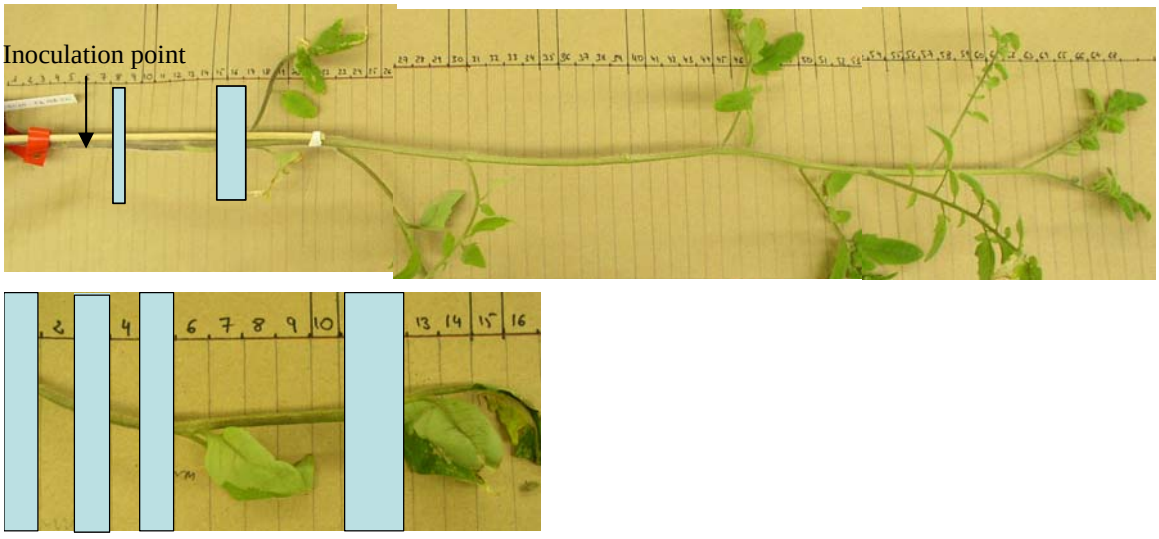
Appendix A

Isolation of fungal mycelium from tomato infected plants

Objective: Detect the presence or absence of fungal mycelium infected plants to evaluate the extent of fungal colonization along the plant stem and leaves.

Materials and Methods: 1 cm sections of 6 week-old infected plants are surface sterilized with a solution of 3% commercial bleach and rinsed 3 times with sterile distilled water. The sections are then dried over sterile filter paper and plated over a selective medium (Potato Dextrose Agar with 50 mg/ml Ampicillin, 34 mg/ml Chlorophenicol, 50 mg/ml Neomycin, 50 mg/ml Streptomycin, 10% Benomyl).

Results:



Map of an infected plant. Sections where *M. perniciosa* fungal mycelium was recovered on selective medium are highlighted in blue.

Discussion: This preliminary experiment demonstrates that the colonization by *M. perniciosa* is localized around the point of infection. In this specific case, the fungus only colonized 15% of the plant main stem. In addition, the fungus colonized 30% of the first leaf, also showing the strongest swelling.

Appendix B

PCR detection of *M. perniciosa* in diseased plants

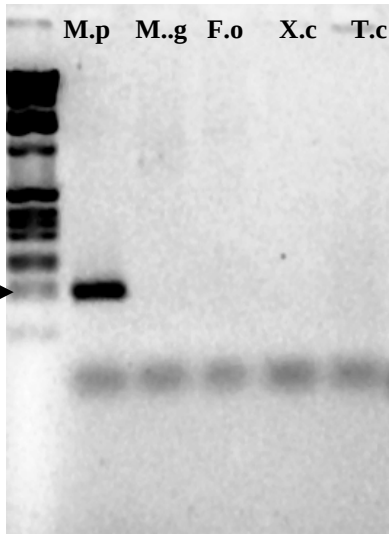
Objective: Design PCR primers based on genomic information of *M. perniciosa* that can detect the presence or absence of the fungus in infected material.

Materials and Methods: The primer sequence was designed based on the genomic sequence of the ITS2 of *M. pernciosa*. To control for amplification of plant genomic DNA, a primer was designed based on the genomic sequence of the actin gene in tomato. The sequence of the primers was:

CPITS2-F	TCCAAACCGAAGTGTTGAGACCTA
CPITS2-R	CTGCCGGCTTTGAACAAGAGTC
ACT-F	CGT ACT GGG CTT TTT GTG ATC CAA
ACT-R	GAA CAA TGT TAC CAT AGA GGT CCT TCC T

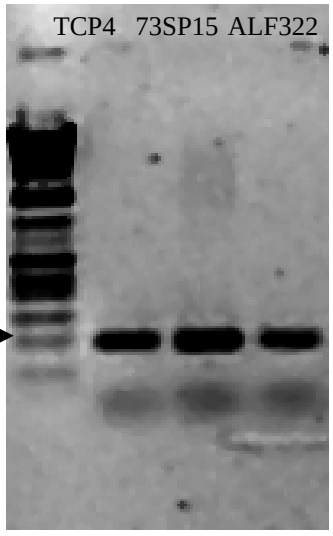
Results:

Primer specificity to *M. perniciosa*:



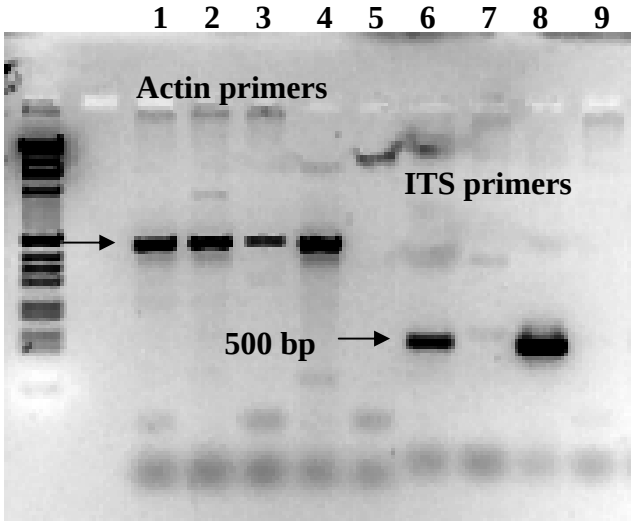
Specificity of the primers. The ITS2 primers were used to amplify genomic DNA from M.p (*M.perniciosa*), M.g (*Magnaporthe grisea*), F.o (*Fusarium oxysporum*), X.c (*Xanthomonas campestris*) and Cacao (*Theobroma cacao*).

Primers amplify different biotypes of M . perniciosa



PCR amplification of strains TCP4 (C-biotype), 73SP15 (S-biotype) and ALF322 (L-biotype), using the ITS primers.

Amplification of M. perniciosa genomic DNA in tomato infected tissue



1=Infected necrotic plant
2= Control plant
3= Tomato genomic DNA
4= Healthy Leaf extract
5= No DNA
6= Infected necrotic plant
7= Control plant
8= M.p genomic DNA
9= Healthy leaf extract

Diagnosis of infected plant: Amplification of various plant extracts. (1) and (6) show the amplification in infected plant extracts. (2) and (7) show the amplification in non inoculated control plant extracts.(3) and (8) represent the positive controls for tomato genomic DNA and *M.p* genomic DNA respectively. (4) and (9) are control extracts prepared by a third person.

Discussion:

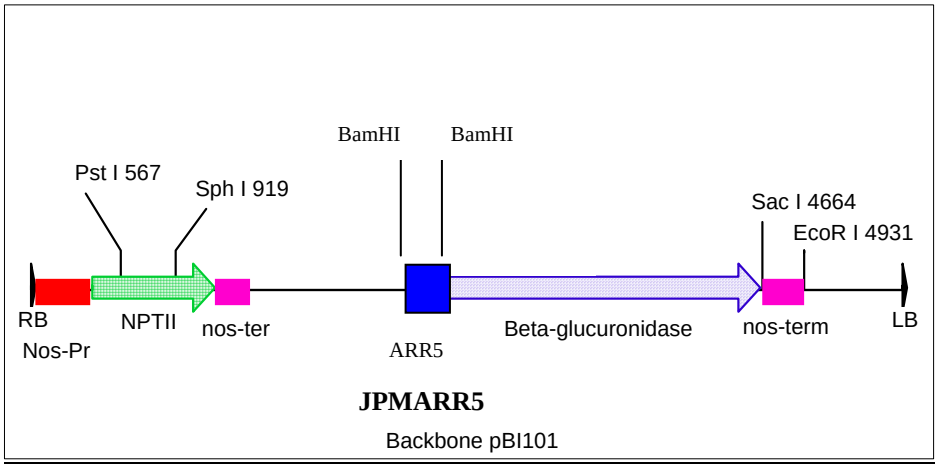
This experiment shows that it is possible to detect the presence of *M. perniciosa* hyphae in infected plants by PCR with fungal specific primers (ITS2). To optimize the system, it would be necessary to make serial dilutions of *M.p* genomic DNA to identify what is the sensitivity of the system.

Appendix C

Stored seeds of transgenic Micro-Tom and NIL lines

Tomato seeds are stored at 4 ºC in paper bags.

Seeds of the ARR5 construct (plasmid JPMARR5)

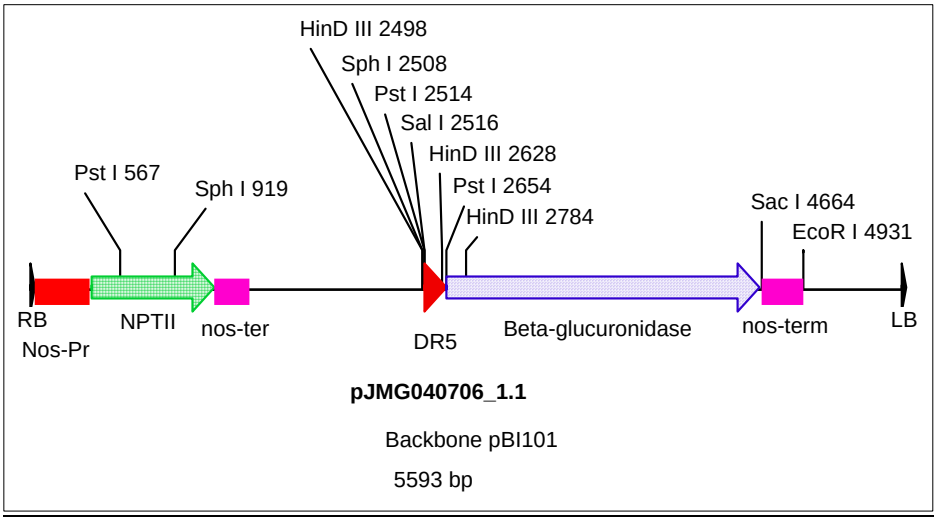


Acclimated transgenic plants were received from the University of California Davis. They were kept in the greenhouse to set fruit. The T1 seeds (first generation) were collected and planted on selective medium on kanamycin (100 mg/ml). The transgenic T1 plants were then grown in the greenhouse to set fruit and T2 seeds (second generation) were collected. Only the seeds of genotype 052234-005 were grown to obtain T3 seeds (third generation).

T1 Genotype	Number of bags	T2 Genotype	Number bags	T3 Genotype	Number of bags
052225-001	1	*			
052225-002	1	052225-002-1	2	**	
		052225-002-2	2		
052234-005	1	052234-005-1	2	052234-005-1'	16
		052234-005-2	2	052234-005-2'	5
		052234-005-3	1	052234-005-3'	11
		052234-005-4	2	052234-005-4'	4
		052234-005-5	1	052234-005-5'	9
		052234-005-7	1	052234-005-7'	8
		052234-005-8	2	052234-005-8'	8
		052234-005-9	3	052234-005-9'	19
		052234-005-11	1	052234-005-11'	9
052230-005	1	052230-005-1	2	**	
		052230-005-2	2		
		052230-005-3	2		
		052230-005-4	1		
052234-007		052234-007-1	2	**	
		052234-007-2	2		
		052234-007-3	1		
052234-009	1	*		**	
052230-004	1	*		**	
0522-34-013	1	*		**	

* Not all T1 plants gave enough seeds to be planted for the T2 generation
** Only one line (052234-005) was used to generate T3 seeds based on the GUS expression assays.

Seeds of the DR5 construct (plasmid pJMG040706_1.1)



T1 Genotype	Number of Bags	T2 Genotype	Number of Bags
DR1	1	DR1_1	8
DR2	1	DR2-1	8
DR3	2	DR3-1	9
DR5	1	DR5-1	9
DR7	2		
DR9	1	DR9-1	6
DR10	1	DR10-1	6
DR11	2	DR11-1	8
DR12	1	DR12_1	10
DR13	2	DR13-1	6

Seeds of the Chitinase transgenic plants (plasmid pGAM00.0511)

Construct map available in Chapter 4

T1 Genotype	Number of Bags
Chi1	2
Chi2	1
Chi3	1
Chi4	1
Chi5	1
Chi6	2
Chi7	1
Chi8	1
Chi9	1
Chi10	1
Chi11	1
Chi12	2
Chi13	1
Chi14	1
Chi15	1
Chi16	1

Seeds of Near Isogenic Lines provided by Nestle

Genotype	Number of Bags
TA496	1
LA1777	1
TA1536	1
TA1128	1
TA1266	1
TA1537	1
TA1328	1

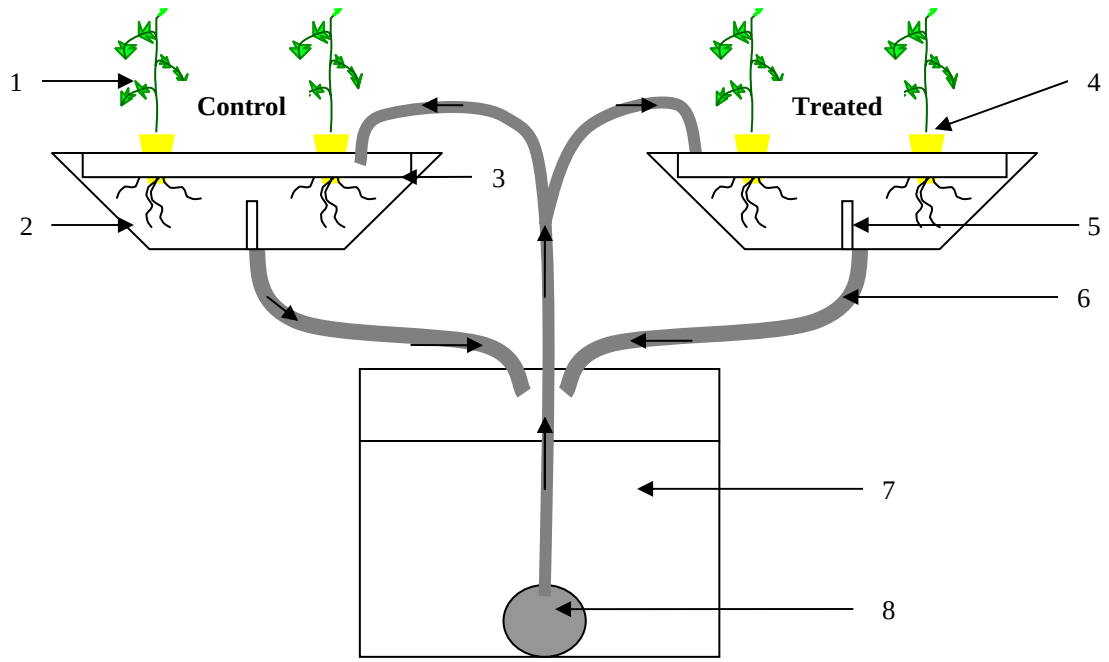
Appendix D

Hydroponic tomato plant system infected with *M. perniciosa*

Objective: Develop a system to grow tomato plants grown hydroponically in a nutrient solution. One set of plants was inoculated with spores of *M. perniciosa*, the other was left non inoculated.

Materials and Methods:

The following figure shows the experimental set up used:

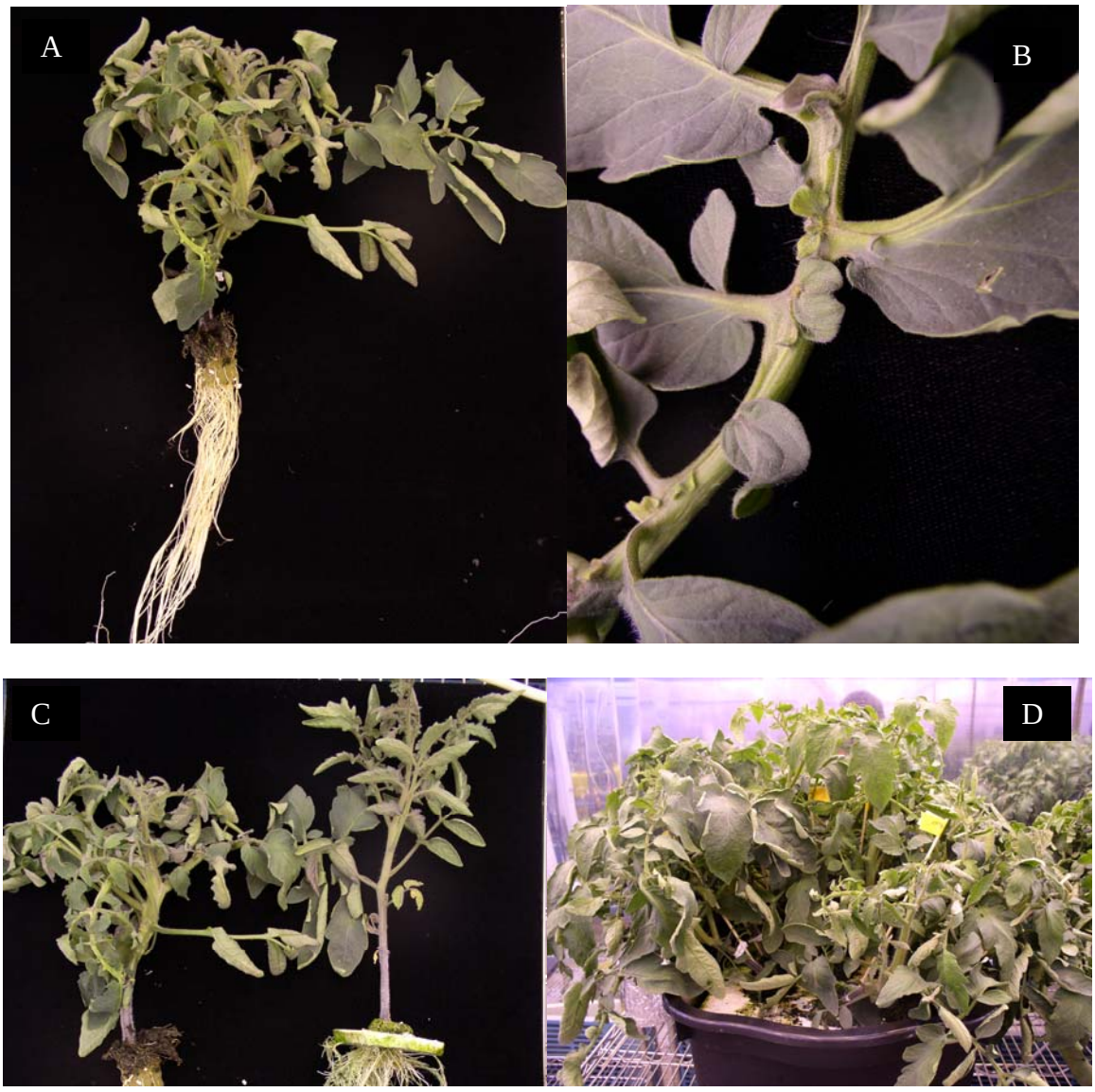


1: Tomato plant
2: Black container
3: Styrofoam piece
4: Rockwool plug
5: Level system
6: Hose
7: Nutrient solution tank
8: Electric pump

The seeds were sterilized and germinated according to the protocols described in Chapter 2 (p). Once the radicule emerged, the seedlings (1) were transferred to the rockwool plugs (4) (Grow cubes Grodan®). The Styrofoam piece (3) was perforated at 6 or 8 different locations in order to place the rockwool plugs. The Styrofoam piece floated on the surface of the nutrient solution inside a container (2) that was painted with black paint to limit light access to the roots. An electric pump (8) poured constantly nutrient solution into each of the containers. A small metal tube (5) placed inside the container would limit overflow by keeping the level of the nutrient solution lower than the top of the container (the overflow went back to the nutrient solution tank through a hose placed underneath the container (6)). The plants were grown at 28 ºC in a high light growth chamber.

Results:

The inoculated plants showed typical witches' broom symptoms a few weeks after infection. The non infected plants also started showing the same type of symptoms. At the end of the experiment all plants were presenting swollen stems, petioles and extremely big leaflets. Some plants had leaflets forming at abnormal locations. The leaflets also presented callus formation along the veins as described for tomato in chapter 2.



Images of the experiment: (A) Infected plant showing typical witches' broom symptoms. (B) A petiole of an infected plant showing the extra leaflets that emerge from abnormal locations.(C) Comparison of an infected tomato plant growing in the hydroponic system (left) and a tomato plant growing in a pot of nutrient solution that was not connected with the hydroponic system (right). (D) Infected plants outgrowing the container.

Discussion

The serendipitous result here was that the plants that were non inoculated showed witches' broom symptoms. This could mean that the infectious agent "travelled" from one tank to the other and affected the control plants. Since basidiospores are known to be short lived and very sensitive to light, it is very unlikely that they survived the high-light conditions. Therefore, the infected plants either produced (1) a volatile compound that promoted the growth of the control plants or that (2) the roots of the infected plants produced a soluble compound that communicated through the nutrient solution tank that was shared between the two containers promoting excessive growth of the control plants. To test this it would be necessary to repeat this experiment by having a two additional set- ups. For hypothesis (1) it would be necessary to compare the effect on the control plants when there is no gaseous exchange between the inoculated container and the control container. For this a "dome" would be placed over the containers to avoid any gaseous contact between them. For hypothesis (2) nutrient solution exchange between infected and control containers could be avoided by having two nutrient solution tanks (one for each container). The effect on the control plants would then be compared between the plants that share nutrient solution and the plants that have an independent nutrient solution intake.

Appendix E

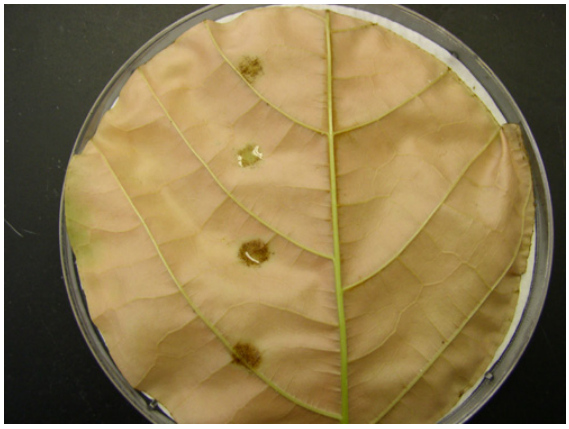
Colletotrichum gloeosporioides infecting cacao leaves

Objective of the study: Determine the differential interactions between *Colletotrichum gloeosporioides* and susceptible and resistant genotypes of *Theobroma cacao*.

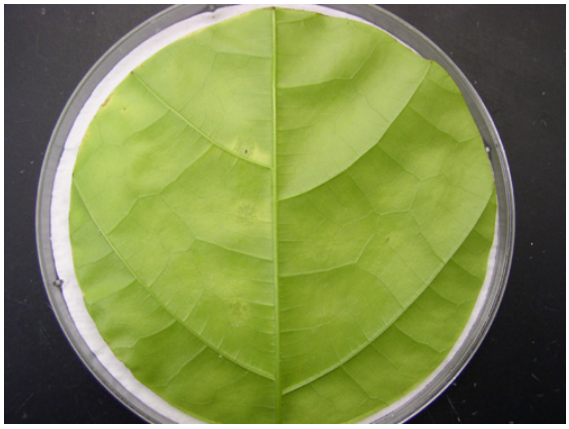
Materials and Methods: Leaf inoculation as described, without wounding, using 1.10^5 spores/mL in water. The leaf discs of Stage C cacao leaved were placed in a Petri-dish containing wet filter paper, and inoculated at four different locations on one side. The other side was inoculated with water for the control. The plates were sealed with paraffin and incubated at 30 ºC for 24 hours.

Results:

General observations:

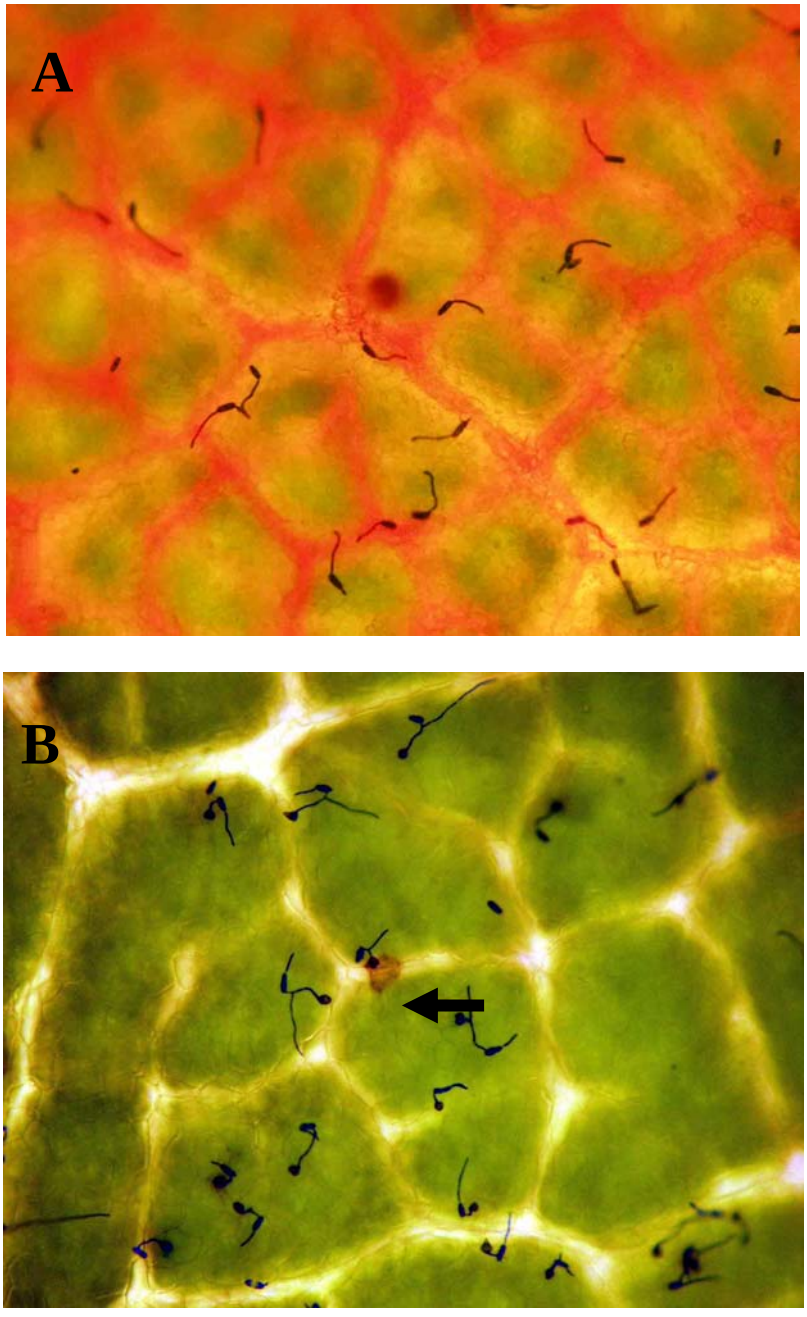


Susceptible clone (ICS1). Arrows represent points of inoculation. Notice the localized necrosis under the inoculum drop.



Resistant clone (SCA6). Arrows represent points of inoculation. Notice the localized yellowing under the inoculum drop.

Microscopic Observations:



C. gloeosporioides conidia forming appressoria few hours after inoculation. (A) On the susceptible clone (ICS1), the conidia germinate but do not form appressorium. (B) On the resistant clone (SCA6), the conidia germinate and form an appressorium. The arrow shows the presence of localized cell death at the site of penetration.

Discussion:

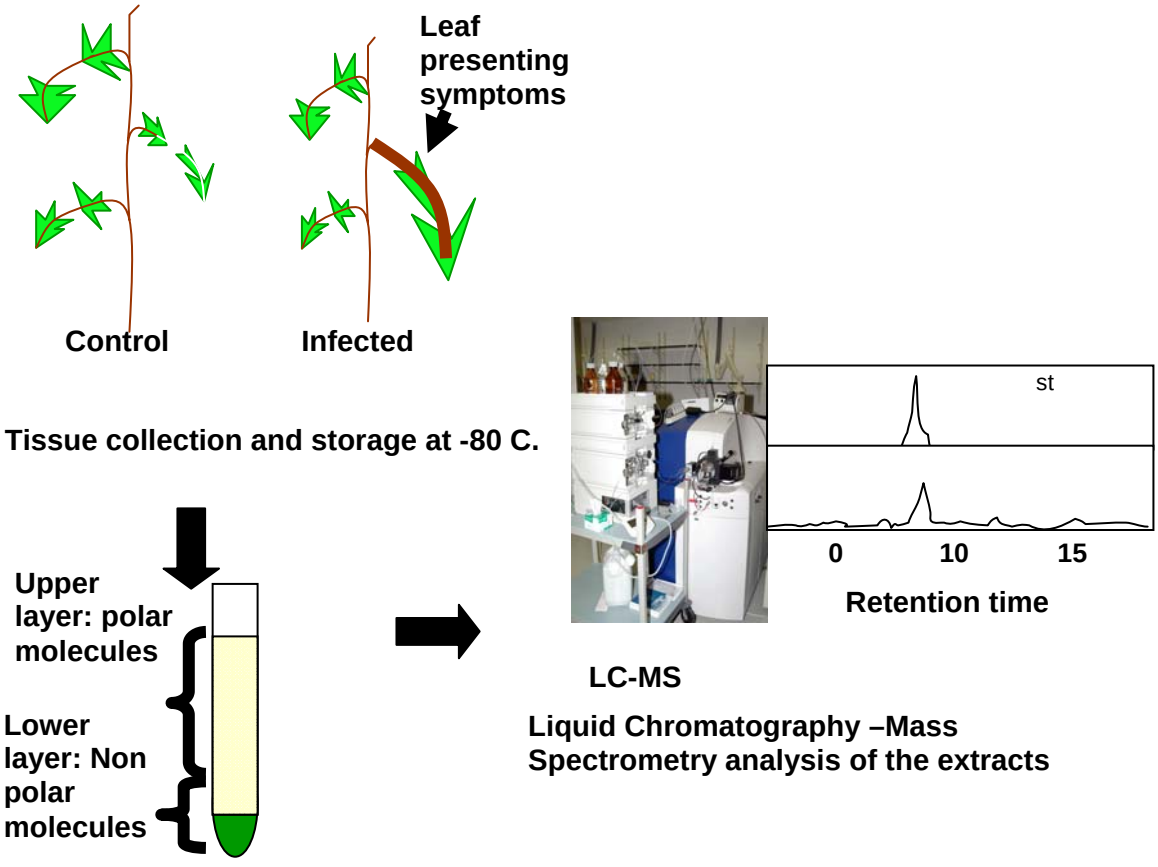
The study of the initial interactions between *C. gloeosporioides* and *Theobroma cacao* showed that there was a different reaction of both pathogen and plant depending on the genotype of *T. cacao* inoculated. On the susceptible genotype, the conidia did not form appressoria but penetrated successfully and formed sporulating lesions. On the resistant genotype, the conidia germinated and formed appressoria, which triggered a localized cell death around the point of appressorium formation. The pathogen could not sporulate in those plants and the infection was successfully contained. This plant reaction seems to be a hypersensitive response (HR) from the plant. However, to prove this hypothesis a series of experiments are needed. First of all, staining for Reactive Oxygen Species would be needed. Then, using specific probes, it could be possible to check for the expression of the genes that are downstream of the HR reaction.

Appendix F

Oxalic Acid measurements performed with Mass Spectrometry

Objective: Quantify the amount of oxalic acid in infected tomato tissue and compare it to non infected control tissue at three time points during the biotrophic phase.

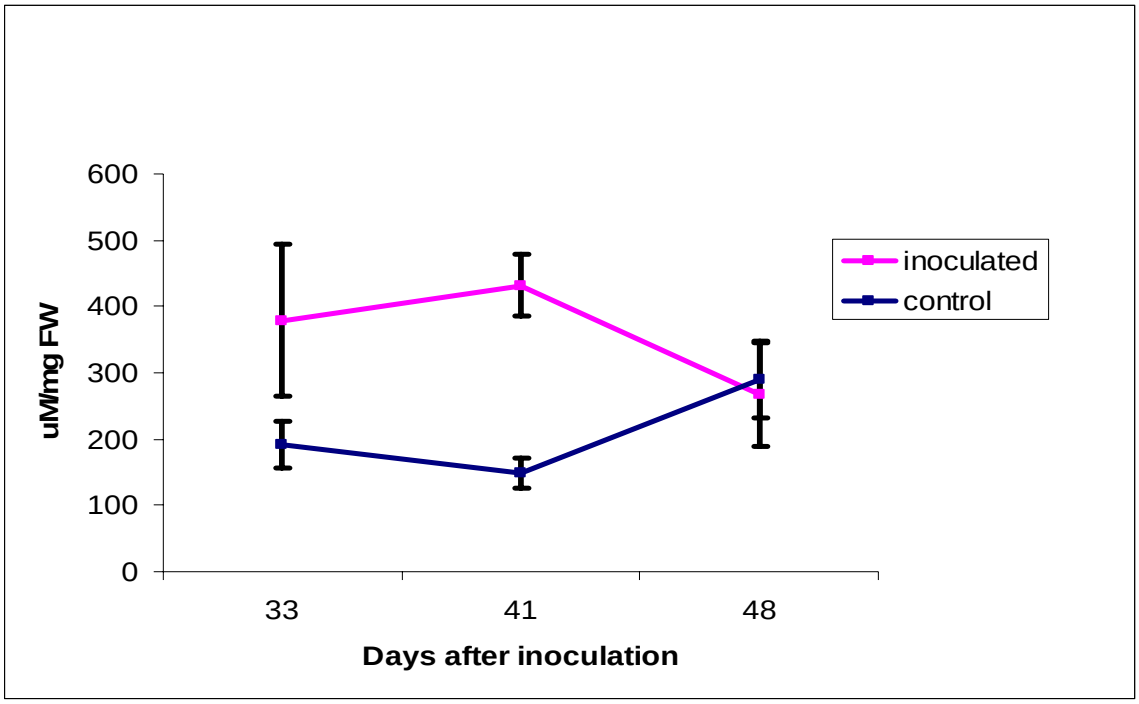
Materials and Methods:



After the chloroform/water/methanol extraction, the oxalic acid was in the upper layer because it is a polar molecule. That phase was then run through the LC-MS with a

standard (pure oxalic acid) to determine the retention time in the chromatography column. Once the peak was identified, the area of the peak was quantified and compared to the area of the standard (of known concentration). The concentration of oxalic acid in µM/mg of fresh weight was then calculated.

Results:



Average concentrations (N=2) of oxalic acid at three different time points after inoculation. The bars represent the standard deviations.

Discussion

This preliminary study shows that at 33 and 41 days after inoculation (dpi), the level of oxalic acid in infected tissues is higher than in the control plants. At 33 dpi, the control plants have about 200 µM/mg FW because tomato naturally produces oxalic acid and forms calcium oxalate crystals in specialized cells (idiobalsts). At 48 dpi, the levels of oxalic acid between infected and control plants are not different. This could be explained by a degradation of the crystals in infected plants that could release oxalic acid in order to trigger cell death and the start of the necrotrophic phase.

To confirm those results, it would be necessary to use more time points particularly at the beginning of the infection to see if oxalic acid increases as the symptoms of the biotrophic phase appear. It would also be necessary to have more biological replicates for every condition.

VITA
Jean-Philippe Marelli

EDUCATION

PhD in Plant Pathology (minor in Science, Technology and Society). **2002-2008**
The Pennsylvania State University. University Park, PA, USA.

MSc in Crop Protection . **1999-2000**
Imperial College. Wye, United Kingdom.

BSc in Agronomy. **1995-2000**
Ecole Nationale Supérieure Agronomique.
French National School of Agricultural Sciences. Toulouse, France.

WORK EXPERIENCE AND ACCOMPLISHMENTS

Graduate Research Assistant **2002-2008**

The Pennsylvania State University, State College, PA

- Developed a leaf bioassay to test the resistance of transgenic cacao plants against *Colletotrichum gloeosporioides.*
- Established a model to study plant and fungal interactions in the witches' broom/cacao pathosystem using tomato.
- Teamed with Cornell and Nestlé's breeders to screen tomato populations with the witches' broom pathogen to find resistance QTLs.
- Taught a Plant Pathology course to 24 undergraduate and graduate students.
- Published papers in referred journals and delivered oral presentations at national meetings.

Junior Researcher **2001- 2002**

Centre de coopération internationale en recherche agronomique pour le développement (CIRAD). Cameroon.

- Supervised a team of technicians and engineers working on an international project on *Phytophthora* pod rot of cacao
- Organized, analyzed and published a 3-year epidemiological data set on *Phytophthora* pod rot of cacao.
- Implemented routine collections of about 300 *Phytophthora megakarya* strains in the field for population biology studies using RAPD markers.

Technical assistant **April-Dec 2001**

CABI Bioscience. Delémont, Switzerland.

- Designed a multi-factorial study to assess the interaction between inter-specific competition and insect herbivory on an invasive weed in Saskatchewan (Scentless Chamomile).