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Biology and epidemiology of *Moniliophthora roreri*, causal agent of Moniliophthora pod rot of cacao

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DEDICATION

To our Heavenly Father and his Son, Jesus Christ, for their endless love and forgiveness.

To Sharon M. Spicer, my sweet angel, my second mom, who will live forever in my heart and thoughts.

To my parents, José Joaquín and Ileana, for their unconditional love and support, for never giving up on me and always encouraging me to fight for every dream I have. I will never stop thanking our Heavenly Father for the family that he prepared for me. I am definitely blessed.

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BIOGRAPHY

The author was born on July 22, 1985, in Turrialba, Cartago, Costa Rica. In 2007, she graduated as an engineer in Biotechnology at the Technological Institute of Costa Rica.

In the year 2006, she came to CATIE as an intern and began her research in Moniliophthora Pod Rot of cacao, specifically in conservation methods of the pathogen's genetic diversity. From 2007 to 2009, she was in charge of the plant pathology area of CATIE´s Cacao Genetic Improvement Program.

In 2011, she obtained her M.Sc. degree in Ecological Agriculture from CATIE, studying the epidemiology of *Moniliophthora roreri.*

Once she completed her master's program, she joined CATIE's doctoral program in Ecological Agriculture with the collaboration of Pennsylvania State University and sponsored by CIRAD, France.

CONTENTS

ABSTRACT

Moniliophthora pod rot (MPR) is one of the main factors limiting cocoa production in Latin America. Currently insufficient information on the biology and epidemiology of the pathogen limits the development of efficient management options to control MPR. The purpose of this study was to identify meteorological and production variables as epidemiological predictors of the MPR disease and, coupled with the existing evidence on the MPR-cacao pathosystem, to develop a conceptual model. Information obtained is strategic for better understanding of the pathosystem, to guide researchers to define new studies and to improve control methods.

The research began with a historical data analysis to determine the influence of the meteorological, productive and genetic resistance variables on the disease over time. This analysis revealed that the resistance of the highly resistant clones is considerably stable and possibly durable; however, for the rest of the clones, disease reaction was shown to be significantly influenced by the environment. Also, temperatures during January, April and May are the only climatic variables that have a significant effect over MPR incidence.

A field trial was then carried out to explain MPR development, onset of symptoms of the disease and fungal sporulation, studying different microclimatic variables for three cacao clones in a range of incomplete resistance. We concluded that water-related variables (positively linked) and temperature (minimum temperature negatively linked while maximum temperature presented a threshold) determine symptom expression for the susceptible clones, while, for the resistant clone CATIE-R4, only temperature (minimum temperature negatively linked and maximum temperature positively linked) showed up as an explicative variable, due to low numbers of CATIE-R4 pods showing symptoms. Differences in resistance among these clones possibly lie in the number of resistant genes accumulated; however, the resistance of the three may be affected by certain environmental conditions.

To separate the microclimatic effects on the infection and the symptoms onset, we studied the relationship between the MPR infection process and the onset of symptoms of three different cacao clones through artificial inoculations. We determined that symptoms/signs onset is close enough to the infection moment that the influence of the microclimate over onset of the symptoms could be extrapolated to the time of infection.

RESUMEN

La moniliasis del cacao es uno de los principales factores que limitan la producción de cacao en América Latina. Actualmente, la insuficiente información sobre la biología y la epidemiología del patógeno limita el desarrollo de mecanismos eficientes para el control de la enfermedad. El propósito de este estudio fue identificar las variables meteorológicas y de producción que actúan como predictores epidemiológicos de la enfermedad y, junto con la evidencia existente sobre el sistema patológico moniliasis-cacao, desarrollar un modelo conceptual. La información obtenida es estratégica para lograr una mejor comprensión del patosistema, así como para guiar a los investigadores a definir nuevos estudios y mejorar los métodos de control.

El estudio comenzó con un análisis de datos históricos para determinar la influencia de las variables meteorológicas, productivas y de resistencia genética sobre la enfermedad a lo largo del tiempo. Este análisis reveló que la resistencia de los clones altamente resistentes es considerablemente estable y posiblemente duradera; sin embargo, para el resto de los clones, su reacción ante enfermedad se vio significativamente influenciada por el ambiente. Además, las temperaturas de enero, abril y mayo son las únicas variables climáticas que tienen un efecto significativo sobre la incidencia de la moniliasis.

Luego, se realizó un ensayo de campo para explicar el desarrollo de la enfermedad, la aparición de los síntomas y la esporulación, estudiando diferentes variables microclimáticas en tres clones de cacao en un rango de resistencia incompleta. Concluimos que las variables relacionadas con el agua (relación positiva) y la temperatura (temperatura mínima con relación negativa mientras que la temperatura máxima presentaba un umbral) determinan la expresión de síntomas para los clones susceptibles, mientras que para el clon resistente CATIE-R4 solo la temperatura (temperatura mínima con relación negativa y la temperatura máxima con relación positiva) se presentó como una variable explicativa, debido al bajo número de mazorcas de CATIE-R4 que mostraron síntomas. Las diferencias en resistencia entre estos clones posiblemente se deben a la cantidad de genes de resistencia acumulados; sin embargo, la resistencia de los tres clones puede verse afectada por ciertas condiciones ambientales.

Para separar los efectos microclimáticos sobre la infección y la aparición de los síntomas, se estudió la relación entre el proceso de infección y la aparición de los síntomas en los tres clones de cacao a través de inoculaciones artificiales. Se determinó que la aparición de los síntomas/signos ocurre bastante cerca al momento de la infección por lo que la influencia del microclima sobre la aparición de los síntomas pueda extrapolarse al momento de la infección.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Cacao (*Theobroma cacao* L) is a highly relevant crop at the global level, both in the agricultural industry and for cosmetics and food. During 2012–2013, 3.931 billion tons of cocoa were produced worldwide. Since the end of 2012, the metric-ton price of cocoa suffered a fall, reaching values close to the USD 2000 in March 2013. However, beginning in January 2014, the price began to pick up, closing with a value of almost USD 3500 in December 2015. Although this price is variable, the increasing demand tied to the benefits of cocoa consumption and cacao's role in biodiversity conservation allows the crop to be profitable (ICCO 2014).

In Africa, approximately 1.2 million farm families depend on cocoa for their survival. In Central America, according to Somarriba et al. (2013), more than 20 000 poor families depend on cocoa as their main source of income. Most of these families belong to different indigenous ethnic groups (Mayas Quekchí, Quiché and Mopán; Mayangna, Bribri, Cabécar, Ngöbe) but also include Afro-Caribbean descendants.

For ancient Mesoamerican cultures (Olmecs, Mayas and Aztecs), cacao was a crop of great value that represented wealth and power. For the Aztecs, cocoa had a religious value and was consumed as a drink called *Xocoalt*, which means "drink of the gods." King Moctezuma was an avid consumer of this drink, which was also used to alleviate stomach aches and flu (López Andrade et al. 2003). That is why in the mid-16th century that the mystical value of cocoa was transferred from the New World to Europe, where the product was extremely well-received. The medicinal use of the crop was documented for relief of fatigue, breathing problems, fever and heart problems. Today, the uses and benefits of cocoa and chocolate are widely known, which has had a strong impact on the food, pharmaceutical and cosmetic industries (Dillinger et al. 2000).

Theobroma cacao L. belongs to the Malvaceae family and is one of the 22 species of the genus *Theobroma*. The species is divided into 10 genetic groups: Amelonado, Contamana, Creole, Curaray, Guiana, Iquitos, Marañón National Nanay and Purús (Motamayor et al. 2008). Its center of origin is located between the Amazon River basin and the Orinoco River and it was later introduced and domesticated in Mesoamerica (Motamayor et al. 2002). It is an evergreen tree with an average height of 5 to 6 meters and dense foliage. Its fruits are called pods and are usually harvested in two major peaks of production. Its productive life is from 25 to 30 years.

Cacao is considered an agroforestry crop since it is usually grown under shade trees, forming systems based on two or more perennial crops. However, there are unshaded, intensively managed cacao plantations in some countries, such as Ecuador. The importance of the shade trees in the perennial crop plantations lies in the fact that these fruit or timber trees offer products/incomes, which are essential when the cocoa prices are low. Shade trees also contribute to the maintenance of the biodiversity in the systems (Beer *et al.* 1998a). According to Beer *et al.* (1998b) and Greenberg (2003), cacao plantations also provide shelter for forest-dependent species and offer other ecosystem services as the promotion of pollination, regulation of existent and potential pests and diseases, facilitation of nutrient recycling, carbon sequestration and protection of available water sources.

Thirty to forty percent of worldwide cocoa production is lost due to different cacao pest and diseases. Table 1.1 summarizes the most important threats in each producing region.

Table 1.1. Most important cacao pest and diseases.

Region	Disease/Pest
West Africa	• Black pod (*Phytophthora megakarya*) • Mirids • Swollen shoot virus
South America	• Witches' broom (including Panama) • Moniliophthora pod rot (except Brazil) • Black pod (*P. palmivora*) • Mirids
Mesoamerica	• Moniliophthora pod rot • Black pod (*P. palmivora*) • Mirids
Southeast Asia	• Vascular-streak dieback • Mirids • Cocoa pod borer

Source: ICCO 2015, http://www.icco.org/about-cocoa/pest-a-diseases.html

As shown in Table 1.1, biological threats vary significantly within regions, resulting in different research focuses. Phytopathological studies are done in a local and isolated way and not as a joint effort, causing unawareness of important threats from other regions that could at any time turn into devastating outbreaks.

At the beginning of the 20th century, more than 50% of global cocoa production (approximately 29 400 tons) occurred in tropical America, followed by the Caribbean, Africa, Asia and Oceania. However, diseases caused a decline in American cocoa production. Today, only 14.4% of world production comes from America, amounting to 522 000 tons of cocoa (ICCO 2014). Moniliophthora pod rot (MPR) is one of the most important diseases in the region; its advance swept many plantations in the countries where it has been reported (Enríquez 2004).

MPR is caused by the fungus *Moniliophthora roreri* (Cif.) Evans et al., (*Basidiomycete, Marasmiaceae*). Its center of origin is in Colombia (Phillips-Mora 2003).

From there, the pathogen spread to 12 countries in tropical America (Phillips-Mora et al. 2006c): Ecuador—formerly considered its place of origin (van Hall 1914) (Rorer 1918), Venezuela, Panama, Costa Rica, Nicaragua, Peru, Honduras, El Salvador, Guatemala, Belize (Phillips-Mora et al. 2006a), Mexico (Phillips-Mora et al. 2006b) and recently Bolivia (Phillips-Mora et al. 2006c) and Jamaica.

Issues around this disease are that the causal agent is in an extremely intense invasive stage, and most of the commercial cacao genotypes established in the region are susceptible. The arrival of this pathogen in countries such as the Dominican Republic and Brazil, and especially in other continents, particularly Africa, would be devastating since the greatest cocoa production in the world is concentrated in West Africa (Phillips-Mora 2003).

Currently there is great interest in reviving the cocoa business in Central America. CATIE (Tropical Agricultural Research and Higher Education Center , Spanish acronym), with projects such as the Cacao Improvement Program, is one of the institutions involved in this process, supporting producers and offering productive alternatives to address the limitations of the crop in the area. Methods currently proposed to control MPR in Central America have been unsuccessful in field applications, sometimes for being inaccessible to smallholders who grow cocoa but also because of the lack of knowledge that still exists about the pathogen. The most viable strategy for confronting this disease is the use of resistant genotypes, but these materials are scarce because multiplication strategies still lag behind the increasing demand. Since arrival of the disease in Central America, a few studies for the effective control of the disease have been done, but none fully explored the biology of *M. roreri* and the epidemiology of the disease. The aggressiveness of the pathogen and the rapid devastation of the cacao farms caused an inactivity of the crop production for nearly 25 years; during which research on the subject was almost null (Phillips-Mora 2003).

Using models to understand the pathogen's life cycles and disease development is common among plant pathologists, but the cacao-MPR system has not been fully described. Leach et al. (2002) attempted to model MPR as a submodel of a management and economical cacao model, which aimed to describe the economic impact of different frequencies of phytosanitary pruning. This model was constructed with historical flowering data and did not

include the influence of the climate, though it did include two theoretical functions, which were clearly affected by the weather. Epidemiological information of this submodel consisted in general descriptions of MPR epidemiology, mainly from Ecuador, based on the perceptions of experts.

With our research, we intended to identify the influence of weather, microclimate and phenology on MPR development and epidemiology in order to fill knowledge gaps and provide information that could be used to model MPR growth. For this, we used two approaches: 1) determining the relationships between weather and yield on disease incidence by using a nine-year database generated by CATIE's Cacao Improvement Program from a 42-clone trial and 2) establishing new experiments focused on determination of the influence of the microclimatic factors over internal and external symptoms and sporulation on three specific clones exhibiting different levels of incomplete MPR resistance.

1.2 RESEARCH OBJECTIVES

1.2.1 General objective

Identify meteorological and production variables as epidemiological predictors of the Moniliophthora pod rot disease to develop a conceptual model of the pathosystem.

1.2.2 Specific objectives

a) Determine the influence of the meteorological, productive and genetic resistance variables on disease incidence over the years.
b) Explain MPR development, onset of symptoms of the disease and fungal sporulation through different microclimatic variables for three cacao clones in a range of incomplete resistance.
c) Study the relationship between the MPR infection process and the onset of symptoms of three different cacao clones through artificial inoculations.

d) Integrate all the pathosystem information (available and new) in a conceptual epidemiological model.

1.3 RESEARCH HYPOTHESES

a) Meteorological, productive and genetic resistance variables interact and have an influence on disease incidence over the years.
b) Different microclimatic variables explain the MPR development, onset of symptoms of the disease and fungal sporulation in interaction with three cacao clones in a range of incomplete resistance.
c) Onset of symptoms and infection process are related.
d) A conceptual epidemiological model could be constructed by integrating all the information (available and new) on the pathosystem to better understand MPR development.

1.4 THEORETICAL FRAMEWORK

1.4.1 Climatic and production factors in plant disease epidemics

To understand and explain epidemics, the basic concept of the disease triangle is fundamental. Disease is a result of the interaction among three components: pathogen, host and environment. Zadoks and Schein (1979) added a new component—crop management, which involves producer interventions in the system and depends on socioeconomic considerations. Thus, the triangle becomes a tetrahedron.

Maximal disease expression relies on 1) the pathogen, which has to be present, expressing pathogenicity, being effective in its dispersal and also presenting reproductive fitness; 2) the host, which needs to be susceptible or be in a susceptible growth stage; 3) the environment, which must be conductive—temperature, organ wetness duration, soil properties, wind and others have to be in the optimal ranges, and 4) crop management, which must be null, detrimental or insufficient to control the disease.

Although these components are well-understood separately, their perfect combination that triggers an epidemic in different systems is hard to define. Physical variables such as temperature, precipitation and humidity have a direct effect on plant growth and development as well as do the action of pests and diseases on the plant (Chelle 2005). As Chelle stated, knowing the actual environment in which plant organs grow may enable advances in the understanding of plant–environment-pathogen interactions.

All of the information concerning these disease tetrahedron components, including the factors that affect each component, could be compiled and organized in plant-disease epidemic models. According to van Maanen and Xu (2003), plant-disease epidemic models summarize the main processes, including all the influencing factors, to verify hypotheses and develop suitable control strategies. Conceptual, explanatory or analytical models are those that identify problems by the method of the cause and effect and aim to quantify the effects of specific events on disease development. These models normally precede the development of complex simulation models. Pathogen, host dynamics (including production trends) and environmental factors are the most important components of these models.

1.4.2 Microclimatic and host physiology effects on plant disease cycle

In order to model a disease, it is necessary to fully understand every factor involved in the pathosystem and how each factor expresses its influence. Table 1.2 synthesizes existing knowledge about some diseases in the different crops intended to be modeled.

Table 1.2. Microclimate variables and host physiology influence over different disease cycles.

Life Cycle Stage	Temperature	Relative Humidity	Wetness	Wind	Physiological Stage
Viability	**Lettuce Downy Mildew (*Bremia lactucae*)** 23°C (9)	**Lettuce Downy Mildew (*Bremia lactucae*)** ≥90% (9)			
Germination	**Coffee Rust (*Hemileia vastatrix*)** 15-30°C (4, 5).	**Sorn Grey Blight Disease (*Pestalotiopsis disseminata*)** 70%	**Coffee Rust (*Hemileia vastatrix*)** 10-48 hours of free water (4, 5).		
	Bean Angular Leaf Spot (*Phaeoisariopsis griseola*) 18-28°C (6).		**Bean Angular Leaf Spot (*Phaeoisariopsis griseola*)** 3 days (6).		
	Sorn Grey Blight Disease (*Pestalotiopsis disseminata*) 23-27°C				
Penetration	**Rose Downy Mildew (*Peronospora sparsa*)** 15-20°C (3).		**Rose Downy Mildew (*Peronospora sparsa*)** 2 hours (3).		
Infection	**Bean Angular Leaf Spot (*Phaeoisariopsis griseola*)** 10-33°C (6).		**Winter oilseed rape Light Leaf Spot (*Pyrenopeziza brassicae*)** Minimum of 16 hours of leaf wetness for infection. Temperature dependant (1).		**Coffee Rust (*Hemileia vastatrix*)** Adult leaves are more susceptible than young leaves (13)
	Grapevine Downy Mildew Pathogen (*Plasmopara viticola*) >11°C (11)		**Bean Angular Leaf Spot (*Phaeoisariopsis griseola*)** 3-4 consecutive wet nights (6).		
	Stone Fruits Brown Rot (*Monilinia fructicola*) 20-25°C (12)		**Grapevine Downy Mildew Pathogen (*Plasmopara viticola*)** >2.5mm of Rainfall (11)		
			Stone Fruits Brown Rot (*Monilinia fructicola*) 12-18h (12)		
Colonization	**Rose Downy Mildew (*Peronospora sparsa*)** 20-25°C (3).				

Symptoms	**Bean Angular Leaf Spot (*Phaeoisariopsis griseola*)** 15-24°C (6).		**Rose Downy Mildew (*Peronospora sparsa*)** Severity increases up 10 hours (3).		
Sporulation	**Bean Angular Leaf Spot (*Phaeoisariopsis griseola*)** 10-30°C (6).	**Oilseed poppy Downy Mildew (*Peronospora cristata*)** 95-96% (2).	**Oilseed poppy Downy Mildew (*Peronospora cristata*)** Rainfall threshold inhibitory 0.2 to 3.0 mm (2).		
	Gray Mold Fruit Rot (*Botrytis cinera*) 17-18°C (7)	**Bean Angular Leaf Spot (*Phaeoisariopsis griseola*)** >71% (6).	**Sudden Oak Death (*Phytophthora ramorum*)** 1-6 days (8).		
Sporulation	**Winter oilseed rape Light Leaf Spot (*Pyrenopeziza brassicae*)** 12-16°C (10)				
	Wheat Rust (*Puccinia recondita*) 23-27°C (14)				
	Grapevine Dead Arm (*Phomopsis viticola*) 21°C (16)		**Grapevine Dead Arm (*Phomopsis viticola*)** Wetness should be constant to promote sporulation (16)		
Dissemination			**Winter oilseed rape Light Leaf Spot (*Pyrenopeziza brassicae*)** Secondary infections due to splash-dispersed conidiospores (1).	**Winter oilseed rape Light Leaf Spot (*Pyrenopeziza brassicae*)** Primary infections due to air-borne ascospores (1).	
			Coffee Rust (*Hemileia vastatrix*) Raindrops up to 1.6mm (5).	**Coffee Rust (*Hemileia vastatrix*)** Main factor of dispersion in full sun conditions (15).	
Deposition					

Sources: (1) Papastamati *et al.* (2002). (2) Scott *et al.* (2008). (3) Aegerter *et al.* (2003). (4) Kushalappa and Eskes (1989a). (5) Kushalappa *et al.* (1983). (6) Allorent and Savary (2005). (7) Sosa-Alvarez *et al.* (1995). (8) Tooley *et al.* (2011). (9) Fall *et al.* (2016). (10) Gilles *et al.* (2000). (11) Kennelly *et al.* (2007). (12) Luo *et al.* (2001). (13) Eskes and Toma-Braghini (1982). (14) Tomerlin *et al.* (1983). (15) Boudrot *et al.* (2016). (16) Anco *et al.* (2012).

As is shown in Table 1.2, germination, infection and sporulation are the phases of the disease cycle most explored. With respect to the microclimatic factors, temperature and wetness are the most studied factors. Wetness is essential for germination and temperature is one of the main factors explaining the latency period. The effects of these variables vary according to the pathogen structures and the type of disease.

Table 1.2 shows that there are phases that need more research, such as deposition, penetration and colonization. In addition, the table highlights the fact that in some research sporulation is not well-differentiated from dissemination and influencing factors are confounded. Sporulation, one of the most important phases, has not been fully explored.

Robust models were built on these pathosystems. For instance, the model proposed by Anco et al. (2012), which includes only the influence of temperature and wetness on the infection (specifically sporulation) of the grape's cane, constitutes a robust model that could improve the warning system: when the influence of the factors is fully understood, the accuracy of the proposed models will increase.

After this analysis, it appeared that every step of the cycle should be analyzed separately, mainly for diseases like MPR that have long incubation and latency periods. In these types of diseases, infection is evident only after several weeks when the symptoms appear, so the diseases can be established without being noticed. The study of the effect of different microclimatic variables on the onset of symptoms and sporulation is the main focus of the present research.

1.4.3 Pathosystem description: Cacao-Moniliophthora roreri

1.4.3.1 Theobroma cacao

Table 1.3 resumes the crop taxonomic description.

Table 1.3. Theobroma cacao *taxonomic hierarchy.*

Kingdom	Plantae
Subkingdom	Viridiplantae
Infakingdom	Streptophyta
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Malvales
Family	Malvaceae
Genus	Theobroma
Species	***Theobroma cacao L.***

Source: *ITIS, 2016*
https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=505487#null

Cacao is a crop native to the tropics and is distributed in different countries of the tropical belt (10ºN and 10ºS) in lands from 0–800 m above sea level (m.a.s.l.). It is found in areas that meet its environmental requirements: temperatures between 18 and 32°C, with a mean of 25°C, abundant and well distributed rainfall (1500–2000 mm) and high relative humidity, from 70 to 100% (Rangel 1982; Wood and Lass 1985). These environmental requirements are, as is commonly observed, the same for the MPR disease. Because of their coevolution, the host and the pathogen share similar environmental requirements.

Most of the cacao varieties planted worldwide are to some degree susceptible to MPR. More than 75% of these varieties come from the Amelonado type, which is highly susceptible to this fungus. Although, resistant genetic material exists, it has not been totally exploited. Since MPR resistance is polygenic, it takes many years to develop and prove a resistant

variety, and the resistant varieties existing are still not being successfully multiplied and distributed worldwide. In addition, the replacement of a cacao plantation could be expensive for producers.

Due to the level of specialization of *M. roreri*, this fungus attacks only the genera *Theobroma* and *Herrania* (Whitlock and Baum 1999); the 22 species of *Theobroma* and the 17 from *Herrania* are susceptible to this fungus (Evans 1981b). Commercially, the most important species affected by this pathogen is *T. cacao*, which is the crop where chocolate and its subproducts are extracted. Cacao is the most merchandised crop of this genus, although *T. bicolor* and *T. grandiflorum* are highly produced and commercialized in Brazil. For these three species, the MPR represents a major threat in tropical America (Wood and Lass 1985).

1.4.3.2 Cacao production in Central America

In Central America, different ethnic groups living on the Caribbean slope grow cacao under low intensive management. Most are smallholders with limited resources and governmental support. Most of the cacao plantations are in buffer zones of the Mesoamerican Biological Corridor (Somarriba Chávez 2015). The crop is established under agroforestry management in a diversified design with other products such as bananas and other fruit crops for family consumption or local sale. A great percentage of the cocoa production is targeted to be sold as organic, fine aroma cocoa, so the crop management is cultural, using mostly organic inputs. Recently, some old plantations have been renewed with improved materials that are resistant to diseases, highly productive and of good cocoa quality.

1.4.3.3 Crop phenology

Niemenak et al. (2010) did a detailed description and codification of the cacao phenology or growth stages:

1. Seed germination/vegetative propagation: from a viable seed to the elongation of the hypocotyl and the growth of buds over the leading shoot.

2. Leaf development on the main shoot of the young plant and on the fan branches: from the cotyledon completely unfolded to the appearance of nine or more flushes completely mature.
3. Main stem elongation, formation of jorquette of fan branches and chupon: from the display of 10% growth of the shoot derived from the seedling until the last chupon reaches its physiological maturity and the growth of its apical meristem is arrested.
4. Fan branch elongation: from the visualization of the jorquette of primary fan branches until the primary fan branch develops 90 or more secondary fan branches.
5. Inflorescence emergence: from the appearance of flower buds (buds primordium of 150 μm wide) until the flower bud growth is completed (buds of 6 mm long and 3 mm large; pedicle 14 mm, still closed).
6. Flowering: from the opening of the first flowers until 90% of the flowers are opened.
7. Development of fruit: from the visualization of fruits at the main stem or branches until the embryos are full-grown and only traces of endosperm remain around the fleshy cotyledons, increase in the external dimension of fruit ceases, and fruits have reached 90% of the final size.
8. Ripening of fruit and seed: from the fruit color changing green or red to yellow or orange until the fruit is fully ripe and attached to the main stem or branches and can be harvested.
9. Senescence: from completed development of the flush and when leaves appear dark green until the postharvest or storage treatments.

1.4.4 Moniliophthora pod rot

According to Evans (1981a), *M. roreri* is indigenous to the northwestern part of South America, with an endemic range from western Ecuador to northwest Colombia. There are reports of the presence of this fungus in ancient populations of wild species of *Theobroma* and *Herrania*, where a natural spread occurred throughout wild hosts (Holliday 1957; Holliday 1971; Thorold 1975; Evans 1981b). Evans (2002) has also suggested that *Theobroma gileri*

could probably be a co-evolutionary host of MPR. For many years, the fungus was confined to the wilderness, but the cultivation of these indigenous forest tree species as monocultures due to the shortage of genetic material led to expansion of the fungus throughout the continent. Phillips-Mora (2003) has found genetic and morpho-physiological evidence of the variation of the *M. roreri* isolates according to the location of the plantations, especially within variations of altitude, precipitation and temperature. Phillips-Mora also reported that with the increment of the distance from the center of origin, isolates from *M. roreri* are less genetically diverse and cacao trees accumulate less resistance genes.

Since the first report of the disease in the early 1900s and for about half a century, MPR was contained in few countries in South America. However, with the arrival of the disease in Panama in the 1950s, this behavior changed and the disease began to spread very quickly. It took only 50 years to invade Central America, finally reaching Mexico (Phillips-Mora et al. 2006c).

The impact of MPR in Central America has been devastating, causing the abandonment of many plantations. This action accelerated the spread of the pathogen in the region. This impact is clearly reflected in the production of each country, which declined immediately after the arrival of the disease (Figure 1.1).

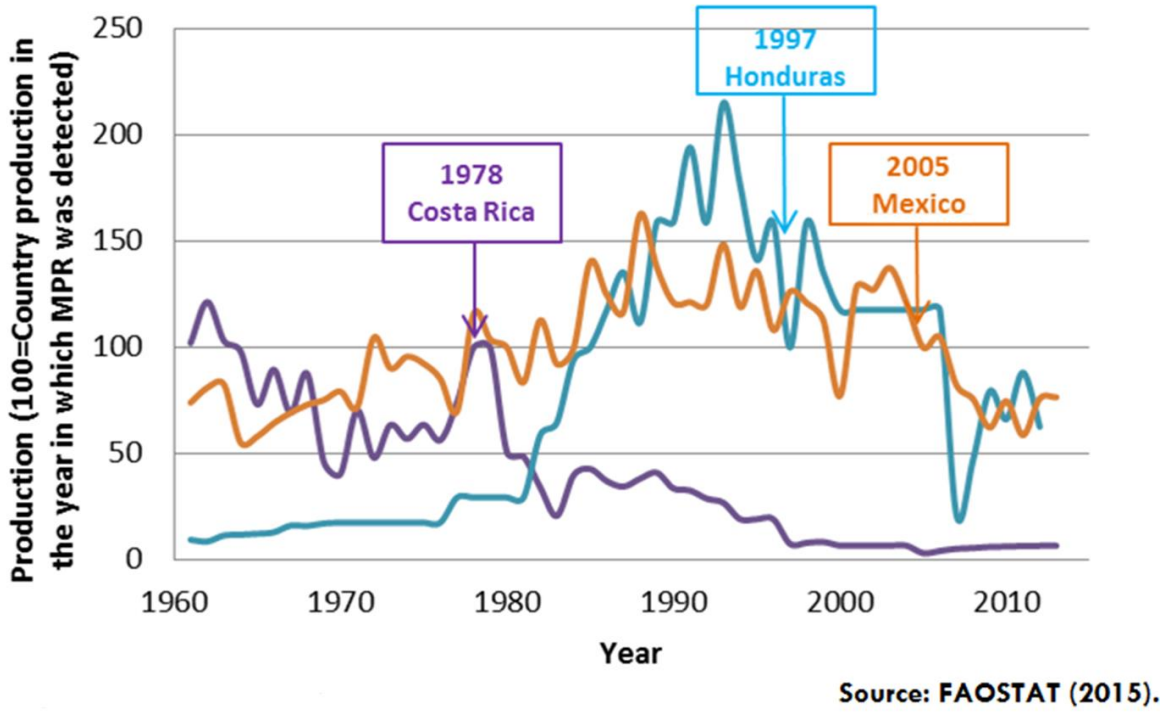

Figure 1.1. Effect of the MPR over the production in three Central American countries.

In light of this panorama, the hypothesis about the devastation that will result from the arrival of this disease to the largest producers of cocoa in West Africa and its worldwide repercussions has a strong foundation, further reinforced by the fact that the predominant African Amelonado variety in West Africa is nearly identical to the highly susceptible Matina variety in Costa Rica (Phillips-Mora 2003).

1.4.4.1 MPR biological knowledge

Little is known about the biology of the fungus and disease development. As for biological characteristics of the fungus, it is known that the hyphae are thin-walled and hyaline. Mycelium has partitions (septa) with dolipores (Figure 1.2a). Spores are formed in simple chains of four to 10 branched spores (Figure 1.2b), with the youngest spore in the chain base (Ram 1989; Thévenin and Trocmé 1996). This last feature makes *M. roreri* related to basidiomycetes (Evans 1986). According to Phillips-Mora (2003), the spores are easily removable. They are thick-walled, pale yellow when immature or dark brown at mature stage, and may be globose, elliptical (Figure 1.2c) or amorphous. The characteristics of these structures vary some depending on strains.

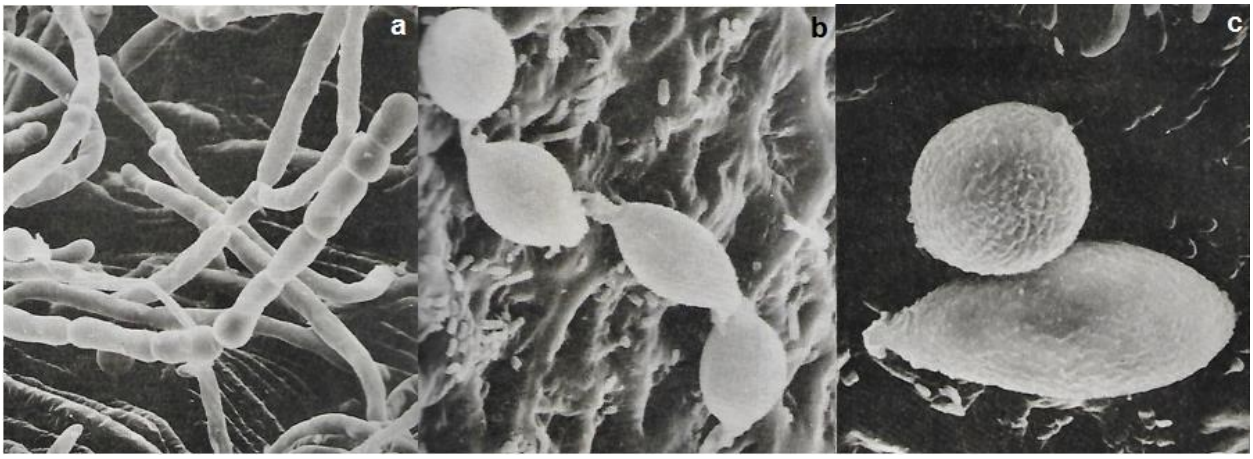

Figure 1.2. Scanning electron micrograms of different structures of Moniliophthora roreri*: a, hyaline hyphae and partitioned mycelium; b, chain of spores; c, two different spore shapes. Photos by Flores (1989).*

The pod is the only known organ susceptible to the fungus (Castaño A 1952; Ampuero 1967; Desrosiers and Suárez 1974). Infection occurs in the early stages of fruit growth; as it grows, the pod becomes more resistant (Enríquez 2004). During initial stages of infection, the fungus invades only the interior of the fruit: external pod damage generally appears 40 to 60 days, though it can take less time. (Bejarano Villacreces 1961). According to Leandro-Muñoz (2011), when the fruit is young and the temperature is higher, this period is shortened.

Sometimes when completely develop or ripe fruits without visible symptoms are opened, their interiors are found to be affected; these rots are called hidden infections. Generally these pods are heavier than healthy fruits (Enríquez 2004). If the pods are less than three months old, the first symptom to appear is a lump, hump or swelling. On older green pods, however, the first symptom of infection is the appearance of small yellow spots and of orange spots on the red ones. Then an irregular brown spot with a yellow halo appears. This symptom is known as chocolate spot (Porras and Enríquez 1998). In warm and humid conditions, the signs of the pathogen appear as a hard white stroma over the chocolate spot. This stroma is the mycelium of the fungus. Large numbers of spores grow on this mycelium, forming a creamy or brown mass. Infected pods that remain attached to trees can sporulate for up to nine months and then start to mummify (Enríquez 2004). Figure 1.3 illustrate the previously mentioned symptoms.

Figure 1.3. Moniliophthora pod rot symptomatology.

M. roreri is considered a hemibiotrophic fungus. Its cycle goes through two stages: 1) a biotrophic phase, from the germination of the spores to the intercellular invasion of the pod and 2) a necrotic phase that causes growth reduction of the pods and finishes with the invasion of the fungus to the cell, causing the appearance of internal and external necrosis (Thévenin and Trocmé 1996). This characteristic is shown in Figure 1.4, which describes the *M. roreri* life cycle.

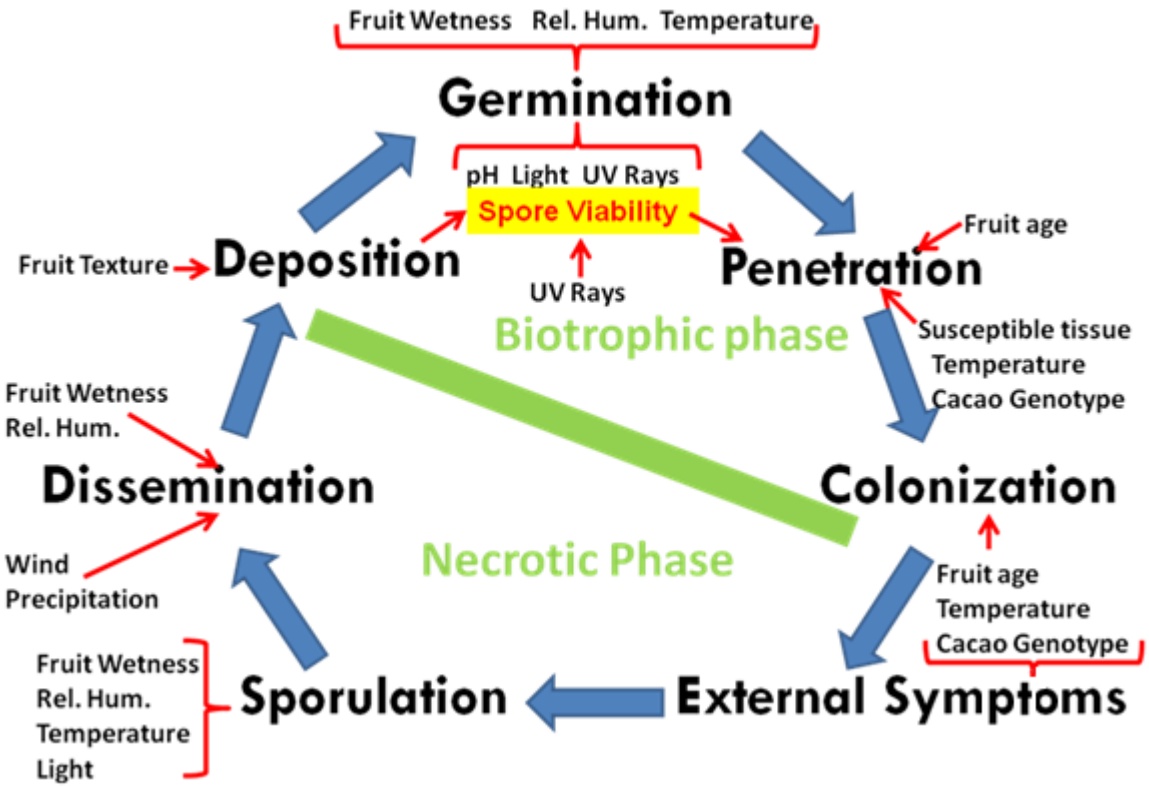

Figure 1.4. Factors affecting the stages of the M. roreri *life cycle.*

A detailed description of the factors affecting each of the stages of the life cycle of the pathogen follow.

Deposition: Fruit texture could affect the probability of a spore to stay on the pod until germination. Rainfall could also affect this stage, since according to Castro (1989), torrential rains can generate a negative effect on the development of disease, perhaps because that rain washes spores off the fruits.

Spore viability: Although not a stage of the fungus life cycle, this parameter is important from spore deposition to penetration. Two different views on this topic exist in the scientific community. The first is that UV rays destroy spores that do not complete their life cycle, based on the fact that solar radiation has a lethal effect on spores of many fungi (Heuveldop et al. 1986). The other point of view is that spores that do not complete their life cycle stay viable, which means that they preserve their capacity to germinate. These spores stay in the environment as a residual inoculum on different parts of the cacao tree (leaves, trunk, and flowers), epiphytes and shade trees or over the plot as a cloud of spores (Ram 1989).

Germination and penetration: Relative humidity above 80% is considered optimal for fungal germination and growth (López 1954; Castro 1989). According to Campuzano (1981a), the highest percentage of *in vitro* germination occurs at 22.5°C and 96% humidity. Chacín (1975), Merchán (1978a), Galindo (1985) and Campuzano (1981a) have indicated that for germ tube development, spores require a water film over the fruit. According to the results of Phillips (1986), the successful use of the wet chamber in artificial inoculations of *M. roreri* is due to the presence of a water film on the surface of the pods during a sufficient period to allow the formation of the germ tube and to achieve penetration by the pathogen. Darkness and low aeration promote the maintenance of this film of water on the fruits longer, and increase the frequency and intensity of disease attack. However, it is known that conditions that favor germination and penetration by the fungus are different from those that favor the release and spread of inoculum (Phillips 1986). Chacín (1975) has noted that the germination of spores of *M. roreri* could occur between pH 4 and 8, considering a pH 6 as the optimum. The higher elongation of the germ tube is between pH 5.5 and 6.5.

Colonization, external symptoms and sporulation: Temperature is the most influential factor on the growth rate of fungi (Moore-Landecker 1996). Its increase causes the rise of the chemical and enzymatic activity that accelerates the synthesis of vitamins, amino acids and other metabolites. However, excessive heat may inactivate these activities and stop the growth. For *M. roreri*, the ideal range for growth and sporulation of the colonies in culture medium V8 is 24 to 28°C (Herrera 1988). According to Hawker (1950), the optimum temperature range for the fungal sporulation is always lower than the optimum temperature range for fungal growth in general. Leandro-Muñoz (2011) has concluded that the air temperature and the pod temperature from the early days of the infection were the only microclimatic variables that were related to the growth rates of the disease in different generations of pods. The rate of growth and formation of propagules are affected by the intensity, duration and quality of light. This stimulatory effect of light is evident in the formation of alternating concentric rings in *M. roreri* isolates when these are exposed to alternating periods of light and darkness. Light favors the formation of reproductive structures (light brown rings with creeping growth) and darkness favors vegetative growth (cream-colored rings, less sporulated and with more aerial growth). Nevertheless, Phillips-Mora (2003) has observed the formation of concentric rings in cultures maintained in total darkness. Although this fungus is not very demanding in terms of light regime, Herrera (1988) has determined that alternating 12 hours of light and darkness provides the peak of sporulation of the colonies on V8. Phillips-Mora (2003) has also recommended a light regime of 12/12 alternating periods of light/darkness in order to stimulate the development of hyphae and vegetative structures of the fungus.

Dissemination: The main mechanism of spore dispersal of *M. roreri* is the wind (Merchán 1981b). However, Barros (1981) stated that spores cannot travel long distances in the air due to their weight, although authors like Naundorf (1954) have indicated that the spread may also occur by water, insects or other animals, including human beings. People who do not realize that pods are infected because the pods show no external symptoms often move them to places without the disease, dispersing propagules and hence the disease (Evans 1986). Release and dispersal of *M. roreri* spores by the wind require lower values of relative humidity and dry conditions. Fallas (1983) has noted that the dispersal of spores of *M. roreri* depends on the conditions of temperature and humidity. Torrential rains also affect the

dissemination of the spores because the stroma gets wet and the spores cannot be dispersed by wind. Dispersion increased at higher temperatures and lower humidity (Castro 1989). Supporting this observation, Schmitz (1984) has noted that in the Atlantic zone of Costa Rica, the maximum number of spores in the air was found between the 10th and 15th hour of the day. According to Campuzano (1981a), the highest rate of spread of the fungus occurs at midday when the air temperature rises and displaces the moisture. However, Leandro-Muñoz (2011) trapped larger numbers of spores at night, at about the 22nd hour of the day. These captures were made at a height of 1.5 m. This led to the hypothesis that during the day, spores lost moisture and went up by air currents within the cocoa plots, while during the night, the spores fell back to the cacao trees due to the higher air relative humidity that increase the spores moisture and therefore its weight. The differences between these results may be due to the complexity of recognition of the spores of *M. roreri* in previous studies.

1.4.4.2 MPR epidemiological knowledge

Compared with other pathogens, the information on the biology and epidemiology of MPR is quite scarce. Moreover, in the information presented, some observations need to be revalidated, as they are not universally accepted. Many of the studies were done several years ago when the technology now available did not exist.

As mentioned, little is known on the epidemiology of the disease. According to Griffiths (1978), epidemiology is more than the life cycle of the pathogen. It encompasses the disease within populations, in the context of pathogen populations interacting with host populations in a variable environment. It is essentially related to numbers and quantities.

The essence of epidemiology is to understand the development of a disease in time and space. Epidemics are based on monocyclic processes. These processes are recurrent and may be repeated several times during the cultivation period. In short, an epidemic is a sequence of monocyclic processes that together make a polycyclic process (Kushalappa and Eskes 1989b).

According to Kushalappa and Eskes (1989b), these monocyclic processes can be defined as threads that trigger morphological changes in the fungus and the host through

conversion and transport of matter and energy. The monocyclic process starts with a viable infectious unit (a spore or set of cells), which gives a mycelial structure that generates a dispersion unit, called propagule. These authors pointed out that in the epidemiology, monocyclic processes (macroprocesses) are subdivided in three mesoprocesses to provide better observation and understanding: 1) sporulation, 2) dissemination and 3) infection. Each of these are also divided into several microprocesses that constitute the growth of the fungus from one stage to the next; an example is the germination, which runs from the spore state to development of the germ tube (a microprocess of the infection mesoprocess).

Monocyclic processes can be described by the survival ratio and the duration of the process, or rate. The rate is a measurement of the speed at which each process is completed (Kushalappa and Eskes 1989b).

According to Tazelaar (1991), *M. roreri* epidemic could be described as a homogenous polycyclic epidemic systematically constructed since all infection cycles are identical. However, Leandro-Muñoz (2011) has presented evidence of the monocyclic behavior of the disease and how the constant availability of susceptible material over time causes the illusion of an epidemic. This research showed that for each generation of pods, there is only one cycle or phase of infection. This result has consequences for management of the disease since it supports the importance of the removal of diseased pods, significantly reducing the very long infective period of the lesions and the amount of initial inoculum for subsequent pod generations.

Epidemic developments are possible from 0 to 1520 m depending on the latitude, with an average rainfall of 780 to 5500 mm (Phillips-Mora 2003) parameters that fit almost all areas where cacao is grown. However, an inverse relationship between disease incidence and altitude of the site has been observed.

Fallas (1983) found a positive correlation between the disease incidence and the amount of rainfall two to three months prior to the infection. He also found a positive correlation between the incidence and periods of high relative humidity and amount of sunlight.

Periods with high precipitation are associated with high incidence of the disease. In areas with precipitation greater than 2500 mm and with relative humidity higher than 90%, incidence can reach values greater than 95% (Castro 1989).

Another factor affecting the incidence of MPR is the availability of susceptible tissue and possibly the pod production dynamic as the young fruits from about two months old are most susceptible to this disease (Porras 1982).

According to Ampuero (1967), mummified pods that are not removed from the trees represent the greatest source of inoculum of the fungus. These can cause various infection waves and can house up to 44 million spores per square centimeter, which means that an infected adult pod can produce up to 7000 billion spores (Campuzano 1981a). It has been suggested that, once released, spores could remain viable in the trunk, leaves, flowers and other parts of the cacao tree or shade trees (Ram 1989). However, González (1981) stated that the spores that fall to the ground do not survive more than three months because they are degraded by soil microorganisms. Ram (1989) has pointed out that in 80 days, up to 20 successive periods of sporulation may occur in a single infected pod.

Figure 1.5 highlights the importance of the pod production dynamic of t cocoa trees on the development of the disease and also shows the importance of locating the initial inoculum in the plots in order to reduce it.

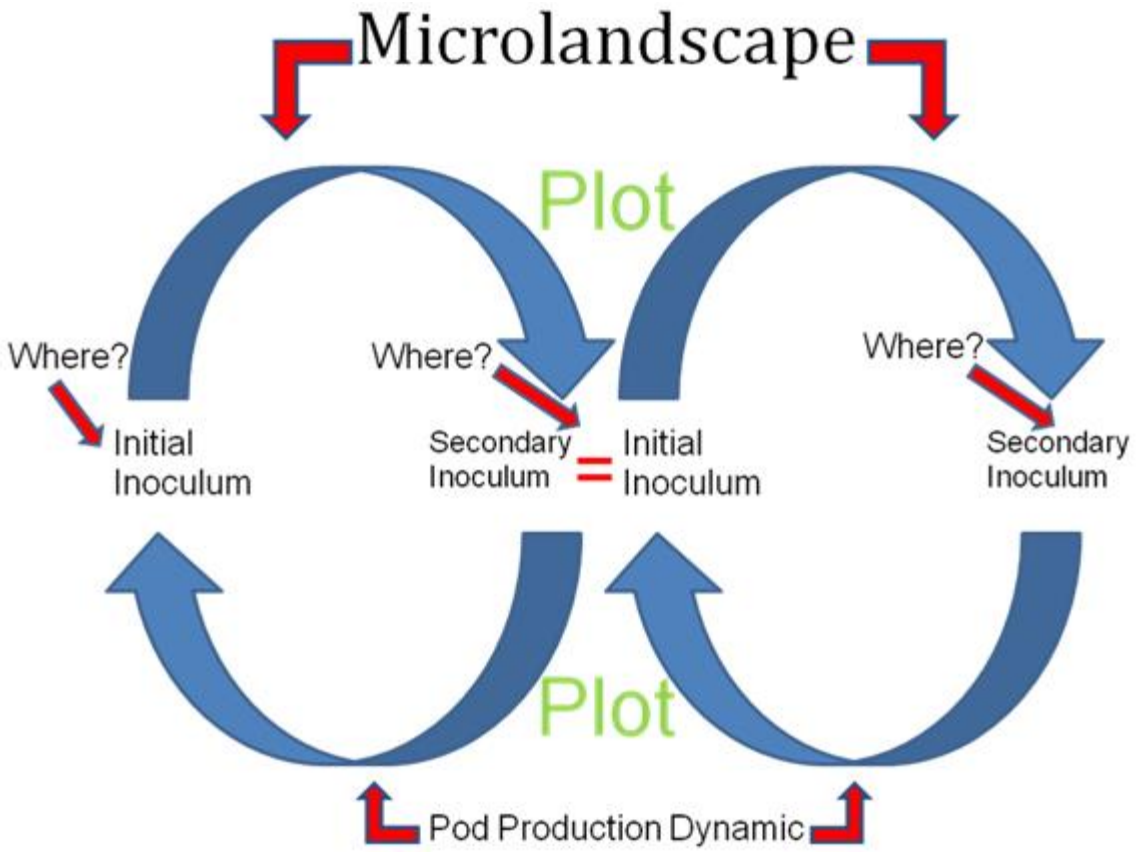

Figure 1.5. Moniliophthora pod rot behavior at plot level and the factors involved.

Although some information is available, study of the basic aspects of the pathogen and the epidemiology of the disease is essential to achieve more effective and lasting control methods. The aim of this research is to help fill some of these gaps.

1.4.5 Available control alternatives

There are different control strategies for MPR, including cultural, chemical, biological and genetic approaches; however, most of these strategies are not accessible to the cocoa producers, who are mostly smallholders (Barros 1980; Evans 1981b; Evans and Prior 1987; Krauss and Soberanis 2001; Arciniegas-Leal 2005). Genetic resistance seems to be the most promising strategy, but is still limited by propagation methods of the resistant clones.

It is considered that challenges presented by the current control strategies stem from lack of biological and epidemiological knowledge of the fungus, due to the lack of research on

the subject influenced by the productive inactivity that came with the abandonment or replacement of cocoa.

1.4.5.1 Genetic control as the most promising control strategy

Cacao in Latin America has broad genetic diversity. According to Phillips-Mora et al. (2013), this diversity has been preserved in collections; however, not all of this genetic pool has been systematically exploited. Most of the commercial varieties have a narrow genetic base that makes them vulnerable to diseases such as MPR.

Conventional genetic improvement of perennial crops such as cacao is challenging since it takes decades for the release of a new variety. However, thanks to the identification of quantitative trait markers, breeding for resistance has been accelerated. CATIE's Cacao Improvement Program had led the search for MPR resistant clones by the evaluation of families resulting from the cross of two resistant clones: UF 273 and UF 712 (Schnell et al. 2007). Clones such as CATIE-R4 and CATIE-R6 are considered to be highly resistant to MPR and to have good production and quality characteristics.

MPR resistance is consider a desirable cacao traits with complex inheritance since they are encoded by multiple genes—a case of polygenicresistance. Though this type of resistance is less complete, it is more durable (Phillips-Mora et al. 2013).

1.4.6 Plant disease management by shade regulation

In cocoa-based agroforestry systems, shade has a major effect on the microclimatic conditions. These conditions directly affect all the components of these systems: the crops, pests and diseases and natural enemies.

To implement shade as a strategy to stabilize the system, it should be optimized since it can influence the interaction between the host plant and pests and diseases through a large number of mechanisms that often act simultaneously. To establish a specific shade level for a certain crop, it is necessary to take in account its management, local environmental conditions and the crop's principal pests and pathogens at the respective site.

According to Avelino et al. (2011), shade has contradictory effects on many diseases in coffee and cacao plantations, favoring some phases of the life cycle and hampering others. These authors have stated that a better understanding of the ecological mechanisms within the agro systems is necessary since it is accepted that increasing the diversity within plantations could suppress the impact of pests and diseases in both of these tropical crops.

Schroth et al. (2000) have presented a review of information that describes the influence of shade on tropical plant diseases; however, these authors noted that the information available tends to be more contradictory than that on insect pests. As for the general aspects of shade, they indicated that the increase of the incidence of pests or diseases in shaded plots is the result of a physical (unspecific) effect, in which shade trees create a suitable microclimate for the respective species. More-shaded positions may differ markedly in microclimatic conditions from more open positions. Shade can reduce the spread of the pathogen propagules from infected plants within these plantations and to healthy plantations by reducing air turbulence, especially affecting plants in the lower canopy. Reduction of air movement may extend the duration of leaf and fruit wetness, favoring the germination of the propagules and facilitating the infection process.

Among other shade effects identified by these authors are the alteration of the quantity and the quality of light. Light can stimulate fungal sporulation and its subsequent release; strong UV exposure also has a lethal effect on microorganisms. Excessive shade, however, may provoke the development of delicate and etiolated plants.

According to Schroth *et al.* (2000), shade regulates air and soil temperatures and intercepts rainfall, reducing the impact of raindrops and spore dispersal from splashing. But Boudrot et al. (2016), in their research on heavily shaded coffee plantations, have found that during rainy events, the foliage of shade trees could provoke the accumulation of water and the development of bigger drops. They indicated that these drops have more kinetic energy accumulated and will fall and hit harder on the leaves, provoking higher dry dispersion of uredospores of *Hemileia vastatrix.*

It is necessary to know that even if the overall shade level of a plot is in the optimum range, pathogens may develop in microniches of high humidity that are created by patches of dense shade, and these may subsequently act as inoculum sources (Schroth et al. 2000). These authors concluded that a homogeneous, intermediate shade is more favorable than a patchwork of heavily shaded and unshaded spots. In the case of MPR, Gidoin et al. (2014) has demonstrated that the incidence of the disease was higher in cacao plots where shade trees had an aggregated spatial distribution.

Schroth et al. (2000) compiled and presented the effects of shade on some cocoa diseases. For example, the incidence of pink disease (*Corticium salmonicolor*) increased under shade in Bahía, Brazil, but disease severity was higher in unshaded fields, presumably due to the increased susceptibility of the plants. In the case of the witches' broom disease, the incidence was reduced by shading, due to the reduction of excessive vigor of the crop, (the pathogen affects flushes), reduction in the fluctuations of air humidity that trigger sporulation, and the reduction of air currents within the stand, which favor autoinfection. However, it was found that excessive shade could enhance the development of the pathogen by increasing wetness. For the same reasons, it also enhanced pathogen's natural antagonists that decompose brooms and mummified pods, thereby reducing the production of new inoculum.

Some pathogens could present a different reaction to shading according to the location, as is the case of *Colletotrichum gloeosporioides*, which cause anthracnose. In general, pruning the cacao trees to reduce humidity is recommended as a way to control fungal diseases. However, in Costa Rica, anthracnose and terminal dieback caused by the same fungus were associated with unshaded fields.

In the case of *Phytophthora palmivora*, causal agent of the black pod disease in America, intermediated shade levels increased the incidence due to the insufficient aeration and high humidity, especially when the temperature is relatively low. This same fungus attacks the stem if it is exposed to full sunlight, due to water stress. Avelino et al. (2011) concluded that the effects of the shade on pests and diseases needs to be better understood since some level of shade could benefit some pathogen and be counterproductive to another and, as in this case, could have contradictory effects within the same disease.

Schroth et al. (2000) concluded that the relationship between shade and MPR has been controversial for many decades because some authors have found the highest incidence of the disease under a very dense shade, explaining why the reduction of excessive shade to reduce humidity and increase the aeration of the plantation has been recommended as a prophylactic control measure. However, according to Díaz Moreno (1957), MPR was found to be somewhat lower under moderate shade than without shade, though this trend could reverse in very rainy years and failed to be statistically significant. Some authors have found no correlation between the disease and shade levels of 17 to 50%.

According to the previous information, it appears that the effect of shade is extremely complex but fundamental to agroforestry systems such as cocoa. For this crop, the shade effect on the microclimate has been documented (Mossu 1990). However, the effect of microclimate on the phenology and the incidence and severity of MPR has not been thoroughly studied. Some research has been performed, but in most of the experiments, shade levels and hence microclimatic data have not been reported. This information is vital to understanding the influence of these variables on the incidence and the severity of a disease (Schroth et al. 2000) and for recommending adapted shade-management practices.

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CHAPTER 2

METEOROLOGY, PRODUCTION TRENDS AND GENETIC RESISTANCE LEVELS ASSOCIATED TO DISEASE INCIDENCE THROUGHOUT THE YEARS*

2.1 INTRODUCTION

An epidemic outbreak occurs when the perfect combination of biotic and abiotic components of the system occurs, but the definition of these components and the description of their influence is difficult to list. Physical variables such as temperature, rainfall and humidity have a direct effect over plant growth and development and also over pests, pathogens and chemicals (pollutants) (Chelle 2005). According to Chelle, knowing the actual environment in which plant organs grow may enable advances in the understanding of plant–environment-pathogens interactions.

The genetic component of the system is also important. Both the host and the pathogen must have ideal genetic traits to enable the infection. Host phenology must be synchronized with the pathogen development in order to attack during the most suitable susceptible stage. In this synchrony, climatic factors have a great influence and, depending on the case, some crops can sometimes escape the attack of a pathogen. This is the case presented by Maddison et al. (1995), in which some genotypes escaped the attack of MPR and witches' broom by growing during the dry season.

Phenotypical reactions of the host against the pathogen are determined by the activation of the resistant genes under two scenarios of plant disease resistance: complete and incomplete. Complete resistance is activated by a few major genes with a strong effect and incomplete resistance is activated by multiple genes with limited effect

* Resistance variability results presented in LXI Annual Meeting of the Instituto Nacional de Innovación y Transferencia de Tecnología Agropecuaria (INTA), Costa Rica: Programa Corporativo Centroamericano para el Mejoramiento de Cultivos y Animales (Central American Corporate Program for Crop and Animal Improvement) in April 2016.

(Poland *et al.* 2009). This second type of resistance is more durable against genetic changes of the pathogen in time. This is the type of resistance possessed by some cacao genotypes against Moniliophthora pod rot. However, changes in the environmental conditions could influence or break an incomplete resistance. According to Bonman (1992), rice blast potential is affected by climatic, edaphic and hydrologic conditions. In an environment conducive to disease development, the level of partial resistance could be insufficient, ending in an incidence increase.

Evidently, environmental factors affect almost every component that triggers an epidemic. Studies of climatic factors influencing plant disease development are common in different plant-pathogen systems since they are needed to understand the interactions between physical and biotic factors and thus prevent new outbreaks or recommend new control strategies. For cultural control measures, Copes and Scherm (2005), for instance, have indicated that climatic factors and their interactions with other factors such as irrigation and evaporation potential influence the magnitude and direction of the plant spacing effect on Rhizoctonia web blight, caused by *Rhizoctonia solani* and binucleate *Rhizoctonia spp*., in compact cultivars of container-grown azaleas (*Rhododendron spp*.). The microclimate effects on natural enemies and their antagonism effects on pests and diseases also need to be clarified and understood before implementation of a biocontrol strategy (Loguercio *et al.* 2009). For cacao, incidence of black pod disease (*Phytophthora palmivora*) has been associated by several authors with a variety of climatic factors such as minimum temperature, rainfall, relative humidity and vapor pressure deficit. It is clear that the duration of surface wetness is critical in determining the probability of infection. This factor is also critical for the Moniliophthora pod rot infection process. Butler (1980) has indicated that individual effects or interactions of these climatic factors have not been characterized or quantified but that all will affect the duration of pod surface wetness.

The influence of different climatic factors has been studied in different pathosystems. Temperature and relative humidity/wetness/rainfall seem to be the most reported factors, perhaps because they are the most explanatory or are the easiest to record and their influence

the easiest to understand. These factors are constantly studied together because they are correlated. Some examples of studies of these two factors follow.

Elmer and Ferrandino (1995) have done artificial inoculations of *Septoria lycopersici* on tomato leaves under controlled conditions to study the effect of the spore density, leaf age, temperature and dew periods on susceptibility to this pathogen. Temperature and relative humidity/dew period duration were found to be the most important factors. The authors reported an optimum temperature for lesion development between 20 and 25°C, with an inferior temperature limit of 10°C. As for relative humidity, after 10 days at 50–60% relative humidity, any dew period was suitable for lesion development. Disease severity increased when the dew period extended beyond 20 hours. These authors also found that the infection process requires dew when leaves are spray inoculated.

For Ascochyta blight caused by *Mycosphaerella pinodes* on pea seedlings, Roger *et al.* (1999) have found that wet–dry–wet cycles are required for infection, with this effect dependent on when the dry period occurred during the infection process—dry periods during germination avoid symptoms appearance. This study concluded that lesion development depended on the duration of the initial wet period as well as the characteristics of both the dry period and the final wet period

Aegerter *et al.* (2003) have studied the effect of various environmental parameters on rose downy mildew caused by *Peronospora sparsa* under controlled conditions and in the field. They concluded that optimal temperatures for infection and colonization of rose leaves in the greenhouse were 15 to 20°C and 20 to 25°C, respectively. Wetness was also crucial for this pathogen. At optimal temperatures, the infection process required only 2 hours of leaf wetness although disease severity increased significantly with an increasing duration of leaf wetness up to 10 hours. Infection of leaves occurred at temperatures as low as 5°C with 8 hours of leaf wetness. In this system, wetness duration was found to be more important than temperature range.

During three potato-growing seasons in 2001 and 2002 in South Africa, trends in weather variables and concentrations of airborne conidia of *Alternaria solani* were monitored in a potato field (Waals *et al.* 2003). Researchers found a distinct seasonal variation with a drop in spore numbers during winter. They also described a favorable effect of interrupted wetting periods for spore formation and dispersal

Gilbert *et al.* (2008) considered that information on survival and the conditions under which ascospores remain viable once released from perithecia may assist in refining disease forecasting models for Fusarium head blight, one of the most important cereal diseases. Based on this premise, they conducted a study and determined that germination rates fell with increasing temperatures at all observation times and at all humidity levels; it was highest at 90% relative humidity, except at 30°C after 48 hours, and lowest at 60% RH. These observations led them to conclude that ascospores are sufficiently robust to constitute a source of inoculum under most environmental conditions encountered during the growing season.

The effect of the different elements of the climate on the cacao phenology and specifically on its productivity has also been studied by Quiroga Gómez (1972), who found that this effect differed according to the genetic material in question.

With respect to the influence of climate on the MPR epidemic, Fallas (1983) has reported that there was a positive correlation between the disease incidence and the amount of rainfall two to three months prior to the infection. He also found a correlation between the incidence and periods of high relative humidity and the amount of hours of sunlight.

Maddison *et al.* (1995) have determined that higher impacts of MPR and witches' broom epidemics could be avoided in places with a long and defined dry-season, since susceptible pods can escape from the pathogen infection due to the absence of suitable fungal climatic conditions, indicating that the resistance shown by some genotypes could be provided by their production trends, with production peaks occurring during periods not conducive to infection.

The availability of susceptible plant material is essential to the start and development of an epidemic. In the case of cacao and pod diseases, for instance, the number of pods produced often depends on numerous environmental factors, and the heritability of this trait, estimated in various situations (Cilas 1991), is relatively weak (< 0.4).In fact, the trait depends on many factors, including flowering, pollination, fruit-setting, cherelle wilt, diseases and insect attacks (Sounigo *et al.* 2003; Nyassé *et al.* 2007), each having specific dependence on environmental conditions. There are cases in which this knowledge is used to deal with diseases, called "escape strategies." To reduce the incidence of the coffee berry disease (known as CBD), early irrigation is applied in order to induce an early berry ripening at a time when the climatic conditions are not suitable for the disease. The crop thus escapes the disease, since its vulnerable stage (green fruits) does not correspond to favorable climatic conditions for the pathogen (Muller *et al.* 2004).

In each of these studies, there is evidence that better knowledge and understanding of the conditions favoring plant epidemics will generate more accurate information to confront the disease and develop appropriate control mechanisms according to the system and the zone, and also identify risk zones in disease-free regions to avoid new outbreaks (Guyot *et al.* 2010). For this, it is necessary to have sufficient detailed field observations and data to study these relationships. Hence, it was decided to use the valuable information collected by CATIE's Cacao Improvement Program to analyze these explanatory relationships.

2.2 RESEARCH OBJECTIVES

2.2.1 General objective

Determine the influence of the meteorological, productive and genetic resistance variables on the disease throughout the years.

2.2.2 Specific objectives

a) Evaluate the resistance variability of 42 cacao clones over 13 years.
b) Rank the microclimatic and production variables that will better explain the incidence of the disease.

2.3 RESEARCH HYPOTHESES

a) The variability of the resistance to MPR of the clones is low; therefore, the resistance, despite being incomplete, is not strongly influenced by external factors.
b) Microclimatic and production variables could explain the variability of the resistance to MPR.

2.4 MATERIALS AND METHODS

2.4.1 Available data

The CATIE Cacao Improvement Program started working in the development of improved new cacao varieties in 1996. According to Phillips-Mora *et al.* (2013), the identification of sources of resistance to MPR and black pod (*Phytophthora palmivora*) and the generation of high-yielding/resistant varieties are the main goals of this program.

L6 or "Experiment on Disease Tolerant Clones" is one of the first-established and more-important clonal trials planted to select superior clones (high productivity, resistance and other attributes). The purpose of this experiment was to study the performance of a clone of previously selected individual trees and identify materials for regional trials. The total trial area is 1.5 ha, planted between July of 1998 and 1999. It follows a complete randomized blocks experimental design, whose treatments are 42 clones selected primarily for their high resistance to disease and/or productivity and four extra clones as the edge of the experiment. It has four repetitions of eight trees each, planted at a distance of 3 x 3 m. Permanent shade is distributed unevenly and is made up of immortelle (poró, *Erythrina poepiggiana*) and guava

(*Inga edulis*) trees. There was no information on the productive potential for MPR-tolerant clones planted in this trial (Phillips-Mora *et al.* 2013).

Evaluation of this trial has provided valuable information during these years. Assessments occurred monthly and were carried out by tree recording parameters such as "number of removed healthy fruits," "seeds' fresh weight" and "number of removed diseased fruits" (MPR and black pod). Disease control occurred during these same evaluations by cutting infected pods and leaving them on the ground without any chemical treatment (Phillips-Mora *et al.* 2013). All this information has been compiled by the Cacao Improvement Program over the past 13 years and was considered a tremendous source for study of the relation of MPR incidence and climate since there is also macroclimate information (temperature and rainfall) available from this same site, recorded at a weather station located in La Lola farm.

2.4.2 Studied variables

Four variables were created for this analysis: 1) "percentage of diseased pods per year" or incidence, which is the ratio of total number of diseased pods and the total pods in all the plots per 100; 2) "total pods per year," which is the sum of all pods produced in a year, including pods affected by MPR and others diseases; 3) "monthly production percentage," which is the percentage of the annual total pods produced in a determined month; 4) "average temperature per month," which is the monthly average of the temperatures recorded, and 5) "total rainfall per month," which is the rainfall recorded by month.

2.4.3 Resistance variability of the clones

The restricted maximum likelihood (REML) method (Corbeil and Searle 1976) was used to estimate the different variances ("clone" and "error" variances) for the percentage of diseased pods. Broad-sense heritability values were estimated for the traits, along with the associated confidence intervals, estimated by the Wald method (Agresti and Coull 1998). Estimations of heritabilities were given by the ratios of genetic variances—i.e., clone variances—and phenotypic variances (Cilas 1991):

$$h_b^2 = \frac{\sigma_G^2}{\sigma_P^2} = \frac{\sigma_c^2}{\sigma_c^2 + \sigma_e^2} \quad (1)$$

Where

h_b^2 = broad sense heritability,

σ_G^2 and σ_P^2 are respectively genetic and phenotypic variances

σ_c^2 and σ_e^2 are respectively clone and error variances

2.4.3.1 Geostatistical methods to correct the spatial effects per year

The aim of this analysis was to correct heritability values taking into account the spatial variability of the MPR incidence, i.e., percentage of diseased pods. An analysis of variance of the "percentage of diseased pods" variable with the effect of "clone" was first made. Then, a Moran's test was done to determine whether there was a spatial relationship between the residuals. When this relationship was found, a semivariogram was done using the spherical model, which better fit most of the years. Then, using a cross-validation function and the model obtained from the semivariogram, a prediction of the value of the residue at a specific point was made without using the actual value. Finally, spatial prediction of residue of the original variable was deleted and the entire process was rerun. Analyses were run using R.

2.4.3.2 Genetic and environmental correlations

Potential yield (total of pods produced) was analyzed jointly to estimate genetic and environmental correlations between the percentage of diseased pods and yield with a multivariate analysis (Hill 1971). Genetic and environmental correlations were also calculated between the percentage of diseased pods in every year of the study.

All quantitative genetic analyses were performed with SAS 9.3.

2.4.4 Analysis of the influence of the climatic and production variables

MPR incidence per production year was the response variable and the explanatory variables were “total pods per year” and “monthly production percentage” as phenological variables and “temperature average per month” and “total rainfall per month” as climatic variables.

The L6 trial was planted in July, so its production year goes from July of the year in course to June of the following year. To explain the incidence per production year (N), the climate interval considered is from January of the previous year (N-1) to March of the present year (N), since it is known that pods could be susceptible only for the first two or three months of life.

Data was analyzed using regression trees (CHAID method) with SPSS statistical software, aiming to rank the predictors of the disease incidence according to their importance.

“Percentage of diseased pods”, which is the variable explained through regression trees, is a quantitative variable. Therefore, the software proceeded to analyze the data by means of an F test with a 95% confidence. Generation of groups with fewer than 50 individuals was not accepted: if the analysis intended to create groups with fewer than 50 individuals, the tree stopped at the previous level. Also, a node had to have at least 100 individuals in order to divide it. The total number of individuals (plots) analyzed using this technique was 1471—the result of 42 clones x 4 repetitions x 9 years (1512 plots) minus some plots that did not produce fruits at certain times. It should be noted that of the 13 years of data, only the past nine years were included in the analysis, considering that during the first four years, cacao trees had not reached their maturity potential and therefore their production trend was not stable (Quiroga Gómez 1972).

2.5 RESULTS

2.5.1 Heritability of the percentage of Moniliophthora pod rot

The broad sense heritability (h^2) for the Moniliophthora pod rot rate, calculated over the 13 years, is very high ($h^2 = 0.86$). This means that 86% of the variability of the resistance within the population of the 42 clones depends on the variability of the genotypes and the other 14% depends on other variations. The heritability for Moniliophthora pod rot rate was estimated for each year (Table 2.1). Heritability was stabilized after the sixth year (mean = 73.8, standard deviation = 0.04). This heritability also seems to be higher when MPR incidence is higher (Figure 2.1).

Table 2.1. Heritabilities (h^2) of Moniliophthora pod rot resistance with a confidence interval at 5%.

Years	h^2 and confidence interval	Mean of Moniliophthora pod rot rate (%)
1	0.794 [0.690; 0.898]	50.89
2	0.681 [0.549; 0.813]	39.09
3	0.494 [0.323; 0.664]	23.00
4	0.849 [0.780; 0.918]	42.41
5	0.587 [0.440; 0.734]	18.10
6	0.702 [0.584; 0.819]	22.84
7	0.708 [0.593; 0.822]	45.89
8	0.702 [0.586; 0.818]	45.61
9	0.741 [0.637; 0.846]	40.18
10	0.773 [0.679; 0.868]	49.69
11	0.797 [0.710; 0.883]	58.68
12	0.745 [0.642; 0.849]	45.50
13	0.808 [0.725; 0.890]	57.58
Total	**0.864 [0.803; 0.925]**	**46.90**

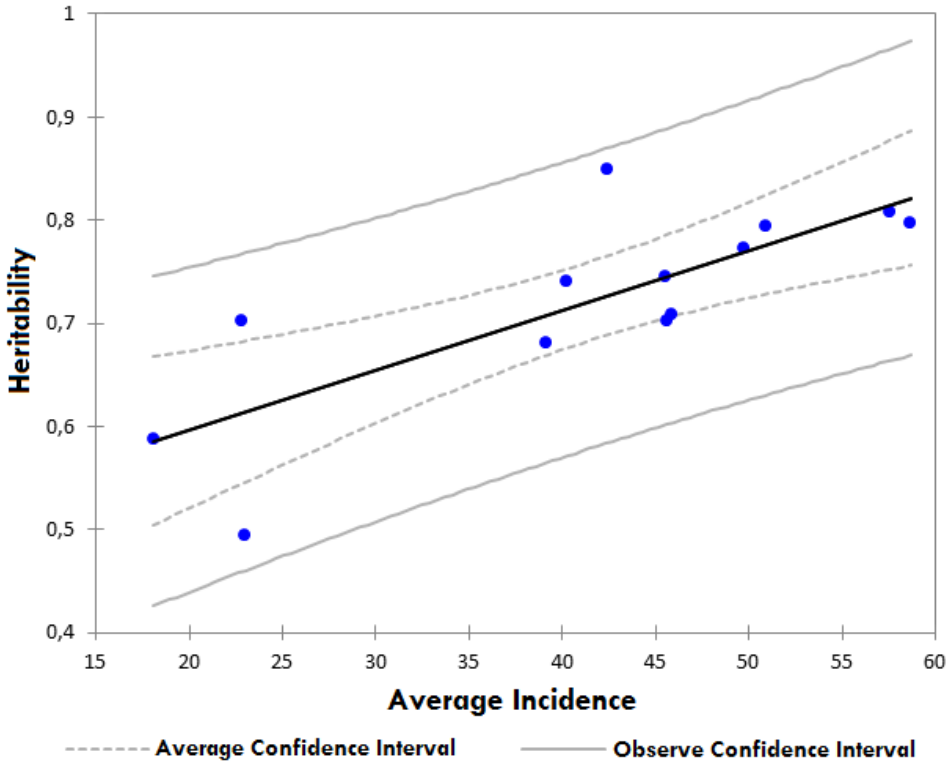

Figure 2.1. Relation between heritability and Moniliophthora pod rot incidence means (R^2=0.611).

The broad-sense heritability for yield, estimated on the same design over 13 years (total of pod produced), is not so high (h^2 = 0.68).

Genetic correlation between Moniliophthora pod rot rate and yield (r_G = 0.18) is not significant, while the environmental correlation between these two traits is positive and significant (r_E = 0.23). Yield and susceptibility to the pathogen are not genetically related, but, for a specific clone, the higher the yield, the higher the Moniliophthora pod rot rate.

Table 2.2 shows the genetic and environmental correlations (lower and upper triangle, respectively) of the annual values of MPR incidence for the 13 years. Values in bold are significant.

Table 2.2. Correlations between annual MPR; lower triangle presents the genetic correlations within the 13 years (y) and upper triangle, the environmental correlations (significant correlations in bold).

	y1	y2	y3	y4	y5	y6	y7	y8	y9	y10	y11	y12	y13
y1	-	0.080	**0.126**	**0.240**	-0.032	**0.106**	**-0.100**	0.045	**-0.107**	0.037	**0.114**	**0.334**	**0.197**
y2	0.031	-	**0.223**	**0.192**	**0.121**	-0.067	**0.107**	**0.130**	-0.005	0.087	0.042	0.075	0.028
y3	0.095	**0.205**	-	0.071	**0.142**	-0.036	0.022	0.075	0.089	**0.105**	**0.164**	**0.160**	0.057
y4	**0.204**	**0.189**	0.042	-	0.076	-0.002	-0.009	0.071	**0.140**	**0.130**	-0.066	**0.215**	0.085
y5	0.100	**0.148**	**0.202**	**0.124**	-	**0.291**	**0.270**	**0.272**	**0.186**	**-0.115**	-0.005	-0.037	**0.514**
y6	0.110	-0.027	-0.009	0.033	**0.212**	-	**0.387**	**0.383**	**0.423**	**0.178**	0.0183	0.082	**0.235**
y7	-0.014	0.091	**0.133**	0.084	**0.179**	**0.252**	-	**0.713**	**0.522**	0.079	**0.273**	**0.184**	**0.419**
y8	0.034	**0.142**	**0.155**	**0.166**	**0.183**	**0.252**	**0.646**	-	**0.486**	**0.235**	**0.340**	**0.217**	**0.509**
y9	-0.075	0.013	**0.134**	**0.205**	0.109	**0.342**	**0.465**	**0.441**	-	**0.309**	**0.208**	**0.196**	**0.392**
y10	0.071	0.113	0.092	0.070	-0.104	**0.277**	**0.148**	**0.356**	**0.388**	-	**0.446**	**0.228**	**0.458**
y11	0.088	0.045	**0.139**	-0.095	0.044	0.0504	**0.363**	**0.470**	**0.256**	**0.442**	-	**0.426**	**0.507**
y12	0.209	0.061	0.068	**0.139**	0.056	**0.155**	**0.324**	**0.353**	**0.342**	**0.233**	**0.432**	-	**0.571**
y13	0.010	0.003	0.048	0.047	0.076	**0.227**	**0.473**	**0.563**	**0.460**	**0.514**	**0.538**	**0.555**	-

2.5.1.1 Spatial effect correction on the heritability per year

Table 2.3 compares the heritability values before and after the spatial correction. Heritability was improved for six years out of 13 (years 5, 7, 8, 9, 11, 12) and was also improved for the whole set of years. However, this increase of heritability was limited and we considered that this correction was not defining, so the original values were kept.

Table 2.3. Heritabilities (h^2) of Moniliophthora pod rot resistance with a confidence interval at 5% after spatial correction (heritabilities increased after spatial correction in bold).

Years	h^2 and confidence interval	Spatial correction
1	0.794 [0.690; 0.898]	0.794 [0.690; 0.898]
2	0.681 [0.549; 0.813]	0.681 [0.549; 0.813]
3	0.494 [0.323; 0.664]	0.494 [0.323; 0.664]
4	0.849 [0.780; 0.918]	0.849 [0.780; 0.918]
5	**0.587 [0.440; 0.734]**	**0.600 [0.456; 0.744]**
6	0.702 [0.584; 0.819]	0.694 [0.574; 0.813]
7	**0.708 [0.593; 0.822]**	**0.727 [0.618; 0.836]**
8	**0.702 [0.586; 0.818]**	**0.739 [0.634; 0.845]**
9	**0.741 [0.637; 0.846]**	**0.754 [0.653; 0.854]**
10	0.773 [0.679; 0.868]	0.769 [0.673; 0.864]
11	**0.797 [0.710; 0.883]**	**0.798 [0.712; 0.884]**
12	**0.745 [0.642; 0.849]**	**0.752 [0.652; 0.854]**
13	0.808 [0.725; 0.890]	0.808 [0.725; 0.890]
Total	**0.864 [0.803; 0.925]**	**0.872 [0.814; 0.930]**

2.5.2 Influential variables ranking

The regression tree in Figure 2.2 presents only two ranking levels: phenotype (clone) and climatic variables (temperature). According to Figure 2.2, “clone” is the most important variable explaining the percentage of diseased pods per plot during the year. Thus, Node 1 clones—CATIE R2, CATIE R4 and CATIE R6 (8, 10 and 12)—have on average the lowest percentage of diseased fruits (8.9%). At the other end, Node 9—CATIE-1000, ICS-44, Pound-7, SCA-6, SCA-12 and RB-41 (18, 29, 33, 36, 37 and 41)—have the highest percentage of diseased pods, with an incidence average of 76.8%.

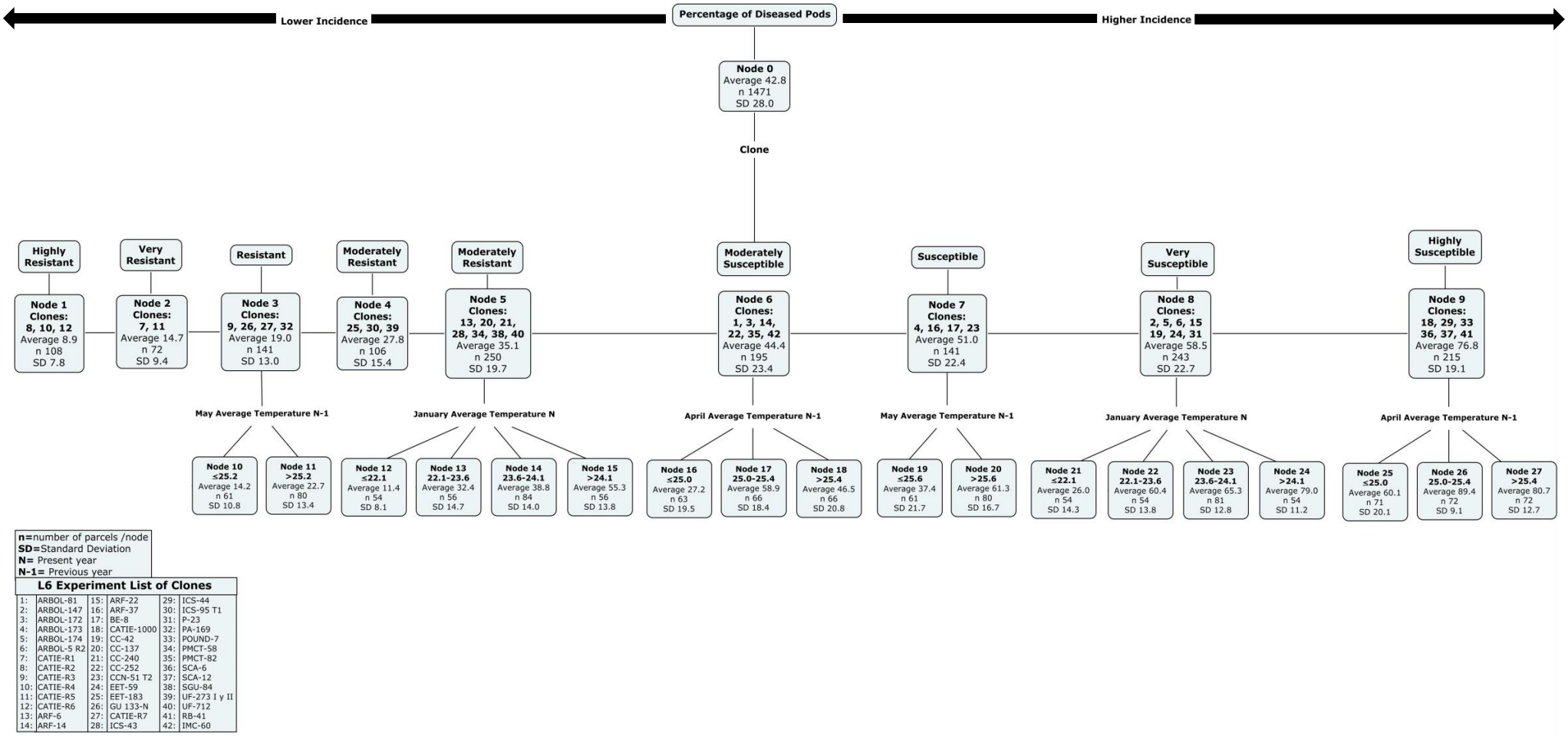

Figure 2.2. Regression tree explaining the percentage of diseased pods per year based on environmental, phenological and annual production variables of the past nine years of the L6 trial.

At the second level of importance, temperature is the only climatic variable that appeared, specifically April and May average temperatures from the previous year (N-1) and January average temperature from the present year (N). For more detail, Node 3 and 7 are divided by "May Average Temperature N-1"; Node 5 and 8, by "January Average Temperature N", and Node 6 and 9, by "April Average Temperature N-1."

In all cases, incidence increased as temperature increased. Critical value for "May Average Temperature N-1" at Nodes 3 and 7 is 25.2 and 25.6°C, respectively, causing an incidence increase from 14.2 to 22.7% and from 37.4 to 61.3%. Nodes 5 and 8 are divided into four groups by "January Average Temperature N," with critical values of 22.1, 23.6 and 24.1°C, which increased the response variable from 11.4 to 55.3% and 26 to 79%, respectively. Finally, critical values for "April Average Temperature N-1" at Nodes 6 and 9 are 25.0 and 25.4°C, increasing the response variable from 27.2 to 46.5% and from 60.1 to 80.7%, respectively.

To better understand this regression tree, a temperature and harvest distribution (Figure 2.3) and some harvest descriptive curves (Figures 2.4 to 2.6) were constructed for the groups of clones (nodes) divided in the tree. Cacao pod age for harvest is about 6 months.

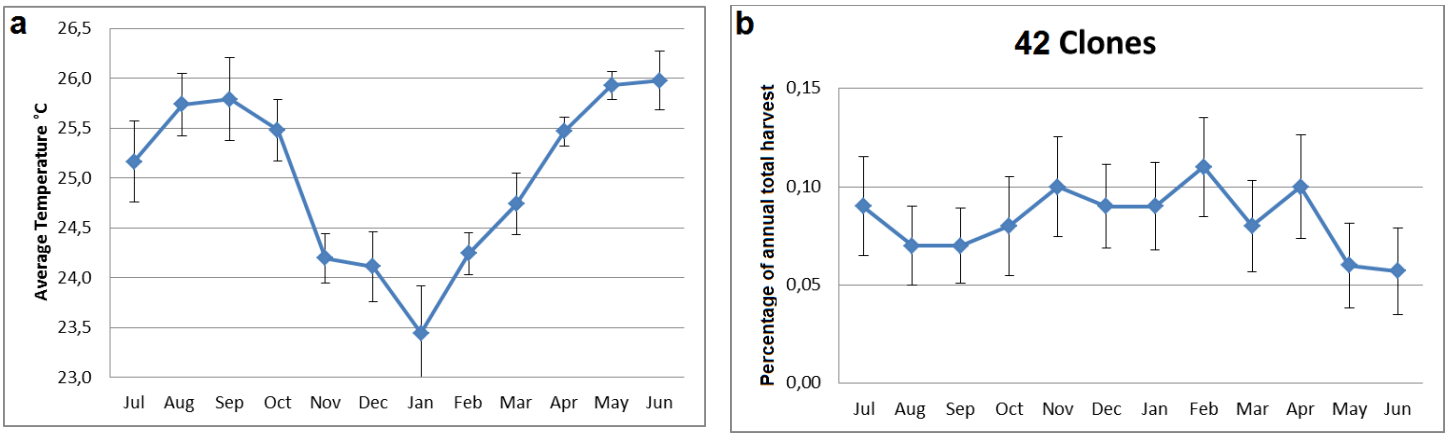

Figure 2.3. Behavior of temperature (a) and harvest distribution (in % from the total harvested pods per year) from the 42 clones of the L6 trial (b) throughout the year; average from the past nine years of the study.

Figure 2.3 allows comparison of temperature and harvest (harvested pods) through the year. November to March is the coolest period of the year (Figure 2.3a). Harvest distribution (Figure 2.3b) presents three peaks: November, February and April. Pods harvested in February and April faced their most susceptible stage (1–3 months old) during the coolest months, from October to January.

As previously mentioned, the variable that better explains MPR incidence for groups 3 and 7 is "May Average Temperature N-1." According to Figure 2.2 Group 3 is classified as a resistant group, and Group 7 as a susceptible group. Figure 2.4 shows the harvest distribution of these two groups produce throughout the year.

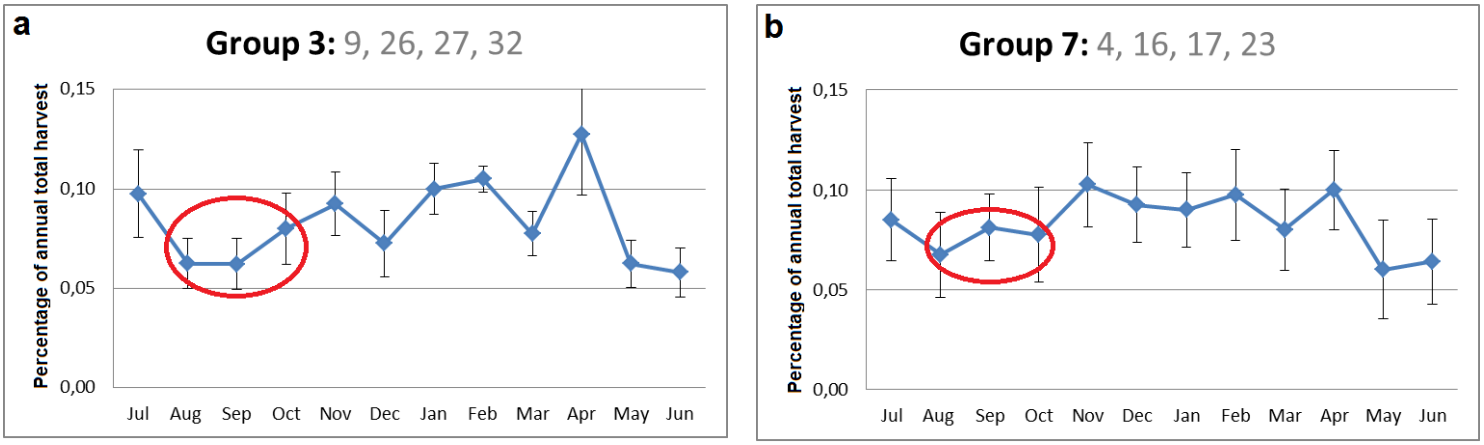

Figure 2.4. Groups 3 and 7 pod harvest distribution throughout the year; see Figure 2.2, for definition of groups (red circles indicate the period where the effect of "May Average Temperature N-1"was shown).

We started from the assumption that "May Average Temperature N-1" could only affect the present pods in the field during this month, especially those who were at the susceptibility stage (1–3 months old). These pods were the ones that were born from March to May. These pods were harvested from August to October (Table 2.4) of the next year (period sorrounded by red circles in Figure 2.4). Analysis was focused on this period.

Table 2.4. Percentage of pods affected by "May Average Temperature N-1".

	Groups of clones according to Figure 2.2					
	Group 3			Group 7		
Month	Harvest %	% Over PHP Average*	Cumulative	Harvest %	% Over PHP Average*	Cumulative
August	0.06	-0.01	0.06	0.07	-	0.07
September	0.06	-0.01	0.12	0.08	+0.01	0.15
October	0.08	+0.01	0.20	0.08	+0.01	0.23

*Period Harvest Percentage (PHP) average from August to October for the 42 clones = 0.07

Cumulative percentage of August, September and October, where "May Average Temperature N-1" affected the pods (Table 2.4) showed that 0.20 and 0.23 from the pods of Group 3 and Group 7, respectively, were present in the field as young pods (susceptible). Table 2.4 also shows that for both groups, the harvest percentages from August to October were close to the average harvest percentage of the 42 clones for these specific months. For Group 3, pod harvest in August and September was below the average by 0.01. October was above the average by 0.01. For Group 7, August corresponds to the average and September and October exceed the average by 0.01 each.

Group 5 is classified as moderately resistant and Group 8 as very susceptible, according to Figure 2.2. For these groups, the determinant variable corresponds to January temperature of the present year, which may have had an effect on pods present as young pods during this month. These pods were the ones harvested from April to June (Figure 2.5).

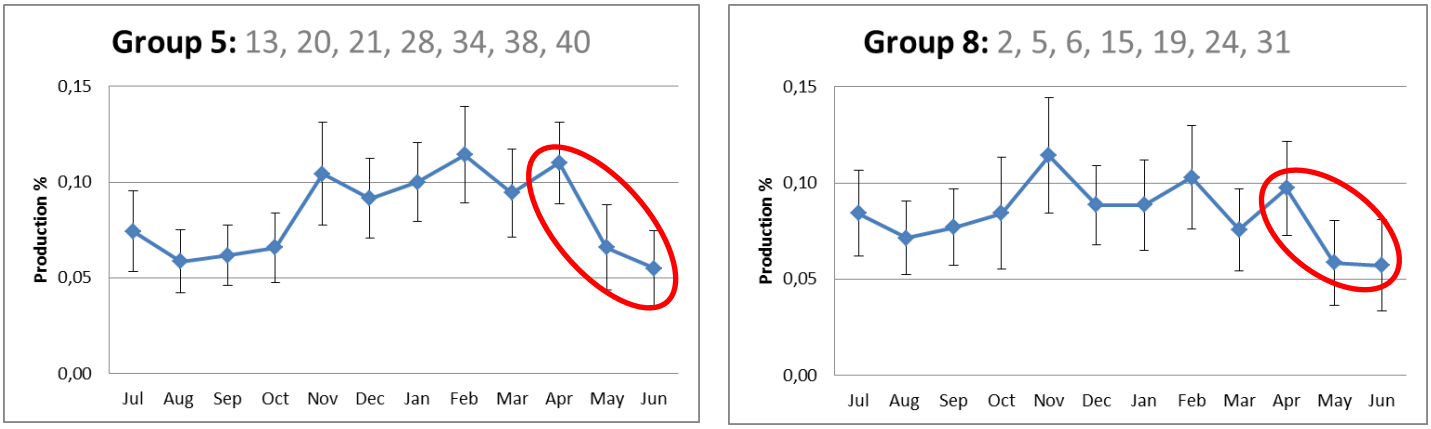

Figure 2.5. Groups 5 and 8 pod harvest distribution throughout the year; see Figure 2.2, for groups definition (fed circles indicate the period where the effect of "January Average Temperature N" was shown).

Table 2.5. Percentage of pods affected by "January Average Temperature N".

	Groups of clones according to Figure 2.2					
	Group 5			Group 8		
Month	Harvest %	% Over PHP Average*	Cumulative	Harvest %	% Over PHP Average*	Cumulative
April	0.11	+0.04	0.11	0.10	+0.03	0.10
May	0.07	-	0.18	0.06	-0.01	0.16
June	0.06	-0.01	0.24	0.06	-0.01	0.22

*Period Harvest Percentage (PHP) average from April to June for the 42 clones = 0.07

Table 2.5 shows that April, May and June only accumulated aproximately a quarter of the pod harvest for Group 5 and Group 8 (0.24 and 0.22, respectively), which were influenced by the January N average temperature. Only the April harvest for both groups exceeded the average harvest percentage of the 42 clones per month by 0.04 and 0.03, respectively.

The April temperature from the previous year is the variable that explains MPR incidence variation of the last two groups (6 and 9) of the regression tree (Figure 2.2), which were classified as moderately susceptible and highly susceptible, respectively. Present young pods during this month were the ones influenced by this variable, which were the pods harvested from July to September of the next year (period sorrounded by red circles in Figure 2.6.

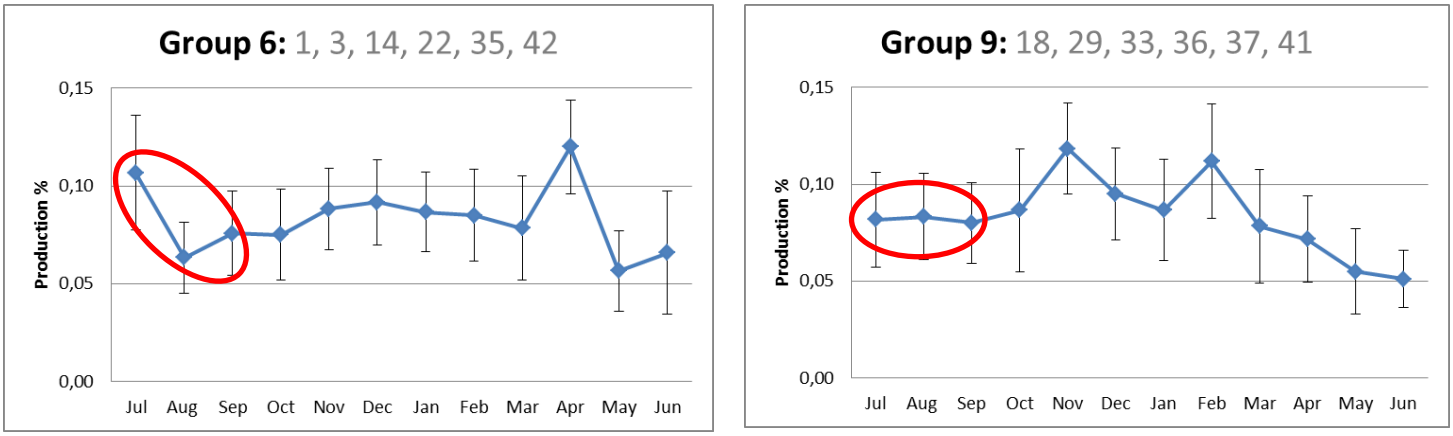

Figure 2.6. Groups 6 and 9 pod harvest distribution throughout the year; see Figure 2.2, for groups definition (red circles indicate the period where the effect of "April Average Temperature N-1" was shown.).

Table 2.6. Percentage of pods affected by "April Average Temperature N-1".

	Groups of clones according to Figure 2					
	Group 6			Group 9		
Month	Harvest %	% Over PHP Average*	Cumulative	Harvest %	% Over PHP Average*	Cumulative
July	0.11	+0.03	0.11	0.08	-	0.08
August	0.06	-0.02	0.17	0.08	-	0.16
September	0.08	-	0.25	0.08	-	0.24

*Period Harvest Percentage (PHP) average from July to September for the 42 clones=0.08

Table 2.6 shows the pod harvest percentages per month affected by April temperature from the previous year. Harvest production during the highlighted periods corresponds to 0.25 for Group 6 and 0.24 for Group 9, which were almost a quarter of the annual production. For both groups, almost all of the monthly harvest percentages were the average from the 42 clones. July for Group 6 was the only month that surpassed the average harvest percentage of the 42 clones per month, by 0.03.

2.6 DISCUSSION

2.6.1 Factors affecting the MPR development

The main idea of the construction of a regression tree is to rank the predictors of the disease incidence according to its importance, in order to identify the most important variables and study their effect in a more specific way in future studies. The genetic material and the temperature of some months appeared to be the most important variables in the disease development for the past nine years of the L6 trial. The effect of these variables follows.

2.6.1.1 Genetic material and resistance expression

As in the results reported by Leandro Muñoz (2011), "clone" was the most explanatory variable of MPR incidence. This means that it has the greatest power to explain the response variable, demonstrating the potential of the genetic component for controlling the disease (Arciniegas L. 2005). This result is confirmed by the high heritability calculated for the MPR incidence.

For this variable, it is important to highlight that the first nodes or groups (1 and 2), which include the most resistant clones of the experiment, are not divided or influenced by any other variable. This behavior could be due to their resistance potential, which is well-maintained through the years despite changes in climate, indicating that the accumulated resistance of these materials is very strong and stable (Phillips-Mora 1996). This is an argument in favor of the durability of the resistance exhibited by these clones, which probably contributed more to the high value of heritability for MPR incidence than others, less resistant. Actually, this behavior was not observed with the rest of the clones, whose response to the disease was not stable and depended on climatic variation, specifically the temperature.

As for durable resistance, Zadoks and Van Leur (1983) have stated that it does not mean that a cultivar or clone will stay free of disease for its entire productive life: it means that for the majority of its productive life, it will on average show a significant grade of resistance. Durability is obtained when the cultivar has accumulated a considerable number of resistance

genes. It also provides the host with protection against genetic changes in the pathogen population. Durability is a feature of incomplete resistance (Crute and Norwood 1978).

Since incomplete resistance allow a limited percentage of damage or infection, it means that reactions against the disease could vary in a limited range, permitting low-incidence values. However, during key moments when environmental conditions could be favorable for the pathogens, incidence could increase, not meaning that the resistance has been broken. Incomplete resistance of coffee against *Hemileia vastatrix* has been reported to vary according to light intensity in one-year-old coffee seedlings (Eskes 1982). Rubiales *et al.* (2012) did a multienvironmental analysis evaluation in their effort to find sources of resistance in a germplasm collection of the faba bean. Their results reveal that incomplete resistance was unstable across environments. And finally, for rice blast disease, Bonman (1992) has concluded that environmental factors such as temperature, duration of leaf wetness, nitrogen fertilization, soil type, and water deficit can influence the resistance expression. Considering the diversity of environments where rice grows, the author concludes that apart from genetic control, other strategies must be applied in highly blast-conductive environments.

According to the results, incomplete resistance of cacao to MPR is variable within the clonal population of cacao trees but the broad sense heritability is high. This validates the development of resistant clones through conventional breeding as an effective and durable control tool.

2.6.1.2 Monthly average temperature effect

For most fungi, temperature is one of the most determinant climatic variables in different stages of their life cycles. Specifically, for *M. roreri* several authors, including Schmitz (1984) have indicated that temperature is a key variable in both dispersal and spore germination. These temperature values under *in vitro* conditions have been reported in different geographic areas and are between 24 and 28°C (López 1954; Chacín 1975; Pérez and Posada 1978; Campuzano 1981b; Evans 1981b; Merchán 1981a; Phillips and Galindo 1985; Ram *et al.* 1987; Herrera 1988).

As an overall result, our regression tree suggests that temperature from the present or previous year is the most important climatic variable in the system, since it has an effect on almost all clones regardless of their resistance level. Also, with the information shown in Figure 2.2, optimum temperature for *M. roreri* can be established at from 22.1 to 25.4°C.

This could be related to the cacao phenology but also to the fungal characteristics, since the MPR attack could be less severe during low temperatures.

The temperature of certain months has a significant effect on the number of diseased pods. If temperatures rise in January, which is the coolest month, the percentage of diseased pods also rises. This is particularly true for Groups 5 and 8 of the regression tree, which had a pod harvest percentage close to the 42 clone-average for April to June, which is the period when the effects of this variable was shown. This means that a sufficient amount of susceptible material was present in January, and a higher incidence resulted when the temperature of this specific month was higher. These two groups of clones were qualified as moderately resistant and very susceptible, respectively, on average. However, these levels of resistance could vary according to January's temperatures. For the other clones, the effect of temperatures on disease incidence was not clear: the relevant month, where temperature was important, was not concurrent either with a period of normally low temperatures or a period of higher production of pods compared with other clones. This was the case with Groups 3 and 7 of the regression tree, qualified as resistant and susceptible, respectively. The MPR incidence increased when the temperature of May increased. The same happened with Groups 6 and 9, qualified as moderately susceptible and highly susceptible, respectively. More analyses or studies are needed to understand this particular relationship.

Based on the resultant knowledge about the importance of the temperature on disease incidence, we proceeded to carry out a study at the level of plot, including other variables (relative humidity, wetness) in order to examine in more detail the effect of microclimate on the epidemic.

2.7 CONCLUSIONS

- "Clone" is the most explanatory variable for the MPR incidence in the experiment.

- Resistance of the highly resistant clones is considerably stable and possibly durable, however for the rest of the clones, resistance is significantly determinate by the environment.

- In general, breeding for resistance is an effective and durable strategy for MPR control.

- Temperature is the only climatic variable that has a significant effect over the MPR incidence.

- High temperatures in January, April and May will increase the probability of having an increase in the MPR incidence from three to six months later.

- January, April and May represent important periods where the farmers should take more aggressive control measures.

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CHAPTER 3

EFFECTS OF MICROCLIMATIC VARIABLES ON THE SYMPTOMS AND SIGNS ONSET*

Part I

3.1 INTRODUCTION

Cocoa diseases are the main threat for production, causing losses estimated at 30 to 40% worldwide (Bowers *et al.* 2001). Cacao has proved to be highly susceptible to new-encounter diseases as well as pests (Evans and Prior 1987). At the beginning of the 20th century, more than 50% of global cocoa production (approximately 29 400 tons) occurred in mainland tropical America, followed by the Caribbean islands, Africa, Asia and Oceania. However, diseases such as Moniliophthora pod rot (MPR) and witches´ broom caused a decline in American cocoa production. Today, only 16% of world production comes from America, which represents 618 000 tons of cocoa (ICCO 2014). MPR has been reported as the most destructive, invasive and difficult-to-control cacao disease in the area (Desrosiers and Díaz 1957; Aranzazu *et al.* 2000). With its advance, the disease has swept many plantations in countries where it has been reported.

MPR is caused by the fungus *Moniliophthora roreri* (Cif.) Evans *et al*, (Basidiomycete, Marasmiaceae). Its center of origin is located in Colombia (Phillips-Mora 2003), and from there the pathogen has spread to 12 countries in tropical America (Phillips-Mora *et al.* 2006c): Ecuador (formerly considered the place of origin) (van Hall 1914; Rorer 1918), Venezuela, Panama, Costa Rica, Nicaragua, Peru, Honduras, El Salvador, Guatemala, Belize (Phillips-Mora *et al.* 2006a), Mexico (Phillips-Mora *et al.* 2006b) and recently Bolivia (Phillips-Mora *et al.* 2015). This disease is considered a threat for cacao production since its causal agent is in a very intense invasive stage, and apparently most of the commercial cacao genotypes established in the region, and in the world, are susceptible. The arrival of this

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pathogen to countries such the Dominican Republic and Brazil, and especially to other continents, particularly to Africa would be devastating (Phillips-Mora and Wilkinson 2007). West Africa is the world's largest cocoa-producing region.

Little is known about the biology of the fungus. According to the description made by Evans *et al.* (1978) *Moniliophthora roreri* has partitioned mycelium (septa) with dolipores and without clamp connections. Hyphae are hyaline and thin-walled. Spores have basipetal formation in simple chains of four to 10 branched units (Thévenin and Trocmé 1996). These features classified this fungus as basidiomycetes (Evans *et al.* 1978). The spores are easily removable, with thick walls, pale yellow in color when immature or dark brown at a mature stage. They could be globose, elliptical or amorphous (Phillips-Mora 2003). The characteristics of these structures vary a little depending on strains.

It is well-described that cocoa pods are susceptible to infection by *M. roreri* (Ram 1989) and some authors have suggested that flowers could be also attacked (Naundorf 1954; Bejarano Villacreces 1961). *Moniliophthora roreri* is considered a hemibiotrophic fungus and its life cycle is completed in two phases: 1) a biotrophic phase, from the germination of the spores to the intercellular invasion of the pod and 2) a necrotic phase causing growth reduction of the pods and finishing with the invasion of the fungus to the cells, causing the appearance of internal and external necrosis (Thévenin and Trocmé 1996).

This pathogen has an extensive incubation and latency period (Evans 1981b). Once the fruit is infected, it may take 40 to 60 days to show external symptoms (Porras and Enríquez 1998). During the early infection stages, the fungus penetrates the pods intercellularly. Once inside, it invades intracellularly, destroying the fruit's internal tissue (Bejarano Villacreces 1961; Desrosiers and Suárez 1974). Subsequently, external symptoms appear, which may occur throughout the pod development (Bejarano Villacreces 1961). Sometimes fruits with no visible symptoms can have a hidden infection and be rotten inside. Generally these pods are heavier than healthy fruits since water starts to accumulate inside. When cells are destroyed, they stop working correctly their internal content is spread around and transpiration is interrupted (Rorer 1918; Merchán 1978a; Campuzano 1981a). Infection normally occurs in the

early stages of fruit growth, and as the organ grows, it becomes more resistant (Ampuero 1967). One possible explanation of the resistance of mature pods is that they are simply harvested before the symptoms become obvious (Suárez 1971). If pods are less than three months old, the first symptom to appear is a lump, hump or swelling (Figure 3.1a). If the infection occurs later, the first symptom will be the appearance of small yellow spots on older green pods and orange spots on the red ones. This symptom is also known as yellowing or early ripening (Figure 3.1b). Appearance of oily or aqueous spots is another early symptom (Figure 3.1c), followed by an irregular brown spot with a yellow halo. This symptom is known as chocolate spot (Figure 3.1d) (Porras and Enríquez 1998). Under warm and humid conditions, pathogen development is observed as a hard white stroma (mycelium) over the chocolate spot. Spores are formed over the mycelium and appear as a creamy or brown mass (Figure 3.1e). Lesions of infected pods that remain attached to trees can sporulate for up to nine months and then pods mummify (Figure 3.1f) (Enríquez 2004).

Figure 3.1. M. roreri *symptoms and signs.* ***Photos by Mariela E. Leandro-Muñoz.***

There are different methods that could be utilized to control this disease. Chemical control has proved to be successful, but the use of chemical fungicides is still not optimized due to the lack of biological and epidemiological information on the pathogen. Periodic removing of the diseased pods from the fields also can be effective but is very time and labor consuming. Increasing the genetic resistance has the potential to be the best approach for long-term and cost-effective control. CATIE-R6 is an example of a genotype that exhibits a high incomplete resistance to MPR, with less than 10% of disease incidence over past 10 years (Phillips-Mora *et al.* 2013). However, in general, incomplete resistance can vary according to weather conditions. There is a small number of resistant varieties and currently the high

demand for planting material cannot be met by the existing clonal propagation infrastructure (Evans and Prior 1987). Existing methods for control of MPR are currently applied within the cacao fields with limited success because they are rarely accessible to smallholder cocoa growers, and also to the limited knowledge on the pathogen biology and disease epidemiology that could help optimize these methods.

Pod infection success is determined by several factors, including pod wetness. It has been reported that the presence of a film of water over the pod is required for spore germination (López 1954; Chacín 1975; Merchán 1978b; Campuzano 1981a; Porras-Umaña and Galindo 1985). High relative humidity (80 to 100%) and warm temperatures (20 to 27°C) are suitable conditions for spore germination and for the fungal penetration (Campuzano Londoño 1980; Phillips 1986; Enríquez 2004). Favorable conditions for sporulation are similar to those required for infection. Sporulation is also dependent on fruit moisture and warm temperatures (20 to 28°C) but this last factor must fluctuate in order to stimulate the sporulation process (Porras-Umaña and Galindo 1985; Herrera 1988). Light is also another important factor in the sporulation process. Alternate periods of light and darkness promote the spore formation in *in vitro* conditions (Herrera 1986).

Weather influence over MPR has been reported by several authors, mostly during the seventies and eighties. Barros Nieves (1977) and Phillips (1986) have concluded that high relative humidity due to excess of shade and poor ventilation within the plantation favors the frequency and intensity of the attack. Merchán (1981c) has established a positive correlation between the MPR incidence and the relative humidity at 60 days before symptoms appearance. In addition, Torres de la Cruz *et al.* (2011) found that MPR incidence is positively associated with periods of relative humidity higher than 90% recorded during the 49 days before symptom appearance. Rainfall has been also studied. Evans (1977) and Porras and González (1982) have reported a positive correlation between disease incidence and the amount of rainfall two to four months before the infection. Also, Maddison *et al.* (1995) concluded that in regions with a well-defined dry season, disease incidence tends to decrease as rain ceases, particularly if flowering decline. Another studied factor is temperature. Torres de la Cruz *et al.* (2011) found that MPR incidence increased when temperatures ranged from 20 to 27°C, 49

days before symptom appearance. Finally, Suárez (1971) has stated that a temperature daily range of 22 to 32°C favors MPR incidence and that cooler temperatures cause less severe attacks as incubation periods become longer. As mentioned, weather influence on the disease has been studied but mostly in very specific studies and in some cases under *in vitro* conditions done several years ago, meaning that available information is still scarce and outdated. Epidemiological field studies are almost nonexistent since the existing studies consist mostly of linear regressions between disease incidence and a single variable in a short period.

Today, new statistical tools are available that are increasingly used in phytopathology and other agronomic disciplines (Garrett *et al.* 2004; Garrett *et al.* 2006). For instance, Bugaud *et al.* (2015) implemented logistic regression models to explore the effect of the preharvest temperature on the chilling susceptibility of banana fruits stored at 13°C and particularly to identify the fruit growth stage that is the most impacted by the chilling injury. Anco *et al.* (2012) used linear and nonlinear models to fit their data in order to examine effects of temperature, wetness duration and interrupted wetness duration over the sporulation rate of *Phomopsis viticola* on infected grapes. Finally, in a cacao system, Ndoumbè-Nkeng *et al.* (2009) used cross-correlation and multiple-regression analyses to better understand the relation between the incidence of Phytophthora pod rot and two environmental factors: rainfall and temperature. To our knowledge, this kind of approach has not been applied to MPR until now.

This study aims to apply advanced statistical modeling to establish the relationships between three microclimatic variables (temperature, relative humidity and wetness) and MPR development. The statistical method applied allows us to 1) determine, with no a priori assumptions, the specific period of the pod life where each microclimatic variable has the greatest influence on disease development and then 2) study all the variables over the determined periods and their interactions together. This research aims to contribute to filling the existing knowledge gaps concerning microclimatic effects on the MPR epidemic by proposing two models based on microclimatic variables to explain appearance of symptoms and sporulation on the pods.

3.2 MATERIALS AND METHODS

3.2.1 Experimental site

The study was conducted at the L6 trial located at CATIE's experimental farm La Lola. The farm is located in 28 Millas, Bataán District, Matina Canton, Limón Province, in the humid and cloudy tropical forest (Holdridge 1967). La Lola is located at 40 m.a.s.l, 10°06' latitude North and 83°23' longitude West, on the Atlantic Coast of Costa Rica. Average rainfall (1949–2010) is 3575 mm with a decrease in March and September. September is the month with less rainfall. Monthly average temperature ranges (1952–2010) were between 20.5 and 30°C. May and June are the warmest months, whereas December and January are the coldest (Phillips-Mora *et al.* 2013). Relative humidity averaged above 91% in the same period. The prevailing climate is influenced by the Caribbean: humid with a not well-defined dry season, quite cloudy with few sunny hours. All these characteristics correspond to the ideal environment for MPR development.

The L6 experiment was planted in 1998 and 1999. Forty-two clones, selected primarily for their high resistance to disease and/or high productivity, were compared in a randomized complete block experimental design, with four replicates of eight trees each. Planting distance is 3 x 3 m. Permanent shade in this experiment is distributed unevenly and is composed by immortelle (poró, *Erythrina poepiggiana*) and guava (*Inga edulis*) trees. Cacao and shade trees receive periodic maintenance pruning (Phillips-Mora *et al.* 2013).

From the 42 clones included in the L6 trial, we used the cacao clone Pound-7 in our study. Pound-7 was selected based on its high susceptibility to MPR and high capacity of fruit production (to assure the presence of pods throughout the year) (Phillips-Mora *et al.* 2013). Its average production is 542 kg/ha/yr (historical data average of 11 years) but its production potential has been severely decreased because of the disease, since its incidence reaches 75%.

3.2.2 Pods measurements

Every week, generations (cohorts) of nascent pods between 3 and 10 cm (1 to 2 months' age) were tagged from 29 May 2012 (the first generation) until 12 June 2013 (the 55th generation), cumulating 2268 pods, corresponding to the entire production of the studied trees. Pods were individually observed for MPR symptoms and signs every week, from week one to 10 after tagging, and every two weeks, after week 10. Different categories of tagged pods were monthly removed from the trees: 1) pods with MPR sporulated lesions, 2) mature healthy pods ready for harvest and 3) pods with symptoms and signs of other diseases (mainly Phytophthora pod rot).

We defined three phytosanitary pod types: 1) healthy, 2) with MPR lesions with no signs of sporulation and 3) with MPR sporulated lesions. We had two categories of phytosanitary status change: from healthy to diseased pods with no signs of sporulation and from diseased with no signs of sporulation to pods with sporulated lesions. These categories are referred to as H→D change and D→S change in the rest of the manuscript, respectively. From the 11th week (pod age of four to five months), pods were evaluated every two weeks, because symptom or sign appearance is not as fast as in younger pods.

3.2.3 Microclimatic data recording and behavior during the evaluation period

Microclimatic data were recorded by a Hobo H21-001 weather station (Onset Computer Corporation, Bourne, MA, USA) positioned within one plot of the trial. A total of nine sensors were installed in the middle of the eight trees of a plot (CC-137, repetition four) at different heights: three for temperature (S-TMB-M006), two of them at 2 m from ground level and one at 1 m; four for wetness (S-LWA-M003) at 1.25 m and two for relative humidity and temperature (S-THB-M008) at 1.5 m. Having several sensors was important to capture the microclimate variability within the plot. Climatic data was recorded every 30 seconds and averaged every 15 minutes. Temperature and relative humidity sensors were previously calibrated and corrections were applied to homogenize the data. In addition, wetness sensors were field calibrated to determine the wet/dry transition point. This transition point was

determined based on the values that recorded the weather station at the moment when the pods and vegetative tissue change from wet to dry in the early morning.

The software HOBOware® Pro was used to collect the data from the data logger weekly. Rainfall was recorded by a rain gauge located near the study site.

Trial meteorological information is presented in Figure 3.2.

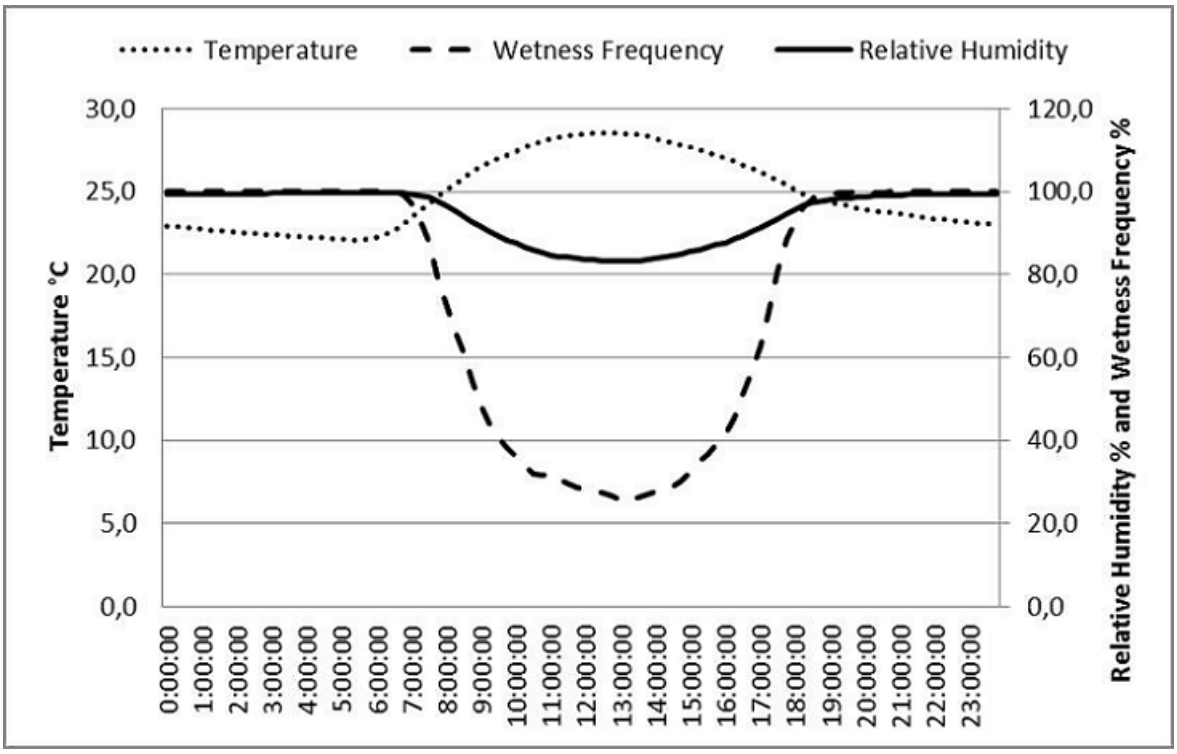


Figure 3.2. Meteorological mean values throughout the day (means of 462 days from 8 May, 2012 to 13 August 2013).

Wetness frequency and relative humidity presented the same behavior throughout the day. This pattern was inverted with the temperature daily pattern. The highest temperatures of the day were registered at noon. The coolest temperatures were recorded in the early morning, about 6:00 and 7:00. Average maximum temperature was almost 29°C and the lowest almost 22°C. All the wetness sensors were wet (frequency = 100%) at night and early morning, from 18:00 to 07:30. During daytime, on average, at least one sensor was wet. The moment with

less wetness was found around 13:30. Relative humidity was always high: on average, above 85%.

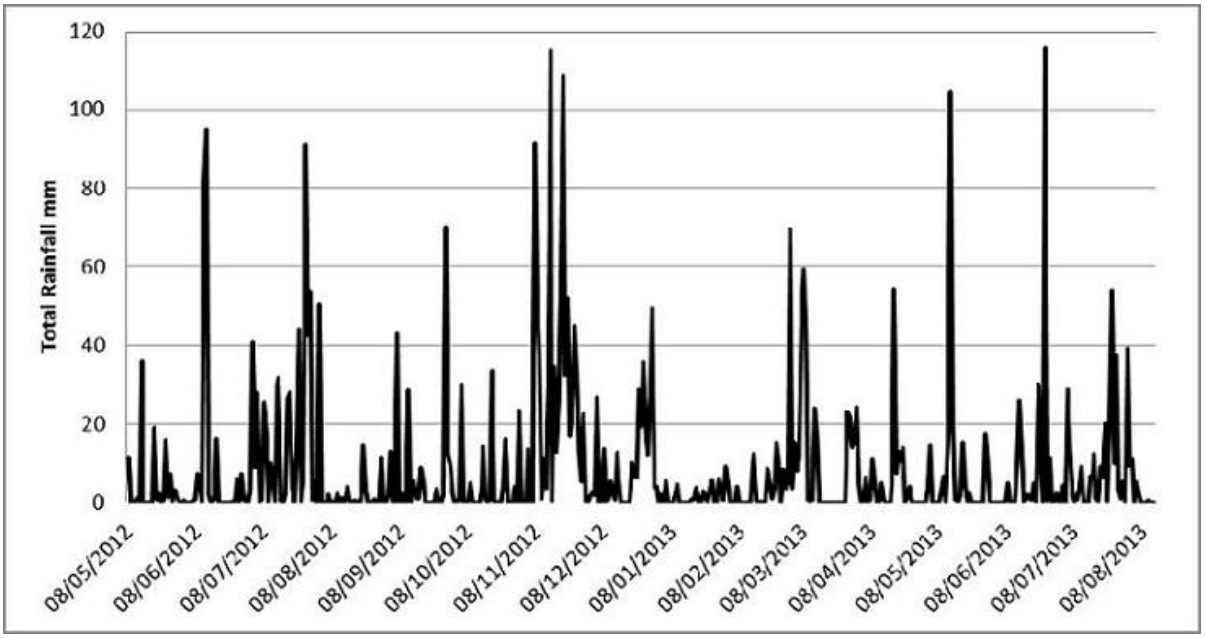


Figure 3.3. Daily rainfall distribution throughout the experimental period.

Daily total rainfall went from 0 up to 115 mm. As shown in Figure 3.3, this factor did not present any pattern. Rainfall was well-distributed during the entire observation period. A dry or rainy season could not be clearly distinguished throughout the year. However, a decrease in rainfall was observed between the beginning of January and the end of February 2013, when daily rainfall did not exceed 15.2 mm.

Our aim was to study the influence of the microclimatic variables on pod status changes. However, a short period after tagging, when changes occurred, needed to be defined to focus the analysis on pods of almost the same age, having the same susceptibility level. We chose a 10-day period for each of the two status change categories described earlier, when the major number of changes occurred, to enrich the statistical analyses. The response variable was the frequency or probability of change in this period. For H→D and D→S changes, we retained the period between 40 and 50 days after tagging (d.a.t.) and between 60 to 70 d.a.t., respectively (Figure 3.4). For the former, 22.4% of changes occurred during this period and for the later, 32.2%.

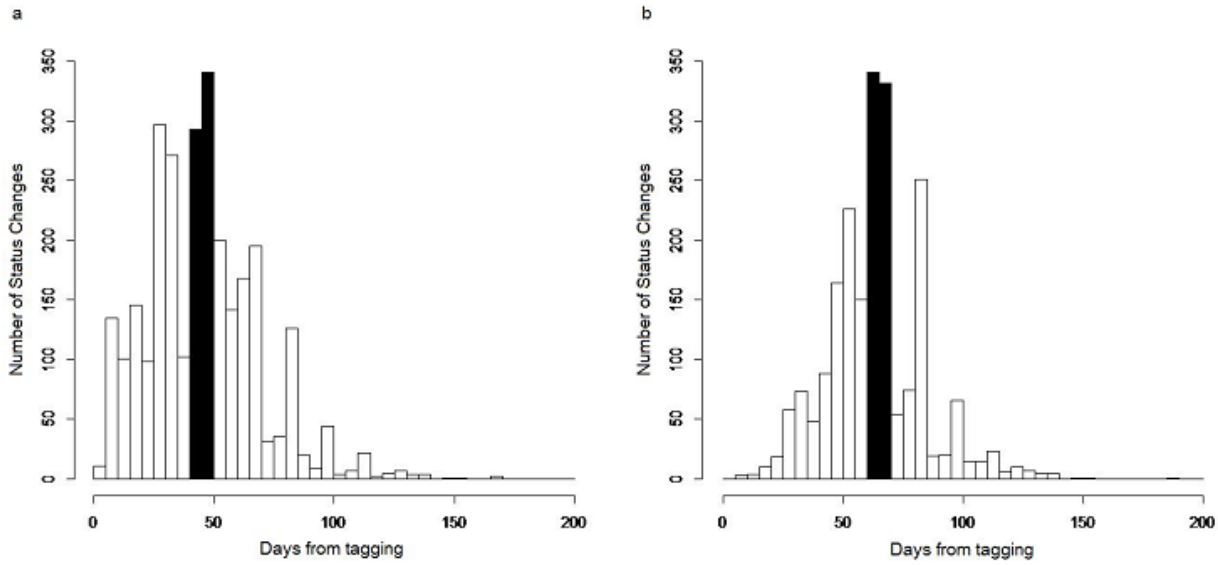


Figure 3.4. Histograms for the selection of the studied periods for Pound-7: a. corresponds to the pod's status change from healthy to diseased with no signs of sporulation; b. corresponds to the pod's status change from diseased with no signs of sporulation to sporulated lesions.

For the Pound-7 clone, 22.4% of H→D changes occurred during the 40–50 d.a.t. period and 32.2% of D→S changes occurred during the 60–70 d.a.t. period.

Similar to what López-Bravo *et al.* (2012) did, we calculated wetness average frequency based on the number of wet sensors among the four wetness sensors used. We preferred using the average frequency of wetness instead of wetness duration, because we considered that it better reflects the real wetness condition in the heterogeneous environment of a cacao plantation.

3.2.4 Data processing

For each pod and for each microclimatic variable, we integrated daily values (mean, sum or frequency, according to the nature of the variable) for all the possible periods between two dates relative to its tagging date (a starting date and duration). The starting date was considered up to 20 days before tagging and the duration of integration was done until the status change (H→D and D→S changes). We integrated daily values of *Wetness average*

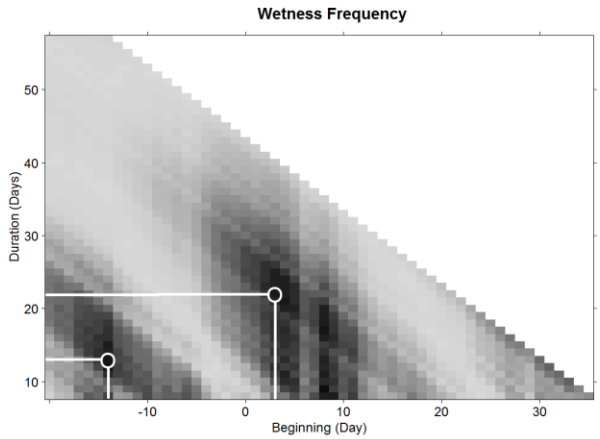
frequency (WF), *Average temperature* (Tmean), *Minimum temperature* (Tmin), *Maximum temperature* (Tmax), *Amplitude of temperature* (Tamp), *Minimum relative humidity* (RHmin) and *Total rainfall*. It was decided not to consider the daily *Maximum relative humidity* (RHmax) because its value at the study site was always 100%, as shown in Figure 3.2. For this same reason, we did not consider the *Amplitude of Relative Humidity* (RHamp) or *Average Relative Humidity* (RHmean), since RHmin was the only variable source of variation.

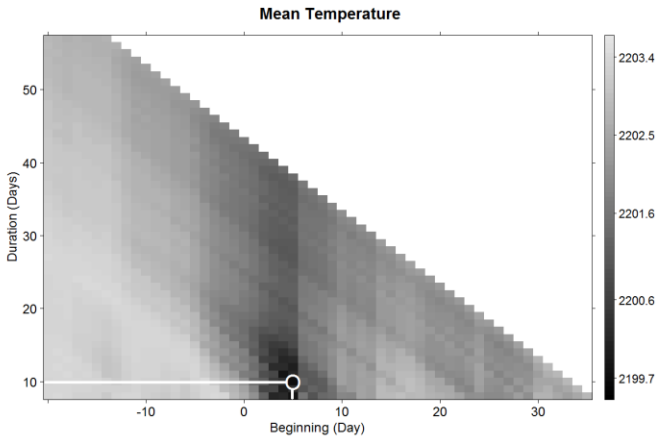
3.2.5 Statistical analyses

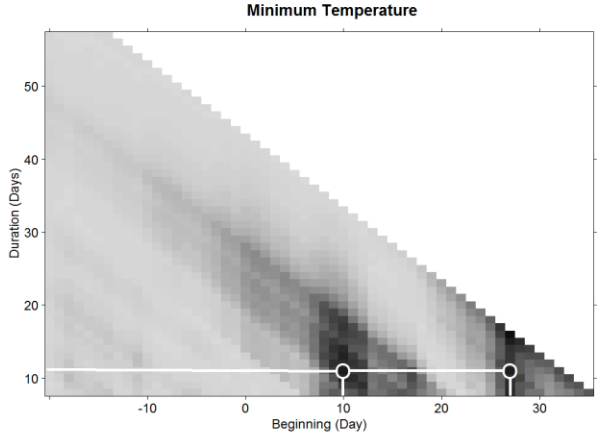
GLMM were used to explain the probability of status change of every observed pod as a function of microclimatic variables, using a binomial distribution (0 = healthy, 1 = diseased, for H→D changes; 0 = diseased, 1 = sporulated for D→S changes). Microclimatic variables were included as fixed factors in models. We included the pod generation (i.e., the week it was tagged) as a random effect to account for the effect of the dynamic of the disease. All models were fitted with the glmer function in the lme4 package (Bates *et al.* 2012), in which the maximum likelihood of parameters is approximated by the Laplace method (Bolker *et al.* 2009).

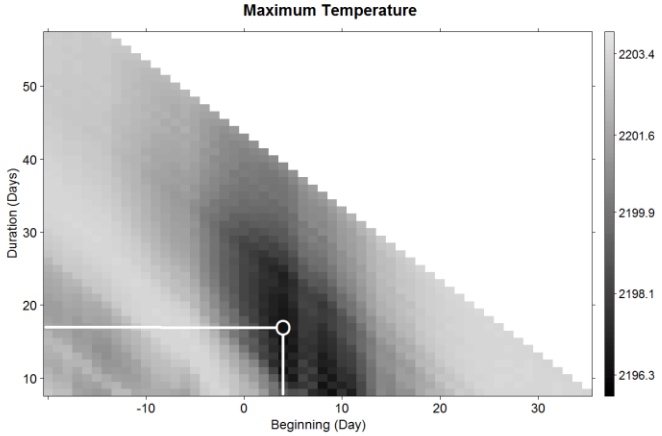
3.2.5.1 Single predictor GLMM analysis

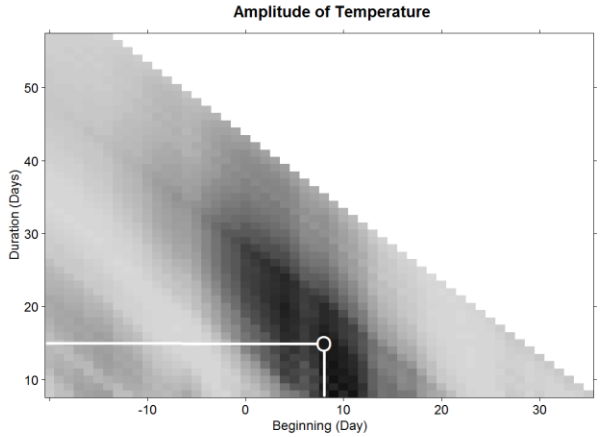
First we performed single variable GLMMs to determine the periods (starting date and duration) where each microclimatic variable better explained the two status changes (Figures 3.5 and 3.6). This was based on the calculation of the AIC value for each variable. The AIC is a criterion that measures the relative quality of a statistical model for a given data set (Akaike 1974). The results of these comprehensive analyses of all possible periods of integration of each climatic variable were presented graphically in a grey scale, corresponding to the AIC value, according to each starting date and duration of integration. The centers of the areas with lowest AIC values were selected as most promising candidate variables for the second phase of the analysis. We then tested the correlation (Pearson coefficients) between candidate variables and kept those with a $R^2 < 0.9$, also considering their potential effect on disease (based on literature) (Appendix Figures A3.1 and A3.2). This step helped us to avoid having highly correlated microclimatic predictors of pod status change in the following complete GLMM analysis.

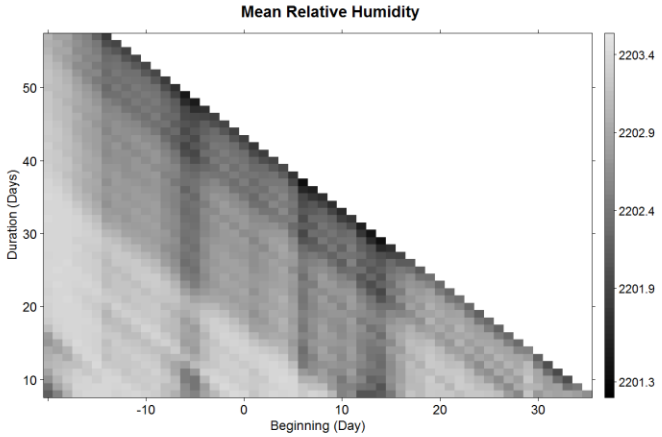

Wetness Frequency
Duration (Days)
Beginning (Day)

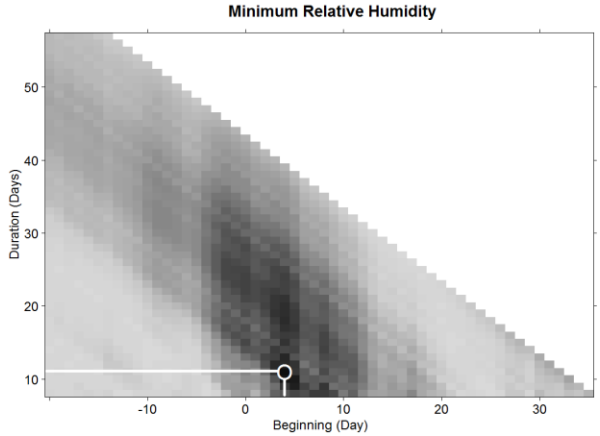

Mean Temperature
Duration (Days)
Beginning (Day)
2203.4
2202.5
2201.6
2200.6
2199.7


Minimum Temperature
Duration (Days)
Beginning (Day)


Maximum Temperature
Duration (Days)
Beginning (Day)
2203.4
2201.6
2199.9
2198.1
2196.3


Amplitude of Temperature
Duration (Days)
Beginning (Day)


Mean Relative Humidity
Duration (Days)
Beginning (Day)
2203.4
2202.9
2202.4
2201.9
2201.3


Minimum Relative Humidity
Duration (Days)
Beginning (Day)

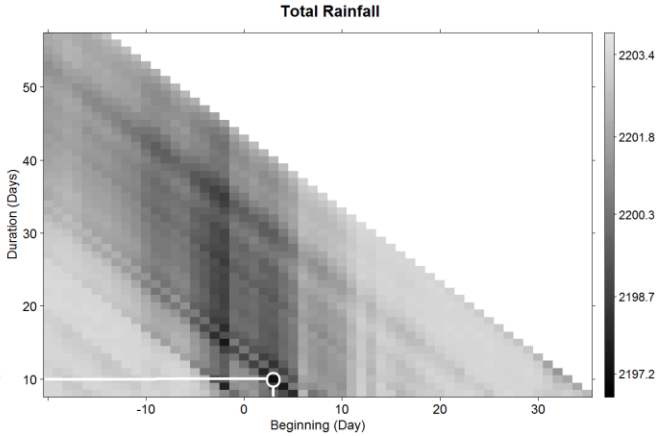

Total Rainfall
Duration (Days)
Beginning (Day)
2203.4
2201.8
2200.3
2198.7
2197.2

Figure 3 5. Period of influence of each daily variable on the pod's status change, from healthy to diseased with no signs of sporulation, 40 to 50 days after tagging.

By period of influence we meant from the starting day respect to tagging and duration from this day. The figure represents the AIC values of the binomial GLMMs explaining the pod status change from tagging, for each period of influence. On the starting date axis, zero corresponds to the tagging date of pods of 3 to 10 cm in length. Circles indicate the lowest AIC value and the best microclimatic predictors of the pod status change (period of influence). The presence of a delimited surrounded black to gray zone indicates a zone of decreasing influence of the variable. Gray scale on the right represents the AIC values. Absence of circle indicates that no clear influence zone was identified.

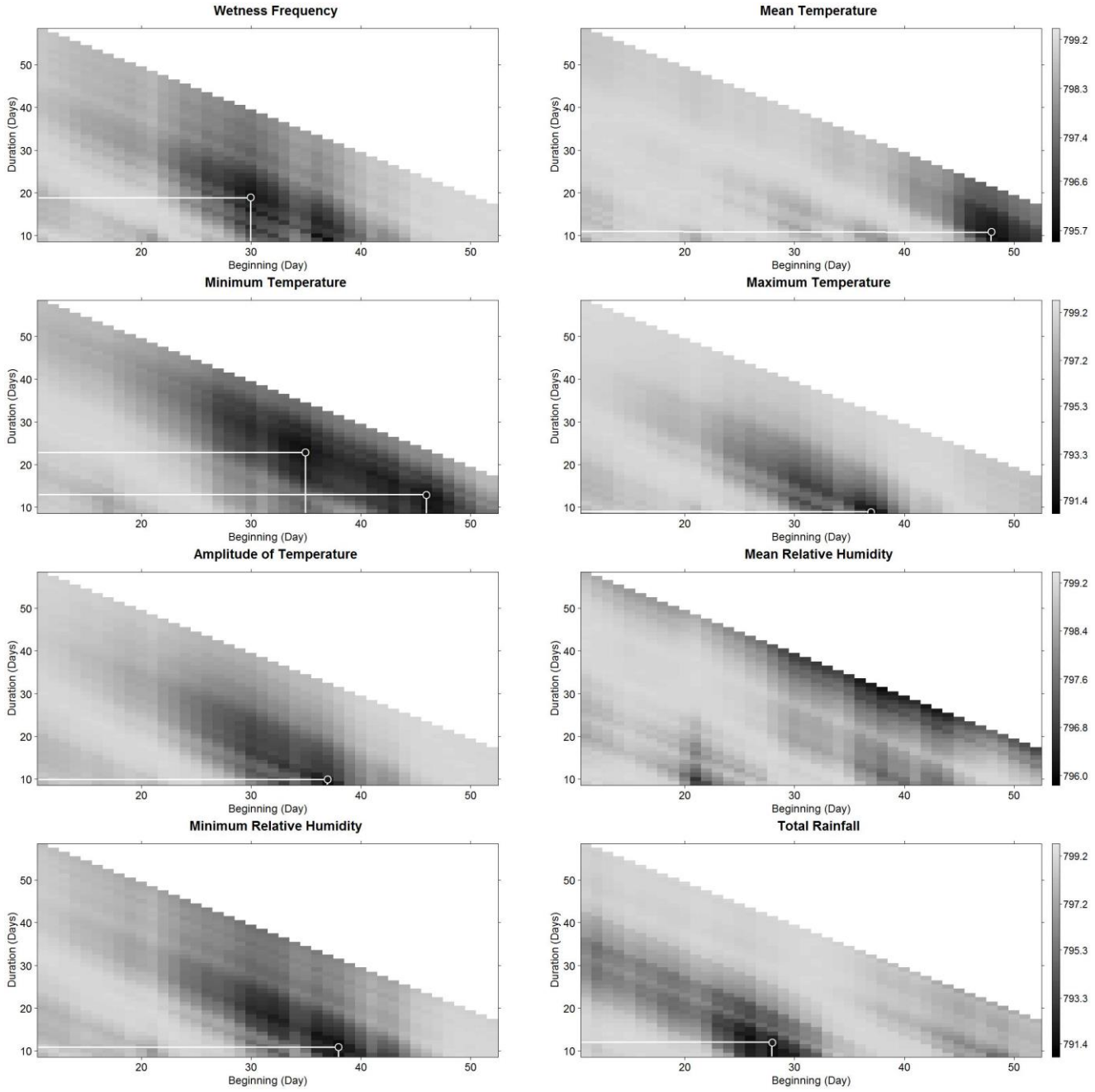


Figure 3.6. Period of influence of each daily variable on the pod status change, from diseased with no signs of sporulation to diseased with sporulated lesions, 60 to 70 days after tagging.

By period of influence we meant from the starting day, with respect to tagging, and duration from this day. The figure represents the AIC values of the binomial GLMMs explaining the pod status change from tagging, for each period of influence. On the starting date axis, zero corresponds to the tagging date of pods of 3 to 10 cm in length. Circles indicate the lowest AIC value and the best microclimatic predictors of pod status change (period of influence). The presence of a delimited surrounded black to gray zone indicates a zone of decreasing influence of the variable. Gray scale on the right represents the AIC values. Absence of circle indicates that no clear influence zone was identified.

3.2.5.2 Complete GLMM analysis

The second step was to build two complete GLMMs (one model for H→D change and one for D→S change) including simultaneously all the candidate variables as fixed factors and *generation* (cohorts of pods produced each week) as random factors. The optimal model was obtained by using a backward model selection process (Drop1 function from R) to eliminate less-significant variables (Zuur *et al.* 2009).

3.3 RESULTS

3.3.1 Selection of the Period of Effect of each Microclimatic Variable

In the case of H→D change, the period of influence of almost all of the variables included the period from two to 24 d.a.t., except for wetness frequency (from the 14th to one day before tagging, Table 3.1), and for minimum temperature (from 28 to 38 d.a.t., Table 3.1). In the case of D→S change, the period of influence of all the variables included the period from 30 to 60 d.a.t. (Table 3.2).

Table 3.1. Selected microclimatic predictors (starting date and duration) of pod status change from healthy to diseased with no sign of sporulation, from 40 to 50 days after tagging.

		Tagging		
		Days *before* tagging	Days *after* tagging	Studied period
ID	Variables	-20 -18 -16 -14 -12 -10 -8 -6 -4 -2	0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38	40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70
$WF_{-14 \text{ to } -1}$	Wetness frequency	-14 -12 -10 -8 -6 -4 -2		
$WF_{3 \text{ to } 25}$	Wetness frequency		4 6 8 10 12 14 16 18 20 22 24	
$Tmean_{3 \text{ to } 15}$	Mean temperature		4 6 8 10 12 14	
$Tmin_{10 \text{ to } 21}$	Minimum temperature		10 12 14 16 18 20	
$Tmin_{27 \text{ to } 38}$	Minimum temperature		28 30 32 34 36 38	
$Tmax_{4 \text{ to } 21}$	Maximum temperature		4 6 8 10 12 14 16 18 20	
$Tamp_{8 \text{ to } 23}$	Amplitude of temperature		8 10 12 14 16 18 20 22	
$RHmin_{4 \text{ to } 15}$	Minimum Relative Humidity		4 6 8 10 12 14	
$TR_{3 \text{ to } 13}$	Total Rainfall		4 6 8 10 12	

Table 3.2. Selected microclimatic predictors (starting date and duration) of pod status change from diseased with no sign of sporulation to diseased with sporulated lesions, from 60 to 70 days after tagging.

		Tagging		
		Days *before* tagging	Days *after* tagging	Studied period
ID	Variables	-20 -18 -16 -14 -12 -10 -8 -6 -4 -2	0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58	60 62 64 66 68 70
$WF_{30\ to\ 49}$	Wetness frequency		30 32 34 36 38 40 42 44 46 48	
$Tmean_{48\ to\ 59}$	Mean temperature		48 50 52 54 56 58	
$Tmin_{35\ to\ 58}$	Minimum temperature		35 38 40 42 44 46 48 50 52 54 56 58	
$Tmin_{46\ to\ 59}$	Minimum temperature		46 48 50 52 54 56 58	
$Tmax_{37\ to\ 46}$	Maximum temperature		38 40 42 44 46	
$Tamp_{37\ to\ 47}$	Amplitude of temperature		38 40 42 44 46 48	
$RHmin_{38\ to\ 49}$	Minimum Relative Humidity		38 40 42 44 46 48	
$TR_{28\ to\ 40}$	Total Rainfall		28 30 32 34 36 38 40	

Based on the results of a pair correlation analysis presented in Appendix Figures A3.1 and A3.2, we discarded *Wetness Frequency* from three to 25 d.a.t. ($WF_{3\ to\ 25}$) and *Amplitude of Temperature* from eight to 23 d.a.t. ($Tamp_{8\ to\ 23}$) for H→D change, and *Amplitude of Temperature* from 37 to 47 d.a.t. ($Tamp_{37\ to\ 47}$) for D→S change.

3.3.2 Best fitted models construction

After model selection, $WF_{-14\ to\ -1}$, $Tmax_{4\ to\ 21}$ and the square of $Tmax_{4\ to\ 21}$ significantly predicted the H→D change (Table 3.3), and $Tmin_{35\ to\ 58}$ and $Tmax_{37\ to\ 46}$ the D→S change (Table 3.4). Model predictions of H→D change (Figure 3.7a) show that $WF_{-14\ to\ -1}$ had a positive relationship with the status change probability, except when $Tmax_{4\ to\ 21}$ was close to 24°C, when the probability of change was constantly null. With respect to $Tmax_{4\ to\ 21}$, there was a quadratic effect with a maximum at 30°C. However, the probability of change was low, with a maximum value of 0.55 predicted by the model. Model predictions of D→S change (Figure 3.7b) show that $Tmin_{35\ to\ 58}$ and $Tmax_{37\ to\ 46}$ are the most explanatory variables (microclimatic predictors) explaining the probability of status change. Increasing temperature amplitude seemed to favor lesion sporulation. Under La Lola weather conditions, the highest change probability (0.9) was found for an average minimum temperature of 20°C (35 to 58 d.a.t.) and an average maximum temperature of 33°C (37 to 47 d.a.t.).

Table 3.3. Results of the analysis of deviance of the best fitted model for pod status change from healthy to diseased with no signs of sporulation, from 40 to 50 days after tagging.

Model	**Df**	**AIC**	**logLik**	**deviance**	**χ2**	**χ2_Df**	**P**
Complete model	5	2182.2	-1086.1	2172.2			
$-WF_{-14 \text{ to } -1}$	4	2186.8	-1089.4	2178.8	6.5338	1	0.01058
$-Tmax_{4 \text{ to } 21}$	4	2194.8	-1093.4	2186.8	14.541	1	0.0001372
$-(Tmax_{4 \text{ to } 21})^2$	4	2193.7	-1092.9	2185.7	13.476	1	0.0002417
Null model	2	2201.4	-1098.7	2197.4	25.145	3	$1.44e^{-05}$

Df degrees of freedom, *AIC* Akaike

Table 3.4. Results of the analysis of deviance of the best fitted model for the pod status change from diseased with no signs of sporulation to diseased with sporulated lesions, 60 to 70 days after tagging.

Model	**Df**	**AIC**	**logLik**	**deviance**	**χ2**	**χ2_Df**	**P**
Complete model	4	2197.1	-1094.6	2189.1			
$Tmin_{35 \text{ to } 58}$	3	2196.4	-1095.2	2190.4	1.2285	1	0.01058
$Tmax_{37 \text{ to } 46}$	3	2200.7	-1097.4	2194.7	5.6032	1	0.0001372
Null model	2	2201.4	-1098.7	2197.4	8.2662	2	0.01603

Df degrees of freedom, *AIC* Akaike

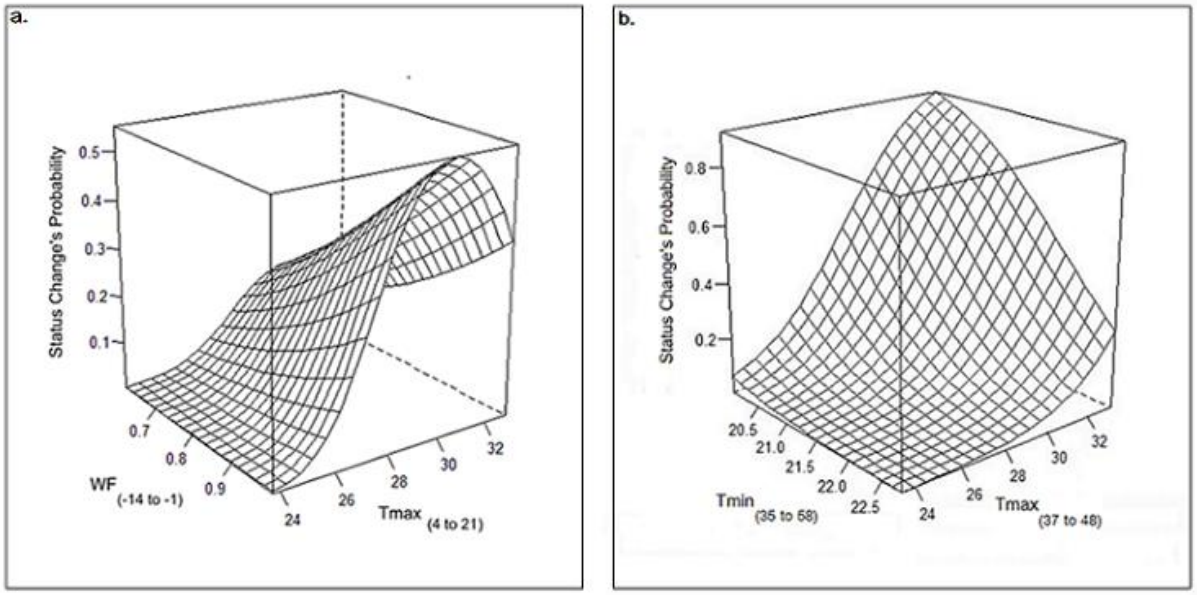


Figure 3.7. Best fitted model predictions: a) status change probability from healthy to diseased pod without sporulation between 40 to 50 days after tagging; b) status change probability from diseased pod without sporulation to diseased pod with sporulated lesions between 60 to 70 days after tagging (numbers in parentheses indicate the range of days of influence of each variable with respect to tagging.

3.4 DISCUSSION

3.4.1 Selected Studied Periods

Pound-7 is an extremely susceptible clone. Symptoms were observed very early (27.9% in the first 30 d.a.t., and for this same period, 4.4% of these pods were already sporulated). This has epidemiological and management implications. The removal of diseased pods, which is targeted toward the control of initial inoculum between pod generations (cohorts), should be accompanied with other control practices against secondary infection within the same pod generation, using protectant fungicides for instance.

However, the highest frequency of pod H→D change is from day 40 to 50 after tagging. This result is in accordance with previous reports indicating that appearance of symptoms occurs 40 to 60 days after infection. Since pods were healthy but in the most

susceptible stage at the starting moment, infection could occur right after tagging, i.e., when pods were about one or two months old (Ampuero 1967). According to Merchán (1981c), the most detectable symptom, the chocolate-colored spot, appears 75 days after infection and this lesion, as Phillips-Mora (2003) has stated, will be covered with mycelium and spores within four to five days. This information also matches our results (Figure 3.6) for D→S change.

3.4.2 Variables' Period of Influence

Our analysis highlighted key variables that affect the change status of pods in specific periods. These changes were observed in a regular, consistent and careful way. With this, it is considered that our results provide accurate information that supports the further conclusions of this work. The periods in which the preselected variables (included in the initial models) presented more influence for H→D change are between day three and day 25 after tagging, except for the wetness frequency. This means that during this period, the fungus is particularly active and its biological machinery and operation is very dependent on environmental conditions. Expression of symptoms appeared about 20 days after these variables produced their influence. For D→S change, the period of greatest influence is located between 28 to 58 d.a.t. This influence period is closer to the status change period (60 to 70 d.a.t.) than before (40 to 50 d.a.t) since the step between symptom appearance and sporulation is faster than between infection and symptom appearance, confirming that difference between the incubation and latency period is short (Evans 1981b).

3.4.3 Pod's Status Change: Healthy to Diseased

3.4.3.1 Wetness and relative humidity effects

Wetness frequency from 14 to one day before tagging is one of the two variables that explain the probability of the H→D change. López (1954), Chacín (1975), Merchán (1978b), Campuzano (1981a) and Galindo (1985) have stated that a film of water over the surface of the pod is necessary for germination to occur, since this factor stimulates the germ tube development. In conditions of high pressure of inoculum, germination and infection are then

likely to happen in very early stages of the pod. As pods of 3 to 10 cm in length were tagged, and assuming that they were between one and two months old, that means that we tagged apparently healthy pods that were already infected.

Relative humidity, despite being related to wetness frequency, did not appear to explain any status change since the experimental site is highly humid and the relative humidity variation is very low. On average, daily relative humidity was above 85%. Scherm and Van Bruggen (1994) have observed a similar situation studying the effect of the fluctuating temperatures on the latent period of lettuce downy mildew. In their experiment, they focused on the temperature since humidity and light were not limiting for the disease growth, but the authors considered that these factors may have had a stronger effect than temperature on the colonization and the latent period of the disease. Similarly, for MPR, relative humidity could have had a higher effect than observed, under conditions of larger variation of this variable. Our results are in accordance to those obtained by Phillips (1986), who demonstrated in pod bagging field experiments the importance of water film on the surface of the pods to allow the formation of the germ tube and to achieve penetration of the pathogen. It is reported that a relative humidity above 80% is optimal for fungus germination and growth (Campuzano 1981a; Castro 1989). These wetness and humidity conditions were normally achieved every day during our experiment.

3.4.3.2 Temperature effects

Maximum temperature was the only temperature variable retained in the model. Similarly to relative humidity, variation of minimum temperature was probably too narrow in the study location (21.7 ± 1.3°C) to explain the probability of pod status change. Maximum temperature, however, had larger variation (30.3 ± 2.7°C), and in some cases could reach detrimental values. Maximum temperature period of influence happened late: four to 20 days after tagging. This variable is likely more important after penetration, for the intra- and intercellular colonization of the pod by the pathogen and thus for the expression of the symptoms (Suárez 1971). We observed an optimum for this variable at approximately 30°C. Herrera (1988) reported that the ideal range for growth and sporulation of the colonies in culture medium V8 is 24 to 28°C, at constant temperatures. This seems compatible with our

results in field conditions. We believe that cool or high temperatures are harmful for the pathogen and inhibit the normal fungal development. While, to our knowledge, there are no reports on high maximum temperature effects on MPR in the field, there are indications that low temperatures and particularly low maximum temperatures are harmful to the disease. For instance, it is known that as altitude increases, the incidence of this disease goes down. Constant temperatures lower than 18°C severely limit the growth and sporulation of the pathogen under *in vitro* conditions (Phillips-Mora 2003).

3.4.4 Pod's Status Change: Diseased to Sporulated

3.4.4.1 Temperature effects

As known, conditions that favor germination and penetration of *M. roreri* are different from those that favor production and release of the inoculum (Phillips 1986). According to Hawker (1950), the optimum temperature range for fungal sporulation is always lower than the optimum temperature range for its growth.

According to our results, sporulation process is determined by the daily temperature amplitude: large temperature amplitudes increase the probability for lesions to sporulate. We hypothesized that the lower temperatures could promote the spore formation, based on the fact that the presence of water is needed for germination, but it must be probed. On the other hand, the higher temperatures could shorten the period between the first symptoms (humps) and the sporulation. This means that temperature could affect in different ways, according to the fungal process. Tomerlin *et al.* (1983) found a similar behavior when studying the effect of the temperature on the development of brown rust on wheat under controlled conditions. These authors have reported that temperature has distinct effects on the different disease stages: latent and infectious periods. In their case, warmer temperatures shortened the latent period but also the infectious period. According to these results, warmer temperatures promote the beginning of sporulation but are detrimental to the continual spore formation. MPR is known to have a very long infectious period that could reach several months, since it could produce different sporulation cycles over the same infected tissue (Enríquez 2004). This fungus has two convenient strategies (van Hall): 1) short period between appearance of symptoms and

spore production and 2) long infectious period. This double strategy makes the disease very difficult to control.

In addition, high thermal amplitudes are more likely to happen in periods with a low number of rainy days, which normally buffer temperatures (López-Bravo *et al.* 2012). Sporulation is then promoted in periods when wind dispersal is also feasible. Spores of *M roreri* are disseminated by a wind-dependent passive mechanism. In order for the spores to be liberated, low relative humidity is required. Without moisture, spores weigh less and could be easily removed from the pods by the air currents (Porras and González 1982; Schmitz 1984; Aylor 1990).

While for most pathogens relative humidity is determinant for the sporulation process, this variable did not appear in our results, perhaps because, as already explained, relative humidity is not a limiting factor in the experimental location. We think that the temperature effect over the sporulation represents valid knowledge to enrich a future epidemiological model. Lalancette *et al.* (2003) have mentioned that, sporulation models for predictive purposes are more efficient when the capacity of spores to be disseminated to new susceptible tissue and the infection potential are high, which is the case for MPR.

3.4.5 Comparing status change probabilities between healthy-to-diseased and diseased to sporulated lesions

Maximum probability value for the H→D change (0.55) is considerably lower than the maximum value predicted for D→S change (0.90). This could be considered understandable since lesions of the diseased pods will likely produce spores whereas healthy pods will not all become diseased. As a consequence, the prediction of new diseased pods is more difficult than the prediction of sporulation, knowing that pods are already infected. The low probability value exhibited by the final model for H→D change indicates that the model is more efficient for predicting unsuitable infection periods than propitious ones.

3.4.6 Methodological approach

Our results constitute a good basis for improving already existing conceptual models such as the one developed by Leach *et al.* (2002). This model is based on management and economics field dynamics and aims to evaluate net returns of different management strategies for Central American farmers, but it presents understandable limitations due to the lack of information on this disease. MPR epidemiological information is restricted since it does not include any microclimatic information. The incorporation of the microclimatic variables into conceptual models requires a precise understanding of the influence of these variables. The strength of our approach consists in the fact that there was no *a priori* on the influential period of each climatic variable on status-change probability. This means that the identification of the influential period is a result of our analysis and not a preset period established subjectively. Such an approach has recently been used in bananas, to study the influence of the temperature on the development of chilling injury during fruit growth (Bugaud *et al.* 2015). It has been also used by Carval *et al.* (2015) to study the effect of rainfall, temperature and biotic variables on the abundance of adult thrips on banana plants. Although this last research is more related to ours, we are the first to use this statistical approach for fungal epidemiological studies. Furthermore, the other advantage and strength of this statistical approach is that it allows analyzing every pod separately and not a proportion of incidence inside the population, offering more robust results and elevating the accuracy and power of the method.

Part II

3.5 INTRODUCTION

The plant immune system differs from the mammal somatic adaptive immune system because it lacks mobile defense cells. Instead, the plant immune system depends on every cell's innate immunity and signal transduction cascades triggered from infection sites. There are two branches of this system; the first one is the pattern-recognition receptor proteins (PRRs) inserted in the cell membrane, which recognize and respond to pathogen-associated molecular patterns (PAMPs), activating PAMP triggered immunity (PTI). The second branch acts largely in the cell's interior. There, most resistance genes (R genes), as members of the nucleotide-binding (NB), leucine-rich repeat (LRR) domain of NOD-like receptors (NLR), encode for effector proteins that can activate the effector-triggered immunity (ETI). This last type of immunity is only effective on obligate biotrophs or hemobiotrophs (Jones and Dangl 2006).

Plant genetic resistance to pathogens could be complete or incomplete. Complete resistance is determined by a single or a few host genes. According to Waller *et al.* (2002), this type of resistance may be overcome by genetic changes in the pathogen. Incomplete resistance is mostly determined by multiple genes with quantitative effects that trigger different reactions to protect the plant material from the fungal damage, reducing the severity of the disease without totally excluding it. The selection pressure of this kind of resistance is lower than the one exerted by major genes of complete resistance. However, incomplete resistance can be affected by the environmental conditions, especially climate (Zadoks and Van Leur 1983).

This situation is well-known in the case of the pathosystem *Coffea Arabica—Mycena citricolor.* All arabica coffee cultivars are susceptible to *M. citricolor;* however, different degrees of susceptibility/resistance have been detected. Cultivars derived from the Timor hybrid, which are resistant to coffee rust caused by *Hemileia vastatrix*, apparently are more susceptible to *M. citricolor* than the others, impeding their use even in certain suboptimal environments for the disease. Nonetheless, as soon as environmental conditions, especially

humidity, approach optimum for the fungus, the differences in susceptibility lessen (Wang and Avelino 1999; Avelino *et al.* 2007). Similarly, Eskes (1982) reports that coffee resistance to coffee rust also varies according to light intensity in one-year-old coffee seedlings. In other pathosystems, Bonman (1992) has stated that incomplete resistance to rice blast disease is greatly affected by the environment, specifically by night temperatures, duration of leaf-wetness, nitrogen fertilization, soil type and water deficit. Rubiales *et al.* (2012) have also found that faba bean resistance against *Ascochyta fabae* is unstable across environments, the result of a multi-environmental analysis evaluation to find sources of resistance in a germplasm collection. Similarly, different behaviors of host genotypes against pathogens in relation with environment (different sites, years, inoculum pressure) have also been highlighted in the case of the cassava pathosystem—*Xanthomonas axonopodis* pv. *Manihotis* (Banito et al. 2008), or *Euthamia graminifolia—Coleosporium asterum* (Price *et al.* 2004).

Therefore, genotype x environment interactions for incomplete resistance seem to be the rule. The mechanisms involved in the expression of incomplete resistance are not clear. Bonman (1992) has considered that environment may act at different levels, over the host physiology, the pathogen or the interaction of these two components. On that issue, Price *et al.* (2004) have developed an interesting proposal by fitting curves of infections levels as a function of inoculum density to logistic models for different genotypes. These authors stated that different shapes of the fitted curves can highlight different mechanisms of environmental influence on incomplete resistance. According to Parlevliet (1979), incomplete resistance can affect the frequency of host penetration, the rate of development from propagule to lesion and/or the number of propagules produced on these lesions. Each of these stages can be influenced by meteorological factors in a different manner, explaining the genotype x environment interactions observed.

Genotype x environment interactions raise question about the specific resistant cultivars that can be distributed in different environments. The use of specific cultivars exhibiting incomplete resistance can be invalidated in specific environments where this resistance is not efficient. This question is also valid for the case of the pathosystem *Theobroma cacao—Moniliophthora roreri,* for which no complete resistance has been found.

However, several degrees of incomplete resistance have been highlighted in different promising host genotypes (Porras Umaña 1985; Phillips 1986) whose resistance could be influenced by different meteorological conditions.

In this study, we analyzed the influence of microclimate on the expression of the incomplete resistance to Moniliophthora pod rot (MPR). We used three different, highly productive clones, with different levels of incomplete resistance characterized by the MPR incidence from 2007 to 2011: 1) Pound-7, with an average incidence of 86%, 2) CC-137, with an average incidence of 43%, and 3) CATIE-R4, with an average incidence of 12%. These clones, on average, maintain their level of resistance, but in specific years, their behavior changes drastically. These differences sometimes have been observed for the three clones and in other cases, only one of them was affected. From this study, we will deduce whether different mechanisms of incomplete resistance are involved in these host genotypes and the conditions for their deployment in the field.

3.6 RESEARCH OBJECTIVE

To compare MPR development, symptoms onset of the disease and fungal sporulation for three cacao clones in a range of incomplete resistance—Pound-7 (highly susceptible), CC-137 (moderately resistant) and CATIE-R4 (highly resistant)—and understand the influence of different microclimatic variables on this development.

3.7 RESEARCH HYPOTHESIS

MPR development, symptoms onset of the disease and fungal sporulation, of three cacao clones in a range of incomplete resistance—Pound-7 (highly susceptible), CC-137 (moderately resistant) and CATIE-R4 (highly resistant)—are under the influence of different microclimatic variables.

3.8 MATERIALS AND METHODS

Data were collected at the same place and in the same moment as data in the first part of chapter 3. In this part, we only include the observations from the other two clones: 1) CC-137, a MPR moderately resistant clone (32% of average incidence) with an average production of 990 kg/ha/yr and 2) CATIE-R4, a MPR highly resistant clone (9% of average incidence) with an average production of 1336 kg/ha/yr. All of these clones are considered as highly productive, thus ensuring the presence of pods throughout the year. This information was obtained from a historical data average of 11 years from the CATIE Cacao Improvement Program (Phillips-Mora *et al.* 2013).

Materials and methods of this part were already described in section 3.2, part I, chapter 3. However, some changes were applied to this section, described below.

3.8.1 Studied periods

The first stage of the analysis considers the identification of the period of major status-change occurrence from healthy pods to diseased pods without sporulation (H→D) and from diseased pods without sporulation to pods with sporulated lesions (D→S). Clones CC-137 and CATIE- R4 had the most number of status changes in the same periods. For H→D, we found the highest number of status changes in a period of 10 days, from 40 to 50 days after tagging (d.a.t) and for D→S, 60 to 70 d.a.t. (Figures 3.8 and 3.9). For the CC-137 clone, 14.6% of H→D changes occurred during the 40 to 50 d.a.t. period and 19.7% of D→S changes occurred during the 60 to 70 d.a.t. period (Figure 3.8). For the CC-137 clone, 14.6% of H→D changes occurred during the 40 to 50 d.a.t. period and 19.7% of D→S changes occurred during the 60 to 70 d.a.t. period (Figure 3.9).

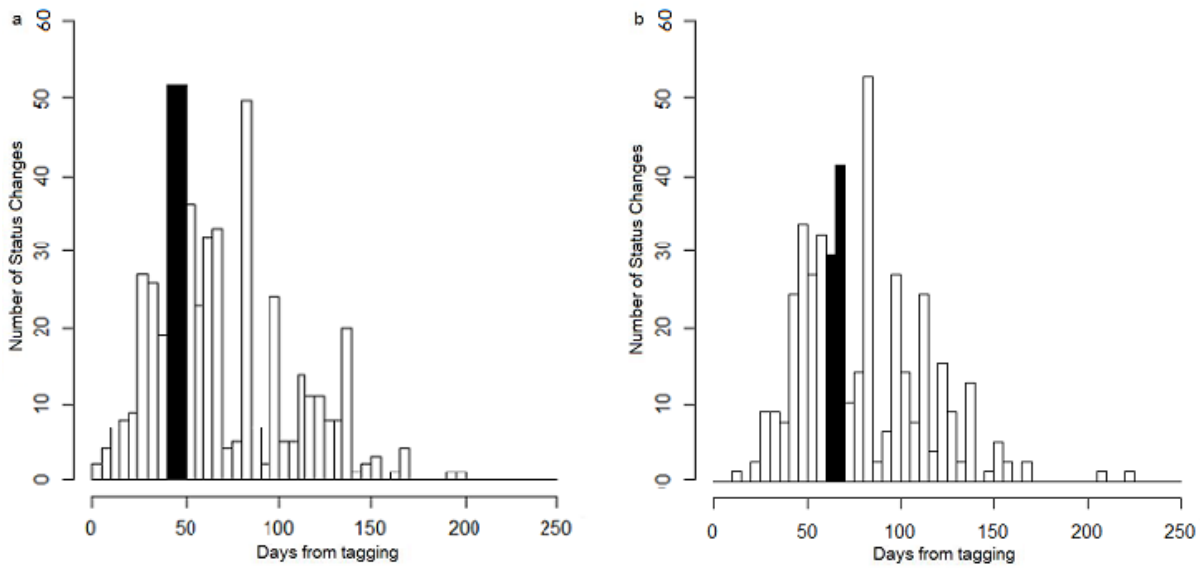


Figure 3.8. Histograms for the selection of the studied periods for CC-137: a. corresponds to pod status change from healthy to diseased with no signs of sporulation, b. corresponds to pod status change from diseased with no signs of sporulation to sporulated lesions.

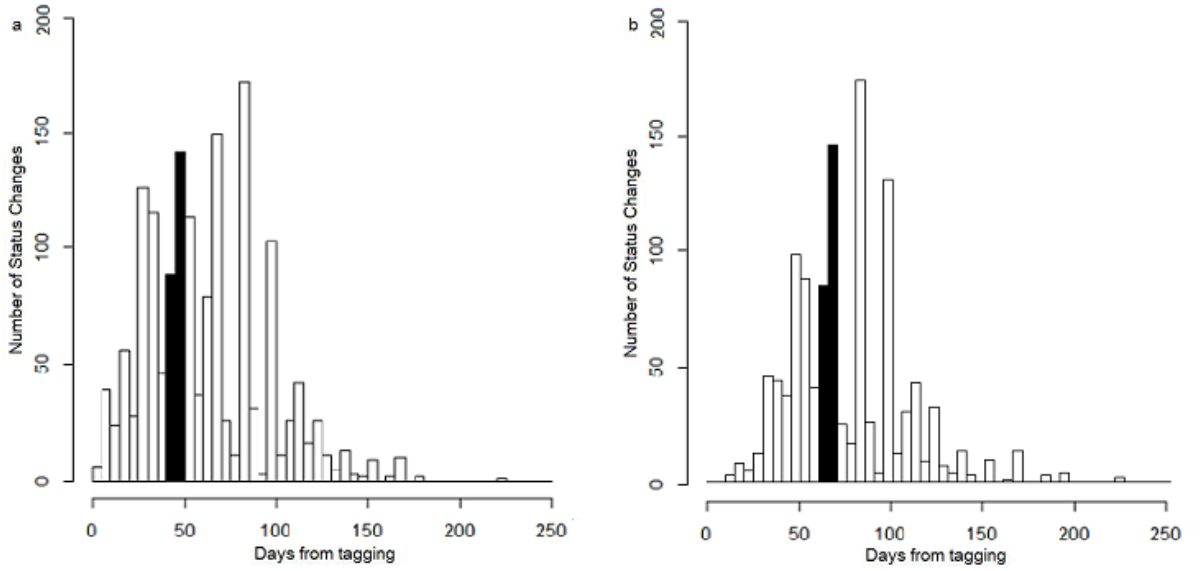


Figure 3.9. Histograms for the selection of the studied periods for CATIE-R4: a. corresponds to pod status change from healthy to diseased with no signs of sporulation, b. corresponds to pod status change from diseased with no signs of sporulation to sporulated lesions.

3.8.2 Statistical analyses

Descriptive analyses were first conducted to highlight different behaviors of disease incidence of each studied clone. For that purpose, incidence curves were built for all of the 55 generations of pods observed in this experiment. Generations with nonconventional clonal behavior were selected in order to illustrate our hypothesis that environment could affect cacao's incomplete resistance to MPR.

The methodological approach was almost the same as implemented in part I (section 3.2). However, in the statistical analyses, a change was made. For the single predictor analysis (section 3.2.5.1) and the complete analysis (section 3.2.5.2), a generalized linear model (GLM) was used instead of a generalized linear mixed model (GLMM), meaning that *generation* was not included in these analyses as a random factor since models did not converge with the GLMM analyses. This could possibly be due to the fewer number of status changes that occurred for CC-137 and CATIE-R4, which are more resistant than Pound-7.

3.8.2.1 Single predictor GLM analysis

In this part, *mean relative humidity, maximum relative humidity* and *amplitude of relative humidity* were not excluded from the analyses. Dates and durations of every variable were selected from Figures 3.10 and 3.11 for the CC-137 clone and from Figures 3.12 and 3.13 for the CATIE-R4 clone.

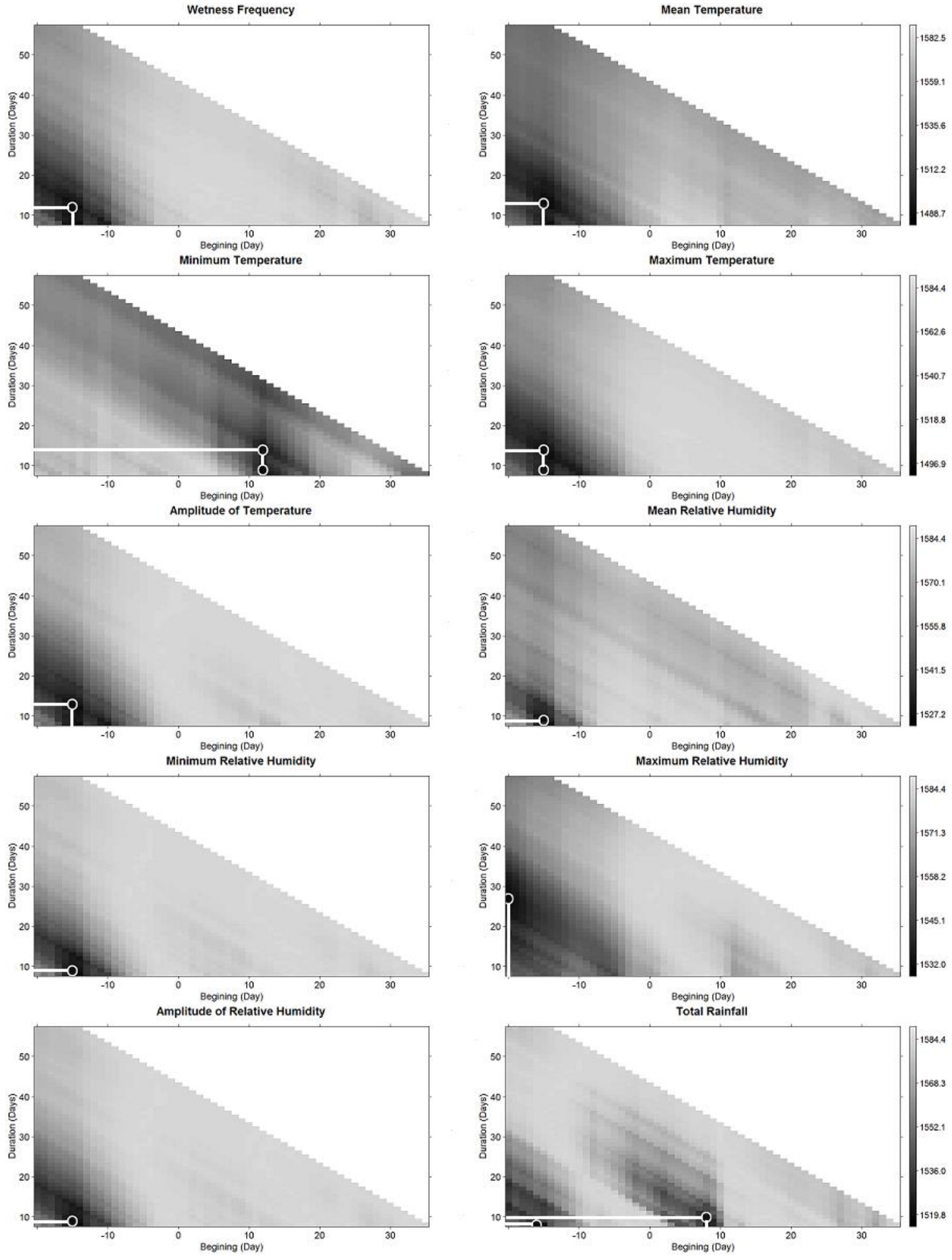
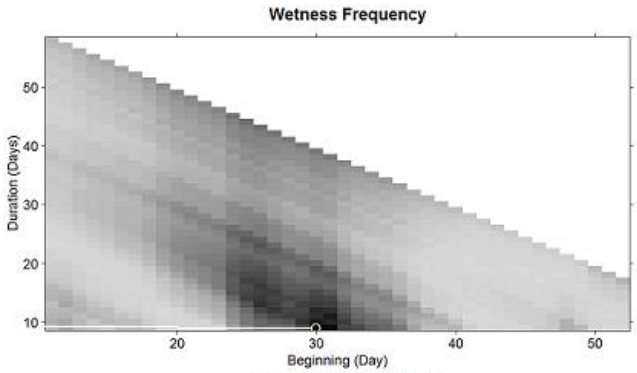
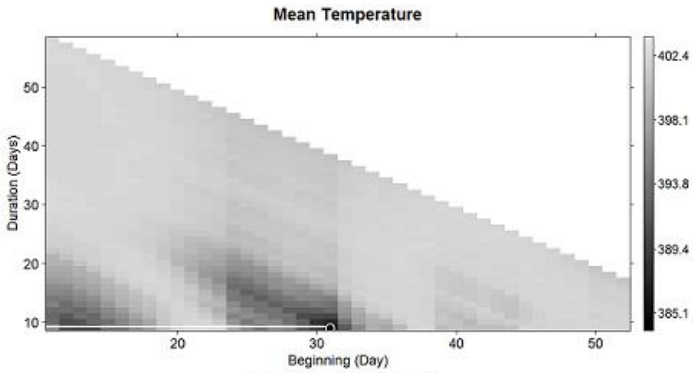
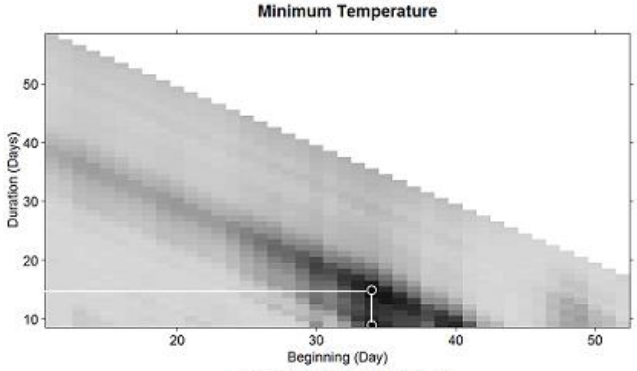

Wetness Frequency
Mean Temperature
Minimum Temperature
Maximum Temperature
Amplitude of Temperature
Mean Relative Humidity
Minimum Relative Humidity
Maximum Relative Humidity
Amplitude of Relative Humidity
Total Rainfall
Duration (Days)
Begining (Day)
10
20
30
40
50
-10
0
10
20
30
1582.5
1559.1
1535.6
1512.2
1488.7
1584.4
1562.6
1540.7
1518.8
1496.9
1584.4
1570.1
1555.8
1541.5
1527.2
1584.4
1571.3
1558.2
1545.1
1532.0
1584.4
1568.3
1552.1
1536.0
1519.8

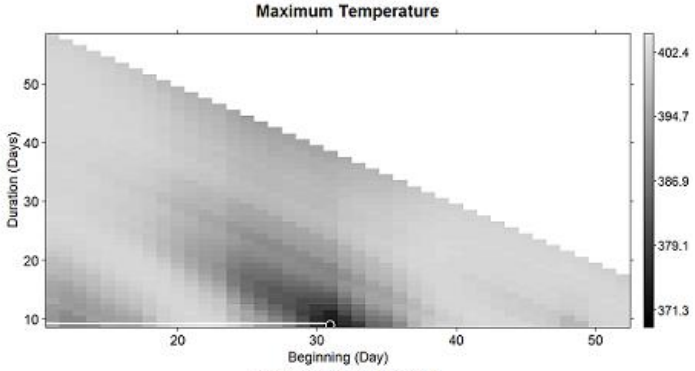
Figure 3.10. Period of influence of each daily variable on pod status change, from healthy to diseased with no signs of sporulation, 40 to 50 days after tagging, for CC-137.

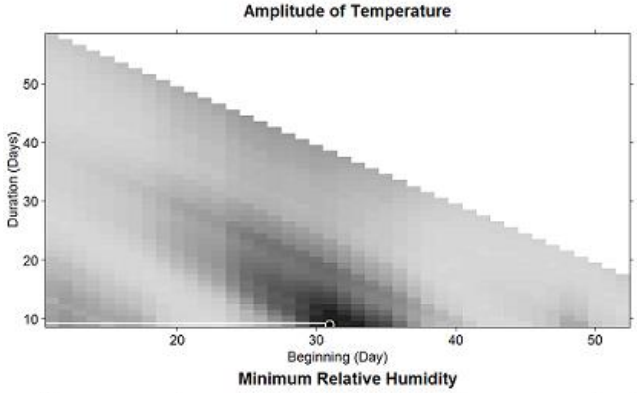
By period of influence we meant from the starting day with respect to tagging and duration from this day. The figure represents the AIC values of the binomial GLMs explaining pod status change from tagging for each period of influence. On the starting date axis, zero corresponds to the tagging date of pods of 3 to 10 cm in length. Circles indicate the lowest AIC value and the best microclimatic predictors of pod status change (period of influence). The presence of a delimited surrounded black to gray zone indicates a zone of decreasing influence of the variable. Gray scale on the right represents the AIC values. Absence of circle indicates that no clear influence zone was identified.

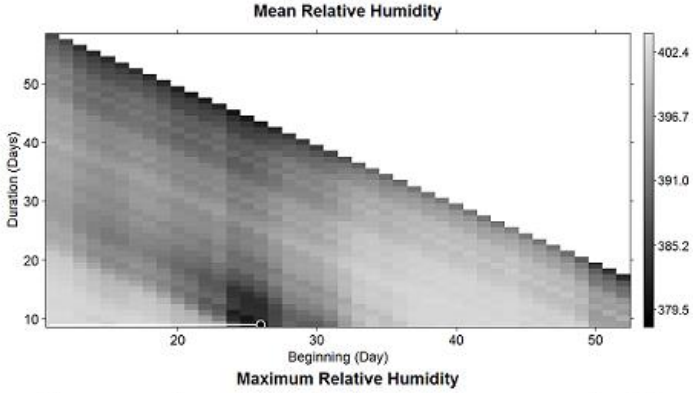

Wetness Frequency
Duration (Days)
Beginning (Day)

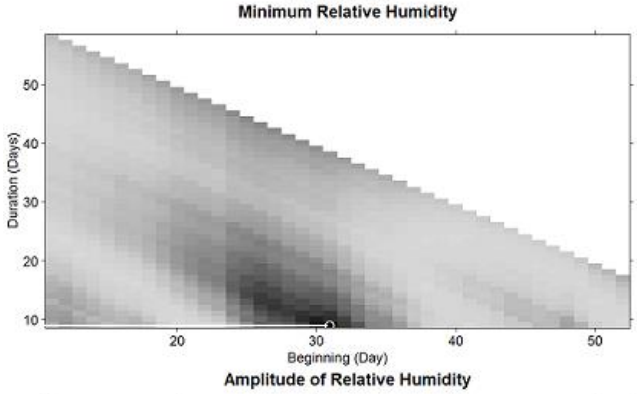

Mean Temperature
Duration (Days)
Beginning (Day)
402.4
398.1
393.8
389.4
385.1

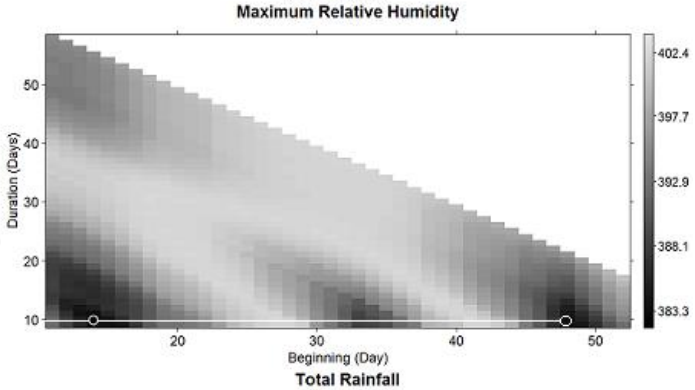

Minimum Temperature
Duration (Days)
Beginning (Day)

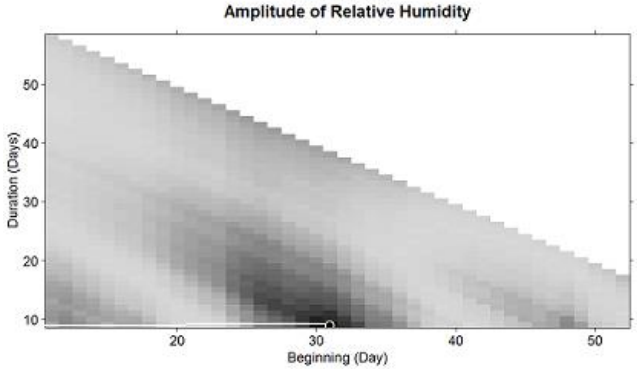

Maximum Temperature
Duration (Days)
Beginning (Day)
402.4
394.7
386.9
379.1
371.3


Amplitude of Temperature
Duration (Days)
Beginning (Day)


Mean Relative Humidity
Duration (Days)
Beginning (Day)
402.4
396.7
391.0
385.2
379.5


Minimum Relative Humidity
Duration (Days)
Beginning (Day)


Maximum Relative Humidity
Duration (Days)
Beginning (Day)
402.4
397.7
392.9
388.1
383.3


Amplitude of Relative Humidity
Duration (Days)
Beginning (Day)

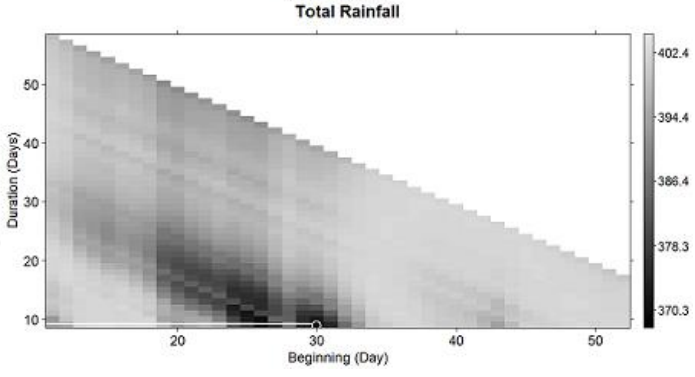

Total Rainfall
Duration (Days)
Beginning (Day)
402.4
394.4
386.4
378.3
370.3

Figure 3.11. Period of influence of each daily variable on pod status change, from diseased with no signs of sporulation to diseased with sporulated lesions, 60 to 70 days after tagging, for CC-137.

By period of influence, we meant from the starting day with respect to tagging and duration from this day. The figure represents the AIC values of the binomial GLM, explaining pod status change from tagging for each period of influence. On the starting date axis, zero corresponds to the tagging date of pods 3 to 10 cm in length. Circles indicate the lowest AIC value and the best microclimatic predictors of pod status change (period of influence). The presence of a delimited surrounded black to gray zone indicates a zone of decreasing influence of the variable. Gray scale on the right represents the AIC values. Absence of circle indicates that no clear influence zone was identified.

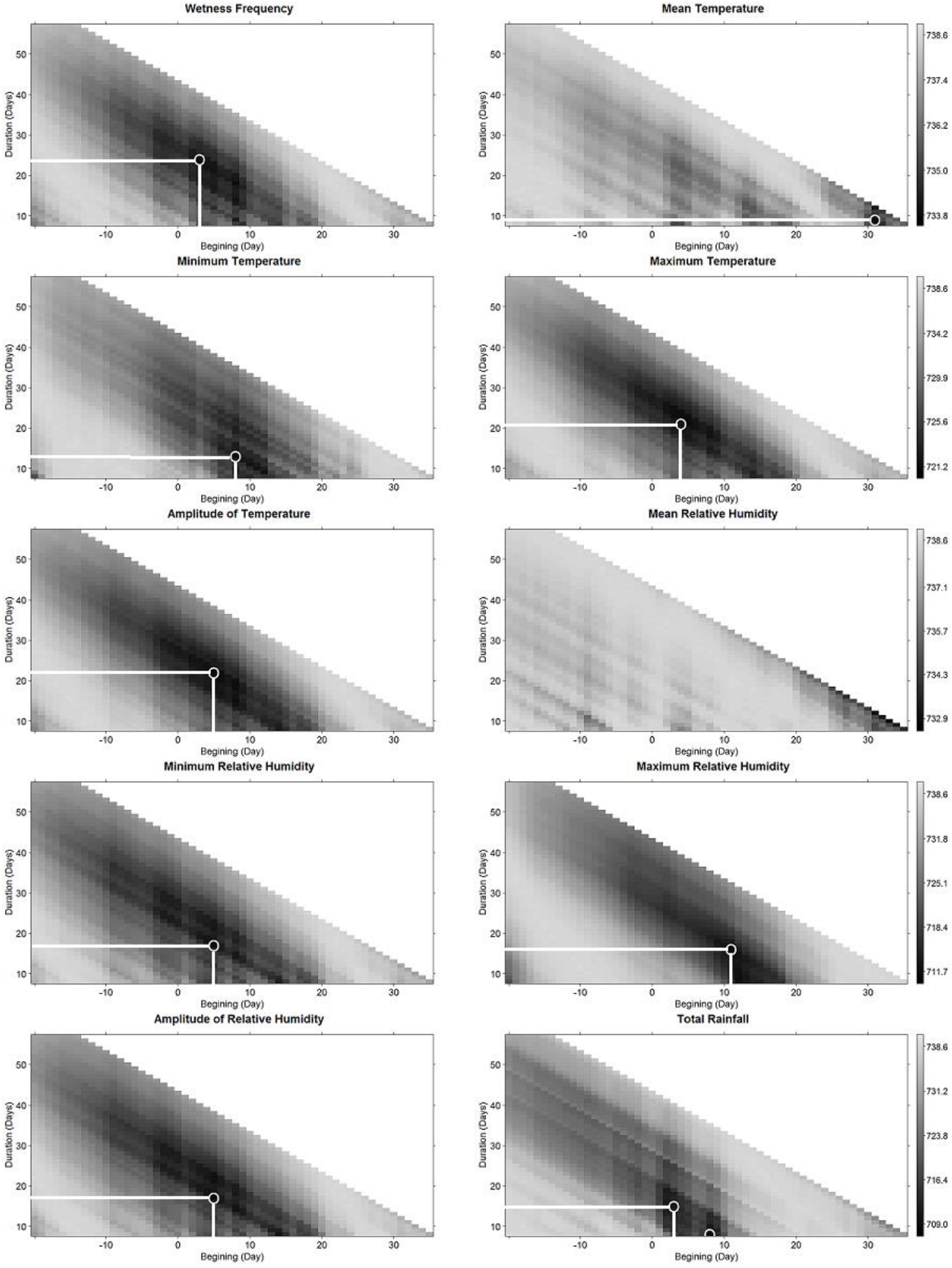

Wetness Frequency
Mean Temperature
Minimum Temperature
Maximum Temperature
Amplitude of Temperature
Mean Relative Humidity
Minimum Relative Humidity
Maximum Relative Humidity
Amplitude of Relative Humidity
Total Rainfall
Duration (Days)
Begining (Day)
10
20
30
40
50
-10
0
10
20
30
738.6
737.4
736.2
735.0
733.8
738.6
734.2
729.9
725.6
721.2
738.6
737.1
735.7
734.3
732.9
738.6
731.8
725.1
718.4
711.7
738.6
731.2
723.8
716.4
709.0

Figure 3.12. Period of influence of each daily variable on pod status change, from healthy to diseased with no signs of sporulation, 40 to 50 days after tagging, for CATIE-R4.

By period of influence, we meant from the starting day with respect to tagging and duration from this day. The figure represents the AIC values of the binomial GLMs explaining pod status change from tagging for each period of influence. On the starting date axis, zero corresponds to the tagging date of pods 3 to 10 cm in length. Circles indicate the lowest AIC value and the best microclimatic predictors of pod status change (period of influence). The presence of a delimited surrounded black to gray zone indicates a zone of decreasing influence of the variable. Gray scale on the right represents the AIC values. Absence of circle indicates that no clear influence zone was identified.

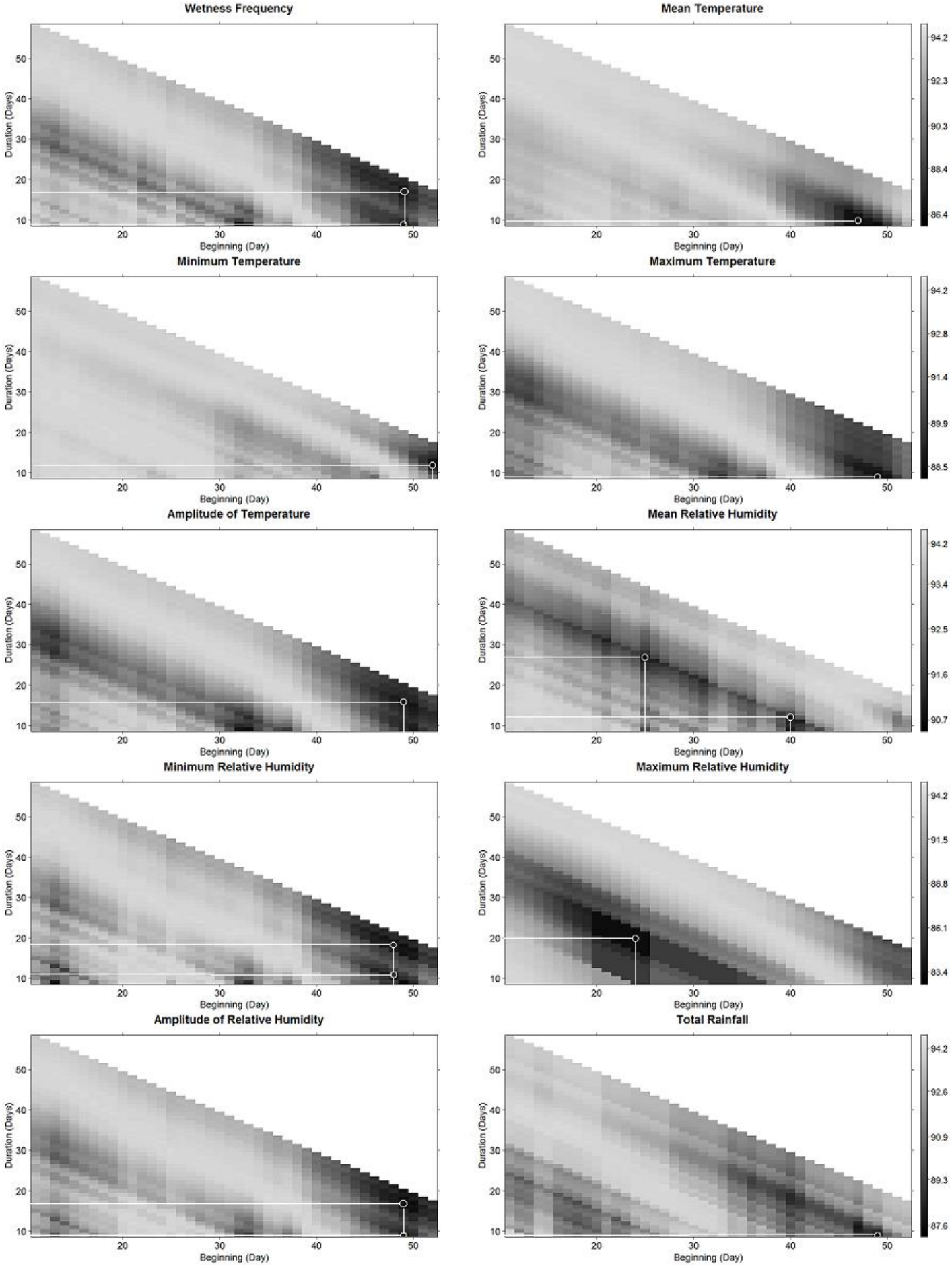

Wetness Frequency
Mean Temperature
Minimum Temperature
Maximum Temperature
Amplitude of Temperature
Mean Relative Humidity
Minimum Relative Humidity
Maximum Relative Humidity
Amplitude of Relative Humidity
Total Rainfall
Duration (Days)
Beginning (Day)
10
20
30
40
50
94.2
92.3
90.3
88.4
86.4
92.8
91.4
89.9
88.5
93.4
92.5
91.6
90.7
91.5
88.8
86.1
83.4
92.6
90.9
89.3
87.6

Figure 3.13. Period of influence of each daily variable on pod status change, from diseased with no signs of sporulation to diseased with sporulated lesions, 60 to 70 days after tagging, for CATIE-R4.

By period of influence, we meant from the starting day with respect to tagging and duration from this day. The figure represents the AIC values of the binomial GLMs explaining pod status change from tagging for each period of influence. On the starting date axis, zero corresponds to the tagging date of pods 3 to 10 cm in length. Circles indicate the lowest AIC value and the best microclimatic predictors of pod status change (period of influence). The presence of a delimited surrounded black to gray zone indicates a zone of decreasing influence of the variable. Gray scale on the right represents the AIC values. Absence of circle indicates that no clear influence zone was identified.

3.9 RESULTS

3.9.1 Resistance clonal behavior during key moments

Figure 3.14 represents four different pod generations (6, 19, 24 and 37), in which some of these clones showed an uncommon resistance behavior. During Generation 6, as shown in Figure 3.14a, the three clones are at low incidence, especially Pound-7, which reduced its incidence by almost half. During Generation 19 (Figure 3.14b), the contrary occurred, and all of the clones showed high incidences, especially CATIE-R4, which reached almost 60% of incidence—completely unexpected. For Generations 24 and 37 (Figures 3.14c and 3.14d), it was observed that CC-137 incidence could fluctuate even when the other two clones presented expected behavior.

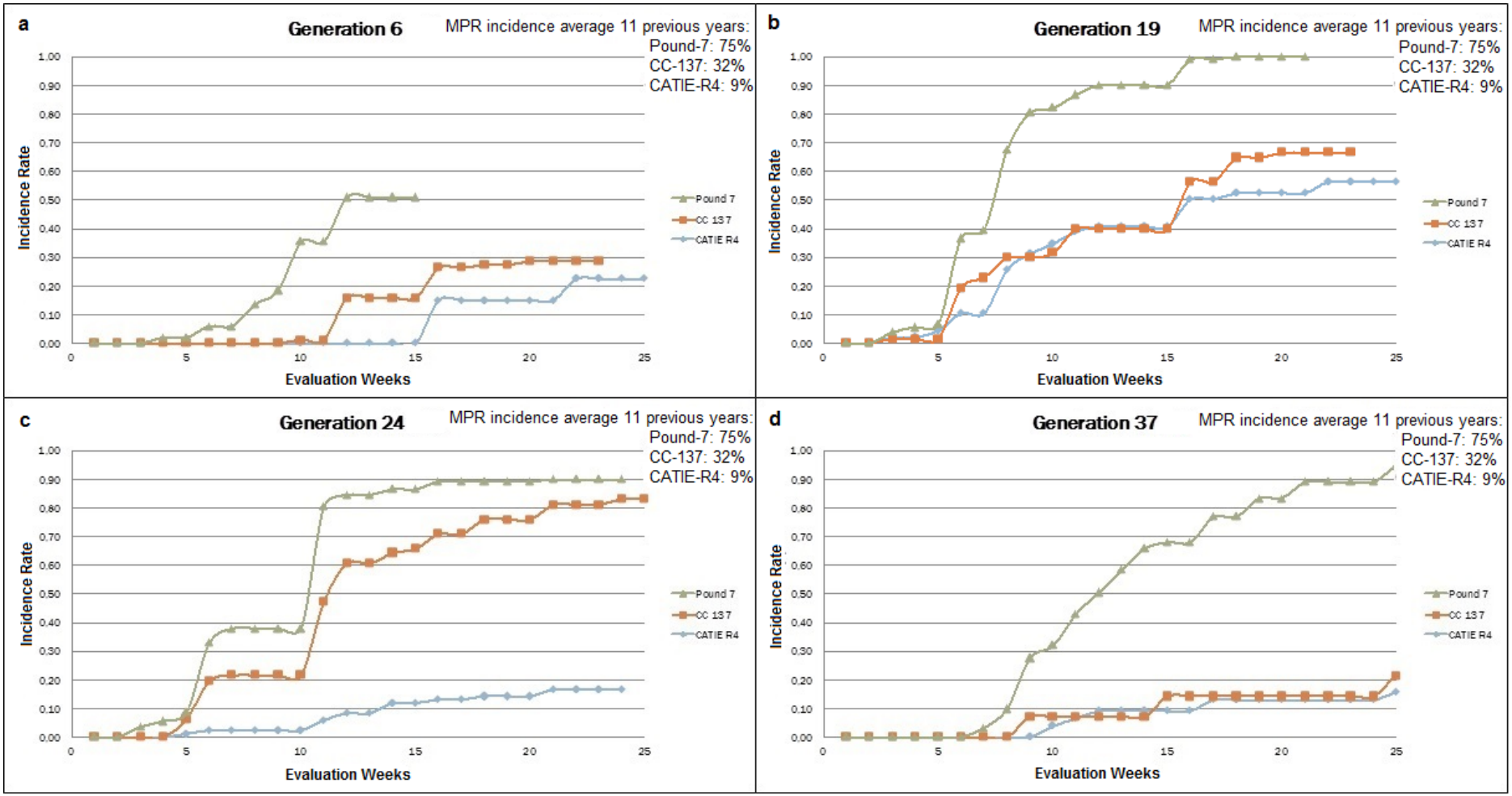


Figure 3.14. Reduction of the incidence of the clones Pound-7, CC-137 and CATIE-R4 compared with their averages: a. Generation 6 (young pods 3 to 10cm in length tagged July 8–14, 2012), b. Generation 19 (young pods 3 to 10 cm in length tagged October 7–13, 2012), c. Generation 24 (young pods 3 to 10cm in length tagged November 11–17, 2012), d. Generation 37 (young pods 3 to 10cm in length tagged February 10–16, 2013).

3.9.2 Selection of the Period of Effect of each Microclimatic Variable

In the case of H→D change of clone CC-137, the period of influence of almost all of the variables included the period from -20 to -2 d.a.t., except for minimum temperature (from 12 to 26 d.a.t., Table 3.5) and total rainfall (from 8 to 18 d.a.t., Table 14). In the case of D→S change, the period of influence of all variables included the period from 28 to 58 d.a.t. (Table 3.6).

Table 3.5. Selected microclimatic predictors (starting date and duration) of pod status change from healthy to diseased with no sign of sporulation, from 40 to 50 days after tagging, for clone CC-137.

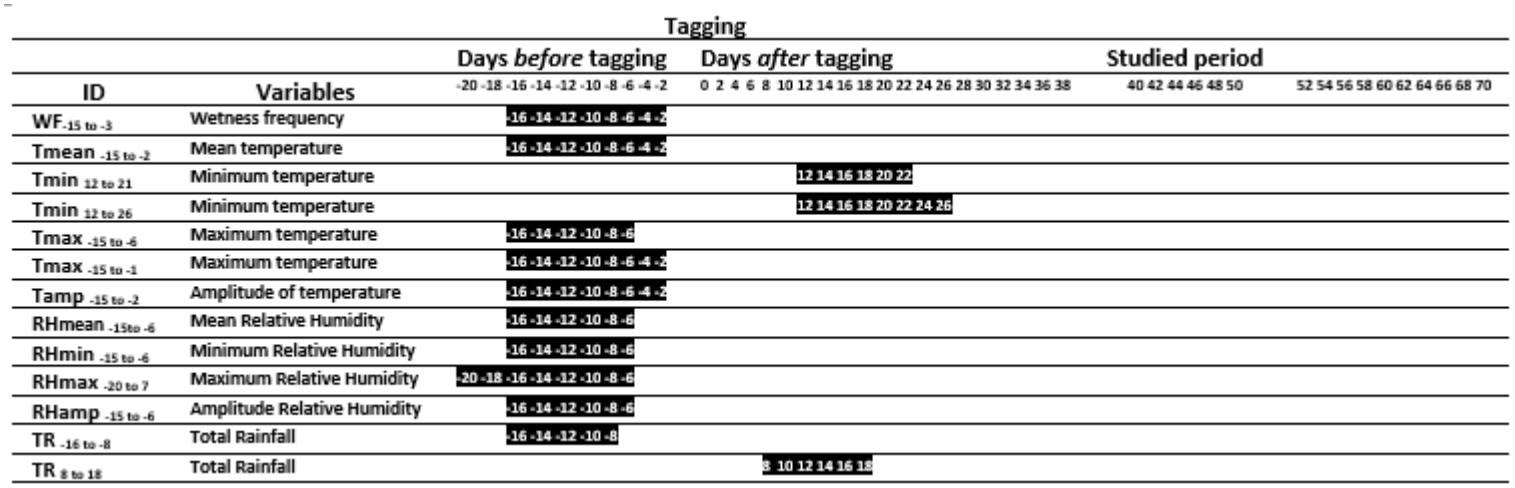
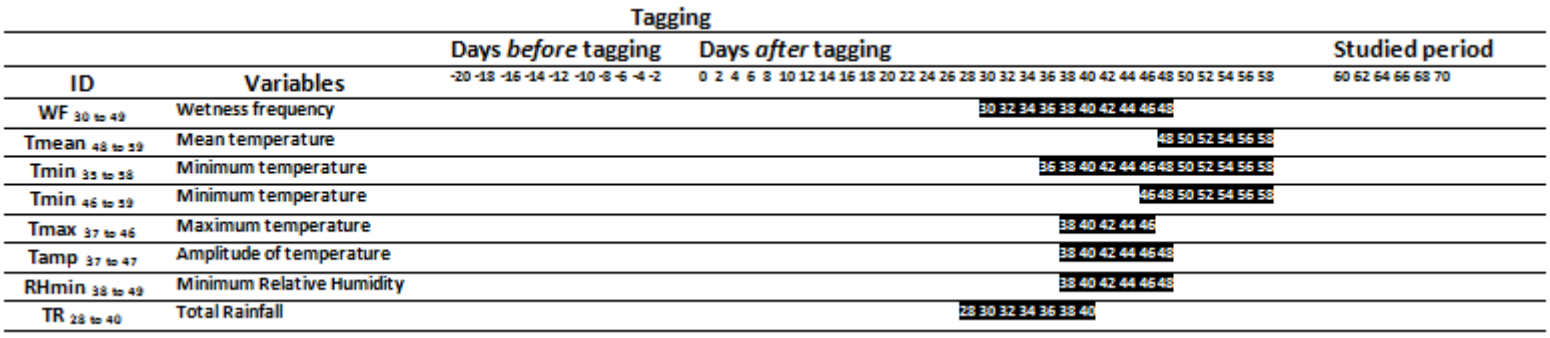


Table 3.6. Selected microclimatic predictors (starting date and duration) of pod status change from diseased with no sign of sporulation to diseased with sporulated lesions, from 60 to 70 days after tagging, for clone CC-137.



In the case of H→D change of clone CATIE-R4, the period of influence, of almost all variables included the period from 2 to 28 d.a.t., except for mean temperature (from 30 to 40 d.a.t., Table 3.7). In the case of D→S change, the period of influence of almost all variables included the period from 46 to 68 d.a.t., except for mean, minimum and maximum relative humidity that included the period from 24 to 52 d.a.t. (Table 3.8).

Table 3.7. Selected microclimatic predictors (starting date and duration) of pod status change from healthy to diseased with no sign of sporulation, from 40 to 50 days after tagging, for clone CATIE-R4.

		Tagging		
		Days *before* tagging	Days *after* tagging	Studied period
ID	Variables	-20 -18 -16 -14 -12 -10 -8 -6 -4 -2	0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38	40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70
$WF_{3\ to\ 27}$	Wetness frequency		2 4 6 8 10 12 14 16 18 20 22 24 26 28	
$Tmean_{31\ to\ 40}$	Mean temperature		30 32 34 36 38	40
$Tmin_{8\ to\ 21}$	Minimum temperature		8 10 12 14 16 18 20 22	
$Tmax_{4\ to\ 25}$	Maximum temperature		4 6 8 10 12 14 16 18 20 22 24 26	
$Tamp_{5\ to\ 27}$	Amplitude of temperature		4 6 8 10 12 14 16 18 20 22 24 26 28	
$RHmin_{5\ to\ 22}$	Minimum Relative Humidity		4 6 8 10 12 14 16 18 20 22	
$RHmax_{11\ to\ 27}$	Maximum Relative Humidity		12 14 16 18 20 22 24 26 28	
$RHamp_{5\ to\ 22}$	Amplitude Relative Humidity		4 6 8 10 12 14 16 18 20 22	
$TR_{8\ to\ 16}$	Total Rainfall		8 10 12 14 16	
$TR_{3\ to\ 18}$	Total Rainfall		2 4 6 8 10 12 14 16 18	

Table 3.8. Selected microclimatic predictors (starting date and duration) of pod status change from diseased with no sign of sporulation to diseased with sporulated lesions, from 60 to 70 days after tagging, for clone CATIE-R4.

		Tagging		
		Days *before* tagging	Days *after* tagging	Studied period
ID	Variables	-20 -18 -16 -14 -12 -10 -8 -6 -4 -2	0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58	60 62 64 66 68 70
$WF_{49\ to\ 58}$	Wetness frequency		48 50 52 54 56 58	
$WF_{49\ to\ 58}$	Wetness frequency		48 50 52 54 56 58	60 62 64 66
$Tmean_{47\ to\ 57}$	Mean temperature		46 48 50 52 54 56 58	
$Tmin_{52\ to\ 64}$	Minimum temperature		52 54 56 58	60 62 64
$Tmax_{49\ to\ 58}$	Maximum temperature		48 50 52 54 56 58	
$Tamp_{49\ to\ 65}$	Amplitude of temperature		48 50 52 54 56 58	60 62 64 66
$RHmean_{25\ to\ 52}$	Mean Relative Humidity		24 26 28 30 32 34 36 38 40 42 44 46 48 50 52	
$RHmean_{40\ to\ 52}$	Mean Relative Humidity		40 42 44 46 48 50 52	
$RHmin_{48\ to\ 59}$	Minimum Relative Humidity		48 50 52 54 56 58	60
$RHmin_{48\ to\ 68}$	Minimum Relative Humidity		48 50 52 54 56 58	60 62 64 66 68
$RHmax_{24\ to\ 44}$	Maximum Relative Humidity		24 26 28 30 32 34 36 38 40 42 44	
$RHapm_{49\ to\ 58}$	Amplitude of Relative Humidity		48 50 52 54 56 58	
$RHapm_{49\ to\ 67}$	Amplitude of Relative Humidity		48 50 52 54 56 58	60 62 64 66 68
$TR_{49\ to\ 58}$	Total Rainfall		48 50 52 54 56 58	

3.9.3 Best fitted models construction

After model selection, for clone CC-137, $WF_{-15 \text{ to } -3}$, $Tmin_{12 \text{ to } 26,}$ and $RHmin_{-15 \text{ to } -6}$ and the square of the three of them significantly predicted H→D change. $Tmin_{34 \text{ to } 49}$, its square value and $TR_{30 \text{ to } 39}$ significantly predicted D→S change. Model predictions of H→D change (Figure 3.15a and 3.15b) show that $WF_{-15 \text{ to } -3}$ and $RHmin_{-15 \text{ to } -6}$ had a positive relationship with the status change probability. On the other hand, $Tmin_{12 \text{ to } 26}$ had a negative relationship with status change probability. As with the clone Pound 7, the probability of change was low, with a maximum value of 0.55 predicted by the model. Model predictions for D→S change (Figure 3.15c) show that $Tmin_{34 \text{ to } 49}$ and $TR_{30 \text{ to } 39}$ were the most explanatory variables (microclimatic predictors) for explaining the probability of status change. Both variables had a negative relationship with the status change probability. In La Lola weather conditions, the highest change probability (0.6) was found when minimum temperature was about 20°C (34 to 49 d.a.t.) and total rainfall was 0 (30 to 39 d.a.t.).

For clone CATIE-R4, $Tmin_{8 \text{ to } 21}$ and its square value and $Tmax_{4 \text{ to } 25}$ significantly predicted H→D change. $Tmax_{49 \text{ to } 58}$ and $RHmean_{40 \text{ to } 52}$ and the square value of both of them significantly predicted D→S change. Model predictions of H→D change (Figure 3.16a) show that $Tmin_{8 \text{ to } 21}$ had a negative relationship with the status change probability. On the other hand, $Tmax_{4 \text{ to } 25}$ had a positive relationship with the status change probability. For this clone, the probability of change was considerably low when compared with the other two clones, with a maximum value of 0.14 predicted by the model. Model predictions for D→S change (Figure 3.16b) showed that $Tmax_{49 \text{ to } 58}$ and $RHmean_{40 \text{ to } 52}$ were the most explanatory variables (microclimatic predictors) for explaining the probability of status change. $Tmax_{49 \text{ to } 58}$ had a negative relationship with the status change probability and $RHmean_{40 \text{ to } 52}$ had a positive relationship. In La Lola weather conditions, the highest change probability (0.5) was found when maximum temperature was about 23°C (49 to 58 d.a.t.) and mean relative humidity was 97% (40 to 52 d.a.t.).

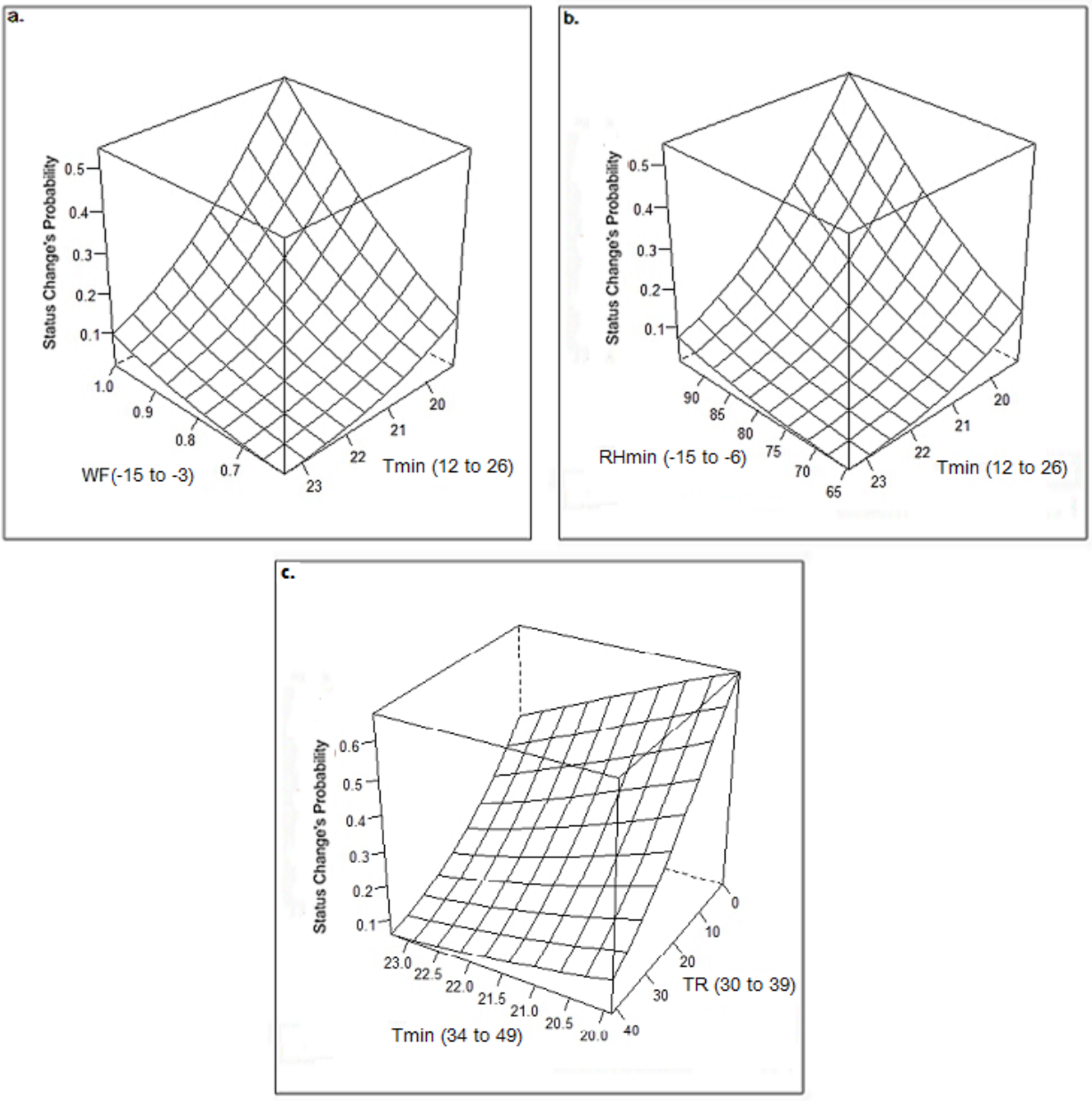


Figure 3.15. Best fitted model predicts for CC-137: a. status change probability from healthy to diseased pod without sporulation between 40 to 50 days after tagging, b. status change probability from healthy to diseased pod without sporulation between 40 to 50 days after tagging, c. status change probability from diseased pod without sporulation to diseased pod with sporulated lesions between 60 to 70 days after tagging. Numbers between parentheses indicate the range of days of influence of each variable with respect to tagging.

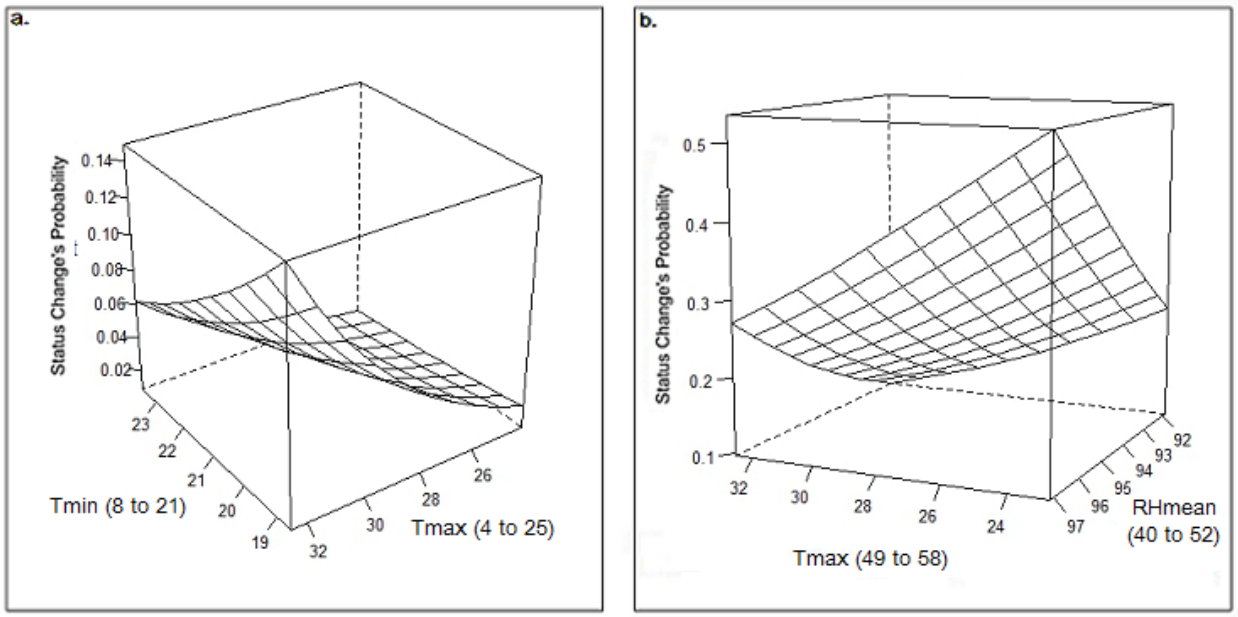


Figure 3.16. Best fitted model predicts for CATIE-R4: a. status change probability from healthy to diseased pod without sporulation between 40 to 50 days after tagging, b status change probability from diseased pod without sporulation to diseased pod with sporulated lesions between 60 to 70 days after tagging. Numbers between parentheses indicate the range of days of influence of each variable with respect to tagging.

3.10 DISCUSSION

3.10.1 Genotype-environment interaction

Figure 3.14 clearly illustrates the existence of an interaction between cacao incomplete resistance to MPR and the environment, showing uncommon behaviors of the three genotypes against the pathogen in determinate generations. Differences within generations are due to the environment, especially climate. Figures 3.14a and 3.14b, corresponding to Generations 6 and 19, respectively, show that the three clones could significantly increase or decrease their MPR incidence as a group due to environmental conditions. Differences in resistance among these clones lie in the number of resistant genes accumulated; however, the resistance of the three may be affected under certain environmental condition. This influence is reported as typical for this type of resistance (Zadoks and Van Leur 1983) and has been described in other pathosystems by several authors (Eskes 1982; Eskes and Toma-Braghini 1982; Bonman 1992; Banito *et al.* 2008; Rubiales *et al.* 2012). On the other hand, Figures 3.14c and 3.14d,

corresponding to Generations 24 and 37, respectively, show a different scenario. Only CC-137, considered a moderately resistant clone, presents an atypical behavior. This result confirms the conclusion made by Porras Umaña (1985) and confirmed by Phillips (1986) that highly resistant or highly susceptible genotypes are very stable, while intermediate clones, such as CC-137, vary according to climatic conditions and inoculum pressure. Our result also agrees with those obtained by Price *et al.* (2004) describing the resistance of *Euthamia graminifolia* against leaf rust.

3.10.2 Resistance mechanisms against MPR

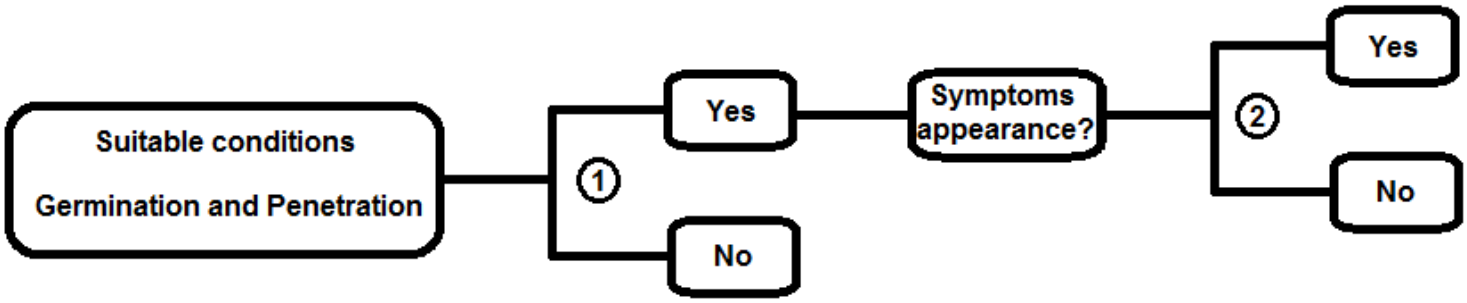


Figure 3.17. MPR infection diagram. 1 and 2 indicate the moment of infection and symptoms onset.

The analyses of the influence of the microclimatic variables highlighted two important events where resistance strategies could be developed. These events are indicated by numbers 1 and 2 in Figure 3.17. Number 1 indicates the infection (fungal germination and penetration), where PAMP-triggered immunity (PTI) could be activated. Number 2 indicates the symptoms onset, where the effector-triggered immunity (ETI) could occur. According to our models (Figures 3.15a, 3.15b and 3.15c), success of these two events responds to the effect of humidity (wetness and relative humidity) and temperature, respectively. However, according to Figure 3.16a, not any water-related variables showed up for clone CATIE-R4, suggesting that germination and penetration are not critical events for this clone. We considered that this is not what is really happening, that the real reason why this influence did not show up is because of the low numbers of CATIE-R4 pods that showed symptoms. Penetration success was not determined in this study, so the only proof we had was the appearance of the symptoms, and thus this influence was left out for CATIE-R4. For this reason, discarding

humidity influence over the infection moment for this clone is not correct. Nonetheless, we considered that none of the clones present PTI as a defense mechanism against spore germination and penetration, i.e. *M. roreri* can easily penetrate the pods. Host resistance mechanisms resulting from the ETI are triggered internally and against colonization, where temperatures influence the success of these strategies. For CATIE-R4, large amplitudes of temperatures favor fungal colonization, evidenced by the appearance of symptoms, although status change probabilities were very low.

For CC-137, water conditions within the first month influenced the moment of infection (germination and penetration). After penetration, low minimum temperatures (about 20°C) elevated the probability of the pods to show symptoms. We considered that this variable influenced the ETI against fugal colonization. However, this resistance strategy was not as effective as the one developed by CATIE-R4, and this could suggest that CC-137 accumulates a minor number of resistance genes, so its ETI against the pathogen is less effective. In addition, the different response of this clone according to the influence of environmental conditions shown in Figure 3.14 suggests that its resistance genes are different from CATIE-R4 and Pound-7 resistance genes.

3.10.3 Resistant clones' stability: the case of CATIE-R4

After field observations and the result of our analyses, CATIE-R4 was found to be a very stable, highly resistant clone. This clone is considered a promising material; however, its resistance stability should be proved in special environments where the large temperature ranges that favor fungal colonization are the norm. The CATIE-R4 resistance strategy consists of the interruption of fungal colonization as an ETI strategy. This interruption also avoids fungal reproduction since we considered that *M. roreri* has difficulties sporulating over CATIE-R4 pods, causing inoculum suppression. For CC-137 and Pound-7, common sporulation was observed normally in the field, with mycelium and spores as commonly reported in the literature (Thévenin and Trocmé 1996). This was not the case of CATIE-R4. Even when most of the CATIE-R4 diseased pods sporulated, this sporulation was different from that in any other susceptible clone. Mycelium and spores were lighter in terms of color

and thickness. Sporulation observed could correspond to other secondary fungi. In this way, effect differences of the variables within clones could be explained. For CC-137, temperatures and rainfall presented normal effects, i.e. status change probability was higher when minimum temperatures and rainfall decreased and maximum temperature increased. In this way, as mentioned in the part I discussion, these conditions were favorable for dissemination. The opposite happened with CATIE-R4, where low maximum temperature and high mean relative humidity elevated the probability of status change. This could be secondary/decomposer fungi that have different environmental requirements, favored by an increment of relative humidity and lower temperatures (Lodge and Cantrell 1995). The CATIE-R4 infection process should be carefully studied to determine what happened in the interior of the pods and whether the apparent sporulation corresponds to *M. roreri.*

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3.12 APPENDIX

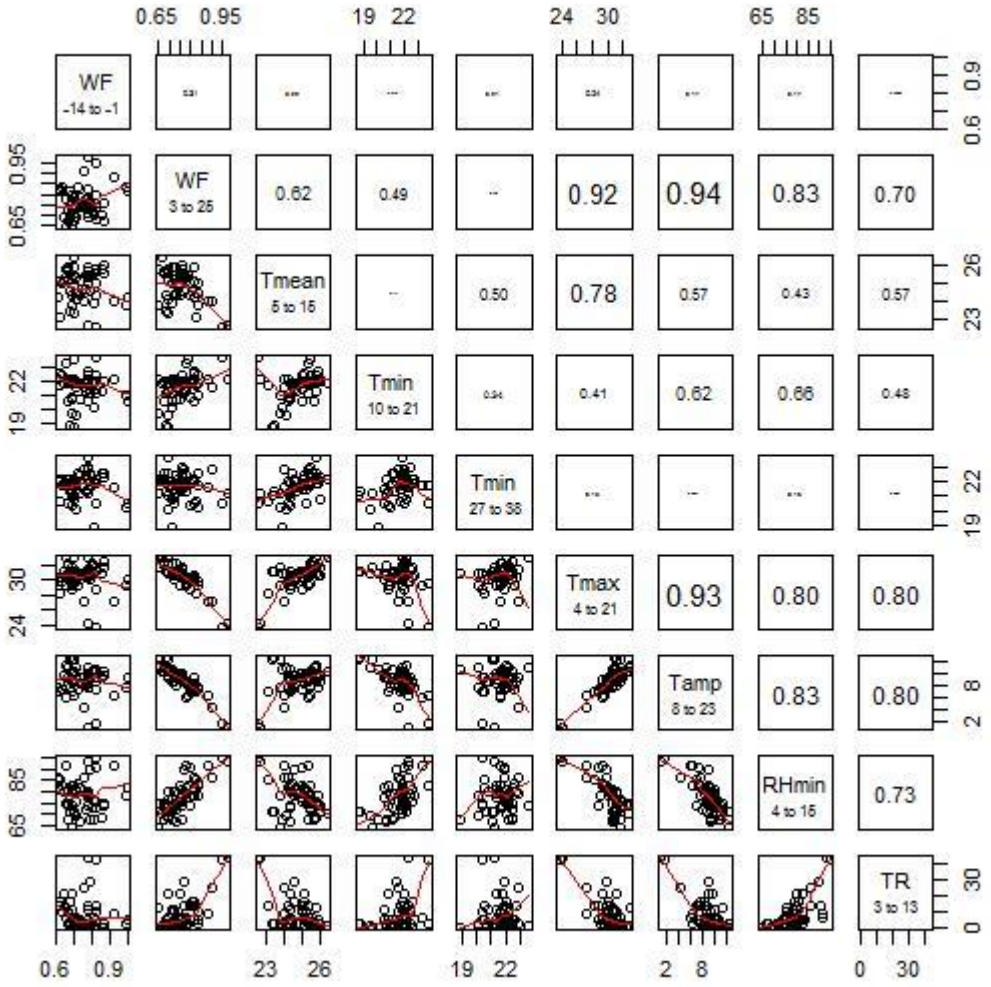

Figure A3.1. Absolute values of Pearson's correlation coefficients (r) within selected microclimatic variables for the status change healthy to diseased without sporulation. The larger the font size, the higher the correlation coefficient.

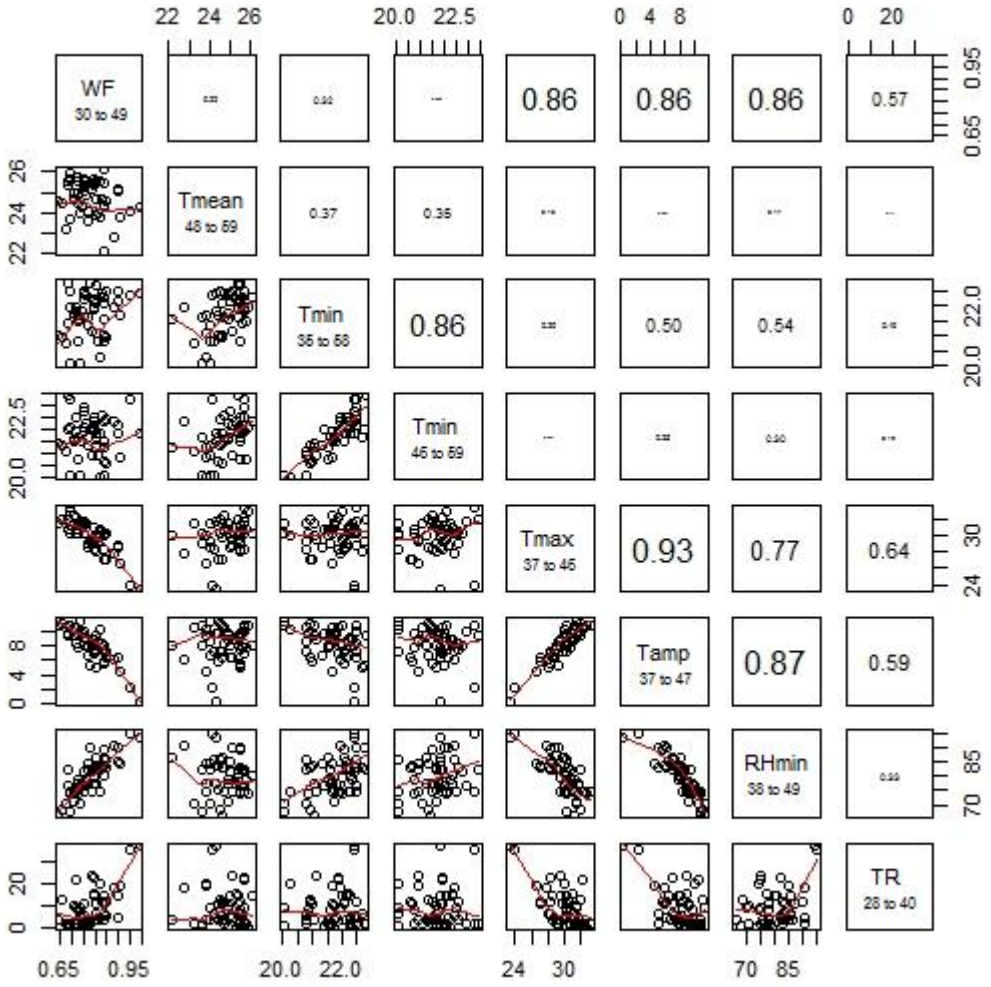

Figure A3.2. Absolute values of Pearson's correlation coefficients (r) within selected microclimatic variables for the status change diseased without sporulation to sporulation. The larger the font size, the higher the correlation coefficient.

CHAPTER 4

RELATIONSHIP BETWEEN MONILIOPHTHORA POD ROT INFECTION AND ONSET OF SYMPTOMS AND SIGNS

4.1 INTRODUCTION

According to Manners (1993), the incubation period (IP) is the period between the inoculation or the host exposure to the pathogen and the appearance of the visible symptoms. The latency period (Evans *et al.*) lasts until the development of infectious lesions. For fungi pathogens, IP is usually shorter than the LP: symptoms appear before the production of the propagules. However, differences between these periods can differ according to biophysical factors, such as in black leaf mold (*Pseudocercospora fuligena*) on fresh market tomatoes: LP-IP = three days on average but can reach seven days, for LP = 21 days, under specific conditions of leaf age, temperature and wetness duration after inoculation (Mersha *et al.* 2014). Sometimes, the difference between the two periods is very short, less than one day, with LP = 16 days as a maximum, for instance in the case of the hawthorn powdery mildew (*Podosphaera clandestina*) (Xu and Robinson 2000). For virus and bacteria pathogens, both periods usually match: symptoms and production of infectious entities occur almost at the same time (Rimbaud *et al.* 2015). However, in specific cases, LP can even be shorter than IP, as in the cacao swollen shoot virus. Symptomless cacao trees, hosting viruses, can be used by mealybugs to spread the disease by moving from branch to branch, from tree to tree or by being dislodged from the branches by wind (Thresh and Owusu 1986).

Just as infection is influenced by climatic conditions, the duration of the incubation and latency periods are also influenced by these conditions. Duration of IP depends on how long it takes to reach the suitable conditions for symptoms development. Lovell *et al.* (2004) studied the effect of the temperature on the LP duration of Septoria leaf blotch in winter wheat. These authors concluded that long periods of cold temperatures (around -2.4°C) decrease the opportunity for plants to resist the pathogen. Another example of the climatic influence over

LP duration is demonstrated by the construction of temperature response curves to simulate latency periods, developed by Wadia and Butler (1994).

IP and LP are important in plant disease science. The latency period is obviously the most important in epidemiological studies, since it represents the time required to produce a new generation of propagules. The shorter this time is, the higher will be the number of propagules produced in a productive cycle and the more intense the epidemic will be. The incubation period is mainly important for studies on vector-borne pathogens, such as viruses and bacteria. Symptoms, but also chemical cues, of infected tissue, help the vector to locate diseased plants as a first step in the transmission of the disease (Mauck *et al.* 2012). Symptoms are also signals for producers to take actions in order to avoid contaminations within host populations. On the contrary, absence of early symptoms or difficulties in identifying them could mislead farmers and researchers about timing, extent and viability of control methods. This often happens with soil-borne pathogens, whose infections are difficult to detect because they primarily affect the invisible part of the host. Long incubation or latency periods are also conditions that can lead to the same failed control (Leclerc *et al.* 2014). However, with time, a disease with a long LP may facilitate the development of resistant hosts. Sinclair (1991) studied the LP of different soybean fungal pathogens. He concluded that long LPs permit the ultimate accumulation of resistance genes in the host, since the degree of host resistance is directly correlated with the duration of the pathogen LP.

Latency period and latent period tend to be confounded in the literature. In order to clarify this terms, we adopt the definitions presented by Verhoeff (1974), who defines the latency period as that in which the pathogen establishes a parasitic relationship with the host but the pathogen stays quiescent or dormant. This relationship turns active after some time. This shift seems to be physiological and could occur when 1) the host tissue reaches or develops the pathogen nutritional requirements, 2) the level of toxins in this tissue decreases, 3) the pathogen reaches its enzyme potential or 4) the environmental conditions stimulate fungal development. Generally, this shift happens with tissue ripening. On the contrary, in the latent period, this parasitic relationship with the host is not established. Penetration occurs, but further growth of the infection hypha is delayed. Some time passes between this fungal stage

and the establishment of a parasitic relationship between the host and the pathogen. Nothing is known about this relationship at this time. The host and the pathogen must be in equilibrium.

During the latency period, the host appears symptomless, but the infection process is already set inside of the susceptible host tissue. Duration of this period could vary according to the pathosystem, and this phase of the disease cycle gains importance relative to its duration. Every pathogen, except necrophilic types, has a latency period. In diseases with long latency periods, for example Botrytis neck rot in onion and Moniliophthora pod rot (MPR) in cacao, symptoms are difficult to detect since infection is not obvious from the beginning. This permits the pathogen to advance in infection progression (Verhoeff 1974; Sinclair 1991).

The incubation period is based on the observation of the first symptoms. Symptoms can be external but also internal with no external expression. This is common in hemibiotrophic fungi such as *Colletotrichum gloeosporioides*, causal agent of papaya, mango and cacao anthracnose, and other species of this genus (Dickman 1993; Nelson 2008; Maeda and Nelson 2014). Producers can sacrifice apparently healthy organs for the observation of internal symptoms and make control decisions according to the results of these observations. However, researchers, particularly epidemiologists, during their studies, are more reluctant to do so. This practice supposes loss of a certain number of healthy organs, with possible effects on the epidemic, when the population of susceptible organs is small. For the case of MPR, epidemiological studies have been conducted (Porras 1982; Fallas 1983; Ram 1989; Tazelaar 1991; Torres de la Cruz 2010) by counting all pods with external symptoms each month during phytosanitary harvests as well as mature pods harvested at the same time that have internal symptoms. However, this method does not allow identification of the presence of internal symptoms in immature pods having no external symptoms, i.e. to identify the closest recognizable moment of infection. In this study, we attempted to highlight the relationships between onset of MPR external symptoms and the onset of internal symptoms, a period fairly close to infection, which could justify the observation of external symptoms only for epidemiological studies. For that purpose, we conducted artificial field inoculations in two different periods, i.e. under two different microclimatic conditions, on three cacao clones

exhibiting different levels of incomplete resistance, and we observed internal and external symptoms at the same time.

4.2 STUDY OBJECTIVES

4.2.1 General objective

Study the relationship between the MPR infection process and the onset of symptoms of three different cacao clones through artificial inoculations.

4.2.2 Specific objectives

a) Describe symptomatology throughout time.
b) Compare internal and external symptomatology within the three clones.
c) Determine how close the external symptoms onset is to internal symptoms onset.

4.3 STUDY HYPOTHESES

a) MPR external symptoms appear in a determined order throughout time.
b) Internal symptomatology and external symptomatology differ according to the cacao clone.
c) External symptoms onset is fairly close to the internal symptoms onset.

4.4 MATERIALS AND METHODS

4.4.1 Experimental site

This experiment was conducted in the L6 trial in CATIE's La Lola Farm located in 28 Millas, Bataán District, Matina Canton, Limón Province. Average rainfall (1949–2010) is 3575 mm with a decrease in March and particularly in September, the month with less rainfall. Monthly average temperature ranges (1952–2010) were between 20.5°C and 30.0°C. May and June are the warmest months, whereas December and January are the coldest (Phillips-Mora *et al.* 2013).

4.4.2 Genetic material

The L6 trial includes 42 clones, of which only three were included in the study: Pound-7, CC-137 and CATIE-R4. These clones were selected to represent the entire scale of susceptibility: a very susceptible clone (Pound-7, with an 11-year average of 86% of incidence); a moderately resistant clone (CC-137, with an average of 32% of incidence); and a highly resistant clone (CATIE-R4, with an average of 9% of incidence). These three clones have good productivity, assuring the presence of pods throughout the year (Phillips-Mora *et al.* 2013).

4.4.3 Methodology

Artificial inoculations of *Moniliophthora roreri* were performed in the field using the standard method proposed by Phillips-Mora (1996). Two events of inoculations were carried out, on May 1 and October 29, 2014. According to the climatic data recorded, the second period of inoculation was cooler and presented less precipitation. For each event, 40 apparently disease-free young pods (one to two months old) of the three clones were inoculated, and eight pods per clone were used as blanks, sprayed with pure water. Inoculum was prepared using the isolate regularly used in the artificial inoculations performed by the

CATIE's Cacao Improvement Program, in a concentration of 120 000 spores/ml. Spore suspension was sprayed over the pods and a plastic bag with a wet paper towel in the bottom was placed over the pod to create a wet chamber effect.

4.4.4 Pod evaluation

External symptoms and signs were observed weekly over a period of 10 weeks, which was the total duration of the experiments. On each date, every external symptom and sign was documented: humps, yellowing, oily dots, chocolate spot, sporulation, mummy (Figure 3.1). Evaluation of internal symptoms started four weeks after inoculation and continued every two weeks by opening lengthwise 10 inoculated pods and two blanks per clone. Table 4.1 presents the standard scale used to assess the internal severity (Brenes 1983).

Table 4.1. Pod internal severity scale for artificial inoculations evaluation (Brenes 1983).

Internal Severity (IS) Values	**Percentage of the pod internal damage**
1	0–20%
2	21–40%
3	41–60%
4	61–80%
5	81–100%

4.4.5 Data analyses

The presence of one or more external symptoms was recorded per pod for each evaluation date. These data were compared with the internal manifestation of the disease for the same dates. Descriptive analyses were performed to compare the internal and external onset of symptoms in each inoculated pod.

4.5 RESULTS

4.5.1 MPR incidence and internal severity values of the three clones in both inoculation events

Figure 4.1 shows the MPR incidence per clone as an average of both artificial inoculation events.

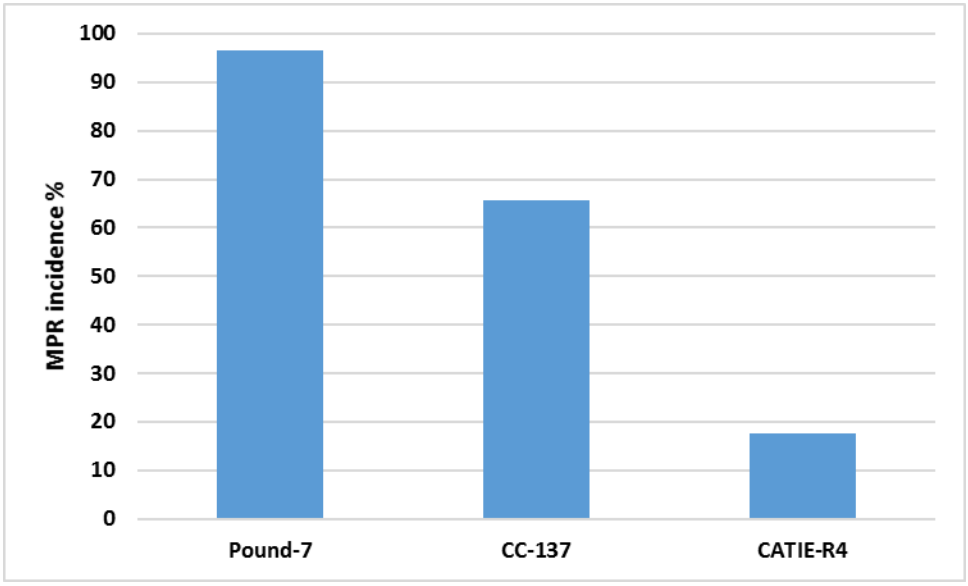

Figure 4.1. MPR incidence of the pods in both events of inoculation for the three clones.

Clone Pound-7 presented an incidence of 96.15%; CC-137, an incidence of 65.6%; and CATIE-R4, an incidence of 17.7%. These values correspond to the common reaction description of these clones against the disease reported by Phillips-Mora *et al.* (2013). Figure 4.2 shows the average internal severity values for each clone, including both inoculation events.

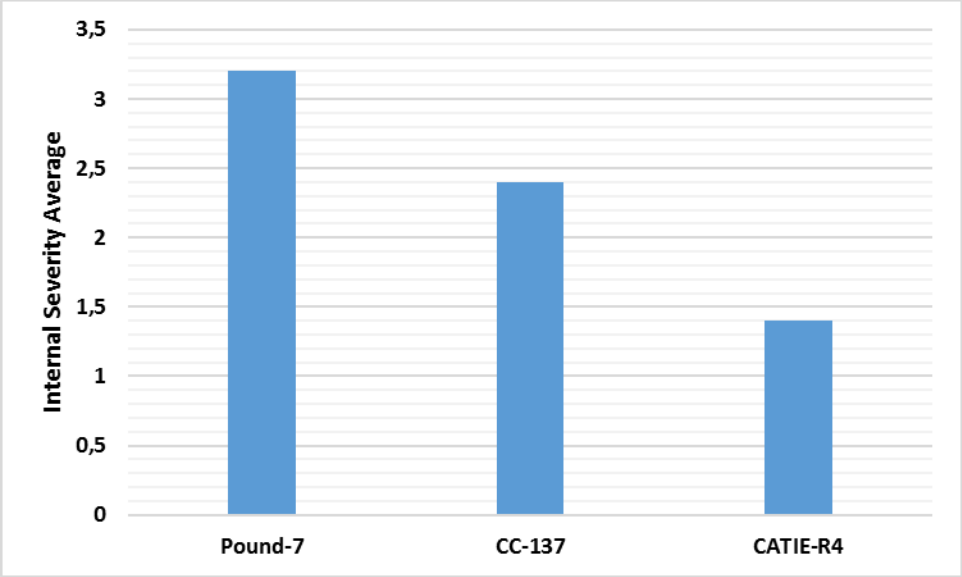

Figure 4.2. MPR internal severity values of the pods in both events of inoculation for the three clones.

Figures 4.1 and 4.2 indicate that the tendency of the reaction of these clones against the fungus is the same. Internal and external expression of the symptoms corresponds.

4.5.2 Internal Severity distribution per clone and inoculation event

The internal severity values distribution for each clone including both inoculation events is shown in Figure 4.3.

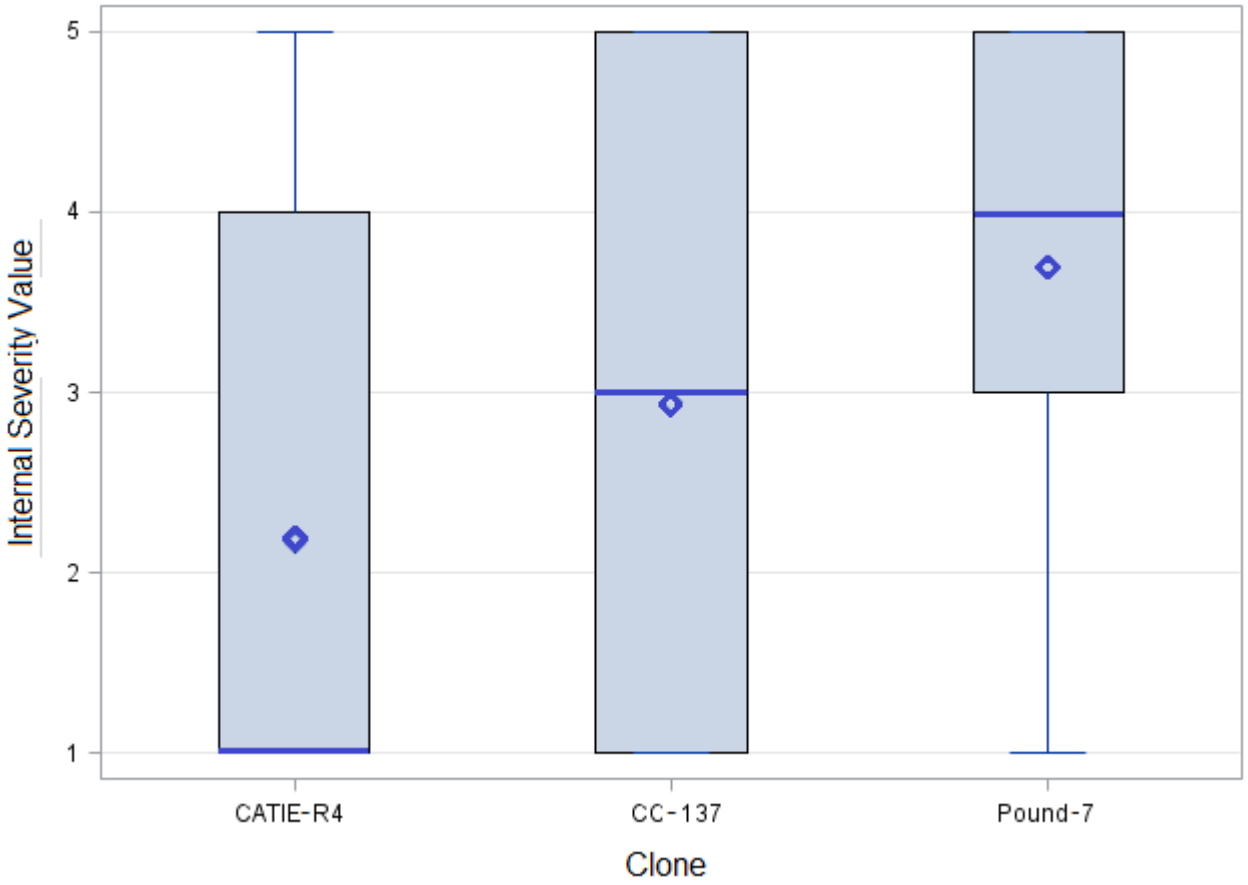

Figure 4.3. Internal severity values distribution per clone including both events of inoculation. Blue bands inside the boxes represent the median or the second quartile. Blue rhombuses inside the boxes represent the mean values.

Most evaluated pods from clone CATIE-R4 showed internal severities between 1 and 4 and a mean value of 2.16. Clone CC-137 presented all the values of internal severity with a mean value of 2.95, and for Pound-7, the majority of evaluated pods presented values between 3 and 5, with a mean of 3.70.

Figure 4.4 represents the internal severity distribution separated by inoculation events.

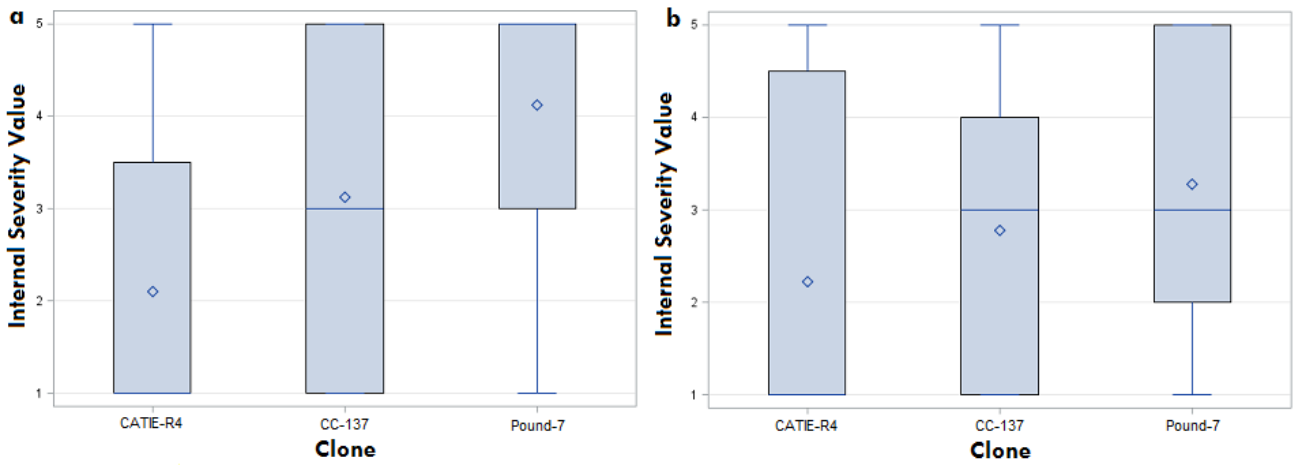

Figure 4.4. Internal severity values distribution per clone. a. Corresponds to the first event of artificial inoculations, from May 1 to July 10, 2014. b. Corresponds to the second event of artificial inoculations, from October 29, 2014, to January 10, 2015. Blue bands inside the boxes represent the median or the second quartile. Blue rhombuses inside the boxes represent the mean values.

Even when the distribution is different in every clone in Figure 4.4a and 4.4b, clonal means follow the expected tendency, where CATIE-R4 is the most resistant and Pound-7, the most susceptible. The incidence was also observed to be lower during the October inoculation event.

4.5.3 Appearance of external symptoms and signs throughout the studied period

Figure 4.5 represents the proportion and type of MPR external symptoms and signs presented within the evaluated pods of the three clones in both events of inoculation.

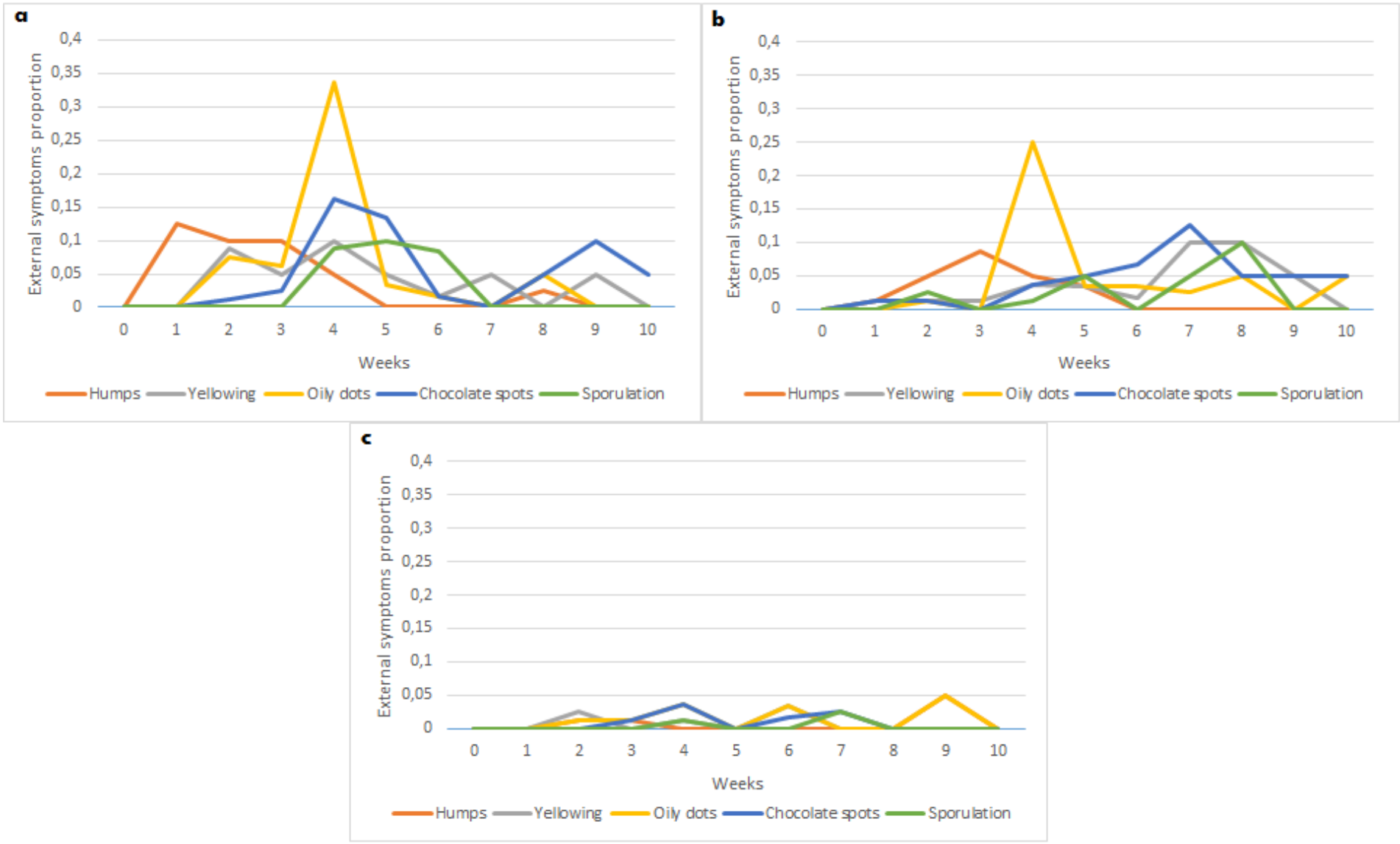

Figure 4.5. Proportion of MPR external symptoms and signs observed per week after the artificial inoculation (0). a. Corresponds to clone Pound-7 at both events of artificial inoculation. b Corresponds to clone CC-137 at both events of artificial inoculation. c. Corresponds to clone CATIE-R4 at both events of artificial inoculation.

Comparing Figures 4.5a, 4.5b and 4.5c, it is evident that Figure 4.5c, which corresponds to clone CATIE-R4, presents a small proportion of every external symptom, so this is not a good figure for studying the evolution of the symptomatology but is very useful in comparing this behavior against clones with contrasting reactions. The Pound-7 clone (Figure 4.5a) shows humps as the first symptom to appear in great proportion, followed by the yellowing and oily dots. Chocolate spot and sporulation appear one or two weeks after these initial symptoms. Clone CC-137 (Figure 4.5b) had a similar behavior, with more symptoms (although in less proportion) appearing at the beginning. Appearance of symptoms and signs seems to be delayed in Pound-7 and CC-137 clones (Figures 4.5a and 4.5b). At week 4, the symptom with the highest proportion was the oily dots, followed by humps and chocolate spot. Sporulation started in the second and third week.

4.5.4 Distribution of external symptoms and signs per clone

The distribution of the external symptoms and signs appearance is illustrated in Figure 4.6. In this case, the three clones present a similar distribution of the external symptoms and signs. The symptom of oily dots is more frequently found in the three clones, accounting for almost a third of the total. Yellowing and chocolate spot follow this first symptom, with percentages from 18 to 25% of the total; the humps, with a percentage of 13 to 21%. Finally, sporulation presents the lowest percentage of the total, from 8 to 12% of the total.

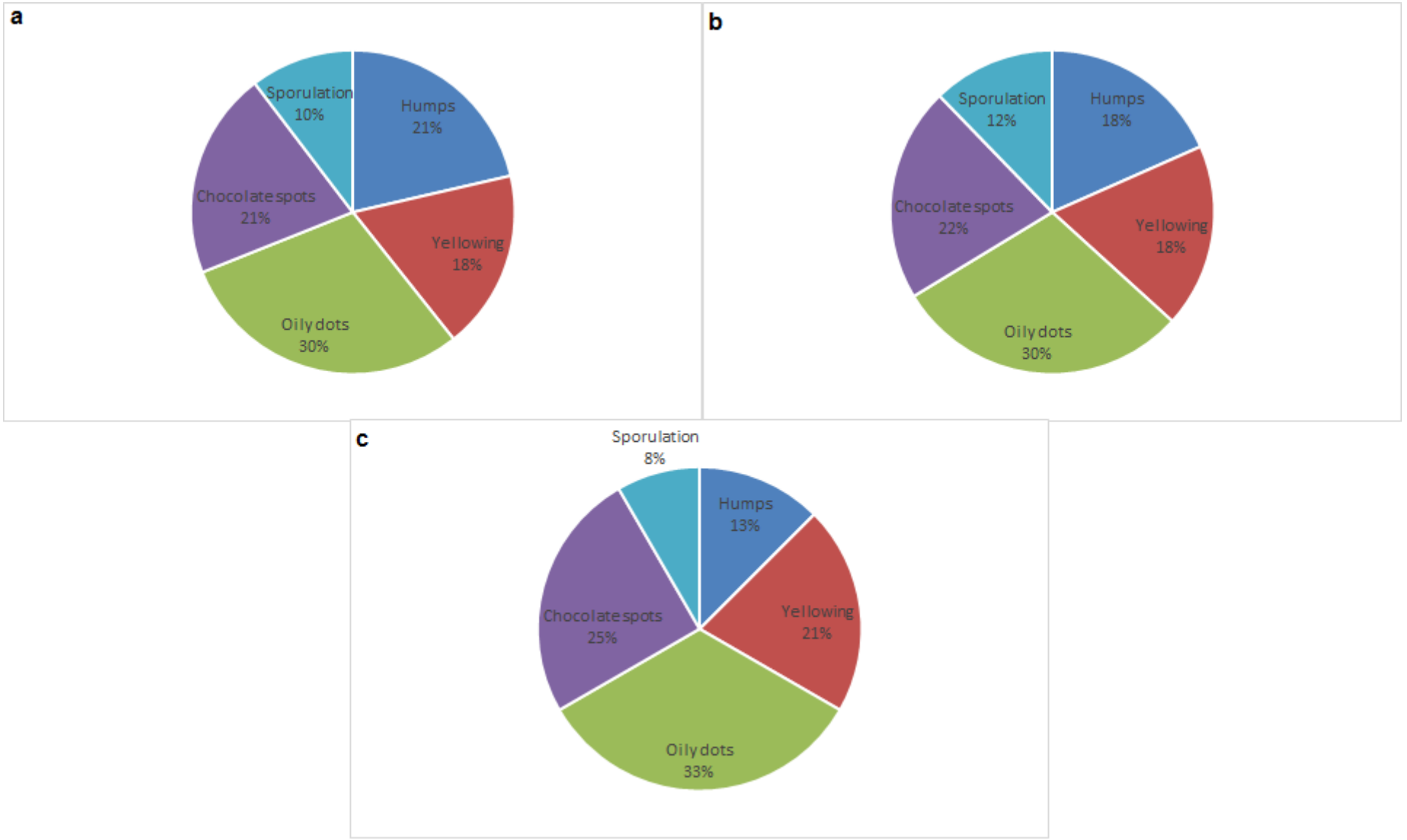

Figure 4.6. Distribution of external symptoms and signs for each clone in both inoculations events. a. Corresponds to clone Pound-7. b. Corresponds to clone CC-137. c. Corresponds to clone CATIE-R4.

Figure 4.7 presents the symptom/signs evolution on the pods that stayed alive during the entire evaluation period (10 weeks). Figure 4.7a, corresponding to clone Pound-7, shows that most of the pods expressed all the symptoms in the expected order, starting with the early symptoms (humps, yellowing, oily dots) and ending in the latest symptoms/signs (chocolate spot and sporulation). For CC-137 (Figure 4.7b), the intermediate clone, this behavior seems to be different, since some pods followed the same order of symptom/signs as Pound-7 and other pods present a delay in the expression of symptoms until six or eight weeks, when these pods then expressed symptomatology and signs rapidly. The pods of resistant clone CATIE-R4 (Figure 4.7c) expressed similar behavior.

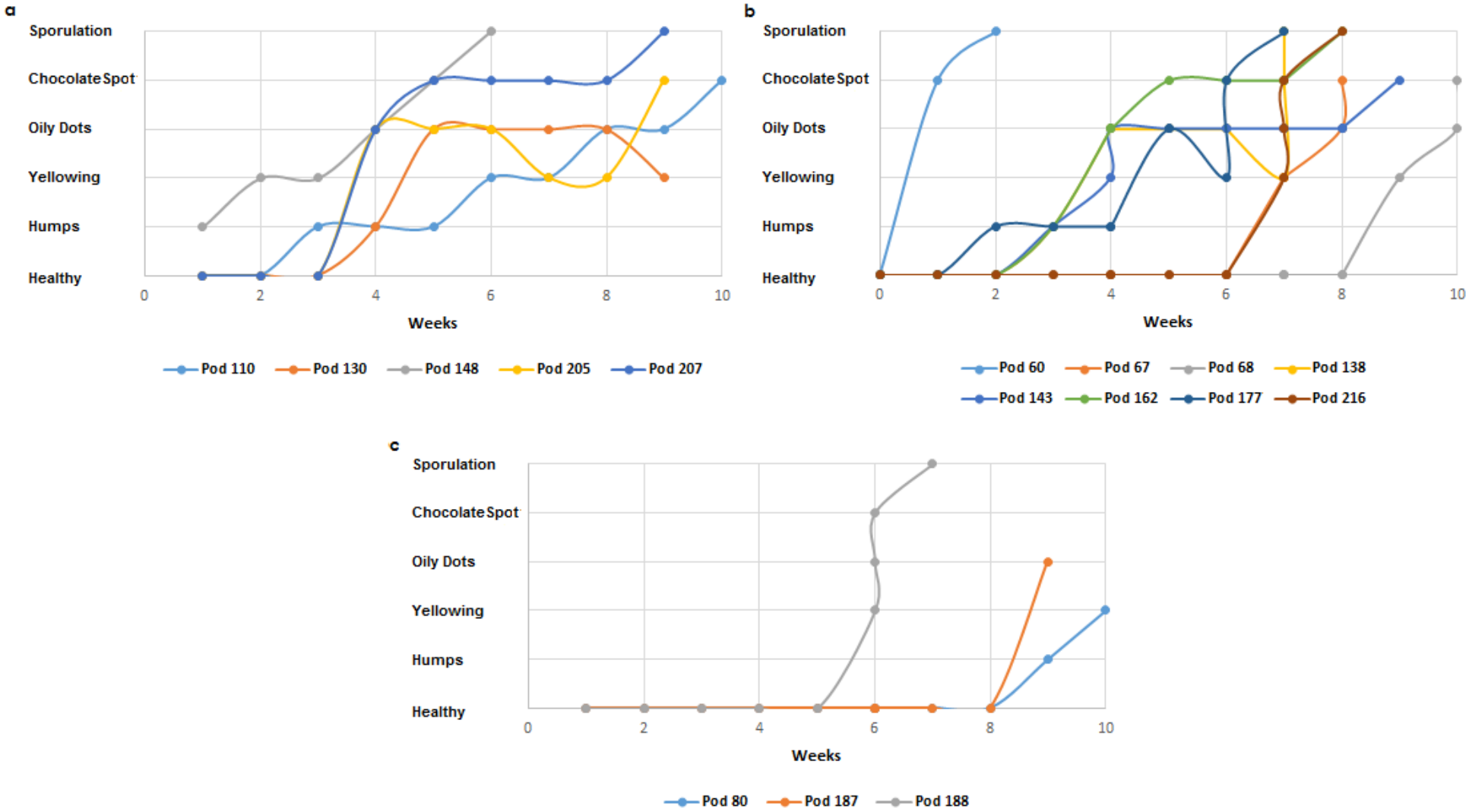

Figure 4.7. Symptom/signs evolution of the inoculated pods for the entire evaluation period. a. Corresponds to clone Pound-7. b. Corresponds to clone CC-137. c. Corresponds to clone CATIE-R4. Differences in the number of pods per clone are due to pod losses given to other factors.

4.5.5 Relationship between external symptoms/signs and internal severity

Tables 4.2, 4.3 and 4.4 present cross-frequency tables of the latest external symptoms and signs that appeared for each internal severity value on the pods of each clone in the last evaluation date of both artificial inoculation events.

Table 4.2. Cross-frequency table of the latest external symptoms and signs that appeared in the pods of the clone Pound-7, by the internal severity values.

	Internal severity values				
Latest external symptoms/signs	**1**	**2**	**3**	**4**	**5**
Oily dots	3	5	6	2	0
Chocolate spot	2	5	3	2	1
Sporulation	0	1	2	3	12

Table 4.3. Cross-frequency table of the latest external symptoms and signs that appeared in the pods of the clone CC-137, by the internal severity values.

	Internal severity values				
Latest external symptoms/signs	**1**	**2**	**3**	**4**	**5**
Humps	2	0	0	0	0
Yellowing	1	0	1	0	0
Oily dots	5	4	3	0	1
Chocolate spot	0	2	4	1	4
Sporulation	0	0	2	5	5

Table 4 4. Cross-frequency table of the latest external symptoms and signs that appeared in the pods of the clone CATIE-R4 ,by the internal severity values.

	Internal severity values				
Latest external symptoms/signs	**1**	**2**	**3**	**4**	**5**
Yellowing	0	0	0	0	1
Oily dots	3	0	0	0	0
Chocolate spot	1	1	1	1	0
Sporulation	0	0	0	1	1

Cross-frequency tables for the susceptible clones Pound-7 and CC-137 (Tables 4.2 and 4.3), show that there is an association between the internal severity values and the latest external symptoms and signs. However, the cross-frequency table for the resistant clone CATIE-R4 (Table 4.4) does not show a clear association between these two variables.

To corroborate these associations, a Fisher's exact test was run for the three clones. Table 4.5 shows the result of this test.

Table 4.5. Fisher's exact test results of the association between the latest external symptoms and signs versus the internal severity values for the three clones.

Clone	**p-value***
Pound-7	0.0002
CC-137	0.0014
CATIE-R4	0.2267

*H_0 = Variables "Latest external symptoms/signs" and "Internal severity values" are independent (p = 0.05).

Results in Table 4.5 confirm that for Pound-7 and CC-137 clones, the latest external symptoms and signs and the internal severity values are not independent. In the case of CATIE-R4, these variables seem to be independent.

According to Table 4.2, which corresponds to the most susceptible clone, Pound-7, the less severe external or earlier symptoms are associated with the lowest internal severity values

(1 and 2), while the more severe or latest symptoms and signs are associated with the highest internal severity values (4 and 5).

4.6 DISCUSSION

4.6.1 Symptoms appearance variation in time and per clone

Our results present more evidence of what has already been reported by other authors: Appearance of symptomatology and signs, both internal and external, varies according to the genetic plant material, environmental conditions, age of the pod in the moment of infection and inoculum pressure (Suárez 1971; Phillips 1986). The inoculum pressure in this study was standardized to 120 x 10^3 spores ml^{-1}. In our study, the genetic plant material seems to be quite determinant in the way that the symptoms and signs were expressed. Pods with a certain level of resistance seem to delay the expression of symptoms or signs, while the susceptible ones trigger the symptomatology during the first weeks.

Appearance of symptoms was highly variable, even among pods of the same clones inoculated simultaneously, as was also reported by (Sánchez 1982; Phillips 1986). Several studies on artificial inoculations of different clones with *M. roreri* drew different results in terms of the numbers of days that the clones took to show the first symptoms. In the present study, we inoculated pods from 30 to 60 days old, and the first symptoms were observed a week after the inoculation. This result is different from the results reported in the literature. Sotomayor (1965) inoculated pods 80 days old and indicates that the appearance of the first symptoms took a mean of 34 days. Aranzazu and Cubillos (1977) inoculated pods of different ages and these pods took 54 to 78 days to show the first external symptoms. Merchán (1981c) inoculated pods 82 to 84 days old, and these pods showed the first external symptoms in 15 to 32 days. Sánchez (1982), after inoculating pods 80 days old, indicated that external symptoms could be detected after 35 days. Finally, Phillips (1986) inoculated 60-day-old pods and reported that first external symptoms were shown in 21 to 24 days. Differences between our

results and these authors' results lies in the age of the pods when inoculated as well as in methodology approach, climate conditions, inoculum pressure and cacao varieties used.

4.6.2 Relationship between symptoms/signs onset and internal infection

Since MPR has a long latency period, it is hard to determine the exact infection moment in natural inoculations (Desrosiers and Suárez 1974; Evans 1981b; Torres de la Cruz 2010). Artificial inoculations provide a useful tool to control the exact moment when infection occurs, and thus this was the methodology used in the present study.

The first evaluation of pod internal damage in this study was done a month after the inoculation, due to the long incubation period of this disease (Torres de la Cruz 2010). As the internal evaluations progressed, external symptoms were more evident. With this consideration, extrapolation of the results of the microclimate influence on disease onset to the moment of infection seems valid. In addition, the correspondence of the MPR incidence reported for these three clones (Pound-7, CC-137 and CATIE-R4) and the results of the internal severity evaluation confirmed by Figures 4.1 to 4.4 validate this extrapolation.

The cross-frequency tables (Tables 4.2 to 4.4) and the Fisher's exact test (Table 4.5) helped to corroborate the strong association between external and internal severity, as has been considered by Sánchez (1982); Brenes (1983); (Phillips 1986). This was very evident for clones CC-137 and Pound-7, though not for CATIE-R4, the most resistant clone, for which a very few number of pods became infected.

4.7 CONCLUSIONS

- Symptoms/signs diversity and order vary according to the genetic plant material, environmental conditions, age of the pod at the moment of infection and inoculum pressure.

- External symptoms and signs and internal damage of the pods are associated.

- Symptoms/signs onset is close enough to the infection moment that the influence of the microclimate over the symptoms onset could be extrapolated to the infection.

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CHAPTER 5

MPR-CACAO CONCEPTUAL MODEL

In conclusion, we submit a summary of all the biological and epidemiological information obtained about the Moniliophthora pod rot (MPR)-Cacao pathosystem, both from the literature and our own results. This information is presented as a conceptual model (Figure 5.1). Due to the complexity of our pathosystem, components of the conceptual model are explained first and then the relationships, in the order that we consider facilitates better comprehension.

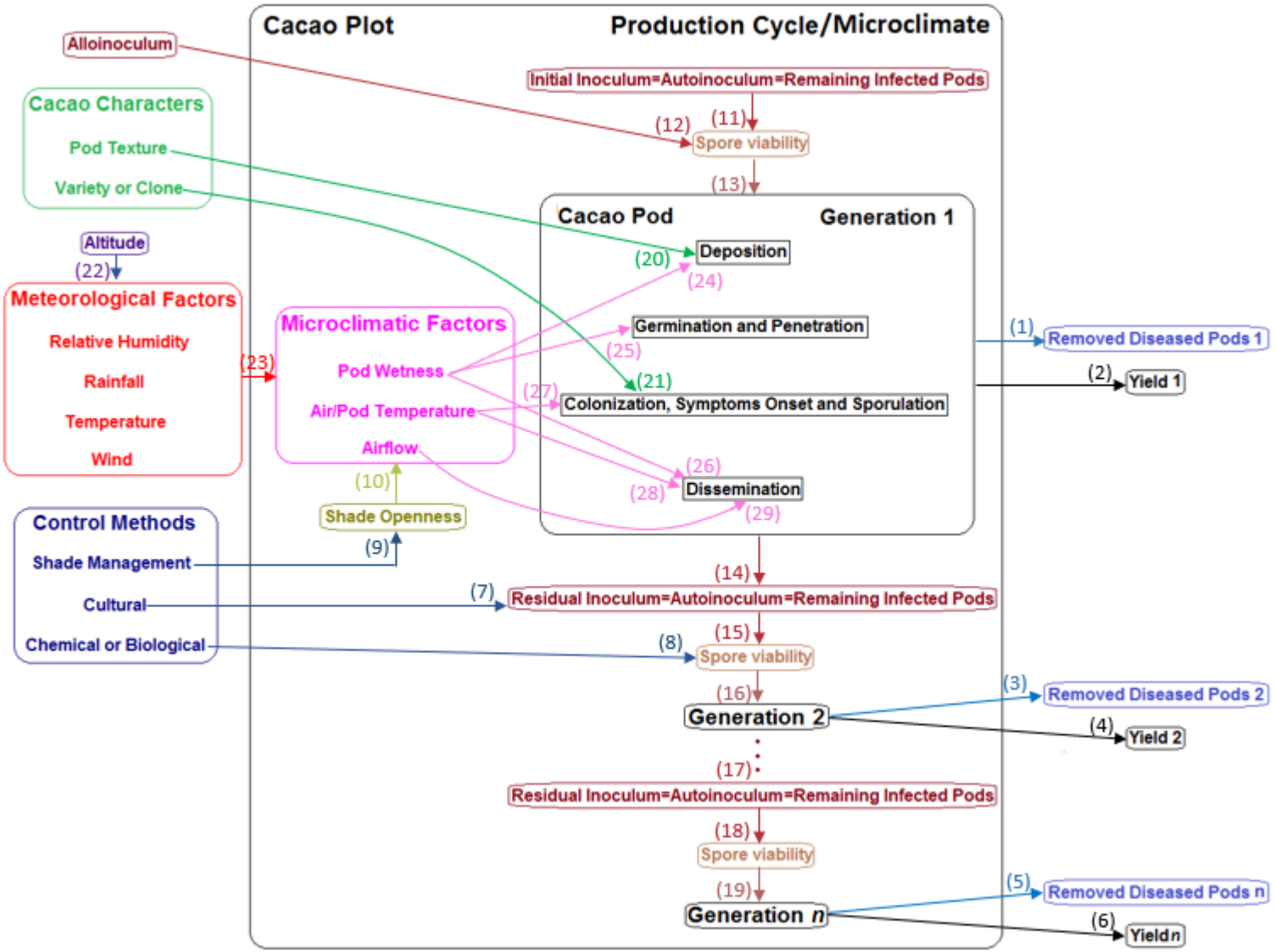

Figure 5.1. Moniliophthora roreri-*Cacao conceptual model. System: Cacao plot, production cycle and microclimate. Numbers indicate relationships within components of the system.*

Figure 5.1 represents the MPR-Cacao pathosystem, where the large text box on the right corresponds to the cacao plot, the production cycle and the microclimate, comprising the system where the relationships to be explained are developed. The smaller box inside the system corresponds to the cacao pod/generation 1 subsystem, which is composed of *Moniliophthora roreri* life-cycle stages that occur on or inside of the pod. The boxes outlined in color represent the system factors and the exits.

For every generation, on and inside of every infected pod, the fungal cycle is developed: deposition, germination and penetration, colonization, symptoms onset and sporulation, and dissemination. From every generation, two components are extracted as exits: removed diseased pods (1), (3) and (5) and the yield (2), (4) and (6), which corresponds to the mature healthy pods extracted from the system for every generation.

Removed diseased pods (1), (3) and (5) are exits resulting from the removal of diseased pods as a cultural (7) control method. Among other MPR control methods are chemical and biological ones (8), which correspond to the application of chemical and biological products that reduces the spore viability. shade management is another control method that directly influences shade openness (9) via pruning of branches in order to give to every tree an adequate structure, regulating the microclimatic factors (10) inside the plot and thus the disease development.

To trigger the MPR infection in a new production cycle, it is necessary to start with an initial inoculum that corresponds to the autoinoculum or the remaining infected pods or mummified pods that were not removed from the trees during the previous cycle or residual inoculum from other parts of the system (Ram 1989). This inoculum is regulated by spore viability (11): not all spores are capable of germinating because of the loss of their viability from factors such as UV light exposure, among others (Heuveldop et al. 1986). The other source of inoculum is the alloinoculum, referring to spores that come from sources located outside of the plot, carried by the air over long distances, and with the ability to cause a disease attacks even when the control methods have been applied correctly within the plot. The alloinoculum is also regulated by the spore viability (12). Spores that survive and reach any susceptible tissue will trigger the infection within generation 1. Once this generation is

over (pod harvest), residual inoculum/autoinoculum (14) is produced by the remaining infected pods that escape from the harvest and the pruning. Viable spores (15) from these pods constitute the inoculum for generation 2 (16). This pattern is repeated until generation *n* (17), (18) and (19).

The severity of the MPR infection depends on the cacao genetic material present in the plot. Every variety or clone reacts differently to MPR (Porras Umaña 1985; Phillips 1986). The pod production dynamic of the genetic material planted in the plot is important. According to our results, clones that produce young pods in periods with conditions unsuitable for the pathogen could escape from the disease or the impact could be reduced. In general, control methods, or their absence within the plot, affect disease impact. Cultural control methods, or the removal of diseased pods, is very important to stopping the epidemic in a crop such as cacao.

With the understanding of how MPR epidemics develop, it is necessary to explain which factors affect the fungal life cycle, and how they affect them. The altitude (22), or the elevation where the plot is located, determines the meteorological factors of the site and thus the microclimatic factors within the plot.

Spore deposition depends on the pod texture (20) and could be affected by rainfall and pod wetness (24), since heavy raining could wash the spores off of the pod surface (Castro 1989). The effect of spore viability occurs during spore germination and fungal penetration since, in order to germinate, the spore must have the capability to develop the germ tube under suitable conditions. Pod wetness is also very important at this stage. According to our results, the probability of a healthy pod becoming diseased increases when wetness frequency and minimum relative humidity also increase. These two variables exert their influence during early stages of pod development, when pods are from two to six weeks old. The presence of a film of water over the pod stimulates germ-tube formation and elongation, permitting fungal intracellular penetration (Campuzano 1981a; Phillips 1986). Fungal colonization, symptoms onset and sporulation vary according to the cacao variety or clone (21). Differences observed within the three clones of our experiments (Pound-7, CC-147 and CATIE-R4), guide us to the conclusion that their resistance strategy consists in the capacity to interrupt internal fungal

development, not the entrance of it. Air/pod temperature (27) has an effect on fungal colonization and symptoms onset. According to our results, large temperature amplitudes increase the probability of a healthy pod to show symptoms. Low minimum temperatures (around 20°C)and high maximum temperatures (not exceeding 30°C) during the second and third month of the pod growth favored the symptoms onset. On the other hand, the probability of a diseased pod to sporulate increased when minimum temperatures were low (20°C) and in the absence of rainfall during the third and fourth month of pod development. Sporulation and dissemination are both affected in this way by rainfall (26) and temperature (28). Airflow (29), as a result of the wind gusts that occur within the plot, also favors the dissemination, since, according to Merchán (1981), wind is the main mechanism for MPR spore dispersal.

With the construction of these conceptual models, the MPR attack on cacao can be better understood. At the same time, these models can guide researchers and help define new studies to further explain the influence of determined factors.

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