FEDERAL UNIVERSITY OF PARANÁ

GHENIFFER FORNARI

VIRULENCE POTENTIAL OF THE *Fonseceae* SPECIES RELATED TO THE CHROMOBLASTOMYCOSIS DISEASES

CURITIBA

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VIRULENCE POTENTIAL OF THE *Fonseceae* SPECIES RELATED TO THE CHROMOBLASTOMYCOSIS DISEASES

Tese apresentada ao curso de Pós-Graduação em Microbiologia, Parasitologia e Patologia, área de concentração em Microbiologia, Departamento de Patologia Básica, Setor de Ciências Biológicas, Universidade Federal do Paraná, como parte das exigências para a obtenção do título de Doutor em Microbiologia, Parasitologia e Patologia.

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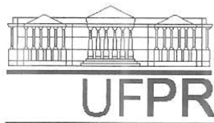

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"Virulence potential of the *Fonsecaea* species associated to the chromoblastomycosis diseases"

por

Gheniffer Fornari

Tese aprovada como requisito parcial para obtenção do grau de Doutor no Curso de Pós-Graduação em Microbiologia, Parasitologia e Patologia, pela Comissão formada pelos professores:

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Curitiba, 27 de junho de 2017.

Dedico

A todos os pacientes portadores da doença Cromoblastomicose;

Aos meus pais Ari e Nelcy;

Ao meu irmão Geovani;

Ao meu esposo Ricardo.

I dedicate

To the Chromoblastomycosis patients;

To my parents Ari and Nelcy;

To my brother Geovani;

To my husband Ricardo.

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I’m thankful to God for enlightening me and giving me strength in this journey.

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“A great discovery does not spring from the brain of a ready and finished scientist, like Minerva jumping fully armed from the head of Jupiter; It is the result of an accumulation of preliminary work”- Marie Curie.

RESUMO GERAL

As leveduras negras associadas à infecção em hospedeiros animais pertencentes à família Herpotrichiellaceae são microorganismos extremamente relevantes quanto a sua natureza ecológica e consequentemente, em relação às interações desenvolvidas com diferentes substratos e hospedeiros. As infecções recorrentes e consistentes causadas por estes agentes indicam uma possibilidade de adaptação ao hospedeiro. Neste contexto, a doença cromoblastomicose caracterizada por uma infecção subcutânea em humanos, causada por diferentes espécies desta família, é decorrente da implantação destes agentes de forma traumática, com posterior adaptação no tecido subcutâneo do hospedeiro através da formação de estruturas diferenciadas, denominadas de corpos muriformes. A hipótese atual baseada em dados epidemiológicos é que os pacientes adquirem a doença principalmente a partir de um trauma causado por material de origem vegetal, como por exemplo, espinhos de plantas. Portanto, o objetivo principal deste estudo foi estabelecer protocolos de isolamento e avaliação do potencial de virulência das espécies do gênero *Fonsecaea* relacionadas à doença cromoblastomicose. Para os testes de virulência foram utilizadas linhagens de procedência clínica, agentes de cromoblastomicose, *Fonsecaea pedrosoi* (CBS 271.37) e *Fonsecaea monophora* (CBS 102248); e a de origem ambiental *Fonsecaea erecta* (CBS 125763) isolada de planta viva, em região endêmica da doença. Para o estudo foram estabelecidos protocolos de infecção utilizando como modelo vegetal, plantas, produtoras de espinhos: a planta rastejante *Mimosa pudica* e a palmeira *Bactris gasipaes*. E, em animais foi adotado como modelo a larva de *Tenebrio molitor* para estudos de virulência e de isolamento ambiental. Testes complementares de imunogenicidade foram realizados utilizando camundongos BALB/c. As plantas eram produzidas *in vitro* com posterior transferência para vasos. Plantas *in vitro* e em vasos eram inoculadas por injeção direta da solução fúngica na concentração 10^2cels/mL no caule e através da contaminação do meio de cultura e ou do solo. Todas as linhagens foram capazes de colonizar os tecidos das duas espécies de plantas produzidas *in vitro* e em vaso. As linhagens clínicas *F. monophora* e *F. pedrosoi* permaneciam na região da epiderme das plantas inoculadas, enquanto que, a espécie associada à planta viva *F. erecta* apresentava um perfil colonizador na planta, semelhante aos isolados endofíticos utilizados como controles neste trabalho. As análises histológicas mostraram que as linhagens clínicas eram capazes de invadir os tecidos das plantas, permanecendo como células leveduriformes e poucas hifas na epiderme, enquanto que a *F. erecta* isolada de planta viva, assim como os endofíticos utilizados como controle, produziam hifas dentro dos tecidos das plantas atingindo a região de vascularização. Nos testes de virulência utilizando *T. molitor* observou-se que as larvas infectadas com *F. erecta* apresentaram menor taxa de sobrevida, seguidas das infectadas por *F. monophora* e *F. pedrosoi*, demonstrando que a espécie procedente de planta viva pode sobreviver dentro do hospedeiro animal. Entretanto, somente a espécie de origem clínica *F. pedrosoi* foi capaz de formar células muriformes, considerada característica clinica da cromoblastomicose. Tais resultados, demonstraram

diferenças na ecologia e virulência destas linhagens, além do potencial dessas larvas como modelo de reprodução da doença. O re-isolamento dos fungos a partir das larvas infectadas após 240 horas demonstrou que *F. erecta* apresentava diminuição significativa, baseado nas UFC/mL, em relação às linhagens clínicas, o que pode ter sido induzida pela alta imunogenicidade desta espécie verificada nos ensaios imunológicos em camundongos BALB/c. O *T. Molitor* foi também avaliado como meio de enriquecimento e isolamento onde foram obtidos a partir de larvas inoculadas com amostras *de coco da palmeira babassu quatro isolados da família Herpotrichiellaceae, identificados como F. erecta (n=2) e F. monophora (n=2) e seis isolados da ordem Capnodiales identificados como Cladosporium cladospoioides (Cladosporiacea) e Pseudocercospora norchiensis (Mycosphaerellaceae*). Estes resultados indicaram a eficácia deste modelo como um método alternativo de isolamento de leveduras negras do meio ambiente. Em conclusão, estes estudos demonstraram que as linhagens de origem clínica podem sobreviver em tecidos vegetais, confirmando a hipótese de infecção traumática destes agentes a partir de substratos vegetais, revelando, no entanto, um comportamento diferenciado das espécies de procedência clinica e daquelas isoladas de plantas vivas. Além disso, os testes realizados em *T. Molitor* mostraram um modelo potencialmente útil para elucidar e comparar a virulência e o dimorfismo desses agentes, e assim, proposto como o primeiro modelo animal para a reprodução de células muriformes.

Palavras-chave: Leveduras negras, Testes de virulência, Isolamento, *F. pedrosoi*, *F. monophora*, *F. erecta*, cromoblastomicose.

GENERAL ABSTRACT

The black yeasts belonging to Herpotrichiellaceae family cause infections in animal hosts and are extremely relevant microorganisms with regard to their ecology and interactions with different substrates and hosts. Recurrent and consistent infections caused by these agents indicate a possible adaptation potential in the host. In this context, chromoblastomycosis is characterized by a subcutaneous infection due to traumatic implantation of these agents within the host tissue, followed by their adaptation through the formation of differentiated structures called muriform cells. According to the current hypothesis that is based on existing epidemiological data, patients acquire this disease mainly from the trauma caused by plant materials (e.g., thorns). Therefore, the aims of this study were to establish protocols for their isolation and to evaluate virulence potential of the *Fonsecaea* species that associate with animals and plants and leads to chromoblastomycosis. For the virulence tests, the clinical strains of chromoblastomycosis agents namely, *Fonsecaea pedrosoi* (CBS 271.37) and *Fonsecaea monophora* (CBS 102248) and an environmental species isolated from living plants in the endemic area known as *Fonsecaea erecta* (CBS 125763) were used. The infection protocols, in plants, were established using the thorny plants, *Mimosa pudica* (creeper) and *Bactris gasipaes* (palm tree), as models. In animals, the *Tenebrio molitor* larvae were chosen for the evaluation of virulence and environmental isolation and BALB/c mice were used for performing complementary immunogenicity tests. The plants were produced *in vitro* with subsequent transfer to culture vessels. These in vitro and in vessel plants were then inoculated by direct injection of the fungal solution (10^2 cells/mL) into the stem and by contamination of the culture medium and/or soil. All strains were able to colonize tissues of both the *in vitro* and in vessel produced plant species. The histological analysis showed that the clinical strains of *F. monophora* and *F. pedrosoi* remained in the epidermal region of the infected plants as yeast cells and hyphae, while the clinical strain of *F. erecta* (associated with the living plant) produced hyphae within the tissues of the plants, reaching the region of vascularization. The colonization profile of *F. erecta* was similar to that of the endophytic isolates, used as controls in this work. In the virulence tests, it was observed that the *T. molitor* larvae that were infected by *F. erecta* had the lowest survival rate followed by the larvae infected with *F. monophora* and *F. pedrosoi*, indicating that the fungal species from a living plant have better survivability inside a host animal. However, it was also observed that only the clinical strain of *F. pedrosoi* was able to form muriform cells, constituting a hallmark of chromoblastomycosis. These results demonstrated that there were differences in ecology and virulence among these strains and also hinted at the potential of these larvae to be used as disease models. The re-isolation of fungi from the infected larvae after 240 h showed a significant decrease in the quantity (in CFU/mL) of *F. erecta*, in relation to other clinical strains. This seemed to be induced due to the high immunogenicity of this species that was seen in immunological assays of BALB/c mice. The *T. molitor* was also employed in methods for enrichment and isolation of chromoblastomycosis causing agents from the environment. A total of four isolates of the family Herpotrichiellaceae, namely, *F. erecta* (n=2) and *F. monophora* (n=2) and six isolates of the order Capnodiales, namely,

Cladosporium cladospoioides (Cladosporiacea) and *Pseudocercospora norchiensis* (Mycosphaerellaceae), were obtained from the larvae inoculated with coconut samples from the babassu palm tree. These results revealed the efficacy of this model as an alternative method for the isolation of black yeast from the environment. In conclusion, this study showed the ability of clinical strains to survive in plant tissues, confirming the hypothesis that these traumatic infection-causing agents are derived from plant substrates. The different behavior of these species originating from the clinical and environmental (living plants) source was also recorded. In addition, the *T. molitor* larvae functioned as a potentially useful model for elucidation of virulence and dimorphism of these agents and thus proposed as the first animal model for the reproduction of muriform cells in research.

Keys-words: Black yeasts, virulence tests, isolation, *F. pedrosoi*, *F. monophora, F. erecta*, chromoblastomycosis.

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LIST OF ACRONYMS

CAPES - Coordination for the Improvement of Post-Graduate Education
CBM- Chromoblastomycosis
CBS - Fungal Biodiversity Centre, Centraalbureau voor Schimmelcultures
CDC- Cell Division Cycle
CFU- Colony-forming unit
CMRP - Microbiological Collections of Paraná Network
CNPq - Brazilian Agency for Scientific and Technological Development
CTAB - Cetyltrimethylammonium Bromide
DNA - Deoxyribonucleic Acid
ITS - Internal Transcribed spacer region
LSU - Large ribosomal subunit
PCR- Polymerase chain reaction
PCZ- Posaconazole
PDT- Photodynamic Therapy
pH- Potential of hydrogen
rDNA- ribosomal Deoxyribonucleic Acid
SSU- Small subunit ribosomal
TEF1 - Translation Elongation Factor 1-alpha
UFPR - Federal University of Parana
UV- Ultraviolet light

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CHAPTER I: OUTLINE OF THE THESIS

1 GENERAL INTRODUCTION

The *Fonsecaea* genus comprises of fungal etiological agents of the chromoblastomycosis a human skin disease. Most agents of this disease are members of a single order in the fungal kingdom, the Chaetothyriales (QUEIROZ-TELLES et al., 2017). These microorganisms have a low competitive capacity when compared to other microorganisms present in the environment, which limits black yeasts to occupy specific habitats (SATOW et al., 2008).

Human chromoblastomycosis (CBM) is caused by the implantation of the fungus and infection of the subcutaneous tissues. This disease is considered to be an orphan and neglected disease that affects low socioeconomic groups mainly. It is characterized by embedded microabscesses with fibrous granulomatous lesions and tissue proliferation with several types of clinical lesions (QUEIROZ TELLES et al., 2016).

After implantation, through the trauma caused by a plant, the fungus establishes in human tissues to form muriform cells with a dark colored thick wall that is assumed to be a structure of adaptation in the host. This disease has been reported worldwide, mainly in the areas of tropical and subtropical climate with high incidence in Brazil, Mexico, Venezuela, Madagascar, and China (XI et al., 2009; NAJAFZADEH et al., 2011 a; QUEIROZ-TELLES et al., 2016).

It is hypothesized that chromoblastomycosis is a traumatic infection that has a higher prevalence among male farm workers, who acquire the disease by contaminated plant thorns or wood cortex. This seems to be the main route of the infection and is supported by clinical reports of infected patients (RIPPON, 1988; SAYAL, et al., 2002; SALGADO, et al., 2004; VICENTE et al., 2008; MENEZES et al., 2008; KHAN et al., 2015).

In Brazil and its main agents are the species of *Fonsecaea* genus. The state of Maranhão in the north of Brazil is considered as one of the regions with the greatest occurrence of this disease (MARQUES et al*.,* 2006) and the prevalence of *F. pedrosoi* and *F. monophora* (VICENTE et al., 2008). Several authors have reported the environmental isolates of these species (IWATSU,

1981; VICENTE et al., 1999, 2008, 2013). In the environmental study, Marques et al., (2006) isolated *F. pedrosoi* that was assumed as the main agent of this disease in the Amazonia areas and reported the source of infection as the coconut shell of Babassu palm tree (*Orbignya phalerata*). According to Silva et al., (1995) during the Babassu harvesting, the workers have the habit of sitting on the fruits of the palm tree, justifying the incidence of lesions on their thighs and regions of the lower limb. Further, Salgado et al. (2004), isolated *F. pedrosoi* from the thorns of *Mimosa pudica* plant after it reportedly caused injury and subsequent skin lesion in a patient. However, this isolate was never identified by molecular methods. These methods are the main tools that are used to clarify the identity and relatedness among *Fonsecaea* species, that are even though very similar in morphology but are different according to molecular studies and hence different in terms of their virulence potentials (HOOG et al., 2000, 2004; SUN et al., 2012; VICENTE et al., 2013; NAJAFZADEH et al., 2011 a; QUEIROZ-TELLES et al., 2016).

Additionally, according to literature, the fungus *Cladophialophora carrionii* is considered the most frequent etiological agent in arid and semi-arid regions of Central and South America (LAVELLE, 1980) and Australia (TREJOS, 1954). According to the authors, the infection in these regions usually associates with trauma by cactus spines (RUBIN et al., 1991; FERNÁNDEZ-ZEPPENFELDT et al., 1994). However, Hoog et al., (2007) demonstrated by molecular studies that the clinical isolates belonged to *C. carrionii* species, whereas the environmental isolates obtained from plant spines cactus (*Stenocereus griseus*) belonged to a new species described as *Cladophialophora yegresii*. Likewise, Vicente et al., in 2013, investigated the occurrence of black fungi in living area of patients with chromoblastomycosis and described two novel species, *F. erecta* and *F. minima*, which were isolated from living plants and were related to the clinical ones. Therefore, the question that remains to be elucidated is whether the environmental and clinical strains associated with chromoblastomycosis represent exactly the same species and genotypes.

Since the discovery of this disease, several attempts have been made in order to develop a model of study in animal and vegetable hosts, aimed at understanding the infection route and clinical evolution of chromoblastomycosis.

Although some models have been established in animal hosts (MARTINEZ et al., 2005; XIE et al., 2010), the chronic infection of human mycosis has not been successful (MARTINEZ et al., 2005). Similar lesions have been found in rats and mice that were infected by the peritoneum and subcutaneous pathway. However, such experimental models tend to cure spontaneously which leads to unreliability (BORELLII, 1972). Thereby, the objective of this work was to understand the environmental origin and the route of infection of the agents causing chromoblastomycosis disease in humans, by evaluating the pathogenic potential of environmental and clinical strains of *Fonsecaea* with the help of virulence tests, using different animal and plant hosts.

This study was, therefore, divided into three chapters. Chapter I consisted of a review addressing the themes black yeasts, taxonomy, and biodiversity, with emphasis on virulence factors and experimental models for reproduction of pathogenesis. In Chapter II, infection models were tested in plants and animals for evaluation of closely related *Fonsecaea* species associated with chromoblastomycosis disease. For the virulence tests the clinical strains of *F. pedrosoi* (CBS 271.37), *F. monophora* (CBS 102248) and *Fonsecaea erecta* (CBS 125763), isolated from a living plant, were used. Chapter III was developed based on the study model proposed in Chapter II, where the *Tenebrio molitor* larvae were evaluated as an alternative source for environmental isolation of *Fonsecaea* species.

2 OBJECTIVES

2.1 GENERAL

The main objective of this study was to establish protocols for isolation and virulence evaluation of the animal and plant associated *Fonsecaea* species related to chromoblastomycosis.

2.2 SPECIFIC

- To establish stablish *Mimosa pudica* plants *in vitro* culture as a model of study;
- Evaluate the development of *Fonsecaea* species inoculated in *Mimosa pudica* and *Bactris gasipaes* (palm tree) plants;
- Analyze the virulence potential of *Fonsecaea* sibling species in animal hosts through inoculation in *Tenebrio molitor* and mice Balb/c.

3 LITERATURE REVIEW

3.1 BLACK YEASTS, TAXONOMY AND BIODIVERSITY

Black yeasts belong to the orders Dothideales and Chaetothyriales (HOOG and MCGINNIS, 1987) and have the characteristic presence of a dark pigment called melanin, in their cell wall. They are also known as dematiaceous fungi, as they are capable of producing melanized yeast cells during a part of their life cycle (HOOG et al., 2000).

The order Dothideales presents adaptive ability in a hostile environment, has rapid growth rate, contains large and multinucleated hyphae and is melanized depending on the stage of development and environmental conditions (VICENTE, 2000). Most species of this family grow in a low concentration of nutrients and are halotolerant or halophilic (HOOG et al., 2006). Some groups such as *Alternaria, Stemphylium, Epicoccum,* and *Ulocladium* have multicellular conidia and transverse and longitudinal septate, whereas groups like *Curvularia* and *Drechslera* have transverse multicellular conidia. Other groups like *Cladosporium*, *Nigrospora, Aureobasidium,* and *Hortaea* belonging to the saprobe genus present only unicellular conidia (HOOG et al., 2000; STERFLINGER et al., 2001).

The members of the order Chaetothyriales cause diseases, such as chromoblastomycosis, mycetoma, and phaeohyphomycosis (MCGINNIS, 1992), in animals. Within this order, the Herpotrichiellaceae family includes fungal species that have highly diversified life style and cause recurrent infection in a variety of vertebrate (HOOG et al., 2014) as well invertebrate hosts with intact immunity, that are commonly affected by these agents, e.g., cold blooded animals (SEYEDMOUSAVI et al., 2014). These agents are slow growing with yeast like appearance and with time they become velvety colonies with mononuclear hyphae showing uniform pigmentation (DIXON, POLAK-WISS, 1991). Some of the main species belonging to this group are from the genus *Exophiala, Cladophialophora, Capronia, Fonsecaea, Phialophora, Rhinocladiella, Veronae,* and *Cyphellophora* (HOOG et al., 2000).

The genus *Cladophialophora* has colonies that are pulverulent, wooly green to grayish-green, are conidiophores or imperceptible and depigmented conidial scars (HOOG, GENE, and FIGUERAS, 2000; HOOG et al., 2004). They are frequently isolated from decomposing organic matter and may be associated with several infections in human and animal hosts (BADALI et al., 2008; VICENTE et al., 2012).

The *Fonsecaea* species, associated with human diseases, are also described in animals, e.g., *Fonsecaea multimorphosa* causes disseminated infection in cats (NAJAFZADEH et al., 2011 a) and *Fonsecaea brasiliensis* causes disseminated infection in crabs (VICENTE et al., 2012). Some species such as *Fonsecaea erecta* and *Fonsecaea minima* have been isolated from the environment (VICENTE et al., 2013). The main micromorphological characteristic of this group is the presence of branched conidiophores, having primary and secondary conidia with predominant variation in morphology (HOOG et al., 2000; VICENTE, 2000). Macromorphologically they present flat or raised velvety colonies of slow growth, with coloration ranging from black, olive to dark gray reverse (AL-DOORY, 1983; DE HOOG, et al., 1998, VICENTE, 2000).

The genus *Exophiala* exhibits typical black-olivaceous colonies, which are reduced, have a viscous center, with yeast growth, and smooth margins. With the passage of time, the colonies become velvety or wooly. They have intercalated cell structures, having a cylindrical shape with relative narrowing; the conidium is subhyaline with a smooth wall. Some chains with spherical cells are formed by the profusion of shoots (HOOG et al., 2000; HOOG; GENE; FIGUERAS, 2004). The members of this family that were isolated from aquatic environments, presenting no thermotolerance, caused diseases in cold-blooded animals such as fishes, amphibians and some invertebrates (HOOG et al., 2011), while other species such as *E. jeanselmei*, *E. spinifera,* and *E. dermatitidis* were found to infect humans (SEYEDMOUSAVI et al. 2013).

The habitats of black yeast are diverse. They are commonly found in tropical and subtropical regions (FIGURE 1), as well as glacial regions (GUNDE- CIMERMAN et al., 2003; STERFLINGER, 2006; BRIDGE; NEWSHAM, 2009), in plant substrates, soil, wood and decomposing organic

matter (MCGINNIS et al., 1999; VICENTE, 2000). In addition, these agents, due to their extremophile nature, are also present in special conditions such as, during the low availability of nutrients, at high temperatures, UV radiation and in the presence of aromatic compounds, osmotic stress or combinations of these factors (STERFLINGER, 2006).

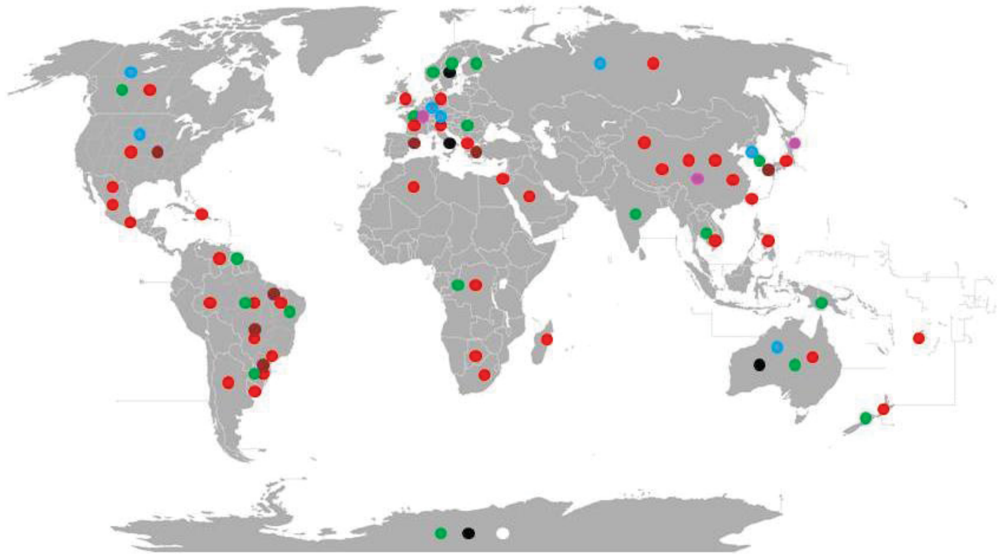

Figure 1. Global distribution of Black yeast based on the report of clinical cases and environmental isolation. The prevalent species ecologies are shown by the balls in the world map. Black: rock-inhabiting; Blue: waterborne; Green: plant-associated; Red: invasive in warm-blooded animals; Brown: soil; Pink: food; White: ice.

The black yeasts and their relatives harbor numerous agents of human infection. Virulence factors involved are not known yet, but given the highly polyphyletic pathogenic potential of the concerned ascomycete order, the Chaetothyriales present a significant predisposition toward growth in human tissue; a capacity that must have arisen during the evolution of this organism (FIGURE 2). The CBM is caused by the genera *Fonsecaea* and *Cladophialophora*, to some extent by species of *Phialophora* and *Exophiala* and less frequently by *Rhinocladiella*. Like other Endemic mycoses, CBM is not a notifiable disease, thus making it difficult to accurately assess its prevalence. Based on the number of cases, it can be noted that the incidence of CBM varies from 1: 6,800 in Madagascar to 1: 8,625,000 in the United States. Most cases are reported in tropical and subtropical regions, mainly between latitudes of 30°N and 30°S (AL-DOORY, 1983; QUEIROZ-TELLES et al., 2011) with more

than 500 cases in China, Brazil, Mexico and Madagascar (QUEIROZ-TELLES et al., 2016) (FIGURE 1).

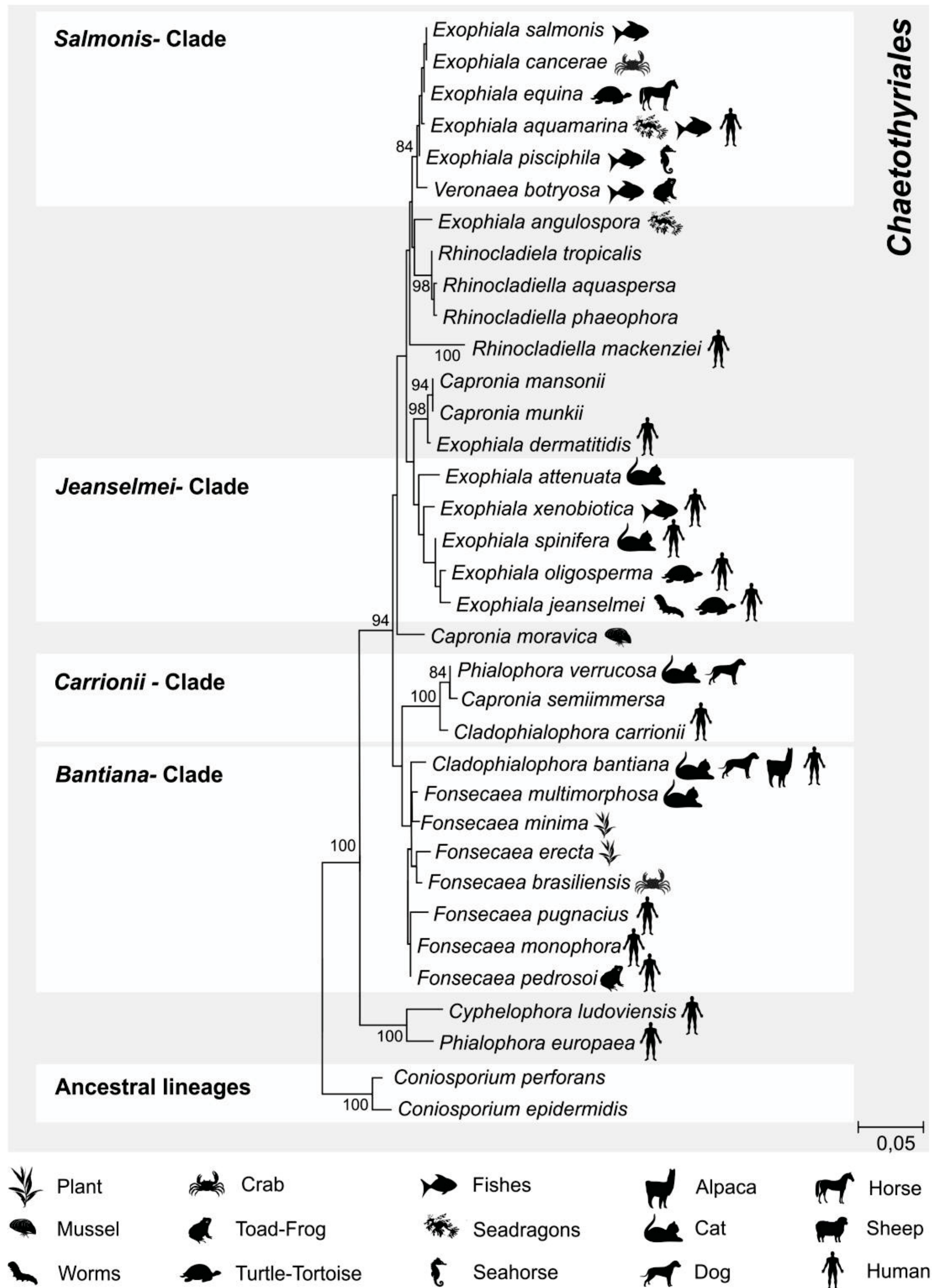

Figure 2. Phylogeny of Chaetothyriales and related Ascomycetes. Tree based on LSU sequences constructed with Maximum likelihood implemented in Mega7 with Kimura's two-parameter model. The bootstrap support was calculated using 100 replicates and values ≥ 80%

are shown in the branches. The main agents and host associated are represented in each family. The symbols before the species represent the animal affected.

According to Hoog et al. (1998), black yeasts cannot be identified by observing the macro and micro morphological structures due to their morphological plasticity. Furthermore, the little difference in the conidial structures, the size variation, and the shape makes it difficult to define the species.

Molecular tools have helped in identifying the species and consequently in clarifying an infection route which helps to build the epidemiological data (HOOG et al., 2006). Such concise results allow the construction of phylogenetic trees that are essential for elucidating the evolution and ecology of these microorganisms (VICENTE, 2000; STERFLINGER, 2006).

Taxonomic studies and the identification of molecular markers, in the species of black yeasts of clinical relevance, have been performed by sequencing the SSU, LSU, ITS, 1-alpha elongation factor (EF1α), β-tubulin and actin (ZENG et al., 2007; BADALI et al., 2008) and CDC42 (SUN et al., 2012). The partial ribosomal DNA (rDNA) large-subunit (LSU) sequences are conserved to show relationships at the level of order or family (VICENTE et al.2013, AZEVEDO et al. 2015). Within Chaetothyriales, the pathogenic species are polyphyletic with dispersion all over the tree (FIGURE 2), wherein the main species involved in CBM are in three clusters. In the *Fonsecaea* cluster, that is included in the “bantiana clade", the prevalent agents of CBM namely *F. pedrosoi*, *F. nubica* and *F. monophora* (HOOG et al., 2000; SUN, et al., 2012; NAJAFZADEH, et al., 2010) are grouped. It also includes the recently described *F. pugnacious*, which is also related to this clade (DE AZEVEDO et al., 2015). Additionally, it was observed that the bantiana clade contained species of *Cladophialophora* and some unknown saprobes species involved in human disease along with the agents of primary brain infection and disseminated infections (SURASH, et al., 2005).

The agents of chromoblastomycosis are also grouped in a separate clade named “carrionii clade” which includes *Cladophialophora carrionii* (HOOG, et al., 2004; BADALI, et al., 2008), the main agent of CBM in semiarid areas, *C. samoensis,* the causal agent of CBM in Samoa (located south of the

equator, between Hawaii and New Zealand in the Polynesian region of the Pacific Ocean) (HOOG, et al., 2007) and *Phialophora verrucosa*, isolated from CBM lesions of chromomycosis reported in northern Africa.(GUGNANI, et al., 1978; SURASH, et al., 2005; HOFMANN, et al., 2005).

Furthermore, the species *Rhinocladiella aquaspersa*, one of the CBM agents (BORELLI, et al., 1972; GONZÁLES, et al., 2013), is located in a separate clade from the ones described above. This separate clade includes the species of *Exophiala* that cause human infection, such as *Exophiala jeanselmei*, *E. dermatitidis* and *E. spinifera* (NAKA, et al., 1986; QUEIROZ-TELLES, et al., 2003; TOMSOM, et al., 2006). The groups of other species that are associated with the environmental occurrence and animal disease (ZENG, et al., 2007; VICENTE, et al., 2012) belong to this clade.

3.2 VIRULENCE FACTORS

Black yeasts can adapt to various environmental conditions; the presence of melanin in the cell wall and the ability to alternate between yeast and filamentous forms (pleomorphism), the production of biofilms and extracellular polysaccharides, allow these yeasts to tolerate conditions like variations in pH and salt concentration, UV radiation and high temperatures (HOOG, 1993; GOSTINCAR, 2011).

The factors that are probably of importance for the pathogenicity of black yeasts include, the presence of melanin and carotene, the formation of thick cell walls and the growth of meristem, the presence of yeast-like, thermotolerant and osmotolerant phases, adhesion, hydrophobicity, assimilation of aromatic hydrocarbons and the production of siderophores (SEYEDMOUSAVI et al., 2014).

The adaptation of these fungi to human hosts, also established as a hostile habitat, is favored by the presence of melanin in their cell wall and their thermotolerance (RIBEIRO et al., 2006). Melanin is a complex polymer, which is widely distributed in nature and is known to be an important virulence factor in the opportunistic and pathogenic dematiaceous fungi (SUN et al., 2012).

The melanin that is synthesized by the DHN and DOPA pathways is known as the eumelanin (LANGFELDER et al., 2003). According to Teixeira et al., (2017), the members of Herpotrichiellaceae possess several melanin-associated genes, suggesting that melanin production can use several pathways (LI et al., 2016). The genomic studies of Herpotrichiellaceae agents have confirmed the presence of multiple laccases, which have several functions. According to these studies, it is possible that the diversification of this enzyme occurred late during the evolution of black yeasts (TEIXEIRA et al., 2017; MORENO, et al., 2017). Apart from the pigmentation function, melanin also plays a role in the degradation of organic pollutants such as lignin and in stress tolerance. The various functions of this enzyme may justify the presence of multiple laccase genes in the genome (BALDRIAN 2006, RODRIGUEZ COUTO & TOCA HERRERA 2006).

Fungal species that are not thermotolerant tend to be saprobic and such species are rarely reported as a cause of infection in humans. Therefore it can be said that the thermotolerance influences the choice of host. During the last decade, some species of *Exophiala* that were isolated from environments without thermotolerance were also seen to be involved in animal diseases (HOOG et al., 2011). A generalized suite of adaptations to extreme environments, including melanin production and meristematic growth, as well as A generalized suite of adaptations to extreme environments, including melanin production and meristematic growth, as well as thermotolerance, is suggested to contribute to pathogenic potential (BADALI et al., 2008).

The ability to grow at 37 °C gives black yeasts the potential to cause diseases. Some clades that are composed of yeasts and grow above 37 °C, like bantiana, dermatitidis, and jeanselmei, can cause systemic infections in humans. Whereas other species that can grow at a maximum temperature of 36 °C, like the carrionii and the europaea can cause only subcutaneous and surface skin infections (HOOG, et al., 2011).

The salmonis clade group members originate in water and are also known as “water-borne” species. It was observed that the maximum temperature for their growth depended on the temperature of the host habitat, for example, the agents from this clade cause diseases in amphibians,

suggesting that the thermotolerance determines the choice of the host. This indicates that fungi can cause disease in cold-blooded animals if they grow at 27 ºC and infections in mammals if they grow above 37 ºC (HOOG et al., 2011; VICENTE et al., 2012; SEYEDMOUSAVI; GUILLOT; HOOG, 2013; VICENTE, et al., 2014).

Fonsecaea brasiliensis is considered as a secondary agent of lethargic crab disease. The etiological agent of this disease is *Exophiala cancerae*, which has been isolated from the sick crabs but never from the mangrove environment (VINCENT et al., 2012). In a study conducted by Hoog et al*.,* (2011), it was verified that the optimal growth temperature for *E. cancerae* is between 24–27 ºC, whereas for *F. brasiliensis* it is 37 ºC (VICENTE et al., 2012). This factor suggests that thermotolerance can determine the host predilection. According to Gostincar et al., (2011) the term poly-extremotolerant could be adopted for these yeasts in order to describe their high ability to colonize a wide variety of environments and to withstand different ecological conditions.

Among the factors for pathogenicity of CBM, muriform cells are the most important. These cells are present either in single or clustered form, consisting of darkly pigmented, thick, transverse, and longitudinal walls. These cells are present only in the order Chaetothyriales and are considered to be invasive with respect to the host tissue (ESTERRE and QUEIROZ-TELLES, 2006), as they are highly resistant to immune system. However, the knowledge regarding how the differentiation occurs in cells is fundamental for the investigation of new and more efficient therapeutic approaches in the treatment of CBM (HAMZA et al., 2003).

Proteolytic enzymes play an important role in the pathogen-host interaction, leading to immunological escape or antimicrobial resistance (KNEIPP et al., 2004; PALMEIRA et al., 2006). According to Kneipp et al., (2004) muriform cells display increased activity of phosphatase enzyme during pathogenicity (KNEIPP et al., 2004; KNEIPP et al., 2008). The CBM agents secrete several hydrolytic enzymes; the muriform cells of *F. pedrosoi* and *R. aquaspersa* show higher phosphatase activity during CBM and thus cause an increase in the cell adhesion (KNEIPP et al., 2004; KNEIPP et al., 2008; KNEIPP et al., 2012). The earlier studies suggest that Ecto-ATPases may favor

the survival of the fungus in environments such as the human body (FILIPPINI et al., 1990; COLLOPY et al., 2006).

In relation to innate immunity, macrophages carry out an important function in the regulation of fungal growth. CBM fungal cells can be detected in intra-cytoplasmic vacuoles of skin macrophages (WALTER et al., 1982; QUEIROZ TELLES et al., 2017). The chronic and granulomatous inflammation is generally characterized by the dominating presence of macrophages in the injured tissue (SEYEDMOUSAVI et al., 2014). Sotto et al., (2004) investigated the cellular immune response in lesional biopsy specimens of patients with CBM and reported that phagocytes are involved in innate immunity against the fungal agents.

Bocca et al., (2006) showed that during infection with *F. pedrosoi*, the macrophages were unable to produce nitric oxide even after stimulation with lipopolysaccharide (LPS) and gamma interferon (IFN-). This was attributed to the host's inability to destroy *F. pedrosoi*, leading to a chronic disease. According to SILVA et al. (2007), phagocytosis of fungal cells by Langerhans cells inhibited the expression of CD40 and CD86. Gimenes et al., (2005), analyzed the production of proinflammatory cytokines by macrophages and found that the peripheral blood mononuclear cells (PBMC) from patients with chromoblastomycosis secreted high levels of tumor necrosis factor alpha (TNF-y), which was not dependent on the clinical form of the disease. Souza et al., (2011) showed that the cytokine response to *F. pedrosoi* was defective due to the lack of fungus recognition by Toll like receptors (TLRs).

Polymorphonuclear neutrophils and dendritic cells are important components in the first line of defense during an infection that arises due to cutaneous or subcutaneous inoculation in tissues. The dendritic cells play an important role in phagocytosis, by serving as major antigen-presenting cells and inducers of adaptive T-cell responses (SOUZA et al., 2009). Mature dendritic cells prime the naive lymphocytes and polarize them toward the T-helper type 1 (Th1) response, whereas immature dendritic cells have been shown to induce tolerance. The severity of diseases caused by chaetothyrialean black yeasts is dependent on Th1/Th2 activation. Patients with severe clinical forms produce interleukin-10 (IL10) with inhibition of IFN-γ and immune response between Th1

and Th2 activation, whereas in mild cases IFN-γ is present along with decreased levels of interleukin-10 (IL10) and increased number of T cells favoring a Th1 profile (MAZO FÁVERO GIMENES et al., 2005; BOCCA et al., 2006).

The immunity in patients with Chromoblastomycosis is impaired, because of which these patients are unable to develop an effective immune reaction to fungal antigens, thereby resulting in disease chronicity (FUCHS and PECHER, 1992). Experimental infection models using athymic mice showed that during the course of infection, granulomas become diffuse and confluent accompanied by a random fungal distribution, suggesting a role for a T-cell-mediated immune response (AHRNES et al., 1989).

In addition to melanin, black yeasts are also known to contain mannose-bearing structures as surface components that activate mannose receptors and help the host to control infections (ALVIANO et al., 2003). Melanin, an immunologically active fungal molecule, activates both humoral and cellular responses that might help to control the infection (ALVIANO et al., 2004). Melanin is potentially involved in the activation of TLR4, with consequent production of the proinflammatory chemokine IL–8 (EL-OBEID et al., 2006). According to Alviano et al.,(2003), the interaction of *Fonsecaea pedrosoi* with phagocytes in the presence of melanin resulted in higher levels of fungal internalization and destruction by host cells, accompanied by greater degrees of oxidative burst (ALVIANO et al., 2004).

The presence of immunoglobulin M (IgM), IgA, and IgG antibodies has been identified in some studies (VIDAL et al., 2004; ESTERRE et al., 2000), but the humoral immune response does not seem to protect from infections caused by black yeasts and their filamentous relatives. Moreover, in vitro studies indicate that the interactions between fungi and anti-melanin antibodies inhibit fungal growth (VIDAL et al., 2004).

Further studies are warranted to evaluate the roles of host genetic susceptibility and immunologic defects in the pathophysiology of black yeast infections. A preliminary study showed that CARD9 mutation was linked to subcutaneous phaeohyphomycosis and Th17-cell deficiencies (WHEELER, et al. 2008). In all patients, the CARD9 mutation was linked to decreased

proportions of cytokines in Th17 with consequently cells impaired immune responses against the infection. Additionally, one more aspect that should be considered in the patients with defects in humoral immunity, such as agammaglobulinemia or hypogammaglobulinemia, is that they might have relatively normal resistance to black yeasts and their filamentous relatives. Nevertheless, vaccination and induction of protective antibodies may provide additional protection in specific groups of patients (NETEA, 2013).

3.3 CHROMOBLASTOMYCOSIS, DISEASE AND DIAGNOSIS

In the middle of the nineteenth century, black yeasts became known by the scientific society as a pathogen of the disease, which was later termed as chromoblastomycosis (CBM). Although the disease was first observed in 1911 by Alexandrino Pedroso, a Brazilian, it was described only three years later by Max Rudolph. Since then, several researchers have dedicated themselves to conduct research on the disease; however, the restricted understanding and knowledge of it has made its identification difficult and complex (HOOG, et al., 2000; MARTINEZ and TOVAR, 2007). Over the years, several terms have been used by the scientific community to denote the disease, but the term chromoblastomycosis, initially used by Terra et al. (1922), has turned out be the most acceptable and is still valid.

The island of Madagascar represents an area endemic to CBM, with 1,323 cases reported between the years 1955 and 1995 (ESTERRE et al., 1996 a). With the presence of two distinct ecosystems in the island, the main agents of CBM are *Fonsecaea pedrosoi* and *Cladophialophora carrionii*. Although CBM is a disease primarily restricted to tropical and subtropical regions, approximately 31 cases have been reported in Europe (PINDYCKA-PIASZCZYNSKA et al., 2014).

In the Asian continent, Japan has the highest incidence of CBM (1 in 416,000 individuals). Since the first description of the disease in 1930, approximately 700 cases have been reported (KANO, 1937; KONDO et al., 2005). In China, research groups have reported the occurrence of several

hundred cases of CBM in 21 provinces (YEW, 1951; DAI et al., 1998). India has long been known for autochthonous CBM infections, with more than 100 cases reported (SHARMA et al., 1999; SAYAL et al., 2002). Within the region of Oceania, approximately 200 cases have been reported in Australia, where the main causative agent is *C. carrionii* due to the climatic conditions. Some cases have also been reported in New Zealand and Solomon Islands (WOODGYER et al., 1992; WEEDON et al., 2013).

The Caribbean region in the Central America has reported around 600 cases of implantation mycosis (TORRES-GUERRERO et al., 2012), where CBM was found to be the main cause of the infection. Of these cases, 450 patients belonged to Dominican Republic (ISA-ISA, 2006). Approximately 100 cases were reported from Cuba (DÍAZ-ALMEIDA et al., 1978; BADALI et al., 2013), and few cases have also been reported from Puerto Rico (CARRIÓN, 1975), Jamaica, and Haiti (BANSAL et al., 1989; ISA-ISA, 2006). The main etiological agent in these countries is *F. pedrosoi* followed by *F. monophora* and *C. carrionii* (CARRIÓN, 1975; BADALI et al., 2013).

Brazil has the highest number of cases in South America; the last decade witnessed the reporting of 872 cases in the northern regions (MELLO et al., 1992; SILVA et al., 1998; QUEIROZ-TELLES et al., 2011; PIRES et al., 2012). However, 332 cases of CBM were reported from other regions of this country (LONDERO et al., 1976; MINOTTO et al., 2001) as well. These included 71 cases in the state of Paraná (southern region) in 11 years; 325 cases in 55 years for Pará (northern region); 13 cases in 3 years for Maranhão (northeastern region); and 73 cases in 28 years for Rio Grande do Sul (southern region, MINOTTO et al., 2001; QUEIROZ-TELLES et al., 2003; QUEIROZ-TELLES et al., 2009; QUEIROZ-TELLES et al., 2011). The principal etiological agent of CBM in Brazil is *F. pedrosoi* (QUEIROZ-TELLES et al., 2016).

Black yeasts serve as the potential causative agents of CBM, which is a chronic skin disease infecting humans and commonly described as causing polymorphic skin lesions. Although the disease majorly affects the cutaneous and subcutaneous tissues, patients with this disease respond inefficiently to the treatment approaches utilized (HOOG et al., 2000; MARTINEZ and TOLVAR,

2007). The diagnosis is based on fungal culture; CBM agents demonstrate an initial slow growth, with deep green colonies that acquire a velvet dark aspect with time (HOOG et al., 2004; QUEIROZ-TELLES, 2015).

Clinically, the CBM initially appears as an erythematous macular skin lesion that progresses to a pink and smooth papular lesion (FIGURE 3). The appearance of peripheral lesions is common, usually originating from autoinoculation or disseminated through the lymphatic vessels (QUEIROZ-TELLES et al., 2009; NAJAFZADEH et al., 2011 a). Over time, the lesion may manifest itself as a papulosquamous lesion and starts evolving with polymorphic aspects, which may be confused with several other infectious and noninfectious diseases. The initial lesion may spread locally and produce satellite lesions (MCGINNIS, 1992; QUEIROZ-TELLES and SANTOS, 2012; QUEIROZ-TELLES, 2015). The CBM lesions are classified as nodular, tumoral (cauliflower-like), verrucous, scarring, and plaque (CARRIÓN, 1950; MCGINNIS, 1983; QUEIROZ-TELLES et al., 2011).

Microscopic analysis has revealed the presence of muriform cells in verrucous lesions, which can often be macroscopically confused with neoplastic formations. The microscopic diagnostic method is applicable within two months of CMB infection. In the third month, the generalized granulomatous inflammation can be observed; the microscope-based identification is not possible at this stage. The differential diagnosis for CBM, which provides a strong evidence for verrucous nodules, can be performed by biopsy. One of the techniques used to collect material for analysis is the fine needle aspiration (FNAB). The biopsy and FNAB may not be effective after the third month, as the muriform cells can no longer be observed; in this case, the gold standard technique is histopathology (XIE, et al., 2010).

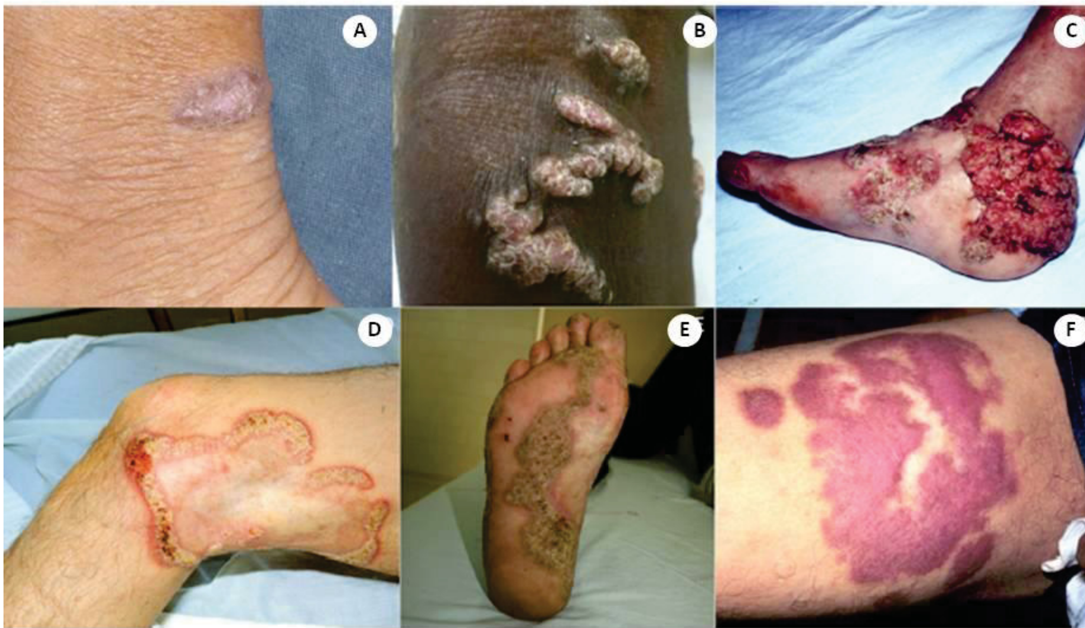

Figure 3. Clinical types of Chromoblastomycosis lesions and the invasive form inside the tissues. (A) Initial lesion within three months after infection in a lower leg. (B) Confluent nodular lesions on a knee. (C) Tumoral (cauliflower-like) lesion on the posterior part of a foot. (D) Cicatricial lesion with verruca showing serpiginous and verrucous contours. (E) Hyperkeratotic verrucous lesion on the sole of a foot. (F) Soft violaceous plaque lesion in the root of a thigh. SOURCE: QUEIROZ-TELLES et al., (2016).

In the case of CBM, spontaneous healing is rare. The treatment approach includes the use of drugs, surgery, and physical therapy; these constitute the major allies in the treatment modality. An amputation is rarely required in the case of CBM since the fungus does not directly reach the muscles and bones and therefore, dissemination usually does not reach the deeper tissues (MARTINEZ and TOVAR, 2007; QUEIROZ-TELLES et al., 2009). In most clinical cases, the various modalities of physical methods available are used as adjuvant therapy in combination with antifungal agents, surgery, thermotherapy, laser therapy, and photodynamic therapy (PDT, QUEIROZ-TELLES et al., 2017).

A study conducted by Bonifaz (2001) suggested certain forms of treatments for CBM. Cryosurgery using liquid nitrogen has been demonstrated to be effective for small lesions. However, contraindications exist for cryosurgery; for example, in the case of larger lesions, treatment with itraconazole or 5-FC is chosen until maximal reduction, upon reaching which, termination of cryosurgery treatment is recommended to cure the previously

reduced lesions. Heat therapy in combination with other treatment modalities has been applied in Japan achieving great results (TAGAMI et al., 1984; TAGAMI et al., 1979). A one-month application of a disposable chemical pocket warmer occluded with a bandage over the lesions 24 h per day resulted in an improvement of lesions as evident by negative microscopic examination and culture results (HIRUMA et al., 1993; YANASE and YAMADA, 1978). The use of local heat therapy for 2 h per day combined with the administration of posaconazole (PCZ) at 400 mg twice a day for an eight-month period led to a significant reduction in lesions, while a combination of heat therapy for 12 h per day and terbinafine (125 to 250 mg daily) led to negative direct examination and culture results within two weeks (TANUMA et al., 2000; WU et al., 2010). However, in some cases, additional surgical therapy was necessary.

Currently, laser therapy with carbon dioxide (CO_2) has been used as monotherapy; in combination with other treatment modalities, lasers promote photocoagulation. Recently, another therapy that has been tried is the photodynamic therapy (PDT) that exerts an effect on CBM similar to that on actinic keratosis or other types of skin cancers. This technique combines visible light photons of an appropriate wavelength to stimulate the intracellular molecules of a photosensitizer, activation of which produces several reactive molecules, including oxygen species, culminating in damage of target cells (LYON et al*.,* 2011, 2013). Having property of minimal invasiveness with few side effects, PDT is considered a promising treatment option; also, it may shorten the time of treatment. Moreover, the effectiveness of PDT against the most common species causing CBM can be verified using an *in vitro* study (LYON, et al., 2013).

3.4 EXPERIMENTAL MODELS

To develop an efficient and chronic animal model, several models have been developed and worked on; however, chronicity is only manifested in humans. The disease in its chronic form is characterized by the presence of muriform cells (XIE, 2010).

Since the description of the disease in 1911, the researchers have been trying to establish a reliable animal model of the disease. However, most attempts were unsuccessful and could not achieve a human-like model. Medlar in 1915 inoculated *F. pedrosoi* into guinea pigs, rats, and mice, thereby producing a non-progressive granulomatous reaction. In 1945, Azulay performed experiments in which *F. pedrosoi* was delivered via intratesticular and intraperitoneal routes but could not produce granulomatous reaction containing sclerotic cells.

In 1966, Simson described the first case of CBM in a horse; subsequently, CBM was found in different animals ranging from frogs to dogs, indicating that it could infect both humans and animals. Similar lesions were detected in rats and mice infected subcutaneously and intraperitoneally, respectively; however, such models developed the acute form of the disease, without success in generation of a chronic model, with a tendency of spontaneous cure, leading to unreliable models (BORELLII, 1972; POLAK, 1984; CARDONA-CASTRO et al., 1999; TEIXEIRA et al., 2006; MACHADO et al., 2010).

A similar CBM infection can be observed in amphibians, such as the toad and frog, in a systemic form (ANVER 1980; BUBE et al., 1992). According to Cicmanec (1973) and Schmidt (1984), these animals appeared to be more susceptible to the disease when they were debilitated or under stress. Velasquez and Restrepo, in 1975, reported two toads (*Bufo marinus*) infected by black yeast. These animals had granulomatous lesions containing brown fungi identical to those found in human chromomycosis infection. After isolation, the isolates shared common antigens with the recognized human pathogens, *F. pedrosoi*, *P. verrucosa,* and *C. carrioni*. Bube et al. (1992) performed a necropsy in the tissues of two marine toads, held in captivity; the animals presented lesions similar to CBM. In the first case, granuloma with necrosis was observed with multinucleated giant cells present in the meninges of the brain, cervical, and thoracic spinal cord, resulting in the compression of spinal nerves in skin, kidneys, and at a single focus in the liver. While in the second case, ulcerating nodules were found in the skin, and the nodules were present in the lungs with granulomas detected in the heart, spleen, bone marrow cavities of

the skull, and in close proximity to spinal nerves. Sclerotic bodies were present in the nasal cavity and the skin. Black yeast was recovered in small numbers from the nodular lesions. Hosoya et al. (2015) reported, in an anuran breeder's water tank in Tokyo, Japan that 10 frogs of at least three species had died in the same facility in a limited period. The animals presented polypoid lesions with ulceration found on the body surfaces. The symptoms did not improve with oral treatment of antifungal agents. Histopathological analysis revealed granulomatous lesions, containing brown hyphae. The result of phylogenetic analysis demonstrated that the query sequence was situated among *Veronaea botryosa*.

However, CBM is assumed to be a human disease due to the scarce reports of this infection in other animals. The most clinical cases of this skin lesion reported in animals have been considered as pheohyphomycosis (PHM), because typical muriform cells were lacking (QUEIROZ–TELLES, 2017).

Regarding the animal model for disease reproduction, Juopperi et al. (2002) reported, in the North Carolina State University (NCSU), a spadefoot toad (*Scaphiopus holbrookii*) for the evaluation of proliferative to ulcerative dermatitis. Histological analyses displayed sclerotic bodies in small clusters and occasionally in the multinucleated giant cells, some brown-pigmented septate hyphae have also been observed. However, isolation of the fungus was not possible because of bacterial overgrowth. The diagnosis of chromomycosis was based on cytologic and histologic findings.

The advances in molecular biology techniques and new immunology tools in the past two decades have expanded the available data regarding interactions of CBM fungus with immune cells. Mazo et al. (2005) demonstrated that the absence of CD4+ T cells induced a more severe form of the disease during experimental infection in mice (TEIXEIRA et al., 2006). According to a study conducted by Siqueira et al. (2017), muriform cells were demonstrated to induce inflammatory response and chronicity during the course of murine CBM.

A study by Xie et al. (2010) tried to induce chronic lesions for more than three months in Wistar rats by *F. monophora*, by infecting the animals intradermally in the ventral region. In another study by Machado et al. (2011), it was possible to observe that mice, infected through plantar cushion region with

conidiogenic cells and conidia, developed lesions for a longer period than when using hyphae and conidia.

Non-mammalian models have been used to investigate fungal virulence, such as *Cryptococcus neoformans* (MYLONAKIS, et al., 2005; SOUZA et al., 2015), *Candida albicans* (FUCHS, et al., 2010; MOWLDS, et al., 2010; SOUZA et al., 2015), *Microsporum gypseum*, *Trichophyton rubrum* (ACHTERMAN, et al., 2011), *Aspergillus flavus* (ST LEGER, et al., 2000), *A. fumigatus* (REEVES, et al., 2004), and *Madurella mycetomatis* (KLOEZEN et al., 2015). The larvae of *Galleria mellonella* and *Tenebrio molitor* are inexpensive to keep, easy to manipulate, and their use may reduce the need for pathogenicity testing in mammals, with a concomitant reduction in potential mammalian suffering (FUCHS, et al., 2010; KLOEZEN et al., 2015). These models could be used to analyze the fungal virulence and pathogenicity of etiologic agents of CBM.

Artificial inoculation in plants was performed in a study by Hoog et al. (2007). The cactus (*Stenocereus griseus*) plants were cultivated from seeds in the greenhouse, followed by inoculation of two species: environmental *C. yegresii* and clinical *C. carrionii*. Both the species persisted in the cactus tissues and upon reaching the spines, produced muriform cells similar to the one described as the invasive form of CBM. However, *C. carrionii* isolate was more virulent than the environmental *C. yegresii*, which was described from the isolation performed in the same species of cactus in the vicinity of a CBM patient’s residence.

In summary, these data indicate that studies to clarify CBM immunopathology, understand the evolutionary cycle of these agents, their environmental origin and infection route are warranted to improve the patients' quality of life and to cure them.

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CHAPTER II: Model for evaluation of *Fonsecaea* species in plant and animal association.

A Model for Trans-Kingdom Pathogenicity in *Fonsecaea* Agents of Human Chromoblastomycosis

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1. ABSTRACT

The fungal genus *Fonsecaea* comprises etiological agents of human chromoblastomycosis, a chronic implantation skin disease. The current hypothesis is that patients acquire the infection through an injury from plant material. The present study aimed to evaluate a model of infection in plant and animal hosts to understand the parameters of trans-Kingdom pathogenicity. Clinical strains of causative agents of chromoblastomycosis *F. monophora* and *F. pedrosoi* were compared with a strain from a living plant belonging to an environmental species (*F. erecta*). Artificial inoculation of *Mimosa pudica* plants and *Bactris gasipaes* palm tree demonstrated the ability of all fungal strains to persist in plant tissues. The clinical strains of *F. monophora* and *F. pedrosoi* were concentrated near the epidermis, whereas *F. erecta* colonized deeper plant tissues, resembling an endophytic behavior. In an invertebrate infection model with larvae of a beetle, *Tenebrio molitor, F. erecta* exhibited the lowest survival rates. However, *F. pedrosoi* produced dark, spherical to ovoidal cells that resembled muriform cells, the invasive form of human chromoblastomycosis. Re-isolation of fungus from the larvae after 240 h of infection yielded low rates of colony forming units (CFUs) for *F. erecta*. An immunologic assay in BALB/c mice demonstrated *F. erecta* to be more immunogenic, as evidenced by antigen recognition of serum from infected animals. In histological sections of the spleen, no changes in the cells were observed in the animals infected with *F. pedrosoi, F. monophora,* and *F. erecta*.

Keywords: *Fonsecaea, F. pedrosoi, F. monophora, F. erecta,* virulence, *Mimosa pudica*, *Bactris gasipaes, T. molitor*, animal model.

2. INTRODUCTION

The fungal genus *Fonsecaea* comprises several etiologic agents of human chromoblastomycosis, a severely mutilating skin disease. The genus belongs to the family Herpotrichiellaceae, which consists of numerous species potentially causing a wide range of recalcitrant infections. Among these are cerebral and disseminated diseases which if untreated mostly lead to the death of the patient (DE HOOG et al., 2000; NAJAFZADEH et al., 2011; GUERRA et al., 2013; DOYMAZ et al., 2015). In general, immunocompromised individuals are more susceptible to fungal infections; however, in black fungi infection is frequently observed in apparently healthy individuals. Vertebrate hosts other than humans include fish and amphibians, other host animals being very rare (DE HOOG et al., 2011).

Chromoblastomycosis is characterized by a chronic involvement of cutaneous and subcutaneous tissues containing the fungal invasive form, the muriform cell (MENDOZA et al., 1993), embedded in microabscesses and fibrosis. The infection often shows skin tissue proliferation, leading to clinically recognizable nodular, tumoral (cauliflower-like), verrucous, scarring, or plaque-like lesions (QUEIROZ-TELLES et al., 2016). The prevalent agents of the disease include *F. pedrosoi*, *F. monophora*, *Cladophialophora carrionii* (NAJAFZADEH et al., 2009; DOYMAZ et al., 2015), and *Rhinocladiella aquaspersa* (GONZÁLEZ et al., 2013). *Cladophialophora carrionii* is found in arid and semi-arid climates (LAVELLE, 1980), while *Fonsecaea* species are endemic to the areas with a warm and humid climate (BADALI et al., 2010b ; NAJAFZADEH et al., 2011).

The causative fungal species seem to be implanted into the host skin through sharp specimens of plant debris, such as thorns, carrying the respective opportunistic agent. In this regard, a report indicated their isolation from plant debris, while non-pathogenic relatives were occasionally derived from living plants (VICENTE et al., 2008). Epidemiological data provided evidence of traumatic infection by puncture of plant material (Queiroz-Telles and SANTOS, 2013; QUEIROZ-TELLES et al., 2016). Marques et al. (2006) isolated *Fonsecaea*-like fungi from the shells of babassu coconuts of the palm tree, *Orbignya phalerata* and Salgado et al. (2004) found a species morphologically resembling *Fonsecaea* on the thorns of a *Mimosa pudica* plant, which the patient identified as the possible source of his disease.

However, molecular studies have demonstrated that environmental strains morphologically similar to clinical strains do not necessarily belong to the same species (CROUS et al., 2006; MOSTERT et al., 2006; VICENTE et al., 2014). Closely related taxa may have a slightly different ecology that seem to interfere with infectious potential and causes them to remain strictly environmental. Rubin et al. (1991) and Fernández-Zeppenfeldt et al. (1994) suggested that *Cladophialophora* might be transmitted by the thorns of the cactus plant (*Stenocereus griseus*) or by wood splinters. However, sequencing of the clinical strains of *C. carrionii* and of a *Cladophialophora* species isolated from cactus thorns surrounding the cottage of a patient by de Hoog et al. (2007)

demonstrated them to belong to a different species, *C. yegresii*, which had never been observed to infect a human host. Upon environmental sampling to recover the clinical species (MARQUES et al., 2006; VICENTE et al., 2008; VICENTE et al., 2014), *F. pedrosoi* and *F. monophora* were only rarely encountered, while the majority of environmental species were not known from human infections. Vicente et al. (2013) described the environmental *Fonsecaea* sibling species as novel taxa, named *F. minima* and *F. erecta*.

The apparent selection by human tissue of *F. monophora* and *F. pedrosoi* remains unexplained. The disease is characterized by the presence of muriform cells inside host tissues, which can also be reproduced *in vitro* in non-pathogenic species (BADALI et al., 2008). De Hoog et al. (2007) demonstrated that both the clinical species, *C. carrionii* and the environmental, *C. yegresii* could produce muriform cells upon their artificial inoculation into cactus plants, which seemed to contradict the observed predilections for animal hosts (VICENTE et al., 2014). In addition, more detailed understanding of the natural ecology and environmental niches of chromoblastomycosis agents is required to monitor the possible routes of infection (GÜMRAL et al., 2014). Adequate infection models in animal and plant hosts are required to build up plausible evolutionary hypotheses. Therefore, the present study compared plant and animal models to evaluate the associations of hosts with clinical and environmental *Fonsecaea* strains to understand the mechanisms involved in the adaptation of fungal animal and plant disease to elucidate routes of infection of this implantation disease.

3. MATERIAL AND METHODS

3.1 VIRULENCE TEST USING PLANTS *AS MODEL*

3.1.1 Strains

The fungal strains were acquired from the reference collection of Centraalbureau voor Schimmelcultures (CBS; housed at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) and Microbiological Collections

of Paraná Network (CMRP, Curitiba, Brazil). i.e. *F. pedrosoi* (CBS 271.37) and *F. monophora* (CBS 102248), both isolated from human chromoblastomycosis, and the environmental species, *F. erecta* (CBS 125763) isolated from a living plant (Vicente et al. 2013). In addition, an endophyte, *Colletogloeopsis dimorpha* (CMRP 1417) isolated from *Mimosa pudica* and a strain of *Cladosporium tenuissimum* (CMRP 1441) isolated as endophyte from *Bactris gasipaes* were utilized as controls. The cultures were maintained on 2% malt extract agar (MEA) and Sabouraud's glucose agar (SGA) at 28 °C.

3.1.2 Plant inoculum preparation

The fungal strains were grown on potato dextrose agar (PDA) at 28 °C for 7 days. Conidial production was enhanced by passing the cells through a liquid PD medium in a shaker at 200 rpm at 30 °C. After 5 days, the cells were allowed to settle; hyphae and conidia were decanted, and the supernatant was filtered through a cell strainer membrane with 40 µm pore size. After repeated washings with phosphate buffered saline (PBS), the inocula were adjusted to the concentration tested and the cell viability was determined by plating of the suspensions on Mycosel agar followed by incubation for 7 days at 28 °C and 37 °C for *Fonsecaea* species, while the control strains were incubated on PDA at 28 °C for 7 days.

3.1.3 ***In vitro*** **and** ***in vessel*** **assays using** ***M. pudica*** **and** ***B. gasipaes***

Seeds of *M. pudica* were provided by the Brazilian Agricultural Research Corporation-EMBRAPA (Manaus, Brazil). Seeds were washed by keeping them for 30 min in flasks under running tap water, then surface sterilized inside a laminar flow chamber with 70 % ethanol for 1 min and 5 % sodium hypochlorite (NaOCl) for 10 min followed by rinsing thrice with autoclaved distilled water. The seeds were incubated in concentrated sulfuric acid for 15 to 20 min followed by rinsing thrice in autoclaved distilled water (SOUZA FILHO et al., 2001; PAIVA and ALOUFA, 2009). Subsequently, the seeds were cultured in agar-solidified Murashige and Skoog (MS) medium (MURASHIGE and SKOOG, 1962)

containing 3 % sucrose for germination. The seeds began to germinate after 48 h under aseptic conditions. An *in vitro* cultured palm plant (*B. gasipaes*) was provided by Vivetech Agrociências using the protocols based on Steinmacher et al. (2011).

In the *in vitro* and *in vessel* assays, both plants tested were inoculated with three *Fonsecaea* species (Group I: *F. pedrosoi,* Group II: *F. monophora,* and Group III: *F. erecta*), the endophyte, *C. dimorpha* (CMRP 1417) was utilized as the positive control and sterile saline (NaCl 0.85 %) was utilized as negative control.

3.1.4 ***In vitro*** **plant inoculation**

Plants of *M. pudica* were inoculated with 10 µL of culture solutions of test strains at concentrations of 10^2, 10^3, 10^4, 10^5, and 10^6 cells/mL, whereas the plants of *B. gasipaes* were inoculated at concentrations of 10^2 and 10^5 cells/mL. Positive and negative controls had equal volumes. The inocula were applied at five different points on the plant stem using 10 µL containing 10^2 cells/mL of each tested strain according to three protocols: (I) direct injection into the stem, (II) stem injury followed by micropipette inoculation, and (III) suspension of culture medium around the root (Fig. 1A). The plants were maintained *in vitro* under controlled temperature at 28 °C.

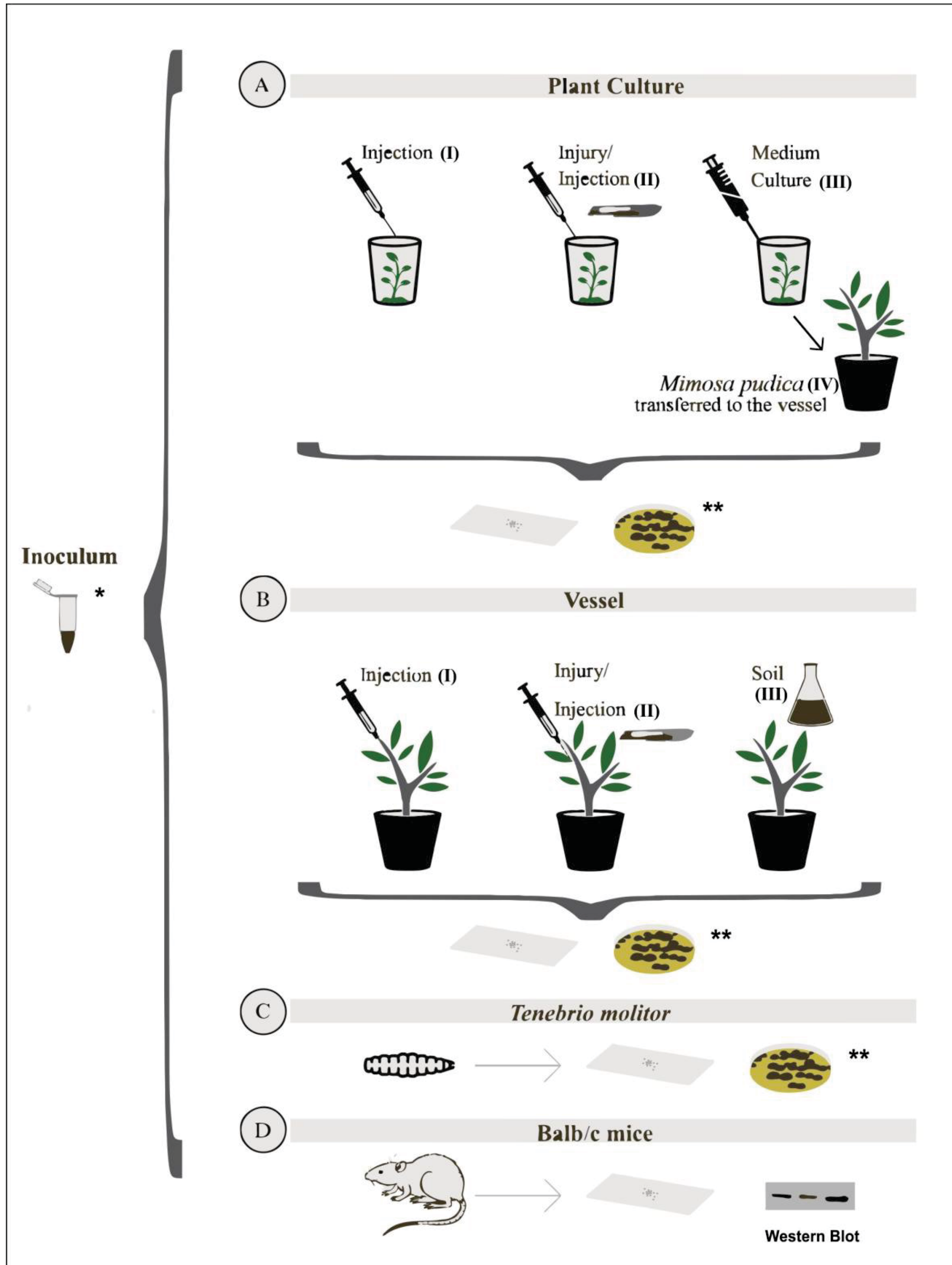


Figure 1: Diagram of inoculation experiments. A. Inoculation of plants *in vitro* (Injection, injury/injection and medium culture) and *M. pudica* transferred to the vessel previously *in vitro* infected; B. Inoculation of plants *in vessel* (Injection, injury/injection and soil); C. Tests in *Tenebrio molitor;* D. Tests in Balb/c mice. *Inoculum of studies strains; **All experiments was monitored by slides and re-isolation in culture in the plate.

3.1.5 **Vessel plant inoculation**

The *M. pudica* plants previously inoculated *in vitro* were transplanted to vessels with soil substrate (Fig. 1A) and maintained at a temperature of 30 °C with 85 % humidity in a Versatile Environmental Test Chamber (VETC; Panasonic MLR–352). Additionally, 61 plants of *M. pudica* obtained by a micropropagation protocol and maintained under aseptic conditions during 60 days were transplanted to the substrate soil containing loam/vermiculite (2:1). In addition, 24 plants of *B. gasipaes* were maintained in soil vessels at a temperature of 30 °C with 85 % humidity in a VETC at the EMPRAPA forest facility. After 15 days of incubation, inoculations were performed following three protocols: (I) direct injection into the stem, (II) injury associated with injection in the stem, and (III) suspension of 250 mL of liquid culture of each strain tested containing 1 × 10^6 cells/mL (Fig. 1B). Histological sections were obtained at days 15, 30, 45, and 60 after infection.

3.1.6 **Plant samples microscopy**

Plant samples were fixed in FAA (50 % formalin, 50 % ethanol, acetic acid, 18:1:1; Johansen, 1940). For inclusion in polyethylene glycol (PEG) 1500, samples were pre-infiltrated with PEG solution and ethanol in the ratio 1:1 at 56 °C for 12 h, followed by infiltration with pure PEG solution for 4 h. The sections of 7 mm thickness were obtained with a rotation microtome equipped with a steel razor and stained with 1 % astra blue and safranin (Bukatsch, 1972) with 0.5 % toluidine blue (O'Brien and Mccully, 1981). The slides were mounted semi-permanently with glycerin gelatin according to Kaiser (Bernal et al., 2015). The slides were observed and photomicrographs were captured using an Olympus microscope equipped with an SC30 camera.

3.1.7 Identification of fungal colony recovered from plant tissue

The re-isolation of fungi from plant tissues was performed by culturing in Sabouraud's medium for two weeks at 28 °C and by flotation technique in

mineral oil (Vicente et al., 2000). DNA extraction was performed with maceration using silica:celite (2:1) in cetyl trimethylammonium bromide (CTAB) and chloroform isoamyl alcohol (CIA, Vicente et al., 2008). The fungal ID was confirmed by rDNA internal transcribed spacer (ITS) sequencing (Vicente et al., 2013).

3.4 VIRULENCE TEST USING *Tenebrio molitor* AS MODEL

3.4.1 **Virulence testing with *Tenebrio molitor***

The larvae of *Tenebrio molitor*, approximately 100 to 200 mg were selected with 10 larvae per group (I, II, III) for the assessment of survival after fungal infection (Fig. 1C). The inocula of 1×10^6 cells/mL in sterile PBS in aliquots of 5 µL were injected using a Hamilton syringe with a 0.75 mm diameter needle into the hemocoel, the second or third sternite visible above the legs, and the ventral portion. Negative controls included sterile PBS and control sham without physical damage (no treatment). The larvae were placed in sterile Petri dishes and kept in darkness at 37 °C. Mortality was monitored once a day for 10 days. The pupae were omitted from the calculation.

To detect melanization, the hemolymph of each larva was collected at 4 h, 24 h, 3 days, 7 days, and 10 days post infection (Scorzoni et al., 2013). The hemolymph was collected in clean 1.5 mL tubes and diluted in a ratio of 1:10 with PBS. The optical density of diluted hemolymph was measured at 405 nm to determine melanization (Perdoni et al., 2014). Each hemolymph sample was measured thrice independently.

3.4.2 ***Tenebrio molitor* tissue burden and histopathology**

Three caterpillars per group (I, II, and III) were weighed and homogenized in 1 mL sterile PBS with a TissueLyser (Qiagen), plated on Mycosel agar, and incubated at 30 °C for 14 days. Fungal growth from caterpillars was measured by the number of colony forming units (CFUs) per mL of solution and the re-isolated fungi were sequenced. The samples were

embedded in Adracanth gum (7 g Adracanth in 100 mL of distilled water, 1 drop of formaldehyde), immersed in liquid nitrogen, and sectioned (8 µm) using steel blades in a cryostat (Leica CM 1850). The samples were observed using an Axio Imager Z2 (Carl Zeiss; Jena, Germany) equipped with Metafer 4/VSlide automated capture software (Metasystems; Altlussheim, Germany) and a CoolCube 1 (MetaSystems) camera.

3.5 IMMUNOGENIC TEST USING THE IMMUNOCOMPETENT BALB/C MICE AS A MODEL

3.5.1 **Immunogenic testing in murine model**

Immunocompetent BALB/c male mice (SPF, 6–8-week-old) were purchased from the animal laboratory of the Federal University of Paraná and maintained under pathogen-free conditions (Fig. 1D). The animal experiments were performed in accordance with the recommendations on animal welfare by the Institutional Ethics Committee of the Federal University of Paraná (approval certificate no. 1002). The experiments were conducted in accordance with international standards. Inocula of *F. pedrosoi* (a), *F. monophora* (b), and *F. erecta* (c) were adjusted to 5×10^7 cells/mL using sterile PBS as the negative control. A volume of 100 µL was injected subcutaneously into the abdomen of the mice. The mice were monitored weekly for up to 30 days post inoculation, on which day histopathological examination of spleen tissue was performed.

Western blot analysis was performed using crude protein extracts of the test species obtained by maceration of mycelia using liquid nitrogen. The protein content was quantified using Bradford’s reagent (Sigma; Saint Louis, MI, U.S.A.). Bovine serum albumin (BSA, Sigma) was used as the standard. The proteins were transferred to nitrocellulose membranes (GE Healthcare; Little Chalfont, U.K.) using the semi-dry Blotter Enhanced Chemiluminescence (ECL) unit (Amersham Biosciences; Piscataway, NJ, U.S.A.). The membranes were blocked using a solution containing 0.05 % PBS-Tween 20, 5 % nonfat milk, and 1 % BSA for 1 h at room temperature followed by incubation with primary antibody (sera from mice infected with strains tested, and control with sera from

uninfected mice), diluted 1:200 in 0.05 % PBST overnight under gentle agitation at 4 °C. After three washes with 0.05 % PBST, the membranes were incubated with anti-mouse IgG linked to horseradish peroxidase (BioRad), diluted at 1:5,000 in 0.05 % PBST for 2 h at room temperature under gentle agitation. The ECL substrate solution Novex (Invitrogen; Carlsbad, CA, U.S.A.) was employed to reveal the membrane using the ImageQuant LAS software.

3.5.2 Murine tissue histopathology

Histopathological samples obtained from mice were fixed in ALFAC solution containing 80 % ethanol, 40 % formalin, and glacial acetic acid for 16 h. Subsequently, the samples were subjected to serial dehydration, embedded in paraffin, sectioned into 5 µm slices, and stained with hematoxylin and eosin (H&E) stain (Cardona-Castro et al., 1996) followed by mounting of the samples with Entellan™. The samples were observed and photomicrographs were captured using an Olympus microscope BX51 equipped with capture software cellSenses (2008, Olympus Soft Imaging Solutions, Münster, Germany) and coupled with a camera model DP72.

4 RESULTS

VIRULENCE IN THE PLANT MODEL

The environmental species, *F. erecta* and the clinical strains of *F. pedrosoi* and *F. monophora* were inoculated into plant hosts using *M. pudica* and *B. gasipaes.* A total of 152 plants were used, of which 112 were inoculated *in vitro* and 40 were transplanted into the vessels containing autoclaved soil as the substrate. The average height of *in vitro* plants varied from 5 to 12 cm and that of vessel plants varied from 30 to 45 cm. Since plants of the *M. pudica in vitro* model were small in height, with structures too delicate to support injury or injection, the infection was conducted by the inoculation of culture medium (Fig. 1A–III). In both the infection models, inocula with densities above 10^2 cells/mL proved to be invasive, leading to weakened and deteriorated plants. In other

words, at this concentration of the inoculum, all studied fungal strains could grow inside *in vitro* plant tissues (Figs. 2, 3); the host plants did not exhibit any visible external lesions during the assay period.

In the *in vitro* plants of *M. pudica* and *B. gasipaes* infected with the clinical species, *F. monophora* and *F. pedrosoi,* presented pseudomycelial cells within the epidermal and cortical tissues, mainly in the intercellular spaces. Hyphae and pseudomycelial cells were concentrated in the parenchyma close to the sclerenchymatic sheath; however, they were absent in the vascular tissue (Fig. 2F, H; Fig. 3H, J). The invasive property of the fungi was demonstrated by their ability to penetrate the epidermis, reaching the cortical region and consequently leading to the separation of parenchymal cells, with the formation of intercellular spaces that were subsequently colonized by the fungus (Fig. 2E, F, H). No colonization of the vascular tissue was observed in the plants infected by the strains of the clinical species; reproductive structures of *F. pedrosoi* were observed to grow on the surface of the plant (Fig. 2G).

In the *in vitro* plants infected with *F. erecta*, smooth, pale brown hyphae with some septa and pseudomycelial cells forming a dense mass in the stalk primary cortex were observed at the location of application of the inoculum (Figs. 2C, 3F). The fungus was present inside the vascular tissue, while the endoderm served as an internal barrier (Figs. 2C, 3F). The developments were similar in both *M. pudica* and *B. gasipaes* plant models. The endophytic fungi, *C. dimorpha* and *Cladosporium tenuissimum* were utilized as the positive controls in *M. pudica*, and *B. gasipaes*, respectively (Fig. 3C, D) and demonstrated similar growth patterns in plant tissues as *F. erecta* (Fig. 3E, F). Identities of the artificially inoculated fungi were confirmed by internal transcribed spacer (ITS) sequencing.

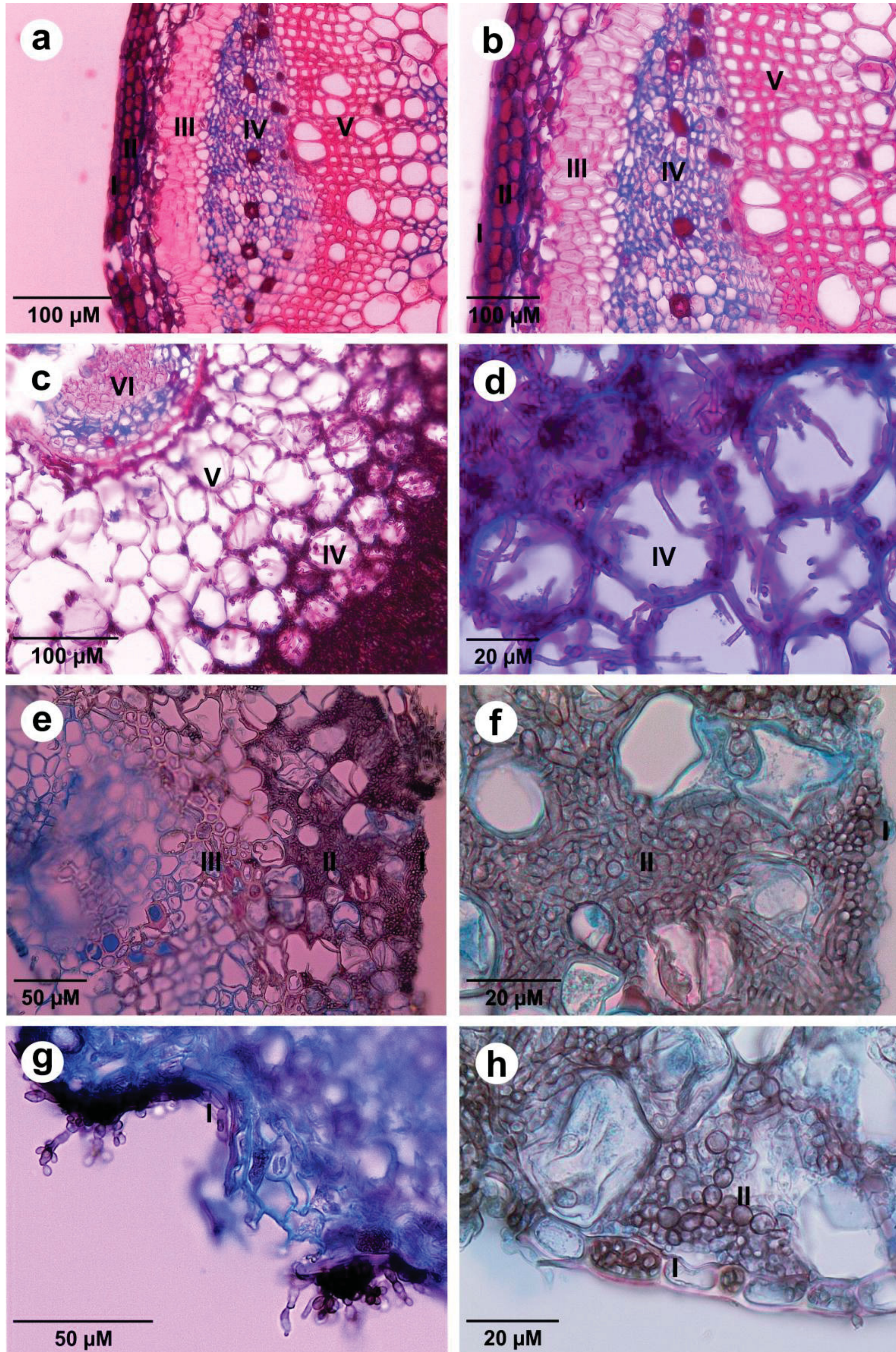


Figure 2: Sections Mimosa pudica micropropagated of plant histological prudish with coloring astra blue and 1% safranin. (A-B) sections stem in plant control without the presence

of fungus; (C-D) root inoculated *F. erecta* upright; (E-F) root inoculated *F. monophora*. (G-H) root inoculated *F. pedrosoi*. I- Epidermis; II- Cortex; III- Fiber sheath; IV- Phoelm; V- Xylem; VI- Medulla.

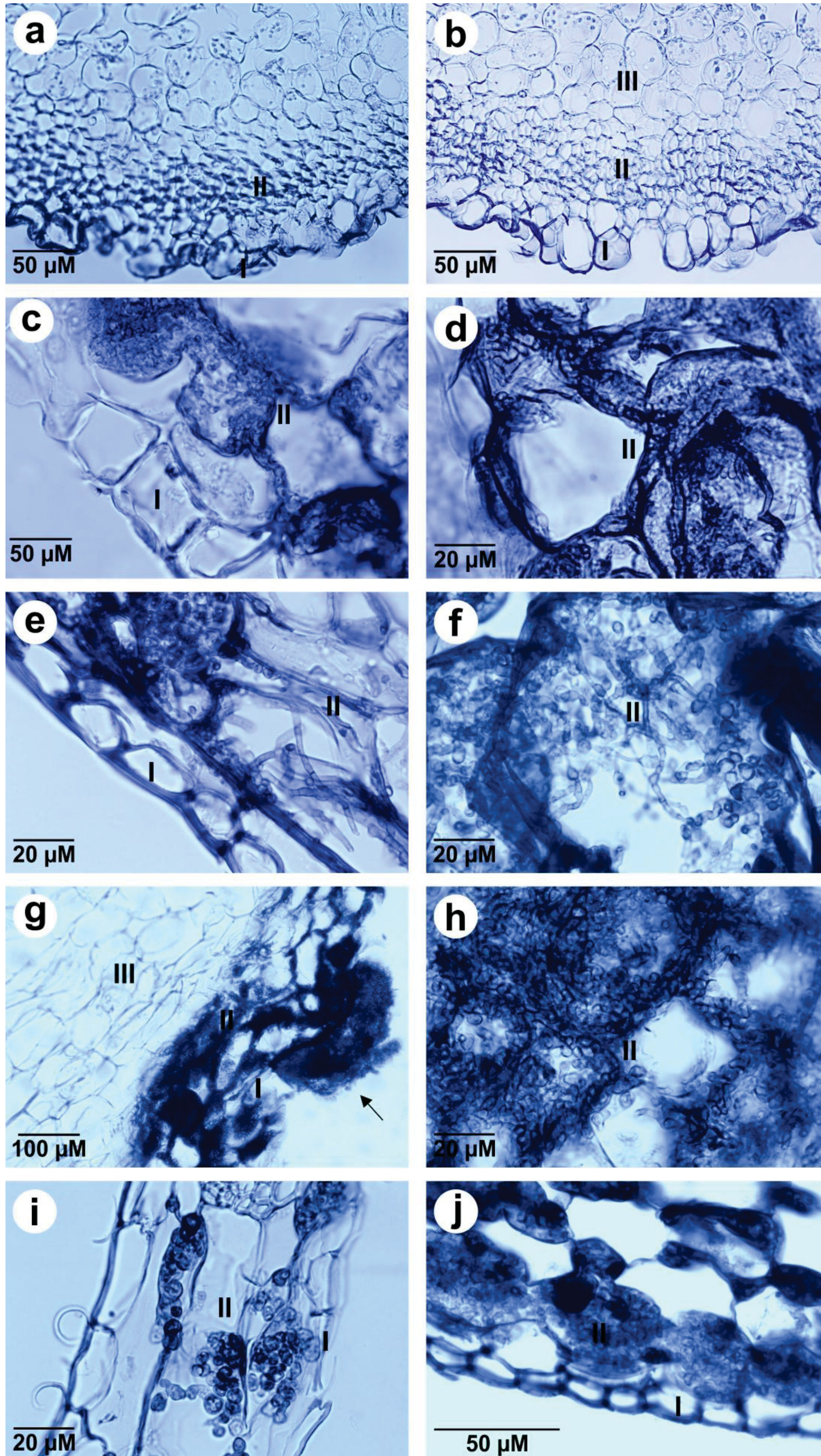
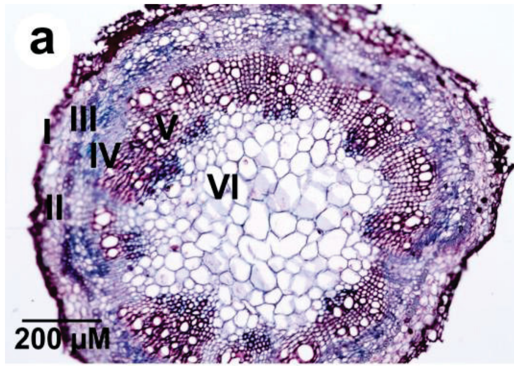

a
II
I
50 μM
b
III
II
I
50 μM
c
II
I
50 μM
d
II
20 μM
e
II
I
20 μM
f
II
20 μM
g
III
II
I
100 μM
h
II
20 μM
i
II
I
20 μM
j
II
I
50 μM

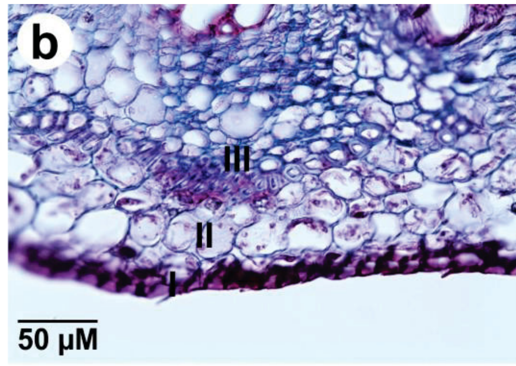
Figure 3: Sections *Bactris gasipaes* of plant histological prudish with coloring 0,5% Toluidine blue safranin. (A-B) sections stalk and root in plant control without the presence of fungus; (C-D) stalk and root inoculated positive control (*Cladosporium tenuissimum*) upright; (E-F) stalk and root inoculated *F. erecta;* (G-H) stalk and root inoculated *F. monophora* (Structures and fungal displaying demostradas by arrow) and (I-J) stalk and root inoculated *F. pedrosoi*. I- Epidermis; II- Cortex; III- Fiber sheath; IV- Phoelm; V- Xylem; VI- Medulla.

Plants of the *in vitro* infected *M. pudica* demonstrated root invasion by all fungi analyzed after 30 days of culture followed by their transfer to vessels (Fig. 1A–IV). After 60 days, both clinical and environmental strains were observed in the root and stalk tissues; epidermal regions and cortical cells contained large fungal concentrations (Fig. 4C, E–G, I) with the cells of *F. erecta* in the vascular tissue (Fig. 4E).

The plants of *M. pudica* previously produced *in vitro* and transferred to vessels for later inoculation by the injection and/or injury respectively (Fig. 1B–I and II) did not display fungal presence in the histological sections of stalk tissue. In contrast, fungi were observed in the stalk tissues of *B. gasipaes* in the vessels and infected by both methods (Fig. 5C, E, G, I).

The plants of *M. pudica* and *B. gasipaes* that were grown *in vitro* and transferred to the vessel were infected via soil (Fig. 1BIII). Pseudomycelial cells and hyphae were observed only in the roots of plants infected by *F. erecta*, which grew as an endophyte similar to the controls (Fig. 4D, F; Fig. 5D, F). The clinical species were unable to invade the plants by this route (Fig. 4H, J; Fig. 5H, J) during the test period of 60 days, implying their ability to invade the plants by this route only during early growth phases via small roots. In contrast, the environmental species could invade the plants through the soil route during all growth phases, indicating a higher ability of invasion and adaptation of these species to living plant tissue.


a
I
III
V
IV
VI
II
200 µM


b
III
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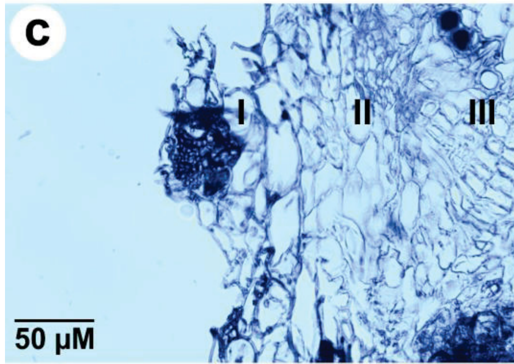
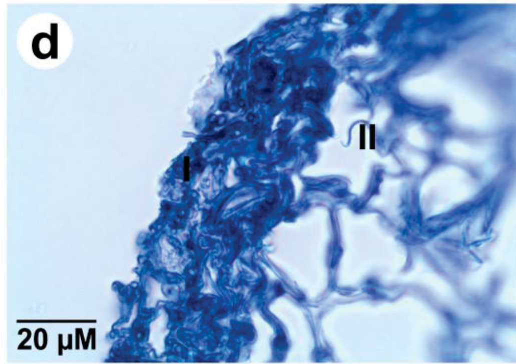
I
50 µM


c
I
II
III
50 µM


d
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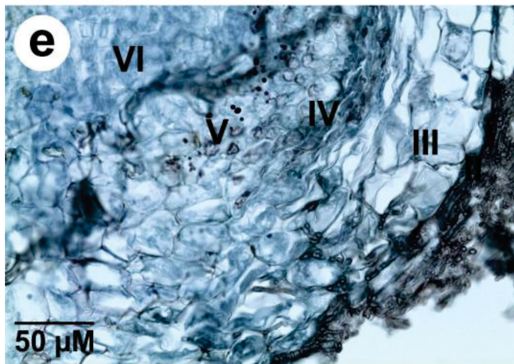
e
VI
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III
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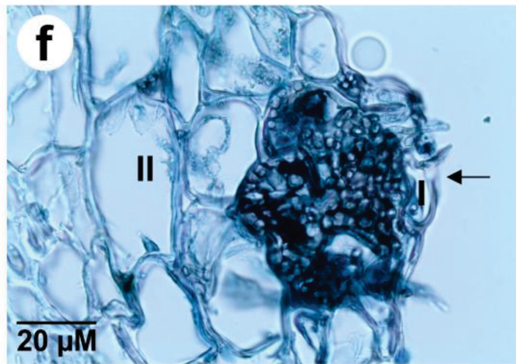
f
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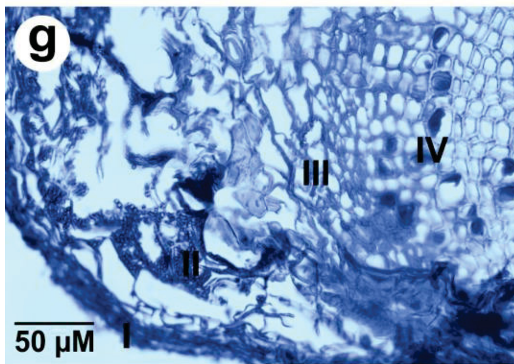
g
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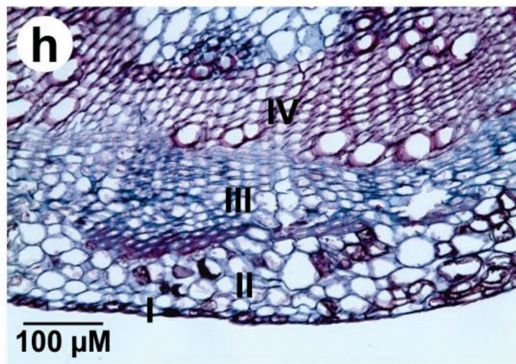
h
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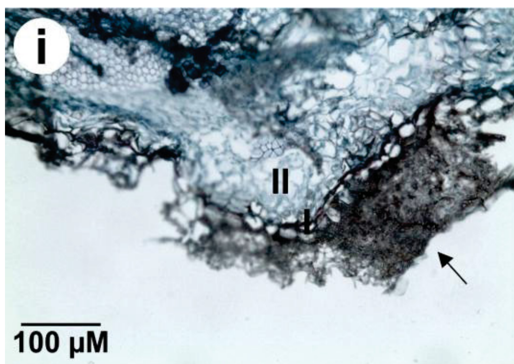
100 µM


i
II
I
100 µM

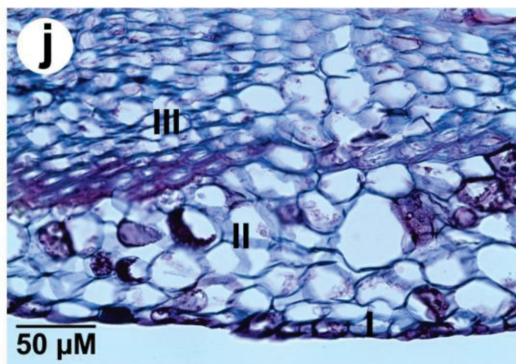
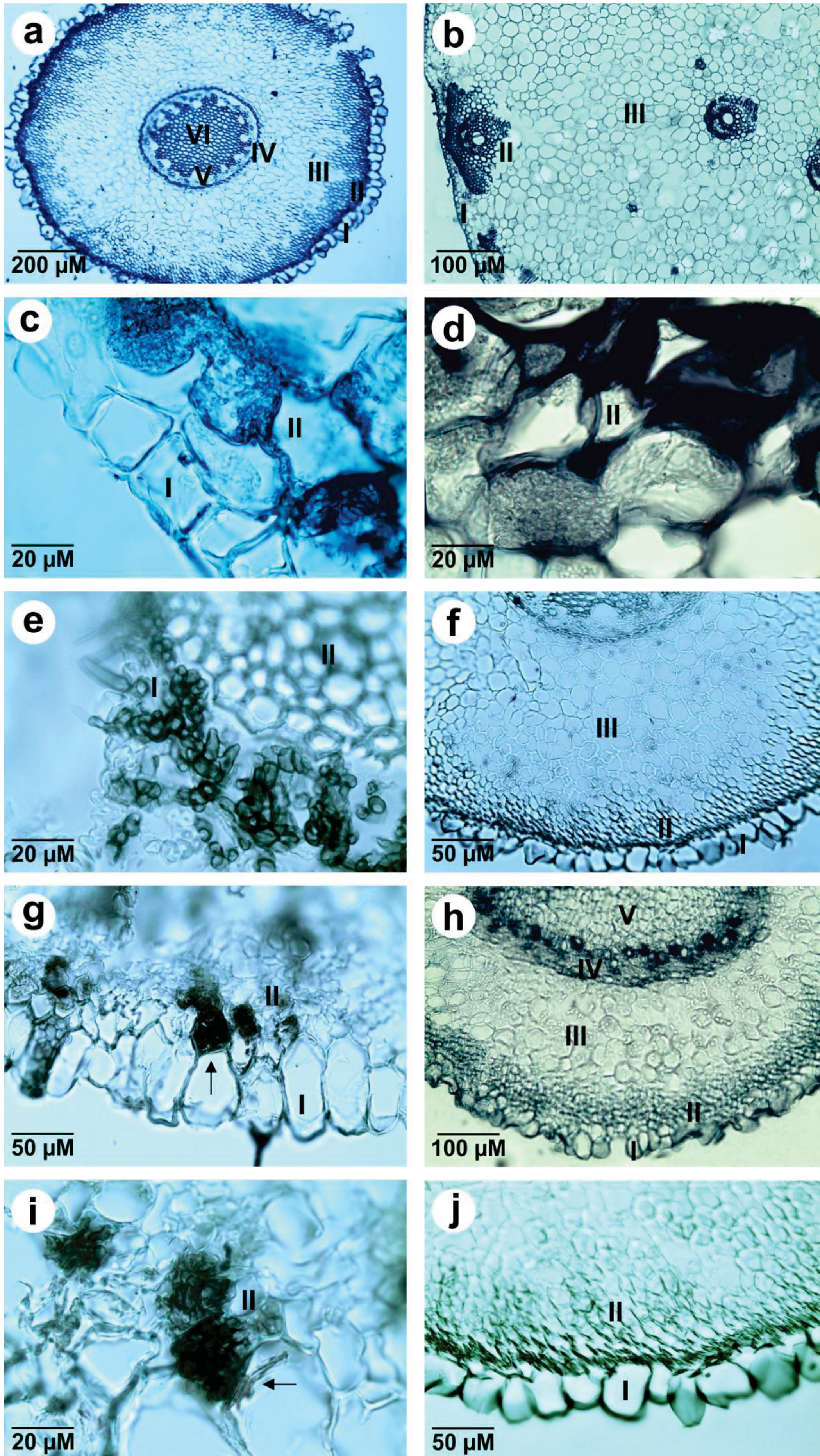

j
III
II
I
50 µM

Figure 4: *Mimosa pudica* in vessel sections histological prudish with coloring 0,5% Toluidine blue safranin. (A-B) cortes stalk and root in plant control without the presence of fungus; (C-D) stalk and root inoculated *Colletogloeopsis dimorpha* (positive control); (E-F) stalk and root inoculated *F. erecta* (Structures and fungal displaying demostradas by arrow); (G and I) stalk inoculated *F. monophora* (Structures and fungal displaying demostradas by arrow) and *F. pedrosoi*; (H and J) root in plant inoculated *F. monophora* and *F. pedrosoi* without the presence of fungus. I- Epidermis; II- Cortex; III- Fiber sheath; IV- Phoelm; V- Xylem; VI- Medulla.


a
VI
IV
V
III
II
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200 µM
b
III
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100 µM
c
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e
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II
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50 µM
g
II
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h
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100 µM
i
II
20 µM
j
II
I
50 µM

Figure 5: *Bactris gasipaes* in vessel sections histological prudish with coloring 0,5% Toluidine blue safranin. (A, B) sections stem and root in plant control without the presence of fungus; (C, D) stalk and root inoculated *Cladosporium tenuissimum* (positive control); (E, G and I) stalk inoculated *F. erecta, F. monophora* and *F. pedrosoi*. (F, H and J) root in plant inoculated *F. erecta, F. monophora* and *F. pedrosoi* without the presence of fungus. I- Epidermis; II- Cortex; III- Fiber sheath; IV- Phoelm; V- Xylem; VI- Medulla. Structures and fungal displaying demostradas by arrow.

VIRULENCE IN THE *TENEBRIO MOLITOR* LARVAE MODEL

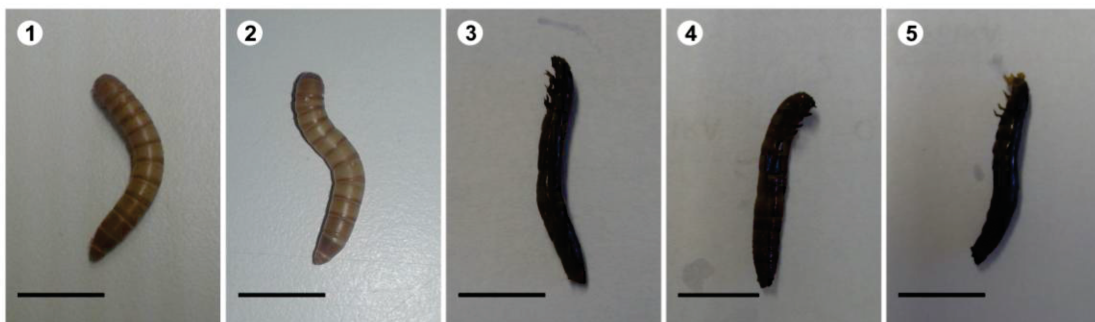
This study is the first of *Fonsecaea* virulence testing using *T. molitor* as an infection model. In all species analyzed, the larvae were successfully infected with inoculum concentrations of 5×10^6 cells/mL. During the 10 days of monitoring, the infected larvae displayed lower survival rates than compared to those in the control groups (Fig. 6B). The larvae infected with *F. erecta* exhibited the lowest survival rates, followed by *F. monophora* and *F. pedrosoi,* demonstrating that all species evaluated were able to survive inside the animal host. The infection rates were significant (F: 33.50; d_f: 4; $p < 0.01$), with larval mortality rates ranging from 41 to 65 % (control: 5 %; Fig. 6C). In the histological sections of larvae, 48 h post inoculation, *F. pedrosoi* was observed to produce spherical or ovoidal cells, 5 to 20 μm in diameter, with a dark, thick, multilayered cell wall resembling muriform cells (Fig. 6F).

Larvae melanization is an intracellular and key defense response to microbial infection. The larvae inoculated with all *Fonsecaea* species presented a gradual increase in the concentration of melanin (pigmentation) within 24 h after inoculation, as assessed both by visual observation and detection of melanization in the hemolymph using spectrophotometry (Fig. 6A). The pigmentation could be observed during the entire 10-day period of analysis (Fig. 6D).

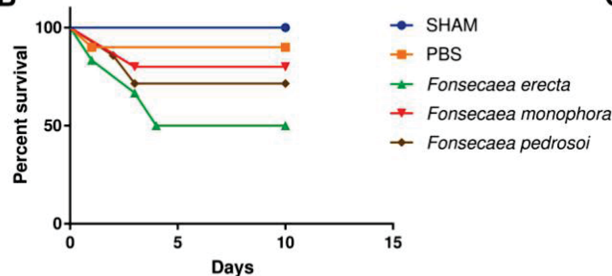
The fungal burden inside the larvae was assessed 24 h post inoculation. Significantly higher CFUs of *F. erecta* in the culture medium than that of *F. monophora* and *F. pedrosoi* were observed (Fig. 6E). The environmental species of *F. erecta* yielded 220 CFUs/mL, while the clinical species, *F. monophora* and *F. pedrosoi* yielded 50 and 38 CFUs/mL, respectively. These results were confirmed by an increase in the number of cells after 72 h, again with *F. erecta* presenting the highest number of 495 CFUs/mL, followed by *F.*

monophora with 97 CFUs/mL and *F. pedrosoi* with 101 CFUs/mL. However, after 240 h, the infected larvae that had survived exhibited a significant reduction in the growth of *F. erecta* with 23 CFUs/mL, while those infected with *F. monophora* and *F. pedrosoi* yielded 77 CFUs/mL and 71 CFUs/mL, respectively, suggesting that the environmental species could survive inside the animal but were controlled over time due to the activation of immune system of the larvae. With the clinical species, a more stable pattern was observed (Fig. 6B).

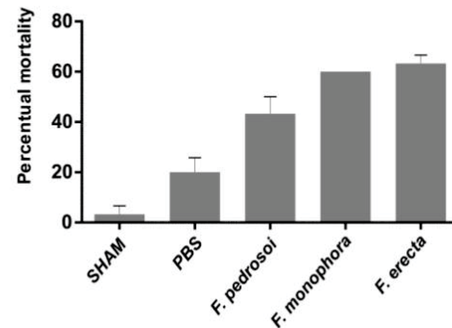
A



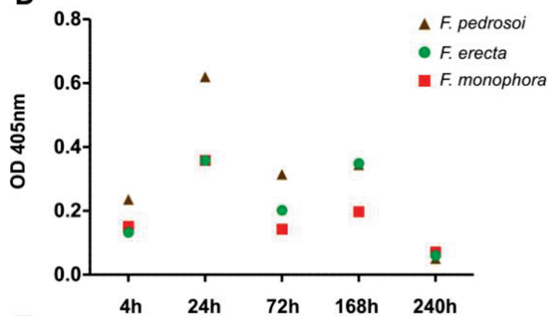
B



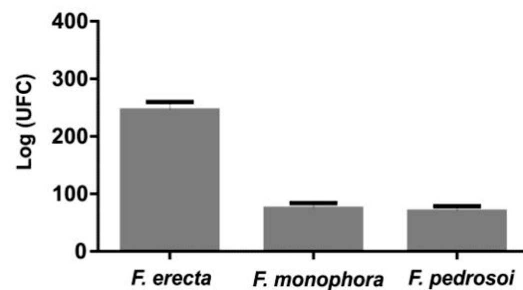
C



D



E



F

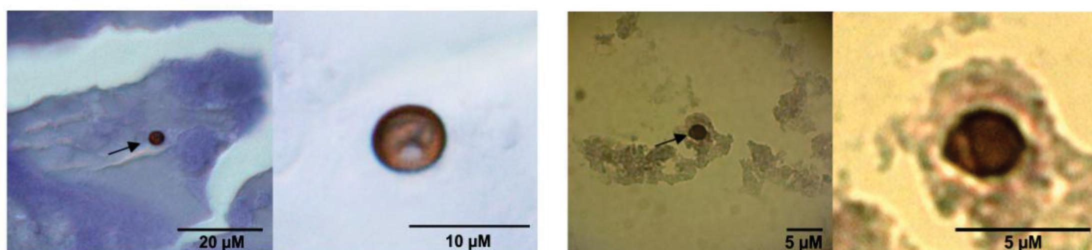
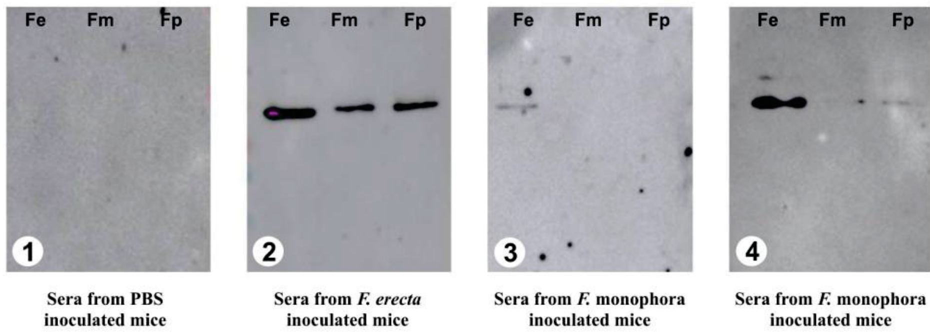


Figure 6: Virulence test using *Tenebrio molitor* infected with *Fonsecaea pedrosoi*, *Fonsecaea erecta* and *Fonsecaea monophora* . In panel A, five larvae PBS and SHAM and infected with *F. erecta; F. monophora; F. pedrosoi* are shown. In graphic B, the survival curves of the larvae are shown. For each experiment two different controls were used: untouched larva (SHAM) and larvae injected with PBS. In graphic C percentual mortality of larvae infected with *F. pedrosoi*, *F. erecta* and *F. monophora* (Fm) is shown. In graphic D, the melanization of the haemolymph is demonstrated by measuring the OD405nm of the haemolymph at time points; 4h, 24h, 72h, 168h and 240h after infection. In graphic E, larvae were homogenized in PBS at time points 4h, 24h, 72h, 168h and 240h after infection with *Fonsecaea* for CFU determination. In panel F muriform cells in larvae infected with *Fonsecaea pedrosoi.*

VIRULENCE IN MURINE MODEL

The plant-associated species, *F. erecta,* appeared to be more immunogenic in mice than the clinical species, as evident by the recognition of the fungal antigens in the serum of infected mice. The sera obtained from mice 30 days post infection with *F. pedrosoi*, *F. monophora,* and *F. erecta* were incubated with the total proteins obtained from the same fungi to detect the antigenic components. Figure 7A depicts that a band of approximately 50 kDa was recognized by the sera. This reaction was more evident when mice were infected with *F. erecta* against all protein extracts. More intense reaction was obtained with *F. erecta* protein extract (Fig. 7A2). At 30 days after systemic inoculation of *F. pedrosoi, F. monophora* and *F. erecta,* no change in the sectioned spleen tissues was observed (Fig. 7B).



B

1 40 μM
2 20 μM
3 40 μM
4 20 μM
5 50 μM
6 50 μM
7 50 μM
8 50 μM

Figure 7: Detection of antigenic components and histologic analysis of infected tissue of Balb/c mice with *Fonsecaea* species. In panel A: Western blot analysis with total proteins from *F. erecta*, *F. monophora* and *F. pedrosoi* against serum from mice infected with the same species. Serum was collected from Balb/c mice 30 days post-infection. In panel B: (1-2) PBS; (3-4) *F. erecta*; (5-6) *F. monophora* and (7-8) *F. pedrosoi*.

5 DISCUSSION

Chromoblastomycosis is supposedly a traumatic fungal implantation disease characterized by the infection of patients from an environmental source (QUEIROZ-TELLES et al., 2017), suggesting the etiologic agents of the disease would be saprobes associated e.g. with thorny plants. Several authors have reported a direct link between the disease and plant thorn inoculation; moreover, plant remains have occasionally been found in the infected tissue of patients (SALGADO et al., 2004).

Not all *Fonsecaea* species are considered as agents of disease, as expressed by large differences in predilection between closely related members of the genus. Upon the isolation of the etiologic agent from clinical samples, *F. monophora* or *F. pedrosoi* are frequently detected, while the isolation from environmental sources has yielded another species spectrum (VICENTE et al., 2013). Environmental searches of the pathogens may not have been conducted yet at their preferred habitats, or perhaps isolation methods were inappropriate.

The rationale of the present study was to establish the degree to which the etiologic agents of chromoblastomycosis could reside in plant material. *Fonsecaea pedrosoi* and *F. monophora* are endemic in humid climates (BADALI et al., 2010b; NAJAFZADEH et al., 2011a), particularly tropical areas, such as the Amazon rainforest (VICENTE et al., 2013). Two plant hosts with similar distribution were selected. *Mimosa pudica* is a creeping annual or perennial herb belonging to the pea family, *Fabaceae*; it is native to South and Central America and currently considered a pantropical weed that is also prevalent in the Asian countries (SOUZA FILHO et al., 2001). It has been associated with thorn trauma, leading to chromoblastomycosis (SALGADO et al., 2004). *Bactris gasipaes* is a palm native to the tropical forests of South and Central America. It is a long-living perennial plant, which on average is

productive for 50 to 75 years. Species of *Palmaceae* have been reported as a habitat of the melanized fungi (GEZUELE et al., 1972; CALIGIORNE et al., 2005). Silva et al. (1995) reported patients presenting with chromoblastomycosis lesions on the buttocks due to an exposure to palm debris while processing babassu coconuts, a common activity in the Maranhao state of Brazil.

Our data demonstrated that both clinical and plant-associated *Fonsecaea* species could cause infection in the two plant models, *M. pudica* and *B. gasipaes* (Figs 2–5) using both *in vitro* and vessel plants as hosts. *Mimosa pudica* represents an attractive model for *in vitro* analysis because of its rapid growth. It was invaded when the fungus was inoculated via culture medium *in vitro*; however, the vessel invasion only occurred when it was previously infected *in vitro* (Fig. 4C, E, G, I). The fine stems of the plant are difficult to manipulate, which interferes with the *in vitro* inoculation. The palm tree, *B. gasipaes,* our second model, with a long life cycle serves as an excellent model once the plant has a more robust stalk, which makes handling easier; the plant could be infected by different methods of inoculation both in vessels and *in vitro*. We believe that the method of inoculation may have influenced our results. The observation that the inoculum in the *in vitro* culture medium applied around the root allowed invasion of several tissues in both plants used (Figs 2 and 3).

The current hypothesis is that patients suffering from chromoblastomycosis acquire the infection via injury from plant material (QUEIROZ-TELLES and SANTOS 2013; QUEIROZ-TELLES et al., 2017). This may be considered a possibility; however, it fails to provide the explanation behind the different isolation rates between the species from humans and plant material. The species must differ significantly in predilection for either host, as exemplified the *Cladophialophora* agents of chromoblastomycosis, the clinical species, *C. carrionii* being restricted to humans while the cactus thorns next to the house of the patient exclusively carried the environmental species, *C. yegresii* (DE HOOG et al., 2007).

In the present study, we expected low invasion rates of plants by the clinical species and low animal virulence in the plant species. Contrary to this, we found *F. erecta* to have the largest CFUs in the larvae model; also, it was

found to be the most immunogenic in mice. It was also observed to be the most invasive in the used plant models, where it developed as an endophyte with deep invasion.

The above-mentioned finding led us to formulate an alternative hypothesis to explain the observed etiology of chromoblastomycosis by *Fonsecaea* species. The clinical species, *F. monophora* and *F. pedrosoi* were seen to remain in the epidermis of the stem in the inoculated plants. The epidermis is responsible for the formation of the thorn, and thus might provide an explanation of the thorn acting as the vehicle of transmission of the ”clinical” species, rather than of *F. erecta* that invaded the deeper tissues with a colonizing profile similar to that of the endophyte controls. The larvae initially displayed no defense against this uncommon invader, but later developed an immune response that could control the infection.

After transcutaneous implantation into a human host, propagules of the agents of chromoblastomycosis become meristematic and form muriform cells (QUEIROZ-TELLES et al., 2017); this is considered to be the pathogenic form of the fungus (ESTERRE and QUEIROZ-TELLES, 2006). Muriform cells can also be produced *in vitro* by the environmental relatives of the agents of chromoblastomycosis, which has never been reported from this disease (BADALI et al., 2009). In the present study, we observed swollen, dark and thick-walled cells that could be considered as a structure similar to the true muriform cells observed in human tissues, where they occasionally have transverse and longitudinal walls.

The mealworm beetle, *T. molitor* is a non-vertebrate animal model that has been used earlier, e.g., in *Candida albicans*, *Cryptococcus neoformans*, and *Madurella mycetomatis* (MYLONAKIS et al., 2005; FUCHS et al., 2010; MOWLDS et al., 2010; KLOEZEN et al., 2015; SOUZA et al., 2015). The larvae infected by *F. erecta* reported the lowest survival rates, but in 240 h, the survived larvae could destroy more *F. erecta* cells than that of *F. pedrosoi.* This suggested that due the fact that *F. erecta* promoted lower survival rates after prolonged periods of infection, higher fungal reduction was achieved, which may be attributed to an increased immune response. In other words, *F. erecta* appeared to be more immunogenic. It may be interpreted that *F. erecta* is less

adapted to animal host tissue and therefore elicits a more pronounced immune response, eventually leading to cure.

In conclusion, the plant infection models employed by our research group suggested that all *Fonsecaea* species were saprobic; however, some were more prone to survive in human tissues, consequently being less immunogenic. Considering the fact that all agents have to be traumatically inoculated to cause disease, it may be speculated that no primary animal pathogenicity is present in any of the species. Unexpectedly, *F. erecta* demonstrated to be a virulent species in the animal model, confirming earlier data by Vicente et al. (2017), who concluded the same using *Galleria mellonella* as the animal model. However, *T. molitor* represented a good model to reproduce the disease. The larvae of *T. molitor* have a longer cycle life than those of *G. mellonella*, and we observed for the first time structures similar to muriform cells produced during infection of human tissue in a larvae model. Assuming that all species concerned are opportunists rather than primary pathogens, we might hypothesize that lower virulence is an indication of higher adaptation to the animal host. This paradigm change complicates the parameter of initial issue destruction as a marker of adaptation to pathogenicity (as opposed to opportunism), since our saprobic species also demonstrated high virulence in animal models, but it was subsequently controlled via higher immune response. The results confirm the role of muriform cells as a pathogenic adaptation in animal tissues.

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CHAPTER III: Enviromental isolation of *Fonsecaea* sibling species using *Tenebrio molitor* (Coleoptera: Tenebrionidae) as a bait.

Enviromental isolation of *Fonsecaea* sibling species using *Tenebrio molitor* (Coleoptera: Tenebrionidae) as a bait

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1. ABSTRACT

The environmental isolation of etiological agents responsible for chromoblastomycosis (CBM) is considered difficult due to the saprobic lifestyle of the fungi. While most of the conventional methods applied are able to retrieve species considering the environment, the frequency of recovery of species related to CBM are specifically low. The aim of this study was to develop an alternative isolation method using *Tenebrio molitor* as bait to recover *Fonsecaea* sibling species from the environment. Six babassu samples previously analyzed in a diversity study and stored at –20ºC were used to investigate the presence of *Fonsecaea* sibling species in babassu. Ten black yeast-like fungi were isolated using *T. molitor* as bait; the analysis of the sequence data assigned the species isolated to the orders Chaetothyriales and Capnodiales. Within Chaetothyriales, we recovered four isolates belonging to the *Herpotrichiellaceae* family, which were identified as *Fonsecaea erecta* and *Fonsecaea monophora*. In Capnodiales, six isolates were identified as *Cladosporium cladosporioides* (*Cladosporiaceae*) and *Pseudocercospora norchiensis* (*Mycosphaerellaceae*). The alternative method using *Tenebrio molitor* has proved to be very effective in isolating *Fonsecaea* sibling species from the environment. The research with this model should be mainly in endemic regions with environmental samples to identify the routes of infection of these black yeasts.
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Keywords: *T. molitor*, fungi, isolation, *Fonsecaea* sibling.

2. INTRODUCTION

Chromoblastomycosis (CBM) is a chronic fungal infection of the skin and subcutaneous regions caused by traumatic fungal elements into the skin (BADALI *et. al.*, 2008) mostly affecting people living in tropical and subtropical areas (QUEIROZ-TELLES *et. al.*, 2017).The verrucous skin lesions with muriform cells, the invasive form of the fungi in the tissue (QUEIROZ-TELLES *et al.*, 2017), are the main characteristics of the infection. It is considered a globally distributed fungal disease, prevalent in tropical and subtropical regions (QUEIROZ-TELLES *et al.,* 2017).

This fungal group has extremotolerant or extremophilic characteristics, being able to tolerate stress factors such as high and low temperatures, osmotic stress, high solar and UV radiation, and can grow at arid, acidic, and hydrocarbon-contaminated sites (STERFLINGER, 2006; SELBMANN *et al.*,

2008, 2011, 2013, 2014a; ZAKHAROVA *et al.*, 2013; DE HOOG*et al.*, 2011; ISOLA *et al.*, 2013a).

CBM is considered a neglected tropical disease as defined by the World Health Organization (WHO), due to its global distribution, impact on the impoverished, and refractoriness (GARNICA *et al.*, 2009; AL-DOORY, 1983; VAN *et al.*, 2014). The mechanism of CBM infection is poorly understood, the current hypothesis is that patients acquire the disease after injury with thorns or wood splinters (RUBIN *et al.* 1991; FERNÁNDEZ-ZEPPENFELDT *et al.* 1994; SALGADO *et al.*, 2004; SILVA *et al.*, 1995). Evidence in a study conducted by Salgado *et al.*, (2004) showed an association between the source of trauma (*Mimosa pudica* thorns) and the onset of the disease in the North-eastern region of Brazil.

Among the etiologic agents of chromoblastomycosis, *Fonsecaea* and *Cladophialophora* species are considered the most common agents (QUEIROZ-TELLES *et al.*, 2015). Sibling species of *Fonsecaea* (family *Herpotrichiellaceae*) are agents of chromoblastomycosis belonging to the bantiana-clade (VICENTE *et al.* 2012), such as *Fonsecaea pedrosoi* and *Fonsecaea nubica,* cause localized infection while *Fonsecaea monophora* and *Fonsecaea pugnacius* *c*ause disseminated infection to the brain (QUEIROZ-TELLES *et al.*, 2015; DE AZEVEDO *et al.*, 2015). The bantiana-clade also contains *Fonsecaea* species associated with animal hosts other than humans, as *Fonsecaea multimorphosa* causing infection in cats (NAJAFZADEH *et al.*, 2011), *F. brasiliensis* associated with disseminated infection in crabs (VICENTE *et al.*, 2012), and also plant related species such as *Fonsecaea erecta* and *Fonsecaea. minima* were isolated from living plant (VICENTE *et al.*, 2014).

The environmental isolation of etiological agents causing CBM is difficult due to the saprobic lifestyle of the fungi. Although most of the conventional applied methods can retrieve the species, the recovery of species related to CBM is low (QUEIROZ-TELLES *et al.*, 2017).

In Brazil, *Fonsecaea pedrosoi* is supposed to be the main etiologic agent of the disease and considered endemic to the state of Maranhão in Northeastern Brazil (VICENTE *et al.*, 2014; DE AZEVEDO *et al.*, 2015). Recently, Nascimento *et al.*, (2017) evaluated black yeast diversity in babassu

samples from an area in Maranhão; although several black yeasts species were recovered; however, none of them was of the genus *Fonsecaea*, which is the causative agent of CBM. The present study focused on the hypothesis that *Fonsecaea* species causing chromoblastomycosis are present in the babassu, which showed a low isolation frequency of these agents by traditional isolation methods. The present work aimed to develop an alternative method using *Tenebrio molitor* as bait to recover *Fonsecaea* sibling species from the environment.

3. MATERIAL AND METHODS

3.1 BABASSU SAMPLES

Six babassu coconut shell samples previously analyzed in a diversity study by Nascimento *et al.*, (2017) and stored at –20ºC were used in this study to investigate the presence of *Fonsecaea* sibling species in babassu coconut.

3.2 INOCULUM PREPARATION

The babassu coconut shell samples were taken in a 1.5-mL microcentrifuge tube and were processed with a TissueLyser (Qiagen), through high-speed shaking with tungsten carbide beads for 5 min at 30 Hz frequency.

An initial solution (0.1 g/L) of each sample (n=6) was prepared using phosphate-buffered saline (PBS) and incubated at room temperature overnight. Afterwards, a serial dilution (1:100) was prepared.

3.3 *Tenebrio molitor* LARVAE AND BAIT MODEL

Larvae weighing approximately 100–200 mg showing no dark spots or grayish marks were selected for the experiments. *T. molitor* larvae were inoculated with 10 µL of each prepared inoculum in the last left pro-leg with a 29G U–100 insulin syringe (BD Diagnostics, Sparks, USA). Two control groups were used: uninoculated larvae and larvae injected with 10 µL of PBS. Each

group evaluated in this study consisted of 15 larvae. The larvae were incubated at 28 °C in Petri dishes containing rearing diet until the metamorphosis to pupal phase. After the change, the pupae were kept at 8 °C.

3.4 PUPA HEMOCELL PREPARATION AND ISOLATION

Initially, a longitudinal incision was made in the pupa and the hemocell was separated. The hemocell from three pupae was transferred to a 1.5 mL microcentrifuge tube containing 300 µL phosphate buffer (PBS) and about 80 mg of a silica mixture (Silica gel H, Merck 7736, Darmstadt, Germany / Kieselguhr Celite 545, Machery, Düren, Germany, 2:1, w/w). The pupa hemocell was disrupted manually using a sterile pestle for approximately 5 min (Figure 1).

Subsequently, the oil flotation method adapted by Vicente *et al.* 2008, was carried out on a smaller scale. The macerated hemocoel was incubated at room temperature for 30 min in 10 mL of a sterile saline solution containing 200 U penicillin, 200 mg/L streptomycin, 200 mg/L chloramphenicol, and 500 mg/L cycloheximide. Next, 2 mL of sterile mineral oil was added, followed by vigorous shaking for 5 min. The flasks were left to settle for 20 min, after which, 100 µL of oil-water interphase was carefully collected, inoculated onto Mycosel agar (Difco, BD), and incubated for 3 weeks at 28 °C. Stock cultures were maintained on slants of 2% malt extract agar.

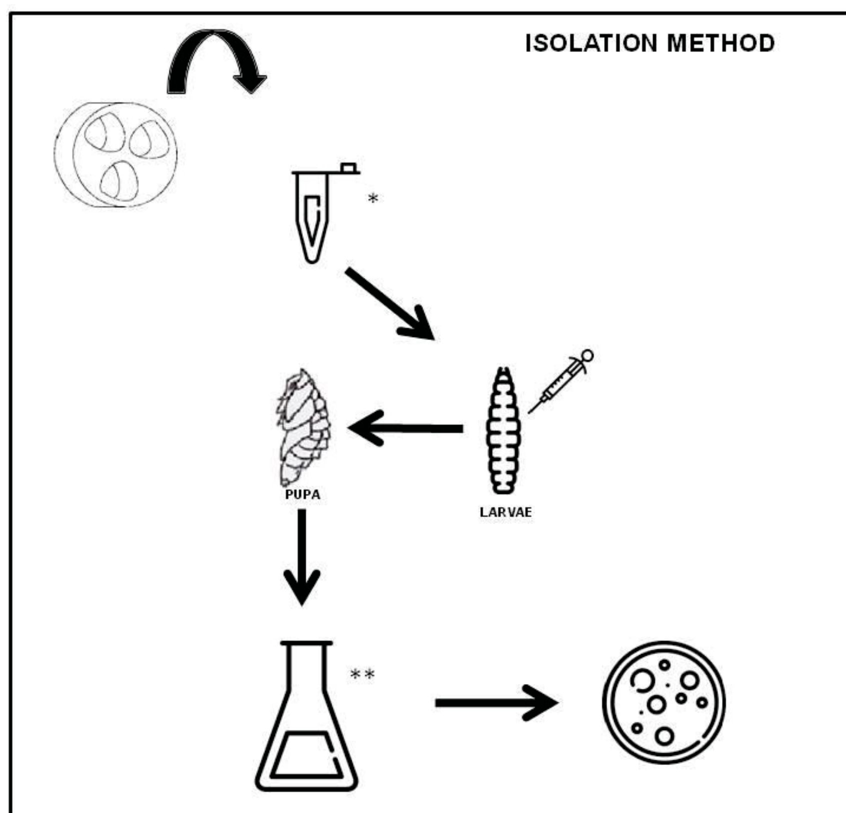

Figure 1: Isolation experiments. The inoculation of *Tenebrio molitor* subsequently by the oil flotation method was carried out on a small scale for the isolation. *Inoculum of babassu coconut; **oil flotation method.

The isolates obtained (Table 1) were deposited to the Microbiological Collections of Paraná Network – CMRP.

3.5 PRELIMINARY IDENTIFICATION

Initial identification was carried out using macro- and microscopic characteristics of the colonies after culture on malt extract agar (MEA, malt extract 20 g/L, glucose 10 g/L, peptone 1 g/L, agar 15 g/L). Subsequently, the molecular identification of the isolates based on the Internal Transcribed Spacer (ITS) rDNA region was done.

3.6 DNA EXTRACTION

The isolates were cultivated on Sabouraud dextrose agar (SDA, dextrose 40 g/L, peptone 10 g/L, agar 15 g/L) for 14 days, and genomic DNA extraction was performed following a previously described method (VICENTE *et al.*, 2008). Approximately 1 cm^2 of culture was transferred to a 1.5 mL microcentrifuge tube filled with 300 µL 2% cetyltrimethylammonium bromide (CTAB) buffer and about 80 mg of a silica-celite mixture (2:1, w/w). The cells were disrupted manually with a sterile pestle for approximately 5 min. To this mixture, 200 µL CTAB buffer was added and incubated at 65 °C for 10 min. Following incubation, 500 µL chloroform:isoamyl alcohol (24:1) was added. The tubes were then centrifuged for 7 min at 20,500 ×g, the supernatants were collected in fresh 1.5 mL microcentrifuge tubes, and 800 µL of ice-cold 96% ethanol was added. DNA was allowed to precipitate overnight at –20 °C and then centrifuged again for 7 min at 20,500 ×g. The pellets were washed with 1 mL ice-cold 70% ethanol, dried at room temperature, and resuspended in 100 µL water. DNA concentrations were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher, Wilmington, U.S.A.).

3.7 AMPLIFICATION AND SEQUENCING

Sequencing of rDNA spanning the internal transcribed spacer region (ITS) was performed using the universal ITS markers (WHITE *et al.*, 1990). PCR reaction mixtures were prepared containing 1 µL template DNA (30 ng), 2.5 µL 10X PCR buffer, 1 µL $MgCl_2$ (2 mM), 2 µL dNTP mix (2.5 mM), 1 µL of each primer (10 pmol), 0.2 µL Taq polymerase (Invitrogen, Brazil) (5 U/mL), and water to bring up the final volume to 25 µL. The PCR reaction was performed in an Axygen MaxyGene thermocycler (Foster City, USA). The amplifications were performed with cycling conditions as follows: 95 ºC for 5 min, followed by 30 cycles of 94 ºC for 45 s, 52 ºC for 45 s, and 72 ºC for 2 min, followed by a final elongation step at 72 ºC for 7 min. The PCR products were visualized by electrophoresis on a 1% (w/v) agarose gel and sent for sequencing (Myleus Biotechnology, www.myleus.com). The sequencing was done on an ABI 3730 DNA Analyzer (Applied Biosystems).

3.8 ALIGNMENT AND PHYLOGENETIC ANALYSES

The molecular identification was performed based on sequencing of the ITS rDNA region. DNA extraction, amplification, and sequencing were performed according to Vicente *et al*., (2008). Sequences were aligned using the SEQMAN package (DNAStar, Madison, U.S.A.).

Comparisons of ITS region were performed using the CBS databank (http://www.westerdijkinstitute.nl/Collections) which searches all major sequence databases including GenBank, and a black yeast research database maintained at CBS. A phylogenetic reconstruction using the maximum likelihood method was carried out. The sequences were aligned using the server version of the MAFFT program (http://www.ebiac.uk/Tools/mafft) and manually corrected using MEGA6. Phylogenetic relationships were estimated using the maximum likelihood method using MEGA V.7 software (KUMAR *et al.*, 2016) to generate the best evolutionary model for this dataset and 1000 bootstrap replicates.

4. RESULTS

Ten black yeast-like fungi were isolated using *T. molitor* as bait (Table 1). From the analyzed samples, black fungi were isolated in four out of six samples of babassu coconut shell (samples 1, 3, 4, and 6); however, no black yeast-like fungi were isolated from samples 2 and 5. Likewise, no black yeast-like fungi were recovered from the pupae in the control groups.

Table 1 – The isolated strains

Species	CMRP Collection number	GenBank Acession number (ITS)
Fonsecaea erecta	CMRP2542	In progress
Fonsecaea monophora	CMRP2543	In progress
Fonsecaea monophora	CMRP2544	In progress
Pseudocercospora norchiensis	CMRP2545	In progress
Pseudocercospora norchiensis	CMRP2546	In progress
Pseudocercospora norchiensis	CMRP2547	In progress
Cladosporium cladospoioides	CMRP2548	In progress
Fonsecaea erecta	CMRP2637	In progress
Cladosporium cladospoioides	CMRP2636	In progress
Cladosporium cladospoioides	CMRP2638	In progress

The occurrence of black yeasts in human-dominated environments has previously been underestimated due to the application of routine microbial isolation methods. Under common laboratory culture conditions, black yeasts are seldom encountered due to their slow growth and limited competitive abilities. Numerous protocols for the selective isolation of black yeasts have been developed during the last decade (an overview is presented in Table 2), which have yielded new data regarding the presence of these fungi in a wide range of artificial environments, sometimes with no obvious counterpart in nature.

Table 2 - Overview of methods applied for environmental isolation of *Fonsecaea* sibling species.

Species	Method	Number of isolates	Source	Geographic	Ecology	Reference
F. brasiliensis	Oil Flotation	16	Rotting wood, soil in rotten, animal tooth decomposition	Brazil	Opportunist	Vicente et al. 2014,
	Oil Flotation	4	Mangrove environment	Brazil	Opportunist	Guerra et al. 2013
	Oil Flotation	4	Crab	Brazil	Opportunist	Vicente et al. 2012
F. erecta	Oil Flotation	4	Living plant	Brazil	Opportunist	Vicente et al. 2000, 2008, 2012,2014
F. mínima	Oil Flotation	6	Living plant	Brazil	Opportunist	Vicente et al. 2014
F. monophora	Oil Flotation	1	Rotting Babassu coconut	Brazil	Pathogenic	Vicente et al. 2014
	Oil Flotation	3	Soil, plant litter	Brazil	Pathogenic	Vicente et al. 2008
F. multimorphorsa	Oil Flotation	1	Babassu coconut pam tree bark		Pathogenic	Vicente et al. 2014
	Oil Flotation	1	Mangrove environment	Brazil	Pathogenic	Guerra et al. 2013
	Oil Flotation	6	Soil cover, Wood, Decaying trunk, Babassu coconut	Brazil	Pathogenic	Najafzadeh et al. 2010
F. pedrosoi	Oil Flotation	2	Rotting wood	Brazil	Pathogenic	Vicente et al. 2014
	Oil Flotation	1	Mangrove environment	Brazil	Pathogenic	Guerra et al. 2013
	Oil Flotation	1	Cambara wood in decomposition	Brazil	Pathogenic	Vicente et al. 2000
	Oil Flotation	2	Babassu coconut shell, decomposing plant matter each	Brazil	Pathogenic	Marques et al. 2006
	Oil Flotation	1	Saprophytic	Japan	Pathogenic	Iwatsu et al. 1981
	Animal bait (Mouse passage)	4	Soil	Venezuela	Pathogenic	Negroni, 1936

The obtained isolates were identified by macro- and micro-morphology and subsequent analysis of the sequence data, which assigned the species isolated to the orders Chaetothyriales and Capnodiales. Within the Chaetothyriales family, we recovered four isolates belonging to the *Herpotrichiellaceae* family, which were identified as *F. erecta* and *F. monophora*. In *Capnodiales,* six isolates were identified as *Cladosporium cladosporioides* (*Cladosporiaceae*) and *Pseudocercospora norchiensis* (*Mycosphaerellaceae*). Among the isolates, *F. monophora* is the only species related to CBM and is a known agent of disseminated human CBM. *F. erecta* is associated with living plants and until now has not been reported as a causative agent of CBM. *Pseudocercospora norchiensis* and *Cladosporium cladosporioides* show a disease-causing association with living plants.

However, this is the first isolation performed using the novel method and the data represent preliminary results at best. Currently, a new experiment using fresh, unfrozen samples of babassu coconut shells collected from Maranhão is underway. In addition, samples of the *Butia capitata* palm tree and *Mimosa pudica* are being included in order to evaluate other plants already associated as a source of these causative agents of CBM.

5. DISCUSSION

Chromoblastomycosis has been reported in the literature as an overlooked disease as defined by World Health Organization (WHO, 2010; QUEIROZ-TELLES *et al.*, 2015) and include a number of endemic diseases that prevail in tropical or subtropical areas worldwide. In the present study, the dematiaceous fungi, *F. monophora* and *F. erecta,* (Table 1) were isolated from natural sources in several areas of the Maranhão state, a region regarded as an important focus of CBM in Brazil (SILVA *et al.*, 1992; MARQUES *et al.*, 2006).

The prevalent species of the humid climate are particularly members of the genus *Fonsecaea*, such as *F. pedrosoi*, *F. monophora, F. nubica* and *F. pugnacius* (NAJAFZADEH *et al.*, 2009; BADALI *et al.*, 2010; DE AZEVEDO *et al.*, 2015). *Fonsecaea pedrosoi* has been isolated from CBM, while *F.*

monophora repeatedly causes brain infection and *F. pugnacius* combines the two disorders by starting as CBM with cerebral dissemination in the same host (DE AZEVEDO *et al.*, 2015). In Brazil, environmental isolation studies demonstrated that clinical species are rarely recovered with few isolates obtained from plant debris (VICENTE *et al.*, 1999, 2008, 2013; SALGADO *et al.*, 2004; MARQUES *et al.*, 2006; SILVA *et al.*, 2009). With respect to living plant sources, Vicente *et al.*, (2013) described the new species *F. erecta* and *F. minima*, which are closely related to the pathogenic species associated with human skin infection found in the bantiana clade, but these living plant associated species were not reported as an agent of CBM. Moreover, the species *P. norchiensis* and *C. cladosporioides* both show an association with living plants and are mainly found causing the disease in plants (CROUS *et al.*, 2013; WANG *et al.*, 2013).

Some earlier studies have shown that different taxa of black yeasts from different environmental samples can efficiently be isolated using the oil flotation isolation technique (IWATSU *et al.*, 1981; MARQUES *et al.*, 2006; SATOW *et al.*, 2008; VICENTE *et al.*, 2008, 2012, 2014; GUERRA *et al.*, 2013). The high success rate and selectivity has been ascribed to the hydrophobicity of the strongly melanized fungal cells (PRENAFETA-BOLDÚ *et al.*, 2006; SATOW *et al.*, 2008). Phylogenetic analysis shows that the isolates usually belong to the Chaetothyriales*,* but only rarely an agent of CBM is found (VICENTE *et al.*, 2008, 2013). However, according to the frequency of isolation of the black yeast in this preliminary study, it was demonstrated that the methods proposed here for recovering the black yeast-like fungi associated with CBM is highly efficient. Moreover, these data confirm the babassu coconut shell as a source of these causative agents, which have been classified as an important risk factor for CBM disease (SILVA *et al.*, 1998; MARQUES *et al.*, 2006).

In a study carried out by Vicente *et al.*, (2008) *Fonsecaea*-like strains were isolated from sharp materials in the immediate vicinity of patients with *Fonsecaea* CBM using the oil flotation method. If CBM is a random process, it remains unclear why clinical cases are dominated by the three species, *F. pedrosoi, F. monophora,* and *F. nubica*, since the disease is never propagated by any of the environmental factors. However, it is possible that their higher

frequency in nature could have much higher chance of inoculation (NAJAFZADEH *et al.*, 2010). Kawasaki *et al.*, (1999) showed that the clinical strains studied belong to a unique group, while environmental isolates are usually clustered in another group. Judging from their results, the three species *F. pedrosoi, F. monophora,* and *F. nubica* must be much more virulent than other taxa that are strictly environmental.

The present study proposes an alternative method using *T. molitor* and demonstrates a very effective method for the isolation of *Fonsecaea* sibling species from the environment. This new isolation method represents a low-cost, easy-to-handle method for use in tests of virulence (SOUZA *et al.*, 2015). However, through this study, we could verify that it is an efficient model of isolation with high growth rate including selectivity for causative agents of CBM. Research with this model should be recapitulated in endemic regions of CBM with environmental samples to decipher further the route of infection of these black yeasts.

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CHAPTER IV: GENERAL DISCUSSION AND FUTURE PERPECTIVES

GENERAL CONCLUSION AND FUTURE PERPECTIVES

The present thesis aimed to evaluate a model of CBM infection in plants and animal hosts (Chapter II) along with designing a new and alternative method of isolation of the causative agents using *T. molitor* as the bait to recover species of *Fonsecaea* from the environment (Chapter III).

Chapter II provided a description of the evaluation of the potential of environmental fungi (*F. erecta*) and clinical fungi (*F. pedrosoi* and *F. monophora*) to infect plant model using *Mimosa pudica* and the palm tree, *Bactris gasipaes* as the models. The histological analyses demonstrated that clinical strains that could invade the plant tissues remained as yeast cells within the host tissues and few hyphae were present in the epidermis. On the other hand, plant associate-strains demonstrated an endophytic nature with a predominance of the hyphae inside the plant tissues that could also reach the vascular system of the plants, which was also observed in positive (endophytic) controls used.

This work was based on the hypothesis that patients suffering from CBM acquired the lesion by infection through a plant material (HOONG et al., 2000; VINCENTE, et al., 2013), and the results demonstrated the ability of the human clinical species of the fungi to survive inside the plant tissues, with maximum concentration in the region responsible for the formation of thorns. These observations implied that the plants could be considered as a part of the cycle of infection.

Further, these results demonstrated that the models evaluated were promising for infection in plants and animals to clarify the hypothesis that CBM was caused by injury through the plant material, as already described by Salgado et al. (2004), Marques et al. (2006), Queiroz-Telles, et al. (2009), and Vicente, et al. (2013).

The tests performed using *T. molitor* as the animal model indicated it to be a potentially useful model to elucidate and compare the virulence and dimorphism of these agents. Thus, it qualified as an excellent model for the reproduction of muriform cells. In the experiments conducted in Balb/c mice, *F. erecta* appeared to be more immunogenic that is considered to be an important

mechanism of defense against fungal infection. These models represent an effective tool to elucidate the immunological aspects of the disease revealing differences in the host responses to the infection caused by the clinical human and/or environmental strains.

In Herpotrichiellaceae family, warm- and cold-blooded hosts with intact immunity are commonly affected by the disease, suggesting the presence of intrinsic virulence factors and temperature independence (SEYEDMOUSAVI et al. 2014). For clarification of the above-mentioned fact, Teixeira et al. (2017) performed a comparative genome analysis to comprehend the fungal ecology, adaptation to harsh and toxic environments, and association with the hosts. The results demonstrated that opportunistic species often possessed different pathways to sequester carbon present in the environment. Therefore, when an opportunistic pathogen colonizes its host, genes associated with acquiring energy might be an advantage.

The main characteristic of CBM is to cause chronic infection in humans, immunological tests in patients with CBM demonstrated the presence of macrophages with fungistatic and non-fungicidal roles, thereby allowing the survival of the fungus inside the macrophages (SOTTO et al., 2004). The study by Sousa et al. (2011) demonstrated that inoculation of conidia of *F. pedrosoi* increased the levels of IL–10 and decreased TNF-α production by spleen cells in mice. *In vitro* study demonstrated a failure of conidia to stimulate macrophages, suggesting that failure of the innate immune system to recognize the pathogen could result in chronic infection. Based on these data, it may be concluded that *F. erecta,* with no such mechanisms, appeared to more immunogenic in mice and depicted lower survival in larvae tests, compared to human pathogenic species, *F. pedrosoi* and *F. monophora*.

According to Vicente et al. (2013), the black yeasts and their relatives harbor numerous agents of human and animal infection, thereby presenting significant predisposition to growth in the animal host tissues. Similarly, these fungal agents occupy specific environmental micro-habitats probably due to their oligotrophism, which permits them to grow at low density on adverse substrates where common saprobes are absent, justifying their low competitive ability toward co-occurring microorganisms. In addition, many species have

composite life cycles, indicating dynamic niches or vectored transmission (SUDHADHAM et al., 2008).

The above-mentioned characteristics of these fungi significantly enhance their isolation by the use of selective methods. Therefore, we proposed a model using *T. molitor* larvae as a means of enriching the Iwatsu technique to select causal agents of CBM as described in Chapter III. The analyses were performed with samples of Babassu used in a previous study performed by Nascimento et al. (2017). The results obtained verified that the proposed model could serve as a promising tool for the isolation of *Fonsecaea* species. Future studies need to be conducted using environmental samples, such as *M. pudica* plants from the endemic region, to improve the understanding of the ecological niche of these fungi.

In conclusion, judgment on the hypotheses tested in the present study indicates the two species, namely, *F. pedrosoi* and *F. monophora*, to be considerably more virulent than *F. erecta*. The studies indicate that the mechanisms of pathogenicity of the causative agents of CBM include thermotolerance, the formation of muriform cells with thick cell walls, cell adhesion, hydrophobicity, and melanin production. Together, these factors are significant contributors of disease chronicity (MACHADO et al., 2010; SUN et al., 2011; SUN et al., 2012; VICENTE et al., 2012; QUEIROZ-TELLES et al., 2016). The virulence assays performed suggested that the species, particularly those causing cutaneous human infection, could grow within the plant tissues. The plant-associated species in the animal models of infection induced higher larval mortality with respective immunogenic response in mice. However, during long periods of infection in larvae, the host immune response was more effective against *F. erecta* cells manifested by a reduction in the number of fungal cells than the pathogenic species of *Fonsecaea*, which formed muriform cells inside the larval tissues, a factor that probably attributed to their survival inside the animal host.

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APPENDIX

MEDIUM CULTURE AND SOLUTIONS USED

Potato Dextrose medium (PDA)

Potato sliced 200 g

Dextrose 20 g

Ágar 15 g

Destilled water 1000 mL

Autoclave 20 min at 121°C.

Final pH, 5.6 ± 0.2.

Potato Dextrose medium (PDA)

Potato slices 200 g

Dextrose 20 g

Destilled water 1000 mL

Autoclave 20 min at 121°C.

Final pH, 5.6 ± 0.2.

Saboraud medium

Glucose 40 g

Peptone 10 g

Ágar 15 g

Destilled water 1000 mL

Autoclave 20 min at 121°C.

Final pH, 5.6 ± 0.2.

Mycosel medium

Dextrose 40g

Peptone 10g

Agar 15g

Cycloheximide 0,5g

Chloramphenicol 0,05g

Autoclave 20 min at 121°C.

Final pH, 5.6 ± 0.2.

Astra Blue and 1%

Astra blue 150mg

Demineralized water with 2 mL acetic acid 1000mL

0,5% Toluidine Blue

Toluidine blue 5mL

1% sodium chloride 45mL

Safranin

Safranin 0,1g

Destilled water 100mL

CTAB

CTAB 2 g

NaCl 8,12 g

EDTA 4 mL

Tris 10 mL

PVP 10g

Destilled water 100 mL

CIA - Chloroform Isoamylic alcohol

Chloroform 96 mL

Isoamylic alcohol 4 mL

Extraction Buffer

Tris-HCl pH 8,0 200 mM

NaCl 250 mM

EDTA 25 mM

SDS (p/v) 1%

TBE 5X

Tris 54g

Boric acid,27,5g

EDTA 0,5M 20 mL

TBE 1X

TBE 5X 100 mL

Ultrapure water 400 mL

PBS

NaCl- 137 mM;

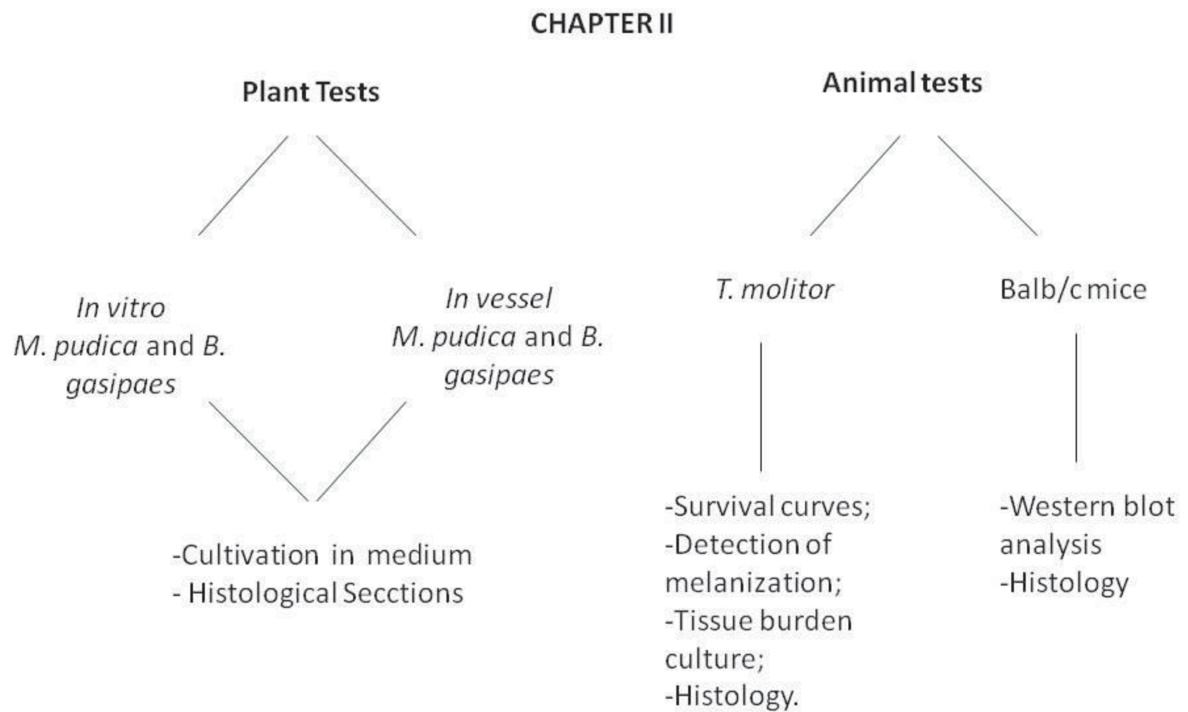
KCl- 2,7 mM;

Na_2HPO_4-10 mM;

KH_2PO_4- 2 mM

Destilled water- 1000mL

METHODOLOGICAL DIAGRAM



CHAPTER III

LIST OF PUBLICATIONS (2013-2017)

1. VICENTE, VANIA A; WEISS, VINICIUS A; BOMBASSARO, AMANDA; MORENO, LEANDRO F; COSTA, FLAVIA F; RAITTZ, ROBERTO T; BOCCA, ANAMELIA L; LEAO, ANIELE; FAORO, HELISSON; TADRA-SFEIR, MICHELI ZVB; BALSANELLI, EDUARDO; SUN, JIUFENG; GOMES, RENATA R; NASCIMENTO, MARIANA MF; **FORNARI, GHENIFFER**; ALMEIDA, SANDRO R; SANTOS, SUELEN S; TEIXEIRA, MARCUS; FELIPE, MARIA SS; PEDROSA, FABIO O; STEFFENS, MB; ATTILI-ANGELIS, DERLENE; NAJAFZADEH, MOHAMMAD J; QUEIROZ-TELLES, FLAVIO; SOUZA, EMANUEL M; DE HOOG, S. Comparative genomics of sibling species of *Fonsecaea* associated with human chromoblastomycosis. Plos genetics. **Subject.**

2. D'AVILA, GERMANA; GOMES, RENATA R.; GONÇALVES, ROSANA; **FORNARI, GHENIFFER;** MAIA, BEATRIZ; GLIENKE, CHIRLEI; DEGENHARDT, JULIANA; RIBAS, MARINA; VICENTE, VANIA A. Molecular identification and antimicrobial activity foliar endophytic fungi reveal new specie of *Diaporthe* from *Schinus terebinthifolius.* Fungal Biology. **Subject**.

3. NASCIMENTO, MARIANA M.F.; VICENTE, VANIA A.; BITTENCOURT, JULIANA V.M.; GELINSKI, JANE MARY L.; PRENAFETA-BOLDÚ, FRANCESC X.; ROMERO-GÜIZA, MAYCOLL; **FORNARI, GHENIFFER**; GOMES, RENATA R.; SANTOS, GERMANA D.; GERRITS VAN DEN ENDE, A.H.G.; DE AZEVEDO, CONCEIÇÃO D.M.P.S.; DE HOOG, G. SYBREN
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5. **FORNARI, GHENIFFER**; VICENTE, VANIA APARECIDA; GOMES, RENATA RODRIGUES; MURO, MARISOL DOMINGUEZ; PINHEIRO, ROSANGELA LAMEIRA; FERRARI, CAROLINA; HERKERT, PATRICIA FERNANDA; TAKIMURA, MARCOS; CARVALHO, NEWTON SÉRGIO DE; QUEIROZ-TELLES, FLAVIO.
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