UNIVERSIDADE FEDERAL DO PARANÁ

CRISTINA ISABEL FERRER VILLENA

TRANSFORMAÇÃO MEDIADA POR *Agrobacterium tumefaciens* DE *Fonsecaea monophora* E *Fonsecaea erecta* PARA MODELO DE ESTUDO CLÍNICO

TRANSFORMATION MEDIATED BY *Agrobacterium tumefaciens* OF *Fonsecaea monophora* AND *Fonsecaea erecta* FOR CLINIC STUDY MODEL

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **CRISTINA ISABEL FERRER VILLENA** intitulada: **Transformaçao mediada por *Agrobacterium tumefaciens* de *Fonsecaea monophora* e *Fonsecaea erecta* para modelo de estudo clínico**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 25 de Maio de 2018.

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RESUMO GERAL

As leveduras negras são fungos melanizados de grande diversidade morfológica pertencentes ao filo Ascomycota, subfilo Pezizomycotina; com a maioria das espécies incluídas nas ordens Chaetothyriales e Dothideales. A ordem Chaetothyriales possui cinco famílias: Chaetothyriaceae, Cyphellophoraceae, Epibryaceae, Herpotrichiellaceae e Trichomeriaceae. Sendo que na família Herpotrichiellaceae estão acomodadas os gêneros *Exophiala*, *Rhinocladiella, Veronea, Capronia, Thysanorea, Dactylospora, Fonsecaea, Cladophialophora e Phialophora*. *Fonsecaea* reconhecidos como agentes etiológicos prevalentes de infecções em humanos e animais. Entre elas a cromoblastomicose uma doença de implantação reconhecida por uma infecção cutânea, subcutânea de evolução crônica e múltiplas formas clínicas em humanos, caracterizada pela presença de corpos esféricos septados no tecido subcutâneo do hospedeiro, denominado de corpos muriformes. Da mesma forma, estas espécies têm sido relatadas como agentes causais da doença feohifomicose caracterizada clinicamente pelo aparecimento de lesões desde superficiais até sistêmicas, com presença de hifas nos tecidos de hospedeiros humanos e animais. A doença cromoblastomicose está principalmente associada ao trauma por fragmentos vegetais. Diversos estudo de isolamento tem sido realizados no sentido de elucidar os nichos e rotas de infecção da doença. Entretanto, o link entre o ambiente e as amostras clinicas ainda não está totalmente esclarecido. Assim, a obtenção de linhagens mutantes marcadas podem auxiliar em estudos de virulência, assim como nos estudos relacionados a investigação da rota de infeção destes agentes. Neste contexto, este estudo objetiva fornecer linhagens mutantes por transformação mediada por *Agrobacterium* (AMT) em espécies proximamente relacionada de *Fonsecaea* de origem clínica e ambiental. Na transformação mediada por *Agrobacterium* (AMT), o co-cultivo foi utilizado conídios de *F. erecta* e *F. monophora* com uma proporção de 1:1, 10:1 e 100:1 (*Agrobacterium*: conidia) a 28 °C por 72 horas. Os plasmideos pAD1625 e pCAMDsRed foram inseridos nos dois fungos. As proporções de 100:1 (*Agrobacterium*:conidia) geraram maior número de transformantes (*F. monophora* e *F. erecta*) do que as proporções 1: 1 ou 10: 1. *F. monophora* transformada com pCAMDsRed apresentou maior número de transformantes. O genoma de DNA do fungo avaliado e modificado aumentou a expressão do gene da higromicina indicado pela fluorescência vermelha moderada ou forte. As duas espécies estudadas apresentaram fluorescência vermelha dentro de *B. gasipaes*, principalmente no tecido da epiderme. Em síntese, este trabalho descreveu AMT de *F. erecta* e *F. monophora* com plasmídeo pCAMDsRed para estudar a interação entre plantas, fungos patogênicos e doenças, e com plasmídeos pAD1625 como um possível modelo para estudo de genes patogênicos.

Palavras-chave: *Fonsecaea monophora*, *Fonsecaea erecta,* cromoblastomicose, *Agrobacterium tumefaciens,* transformação.

GENEREAL ABSTRACT

Black yeasts are melanized fungi of morphological diversity belonging to the Ascomycota phylum, Pezizomycotina subphylum; with most of the species included in the orders Chaetothyriales and Dothideales. The Chaetothyriales order has five families: Chaetothyriaceae, Cyphellophoraceae, Epibryaceae, Herpotrichiellaceae, and Trichomeriaceae. In the Herpotrichiellaceae family, the genera *Exophiala, Rhinocladiella, Veronea, Capronia, Thysanorea, Dactylospora, Fonsecaea, Cladophialophora,* and *Phialophora* are accommodated. *Fonsecaea* are recognized as prevalent etiological agents of humans and animals infection such as chromoblastomycosis, an implantation disease recognized by a cutaneous and subcutaneous infection of chronic evolution and with multiple clinical forms in humans, characterized by the presence of spherical septate bodies into the subcutaneous tissue of the host tissues called as muriform cells. Likewise, the species have also been reported as causative agents of phaeohyphomycosis, a disease characterized clinically by superficial to systemic infection, with presence of hyphae inside the tissues of human and animal hosts. The Chromoblastomycosis disease is mainly associated to the trauma caused by plant fragments. However, the link between the environment and the clinical pictures is still not fully understood. Therefore, obtain target mutant strains would support the studies about virulence infection route of these agents. In this context, the aim of this study is to provide mutant strains by *Agrobacterium*-mediated transformation (AMT) in *Fonsecaea* sibling species obtained from the environmental and clinical source. In Agrobacterium-mediated transformation (AMT) the co-cultivation was utilized conidia of *F. erecta* and *F. monophora* with a ratio 1:1, 10:1 and 100:1 (*Agrobacterium*: conidia) at 28 °C by 72 hours. pAD1625 and pCAMDsRed plasmids were inserted into both fungi. The 100:1(*Agrobacterium*: conidia) ratios generated many numbers of transformant (*F. monophora* and *F. erecta* transformed) than 1:1 or 10:1 ratios. *F. monophora* transformed with pCAMDsRed showed higher number of transformations. The DNA genomic of fungus evaluated transformed were amplified the hygromycin gene, and expressed moderate or strong red fluorescence. The two species studied showed red fluorescence inside of *B. gasipaes,* mainly in the tissue of the epidermis. In summary, this work described AMT of *F. erecta* and *F. monophora* with the pCAMDsRed plasmid to study the interaction between plant and disease fungi, and pAD1625 as a possible model of deletion of pathogenic genes.

Keywords: *Fonsecaea monophora*, *Fonsecaea erecta,* chromoblastomycosis, *Agrobacterium tumefaciens,* transformation.

FIGURE LIST

CHAPTER I

CHAPTER III

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ATTACHMENT

LIST OF ACRONYMS

ANOVA	-	Variance analysis
AS	-	Acetosyringone
CBM	-	Chromoblastomycosis
CMRP	-	Microbiological Collections of the Paranaense Network
DNA	-	Deoxyribonucleic acid
GFP	-	Green fluorescent protein
IM	-	Induction medium
ITS	-	Internal transcribed spacer
LB	-	Luria Bertani
MES	-	(2-N-morpholine)-ethane sulfonic
NTDs	-	Neglected tropical diseases
PMT	-	Protoplast mediated transformation
RNA	-	Ribonucleic acid
SDA	-	Agar Sabouraud dextrose
TI	-	Tumor-inducing plasmid

SUMMARY

1 GENERAL INTRODUCTION

Black yeasts-like-fungi is a heterogeneous group of fungi that belongs to Chaetothyriales and Dothideales orders (Ascomycota) (TEIXEIRA et al., 2017; DE HOOG et al., 2000; DE HOOG, MCGINNIS, 1987), which the main characteristic is the presence of melanin in the cell wall of the vegetative and reproductive cells (CHOWDHARY et al., 2015; DIXON, POLAK-WISS, 1991). Black yeasts belonging to Dothideales order exhibit adaptive ability for survival in hostile environmental conditions (STERFLINGER et al., 1999). Chaetothyriales order comprises black yeast-like fungi with a vast and diverse ecology (DE HOOG, 2014; ZHAO et al., 2010), and includes species with clinical relevance, pathogens and opportunists of human infection, causing diseases such as Chromoblastomycosis, Mycetoma and Phaeohyphomycosis (BONIFAZ et al., 2013; VICENTE et al., 2008), species associated with infection in cold-blooded animals (GUERRA et al., 2013; VICENTE et al., 2012), ant;associated species (HUBKA et al., 2014; VOGLMAYR et al., 2011), epiphytic and epilithic species (CROUS et al., 2006). In addition, the pathogenicity and virulence along Chaetothyriales fungi differ significantly between the species and many factors are involved (SEYEDMOUSAVI et al., 2014).

The black yeasts from the Chaetothyriales order are composed of five families: Chaetothyriaceae, Cyphellophoraceae, Epibryaceae, Herpotrichiellaceae and Trichomeriacea, which includes species related to clinical cases and from the environment, which are able to adapt to a wide diversity of lifestyles, even in extreme and adverse environments, like the desert, saline, glaciers, exposed rocks, anthills, man-made environments such as dishwashers and saunas (ZUPANCIC et al., 2016; VOGLMAYR et al., 2011; MATOS et al., 2002; GUNDE-CIMERMANA et al., 2000).

The Herpotrichiellaceae family groups pathogenic and opportunistic fungi from human and animal hosts, such as *Fonsecaea monophora, F. pedrosoi, F. nubica, Cladophialophora carrionii, Phialophora verrucosa*, and *Rhinocladiella aquaspersa, F. brasiliensis and F. multimorphosa* (GONZALEZ et al., 2013; VICENTE et al., 2012; NAJAFZADEH et al., 2011; HOOG et al., 2004). Likewise, in this family are included environmental fungi such as *F. erecta, F. minima* and other fungi (VICENTE et al., 2013). *Fonsecaea pedrosoi* and *F. nubica* are cryptic species associated to Chromoblastomycosis. However, *F. monophora and F. pugnacious* have been related as agent of Phaeohyphomycosis causing infection in brain and

other organs (AZEVEDO et al., 2015). *Fonsecaea* species can grow in humid (sub) tropical climates, (QUEIROZ-TELLES et al., 2017) such as: Brazil (GOMES et al., 2016; SALGADO et al.,2004; VICENTE, 2000; QUEIROZ-TELLES, 1997; SILVA et al., 1992), China (CHEN et al., 2016; NAJAFZADEH et al., 2010; XI et al., 2009), India (AGARWAL et al., 2017), and other countries. Also in non-tropical regions, these are rare cases (OUÉDRAOGO et al., 2017).

Chromoblastomycosis is the most prevalent implantation mycosis infection leading to the hyperproliferation of host tissue, the etiology agents are members of Chaetothyriales order (QUEIROZ-TELLES et al., 2017). It is characterized by verrucose skin lesions, occasionally developing cauliflower-like eruptions, with the formation of the muriform cell (AZEVEDO et al., 2015).

Many species have composite life cycles, indicating dynamic niches or vectored transmission (SUDHADHAM et al., 2008). In the environment, they seem to occupy specific micro-habitats, probably stimulated by low competitiveness against competing microorganisms, as judged by the fact that isolation is significantly enhanced by using selective methods. Their oligotrophism (SATOW et al., 2008) allows them to thrive and maintain at low density on adverse substrates where common saprobes are absent (DE HOOG et al., 1997). The infection process and route of the disease have been insufficiently clarified (VICENTE et al., 2013, 2008; SALGADO et al., 2004; VICENTE, 2000). Agents of human chromoblastomycosis are assumed to be traumatically inoculated into the skin with sharp environmental materials such as plant thorns or wooden splinters carrying the respective opportunist (VICENTE et al., 2014; DE HOOG et al., 2007; SALGADO et al., 2004). However, the agents of disease may be morphologically indistinguishable from their environmental counterparts (VICENTE et al., 2014).

Nevertheless, the main biological issues revolve around the dimorphism and pathogenicity of *Fonsecaea* species related to human/animal infection and the closely related plant-associated species. Therefore, tools are mandatory to evaluate the link between clinical and environmental *Fonsecaea* species, in order to understand the mechanisms involved in the species adaptation in the plants and animals tissues, and the route of infection of this disease, which is associated with a trauma caused by plants. (FORNARI, 2017).

In this context, the genetic transformation techniques are a precondition that enables to target and modify genes efficiently and may reveal the function of target

genes (LI et al., 2017). The method is enable produce mutants with specific tags and fluorescent genes, which may facilitate, for instance, the visualization of strains inside human and animal tissues. There are some general methods of genetic transformation for fungi, including protoplast-mediated transformation, *Agrobacterium*-mediated transformation, electroporation, biolistic method and shock-wave-mediated transformation (Li et al., 2017).

The *Agrobacterium tumefaciens* is a bacteria Gram-negative that can transfer DNA of interest into the host genome. The *Agrobacterium*-mediated transformation (AMT) could provide important information to understand the process and route of disease. The AMT is a method with higher transformation frequencies for most fungi compared with conventional methods (MICHIELSE et al., 2008). Therefore, AMT could be an effective method for insert fluorescence gene in *F. monophora* and *F. erecta*, being a tool to determinate the presence or absence of fungal cells inside of the plant *Bactris gasipaes,* which is a palm native of the tropical forests and had been reported habitat of melanized fungi (CALIGIORNE et al., 2005). Thus, the *Agrobacterium*-mediated transformation represents a new strategy to obtain target strains in order investigate the route and process of implantation disease, and as method to knockout of genes.

Therefore, the main objective of this work is to transform mediated by *Agrobacterium tumefaciens* of *F. monophora* and *F. erecta* for clinic study model.

2 OBJECTIVE

2.1 GENERAL

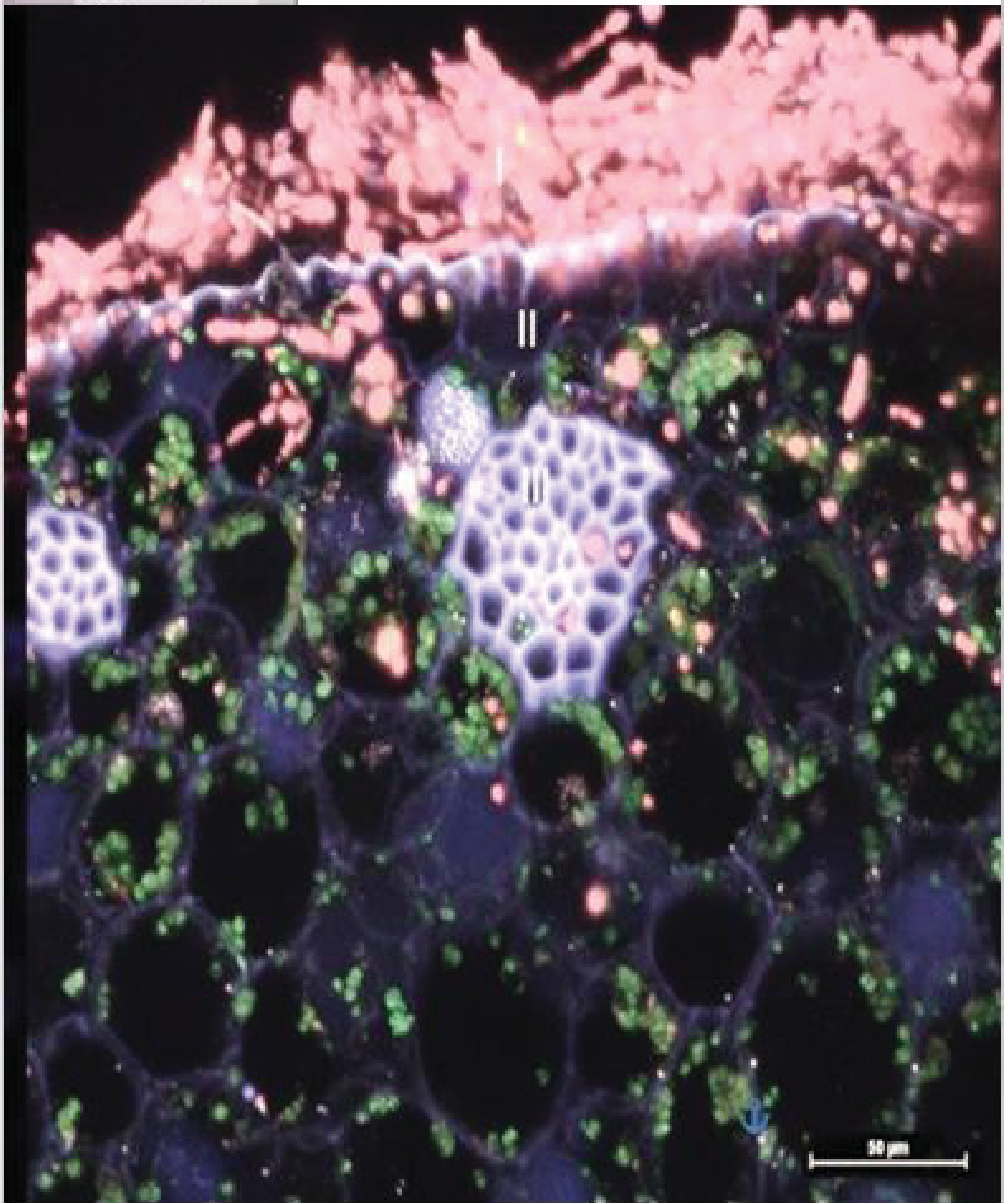
- Transform *Fonsecaea monophora* and *Fonsecaea erecta* strains by *Agrobacterium* for clinic study model.

2.2 SPECIFIC

- Determinate *F. monophora* and *F. erecta* susceptibility to the dominant selective markers;
- Transform of the *F. monophora* and *F. erecta* mediated by *Agrobacterium tumefaciens* with the pAD1625 plasmid and pCAMDsRed plasmid;
- Determinate mitotic stability of the transformed colonies;
- Analyze the expression of the reporter pCAMDsRed;
- Analyze PCR of the reporter pCAMDsRed and pAD1625;
- Inoculate *F. erecta* and *F. monophora* transformed with pCAMDsRed plasmid in the plant *Bactris gasipaes*;
- Analyze the expression of the reporter pCAMDsRed in *B. gasipaes*.

CHAPTER 1

OUTLINE OF THE THESIS



1 LITERATURE REVIEW

1.1 BLACK YEASTS TAXONOMY AND BIODIVERSITY

Black yeasts are melanized (dark coloration), no-lichenized fungi, with wide morphological diversity (TEIXEIRA et al., 2017), with mycelia and yeast-cells development during its growth (STERFLINGER, 2006). Besides, they are characterized by slow growth in traditional cultures and some species are dimorphic yeast, which depended on environmental condition (SEYEDMOUSAVI, 2014; VICENTE, 2000). The little differences structure morphological of black yeasts, the size and form are varied depending of the species. Therefore, it is very difficult to determinate the taxonomic classification and the biology molecular methods will help to classify the taxonomic (SATOW, 2008).

They are included in Ascomycota phylum and Pezizomycotina subphylum; the principal order is Chaetothyriales (Chaetothyriomycetidae subclass) and Dothideales. The Chaetothyriales has five families: Chaetothyriaceae, Cyphellophoraceae, Epibryaceae, Herpotrichiellaceae, and Trichomeriaceae (TEIXEIRA et al., 2017).

The Chaetothyriales order has very different habitats; the black yeasts are special because the many species inhabit extreme environment (high temperature, acidic and dry conditions), poor (scarce of nutrients), toxic chemicals and extreme environment (DE HOOG, 2014; ZHAO et al., 2010). The species also are associated with plants, plant debris, soil, decomposing plants, including thorn, leaves, bark, fruits and wood (STERFLINGER, 2006; MCGINNIS, 1992). The Satow in 2008 related that some black yeasts species can growth in hydrocarbons soil.

The Chaetothyriaceae family includes species that are epiphytes, colonizing the surface leaves, with mycelium growing in host cuticle without penetrating other tissues. The members are distributed in the tropical environment and the principal generous are *Ceramothyriu, Chaetothyrium, Vornaxia* (CHOMNUNTI et al., 2012b).

The Cyphellophoraceae family includes species that are known by their asexual morphs, with conidia may be hyaline and one-celled, the members could be opportunists on human skin and nails, such as *Cyphellophora* and *Phialophora* (TEIXEIRA et al., 2017).

The Epibryaceae family was proposed by Gueidan in 2014, some genus are

Epibryon, Cladophialophora, Epibryaceae and they are associated to the plant (TEIXEIRA et al., 2017). The Trichomeriaceae family has epiphytes species that live in trees, and/or saprobes species, and the most important genus is *Trichomerium* (CHOMNUNTI et al., 2012a). The Herpotrichiellaceae family is a dual ecology that includes saprobic species on plant debris and clinical species (TEIXEIRA et al., 2017; REBLOVA et al, 2013; ZHAO et al., 2010). They have an ability to adapt to extreme environments, and they exhibit a pathogenic potential in human and cold- or warm-blooded vertebrates (TEIXEIRA et al., 2017; ZHAO et al., 2010). Some species are the main etiological involved in infections of cutaneous and subcutaneous tissue of humans and animals. The human's diseases are Chromoblastomycosis and Phaeohyphomycoses (REBLOVA et al, 2013; MARQUES et al, 2006).

The family Herpotrichiellaceae have genera such as: *Exophiala, Rhinocladiella, Veronea, Capronia, Thysanorea, Dactylospora, Fonsecaea, Cladophialophora* and *Phialophora.* This family is divided phylogenetically into six clades identified in the Figure 1. These are *R. mackenziei*, bantiana, carrion, jeanselmei, dermatitidis and salmonis - clade (TEIXEIRA et al., 2017).

The *Fonsecaea* and *Cladophialophora* genera are included in bantiana-Clade (VICENTE et al. 2013; TEIXEIRA et al., 2017and VICENTE et al. 2017) with a wide diversity in ecology. The *F. pedrosoi* and *F. nubica* (Najafzadeh et al., 2010; CHEN et al., 2016) are prevalent etiologic agents of chromoblastomycosis (CLEINMAN et al., 2017) and *F. monophora* also can disseminated phaeohyphomycosis of the brain or other organs in humid tropical and subtropical climates; and newly *F. pugnacius* has been described as a chromoblastomycosis agent, that also disseminated with hyphae to brain (AZEVEDO et al., 2015). Vicente et al. (2013) described environmental species such as *F. erecta* and *F. minima*, isolated from organic material in endemic areas of Brazil, so too *F. brasiliensis* is associated with infections of cold-blooded animals and organic material (VICENTE et al., 2012).

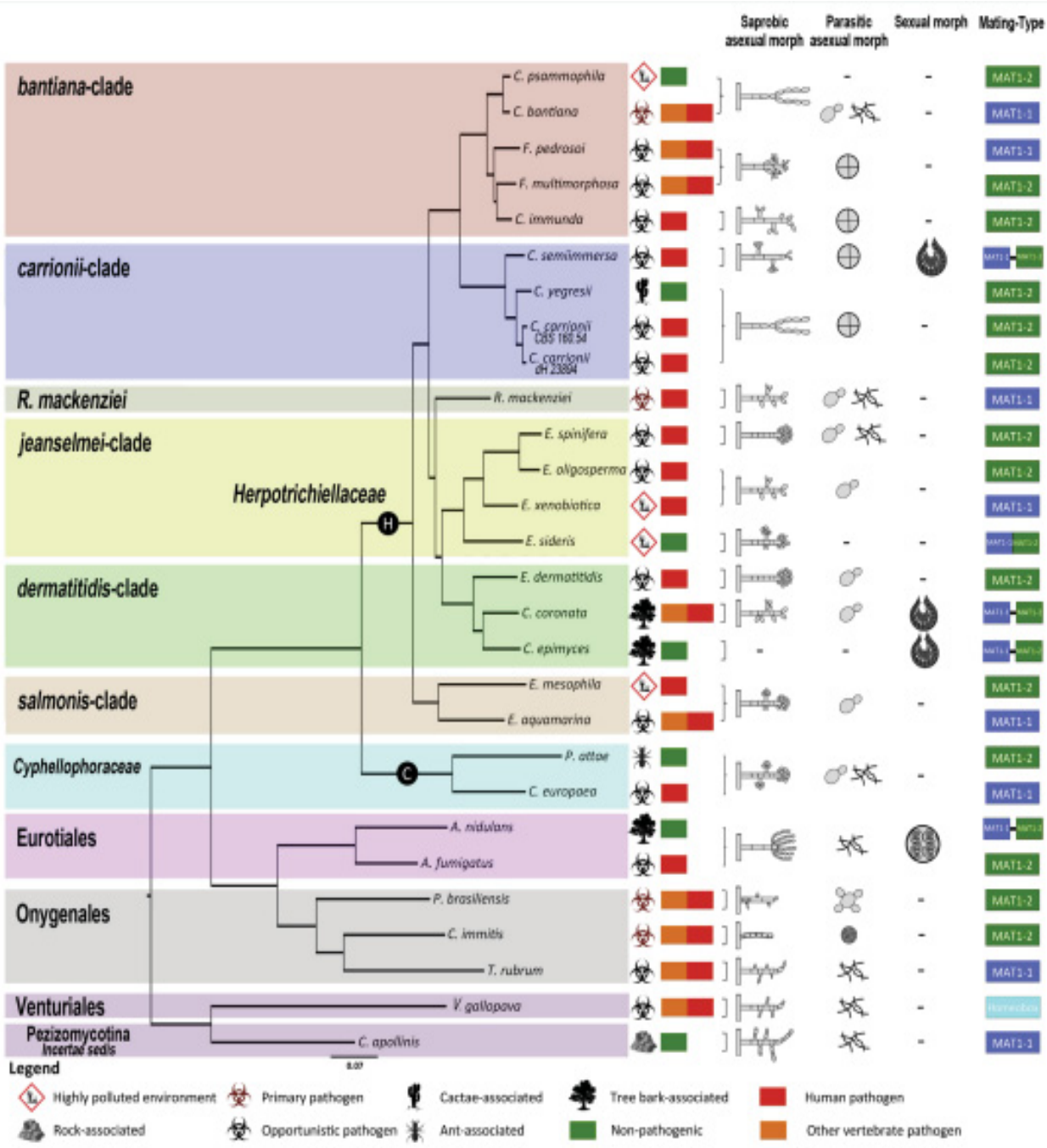


Figure 1 - Phylogenetic tree of Chaetothyriales constructed with Maximum Likelihood.

SOURCE: TEIXEIRA et al., 2017.

De Hoog et al. (2004) described *F. monophora* as colonies restricted, spreading moderately, lanose to velvety, olivaceous to black, with black reverse. Hyphae septate, pale olivaceous. Conidiophores erect, mostly arising at right angles from supporting hyphae, unbranched or branched, concolorous with hyphae or slightly

darker. Conidiogenous cells short-cylindrical, olivaceous, arranged in loosely branched systems, with prominent denticles bearing ellipsoidal, pale olivaceous conidia in short chains comprising 1 to 5 cells; conidia of variable size, smooth- and thin-walled; conidial scars slightly darkened. Additional, multicelled, sessile conidial chains morphologically fitting the genus *Cladophialophora* often present (Figure 2).

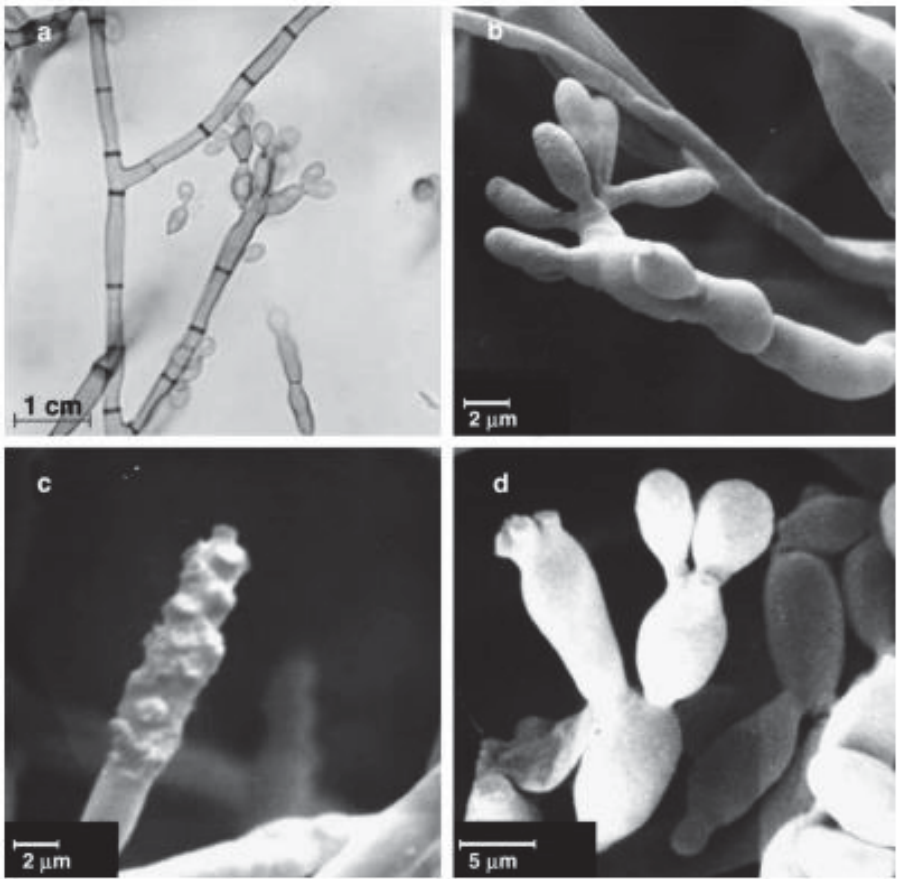


Figure 2 - Micromorphology of *F. monophora* (CBS 269.37), (a) Conidial apparatus bearing short conidial chains. (b) Conidiogenous cell with conidia. (c) Blunt denticles on conidiogenous cell. (d) Series of conidia, one with denticles.
SOURCE: DE HOOG et al., 2004

Fonsecaea erecta was described by Vicente et al. (2013), the colonies on MEA at 30 °C are velvety to downy, olivaceous brown; reverse olivaceous black. Hyphae smooth-walled, pale olivaceous brown, 3 to 4 µm wide, regularly septate every 7–17 µm. Conidiophores erect, olivaceous brown, apically densely branched; conidiogenous cells pale olivaceous, 18 to 25 µm long, usually with some septa and with prominent denticles. Conidia pale olivaceous brown, barrel-shaped, in short

chains containing 1 to 4 conidia, with brown scars. Germinating cells, phialides and chlamydospores absent. Teleomorph unknown. Optimal growth at 27-33 °C, scant growth at 37 °C, no growth at 40 °C (Figure3).

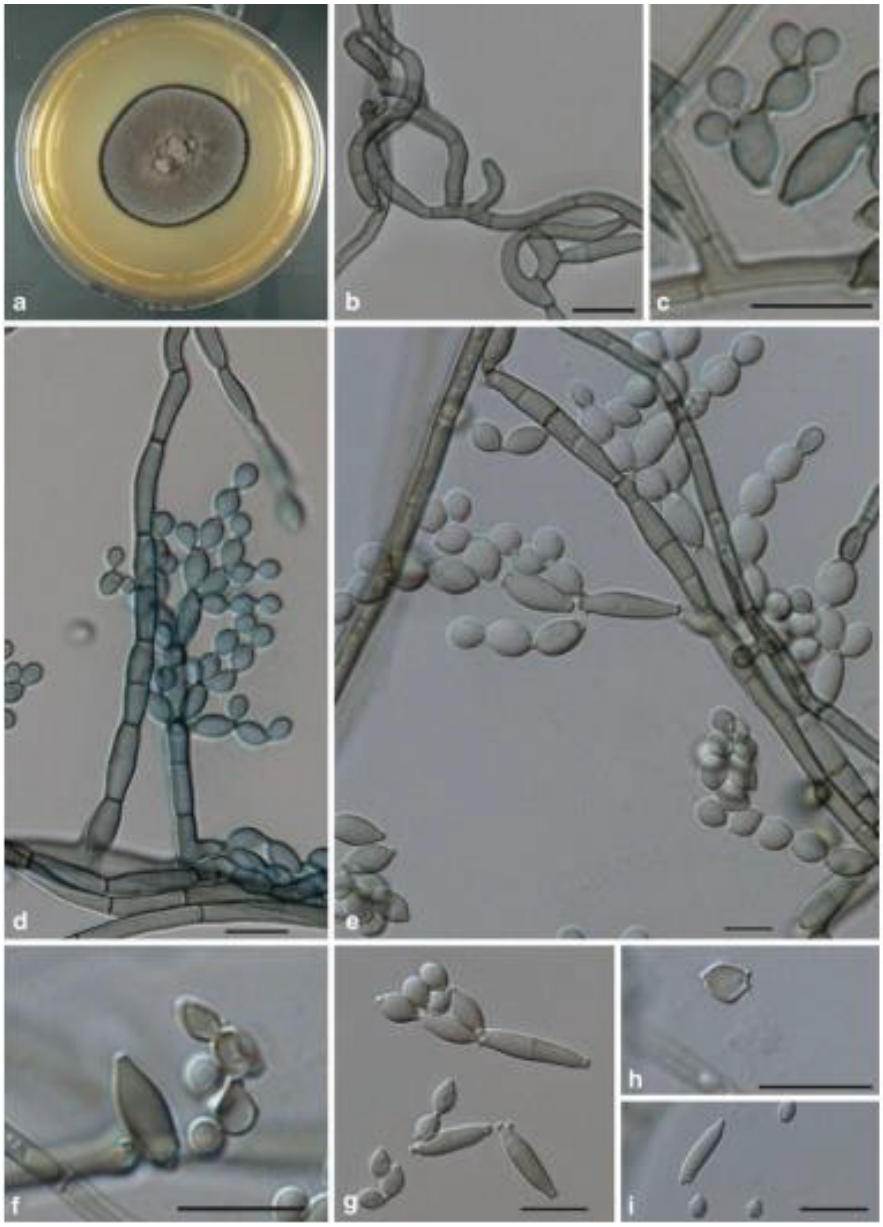


Figure 3 - *Fonsecaea erecta* (CBS 125763) microscopic and macroscopic morphology. (a) Colonies on MEA. (b) Hyphae. (c) Conidia. (d) Conidiophores. (e to i) Conidia. Scale bars=10 µm

SOURCE: VICENTE et al., 2013

1.2 CHROMOBLASTOMYCOSIS AND PHAEOHYPHOMYCOSES DISEASE

The name “Chromoblastomycosis” was established by Terra et al., in 1922. Rudolph reported the first case of Chromoblastomycosis in 1914; the patients lived in State of Minas Gerais and Sâo Paulo in Brazil, and another patient that was living in the United States (MCGINNIS, 1983).

According to the current concept, chromoblastomycosis is a neglected and occupational disease (QUEIROZ-TELLES et al., 2017). It is chronic fungal infection of skin and subcutaneous tissues, caused by several different dematiaceous fungi (KURIEN et al., 2018; SMITH et al., 2017; SUBHADASHANI et al., 2017; VENTURA et al., 2017; CHOWDHARY et al., 2015; GARNICA et al., 2009). The infection produces to the formation of slow-growing verrucous-looking skin and subcutaneous lesions that may emerge as tumor-like ulcerative eruptions (KURIEN et al., 2018; VICENTE et al., 2012; NAJAFZADEH et al., 2011, 2010, 2009; DE HOOG et al., 2004). The lesions are pruritic, and some patients feel pain (KURIEN et al., 2018; BADALI et al., 2008).

The disease is characterized by the fungi form sclerotic or muriform cells, which are septate bodies, brown color and 5 to 12 μm diameter (KURIEN et al., 2018; BONIFAZ et al., 2001; VINCENT, 2000; MCGINNIS, 1983), that it is found as a “black spots” on the surface of the host lesion (DA SILVA et al., 2008, 2002; MCGINNIS, 1983). The first known etiologic agent of the Chromoblastomycosis was *Phialophora verrucosa* (MCGINNIS, 1983). The most common agent pathogens as *Fonsecaea pedrosoi* and *Cladophialophora carrionii,* they are in tropical and subtropical areas (GARNICA et al., 2009). Other fungi are *Rhinocladiella aquaspersa, Exophiala jeanselmei, E. spinifera, E. dermatitidis* (GARNICA et al., 2009)*, F. nubica* (Najafzadeh et al., 2010), *F. monophora* (XI et al., 2009), *F. pugnacius* (AZEVEDO et al., 2015).

Chromoblastomycosis (CBM) has a worldwide occurrence, being more prevalent in tropical and subtropical climate (LI et al., 2016), such as Brazil (GOMES et al., 2016; SALGADO et al.,2004; VICENTE, 2000; QUEIROZ-TELLES, 1997; SILVA et al., 1992), China (CHEN et al., 2016; NAJAFZADEH et al., 2010; XI et al., 2009), India (AGARWAL, et al., 2017), and other countries. Also in non-tropical regions, these are rare cases (OUÉDRAOGO, et al., 2017).

In the Figure 4 it was showed the clinical cases report around the world in Central, South and North America, Cuba, Jamaica, Martinique, India, South Africa, Madagascar, Australia, Northern Europe, China, Japan and Malaysia. In South America, it is found in Brazilian Amazon, Southern Brazil (KURIEN et al., 2018), Peruvian Amazon (VENTURA et al., 2017), Northern Venezuela, Uruguay, Northern Argentina, Colombia. It is common in agricultural workers (KURIEN et al., 2018).The susceptible age is apparently all ages, but the majority patients are between 30 and 50 years and males (SANTOS et al., 2007; MCGINNIS, 1983).

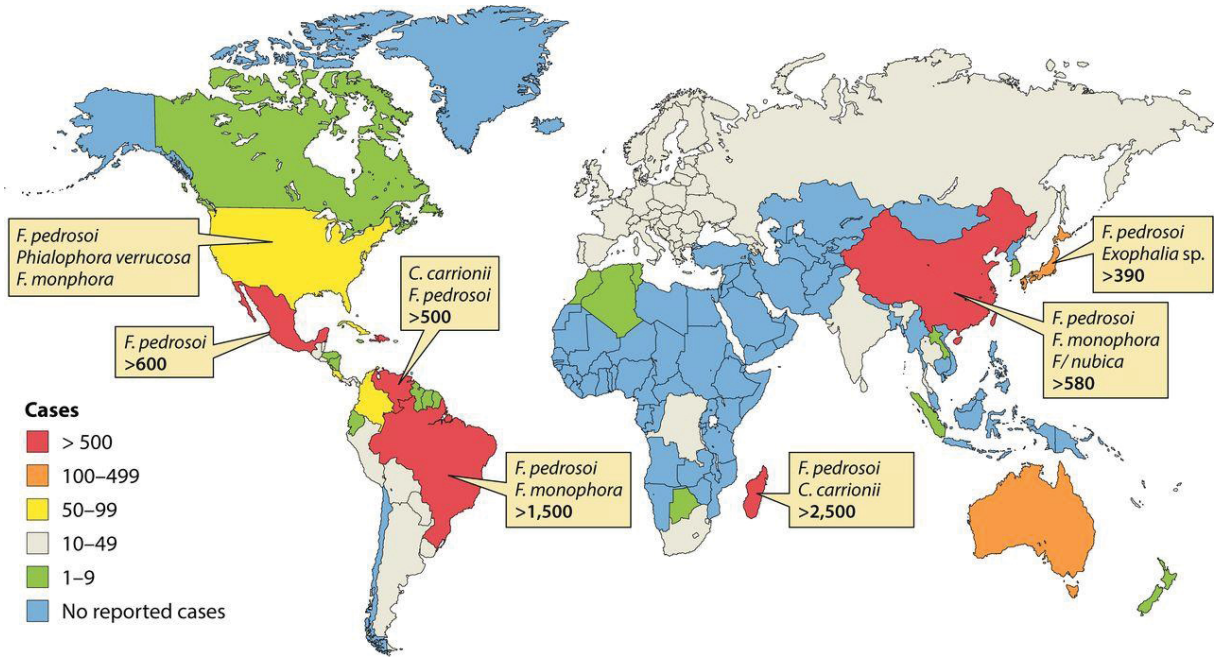


Figure 4 - Chromoblastomycosis global distribution.

SOURCE: QUEIROZ-TELLES et al., 2017.

Ajello et al. named Phaeohyphomycosis in 1974. It is also caused by dematiaceous fungus, in comparison with chromoblastomycosis has hyphae and muriform cells absent, and it is not limited to the skin or subcutaneous tissues, it can involve any organ or system such as the sinuses, lungs and brain with hyphae in tissue (SEYEDMOUSAVI et al., 2013; GARNICA et al., 2009). The pathogenic agents have more tropism for the nervous system, so in the brain tissue has hyphae growth and typically patients die (AZEVEDO et al., 2015). Phaeohyphomycosis agents are *E. jeanselmei, Wangiella dermatitidis, Bipolaris* species (GARNICA et al.,

2009); *Fonsecaea monophora* (DOYMAZ et al., 2015) and *F. pugnacius* (AZEVEDO et al., 2015). This infection is common in patient with transplant recipients due to the underlying immunosuppression (WELFRINGER et al., 2017).

The chromoblastomycosis and subcutaneous phaeohyphomycosis may be acquired by the inoculation of pathogen agents following penetrating trauma, or in the case of phaeohyphomycosis with secondary dissemination is almost exclusively in immunocompromised hosts (GARNICA et al., 2009). Azevedo et al. (2015), reported a patient with phaeohyphomycosis for *F. pugnacius* in immunocompetent hosts. The pathogenic agents have some virulence factors; such as the presence of melanin and carotene, the formation of muriform cells inside the subcutaneous tissues, thermotolerance, adhesion and hydrophobicity.

The melanin pigment can block the effects of hydrolytic enzymes and therefore it has an inhibitory effect on a receptor-mediated phagocytosis, in addition, the carotenoids can shield molecules or sensitive organelles. Some fungus produce hyphae forms in nature and tissue, but in the infected tissues it exists almost exclusively as muriform cells (muriform cells are swollen, iso diametrically enlarging cell with thick cell walls); these cells seem to be form by environmental conditions. Thermotolerance of the black yeast is important because may cause systemic or disseminated infections in mammals when the black yeast growth 35 to 37°C. Another characteristic is adhesion to epithelial cells with formation of muriform cells and hydrophobicity that help for airborne dispersal (SEYEDMOUSAVI et al., 2014).

Treatment of Chromoblastomycosis and Phaeohyphomycosis is a challenge because they have low cure rates. Chromoblastomycosis disease can be treated with itraconazole (QUEIROZ-TELLES, 1997) alone or combined with flucytosine, cryosurgery or heat, fluconazole, amphotericin B and terbinafine. In this case, the success of therapy depends on the causal agent and the severity of the disease. And the other hand, the Phaeohyphomycosis treatment is with oral azoles such as: itraconazole (NAJAFZADEH et al., 2010), voriconazole and posaconazole; however, the treatment in immunosuppression patients is a challenge, because the disease can reappear if the patient remains immunosuppressed (OUÉDRAOGO et al., 2017; GARNICA et al., 2009).

Currently, Queiroz-Telles et al. (2017) reported that treatment with physical methods in combination with antifungal agents. The physical methods can be surgery, cryotherapy, thermotherapy, laser therapy, and photodynamic therapy

(PDT). The conventional surgery is the best physical methods and can be used with itraconazole or terbinafine; the cryotherapy uses liquid nitrogen for small lesions and can combine with itraconazole or terbinafine; thermotherapy with chemical pocket wamer, which can be used with posaconazole and terbinafine; laser therapy with CO2 can be used as monotherapy or in combination. The antifungal agents can be itraconazole, posaconazole, voriconazole and isavuconazole exhibit the best in vitro activity against agents of CBM.

1.3 TRANSFORMATION AND TRANSFORMATION MEDIATED BY *Agrobacterium tumefaciens*

Genetic transformation is the process of inserting exogenous DNA fragments in host cells, with the objective of expression of exogenous DNA and genotypic alteration, which helps to study different aspects of the molecular biology of microorganisms (LI et al., 2017).

The transformation can be divided in stages (MULLINS et al., 2001): (I) induction of competent cell; (II) introduction of exogenous DNA; (III) selection of transformed cells and stable replication. The methods of transformation are: protoplast mediated transformation (PMT), lithium acetate treatment, electroporation, biolistic, and *Agrobacterium tumefaciens* – mediated transformation (AMT). The advantages and disadvantages of the diversity transformation methods for filamentous fungi have been reviewed (LI et al., 2017; MICHIELSE et al, 2008).

The PMT is a common method to transform fungi, which needs a large number of competent fungal protoplasts. To generate protoplasts is used some enzymes to remove fungal complex cell wall components, and then for fusion of exogenous nucleic acids and protoplasts is used some chemical reagent (PEG) (LI et al., 2017). In the biolistic transformation, the DNA is coated with tungsten, introducing with high speed into fungal cells; this requires special equipment (MARTIN, 2015). In addition, the electroporation transformation is a simple and rapid method, which the exogenous nucleic acid can be transferred inside cells mediated by the blow of high voltage electric charges stored in the condenser (LI et al., 2017).

Around 30 years ago was the first of transformed filamentous fungi (*Neurospora crassa*), but previously was transformed the yeast *Saccharomyces cerevisiae*, besides the *Saccharomyces* plasmid is different, and was developed news vectors

(MARTIN, 2015). The use of protoplasts for transformation has been used for several filamentous fungi (RUI-DIEZ, 2002), but the frequency of transformation is low, and to improve the transformation of filamentous fungi have established other alternative methods such as: electroporation, biolistic transformation and *Agrobacterium*-mediated transformation (MEYER, 2007). The transformation of dematiaceous fungus was realized, Peng et al. (1995) reported transformants by electroporation and PEG methods of *Wangiella dermatitidis* (*Exophiala dermatitidis*); and Florencio et al. (2017) reported transformation mediated by *Agrobacterium tumefaciens* and biolistic of *Fonsecaea pedrosoi*, with more efficiency in AMT.

The AMT is a transformation made by a soil bacterium (*Agrobacterium tumefaciens*). *Agrobacterium tumefaciens* is a Gram-negative bacterium that can cause plants disease. The bacterium can pass its DNA (T-DNA), which is transferred with a tumor-inducing plasmid (Ti) to plant cells and alter the plant genome. The first transformations were made only in one species of plant, but over time, the researchers have improved to make the transformation of other species such as bacteria, fungi and some mammalian cells (LI et al., 2017; MICHIELSE et al, 2008).

The AMT is a method of excellent alternatives for fungal transformation. It requires a binary vector system (Figure 5), which mainly contains besides its chromosome two plasmids (plasmid Ti, but only contains a region of virulence with *vir* genes and does not contain the T-DNA, and binary vector that contains the T-DNA). The virulence region gives the machinery (proteins) to transfer the T-DNA (MICHIELSE et al, 2008).

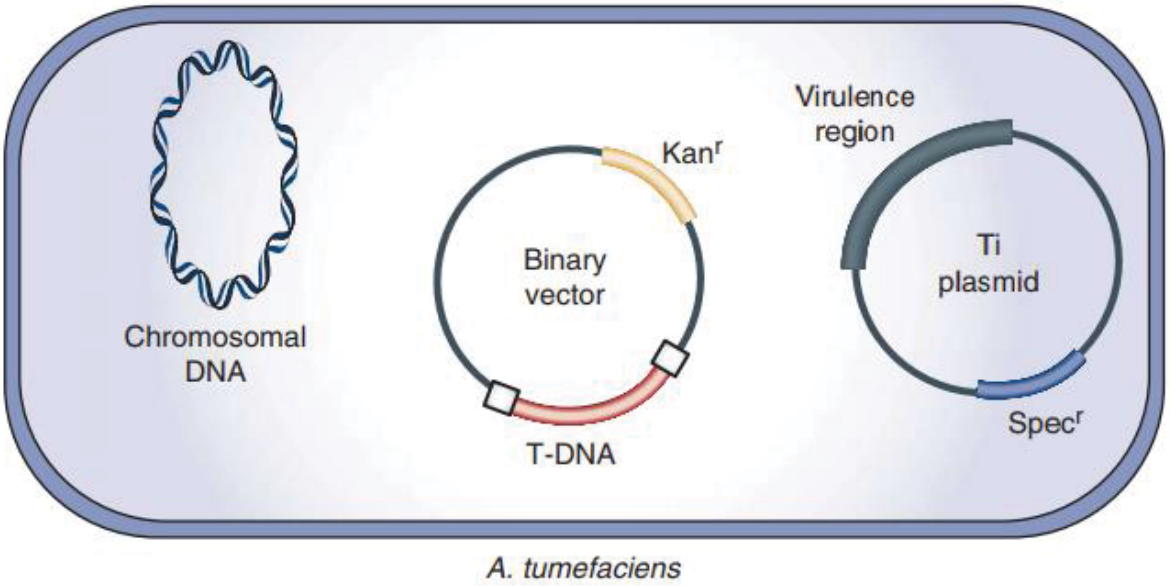


Figure 5 - Schematic overview of the binary vector system for *Agrobacterium*-medited transformation.

SOURCE: MICHIELSE et al., 2008.

There are some factors that will influence yeast transformation, one of the most important is acetosyringone (yeast-cocultivation period) it will act on the induction of vir genes, which are required for the transfer of T-DNA (MICHIELSE et al., 2008). Another very important factor is the use of starting material, which is usually the conidia because the transformation is more efficient than other material (MICHIELSE et al., 2004). In addition, the *A. tumefaciens* strain is important and the culture conditions for each strain (MICHIELSE et al., 2008). The relationship between *Agrobacterium* concentration and spore concentration is important and we should experience the effect of different concentrations. Finally, the temperature and co-cultivation time of transformation are important, since there will be a better transformation at a low temperature (22-25 ° C) and 2-3 days (MICHIELSE et al., 2005).

The principal advantages of the AMT are the versatility it provides in choosing which starting material to transform (MULLINS et al., 2001), form a stable and efficient tramsformants, that is suitable for knock-in mutations, because DNA is inserted randomly, with a single copy in genomes (LI et al., 2017). However, the principal disadvantages are: that need to consider the factors into of the transformation process and the time, which is a lot of time invested (MEYER, 2007).

In addition, among transformation methods, the T-DNA plasmid has marker and reporter genes. The marker gene allows selecting the transformed strains. Drug resistance markers are the most used with filamentous fungi, in this case, the wild strain has to be sensitive to an antibiotic (marker gene) (RUI-DIEZ, 2002). The resistance marker most used is Hygromycin B (AUSTIN et al., 1990), is an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus* with broad-spectrum activity against both procaryotic and eucaryotic cells (RAO et al., 1983). It inhibits translocation of mRNA and tRNAs on the ribosome (BOROVINSKAYA et al., 2008).

The reporter genes as fluorescence can be used as a diagnostic tools in order to detect the presence/absence of fungal cells inside the plant tissues (MULLINS et al., 2001). The fluorescent markers (GFP and DsRed) do not require cofactors or substrates (NAHALKOVA et al., 2003). They can be observed *in vivo* of individual and population cells (LORANG et al., 2001).

The green fluorescent protein (GFP) was isolated of *Aequorea victoria*. This protein does not need additional cofactor. This protein has 238 amino-acids, that absorbs light since 395 to 475 nm, and the maxim of 508 nm (HEIM et al., 1996). The red fluorescent DsRed was isolated from *Discosoma sp.,* it also does not need cofactor, and absorbs light since 558 to 583 nm. DsRed has advantages compared to GFP for use as a single color fluorescent marker since it provides a higher signal-to-noise ratio and it is relatively resistant to photobleaching (NAHALKOVA et al., 2003). The GFP and DsRed are produced and accumulated into the cytoplasm of cells, emitting fluorescence (MAOR et al., 1998).

The genome engineering is a tool, which helps to study genes different. It can insert, delete, modify or replace DNA in the genome of wild strain. It needs efficiency and reliable methods that allow researchers to determine how the genotype influences the phenotype. The homologous recombination is a powerful method that helps to evaluate the function gene. However, these techniques present obstacles such as low efficiency for the correct insertion, the possibility of adverse mutagenic effects and the creation of strategies of selection/screening (GAJ et al., 2013).

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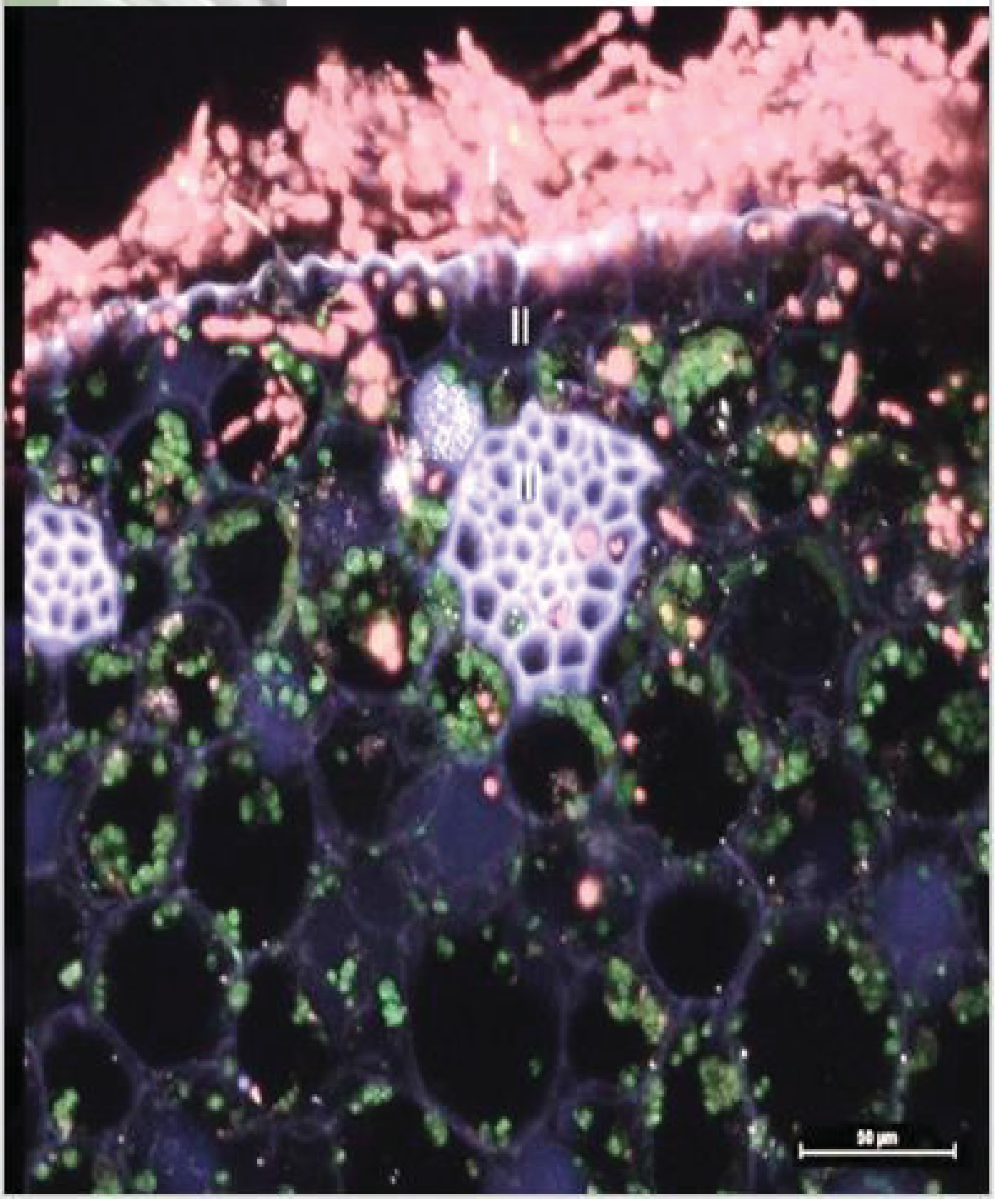
CHAPTER 2

Agrobacterium tumefaciens - MEDIATED TRANSFORMATION OF THE *Fonsecaea monophora* AND *Fonsecaea erecta* FOR CLINIC STUDY MODEL



Agrobacterium tumefaciens - mediated transformation of the *Fonsecaea monophora* and *Fonsecaea erecta* for clinic study model

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1. ABSTRACT

Fonsecaea genus contains etiologic agents of human chromoblastomycosis, a human cutaneous subcutaneous implantation disease with a worldwide distribution associated with trauma caused by plant. However, the invasive potential differs significantly among species. The species *F. monophora* have been reported as cause of cutaneous and subcutaneous chromoblastomycosis and eventually associated to primary brain infection and supposed to originate from the environment, although its isolation from nature is difficult. The epidemiology of the disease has not been fully elucidated and so, questions related to its infection route and virulence have to be elucidated. The environmental specie *F. erecta* was isolated from organic material and living plant in endemic areas of Brazil. Thus, this work described *Agrobacterium tumefaciens*-mediated transformation (AMT) of *F. erecta,* an environmental species isolated from living plant, and the pathogenic species *F. monophora* with the pCAMDsRed plasmid in order to determine whether a link could be made between environmental and clinical strains. Here we are proposing the pAD1625 plasmid, such as a model to study deletion of gene or multiple gene for virulence and pathogenicity studies. The co-cultivation was utilized conidia of *F. erecta* and *F. monophora* with a ratio 1:1, 10:1 and 100:1 (*Agrobacterium*: conidia) at 28 °C by 72 hours. The pAD1625 and pCAMDsRed plasmids were inserted into both fungi. The *Agrobacterium*: conidia ratios of both fungi (*F. erecta* and *F. monophora*) with both plasmids (pAD1625 and pCAMDsRed plasmid) from 1:1 to 100:1 led to a 4-25 fold increase in transformation efficiency. Confirmation of transformation was realized by amplification the hygromycin gene, and the fluorescence expression, which was variable between transformed fungi. Both fungi were able to colonize *Bactris gasipaes,* allowing to understand that they have invasive property to penetrate the epidermis.

Key words: Chromoblastomycosis, agrotransformation, plasmid, expression, pAD1625, pCAMDsRed

2. INTRODUCTION

Chromoblastomycosis is a neglected and occupational disease (QUEIROZ-TELLES et al., 2017). It is a chronic fungal infection of the skin and subcutaneous tissues, caused by several dematiaceous fungi (KURIEN et al., 2018; SMITH et al., 2017; SUBHADASHANI et al., 2017; VENTURA et al., 2017; GOMES et al. 2016; AZEVEDO et al., 2015; CHOWDHARY et al., 2015; GARNICA et al., 2009). The infection produces to the formation of slow-growing verrucous-looking skin and subcutaneous lesions that may emerge as tumor-like ulcerative eruptions (KURIEN et al., 2018; VICENTE et al., 2012; NAJAFZADEH et al., 2011, 2010, 2009; DE HOOG et al., 2004). In comparison with phaeohyphomycosis, other disease associated to this species, it has hyphae and is not limited to the skin or subcutaneous tissues; it can involve any organ or system such as the sinuses, lungs and brain (SEYEDMOUSAVI et al., 2013; GARNICA et al., 2009). The chromoblastomycosis and phaeohyphomycosis may be acquired by the inoculation of pathogen agents following penetrating trauma, or in the case of phaeohyphomycosis with secondary dissemination (GARNICA et al., 2009). However, the infection process and path of dispersion are still insufficiently clarified (AZEVEDO et al., 2015; VICENTE et al, 2013, 2008; VICENTE, 2000).

The *Fonsecaea* species and their relatives are anamorphic Ascomycetes belonging to the family Herpotrichiellaceae (order Chaetothyriales) gathering numerous agents of human infection with significant predisposition to growth in human tissue (VICENTE et al., 2017; VICENTE et al., 2013). *Fonsecaea* is the second prevalent genera containing etiologic agents of human Chromoblastomycosis and comprises cryptic entities (*F. pedrosoi, F. monophora* and *F. nubica*) potentially causing the diseases (NAJAFZADEH et al., 2010; DE HOOG et al., 2004). However, the invasive potential differs significantly among species (SEYEDMOUSAVI et al., 2011; BADALI et al., 2008; DE HOOG et al., 2006;). *Fonsecaea pedrosoi* and *F. nubica* are narrowly associated with chromoblastomycosis, while *F. monophora* is also involved in disseminated phaeohyphomycosis of brain and other organs (KOO et al., 2010; NAJAFZADEH et al., 2010; SURASH et al., 2005). Recently it was described *F. pugnacius* combines that causes two disorders starting as

chromoblastomycosis with cerebral dissemination in the same host (AZEVEDO et al., 2015).

An effective way to study the disease causing mechanisms of these organisms is to disrupt their genes, as a targeted and random manner,and also as to isolate mutants exhibiting altered virulence (MULLINS et al., 2001). Thereby, it represents a tool to understand the molecular basis of virulence and host specificity.

The transformation mediated by *Agrobacterium* (AMT) is an excellent alternative of method for fungal transformation, because it has higher transformation frequencies, not need equipment, the starting materials can be variable and the fungi has a single-copy of DNA (MICHIELSE et al, 2008). The marker gene such as hygromycin can be used like a model for construction of cassettes to delete one or more genes. The fluorescents reporter genes can be as diagnostic tools to signal the presence/absence of fungal matter in the plant (MULLINS et al., 2001), which helps to the visualization inside the plant and the formation of characteristic structures of the disease. Hence, the AMT is a good method for insert fluorescence gene and being a tool to determinate the presence or absence of these fungis inside of plant. Therefore, this study aimed to transform *Fonsecaea* sibling species mediated by *Agrobacterium tumefaciens* in order to obtain an effective tool to understand the infection process using the palm native *Bactris gasipaes* as a model. In this context, it was described AMT of *F. erecta,* an environmental species isolated from living plant, and the pathogenic species *F. monophora* transformed with the pCAMDsRed plasmid and pAD1625 plasmid, can contribute in studies to determine a link between environmental and clinical strains and as a possible model for the elimination of pathogenic genes.

3. MATERIALS AND METHODS

3.1 Strains, plasmids and growth conditions

Agrobacterium tumefaciens strain EHA 105, with binary vector pCAMDsRed (DsRed-Express), which is composed of *DsRed*- Express gene (reporter gene), hygromycin phosphotransferase gene (marker gene, hygromycin resistance), and kanamycin gene (kanamycin resistance for bacteria) (DOS SANTOS et al., 2016).

And other *Agrobacterium tumefaciens* strain EHA 105, with binary vector pAD1625 which contains hygromycin genes (marker gene, hygromycin resistance), and ampicillin gene (ampicillin resistance for bacteria) (FLORENCIO et al., 2018). *Agrobacterium tumefaciens* was grown at 28 °C in Luria Bertani (LB) supplemented with 100 mg/mL kanamycin (pCAMDsRed) or 100 µg/mL ampicillin (pAD1625). *Fonsecaea monophora* (CBS 269.37) and *F. erecta* (CBS 125763) strains were stored in SDA, pH 5.6 and were provided by "Microbiological Collections of the Paranaense Network" (CMRP) at the University Federal of Paraná, Curitiba, Paraná, Brazil.

3.2 *Fonsecaea monophora* and *F. erecta* susceptibility to the dominant selective markers

For evaluate the susceptibility of hygromycin, the fungus was inoculated by the single-point methodology in Sabouraud Dextrose Agar (SDA) supplemented with different concentrations of hygromycin (25, 50, 75, 100 and 150 µL.mg-1). This test was performed in duplicate and conditioned at 28°C and evaluated at 3, 7, 14, 21 and 30 days after inoculation. A sensitivity to hygromycin and estimated as a base without mycelia growth (RODRIGUES, 2010). For negative control was used *F. pedrosoi* CBS 271.37 and positive control *F. pedrosoi* with FpgGFP plasmid.

3.3 Fonsecaea sibling species *Agrobacterium tumefaciens*-mediated transformation

The *Agrobacterium*-mediated transformation protocol was described by Florencio et al. (2018) with few modifications. *Fonsecaea monophora* and *F. erecta* were inoculated in potato broth with chloramphenicol, were grown for 7 days at 28°C on shaker at 150 RPM, centrifuged and resuspended induction medium (IM) plus 40mM of (2-N-morpholine)-ethane sulfonic acid (MES) and 0.2 mM of 3',5'-dimethoxy-4'-hydroxyacetophenone (AS). The isolates of *A. tumefaciens* was grown for overnight in Luria Bertani (LB) liquid medium supplemented for kanamycin (pCAM-DsRed plasmid) and ampicillin (pAD1625 plasmid), at 28 °C on shaker at 200 RPM overnight. The bacterias were centrifuged, washed and resuspended in 10 mL

of IM+MES+AS grown for approximate 7 hours 28 °C on shaker at 150 RPM until reaching a density 0.5 to 0.8 at OD_{600nm}. For co-cultivation, yeast cells of *F. monophora* and *F. erecta* were mixed with *A. tumefaciens* cells at varying ratios (1:1, 10:1, 100:1). The cells mix were inoculated in induction medium solid for 3 days at 28 °C. After the co-cultivation, the colonies were scraped and washed with a physiological saline solution to inoculate in SDA plus 100 µg/mL hygromycin and cefotaxime, incubated for 7 days at 28 °C.

3.4 Mitotic stability of the transformed colonies

The mitotic stability of random colonies transformed was performed for single-point methodology in SDA at 28 °C for 1 week. After, were inoculated another 4 times, and finally, the fungus would have to grow in SDA supplemented with hygromycin for the evaluation of mitotic stability (RODRIGUES, 2010).

3.5 PCR assays (Polymerase Chain Reaction)

For the detection was selected randomly four transformants of each fungus (*F. erecta* and *F. monophora)* from the two different plasmids (pCAMDsRed and pAD1625).Total DNA was extracted according to the method described by Vicente et al. (2008). The amplification reactions of 16 selected transformants were performed using primers: hph1 (5'AGCGTCTCCGACCTGATG3') and hph2 (5'CGACGGACGCACTGACGG3'), with the conditions described by Malonek and Meinhardt (2001). The wild-type strains were used as the negative control and the transformed strain with GFP (FLORENCIO et al., 2018) was used as the positive control.

3.5 Fluorescence test of transformants

Eight transformants (four *F. monophora* and four of *F. erecta* strains) were randomly selected and cultured on SDA with 100 µg/mL hygromycin and were incubated at 28° C for 7 days. The red fluorescence of DsRed was detected using a

Nikon confocal microscopy A1RSiMP (NIKON, Tokyo, Japan) with the following filter settings: 405 nm excitation and 425-475 nm emission. Images were saved and processed using NIS-Elements Viewer 4.20 software. The wild-type strains were used as negative controls.

3.6 Inoculation of *F. monophora* and *F. erecta in vitro Bactris gasipaes*

Bactris gasipaes was provided by the Brazilian Agricultural Research Corporation - EMBRAPA. *In vitro* plant was in culture medium MS (Murashige & Skoog, 1962), were inoculated with 20 µL of physiological saline solution with transformed strains and with wild strains (negative control) at concentration 10^7 cells/mL of each fungus (*F. monophora* and *F. erecta*). The experiments were by duplicate per group. The inoculum was applied as suspension of culture medium around the root. The plants were maintained at room temperature and the infection was monitored by Nikon confocal microscopy.

3.7 Statistical analysis

Analysis of variance (ANOVA) of the results was performed, when the F test was significant, subsequent comparisons between ratios different were made using the Tukey test.

4. RESULTS

4.1 *Fonsecaea monophora* and *F. erecta* susceptibility to the dominant selective markers

In order to proceed the transformation *Fonsecaea* sibling species by agrobacterium, the *F. monophora* (CBS 269.37) and *F. erecta* (CBS 125763) were tested to hygromycin susceptibility. In the susceptibility test was observed the

residual growth of *F. monophora* and *F. erecta* at concentrations of 25 µg/mL and the strains were completely inhibited at concentrations 50, 100 and 150 µg/mL (Figure 6). The MIC concentration was determined as 25 µg/mL of hygromycin, but 100 µg/mL was the concentration which was used for the selection of transformants by *Agrobacterium tumefaciens* – mediated.

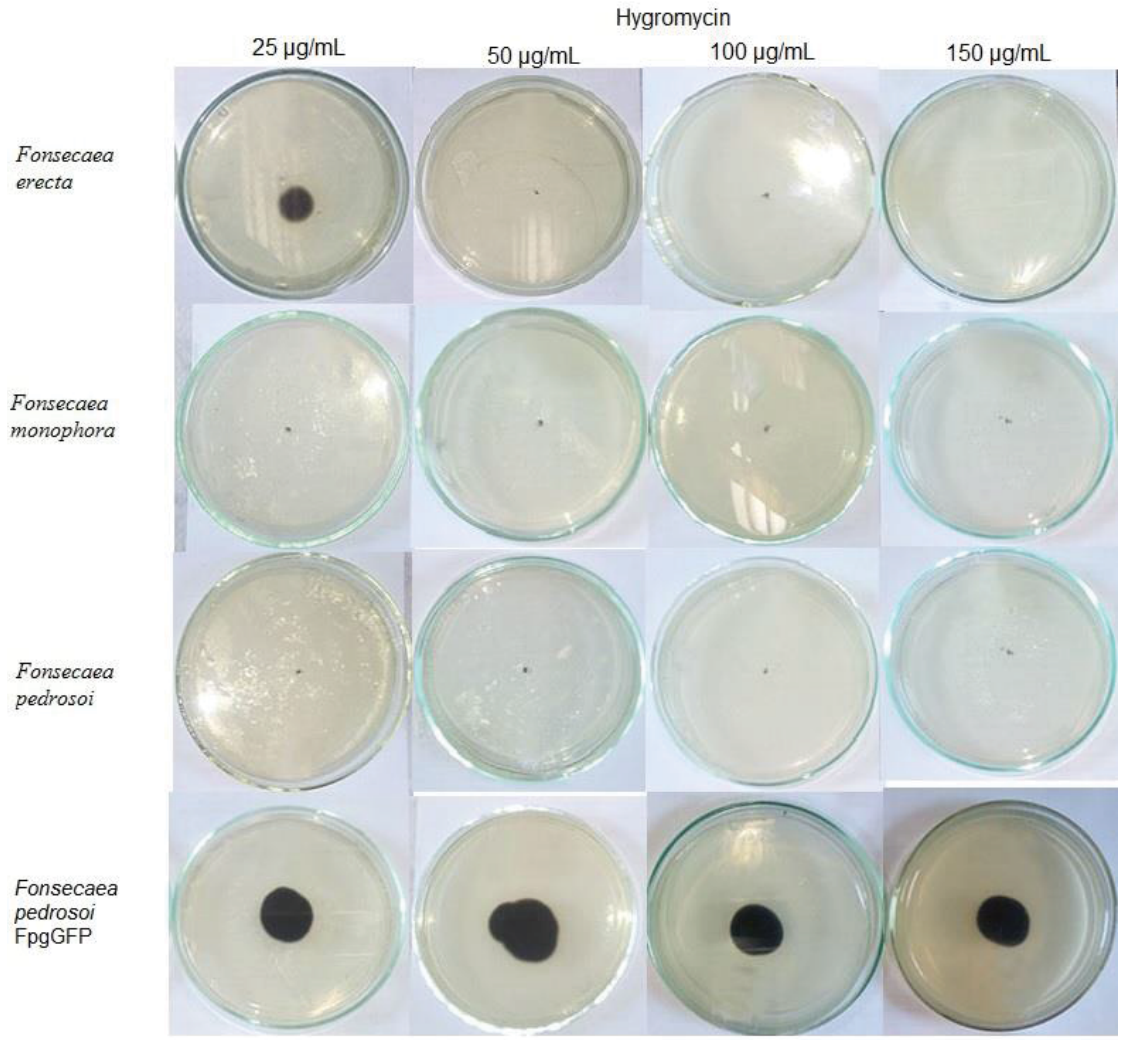

Figure 6 - Susceptibility of *F. monophora* and *F. erecta* to Hygromycin B using *F. pedrosoi* as a negative control and *F. pedrosi* with FpgGFP plasmid as a positive control. All the strains were inoculated by single point method at hygromycin concentrations (25, 50, 100 and 150 µg/mL). The plates were incubated at 28° C for 30 days.

4.2 *Fonsecaea* sibling species *Agrobacterium tumefaciens* - mediated transformation

The clinical strain *F. monophora* and environmental strain *F. erecta* were transformed by *Agrobacterium* with the pAD1625 and pCAMDsRed plasmids. The Figure 7 shows the frequency of transformants per 10^7 conidia for both plasmids tested and both fungus (*F. monophora* and *F. erecta*), after 72h of co-cultivation with 1:1, 10:1, 100:1 of ratios (bacteria: conidia). The AMT with EHA105 harboring pAD1625 plasmid with *F. erecta* provided statistically increased number of transformants with respect to pCAMDsRed plasmid, but *F. monophora* with pCAMDsRed plasmid provided statistically increased number of transformants with respect to pAD1625. Concerning to the transformation efficiency in both strains after co-cultivation of EHA105/pAD1625. *Fonsecaea monophora* with EHA105/pCAMDsRed for 72 hours it was observed that the best ratio of the bacteria related to conidia was 100:1 significant statistical difference. Nonetheless, *F. erecta* with pCAMDsRed plasmid has significant statisticals difference between 1:1, 10:1 and 100:1 ratios (Figure 7).

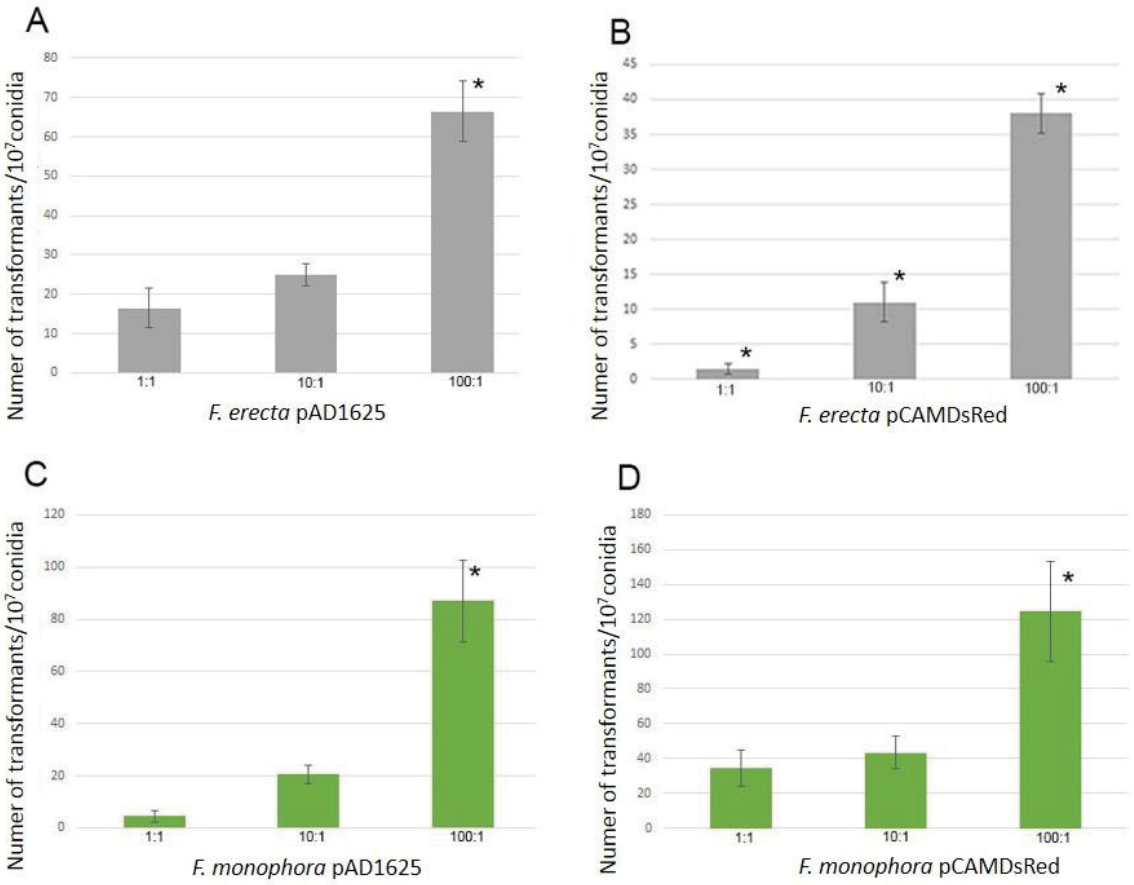

Figure 7 - Number of transformants per 10^7 conidia obtained by AMT. Comparison of transformation efficiency between 1:1, 10:1 and 100:1 (bacteria:conidia) ratio of *F. erecta* with pAD1625 (A) and pCAMDsRed (B) plasmids, and *F. monophora* with pAD1625 (C) and pCAMDsRed (D) plasmids. The statistical test applied was One-way ANOVA and Tukey post-test.

* significant statistical difference with 95% confidence

The transformants that were tested for mitotic stability, an average of 100% were mitotically stable for resistance to hygromycin at a concentration of 100 ug/mL after five generations. The transformants conserved colonies of parental morphology and velvet-melanized aspect on SDA plates.

4.3 PCR assays (Polymerase Chain Reaction)

In the PCR assay the 500 bp fragment of the *hph* gene was amplified, demonstrating that the plasmids (pCAMDsRed and pAD1625) were inserted into *F. erecta, F. monophora* and the wild type strain without amplification (Figure 8A-B).

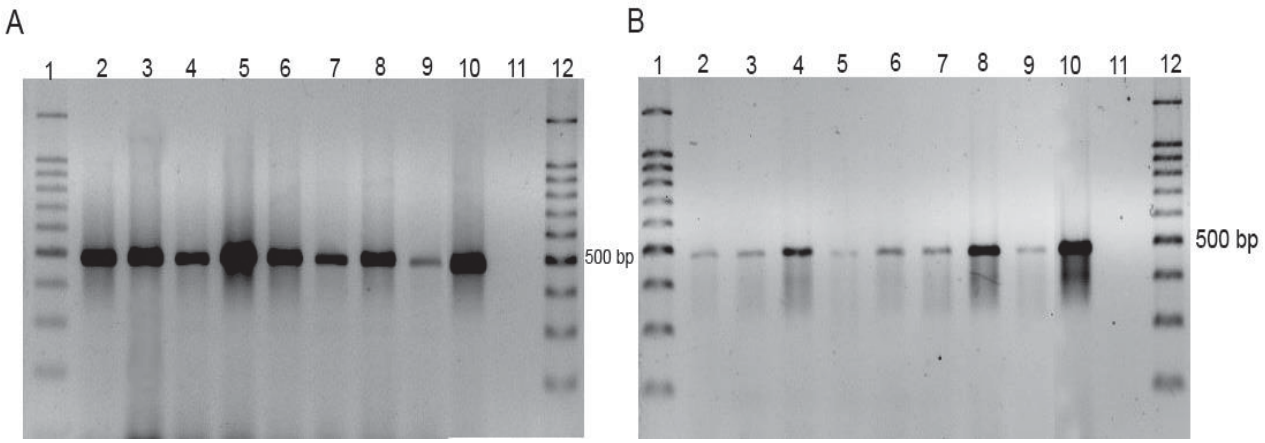

Figure 8. Agarose gel electrophoresis showing presence of *hph* gene of the pAD1625 plasmid **(A)** and pCAMDsRed plasmid **(B)** in transformed colonies. In the **(A):** *F. erecta*: 1-pAD1625 (2), 2-pAD1625 (3),3-pAD1625 (4), 4-pAD1625 (5) and *F. monophora*: 1-pAD1625 (6), 2-pAD1625 (7), 3-pAD1625 (8), 4-pAD1625 (9). Isolated wild-type of *F. monophora* was used as a negative control (11), *F. pedrosoi*

FpgGFP was used as a positive control (10), and 100-bp ladder (1,12). **(B)** *F. erecta:* 1-pCAMDsRed (2), 2-pCAMDsRed (3),3-pCAMDsRed (4), 4-pCAMDsRed (5) and *F. monophora*: 1-pCAMDsRed (6), 2-pCAMDsRed (7), 3-pCAMDsRed (8), 4-pCAMDsRed(9). Isolated wild type of *F. erecta* was used as a negative control (11), *F. pedrosoi* FpgGFP was used as a positive control (10), and 100-bp ladder (1,12).

4.4 Fluorescence test of transformants

The transformed colonies were observed using fluorescence microscopy for the expression of DsRed of the pCAMDsRed plasmid (Figure 9 and Figure 10) of *F. erecta* and *F. monophora.* The transformed strains showed fluorescence in the hyphae, cytoplasm and conidia. In addition, the wild strains types did not present fluorescence.

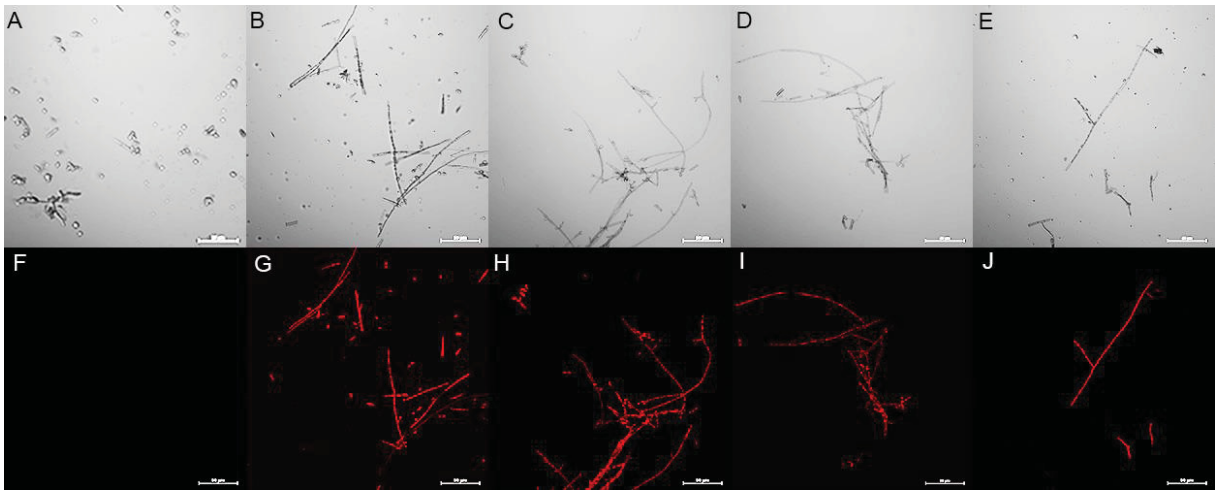

Figure 9 - Photomicrographs of wild and transformed strains of *F. erecta.* Confocal microscopy images of wild-type (A and F), pCAMDsRed (B and G), pCAMDsRed (C and H), pCAMDsRed (D and I) and pCAMDsRed (E and J).

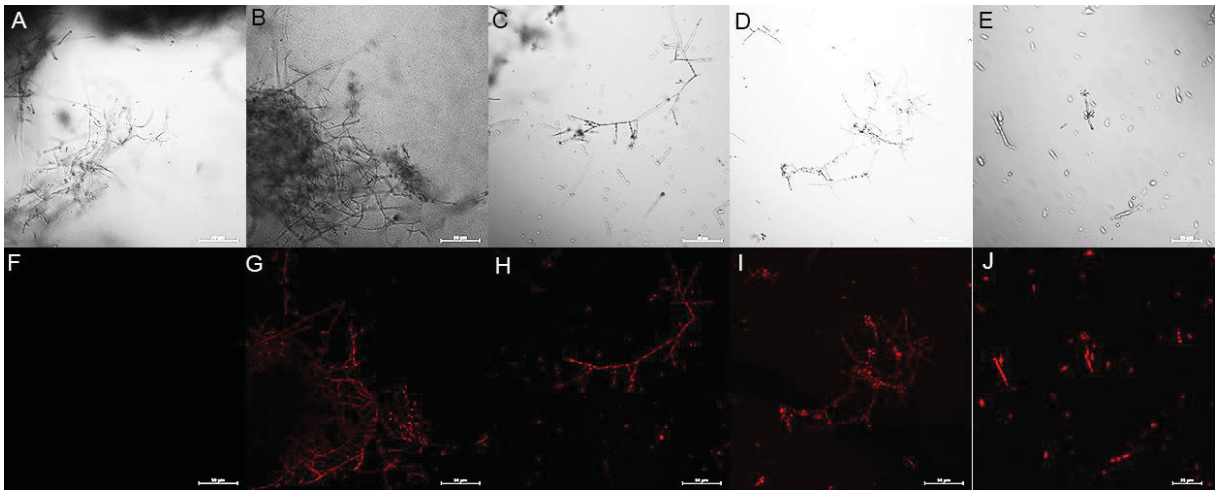

Figure 10 - Photomicrographs of wild and transformed strains of *F. monophora.* Confocal microscopy images of wild-type (A and F), pCAMDsRed (B and G), pCAMDsRed (C and H), pCAMDsRed (D and I) and pCAMDsRed (E and J).

4.5 Inoculation of *F. monophora* and *F. erecta in vitro Bactris gasipaes*

The transformants of *F. erecta* (E and J pCAMDsRed) and *F. monophora* (E and J pCAMDsRed) with fluorescence were inoculated to the plant. The wild strains was used as comparative pattern. In the Figure 11 was present the *F. erecta* inside tissues plant.

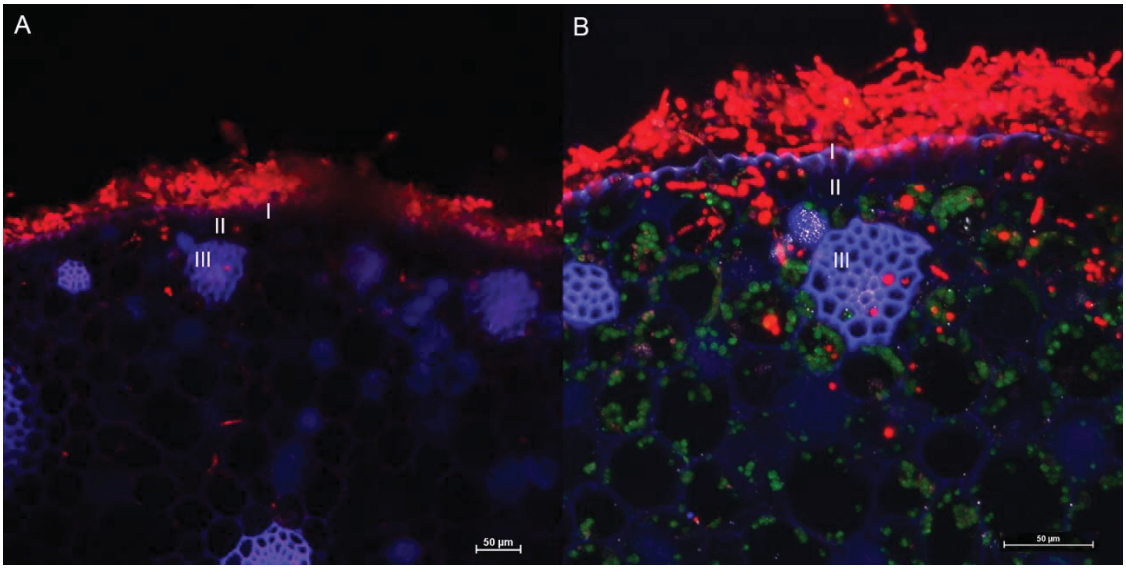

Figure 11 - Photomicrographs of *B. gasipaes* plants inoculated with transformed *F. erecta* expressing *DsRed* gene. Confocal Microscopy images 20X (A) and 40X (B).

Epidermis (I), parenchyma (II) and vascular tissue (III).

Likewise, it was possible to observe *F. monophora* colonizing the intercellular spaces (Figure 12). According to the results was possible to affirm that both strains are able to survive inside the tissues plants.

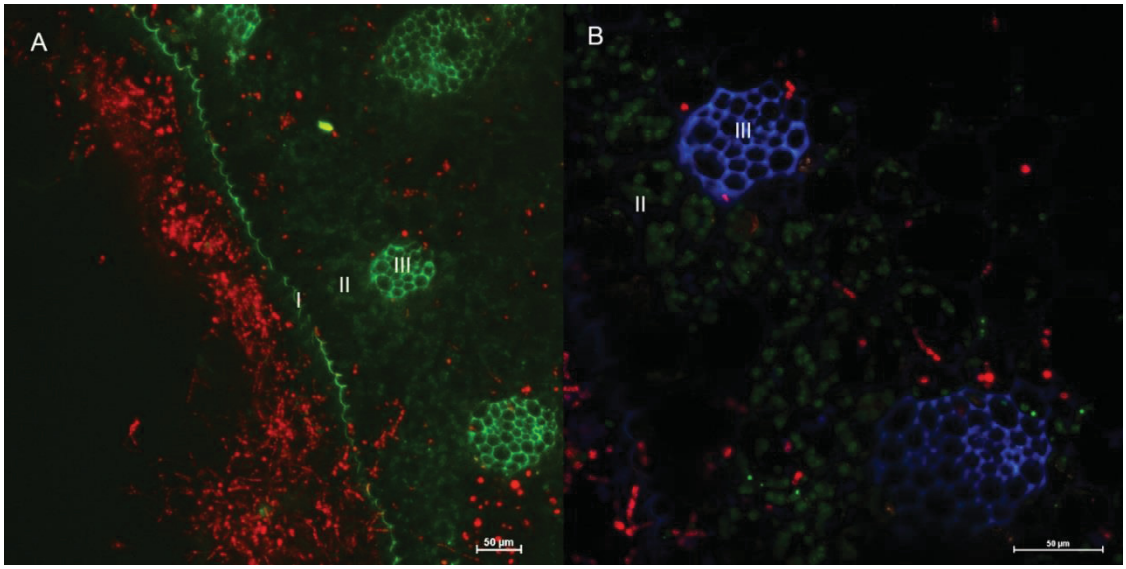

Figure 12 - Photomicrographs of *B. gasipaes* plants inoculated with transformed *F. monophora* expressing *DsRed* gene. Confocal Microscopy images 20X (A) and 40X (B). Epidermis (I), parenchyma (II) and vascular tissue (III).

The wild fungus did not show fluorescence. The fungus were inoculated in media culture with plants (*B. gasipaes*) produced in vitro. *Fonsecaea erecta* and *F. monophora* were observed to grow on the surface of the epidermis, parenchyma and vascular tissue of the plant. Both clinical and environmental strains demonstrated ability to invade the tissues plant.

5. DISCUSSION

Chromoblastomycosis (CMB) is a neglected tropical disease (NTDs), which is dematiaceous fungus infection, characterized by the presence of muriform cells, in comparisons with phaeohyphomycosis that presents hyphae. CMB and

phaeohyphomycosis are tropical/subtropical diseases, most of the cases have been described in Brazil especially in the northern regions. These diseases are produced by traumatic inoculation of the fungi by implantation of an environmental source (QUEIROZ-TELLES et al., 2017).

The fungi and another microorganism can infect or live as saprobic or parasitic form (OMACINI et al., 2001). Many investigations have shown that dematiaceous fungi are isolated from different natural sources. However, only few reports of the environmental occurrence of the clinical species. For instance Vicente et al. (2013) report some few isolates of *F. pedrosoi* (n=2) and *F. monophora* (n=1) from debris plant. SALGADO et al., 2004 reported the same fungus species of the patient in the thorn of the *Mimosa pudica* plant.

Fonsecaea species such as *F. monophora* and *F. pedrosoi* were isolated from the patient while *F. erecta* and *F. minima,* "saprobes" agents, still were not found in CBM lesions (Vicente et al., 2013). Moreover, the specific environment of pathogenic fungi is still unclear. .

The present study demonstrated the *Agrobacterium tumefaciens*-mediated transformation (AMT) of *F. erecta* and *F. monophora* introducing two transformed strains expressing the DsRed gene into the plant, which will help elucidate the interaction between fungi and plant as well the process of infection. Likewise, the transformation with pAD1625 plasmid represents a model to study functions of genes. The marker gene (hygromycin) can be employed for the construction of cassette deletion of one or more genes.

The AMT is a tool, which helps to transform fungi that cannot be transformed by other methods (MARTIN, 2015). In this study was showed that AMT demonstrated to be an excellent method to transform this group of fungi, because it has higher transformation frequencies, not need equipment, the starting materials can be variable and the fungi has a single-copy of DNA (MICHIELSE et al., 2008).

The variables used for AMT were chosen based on the work of Florencio et al. (2018), where they described that: had more frequency of transformants with 10^8 conidia than 10^6 conidia, and the co-cultivation duration indicated more frequency of transformants at 72 hours than 48 hours. Consequently, the variables were 10^7 conidia and 72 hours of co-cultivation AMT demonstrated excellent results.

Agrobacterium tumefaciens EHA105 is an excellent strain to produce transformants because EHA105 was constructed a super-virulent strain. Besides that

AS is very important to induce expression of *vir* gene, and fungi are not produced (ZHANG et al., 2008). The factors can change the frequency of transformants, hence is noted that changing the *Agrobacterium*: conidia ratios of both fungis (*F. erecta* and *F. monophora*) with both plasmids (pAD1625 and pCAMDsRed plasmid) from 1:1 to 100:1 led to a 4-25 fold increase in transformation efficiency; and 100:1(*Agrobacterium*: conidia) ratios generated many numbers of transformant (125 *F. monophora* transformed) with co-cultivation of 72 hours with *A. tumefaciens* EHA 10 with pCamDsRed plasmid; Abuodeh et al. (2000) also described an increase in transformation efficiency with *Coccidioide immitis* .

Abuodeh et al. (2000) described that pAD1625 plasmid improved transformation rates in fungus because the plasmid has 2 mutations, which permits constitutive expression of *vir* genes and others that helps to have high plasmid copy numbers. The pCAMDsRed plasmid in this study was efficient for *F. monophora* than *F. erecta,* this can be explained by the greater compatibility of the plasmid promoter (PgpdA), likewise, it was observed in the study of Eckert et al. (2005), where PgpdA promoter expressed any gene.

All the *F. erecta* and *F. monophora* transformants were stable after five rounds of growth; even as many investigations of AMT described transformants mitotically very stable (RHO et al., 2001; FIGUEIREDO et al., 2010). The marker gene (hygromycin) allows selecting the transformed strains. Drug resistance markers are the most used with filamentous fungi (RUI-DIEZ, 2002). The evaluated transformants of both fungi amplified the hygromycin gene of pAD1625 and pCAMDsRed plasmids.

The reporter genes as fluorescence can be as diagnostic tools in determining the presence/absence of fungal matter in the plant (MULLINS et al., 2001). The fluorescent markers, such as GFP and DsRed are widely used because they do not require cofactors or substrates (NAHALKOVA et al., 2003), can be observed *in vivo* of individual and population cells and have low toxicity (LORANG et al., 2001). The DsRed protein (pCAMDsRed) presented a very good expression; Eckert et al. (2005) also reported more expression in DsRed protein than GFP protein; as mentioned above, it can be understood for the compatibility with plasmid promoter (PgpdA). The red fluorescence was microscopically detected in the hyphae and conidia of all transformants evaluated (Figure 9 and Figure 10). Eckert et al. (2005), described the older mycelium and some collapsed cells can have reduced expression or without

expression of DsRed.

The *in vitro* plant (*Bactris gasipaes*) was inoculated with transformed *F. erecta* and with *F. monophora,* the expression of the DsRed protein was strong. *Fonsecaea* species are endemic fungi in humid climates (VICENTE et al., 2013). The *B. gasipaes* is a palm native to the tropical forests and had been reported habitat of the melanized fungi (CALIGIORNE et al., 2005). The inoculation of both fungi displayed that transformed strains were able to grow inside tissue of *B. gasipaes* plant. The inoculum *in vitro* was applied around the root according to Fornari, (2017) that described clinical and environmental *Fonsecaea* species able to invade the tissues plant and demonstrated that the fungi survive inside the plant after histopathological studies by fixation.

However, here we propose a new method with allows to observe the fungi inside the plant without any kind of colorant. The invasion of several tissues and raise the possibility of a way to investigate the route of this implantation, and proposed alternative hypothesis is that the patients with chromoblastomycosis are infected by injury from plant material.

The photomicrographs (Figure 11 and Figure 12) of *B. gasipaes* plants inoculated with *F. erecta* and *F. monophora* shows that both fungi have invasive property to penetrate the epidermis, It demonstrated that hypothesis described by Fornari (2017) may be close to disease reality because the epidermis tissue forms thorns.

In conclusion, *Fonsecaea erecta* and *F. monophora* were completely inhibited at concentrations of 100 µg/mL hygromycin. *Fonsecaea monophora* with pCAMDsRed plasmid provided statistically increased number of transformants with respect to pAD1625 and *F. erecta* with pAD1625 plasmid provided statistically increased number of transformants with respect to the pCAMDsRed plasmid. For both fungi, bacteria: conidia ratio from 1:1 with 10:1 do not have a significant statistical difference, nevertheless, with 100:1 ratio has a significant statistical difference in transformation efficiency after co-cultivation. An average of 100% was mitotically stable for resistance to hygromycin. The fragment of 500 bp of the *hph* gene was amplified by PCR, representing the *hph* gene, but not the wild-type strain as expected. The transformed strains showed fluorescence in the hyphae cytoplasm and conidia. In addition, the transformed *F. erecta* and *F. monophora* were successfully inoculated into *B. gasipaes* and colonized intercellular spaces, showing

the invasive property of the fungi to penetrate the epidermis, reaching parenchyma and vascular tissue of the plant.

6. ACKNOWLEDGMENTS

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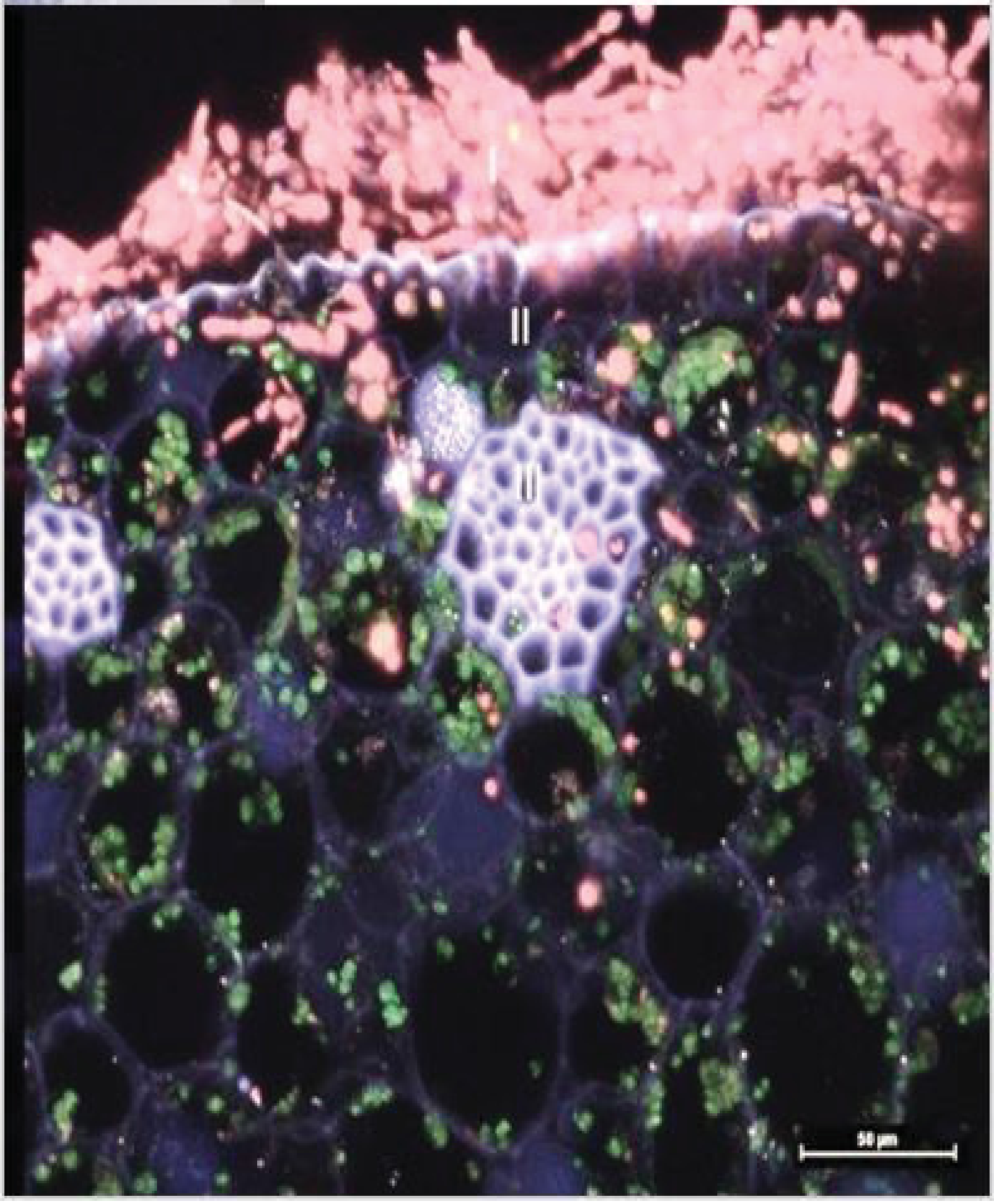
CHAPTER 3

CONCLUSION



CONCLUSSIONS

- *Fonsecaea erecta* and *F. monophora* are susceptible to hygromycin, the residual growth of *F. monophora* and *F. erecta* were at concentrations of 25 µg/mL and were completely inhibited at concentrations 50, 75, 100 and 150 µg/mL. The used concentration was 100 µg/mL of hygromycin.
- *Fonsecaea monophora* with pCAMDsRed plasmid provided statistically increased number of transformants with respect to pAD1625. Bacteria: conidia ratio from 1:1 with 10:1 do not have a significant statistical difference, nevertheless, 100:1 ratio has a significant statistical difference in transformation efficiency after co-cultivation. *Fonsecaea erecta* with pAD1625 plasmid provided statistically increased number of transformants with respect to the pCAMDsRed plasmid. Bacteria: conidia ratio from 1:1 with 10:1 do not have a significant statistical difference, nevertheless, 100:1 ratio has a significant statistical difference in transformation efficiency after co-cultivation.
- An average of 100% was mitotically stable for resistance to hygromycin at a concentration of 100 ug/mL after five generations.
- The transformed strains showed fluorescence in the hyphae cytoplasm and conidia. In addition, the wild types did not present fluorescence.
- The fragment of 500 bp of the *hph* gene was amplified by PCR, representing the *hph* gene, but not the wild-type strain as expected.
- The transformed *F. erecta* and *F. monophora* were successfully inoculated into *Bactris gasipaes*.
- The *F. erecta* and *F. monophora* transformed colonized intercellular spaces, showing the invasive property of the fungus to penetrate the epidermis, reaching parenchyma and vascular tissue of the plant.

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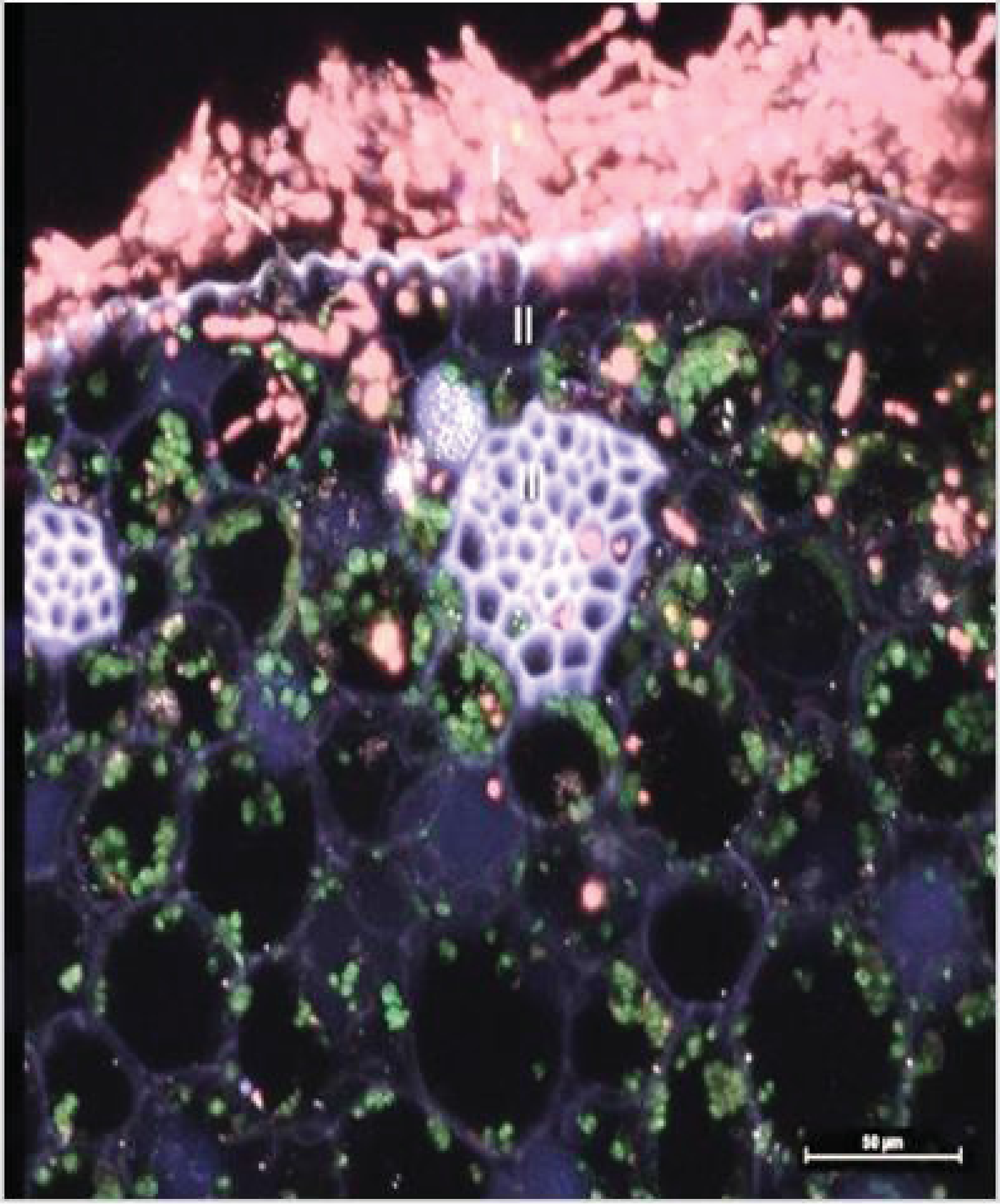
ATTACHED



1. METHODOLOGY DETAILS

1.1 *Agrobacterium tumefaciens* - mediated transformation

Fonsecaea monophora and *F. erecta* were inoculated in potato broth with chloramphenicol, they were grown for 7 days at 28°C on a shaker at 150 RPM, centrifuged, filtered with glass wool, washed with physiological saline solution and resuspended induction medium (IM) plus 40mM of (2-N-morpholine)-ethane sulfonic acid (MES) and 0.2 mM of 3',5'-dimethoxy-4'-hydroxyacetophenone (AS). The isolates of *A. tumefaciens* was grown for overnight in Luria Bertani (LB) liquid medium supplemented for kanamycin (pCAM-DsRed plasmid) and ampicillin (pAD1625 plasmid), at 28 °C on a shaker at 200 RPM overnight. The next day the bacteria were centrifuged, washed with physiological saline solution and resuspended in 10 mL of IM+MES+AS grown for approximate 7 hours 28 °C on a shaker at 150 RPM until a density 0.5 to 0.8 at OD_{600nm}. For co-cultivation, yeast cells of *F. monophora* and *F. erecta* were mixed with *A. tumefaciens* cells at varying ratios (1:1, 10:1, 100:1). The cells mixtures were centrifuged and inoculated in induction medium solid for 3 days at 28 °C. After the co-cultivation, the colonies were scraped and washed with a physiological saline solution to inoculate in SDA plus 100 µg/mL hygromycin and cefotaxime, incubated for 7 days at 28 °C.

2. RESULTS DETAILS

2.2 *Fonsecaea* sibling species *Agrobacterium tumefaciens* - mediated transformation

***Fonsecaea erecta* with pCAM-DsRed plasmid**

Table 1 - Summary obtained by *Agrobacterium* mediated transformation of *F. erecta* with pCAM-DsRed plasmid.

Groups	*Count*	*Sum*	*Average*	*Variance*
(1:1)	2	3	1.5	0.5
(1:10)	2	22	11	8
(1:100)	2	76	38	8

Table 2 - Variance analysis obtained by *Agrobacterium* mediated transformation of *F. erecta* with pCAM-DsRed plasmid.

Origin of variations	*Sum of squares*	*Degrees of freedom*	*Average of squares*	*F*	*Probability*	*Critical value for F*
Between groups	1434.333	2.000	717.167	130.394	0.001	9.552
Within the groups	16.500	3.000	5.500			
Total	1450.833	5.000				

Table 3 - Multiple comparison with Tukey test obtained by *Agrobacterium* mediated transformation of *F. erecta* with pCAM-DsRed plasmid.

(I) proportions	*(J) proportions*	*Difference of means (I-J)*	*Standard error*	*Sig.*	*95% confidence interval*	
					Lower limit	*Upper limit*
1:1	1:10	-9,500	2,345	,054	-19,300	,300
	1:100	-36,500*	2,345	,001	-46,300	-26,700
1:10	1:1	9,500	2,345	,054	-,300	19,300
	1:100	-27,000*	2,345	,003	-36,800	-17,200
1:100	1:1	36,500*	2,345	,001	26,700	46,300
	1:10	27,000*	2,345	,003	17,200	36,800

*. La diferencia de medias es significativa en el nivel 0.05.

***Fonsecaea erecta* with pAD1625 plasmid**

Table 4 - Summary obtained by *Agrobacterium* mediated transformation of *F. erecta* with pAD1625 plasmid.

Groups	*Count*	*Sum*	*Average*	*Variance*
(1:1)	2	33	16.5	24.5
(1:10)	2	50	25	8
(1:100)	2	133	66.5	60.5

Table 5 - Variance analysis obtained by *Agrobacterium* mediated transformation of *F. erecta* with pAD1625 plasmid.

Origin of variations	*Sum of squares*	*Degrees of freedom*	*Average of squares*	*F*	*Probability*	*Critical value for F*
Between groups	2863.000	2.000	1431.500	46.177	0.006	9.552
Within the groups	93.000	3.000	31.000			
Total	2956.000	5.000				

Table 6 - Multiple comparison with Tukey test obtained by *Agrobacterium* mediated transformation of *F. erecta* with pAD1625 plasmid.

(I) proportions	*(J) proportions*	*Difference of means (I-J)*	*Standard error*	*Sig.*	*95% confidence interval*	
					Lower limit	*Upper limit*
1:1	1:10	-8,500	5,568	,396	-31,766	14,766
	1:100	-50,000*	5,568	,006	-73,266	-26,734
1:10	1:1	8,500	5,568	,396	-14,766	31,766
	1:100	-41,500*	5,568	,010	-64,766	-18,234
1:100	1:1	50,000*	5,568	,006	26,734	73,266
	1:10	41,500*	5,568	,010	18,234	64,766

*. La diferencia de medias es significativa en el nivel 0.05.

***Fonsecaea monophora* with pCAM-DsRed plasmid**

Table 7 - Summary obtained by *Agrobacterium* mediated transformation of *F. monophora* with pCAM-DsRed plasmid.

Groups	*Count*	*Sum*	*Average*	*Variance*
(1:1)	2	69	34.5	112.5
(1:10)	2	87	43.5	84.5
(1:100)	2	249	124.5	840.5

Table 8 - Variance analysis obtained by *Agrobacterium* mediated transformation of *F. monophora* with pCAM-DsRed plasmid.

Origin of variations	*Sum of squares*	*Degrees of freedom*	*Average of squares*	*F*	*Probability*	*Critical value for F*
Between groups	9828.000	2.000	4914.000	14.209	0.030	9.552
Within the groups	1037.500	3.000	345.833			
Total	10865.500	5.000				

Table 9 - Multiple comparison with Tukey test obtained by *Agrobacterium* mediated transformation of *F. monophora* with pCAM-DsRed plasmid.

(I) proportions	*(J) proportions*	*Difference of means (I-J)*	*Standard error*	*Sig.*	*95% confidence interval*	
					Lower limit	*Upper limit*
1:1	1:10	-9,000	18,597	,884	-86,71	68,71
	1:100	-90,000*	18,597	,034	-167,71	-12,290
1:10	1:1	9,000	18,597	,884	-68,71	86,71
	1:100	-81,000*	18,597	,045	-158,71	-3,290
1:100	1:1	90,000*	18,597	,034	12,290	167,71
	1:10	81,000*	18,597	,045	3,290	158,71

*. La diferencia de medias es significativa en el nivel 0.05.

***Fonsecaea monophora* with pAD1625 plasmid**

Table 10 - Summary obtained by *Agrobacterium* mediated transformation of *F. monophora* with pAD1625 plasmid.

Groups	*Count*	*Sum*	*Average*	*Variance*
(1:1)	2	9	4.5	4.5
(1:10)	2	41	20.5	12.5
(1:100)	2	174	87	242

Table 11 - Variance analysis obtained by *Agrobacterium* mediated transformation of *F. monophora* with pAD1625 plasmid.

Origin of variations	*Sum of squares*	*Degrees of freedom*	*Average of squares*	*F*	*Probability*	*Critical value for F*
Between groups	7656.333	2.000	3828.167	44.342	0.006	9.552
Within the groups	259.000	3.000	86.333			
Total	7915.333	5.000				

Table 12 - Multiple comparison with Tukey test obtained by *Agrobacterium* mediated transformation of *F. monophora* with pAD1625 plasmid.

(I) proportions	*(J) proportions*	*Difference of means (I-J)*	*Standard error*	*Sig.*	*95% confidence interval*	
					Lower limit	*Upper limit*
1:1	1:10	-16,000	9,292	,332	-54,827	22,827
	1:100	-82,500*	9,292	,006	-121,327	-43,673
1:10	1:1	16,000	9,292	,332	-22,827	54,827
	1:100	-66,500*	9,292	,011	-105,327	-27,673
1:100	1:1	82,500*	9,292	,006	43,673	121,327
	1:10	66,500*	9,292	,011	27,673	105,327

*. La diferencia de medias es significativa en el nivel 0.05.