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MODE OF ACTION OF *Phialomyces macrosporus* AS A BIOCONTROL AGENT IN COFFEE INFECTED WITH *Colletotrichum* sp.

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Orientador

Dr. Mario Sobral de Abreu

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ABSTRACT

Colletotrichum sp. is a phytopathogenic fungus that gradually weakens both coffee seedlings and adult plants, reducing the quality of the berries. Without commercial products in the market for controlling this disease, the prospection of different biocontrol agents is a viable option. The *Phialomyces macrosporus* fungus stood out as a preventive control treatment for *Colletotrichum* in coffee seedlings, however, its mode of action has not yet been investigated. We aimed at evaluating the mode of action of *P. macrosporus* as a biocontrol agent in coffee seedlings infested with *Colletotrichum* sp. The application of *P. macrosporus* done seven days before the inoculation of *Colletotrichum* sp. reduces disease severity in 32-41%. The application of *P. macrosporus* on the leaves of the coffee seedlings, with and without using moist chamber, increased the permeability and rupture of the cuticle. The disturbance caused by *P. macrosporus* in the cuticle was followed by increased activities of PAL, CAT and POX, as well as the buildup in total phenol content and deposition of lignin. No evidence of mycoparasitism was observed either *in vivo* or *in vitro*. Due to the saprophytic nature of *P. macrosporus*, a decrease in the sporulation of the pathogen was observed 21 days after the application on the necrotic lesion. Nutrient competition stood out as another plausible mode of action, since both fungi presented similar niche overlap index for the use of carbon and nitrogen sources. Therefore, the saprophytic fungus *Phialomyces macrosporus* interfered with *Colletotrichum* sp. infection and survival on coffee seedlings by means of a combination of induced resistance and nutrient competition.

Keywords: *Coffea arabica* L.. blister spot. ISR. PR. Antioxidants. phenylpropanoids.

RESUMO

Colletotrichum sp. é um fungo fitopatogênico que, gradualmente, fragiliza as mudas de café, plantas adultas e qualidade do fruto. Sem um produto comercial no mercado, para controle desta doença, a bioprospecção de diferentes agentes de biocontrole é uma opção viável. O fungo *Phialomyces macrosporus*, como tratamento preventivo, destacou-se no controle de *Colletotrichum* em mudas de café, entretanto os mecanismos de ação não foram estudados. O objetivo deste trabalho foi avaliar os mecanismo de ação de *P. macrosporus* como agente de biocontrole em mudas de café infestadas com *Colletotrichum* sp. A aplicação de *P. macrosporus*, sete dias antes da inoculação de *Colletotrichum* sp., reduziu em 32-42% a severidade da doença. A aplicação de *P. macrosporus* nas folhas das mudas de café com e sem câmara úmida aumentou a permeabilidade e ruptura da cutícula. A perturbação provocada por *P. macrosporus* na cutícula foi acompanhada pelo aumento na atividade de FAL, CAT e POX e o acúmulo de fenóis totais e deposição lignina. Não há provas de microparasitismo *in vitro* ou *in vivo*. Em razão da natureza saprofítica de *P. macrosporus*, 21 dias depois da aplicação no tecido necrosado, foram observados conídios de *P. macrosporus* e redução de produção de conídios de *Colletortichum*. Competição por nutriente foi outro mecanismo de ação estudado, ambos os fungos demonstram similaridade ecológica na utilização de fontes de carbono e nitrogênio. Portanto, o fungo saprofítico *Phialomyces macrosporus* controla *Colletotrichum* sp. em mudas de café s de uma combinação de indução de resistência e competição por nutrientes.

Palavras-chave: *Coffea arabica* L.. Mancha manteigosa. ISR. PR. Antioxidantes. fenilpropanoides.

LISTA DE FIGURAS

APÊNDICES

LISTA DE ABREVIATURAS

AUDPC	Área Abaixo da Curva do Progresso
Bion	Acibenzolar-S-methyl
CAT	Catalase
CBD	Coffee Berry Disease
CC	Cenoura e Milho
CCA	Cenoura, Milho e Agar
Col	*Colletotrichum*
EtOH	Etanol
Inj.	Ferimento
MEA	Malta Extrato Agar
NOI	Niche Overlapping Index
PAL	Fenilalanine amonia-lyase
Phm	*Phialomyces macrosporus*
POX	Peroxidase Guaiacol
PVP	Polyvinylpyrrolidone
SEM	Microscopia Eletrônica de Varredura
sp.	Espécie
subsp.	Subespécies

LISTA DE SÍMBOLOS

ºC	Graus Celcius
kg	Quilograma
min	Minuto
mL	Mililitro
mM	Milimolar
nm	Nanometro
rpm	Revoluções por minuto
µL	Microlitro

SUMÁRIO

1 INTRODUCTION

Coffee (*Coffea arabica* L.) is the main international agriculture trade product for several countries in Latin America, Africa and Asia (DAMATTA, 2004). The world coffee production for 2014/2015 was 149 millions of bags (60 kg), whereas European Union, United State and Japan import 60% of the coffee production. The coffee represents to Brazil a highly important commodity for the economy, because is the first producer of coffee and the third consumer in the world (COMPANHIA NACIONAL DE ABASTECIMENTO, 2015). However, the last three years had observed a decrease of 4.8 million of bags (60 kg) in the production (COMPANHIA NACIONAL DE ABASTECIMENTO, 2015). The biennial nature of coffee trees combined with abiotic and biotic factors can explain the decrease in the production (COMPANHIA NACIONAL DE ABASTECIMENTO, 2015). Several biotic factors had been described affecting the production in the coffee, standing out the halo blight (*Pseudomonas syringae* pv. *garcae*), coffee rust (*Hemileia vastatrix* Berk & Br.), brown eye spot (*Cercospora coffeicola* Berkeley & Cooke), phoma leaf spot (*Phoma tarda* (R.B. Stewart) H. Verm) and anthracnose (*Colletotrichum* sp. Complex).

The phytopathogenic fungus *Colletotrichum* in coffee has a widely distribution in the area of coffee production in a diverse set of diseases such as anthracnose, dieback, coffee berry disease (CBD) and blister spot. Several species of *Colletotrichum* are reported affecting coffee plant (ALVES; CASTRO, 1998; PRIHASTUTI et al., 2009; WALLER et al., 1993). Among the species associated with coffee, damage caused can vary by the specie affecting the plant (WALLER et al., 1993), climatic condition (CHEN et al., 2003) and the cultivar affected (SERA et al., 2005) and infection causes diverse symptoms such as anthracnose, dieback, coffee berry disease (CBD) and blister spot. In Brazil, the principal specie of *Colletotrichum* affecting the coffee plant is a

Colletotrichum complex. The incidence in field are significantly low nevertheless they can cause an extreme deterioration in plant production and some case a total lost of the plant crop (PASIN; ALMEDA; ABREU, 2009).

Different alternatives have been used in the crop, the application of abiotic elicitor (chemical compound) and/or biotic agents (microorganism). Among the different abiotic elicitor we have Acibenzolar-S-methyl (Bion) this product is used as a chemical inducer enabling the plant protection against different pathogen such as fungal, virus and bacteria infections (BONALDO; PASCHOLATI; ROMEIRO, 2005) and has demonstrated that in coffee seedlings increase the activity of different pathogen-related protein (GUZZO; HARAKAVA; TSA, 2009).

In the past 10 years, the biological control on coffee has concentrated on using bacterial and fungal antagonists showing a promise alternative to minimize the application of chemicals compound (CARVALHO et al., 2012; HADDAD et al. 2009; HADDAD et al., 2014; HAILU; ALEMU, 2010; MULAW; KUBICEK; DRUZHININA, 2010; MULATO; MEGERSA; ALEMU, 2013;. SHIOMI et al., 2006). The applications of saprophytic fungal can interfere in the progress of the disease by nutrient competition and mycoparasitism (ANDREWS, 1992; DRUZHININA et al., 2011). In addition when the saprophytic fungal colonize the surface, segregate different metabolites such as cutinase for better attachment to the leaf surface triggering defense response of the plant against the pathogen (ESPINO-RAMMER et al., 2013; SINGH et al., 2014). These responses may include higher accumulation of defense enzyme and deposition of lignin and phenolic compound in the cell wall (SINGH et al., 2014; YACOUB et al., 2016). The objective of this work was to evaluate the mode of action of the fungus *Phialomyces macrosporus* in the biocontrol of coffee anthracnose caused by *Colletotrichum* sp.

2 LITERATURE REVIEW

2.1 Coffee seedling

The coffee belongs to the Family Rubiaceae, Genus *Coffea*, in which cluster more than 126 species of coffee (LASHERMES et al., 2011). Among the 126 species, *Coffea arabica* L. and *Coffea canephora* Pierre, stand out as the most cultivated in the areas of coffee production. Being *C. arabica*, the principal specie cultivated and with more variety and cultivars. Brazil is the main producer in the world of coffee, with 42.14 millions bags (60 kg) in the year 2014/2015. The production of coffee is distributed in 15 states: Acre, Bahia, Ceará, Espiruto Santo, Goiás, Distrito Federal, Mato Grosso do Sul, Minas Gerais, Pará, Paraná, Pernanbuco, Rio de Janeiro, Rondônia and São Paulo (MINISTÉRIO DA AGRICULTURA, 2012). Minas Gerais stand out as the highest coffee producer in Brazil, with 51.86% of the national production (COMPANHIA NACIONAL DE ABASTECIMENTO,2015).

The coffee represents to Brazil a highly important commodity for the economy, because is the first producer of coffee and the third consumer in the world, however in the last three years had observed an important decrease of 4.8 million bags (60 kg) in the production (COMPANHIA NACIONAL DE ABASTECIMENTO, 2015). The biennial of coffee combined with abiotic and biotic factors can explain the decrease in the production (COMPANHIA NACIONAL DE ABASTECIMENTO, 2015). Several biotic factors had been described affecting the production in the coffee, standing out the halo blight (*Pseudomonas syringae* pv. *garcae*), coffee rust (*Hemileia vastatrix* Berk & Br.), brown eye spot (*Cercospora coffeicola* Berkeley & Cooke), Phoma leaf spot (*Phoma tarda* (R.B. Stewart) H. Verm) and anthracnose (*Colletotrichum* sp. Complex).

2.2 Genus *Colletotrichum*

The genus *Colletotrichum* Corda is among the most widely foliar endophyte, saprophytic or plant pathogen fungus (BAILEY; JEGER, 1992; KURMAR; HYDE, 2004; PHOTITA et al., 2001; PHOTITA et al., 2004). The fungus present acervuli, within the acervuli present setae, conidia are smooth-walled, hyaline and cylindrical with obtuse end (SUTTON, 1992). Pathogenic species of *Colletotrichum* present a hemibiotrophic strategy; first act as a biotrophic fungus penetrating the tissues and colonizing one cell. After the infection present a destructive necrotrophyc phase (CANNON; BRIDGE; MONTE, 2000; HYDE et al., 2009; O'CONNELL et al., 2004; SHEN; GOODWIN; HSIANG, 2001).

2.2.1 *Colletotrichum* in coffee

The genus *Colletotrichum* in the coffee seedling is widely distributed in all the areas of coffee production (PARESQUI et al., 2003). Several species of *Colletotrichum* had reported affecting coffee seedlings (ALVES; CASTRO, 1998; PRIHASTUTI et al., 2009; WALLER et al., 1993). Among that species associated to coffee, the damage caused can vary by the specie affecting the plant (WALLER et al., 1993), climatic condition (CHEN et al., 2003) and cultivar affected (SERA et al., 2005) and cause diverse set of symptoms such as anthracnose, dieback, coffee berry disease (CBD) and blister spot (ALVES; CASTRO, 1998; DORIZZOTO, 1993; FREITAS et al., 2011; NECHET, 1999; WALLER, 1993), affecting all the coffee tissue: leaf, fruit, flower and branches.

In 1901, Noack describe the first *Colletotrichum* affecting coffee leaves in Brazil denominated as *C. coffeanum*. In Kenya, was described a different *Colletotrichum* affecting the coffee berries denominated "virulans"

(MCDONALD, 1926) or "a form of *C. coffeanum*" (NUTMAN; ROBERTS, 1960) which was associated to CBD. Waller et al. (1993), with a series of subtract utilization and pathogenic test distinguish that the casual agent affecting the coffee berry disease was *C. kahawae*. In recent years, was found in New Zealand, Brazil and Australia a *C. kahawae* affecting different plants. However, with pathogenic test have shown that the *C. kahawae* from New Zealand, Brazil and Australia was not able to cause CBD (SILVA et al., 2006) and with GS gene sequence was able to distinguish the two subspecies within *C. kahawae* (WEIR; JOHNSTON; DAMM, 2012). The isolate not able to infect the coffee berry was referred as *C. kahawae* subsp. *cigarro* and isolate able to infect the coffee berry was referred as *C. kahawae* subsp. *kahawae* (WEIR; JOHNSTON; DAMM, 2012).

The disease most studied so far in Brazil is the blister spot, caused by a *Colletotrichum* sp. complex. The first report of blister spot was in Costa Rica, the disease was considered as a virus (WELLMAN, 1957). In 1958, Bintacourt made the first report of blister spot in Brazil affecting leaves of *C. arbica* L, later reports, affecting *C. canephora* (MANSK; MATIELLO, 1977). Vargas and Gonzales (1972) scarified the agent that causes the blister spot was *Colletotrichum* and not a virus. Orozco (2003), with a series of biochemical and phylogenetic assays determinate that *Colletotrichum gloeosporioides* was the pathogen involved in the blister spot disease. In 2011, Freitas and collaborators determinate a different *Colletotrichum* affecting the coffee from Espirito Santa and Bahia, with the phylogenetic study *C. boninense* was associated with the dieback.

2.2.2 Blister spot

The symptoms of blister spot in coffee occurs in new leaves, with the emergence of green-yellowish circular spots with an oily aspect, the diameter of the spots are between the 2-10 mm. In severs attacks, the spots collapse and the plant have premature loss of the leaves (BITANCOURT, 1958; VARGAS; GONZÁLES, 1972; WELLMAN, 1957).

The dissemination of *Colletotrichum gloeosporioides* in the plantation of coffee is by infested seed; apparently in healthy coffee plantations appear small foci of plants with blister spot (CARVALHO et al., 2012; FERREIRA et al., 2009; OROZCO, 2003). Orozco (2003), planted seeds of coffee that present the symptoms of blister spot, insolating the fungus from the seedlings. The seeds affected by *Colletotrichum gloeosporioides* compromise the germination and development of the seedling and the intensity of *Colletotrichum* in the coffee seed and the seedlings will depend in the coffee variety affecting (CARVALHO et al., 2012).

2.3 Biological control

Biological control is defined as a reduction of the inoculum density or determining activity of disease caused by a pathogen or parasite in their activity or dormancy states, by one or more organism conducted naturally or through manipulation of environment, host or antagonist, or the massive introduction or more antagonists (BAKER; COOK, 1974). To succeed the antagonist in the biocontrol of the pathogen they apply one or more mode of action (LO et al., 1998), which may result in inducing plant resistance, nutrient competition and mycoparasitism (ANDREWS, 1992; LO et al., 1998; LORITO et al., 1994;

OLMEDO; CASAS-FLORES, 2014; SCHWAN-ESTRADA; STANGARLIN; PASCHOLATI, 2008).

2.3.1 Mode of action

Induced resistance is a mode of action in which the plant when exposed to an abiotic or biotic elicitor activates an arsenal of plant defense responses that cope with pathogen infection (RESENDE et al., 2004). This mechanism encompasses phenol compounds, tissue lignification and pathogen-related proteins (REGLINSKI et al., 1997, SCHWAN-ESTRADA; STANGARLIN; PASCHOLATI, 2008). Among the different abiotic elicitor we have Acibenzolar-S-methyl this product is used as a chemical inducer enabling the plant protection against different pathogen such as fungal, virus and bacteria infections (BONALDO; PASCHOLATI; ROMEIRO, 2005) and had been demonstrated that in coffee seedlings increase the activity of different pathogen-related protein (GUZZO; HARAKAVA; TSAI, 2009). The biotic agents and their products have received a particular attention as potential to biocontrol the pathogen and secrete different enzyme and secondary metabolites that act as elicitor to plant defense, promoting the expression of pathogen-related proteins of the plant before the attack of the pathogen increase the plant immune responses (DJONOVIĆ et al., 2003; DRUZHININA et al., 2011; HJELJORD; STENSVAND; TRONSMO, 2000; VINALE et al., 2008; YEDIDIA et al., 2003).

Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) is the enzyme most intensively studied in plants due to the importance of phenolic compounds and lignin formation (SCHWAN-ESTRADA; STANGARLIN; PASCHOLATI, 2008). This enzyme is responsible for the conversion of L-phenylalanine into trans-cinnamic acid. The trans-cinnamic acid can be incorporated into many

different phenolic compounds, which are present in the formation of esters, coumarins, flavonoids and lignins (ASANO et al., 2004).

The peroxidase (POX; EC 1.11.1.7) is an important protein that are related to the pathogenesis and which is generated from different genes. These proteins can oxidize phenolic compounds and increase the rate of polymerization of the lignin-like substances, which are deposited in the cell and appear to interfere with subsequent growth and development of the pathogen (AGRIOS, 2005). Also, they are involved in the detoxification of reactive oxygen species (ROS) and the redox various substrates using hydrogen peroxide (H_2O_2) (KAWAOKA et al., 2003).

The lignin in the cell wall provides mechanical support and performs protective functions in plants. The lignification can prevent fungal growth on plant tissues in several ways: mechanical barrier to the progress of establishment and development of the pathogen; modifying the cell wall, making it more resistant to the attack of hydrolytic enzymes; increased resistance of the walls to the diffusion toxins produced by pathogens and prevent the host nutrients to be used by the attacker (CAVALCANTI et al., 2005).

Maia (2012), Ogoshi (2011) and Santos Neto (2011), in their studies with coffee resistant genotype, revealed the activity of POX as possible resistance of coffee plant to *C. gloeosporioides*, however the activity of PAL was not activated during the experiment. Gichuru, Mwang'Ombe and Gupta (1997), in their biochemistry studies, revealed the activity of POX after the inoculation of *C. kahawae*. Durand et al. (2009), relate a low increase in the activity of PAL in CBD. The low activity of PAL found in CBD and *C. gloeosporioides* might not associated to the resistance to the pathosystem, however PAL is the precursor of phenol compounds and lignin formation an important mechanism of protection in resistant genotype to CBD (LOUREIRO et al., 2012).

Cutinase are serine esterases know as cutin hydrolysis was first discovered from phytopathogenic fungi to degrade the cuticle (PURDY; KOLATTUKUDY, 1973). This enzyme is used from different pathogenic fungus as a strategy to penetrate directly the plant (PIO; MACEDO, 2009). Recently, in the genus *Trichoderma* was revealed the capacity to produce cutinase, this capacity can be associated to the saprophytic lifestyle to decompose different subtract and for attachment to different hydrophobic surfaces (ESPINO-RAMMER et al., 2013) and this perturbation in the surface of the plant might triggered different immune response of the plant (L'HARIDON et al., 2011).

The mycoparasitism is a biotrophic interaction in which an organism benefits at expenses from other organism (DRUZHININA et al., 2011). This property is commonly within the fungal family *Hypocreaceae* (DRUZHININA et al., 2011). They display complex interactions that involve such steps as host recognition, attachment to the host, coiling around the host hyphae and secretion of different metabolites and enzymes for direct consumption of nutrients from the host hyphae (HARMAN et al., 2004; KUBICEK et al., 2011; OLMEDO; CASAS-FLORES, 2014). The capacity of mycoparasitism was evaluated with different test of antagonism, antagonist hyphae coiling around the host and evidence of death host hyphae (ZHANG et al., 2015)

Competition is considered a case of indirect interaction of the pathogen with the biocontrol agent where either of them delete source of nutrients or physical space (LORITO et al., 1994). The nutrient competition plays an important role in suppression of diseases and may occur when the biocontrol agent absorb any sustenance on the surface limiting pathogen growth (ANDREWS, 1992). Generally, the biocontrol has greater ability to obtain or use substances deposited in the surface of the plant than pathogen (NELSON, 1990). The nutrient competition is evaluated with microplates BIOLOG-FF that

containing 95 wells, each well contain a different carbon and nitrogen source (CHUN; SCHNEIDER; CHUNG, 2003; JANISIEWIES; USALL; BORS, 1992; KHALIL; ALISANIUS, 2009; SINGH, 2009).

2.4 The genus *Phialomyces*

The genus *Phialomyces* Misra & Talbot has four described species: *P. striatus, P. fusiform, P. microsporus,* and *P. macrosporus* (DELGADO-RODRIGUEZ; DECOCK, 2003; MISRA; TALBOT, 1964; ZHANG; ZHANG; GENG, 2010). The fungus is found in different soils around the world as saprophytic and endophytic in seed (BEZERRA et al., 2012; DUNN; BAKER, 1984; GOULART; PAIVA, 1992; MATEUS et al., 2001; PEREA, 2013; TOKURNASU; AOIKE, 2002; WAHEGAONKAR et al., 2011).

P. macrosporus is a Ascomycete, characterized by hyaline hyphae and septate; phialides hyaline, subcylindrical with a median constriction, narrowed towards the base, abruptly attenuated, short neck and relatively large at the apex-shaped bottle, usually monoverticillate but sometimes biverticillate; conidia aseptate usually subglobose to slightly ellipsoid, black and conidia formed chain up to 26 spores. The colony has a moderate growth and color presented is an olive-greenish to black (MISRA; TALBOT, 1964).

The mechanism involved by *P. macrosporus* in the biocontrol of phytopathogens are production of volatile, induce of resistance, and growth promotion under stress condition (BOTREL, 2013; OLIVEIRA et al., 2013; PIEROZZI, 2013; PINTO, 2013). However, to control the blister spot, only direct antibiosis and volatile was proven, the other mechanisms involved in control of the disease have yet to be validated (PINTO, 2013).

3 MATERIALS AND METHODS

3.1 Plant production

Coffee seeds (variety "Caturra IAC-99") were peeled off, disinfested and soaked in sterile distilled water (3 days). White sand was used for period germination. The seedlings of two months were transplanted into plastic bag of 250 x 90 mm with a mixture of soil, white sand and cattle manure (2:1:0.5). The seedlings were maintained in the greenhouse throughout the duration of the experiment.

3.2 Preparation and production of inoculum

The strain of *P. macrosporus* was obtained from the Microorganisms Culture Collection of Bahia (CCMB) – the accession number CCMB 0053/07 – University State of Feria de Santana, BA, Brazil. The strain was cultivated in CCA (Carrot 30 g, Corn 30 g, Agar 20 g in one liter of distilled water) at 25ºC. After 10 days of incubation, was removed two disks (50 mm) containing conidia and mycelium and added in an Erlenmeyer flask containing 100 mL of liquid CC (Carrot 30 g, Corn 30 gr in one liter of distilled water), maintained for 10 d in agitation (120 rpm at 25 – 27ºC), the final content was triturated and adjusted to 1 x 10^6 conidia mL^1.

The strain of *Colletotrichum* sp. was obtained from the collection from the Laboratory of Diagnose and Disease Control – the accession number I-24 – Federal University of Lavras, MG, Brazil. The strain was cultivated in MEA (Malt Extract 20 g, Agar 20 g in one liter of distilled water) at 25ºC. After 8 days of incubation, the mycelium and conidia was collected with distilled water and adjusted to 2 x 10^6 conidia mL^1.

3.3 Test of cuticle permeability

The extraction and quantification of chlorophyll was performed according the protocol of Sieber et al. (2000). The leaves of coffee were cut at the petiole, weighed and immersed in 30 mL of ethanol at 80%. Chlorophyll was extracted in dark at room temperature with gentle agitation (90 rpm). Aliquots were removed at 2, 5, 10, 20, 30 and 40 min after immersion of ethanol. The chlorophyll content was determined by measuring absorbance at 647 and 660 nm and the mM concentration of total chlorophyll per gram of fresh weight of tissue was calculated with the equation: $(7.93x(A_{660}\ nm) + (19.53x(A_{647}\ nm))\ g^{-1}$. The toluidine blue and calcoflour staining test was performed according the protocol of L'Haridon et al. (2011). The toluidine blue test was carried out by placing 6 mL of 0.025% toluidine blue solution on the leaf surface, was incubated in high humidity for 2 h. The leaves were washed gently with distilled water to remove the excess of the toluidine blue solution. The staining with calcoflour white, the leaves were bleached in absolute ethanol overnight, equilibrated in 0.2 M $NaPO_4$ (pH 9) for 1 h, and incubated for 10 min in 0.5% of calcoflour white in 0.2 M $NaPO_4$ (pH 9). Leaves were rinsed in $NaPO_4$ buffer to remove excess of calcoflour white and viewed under UV light.

3.4 Mode of application, control of the disease and sampling for biochemical analysis

The suspension of *P. macrosporus* and Acibenzolar-S-methyl were sprayed seven day before the inoculation of *Colletotrichum* sp. The inoculation of *Colletotrichum* sp. was performed according the protocol of Maia et al. (2013), placing a droplet of 10 µL suspension of conidia (2×10^6 conidia mL^{-1}) on two different area of the abaxial side of leaf and on the area of inoculation

was place a disk of moistened filter paper (1.3 cm of diameter) to simulate a moist micro-chamber. The inoculation was made in the fifth pair of leaves.

The efficiency from *P. macrosporus* and Acibenzolar-S-methyl was evaluated through the quantification of the disease severity over the time (5, 10, 15 and 20 days after the inoculation of I-22). The severity was determinate by the scale modify of Maia et al. (2013), where 0= absence of necrosis, 1= small necrotic lesion, 2= more than two necrotic lesion, the lesion diameter exceed 0.5 mm, 3= extensive brownish lesions with numerous black or dark lesions points, The lesion diameter exceed more than 50% of inoculated area, 4= The inoculated area completely necrotic. The data obtained from the severity was calculated the area under the disease progress curve (AUDPC) by equation of Shaner and Finney (1977).

The sampling for biochemical analysis, control and the leaves treated were collected at 0, 24, 48 and 96 h. The leaves were cut at the petiole, immersed in liquid nitrogen and stored in deep freezer (-80ºC) until assessment of defenses enzymes analysis at later date.

3.5 Assessment of defenses enzymes and total phenols compounds and lignin

For the defenses enzymes assay, approximately 900 mg of samplings were ground to a fine powder, immediately for each enzyme was collected nearly 200 mg of sampling and store at -80ºC until enzyme extraction.

For the total phenols compounds and lignin assay, approximately 200 mg of leaves sample were ground to a fine and store at -80ºC until phenols compounds and lignin extraction. The samples were lyophilized for 76 hours.

3.5.1 Defenses enzymes

Approximately 900 mg of leaves sample were macerated in liquid nitrogen for the assessment of defenses enzymes. Approximately 200 mg of leaf sample was transferred to Eppendolf tube. For extraction of Phenylalanine ammonia-lyase (PAL) was performed according the protocol of Mori, Sakuraiand Sakuta (2001). Placing 700 µL of extraction buffer (50 mM of $NaPO_4$ [pH 6.5], 1 mM PMSF and 1% PVP) in the sample and centrifuged at 14000 g for 25 min at 4ºC. The supernatant was used for assessment, 5 µL of enzymatic extract was mixture with 145 µL Tris-HCl [pH 8.8], 50 µL of 40 mM phenylalanine and incubated for 20 min with lecture of 2 in 2 min at 37ºC. The PAL content was determined by measuring absorbance at 280 nm and expressed as Δ 280 nm mgP^{-1} Min^{-1}. For activity of Peroxidase was placed 1200 µL the extraction buffer (400 mM of KH_2PO_4 [pH 7.8], 10 mM of EDTA, 200 mM of ascorbic acid and distilled water) in the sample and centrifuged at 14000 g for 25 min at 4ºC. For Peroxidase of guaiacol (POX), quantification was performed according the protocol of Urbanek, Kuzniak-Gebarowska and Herka (1991). The supernatant was used for assessment, 10 µL of enzymatic extract was mixed to 100 µL of 100 mM KH_2PO_4 [pH 7.0], 45 µL of 50 mM guaiacol and 45 µL of 125 mM H_2O_2 and incubated for 10 min with lecture of 1 in 1 min at 30ºC. The POX content was determined by measuring absorbance at 480 nm and expressed as Δ 480 nm mgP^{-1} Min^{-1}. For activity of Catalase (CAT), quantification was performed according the protocol of Havir and McHale (1987). The supernatant was used for assessment, 10 µL of enzymatic extract was mixture with 100 µL of 200 mM of KPO_4 (pH 7), 250 mM H_2O_2 and 80 µL distilled water and incubated by 3 min 1 in 1 min at 25ºC. The CAT content was determined by measuring the rate of decrease in absorbance at 240 nm and the molar extinction coefficient of 18 mM^{-1} cm^{-1}.

3.5.2 Total phenols and lignin

Approximately 200 mg of leaves sample were macerated in liquid nitrogen for the assessment of total phenols and lignin. The samples were lyophilized for 76 hours. Approximately 30 mg of the lyophilized samples were transferred to Eppendolf tube of 2 mL, homogenized with 1.5 mL of methanol at 80%, agitated for 15 h in dark and centrifuged at 12,000 rpm for 5 min. The supernatant was used for total phenols and the solid residue was used for lignin.

For activity of lignin quantification was performed according the protocol of Doster and Bostock (1988). The solid residue was added 1.5 mL of methanol at 80% agitated to homogenized, centrifuged at 12,000 rpm for 5 min, the supernatant was discarded and the solid residue was dry for 48 h at room temperature. The samples were added 1.5 mL of tioglicolic acid and chloridic acid (HCl) 2M (portion 1:10), agitated gently and placed in water at 90ºC for 4 h, centrifuged at 10,000 rpm for 10 min, the supernatant was discarded and used the solid residue. The solid residue was added 1.5 mL of distilled and deionized water, centrifuged at 10,000 rpm for 10 min, the supernatant was discarded and the solid residue was resuspended with 1.5 mL of hydroxide of sodium (NaOH) 0.5 M and maintained in agitation for 15 h at dark. The samples were centrifuged at 10,000 rpm for 10 min, the supernatant was transferred to a new Eppendolf tube of 2 mL and added 200 μL of HCl concentrated and maintained for 4 h at 4ºC. the samples were centrifuged at 10,000 rpm for 10 min, the supernatant was discarded and the solid residue resuspended in 2 mL of NaOH 0.5 M. The lignin content was determined by measuring absorbance at 280 nm and expressed as μg of soluble lignin of milligram of dry mass.

For activity of soluble total phenol quantification was performed according the protocol of Spanos and Wrolstad (1990). A 150 μL supernatant of methanol was blend with 150 μL of Folin-Ciocalteau 0.25 N for 5 min,

homogenized with 150 µL of Na_2CO_3 1 M for 10 min and diluted with distilled and deionized water for 1 h at room temperature. The total phenol content was determined by measuring absorbance at 725 nm and expressed as µg of chlorogenic acid of milligram of dry mass.

3.6 Carbon source utilization

The carbon source utilization between *P. macrosporus* and *Colletotrichum* sp. was performed according the protocol of Kraus et al. (2004). Both strains were inoculated on 2% malt extract agar plates and incubated 10 days at 25ºC or until sufficient conidiation for inoculation on the Biolog microplates. The conidia of *P. macrosporus* and *Colletotrichum* sp. were collected spreading distilled water over the mycelium. The concentration used to inoculate on the microplates was $1x10^6$ conidia ml^{-1}, one hundred µL of suspension was inoculated into each 96 wells of the Biolog FF MicroPlate ™ (Biolog Inc., Hayward, California). Microplates were incubated at 25ºC, after 72 h the absorbance was read at 490 nm (mitochondrial activity) and 750 nm (mycelium growth) using microplate reader. The absorbance data of 490 nm and 750 nm were analyzed separated.

A niche overlapping index (NOI) was calculated for the assay based on the number of carbon and nitrogen sources utilized by *P. macrosporus* that were also utilized by *Colletotrichum* sp., divided by the total number of nutrient sources utilized by *Colletotrichum* sp. (THORNTON; SAVELLE; SCHERM, 2008).

3.7 Microbial antagonism assay

The microbial antagonism *in vitro* between *P. macrosporus* and *Colletotrichum* sp. was performed according the protocol of Larralde et al. (2008). Both strains were inoculated on the side of the plate in 2% malt extract agar, incubated for 7, 14 and 21 d at 25ºC with 12 h light and determinate the antagonistic capacity by the scale of Bell, Wells and Markham (1982), where 1= biocontrol completely covers the surface of the medium where the pathogen is growing, 2= biocontrol covers two thirds the surface of the medium where the pathogen is growing, 3= biocontrol and the pathogen each colonize about half of the surface and none of them seems to dominate, 4= pathogen covers two thirds the surface of the medium where the biocontrol is growing, 5= pathogen completely covers the surface of the medium where the biocontrol is growing. When the mycelium of the fungus crossed over, were removed samples for optical microscope and scanning electron microscope (SEM) for evidence of mycoparasitism. For microbial antagonism *in vivo* between *P. macrosporus* and *Colletotrichum* sp., the inoculation of *Colletotrichum* sp was performed according the protocol of Maia et al. (2013) previously descripted. After the appearance of the first symptoms of necrosis on the leaf was placed a droplet of 10 µL suspension of conidia the *P. macrosporus* (1×10^6 conidia mL^1). After 7, 14 and 21 d of inoculated *P. macrosporus*, the production of conidiomata was evaluated on the necrotic area and recovery on the affected and healthy tissue. The Trypan blue staining was performed according the protocol of Zhang et al. (2015). In the confrontation plates were spread 15 ml of Trypan blue solution at 0.1%, with a glass rod the dye were spread to ensure homogenous access all the mycelia, after 10 min of incubation at room temperature the plates were rinsed with distilled water and photographed.

3.8 Statistical analysis

Values from different experiments shown in figures were submitted to one-way analysis of variance (ANOVA) and followed by Scott Knott test. Different letters above each bar represent statically significance differences ($P<0.05$).

4 RESULTS

4.1 Reduction of necrotic area

P. macrosporus and Acibenzolar-S-methyl protected the leaf from *Colletotrichum* sp. in both assessment (Fig. 1), reducing the severity of the assessment one in 32.8 and 11.4% and in assessment two in 40.4 and 18.1%, respectively compared to the control.

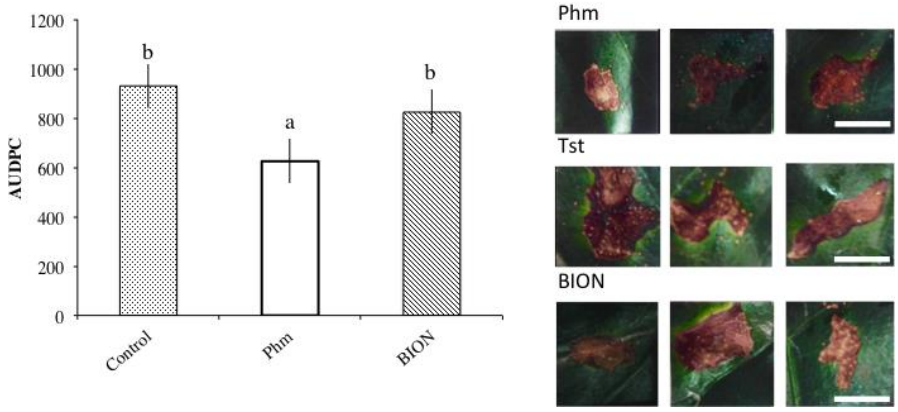

Figure 1 Effect of *P. macrosporus* on *Colletotrichum* sp. infected on coffee leaf. Scale bar: 50 mm. Mean followed by the same letter are statistically the same (Scott-Knock, P<0.05)

4.2 Cuticle permeability

The permeability of the cuticle increases with the application of *P. macrosporus* with and without moist chamber for 12 h, observing greater damage, measured by the release of chlorophyll (Fig. 2A), which did not happen in the moist chamber for 24 h, the damage was similar (Fig. 10). The higher release of chlorophyll was observed in the moist chamber for 12 h, consequently using the test of cell wall stain calcoflour and toluidine blue was observed higher

stain in that treatment (Fig. 2B-C), confirming that *P. macrosporus* increase the permeability and the rupture of the cuticle (Fig. 2D)

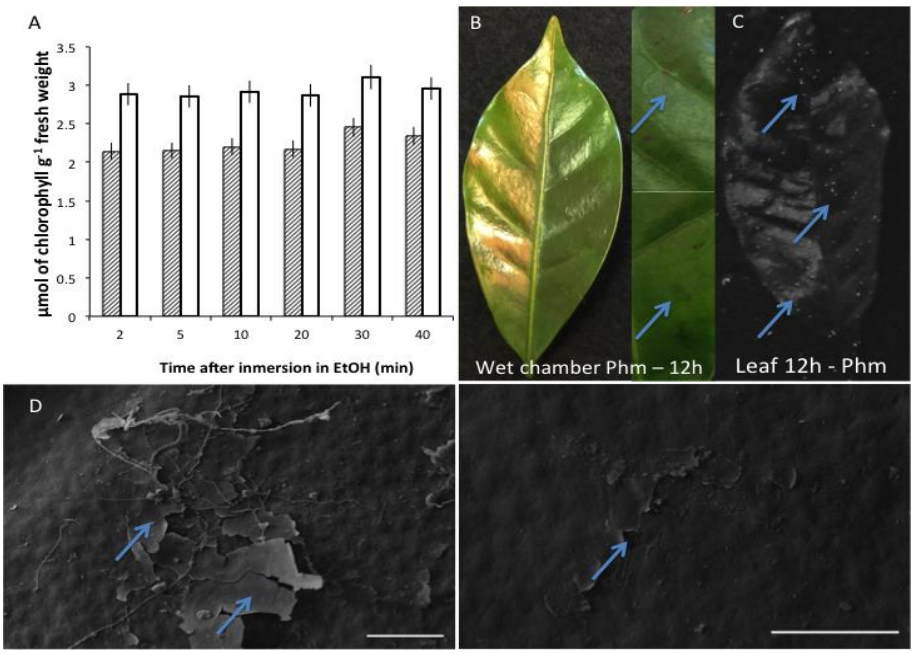

Figure 2 Cuticle permeability after *Phialomyces macrosporus* inoculation under 12 h of humid condition. (A) Chlorophyll extraction test (B) Calcoflour straining test. (C) Blue toluidine test. (D) Electro scanning picture from the cuticle. Bar scale = 100 µm

4.3 Effect of *P. macrosporus* on PAL activity

In the experiment of PAL activity, the treatments Phm and Bion stimulate the activity of PAL before the beginning of the experiment, which was 60 and 78% higher, respectively, than the control (Fig. 3E). The activity of PAL in the treatment Phm + Col, show a decrease in the level, still the levels of PAL remains higher compared to the treatment I-22 (Fig. 3A). For the activity of PAL in the treatment Bion + Col, there was a sharp decrease in the levels (Fig. 3B).

The expression of PAL in the treatment control + Inj, there was an increase in the levels of PAL not observed in the treatment control (Fig. 3C). The expression of PAL in the treatments Phm and Bion challenged and not challenged with Col, there was a decrease in the levels, stills Phm + Col and Phm present higher levels of PAL compared to Bion + Col (Fig. 3D).

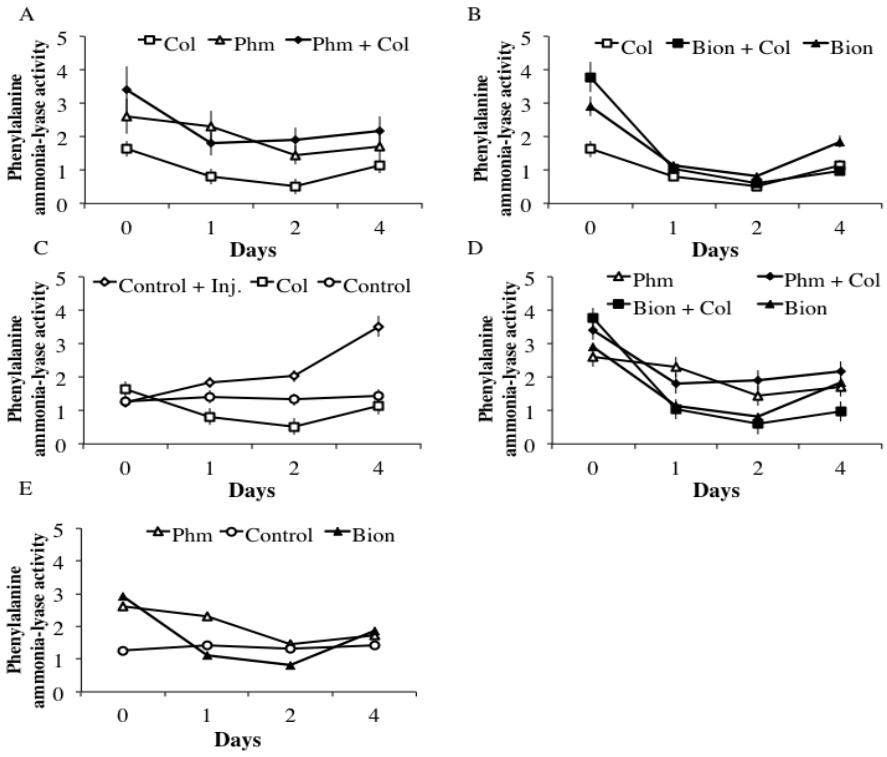

Figure 3 Phenylalanine ammonia-lyase activity in leaf of coffee seedling. Control + Inj.: Control with injury; Col: *Colletotrichum* sp. + injury; Phm: *Phialomyces macrosporus* without injury; Phm + Col: *Phialomyces macrosporus* challenged *Colletotrichum* sp. + injury; Bion: Acibenzolar-S-methyl without injury; Bion + Col: Acibenzolar-S-methyl without injury challenged *Colletotrichum* sp. + injury; Control: Control without injury

4.4 Effect of *P. macrosporus* on POX and CAT activities

In the experiments of POX activity, the treatments Phm and Bion did not stimulate the activity of POX before the beginning of the experiment (Fig. 4E). The activity of POX in the treatment Phm + Col, there was an increase in the levels first and second days after inoculation of I-22 (Fig. 4A). The activity of POX in the treatment Bion + Col, there was increase in the levels second and fourth days after inoculation of Col (Fig. 4B). The activity of POX in the treatment control + Inj, there was an increase in the levels of POX in the first day not observed in the treatment control (Fig. 4C). The expression of POX in the treatments Phm and Bion challenged and not challenged with Col, there was a sharp increase in the second days in the levels of Phm + Col not observed in the treatment Bion + Col (Fig. 4D).

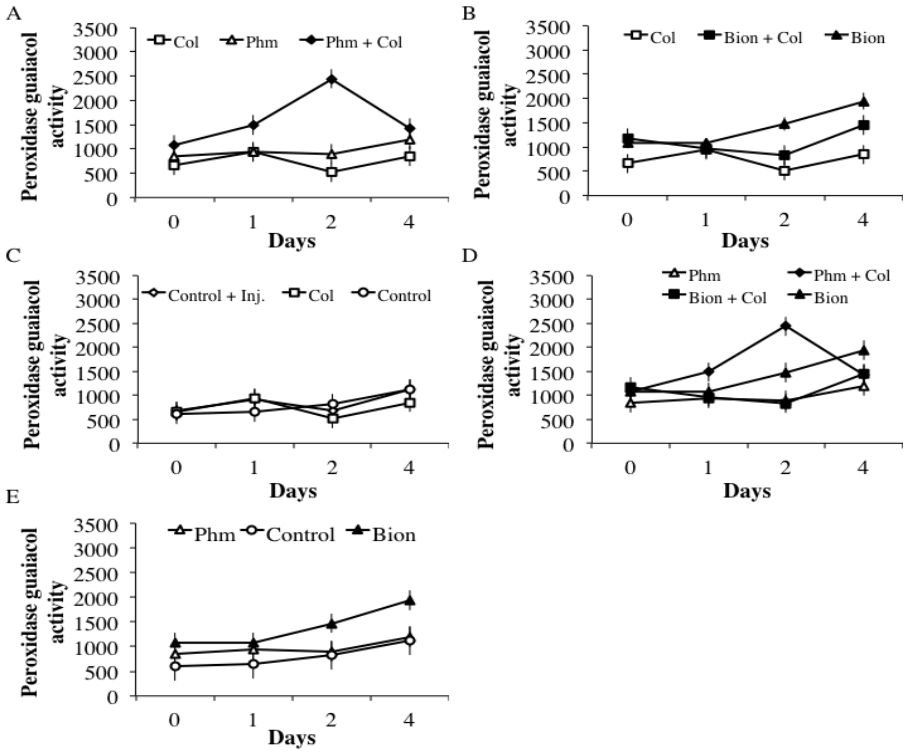

Figure 4 Peroxidase guaiacol activity in leaf of coffee seedling. Control + Inj.: Control with injury; Col: *Colletotrichum* sp. + injury; Phm: *Phialomyces macrosporus* without injury; Phm + Col: *Phialomyces macrosporus* challenged *Colletotrichum* sp. + injury; Bion: Acibenzolar-S-methyl without injury; Bion + Col: Acibenzolar-S-methyl without injury challenged *Colletotrichum* sp. + injury; Control: Control without injury

In the experiments of CAT activity, the treatments Phm and Bion did not stimulate the expression of CAT before the beginning of the experiment (Fig. 5E). The activity of CAT in the treatment Phm + Col increased in the second and fourth days compared to control Col (Fig. 5A). The activity of CAT in the treatment Bion + Col increased at the second and fourth days after inoculation of Col (Fig. 5B). The activity of CAT in the treatment control + Inj, there was an increase in CAT activity in the second and fourth days not observed in the

control treatment (Fig. 5C). The activity of CAT in the treatments Phm and Bion challenged or not with Col, there was a sharp increase at the second day for Phm + Col, which was not observed for Bion + Col (Fig. 5D).

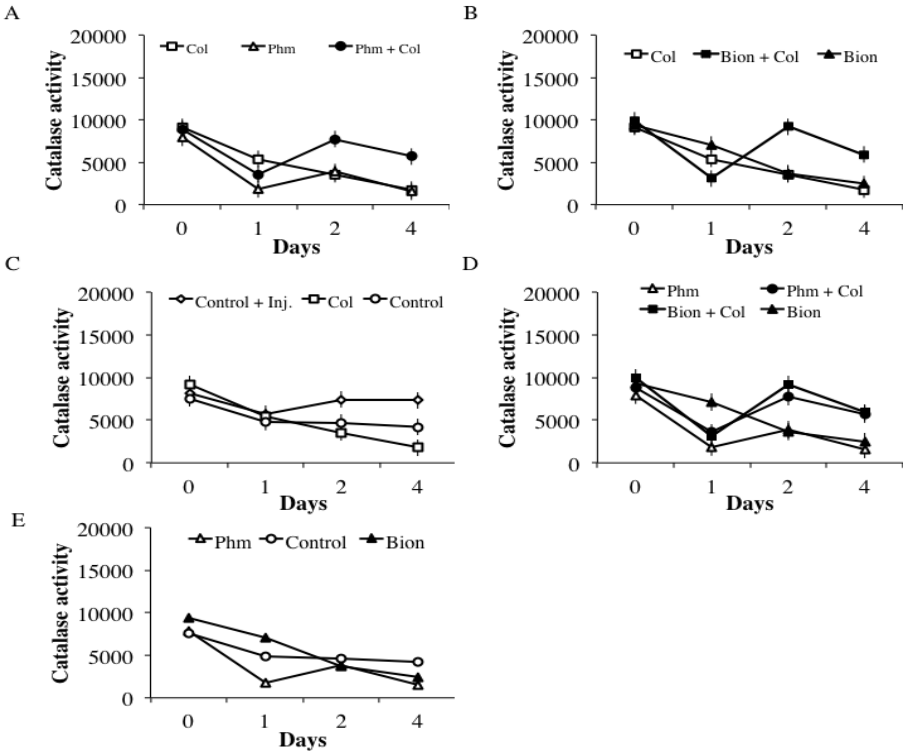

Figure 5 Catalase activity in leaf of coffee seedling. Control + Inj.: Control with injury; Col: *Colletotrichum* sp. + injury; Phm: *Phialomyces macrosporus* without injury; Phm + Col: *Phialomyces macrosporus* challenged *Colletotrichum* sp. + injury; Bion: Acibenzolar-S-methyl without injury; Bion + Col: Acibenzolar-S-methyl without injury challenged *Colletotrichum* sp. + injury; Control: Control without injury

4.5 Effect of Phm in the deposition of lignin

The treatments Bion and Phm did not increase the deposition of lignin before the experiment; still 96 hours after the inoculation of Col the deposition of lignin was the same for all the treatments (Fig. 6A-B). The deposition of lignin between hours, there was an increased deposition in the treatment Control + Inj., Phm and Phm + Col and a decrease in the treatment Col (Fig. 6C).

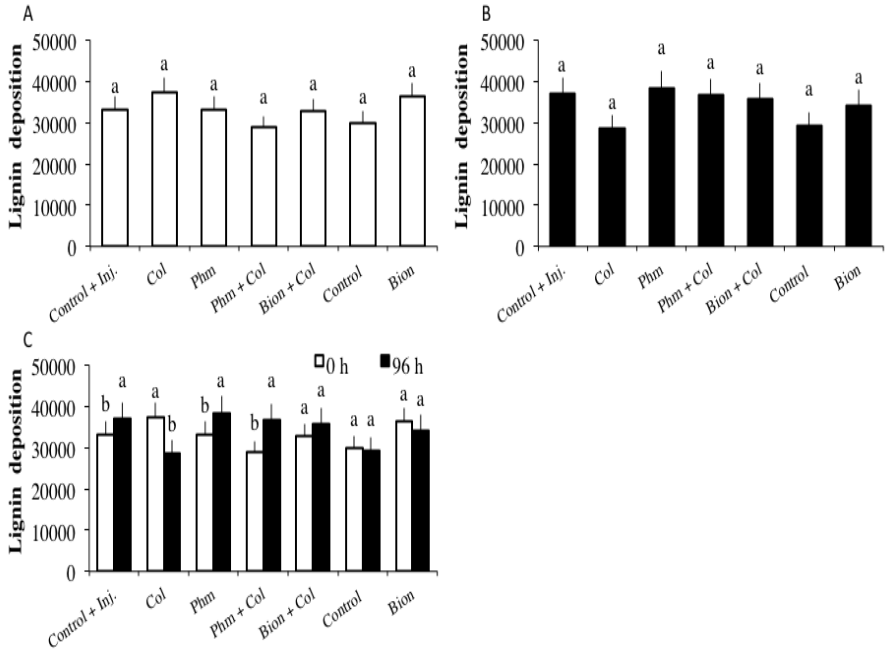

Figure 6 Effect of the treatments in the deposition of lignin on leaf of coffee seedling. (A) Lignin at 0 h. (B) Lignin at 96 h. (C) Comparison of Phenol content for both sampled time points. Control + Inj.: Control with injury; Col: *Colletotrichum* sp. + injury; Phm: *Phialomyces macrosporus* without injury; Phm + Col: *Phialomyces macrosporus* challenged *Colletotrichum* sp. + injury; Bion: Acibenzolar-S-methyl without injury; Bion + Col: Acibenzolar-S-methyl without injury challenged *Colletotrichum* sp. + injury; Control: Control without injury. Mean followed by the same letter are statistically the same (Scott-Knock, $P<0.05$)

4.6 Effect of Phm in the phenol content

The treatments Bion and Phm did not increase the levels of phenol content before the experiment, still 96 hours after the inoculation of Col the levels of phenol content was the same for all the treatments (Fig. 7A-B). The level of phenol content compare between hours, there was an increase of deposition in the treatment Control + Inj. and Phm + Col (Fig. 7C).

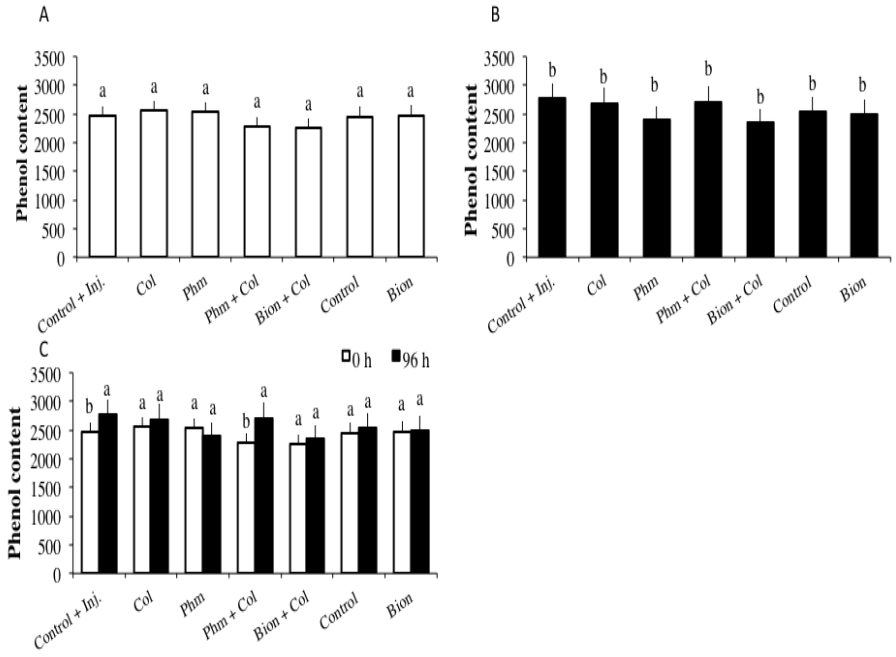

Figure 7 Effect of the treatments in the phenol content on of coffee leaves. (A) Phenol content at 0 h. (B) Phenol content at 96 h. (C) Comparison of Phenol content for both sampled time points. Control + Inj.: Control with injury; Col: *Colletotrichum* sp. + injury; Phm: *Phialomyces macrosporus* without injury; Phm + Col: *Phialomyces macrosporus* challenged *Colletotrichum* sp. + injury; Bion: Acibenzolar-S-methyl without injury; Bion + Col: Acibenzolar-S-methyl without injury challenged *Colletotrichum* sp. + injury; Control: Control without injury. Mean followed by the same letter are statistically the same (Scott-Knock, $P<0.05$)

4.7 Microbial antagonism assay

Measure of antagonism by *P. macrosporus* over *Colletotrichum* sp. was carried out during 21 days of incubation; during that time the mycelium of *Colletotrichum* sp. was not inhibited or colonized by *P. macrosporus* (Fig. 8A-C). Although, with the observation at compound microscope and SEM *in vivo* and *in vitro* was not observed an evidence of mycoparastism by *P. macrosporus* (Fig. 8 D-F). However, *in vivo* at 21 days of inoculation of *P. macrosporus* was found conidia over the necrosis and less conidia of *Colletrotichum* (Fig. 8 E). The recovery of *P. macrosporus* from the necrotic area was 45% and was only recovered from the necrotic area; this may be explained by nutrient competition (Table 1) and the saprophytic nature of *P. macrosporus.*

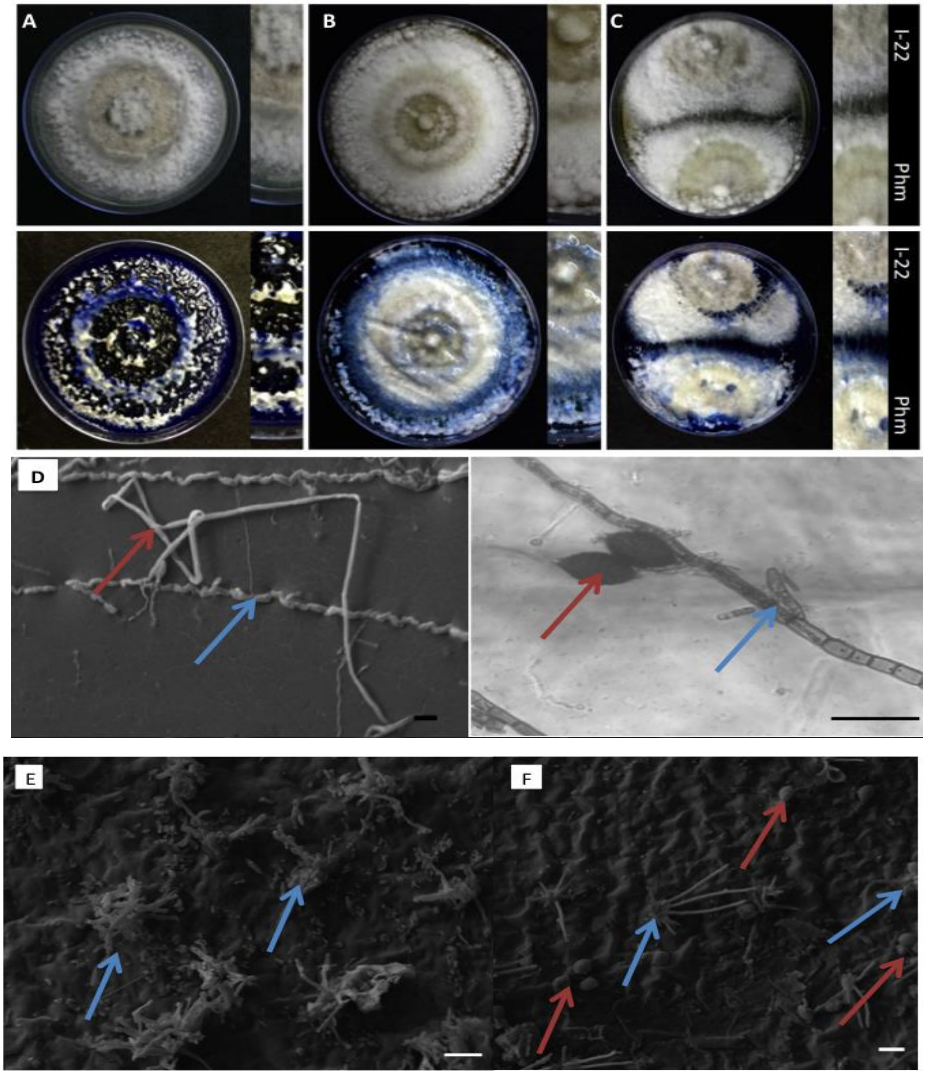

Figure 8 Antagonism test of *Phialomyces* and *Colletotrichum*. (A) *Colletotrichum*, (B) *Phialomyces*, (C) 21 days of antagonism, (D) Hyphae interaction of *Colletotrichum* and *Phialomyces macrosporus in vitro* assay. (E) Symptoms and signals in leaf by *Colletotrichum* vivo assay, (F) Interaction of *Colletotrichum* and *Phialomyces macrosporus in vivo* assay at 21 days after Phm inoculation. Red arrow indicates *Colletotrichum* and blue arrow indicates *Phialomyces*. Bar scale: 20 μm

4.8 Carbon and nitrogen source utilization

Colletotrichum sp. utilized the 95 carbons and nitrogen sources and grows in the 96 wells of the FF microplate, *P. macrosporus* also utilized the same nutrient sources and grows in the 96 wells. The niche-overlapping index (NOI) was 1 showing that *P. macrosporus* used the same amount of carbon sources than *Colletotrichum* sp. (Table 1). Analyzing the 96 wells individually the utilization of carbon and nitrogen source between fungal was found *Colletotrichum* used α-D-Lactose, Adonitol, Amydgalin, D-Arabionose, D-Lactic acid Methyl ester, D-Raffinose, Glycerol, L-Fucose, L-Rhamnose, LSorbose, Stachyose, Water and γ-Hydroxy-Butric acid and *Phialomyces* used 2-Keto-D-Gluconic acid, α-D-Glucose, Adenosime, D-Cellobiose, D-Glucuronic acid, D-Malic acid, D-Ribose, Fumaric acid, L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic acid, L-Phenylalanine, L-Pyroglutamic acid, L-Serine, Fumaric acid, L-Threonine, N-Acetyl-D-Mannosamine, Putrescine, Quinic acid, Succinamic acid, Succinic acid, Uridine and γ-Animo-Butyric acid (Fig. 9).

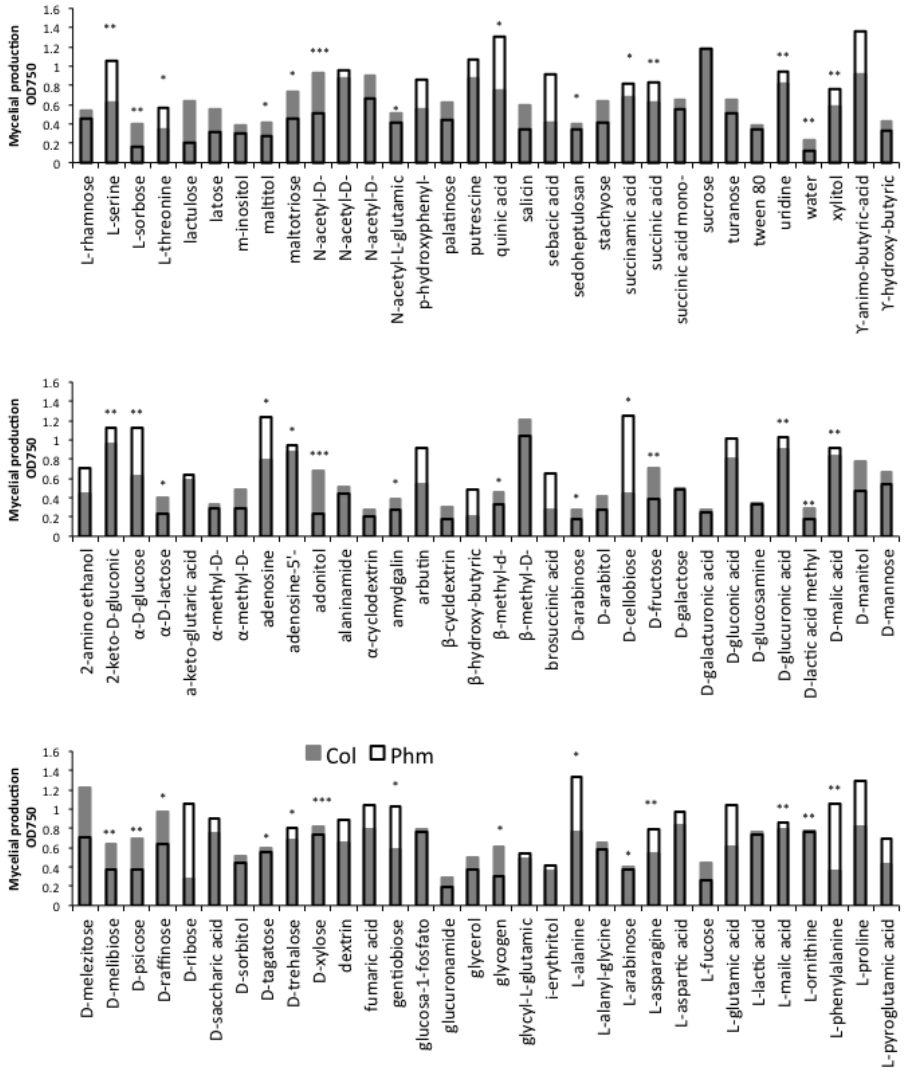

Figure 9 Carbon and nitrogen source profile of Phm against I-22 in biolog FF microplates after 72 h of incubation. * = P<0.05, ** = P<0.01, *** = P<0.001

5 DISCUSSION

The application of saprophytic fungus and their products to plants are potential biocontrol agents of plant diseases acting directly on the pathogen or triggering the plant immune responses (HJELJORD; STENSVAND; TRONSMO, 2000; YEDIDIA et al., 2003; DJONOVIĆ et al., 2003; ALFANO et al., 2007; SINGH et al., 2014; YACOUB et al., 2016). The saprophytic fungus secrete a number of metabolites that act as elicitors to promote plant defense, promoting the expression of defense-related enzymes prior to the pathogen attack (DRUZHININA et al., 2011; LORITO et al., 2010; VINALE et al., 2008). This enzymatic machinery is produced during the phylloplane or rhizosphere colonization, can be recognized by the plant host as molecular-associated microbial pattern (MAMP) but has major role in plant tisse penetration into the epidermis and outer part of the plant (CHET, 1999; LO et al., 1998; VINALE et al., 2008; YEDIDIA; BENHAMOU; YEDIDIA et al., 2000). However, to settle onto the upper or low epidermis the saprophytic fungus needs to find natural openings or penetrate through the cuticle. The cuticle was considered as a barrier against water loss, irradiation or pathogens (NAWRATH, 2006) nevertheless in recent study the cuticles can be considerate as important part of a sensing device recognizing the microbe-associated molecular patter (MAMPs) and damage-associated molecular patterns (DAMPs) signals (L'HARIDON et al., 2011). In the present investigation, was observed *P. macrosporus* increases the permeability of the cuticle (Fig. 2 A-D). This type of degradation and perturbation in the cuticle made by *P. macrosporus* is recognized as DAMPs and MAMPs elicitors and potentially activated the immune response measured by PAL (Fig. 3), POX (Fig. 4) and CAT (Fig. 5) in the plant (HENRY; THONART; ONGENA, 2012; NEWMAN et al., 2013).

The Phenylalanine ammonia-lyase precursor of Phenylpropanoid pathway is an important response in the plant defense against different pathogens and it is involved in the biosynthesis of Salicylic acid and different toxics compounds to the pathogen (CHAMAN; COPAJA; ARGANDONA, 2003; LA CAMERA et al., 2004). In the present study, the level of PAL shown by *P. macrosporus* prior the inoculation of *Colletotrichum* sp. was superior and sustained high levels after inoculation of *Colletotrichum* sp. (Fig 3 A, E). This result corroborated with previous reports on the application of biologicals prior the pathogen inoculation and sustained high PAL levels (DJONOVIĆ et al., 2003; GAJERA et al., 2015; MUTHUKUMAR; VENKATESH, 2014; SINGH et al., 2014). Another important plant defense enzyme is Peroxidase; it is involved in the biosynthesis of ethylene, lignification, stress response, salt tolerance and reactive oxygen species (ROS) detoxification (ALMAGRO et al., 2009; VAN CAMP et al., 2009). ROS play an important role in the plant defense against different pathogens. These molecules of ROS are highly toxic to the pathogen (LIU et al., 2010; MITTLER et al., 2004). The accumulation of ROS during the plant defense is biphasic. The first phase of ROS accumulation is associates with infection of plant by avirulent or virulent fungus with a rapid but weak accumulation and the second phase of ROS accumulation is associated only with the infection by avirulent fungi and is an induced response dependent on the increased transcription of mRNA encoding ROS-generating enzyme (MITTLER et al., 2004). The ROS accumulation in the plant can lead to the destruction of cell plant and the plants have a complex and efficient system to reduce oxidative burst induced by the pathogen. The antioxidant enzyme, such as POX and CAT are vital in this defense system and contributed to detoxification of ROS (SPYCHALLA; DESBOROUGH, 1990). Therefore, the importance of *P. macrosporus* increasing the activity of POX and CAT to scavenge the radical -OH and O_2 produced by the ROS accumulation in the

infection of *Colletotrichum* sp. at 24 and 48 h. This low activity of POX and CAT at 24 h can be associated to the biotrophic phase of *Colletotrichum* infecting one cell, after the biotrophic is concluded the destructive necrotrophic phase began with secondary hyphae colonizing the surrounding cell observing an intense activity at 48 h, at the same time was observed the beginning of the necrosis in the leaf and may explain the increment of the enzyme.

The cell wall is an important barrier against the pathogen colonization and is mainly consisted of cellulose, hemicellulose and pectins and some proteins (CARPITA; MCCANN, 2000; MIEDES et al., 2014). The cell wall acts as a passive barrier for the progression of the pathogen, after the degradation of the cell wall the plant will release an arsenal of antimicrobial compounds (VORWERK; SOMERVILLE; SOMERVILLE, 2004). This antimicrobial compounds are rapidly deposited in the cell walls as lignin, lignin-like and phenol compounds (SATTLER; FUNNELL-HARRIS, 2013). An important mechanism of plant defense regulated by the coffee when is attack by *C. kahawae* subsp. *kahawae* is the rapid deposition of lignin and phenol compounds (LOUREIRO et al., 2012; SILVA et al., 2006). In the study, no difference was found in the deposition of lignin and soluble phenol compounds between treatments, nevertheless the deposition of lignin and soluble phenol compounds in the treatment of Phm + I-22 in the 0 h to 96 h increased in 21 and 16%, respectively.

The invasion of the leaf tissues by *Colletotrichum* sp. might have resulted in the decreased activity of PAL, POX, CAT, phenol and lignin, whereas increased PAL by *P. macrosporus* before inoculation of *Colletotrichum* sp. and a later increase at 24 and 48 h after inoculation of POX and CAT and higher deposition of lignin and phenol in the cell wall might have prevented the progress of the disease in the tissue.

The phyllosphere is generally considered to be a nutrient limited environment (ANDREWS, 1992), to succeed in this adverse environment the fungi used different strategies such as hyperparastism and nutrient competition (ANDREWS, 1992). Hyperparasitism are classified in two groups. The first group compromise the biotrophic fungus, this kind of interaction is highly specific. The second group has a wide range of host the necrotrophic fungus, where this group is considered the most effective for biological control. The event of mycoparasitism is a complex process, which involves different events, the hyphae of the antagonism enter in contact with the fungal prey and coiling around succeed by a formation of an appressoium-like that penetrates the host hyphae (OLMEDO; CASAS-FLORES, 2014). In the study, was observed that *P. macrosporus* do not have a direct antagonism to *Colletotrichum* sp. (Fig. 8A-E) and no evidence of mycoparasitism during 21 days of evaluation. However, *in vivo* after 21 days after the inoculation of *P. macrosporus* over the necrosis of *Colletotrichum* sp. was observed conidia from the antagonism and a sharp decrease of the conidia of the pathogen. To understand the emergence of conidia from *P. macrosporus* over the necrosis of *Colletotrichum* sp. without a clear evidence of mycoparasitism, a competitive interaction between fungal was checked. The niche overlapping index present in the study (NOI=1) exhibit a high degree of ecological similarity indicating both fungal can compete for the same nutrient in the leaf surface. Furthermore, *in vitro* assays *Colletotrichum* sp. has a good sporulation in the presence of glucose, xylose, asparagine and proline (DESHMUKH et al., 2012; KUMARA; RAWAL, 2008; SANGEETHA; RAWAL, 2008). Although, these carbon and nitrogen sources are present in coffee grain and leaf in high concentration (CAVATTE et al., 2012; KY et al., 2001; ROGERS et al., 1999). The introduction of *P. macrosporus* over the necrosis and the higher activity on those compounds (Fig. 9), might explain the sharp decrease of the conidia from *Colletotrichum* sp. and an emergence of

conidia from *P. macrosporus* over the necrosis (Fig. 8F). Unfortunately, this interference from *P. macrosporus* against *Colletotrichum* sp. will only occur in the necrosis. *Colletotrichum* sp. use an intracellular hemibiotrophic strategy having a biotrophic phase and succeeded by a destructive necrotrophyc phase. This necrotrophyc phase culminates in absorbing the nutrients deposited on the death tissues and used for the reproduction of the pathogen (PERFECT et al., 1999). The saprophytic fungus such as *P. macrosporus* obtains his nutrient over death organic matter and the necrotrophic phase of *Colletotrichum* gave the niche to develop.

Therefore, *P. macrosporus* have potential to control *Colletotrichum* sp. on coffee seedling with probable combination of mode of action by inducing the plant defense and nutrient competition.

6 CONCLUSION

Phialomyces macrosporus induces an increase of PAL, POX and CAT activities of the plant defense enzymes and responses against *Colletotrichum* sp.

Phialomyces macrosporus does not parasite *Colletotrichum* sp.

Phialomyces macrosporus has ecological similarity and potentially competes for nutrients with *Colletotrichum* sp.

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7 FINAL CONSIDERATION

In the present study, the application of *Phialomyces macrosporus* on the coffee seedling enhances the biocontrol against *Colletotrichum* sp. with a combination of plant defense and nutrient competition. The capacity to increase the plant defense (PAL, POX, CAT, lignin and phenol compounds) and the interference in the carbon sources generates important information for new strategies of control in coffee.

Despite the promising result obtain from the work more study is needed. In the phenol compound study, a different measure is need to be investigated because the reactive Folin-Cicalteu does not separate correctly the phenolic compound over the interference (mainly sugar compounds). In the lignin study, a different measure is needed such as microscopy to ensure a higher deposition in the cell wall. Different protein related to pathogenesis is needed to be aboard such as Chitinase and 1,3-Glucanase, ROS and callose activity and the expression of the enzyme in RT-qPCR.

APPENDIX

Table 1 Micelial growth on 95 carbons sources and water of I-22 and Phm in biolog FF microplates at 25ºC in darkness for 72 h

Carbon Source	I-22	Phm
Alfa- cyclodextrin	+	+
glucosa-1-fosfato	+++	+++
d-manitol	+++	++
d-ribose	+	+++
y-animo-butyric-acid	++++	+++++
d-saccharic acid	+++	+++
glycyl-L-glutamic acid	+++	+
tween 80	++	++
B-cycldextrin	+	+
glucuronamide	+	+
d-mannose	+++	++
salicin	++	+
brosuccinic acid	+	++
sebacic acid	+	+++
l-ornithine	+++	+++
n-acetyl-d-galactosamine	++++	++
dextrin	+++	+++
d-glucuronic acid	++++	++++
d-melezitose	+++++	+++
sedoheptulosan	+	+
fumaric acid	+++	++++
succinamic acid	+++	+++
l-phenylalanine	+	++++
n-acetyl-d-glucosamine	++++	+++
i-erythritol	+	++
glycerol	++	+
d-melibiose	+++	+
d-sorbitol	++	++
b-hydroxy-butyric acid	+	++
succinic acid	+++	+++
l-proline	+++	+++++
n-acetyl-d-mannosamine	++++	++
d-fructose	+++	+
glycogen	++	+
a-methyl-d-galactoside	+	+

Carbon Source	I-22	Phm
l-sorbose	+	+
y-hydroxy-butyric acid	+	+
succinic acid mono-methyl ester	+++	++
l-pyroglutamic acid	+	+++
adonitol	+++	+
l-fucose	++	+
m-inositol	+	+
b-methyl-d-galactoside	++	+
stachyose	+++	++
p-hydroxyphenyl-acetic acid	++	+++
n-acetyl-lglutamic acid	++	++
l-serine	+++	++++
amydgalin	+	+
d-galactose	+++	+
2-keto-d-gluconic acid	++++	++++
a-methyl-d-glucoside	++	++++
sucrose	+++++	+++++
a-keto-glutaric acid	++	++
alaninamide	++	++
l-threonine	+	++
d-arabinose	+	+
d-galacturonic acid	+	+
a-d-lactose	+++	+
b-methyl-d-glucoside	+++++	++++
d-tagatose	++	++
d-lactic acid methyl ester	+	+
l-alanine	+++	+++++
2-amino ethanol	+	+++
l-arabinose	+	+
gentiobiose	++	++++
lactulose	+++	+
palatinose	+++	++
d-trehalose	+++	+++
l-lactic acid	+++	+++
l-alanyl-glycine	+++	++
putrescine	++++	++++
d-arabitol	+	+
d-gluconic acid	+++	++++
maltitol	+	+
d-psicose	+++	+
turanose	+++	++

Carbon Source	I-22	Phm
d-malic acid	+++	+++
l-asparagine	++	+++
adenosine	+++	+++++
arbutin	++	+++
d-glucosamine	+	+
latose	++	+
d-raffinose	++++	++
xylitol	++	+++
l-mailc acid	+++	+++
l-aspartic acid	+++	+++
uridine	+++	+++
d-cellobiose	+	+++++
a-d-glucose	+++	++++
maltotriose	+++	++
l-rhamnose	++	++
d-xylose	+++	+++
quinic acid	+++	+++++
l-glutamic acid	++	++++
adenosine-5'-monophosphate	++++	+++
water	+	+

+, positive; -, negative; NOI; 1

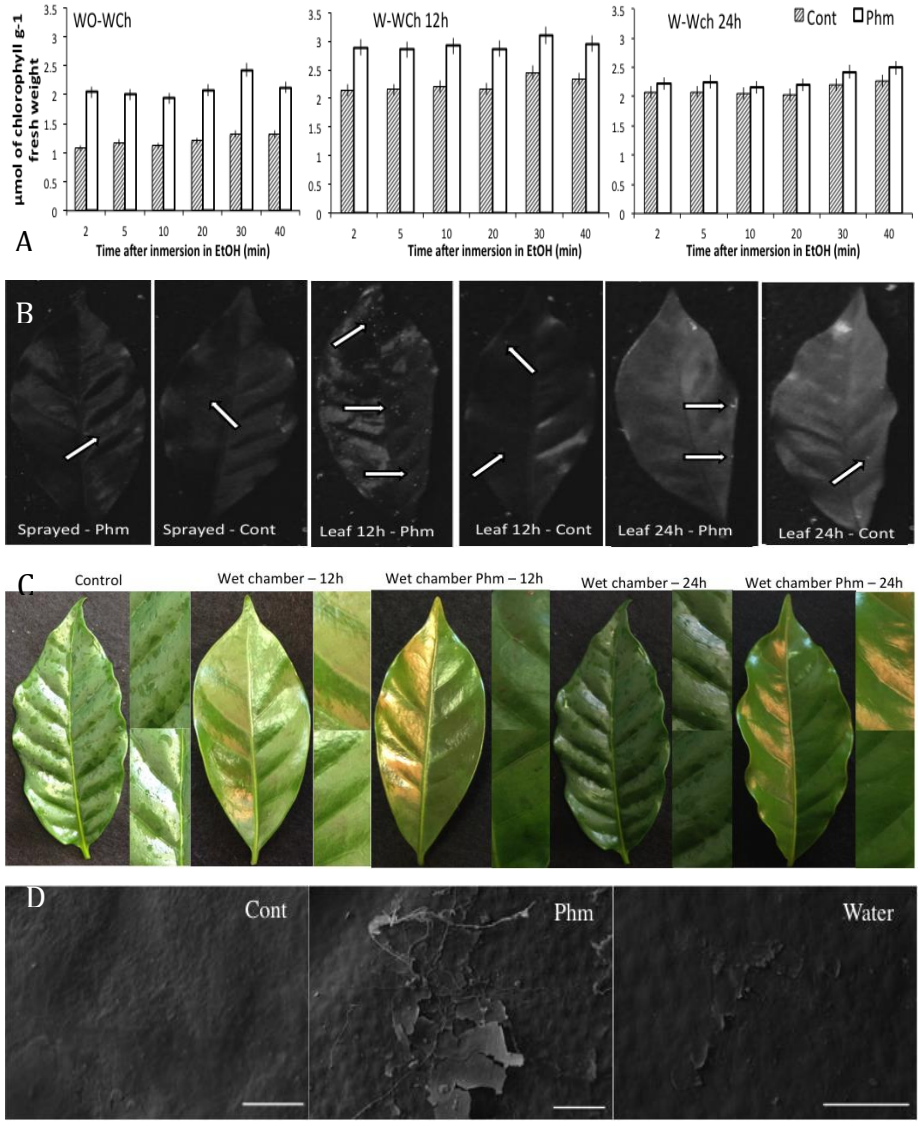

Figure 1 Cuticle permeability after *Phialomyces macrosporus* inoculation under humid condition. (A) Chlorophyll extraction test (B) Calcoflour straining test. (C) Blue toluidine test. (D) Electro scanning picture from the cuticle. Bar scale = 100 µm