ENZYMATIC ACTIVITY AND PROCESS OF INITIAL INFECTION OF GUARANA PLANT (*Paullinia cupana*) BY PATHOGENIC AND ENDOPHYTIC STRAINS OF *Colletotrichum guaranicola*

ACTIVIDAD ENZIMÁTICA Y PROCESO DE INFECCIÓN INICIAL DE LA PLANTA DE GUARANÁ (*Paullinia cupana*) POR CEPAS PATOGÉNICAS Y ENDOFÍTICAS DE *Colletotrichum guaranicola*

Marcely Andrade da Silva[1], Cledir Santos[2]*, Francisco Pérez-Nevado[3], Nelson Lima[4] y Jânia Lilia da Silva Bentes[1]

[1]Federal University of Amazonas-UFAM, - Coroado, Manaus, Amazonas, Brazil. [2]Universidad de La Frontera, Department of Chemical Sciences and Natural Resources, Temuco, Chile. [3]Universidad de Extremadura, Escuela de Ingenierías Agrarias, Departament of Animal Production and Food Sciences, Badajoz, Spain. [4]Universidade do Minho, Campus of Gualtar, Biological Engineering Centre, Micoteca da Universidade do Minho, Braga, Portugal.

**Corresponding author* (cledir.santos@ufrontera.cl)

SUMMARY

Anthracnose of the guarana plant (*Paullinia cupana*) is a disease caused by *Colletotrichum guaranicola* Albuq. Few studies have analyzed the origin, the form of penetration and the colonization of endophytic fungi in the guarana plant. This study aimed to determine differences in the enzymatic activities of endophytic and pathogenic strains of *C. guaranicola* during the initial infection process in leaves of clones sensitive to guarana anthracnose. Fourteen pathogenic and nine endophytic strains of *C. guaranicola* were isolated from guarana leaves with and without anthracnose symptoms, respectively. The enzymatic activities of protease, amylase, polyphenol oxidase, lipase, pectinase, and cellulolytic activities were evaluated for each fungal strain. The experimental design for each enzymatic assay was completely randomized with five replicates. The enzymatic evaluation was based on the measurement of halo produced by the enzyme reaction in each assay. Conidia were evaluated during germination and appressoria formation. Results did not detect enzymatic difference between pathogenic and endophytic strains. Data prior to germination showed differences between endophytic and pathogenic strains. Colonization of plant tissues by pathogenic strain was evidenced after the appearance of disease symptoms 48 hours after inoculation (hai), while for endophytic strains the presence of appressoria was observed on the surface of the epidermis 48 hai, and colonization of cells was not observed.

Index words: *Colletotricgum guaranicola*, *Paullinia cupana* var. *sorbilis*, pathogenesis.

RESUMEN

La antracnosis de la planta de guaraná (*Paullinia cupana*) es una enfermedad causada por *Colletotrichum guaranicola* Albuq. Pocos estudios han analizado el origen, la forma de penetración y la colonización de hongos endofíticos en la planta de guaraná. El presente estudio tuvo como objetivo determinar las diferencias en las actividades enzimáticas de cepas endofíticas y patogénicas de *C. guaranicola* durante el proceso de infección inicial en hojas de clones sensibles a la antracnosis de guaraná. Se aislaron 14 cepas patogénicas y nueve endofíticas de *C. guaranicola* desde hojas de guaraná con y sin síntomas de antracnosis, respectivamente. Se evaluaron la actividad enzimática de proteasa, amilasa, polifenol oxidasa, lipasa, pectinasa y la actividad celulolítica para cada cepa del hongo. El diseño experimental para cada ensayo enzimático fue completamente al azar con cinco repeticiones. La evaluación enzimática se basó en la medición del halo de reacción enzimática formada en cada ensayo. Se evaluaron las conidias durante su germinación y formación de apresorios. Los resultados no detectaron diferencia enzimática entre las cepas potogénicas y endofíticas. Los datos previos a la germinación mostraron diferencias entre las cepas endofíticas y potogénicas. La colonización de los tejidos de la planta por cepas patogénicas se evidenció tras la aparición de síntomas de la enfermedad después de 48 horas de la inoculación (hdi), mientras que para las endofíticas se observó la presencia de apresorios en la superficie de la epidermis 48 hdi y no se observó la colonización de las células.

Palabras clave: *Colletotrichum guaranicola*, *Paullinia cupana* var. *sorbilis*, patogénesis.

INTRODUCTION

The anthracnose caused by *Colletotrichum* spp. is one of the most destructive crop diseases worldwide (Meng *et al*., 2020) and in the guarana plant (*Paullinia cupana* var. *sorbilis*) caused by *Colletotrichum guaranicola* Albuquerque, is one of the main limiting factors for the expansion of the production of guarana in the State of Amazonas, Brazil (Silva *et al*., 2018). The lifestyle patterns of *Colletotrichum* species are highly regulated by specific gene families and biochemical interactions that occur through specific enzymes and secondary metabolites produced at the host-pathogen interface (De Silva *et al*., 2017).

Pathogenicity is due to the ability of the microorganism to produce extracellular enzymes that can modify the cellular components present in the plant tissues (Armesto *et al*., 2019; Parveen *et al*., 2017). Phytopathogenic fungi secrete a variety of enzymes such as cutinases, pectinases, hemicellulases and cellulases, which will act in the degradation of the plant cuticle and the cell wall, colonization, and consequently in the onset of symptoms (Armesto *et al*., 2019). Along with hydrolytic enzymes, fungal oxidases such as polyphenol oxidase (PPO), which

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is recognized to be involved in necrotic lesions, might play an important role in this process (Boeckx *et al*., 2015).

Comparative studies of the enzymatic activity of pathogenic and non-pathogenic *C. guaranicola* and its relationship with the infection process facilitate the understanding of the enzymatic mechanisms related to pathogenicity and could provide information for the establishment of pathogen management strategies and knowledge about the pathogen-host interaction. Based on this, the present study aimed to evaluate the enzymatic activity and the initial infection process of endophytic and pathogenic strains of *Colletotrichum guaranicola* in the guarana plant.

MATERIAL AND METHODS

Fungal strains

Fourteen pathogenic and nine endophytic strains were evaluated. The fungal strains were obtained from guarana leaves collected in fields at the municipalities of Manaus, Maués, Presidente Figueiredo and Rio Preto da Eva (State of Amazonas, Brazil) (Table 1). For the isolation of endophytes, healthy and undamaged guarana leaves were used according to Petrini (1996). The pathogenic strains were obtained from leaves with typical anthracnose symptoms. The indirect isolation was performed on potato dextrose agar medium (PDA, Difco) at 27 °C (Alfenas and Mafia, 2007). The identification of the strains was carried out using morphological characters of 100 conidia and 100 appressoria of each strain and was compared with descriptions in literature (Albuquerque, 1960; Bentes and Barreto, 2004).

Enzyme assays

Protease, amylase, and pectinase of endophytic and pathogenic strains were evaluated according to the protocol described by Valente *et al*. (2002) with some adaptations for pectinolytic assays. In this case, the analysis was performed with the substitution of CTAB by Congo red 0.05 % (Sigma-Aldrich). PPO and lipolytic activities were assessed according to the protocol of Trigiano *et al*. (2016). Cellulolytic activity was determined according to the protocol of Nogueira and Cavalcanti (1996).

Protease activity was evaluated using a culture medium containing skimmed milk in its constitution [0.25 g KH_2PO_4, 0.125 g KCl 0.05 g $MgSO_4 \cdot 7H_2O$, 0.025 g $CaCl_2$, 6.25 mL 22.5 % skimmed milk (Parmalat), 2.5 g glucose, 250 mL distilled water; 3 g agar; pH 5.4]. The observation of the presence of lighter concentric halos around the colony was an indication of the extracellular protease activity.

Amylase production was detected on medium containing soluble starch (0.25 g soluble starch, 0.5 g yeast extract, 0.25 g K_2HPO_4, 0.125 g $MgSO_4 \cdot 7H_2O$, 25 µg thiamine-HCl, 4.25 g agar, and 0.25 mL of the micronutrient solution composed of 0.127 g $FeNH_4(SO_4)_2 \cdot 12H_2O$, 0.178 g $ZnSO_4 \cdot 7H_2O$, 0.074 g $MnSO_4 \cdot 4H_2O$, 0.008 g $CuSO_4$, 0.01 g $CoSO_4$, 0.01 g H_3BO, 100 mL of distilled water, pH 7.3 ± 0.2); after incubation, the plates were flooded with iodine solution.

Pectinase activity was determined by cultivating the fungi in pectin agar medium [0.25 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.75 g Na_2HPO_4, 0.025 g $FeSO_4 \cdot 7H_2O$, 0.13 mg $CaCl_2 \cdot 2H_2O$, 125 mL distilled water, 2.94 g sodium citrate, 3 g agar, 1.25 g pectin, pH 4.5]. To detect pectinase production the fungal culture plates were flooded with a 0.05 % Congo red solution (2.5 g L^{-1}) supplemented with 0.1 M Tris-HCl buffer pH 8.0 for 30 min at room temperature (25 ± 2 °C). The solution was then discarded and the activity detected by the formation of a light halo surrounded by a red zone.

PPO activity was determined using gallic acid as substrate (1000 mL distilled water, 15 g malt extract, 20 g agar and 5 g gallic acid). PPO production was observed after 24 and 48 hours; its evaluation was made by measuring the halo around the colonies.

The lipase production was evaluated in culture medium using Tween 20 as substrate (10 mL tween-20-sorbitol monolaurate-, 8 g peptone, 0.1 g $CaCl_2 \cdot H_2O$, 20 g agar, 990 mL distilled water). After the incubation period, the lipase activity was observed by the presence of a white flocculent precipitate visible underneath and in front of the mycelial layer, where calcium crystals formed.

Cellulolytic activity was evaluated using a medium supplemented with the soluble carboxymethylcellulose (CMC) (Synth) as the sole source of carbon [3.0 g $NaNO_3$, 1.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 10 g CMC, 20.0 g agar, 1000 mL distilled water]. Plates containing 5-mm diameter mycelium discs were incubated for 4 d at 28 °C. The samples were then subjected to heat shock for 16 h at 50 °C. Plates containing fungal cultures were flooded with 10 mL of Congo red solution (0.05 %) supplemented with 0.1 M Tris-HCl buffer pH 8.0 for 30 min at 25 ± 2 °C. The solution was then discarded and each enzymatic activity was detected by the formation of a light halo surrounded by a red zone.

The experimental design was completely randomized with five replicates of the 23 fungal strains. PDA discs without fungi were deposited on Petri dishes with culture medium for each enzyme and used as control. A semi-quantitative evaluation was performed by measuring the degradation halo formed in each assay. The degradation

halo values were statistically analyzed, and the means compared by the Scott-Knott test (P ≤ 0.05) using the ASSISTAT version 7.7 software (Silva and de Azevedo, 2016).

Infection process of endophytic and pathogenic *Colletotrichum guaranicola*

To detect differences in tissues pre-penetration and colonization that could be related to the pathogenicity mechanism, the initial infection process of pathogenic and endophytic *C. guaranicola* in leaves of a susceptible guarana clone was studied. To select strains of pathogenic and endophytic fungi, a pathogenicity test was carried out with the whole set of strains. Each fungus was cultured on PDA (Difco) medium for 10 d at 25 ± 2 °C. Healthy guarana seedlings of clone 300 were used. The inoculation was performed on young leaves in phenological stages 1 to 3, as previously described by Bentes and Matsuoka (2002).

Four 5-mm diameter fungal colony plugs were deposited on the adaxial side of leaves without lesions. Three leaves of each seedling were inoculated and kept in a humid chamber for 48 h. The infection was assessed for 7 d,

Table 1. Enzymatic activity of *Colletotrichum guaranicola* strains by diffusion in specific solid substrates

Strain (sample origin)	Enzymatic activity [Degradation halo (mm)]					
	Lipase	Amylase	Polyphenol oxidase	Cellulase	Pectinase	Protease
Pathogenic						
I 01 (1)†	0.00 c	3.63 d	0.96 a	0.26 a	0.33 a	0.30 a
I 02 (1)	0.00 c	4.96 a	0.83 a	0.05 c	0.23 b	0.24 a
I 04 (2)	0.32 b	4.56 b	0.00 d	0.03 c	0.43 a	0.32 a
I 06 (2)	0.57 a	4.70 b	0.43 b	0.05 c	0.23 b	0.36 a
I 07 (1)	0.00 c	3.96 c	0.20 c	0.10 b	0.23 b	0.33 a
I 08 (3)	0.28 b	4.23 c	0.00 d	0.00 d	0.26 b	0.27 a
I 09 (3)	0.00 c	3.63 d	0.00 d	0.00 d	0.30 b	0.21 a
I 10 (3)	0.00 c	4.76 b	0.00 d	0.00 d	0.43 a	0.17 a
I 11 (3)	0.32 b	5.20 a	0.21 c	0.00 d	0.10 b	0.30 a
I 12 (3)	0.26 b	5.00 a	0.43 b	0.00 d	0.10 b	0.28 a
I 461 (1)	0.00 c	4.60 b	0.55 b	0.00 d	0.20 b	0.25 a
P11 (4)	0.30 b	5.00 a	0.81 a	0.05 c	0.40 a	0.26 a
P17 (4)	0.00 c	4.96 a	0.00 d	0.00 d	0.30 b	0.20 a
P18 (4)	0.50 a	5.26 a	0.00 d	0.00 d	0.30 b	0.23 a
Endophytic						
I 13 (1)	0.00 c	5.13 a	0.00 d	0.06 b	0.16 b	0.34 a
I 16 (4)	0.34 b	4.36 c	0.26 c	0.00 d	0.36 a	0.20 a
I 17 (4)	0.30 b	4.00 c	0.43 b	0.00 d	0.16 b	0.17 a
P 01 (3)	0.41 b	4.70 b	0.33 c	0.06 b	0.16 b	0.28 a
P 07 (4)	0.53 a	4.36 c	0.28 c	0.05 c	0.23 b	0.32 a
P 09 (4)	0.00 c	4.16 c	0.00 d	0.08 b	0.20 b	0.26 a
P 10 (3)	0.27 b	4.10 c	0.45 b	0.05 c	0.56 a	0.24 a
P 12 (3)	0.41 b	4.73 b	0.76 a	0.03 c	0.36 a	0.27 a
P 13 (3)	0.00 c	4.03 c	0.00 d	0.00 d	0.26 b	0.21 a
CV (%)	5.09	2.35	8.22	2.07	7.71	5.48

Means followed by the same letter in the same column do not differ from each other (Scott-Knott test, P ≤ 0.05). CV: coefficient of variation. †Sample origin: (1) Manaus, (2) Rio Preto da Eva, (3) Presidente Figueiredo, (4) Maués.

based on the presence or absence of symptoms. Conidia germination and appressorium formation were quantified in 100 conidia for each strain, the leaves inoculated with pathogenic and endophytic strains of *C. guaranicola* were selected according to the results of the pathogenicity test.

The inoculated samples were collected at 12, 24, and 48 hours after inoculation (hai). After this period, symptoms were visible in plants inoculated with the pathogenic strain, evidencing the colonization of tissues. The collected samples were cut into 0.5 cm^2 fragments and processed according to the methodology described by Longo *et al*. (1994). Conidia were observed in light microscope (Carl Zeiss, Jena, Germany) and photographed with the AxioCAM ERc 5s camera with a 40x objective.

The experimental design was completely randomized with four replicates; the experimental unit was one plant. The control consisted of plants that received PDA culture medium discs without fungal inoculum. Data were submitted to analysis of variance (ANOVA) and the Scott-Knott ($P \leq 0.05$) using the ASSISTAT version 7.7 program (Silva and de Azevedo, 2016). Inoculated samples were collected at 48 hai and cut into pieces of 0.5 cm^2. Samples were then fixed on a solution composed of 10 mL 35 % formaldehyde, 50 mL 95 % ethyl alcohol, 5 mL glacial acetic acid and 35 mL distilled water, for 48 h. Samples were preserved in 70 % ethyl alcohol aqueous solution for 48 h.

Samples were dehydrated in a series of aqueous ethanol solutions at 30, 50, 80 and 100 % (v/v) under vacuum. Dehydrated samples were then embedded into historesin (Leica) and successively submitted to alcohol and historesin at the following concentrations: 1:1 (v/v, 2 h), 1:3 (v/v, 2 h), neat historesin for 48 h. Embedded samples were cut on a rotary microtome (Leica RM 2145, Nussloch, Germany) into 6-µm thick sections. Histological sections were mounted under microscope slides and stained with 0.1 % toluidine blue. The slides were observed with a Carl Zeiss light microscope and photographed with the AxioCAM ERc 5s camera with a 40x objective.

RESULTS AND DISCUSSION

Enzymatic assay

The enzyme activities of *C. guaranicola* strains are presented in Table 1. All strains showed amylase activity with degradation halo ranging from 3.63 to 5.26 mm (Table 1). The largest halo degradations were observed within the pathogenic set and one endophytic strain (I 13) showing a halo of 5.13 mm. Similarly, Khodadadi *et al*. (2020) evaluated the production of extracellular enzymes of *Colletotrichum* isolates on starch agar plates and showed that all the species produced halo zones reflecting amylase activity after exposure to iodine.

According to Velho *et al*. (2018), most pathogens utilize starch to perform their metabolic activities, and the degradation occurs directly by the action of amylases. These enzymes promote starch degradation, which can likely be used as an energy source during growth and sporulation (Armesto *et al*., 2019).

The medium used for polyphenol oxidase had as main constituent gallic acid and the presence of a dark halo around colonies indicating PPO activity. Fourteen out of 23 strains (eight pathogenic and six endophytic) presented positive results for the assessed enzymes. The halo diameter ranged from 0.21 to 0.96 mm (Table 1), with no distinction between pathogenic and endophytic. Tozze Júnior *et al*. (2016), characterising the aggressiveness and enzymatic activity of *Colletotrichum* spp. associated with avocado (*Persea americana*) anthracnose, detected laccase activity in only 14 of the 30 isolates studied, with the distinction of six groups. According to the authors, there is evidence of the involvement of laccase in the activation of quiescent infections promoted by *C. gloeosporioides* in avocado during fruit ripening, in addition to the action of these enzymes in the detoxification of antifungal compounds and tannins present in the host.

According to Armesto *et al*. (2019), the greater expression of amylase and laccase activities in fungi may be related to their high needs for nutrients in the tissue colonization process. Amylase is directly related to energy requirement and laccase (polyphenol oxidase) with the penetration process.

Lipase activity was detected by the presence of a white precipitate below the mycelial layer, as a result of the formation of calcium salt crystals of the lauric acid released by the enzyme (Hankin and Anagnostakis, 1975). Strains I 06 and P18 (pathogenic), and P07 (endophytic) showed the greatest degradation halo for the lipase enzyme. For seven pathogenic and three endophytic strains (Table 1) degradation halos of lipase were not observed. Lipase represents an important enzyme complex in pathogenesis for decomposing host cellular components as phospholipids in the plasma membrane. The use of Tween 20 to screening fungi for lipase activity is a simple and appropriate method. However, oil substrate and their constitutive effect on extracellular lipase that varies from one to another organism. Lipase production can also be influenced by diverse factors such as carbon and nitrogen sources, pH in culture media, temperature, oxygen levels, and agitation (Geoffry and Achur, 2018).

In this study the differences observed on lipase production by pathogenic and endophytic isolates of *C. guaranicola* can be affected by intraspecific variations between isolates, as demonstrated by Armesto (2019) on *C. gloeosporioides* and coffee plants pathosystem, where the samples showed different enzymes profiles that may be associated to virulence.

Only for 12 strains (six endophytic and six pathogenic) showed cellulolytic enzyme production, with the formation of a small degradation halo (Table 1). Khodadadi *et al.* (2020) observed that all isolates belonging to *C. noveboracense*, *C. fioriniae* and *C. chrysophilum* showed cellulolytic activity after five days of incubation on PDA as a yellow halo around the colony in plates, including CMC stained with Congo red solution and NaCl. According to Acosta-Rodríguez *et al*. (2005), *C. lindemuthianum* was more efficient on medium supplemented with cellulose than on the CMC medium. In the first one, cellulolytic activity was detectable at 3 days of incubation, while after 13 days of growth in the CMC medium only about 35 % of the result was reached with cellulose.

Either the CMC medium could not have favoured the production of the enzyme or the evaluation time may not have been sufficient to verify the formation of the halo. Overall, these authors observed different cellulolytic enzyme production profiles between pathogenic and non-pathogenic *C. lindemuthianum* strains, suggesting that this enzyme has a role in the degradation of the plant cell wall during the infection onset. All the fungal strains assessed in the present study had positive results for pectinolytic enzymes, with halos ranging from 0.10 to 0.56 mm (Table 1). There were no differences between pathogenic and endophytic strains. High pectinolytic activity is likely related to pectin, which is a major plant component that could induce the fungus to produce a larger amount of these enzymes (Armesto *et al*., 2019). In the present study, the proteolytic enzyme was evident for all fungal strains. Degradation halos varied from 0.17 to 0.36 mm and there were no statistical differences between pathogenic and endophytic strains (Table 1).

Proteases are abundant in fungi, they generate peptides or amino acids to support fungal growth and contribute to degradation of the plant cell wall (González *et al*., 2016). Redman and Rodriguez (2002) reported a direct relationship between protease activity and virulence in *Colletotrichum coccodes*; according to the authors, extracellular protease activity was required for pathogenicity and virulence, and the elimination of protease activity transformed the virulent pathogen into a non-pathogen.

In the present study, pathogenic and endophytic strains were not distinguished based on the production of extracellular enzymes; both amount and rate of enzyme produced can interfere with the plant infection process resulting in success or failure in the establishment of the pathogen in the host.

Parveen *et al*. (2017) stated that differences in enzyme production by fungal pathogens can represent their virulence and specificity in causing diseases. Based on these data, it seems reasonable to suggest that these enzymatic activities should not be used as markers of virulence for these fungal isolates, considering that specific conditions in the host tissue, such as temperature and pH, are also important factors for regulation of gene expression and subsequent enzyme production (Velho *et al*., 2018).

Pre-penetration events

All pathogenic and endophytic strains were assessed for pathogenicity and strains were selected for the infection and colonization studies. None of the endophytic strains developed infection symptoms in the plant after 7 days of inoculation; however, in tomato (*Solanum lycopersicum*) infected with *Colletotrichum coccodes*, the infection symptoms appeared before 7 days after inoculation, mainly in fruits inoculated with artificial wound (Nieto-Angel *et al.,* 2019).According to the statistical analysis, significant differences were observed in the injured area size caused by the pathogenic strains (Table 2).

Brader *et al*. (2017) claim that commonalities and differences exist in colonization routes for both pathogenic and non-pathogenic endophytes. The pathogenic strain

Table 2. Injured area in guarana leaves inoculated with mycelial discs containing *Colletotrichum guaranicola* pathogenic strains.

Strain	Injury area (cm^2)	Strain	Injury area (cm^2)
I 01	1.56 e	I 10	2.96 b
I 02	2.40 c	I 11	2.26 c
I 04	2.73 b	I 12	2.80 b
I 06	3.53 a	I 461	1.90 d
I 07	2.93b	P11	2.26 c
I 08	2.53 c	P17	2.36 c
I 09	2.26 c	P18	2.46 c
CV %	4.86		

Means with the same letter do not differ from each other (Scott-Knott test, $P \leq 0.05$). CV: coefficient of variation.

I 06, the most aggressive one observed in the present study, was selected along with the endophytic strain P10 for the study of initial infection and colonization process in guarana leaves. Symptoms were observed only on leaves inoculated with the pathogenic *C. guaranicola* strains after 48 hai. It appeared as small, reddish stains that indicate cell death. Fungal re-isolation corroborated the hypothesis that different endophytic *C. guaranicola* strains can stay in the endophytic association in guarana without manifesting symptoms. In addition, pathogenic strains induced symptoms appearance within 48 hai.

There was significant difference (Scott-Knott test, $P \leq 0.05$) regarding germination and appressorium formation between endophytic and pathogenic strains (Figure 1A). Conidia germination was observed 12 hai for 84 and 66 % of the pathogenic and endophytic strains, respectively. Overall, pathogenic and endophytic strains reached 100 and 82 % germination respectively at 48 hai. Regarding appressoria formation, 76 % conidia of pathogenic strains formed appressorium at 12 hai and reached 100 % at 48 haI, while for endophytic strains 48 % were observed at 12 hai and 70 % at 48 hai (Figure 1B).

No difference was observed in the morphology of appressorium produced by endophytic or pathogenic strains. Appressoria presented globose to subglobose form with the presence of melanin. According to Ryder and Talbot (2015), the presence of melanin in the appressorium keeps the pressure of turgor due to a lowering in the porosity of the appressorium cell wall. Fungal melanin does not only serve as protection against environmental stresses but also determine virulence in some pathogenic fungi (Ludwig *et al*., 2014). Melanin impregnation in the appressoria wall enables *Colletotrichum* species to build up high osmotic pressure and finally penetrate itself into the cell wall (Reboledo *et al*., 2015).

In the genus *Colletotrichum*, conidia germination and appressoria formation occur between 3 and 48 h after conidia deposition (Araujo and Stadnik, 2013). Its speed is strongly influenced by environmental factors and chemical or physical signs present on the surface of the host. Bentes and Matsuoka (2002) observed that the beginning of conidia germination for pathogenic *C. guaranicola* occurred between 6 and 12 hai, reaching the maximum of germination in the period of 24 hai (94.7 %). The same authors also observed that appressoria formation reached its maximum within 24 hai (94 %).

Colonization of plant tissues by the pathogenic strain I 06 was evidenced by the appearance of symptoms at 48 hai. No symptoms were observed seven days after inoculation in the plants inoculated with the endophytic strain P10. Evaluation of sections of leaf tissue showed colonization by the pathogenic strain I 06 in the cells of the epidermis and palisade parenchyma. Intracellular hyphae presence and parenchyma cells degradation were observed (Figure 2).

Pre-penetration and colonization processes by pathogenic *C. guaranicola* strains in guarana resembled those observed for other *Colletotrichum* species (Barimani *et al*., 2013; Bentes and Matsuoka, 2002; Jayawardena *et al*., 2016). Appressoria presence was observed on the surface of leaf epidermis in the samples inoculated with the endophytic strain P10 but no cell colonization was observed in the sections analyzed at 48 hai (Figure 2). Delay in tissue colonization by the endophytic strain (P10), compared to the pathogenic one (I 06), can be related to pathogenicity mechanisms, which make the pathogenic strain more efficient in degrading the host natural barriers and colonizing cells.

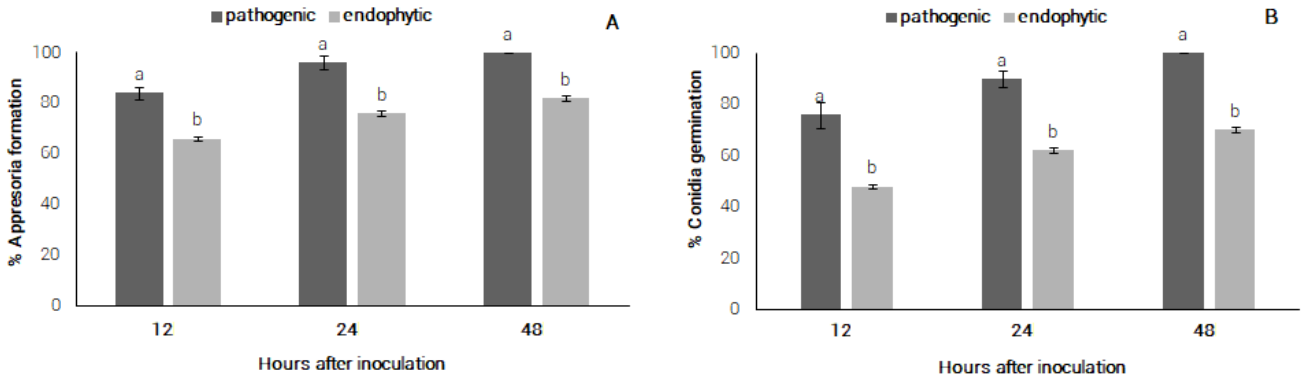


Figure 1. A) pathogenic and endophytic conidia germination of *C. guaranicola*, B) appressoria formation by endophytic and pathogenic *C. guaranicola* strains.

Despite that results show no *in vitro* variation in enzymatic activity among strains, the pathogenic strains were more efficient in infecting and colonizing guarana tissues, resulting in symptoms at 48 hai. The pre-invasion perception of plant-derived signals reprograms the fungal gene expression, a genome-wide expression, and secondary metabolism enzymes are induced before penetration and tissue colonization (O'Connell *et al*., 2012). In presence of host tissue, some enzyme can be produced in different amount and it might be related to the variable ability of different *Colletotrichum* strains to effectively penetrate and spread into host plant tissues performing a higher level of virulence.

The lifestyle patterns of *Colletotrichum* species are highly regulated by both specific gene families and biochemical interactions that occur through specific enzymes and secondary metabolites produced at the host-pathogen interface (De Silva *et al*., 2017). Results observed in this study were similar to those reported by Horowitz *et al*. (2002), who studied colonization of pathogenic and endophytic strains of *C. acutatum* on strawberry; they observed that pathogens quickly developed into the host tissues, causing tissue necrosis four days after inoculation.

Endophytes conidia presented germination by forming a germinative tube and giving rise to the appressoria, that failed to penetrate the leaf tissue, resulting in an epiphytic growth without invasion into the plant. Penetration of endophytic was observed approximately seven days after inoculation and was restricted to the intercellular spaces of subcuticular layer of the hosts, without causing any visible damage. According to Freeman and Rodriguez (1993), a single gene mutation transformed a *Colletotrichum magna* pathogenic strain into an endophytic one, leading to slight differences between both strains. Overall, the effect of pre-penetration and penetration of *Colletotrichum* species appear to be similar; however, there are differences between species in the mechanisms of adhesion, melanization and cutinase production for penetration into the cuticle of the plant (Perfect *et al*., 1999).

CONCLUSIONS

There was no enzymatic difference between the pathogenic and endophytic strains evaluated in the present study. Pre-germination data showed differences between endophytic and pathogenic strains. Tissue colonization by pathogenic strain was evidenced by the appearance of symptoms 48 hai while for the samples inoculated with endophytic strains, the presence of appressoria was observed on the surface of the epidermis 48 hai, and no cell colonization was observed. The effect of pre-penetration and penetration of *Colletotrichum* species appears to be similar; even though, there are differences between species in the mechanisms of adhesion, melanization and cutinase production for penetration into the plant cuticle.

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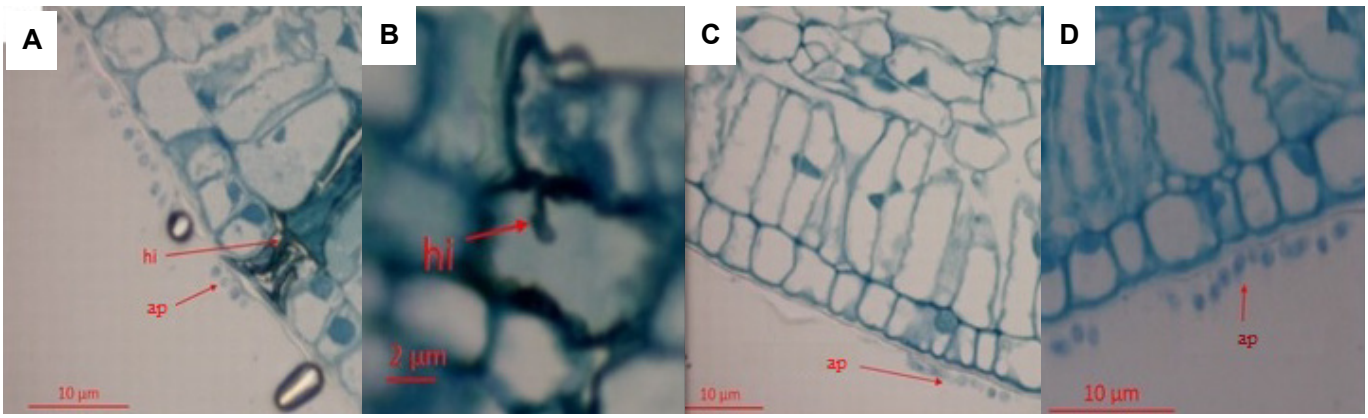


Figure 2. Process of *C. guaranicola* pathogenic and endophytic colonization in leaves of susceptible guarana (clone 300) within 48 hai. A) hyphae (hi) of the *C. guaranicola* pathogenic strain colonizing the tissues of the host plant, B) infection process of pathogenic strain with presence of appressoria (ap) on the surface and penetration hypha, C) and D) endophytic appressoria on the leaf surface of the host plant.

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