ANTAGONISTIC ACTIVITY OF FUNGI FROM ANTHRACNOSE LESIONS ON *PAULLINIA CUPANA* AGAINST *COLLETOTRICHUM* sp.

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SUMMARY

Anthracnose is a cosmopolitan disease caused by *Colletotrichum* spp. that affects many crops worldwide. Observations have shown that anthracnose leaf lesions may be colonized by several non-pathogenic microorganisms. The relationship of these microorganisms with the pathogen as well as their potential as biocontrol agents is not well known. Guarana (*Paullinia cupana* Mart. var. *sorbilis*) is a typical native Amazon crop with unknown microbial diversity. Guarana productivity has been reduced by a fungal disease caused by *Colletotrichum* sp. In this study, we isolated 15 fungi from guarana anthracnose leaf lesions that belong to five genera: *Fusarium*, *Phomopsis*, *Leptosphaeria*, *Microdochium* and *Pestalotiopsis.* Four isolates from the *Fusarium* sp. (C6 and C10), *Pestalotiopsis* sp. (C3), and *Microdochium* sp. (P7) consistently inhibited anthracnose fungal growth *in vitro*. Except for the *Microdochium* sp. (P7), these isolates were also able to inhibit the growth of the pathogen in *in vivo* assays using detached guarana leaves. Some mechanisms related to the growth inhibition of this pathogen were studied. *Fusarium* sp. (C6) produced chitinases; *Fusarium* sp. (C6, C10) and *Pestalotiopsis* sp. (C3) produced antagonistic volatile organic compounds. These three isolates also inhibited the growth of *Fusarium* spp., a pathogen of several plant species, suggesting their potential broad range of growth inhibition of other phytopathogens.

Keywords: biological control, saprophytes, chitinase, volatile organic compounds, detached leaf bioassay.

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INTRODUCTION

Guarana (*Paullinia cupana* Mart. var. *sorbilis*) is an Amazon native crop whose seeds serve as ingredient for industrial products with stimulant properties due to their high concentration of caffeine. Guarana plants are commercially cultivated exclusively in Brazil, which supplies raw material to the carbonated soft-drink and natural products markets. Approximately 20% of guarana production is designated for the international market (Araújo *et al*., 2009). The yield in 2013 reached 3,676,000 tons of guarana beans (IBGE, 2014).

Guarana productivity has been strongly affected by the presence of the anthracnose disease caused by *Colletotrichum* sp. fungi, which was first described as *C. guaranicola* (Albuquerque, 1960). However, the classification of guarana's anthracnose pathogen at the species level is hampered by the scarcity of information about this pathogen and the complexity of the classification of species of the *Colletotrichum* genus (Sutton, 1992; Bentes *et al*., 2011).

Anthracnose in guarana is characterized by the appearance of rounded necrotic orange-colored lesions on leaf surfaces (Silva *et al*., 2004). Typical leaf lesions of anthracnose in guarana are also colonized by other pathogens and microorganisms that take advantage of the environmental conditions created by the phytopathogens (Przybyl, 2002; Newton *et al*., 2010). These microorganisms are saprophytes, or weak parasites, and their potential in the biological control of pathogens occupying a common leaf niche has been recently explored (Przybyl, 2002; Kaur *et al*., 2011; Arriagada *et al*., 2012).

There are several mechanisms related to fungal-fungal antagonism. Competition for nutrients and space in the plant host or soil can be critical to the establishment of a pathogen (Handelsman *et al*., 1996; Compant *et al*., 2005; Kaur *et al*., 2011); the production of antifungal compounds that directly affect the pathogen, including chitinase, volatile organic compounds (VOCs), antibiotics or other important metabolites (Fravel *et al*., 1988; Zhang *et al*., 2011; Arriagada *et al*., 2012; Zhi-Lin *et al*., 2012); mycoparasitism, where the pathogen is directly attacked by a specific fungi (Milgroom and Cortesi, 2004; Heydari and

Pessarakli, 2010) and the induction of plant defense mechanisms through the production or release of compounds that induce resistance responses (Harman *et al*., 2004). Among these mechanisms, chitinase and VOCs have been extensively investigated (Schoeman *et al*., 1999). Toyama and Ogawa (1968) investigated the mycolytic activity of *Trichoderma viride*, a well-known biological control agent, and identified chitinase activities as an important step in the breakdown of the fungal cell wall in *Penicillium chrysogenum*. Dennis and Webster (1971) suggested that *Trichoderma* isolates may produce several active volatile metabolites related to boilogical control (Schoeman *et al.,* 1999).

Interactions may occur among fungi that live in the same niche, for example, the same plant species, or by the introduction of a foreign antagonistic agent capable of reducing the incidence of a particular disease (Widmer and Dodge, 2013). However, fungi that naturally inhabit leaf lesions, living in the same locale as phytopathogens, have some advantages as biocontrol agents (Newton *et al*., 2010). Therefore, this study had the following objectives: (i) to isolate the fungal community from guarana anthracnose lesions, (ii) to study the interaction of this fungal community with *Colletotrichum* sp. and other phytopathogens, and (iii) to investigate possible fungal strategies to control phytopathogens, such as the production of the antifungal compounds chitinase and VOCs.

MATERIAL AND METHODS

Fungal isolation. *Paullinia cupana* var. *sorbilis* leaves from 10 plants with typical anthracnose's symptoms were randomly sampled in November 2010 from a commercial field located in Maués/Amazonas State (3°26'57" S and 57°39'5" W), Brazil. Samples were immediately stored in paper bags for further fungal isolation. The guarana trees were two years old. The annual average temperatures of this region range between 21.4 and 31.1°C, and its mean annual relative humidity is approximately 80%.

All leaves were washed in running tap water. The plant tissues were rinsed with 70% ethanol, surface disinfected with sodium hypochlorite solution (3% available Cl^-) for 3 min, rinsed once in 70% ethanol, and twice in sterile distilled water. The disinfection process was checked by plating water aliquots from the final rinse solutions onto potato dextrose agar (PDA, Difco) (Araújo *et al*., 2001). After the surface disinfection, small leaf fragments (0.5 cm^2) were cut, leaving the edge of necrotic tissue exposed, and placed on water agar medium (WA), containing 10 g l^{-1} agar in distilled water. Petri dishes were incubated at 28°C, and fungal growth was evaluated; subsequently, the sample was subcultured onto a PDA Petri dish. Fungi with different morphology were selected and subcultured in water following Castellani´s method, for further analysis.

DNA extraction and fungal identification. Fungal isolates from one sample of every morphotype were incubated in 50 ml flasks of Potato-Dextrose Broth (PDB, Difco) by continuous shaking (150 rpm) for 5 days at 28°C, 14 h photophase. The fungal suspensions were filtered to separate the mycelium that was macerated in liquid nitrogen. Powdered mycelium was put into a microtube, and 500 µl TE buffer (24 mM EDTA, 250 mM NaCl, 200 mM Tris-HCl, 1% SDS, pH 8.0) was added before incubation at 65ºC for 20 min. The solution was centrifuged for 10 min at 12,000 *g*, then the supernatant was transferred to a fresh tube and 1 vol chloroform:phenol (1:1) was added. The suspension was mixed by inversion and centrifuged for 5 min at 16,000 *g*. The supernatant was transferred to a fresh microtube, and 1 vol chloroform:isopropanol (4:1) was added and gently mixed. The suspension was centrifuged for 5 min at 16,000 *g*, then 0.1 vol NaCl (5 M) and 0.6 vol isopropanol were added to precipitate the DNA. The mix was incubated for 10 min at 6°C and then centrifuged for 15 min at 10,000 *g*. The supernatant was removed, and the pellet (DNA) was washed twice with 70% ethanol, dried at 40°C for 20 min and finally resuspended in 50 µl sterile water. After DNA extraction, the amplification of ITS1-5.8S-ITS2 rDNA was carried out using the primer set ITS-1 (5'-TCCGTACCTCAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3'), annealing at specific positions on the 18S and 28S rDNA (White *et al*., 1990). The PCR contained 5 ng of template DNA, 0.4 µM of each primer, 3.7 mM of $MgCl_2$, 1 mM of each dNTP, 2.5 U of *Taq* DNA polymerase, and 1× Taq buffer in a 50 µl final volume. Amplifications were conducted in a Peltier Thermal Cycler PTC200 (MJ Research Inc., Canada) with an initial denaturation of 4 min at 94°C followed by 24 cycles of 30 s denaturation at 94°C, 30 s primer annealing at 55°C, 30 s extension at 72°C and a final extension of 10 min at 72°C. Aliquots of 2 µl PCR products were analyzed by electrophoresis with the 1 Kb DNA molecular ruler 1 k DNA (Invitrogen, USA) in a 1% agarose gel and stained with ethidium bromide for visualization under UV light.

The ITS1-5,8S-ITS2 amplified fragments, approximately 400 bp in length, were purified using the polyethylene glycol method according to Lis (1980) and sequenced by the Human Genome Research Center, Institute of Biosciences, University of São Paulo, SP, Brazil. Sequences were deposited in GenBank under the accession numbers KC847976-KC847990.

Molecular analysis. The obtained sequences were used for fungal identification by alignment with the GenBank database using the BLASTn tool from NCBI (National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). A comparative analysis dendogram was built using the MEGA 5.0 program (Tamura *et al*., 2011). Clustering was calculated according to the Neighbor-Joining

(NJ) method with 1000 bootstrap replicates based on genetic distances calculated by the Jukes-Cantor model.

***In vitro* antagonistic activity.** Antifungal activity of isolates was evaluated by dual culture experiments against one phytopathogen isolate, the *Colletotrichum* sp. (L3). The phytopathogen, which was isolated from a typical anthracnose lesion, was kindly provided by Dr. Pedro Queiroz Costa Neto, Federal University of Manaus. The Petri dish, containing the fungi growth on PDA medium, had two 5 mm diameter mycelial disks removed from the edge of young cultures that were transferred to a new plate. One disk belonged to one antagonistic fungi, and the other was the *Colletotrichum* sp. L3 culture, placed 30 mm apart from the other disk. The dual culture plates were incubated at 28°C for 15 days under 14 h photophase. The control treatments consisted of Petri dishes with one disk of *Colletotrichum* sp. L3 without any other fungal isolates. The assay was performed with 4 replicates of every combination of pathogen L3 and fungal isolate. Each replicate consisted of one Petri dish. Mycelial growth inhibition was determined according to Lahlali and Hijri (2010); the radial fungal growth towards the antagonistic fungus (Ri) and a control plate (Rc) were measured and the mycelial growth inhibition was calculated according to the following formula: (Rc–Ri)/Rc × 100.

The antagonistic ability was also assessed using a rating scale (Badalyan *et al*., 2002) for three main types of interactions: types A and B were classified as deadlock or mutual inhibition, in which neither organism was able to overgrow the other at mycelial contact (A) or at distance (B), and type C was characterized by the overgrowth between the fungi (replacement), without initial deadlock.

Detached leaf bioassay. Guarana leaves were collected from healthy plants at the University of São Paulo, ESALQ/USP, in Piracicaba-SP, Brazil (22° 42' 44.23'' S and 47° 38' 2.61'' W) in April 2014. The leaves were wiped clean by rising in 70% ethanol for 10 s and then washed twice with sterilized distilled water. For the bioassay, five leaves were randomly inoculated with all treatments. Each treatment was performed with half of a 5 mm disc of the pathogen *Colletotrichum* sp. L3 and half of a 5 mm disc of the *in vitro* antagonistic fungi. A positive control was performed with half of a 5 mm disc of pathogen *Colletotrichum* sp. L3 and the other half with PDA media, and a negative control was performed with a 5 mm disc of PDA media. Leaves were incubated at room temperature with high humidity and natural light. The diameter of the leaf tissue lesions was measured after five days. Fungi that showed antagonist activity *in vitro* and *in vivo* against *Colletotrichum* sp. L3 were evaluated for the production of antifungal compounds.

Chitinolytic activity. Antagonistic fungi were grown in 50 ml PDB supplemented with 0.5% insoluble chitin (Sigma) and incubated at 28°C for 5 days under agitation (150 rpm) under 14 h photophase. After fungal growth, the crude protein extract was obtained by centrifugation (12,000 *g* for 5 min). The crude extracts were maintained at –20°C for further analyses of different chitinases.

Table 1. List of plant pathogenic fungi assayed in this study.

Identification	Isolate[a]	Host
Ceratociste padadoxa	CP-01	Sugarcane
Fusarium verticillioides	FM	Sugarcane
Fusarium oxysporum	R2238	Pea
Rhizoctonia solani	RS-01	Bean
Fusarium verticillioides	T4	Corn

[a] Isolates obtained from culture collection "Laboratory of Genetics of Microorganisms", Department of Genetics, ESALQ/USP, Piracicaba, SP, Brazil.

The endo-chitinolytic activity was assayed using CM-Chitin-RBV (Loewe) in 50 mmol l^{-1} Tris-HCl buffer (pH 7.5) at 40°C for 2 h. The reaction was stopped by the addition of HCl 2 N (25 µl) and incubated at 4°C for 10 min. The values of chitin degradation were quantified by the absorbance reading from ELISA plates in a spectrophotometer (Ultrospec 3000; Pharmacia/Biotech) for CM-chitin absorbance at 550 nm.

The *N*-acetyl-β-D-glucosaminidase, chitobiosidase and chitotriosidase activities were measured by enzymatic hydrolysis of the substrates: *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-*N*,*N*'-diacetyl-β-D-chitobiose and *p*-nitrophenyl-*N*,*N*',*N*''-triacetyl-β-D-chitotriose (Sigma), respectively. The reagents were dissolved in a 10% (v/v) dimethylsulfoxide solution. The fungal protein extract (40 µl) was added to 25 µl of chitin substratum. The reaction was incubated at 37°C for 2 h and then stopped by the addition of 100 µl Na_2CO_3 0.4 N. The enzyme activity was measured in absorbance from ELISA plates at 550 nm. All experiments were performed in triplicate. The relative enzyme activity (EA) was measured in absorbance per milliliter of substrate reaction per hour × 10^3 (Guzzo *et al*., 1999; Quecine *et al*., 2008).

Volatile organic compound production. The antagonistic fungi were also evaluated for the production of VOCs against *Colletotrichum* sp. (L3), according to Lahlali and Hijri (2010). Briefly, PDA plates were inoculated in the center with a 0.5 cm diameter mycelial disc, containing mycelia from both antagonistic fungus and pathogen, with one plate inverted and place on top of the other plate. The two plates were sealed with a double layer of parafilm. Controls were performed by replacing the antagonistic fungi with agar discs. The plates were placed at 28°C for 7 days and the fungal growth diameter measured. The assay was performed using four biological replicates. The effect of VOC production was determined measuring the diameter of the phytopathogen growth towards the antagonistic fungus according to the formula described by Lahlali and Hijri (2010).

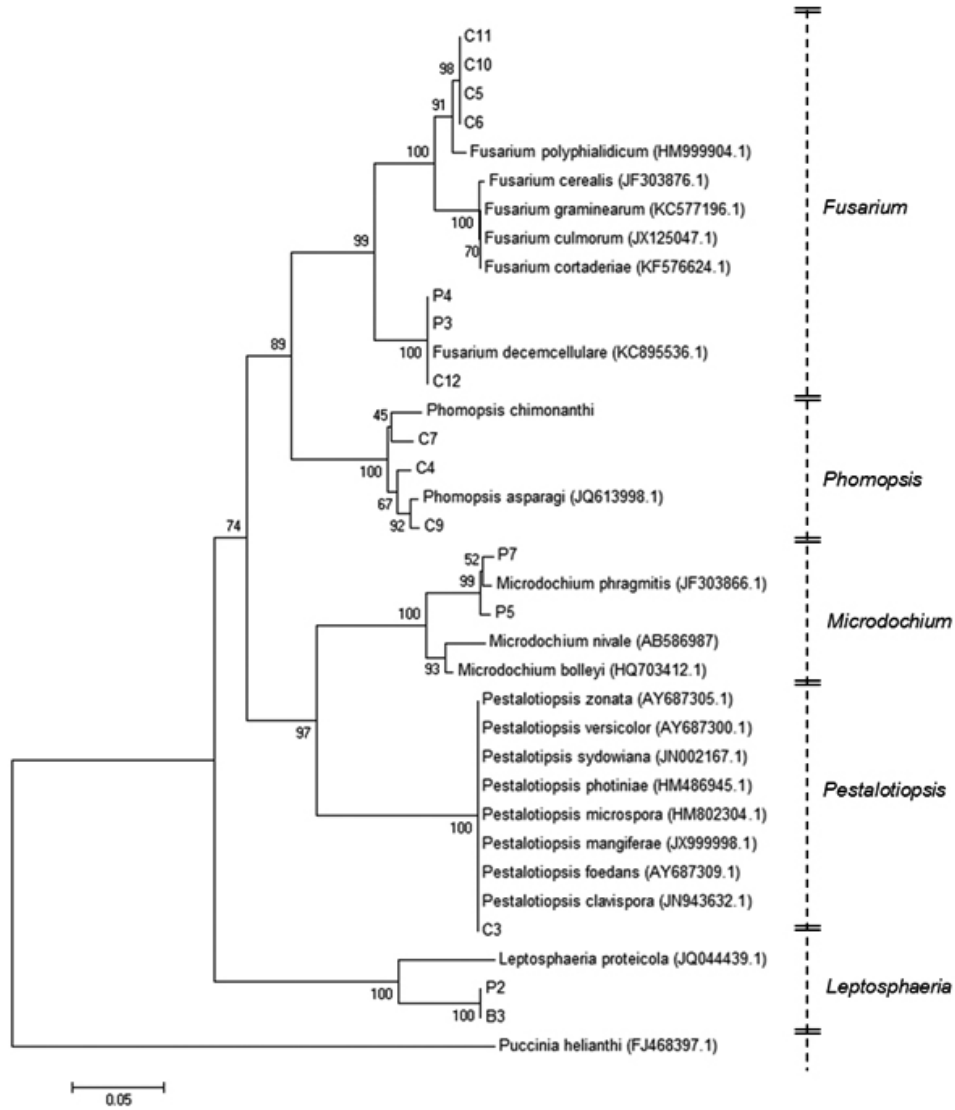

Fig. 1. Neighbor-joining tree, based on Jukes and Cantor's model, obtained from the analysis of rDNA ITS sequences of 15 fungi isolated from guarana plants. Reference sequences from GenBank were used to compare the relationships among the isolates. The Basidiomycota *Puccinia psidii* (FJ468397.1) was used as the out-group. Bootstrap values (n = 1,000 replicates) are shown at the intersections.

Phytopathogen growth inhibition. The antagonistic activity of *Colletotrichum* sp. fungi was also evaluated in comparison with other phytopathogens. The following phytopathogenic fungi were included in the study: *Ceratocystis paradoxa* (CP-01) and *Fusarium verticillioides* (FM) from sugarcane, *Fusarium oxysporum* (R2238) from pea, *Rhizoctonia solani* (RS 01) from bean, and *F. verticillioides* (T-4) from corn (Table 1). These fungi were maintained on PDA at room temperature. All isolates belonged to the Culture Collection at the Laboratory of Microbial Genetics, Department of Genetics, ESALQ/USP, Piracicaba, SP, Brazil. The antagonistic activity was measured under *in vitro* conditions via the dual cultural experiment, where two 5 mm diameter mycelial disks were removed from the edge of young cultures, one from isolated fungi and the other from phytopathogens, and inoculated 30 mm apart on Petri dishes containing PDA medium. The inoculated plates were incubated at 28°C for 15 days under 14 h photophase. The evaluations were made according to the Badalyan *et al.* (2002) scale.

Statistical analysis. All data were analyzed for significance ($P < 0.05$) using the SAS (version 8.1) software package [SAS] Statistical Analysis Systems (http://www.sas.com/en_us/home.html). A completely randomized analysis was employed in all assays. To quantify the *Colletotrichum* sp. L3 growth inhibition *in vitro* based on the diameter of mycelia pathogen growth towards the antagonistic fungus compared with control, we performed the Tukey's test. The same test was used to analyze the effect of VOCs produced by isolated fungi. This was determined by measuring the pathogen mycelial growth compared to the control. The percentage data were arcsin-square-root transformed to stabilize the variance, and Tukey's test was

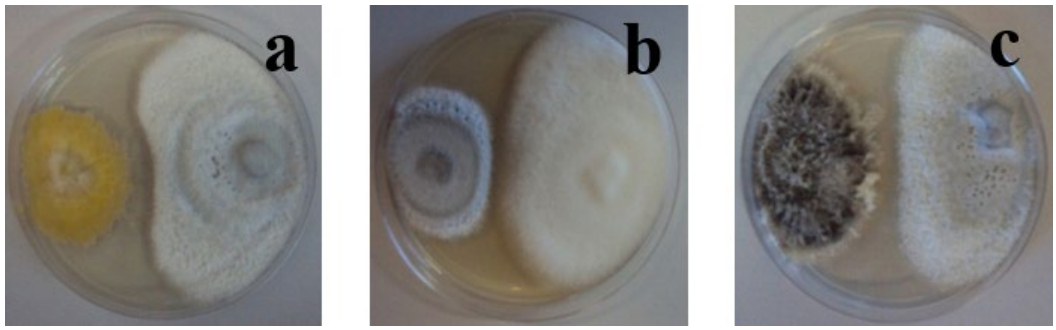

Fig. 2. *In vitro* antagonism of *Colletotrichum* sp. by **(a)** *Fusarium* sp. P3, **(b)** *Fusarium* sp. C10 and **(c)** *Microdochium* sp. P5 isolated from leave anthracnose's lesion after 14 days of incubation on PDA media at 28°C. The pathogen *Colletotrichum* sp. L3 is on the right.

also performed. Finally, for the *in vivo* assay, the reduction of anthracnose lesion size by fungal isolates was statistically tested against the positive control by Student's t-test. The asterisks in the figure indicated significant differences (*p-value* = 0.01). Bars in the figures represent the means +/– standard errors of the replicates.

RESULTS

Fungal isolation and identification. After 15 days of incubation on WA, 15 fungal isolates were obtained from the anthracnose lesions on guarana leaves. Between 1 or 2 fungal isolates were recovered from each lesion. According to the molecular identification by partial sequence of ITS1-5,8S-ITS2, all isolates belonged to the Ascomycota phylum; 13 fungi belonged to the Sordariomycetes class, and two isolates were from the Dothideomycetes class. The *Fusarium* genus was the most abundant (7 isolates), followed by *Phomopsis* (3 isolates), *Leptosphaeria* (2 isolates), *Microdochium* (2 isolates) and *Pestalotiopsis* (1 isolate). The *Fusarium* isolates clustered into two different clades; C5, C8, C10, C11 clustered with *F. polyphialidicum*, and C13, P3 and P2 with *F. decemcellulare*. Similar results were found with *Phomopsis* sp.; C4 and C9 clustered with *P. asparagati* and C7 with *P. chimonatini* (Fig. 1). *Microdochium* isolates (P5, P7) showed the highest similarity with the *M. phragmitis* and *Leptophaeria* isolates (B3, P2) with *L. proteicola*. The isolate *Pestalotiopsis* C3 did not differentiate between the species.

Antagonistic activity. Among the 15 isolates, 4 clearly inhibited L3 growth *in vitro*. *Microdochium* sp. (P7) showed the highest antagonistic activity, followed by *Fusarium* (C6 and C10) and then *Pestalotiopsis* (C3) (Fig. 2; Table 2). Based on the study by Badalyan *et al.* (2002) on the antagonistic activity scale, almost 87% of the fungi showed deadlock (mutual inhibition, where neither organism was able to overgrow the other); however, 46% showed deadlock at contact (A), 20% at distance (B) and for 13%, it was not possible to distinguish between A or B. Only the isolate *Leptosphaeria* (P2) showed partial replacement after initial deadlock with mycelia contact. After the mutual analysis of fungal growth inhibition measurements with the Badalyan *et al.* (2002) antagonistic activity scale, four isolates *Fusarium* sp. (C6, C10), *Pestalotiopsis* sp. (C3) and *Microdochium* sp. (P7) were selected as potential biocontrol agents and evaluated via an *in vivo* assay.

Table 2. Antagonistic activity of fungal isolates from leaves of guarana antrachnose's lesions against *Colletotrichum* sp. L3.

Isolates	Identification	Inhibition (%)*	Antagonistic ability**
C3	*Pestalotiopsis* sp.	14.3 b	A
C4	*Phomopsis* sp.	0.0 c	A
C5	*Fusarium* sp.	0.0 c	A
C6	*Fusarium* sp.	25.0 ab	A
C7	*Phomopsis* sp.	14.3 b	N.D.
C9	*Phomopsis* sp.	0.00 c	A
C10	*Fusarium* sp.	28.6 ab	A/B
C11	*Fusarium* sp.	0.0 c	B
C12	*Fusarium* sp.	0.0 c	N.D.
B3	*Leptosphaeria* sp.	0.0 c	A
P2	*Leptosphaeria* sp.	0.0 c	C
P3	*Fusarium* sp.	0.0 c	B
P4	*Fusarium* sp.	0.0 c	A/B
P5	*Microdochium* sp.	0.0 c	A
P7	*Microdochium* sp.	35.7 a	B

*Average of four replicates from the dual plate assay measuring pathogen inhibition growth, which was determined by measuring the diameter of pathogen growth towards the antagonistic fungus and compared with the control plate. Values with the same letter within a column are not significantly different (P > 0.01) according to Tukey's test.

**Antagonistic ability scale: type A and B - deadlock or mutual inhibition at mycelial contact or at distance, respectively, and type C - overgrowth between the fungi (replacement), without initial deadlock (according to Badalyan *et al.*, 2002).

Regarding the leaf bioassay, after 5 days, it was possible to verify typical lesions of anthracnose in all treatments. No lesions were observed in the control. Differences were found in the size of the lesions comparing the treatments and positive control (Fig. 3). The antagonistic fungus *Microdochium* sp. (P7) showed the highest reduction in mycelial growth for the pathogen in the *in vitro* assay; however, it showed no inhibition *in vivo*. Furthermore, the presence of *Fusarium* sp. isolates (C6 and C10) and *Pestalotiopsis* sp. (C3) significantly reduced the size of the lesion caused by the pathogen.

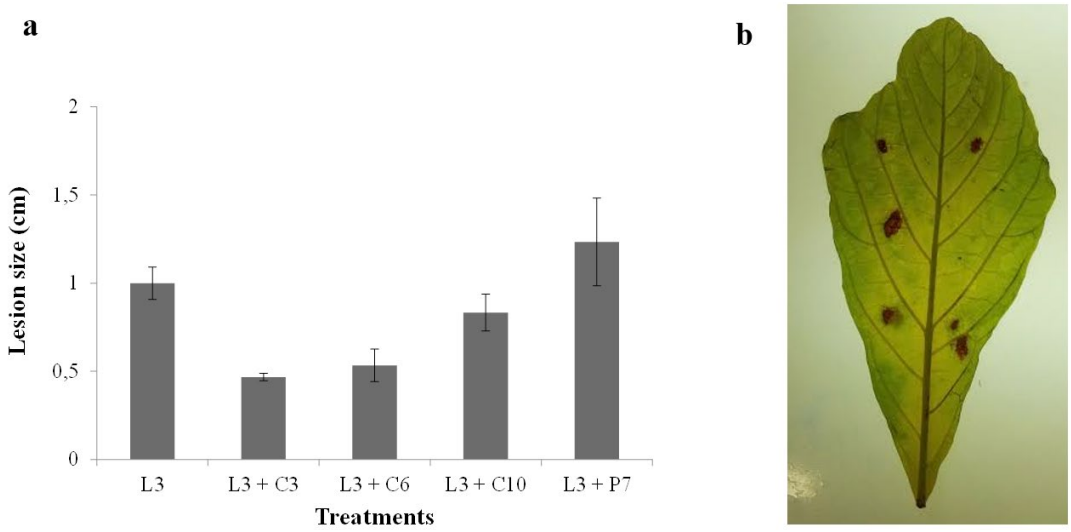

Fig. 3. Suppression of anthracnose symptoms caused by *Colletotrichum* sp. (L3), *Microdochium* sp. (P7), *Fusarium* (C6 and C10) and *Pestalotiopsis* sp. (C3) during the *in vivo* assay. **(a)** Lesion size (cm) during the co-inoculation of pathogen L3 and the antagonistic fungi. The results are expressed as the means +/- standard errors of five replicates. Values with asterisks (**) differ statistically compared with the control treatment (*p-value* = 0.01) according to Student's t-test. **(b)** Guarana leaf bioassay containing all treatments: L3 - only pathogen inoculation, L3 + P7 - pathogen and *Microdochium* sp. (P7), L3 + C6 - pathogen and *Fusarium* sp. (C6), L3 + C3 - pathogen and *Pestalotiopsis* sp. (C3), and NC - negative control (PDA medium disc).

Fungal growth inhibition mechanisms. *Chitinase assay.* Among the selected potential biocontrol agents only one isolate from *Fusarium* (C6) showed chitinolytic activity: 15 EA was detected using the CM-Chitin compound and 0.1375 EA using the *N*-acetyl-β-D-glucosaminidase compound.

Volatile organic compound production. Fusarium sp. (C10) showed the highest reduction of phytopathogen growth (26.04%) by VOCs, followed by *Fusarium* sp. (C6) (20.27%) and *Pestalotiopsis* sp. (C3) (5.32%) (Fig. 4).

Inhibition potential of phytopathogen. Among the selected isolates, *Pestalotiopsis* sp. (C3) and *Fusarium* sp. (C6 and C10) showed similar responses against *Fusarium* spp. phytopathogens, deadlock at mycelia contact (A) to *F. verticillioides* (FM) or at distance (B) to *F. oxysporum* (R2238) and *F. verticillioides* (T4). Two pathogens, *C. paradoxa* and *R. solani*, overgrew the three fungal isolates (Table 3).

DISCUSSION

In this study, 15 fungal species were isolated from guarana anthracnose leaf lesions, with *Fusarium* being the most abundant genus, followed by *Phomopsis, Microdochium, Pestalotiopsis* and *Leptosphaeria* that by diverse mechanisms of inhibition fungal growth, showed different degrees in the control of *Colletotrichum* sp. *in vitro* and *in vivo* assay. All of these genera are defined as endophytes in many plants worldwide (Fröhlich and Hyde, 1999; Cannon and Simmons, 2002; Morakotkarn *et al*., 2007).

Promputtha *et al.* (2007) studied the fungal community from *Magnolia liliifera* and reported the dual behavior of the endophytic and saprophytic states of *Fusarium* and *Phomopsis* isolates that changed their ecological strategies during host senescence. Recently, Sia *et al*. (2013) reported the isolation of the endophytic fungi *Pestalotiopsis* sp. and *Phomopsis* sp. from healthy guarana leaves, suggesting that the fungi isolated in this study may also change their ecological role from that of an endophyte to that of a saprophyte.

To our knowledge, all isolated fungi from guarana anthracnose lesions were previously described as saprophytes, except *Leptosphaeria* sp. This genus has been commonly reported to be a pathogen, causing blackleg in crucifers (Goodwin and Annis, 1991; West *et al*., 2001).

Some saprophytes have been considered biological control agents (Przybyl, 2002; Kaur *et al*., 2011; Arriagada *et al*., 2012). We also observed that three isolated fungi from anthracnose lesions on guarana leaves clearly showed an *in vivo* potential to control the phytopathogen *Colletotrichum* sp., highlighting the genera *Fusarium* and *Pestalotiopsis*. Both genera were previously reported to be biocontrol agents: *F. equiseti* reduced cucumber anthracnose severity caused by *C. orbiculare* (Saldanejo and Hyakumachi, 2011), and *P. neglecta* was reported to be an antagonist fungus against anthracnose disease in apricots (Adikaram and Karunaratne, 1998).

Interestingly, *Pestalotiopsis* sp. (C3) showed the lowest *Colletotrichum* growth inhibition *in vitro*, but the *in vivo* assay showed it as the most promising for the control of guarana anthracnose. It had a low production of VOCs and no detectable chitinase activity, suggesting that other mechanisms may be involved in its disease control strategy.

Fungi may interact with phytopathogens as agents of control via a diverse range of mechanisms, such as i) competition for nutrients and space, ii) the production of

Table 3. Evaluation of the antagonistic ability of fungi isolated from anthracnose lesions on guarana leaves.

Phytopathogen	Fungal isolates's antagonistic ability*		
	Pestalotiopsis sp. (C3)	*Fusarium* sp. (C6)	*Fusarium* sp. (C10)
Ceratociste padadoxa	C	C	C
Fusarium verticillioides (FM)	A	A	A
Fusarium oxysporum	B	A	B
Fusarium verticillioides (T4)	A/B	B	B
Rhizoctonia solani	C	C	C

*Antagonistic ability scale according to Badalyan *et al.* (2002): type A and B - deadlock or mutual inhibition, at mycelial contact or at distance, respectively; type C - overgrowth between the fungi (replacement), without initial deadlock.

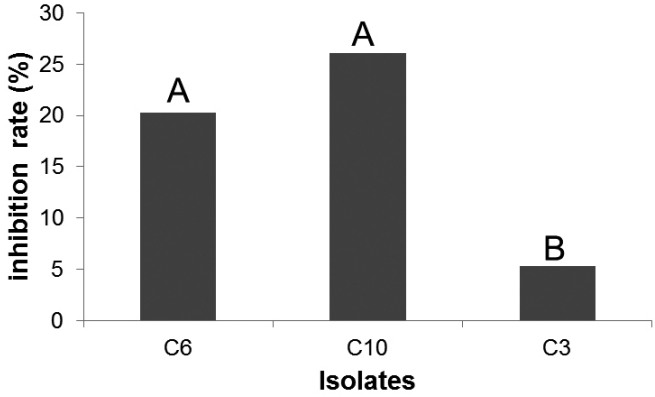

Fig. 4. Effect of volatile organic compounds from antagonistic fungi on the mycelial growth of *Colletotrichum* sp. The results are expressed as the means +/– standard errors of four replicates for each fungus. Values with the same letter are not significantly different (P > 0.01) according to Tukey's test.

antifungal compounds (metabolites, antibiosis), iii) parasitism, and iv) the induction of plant defense mechanisms (Schoeman *et al*., 1999; Alabouvette *et al*., 2006; Heydari and Pessarakli, 2010). We evaluated the role of two related compounds involved in biological control success: chitinases and VOCs.

Chitinase production has been considered one of the major mechanisms for the control of plant pathogenic fungi (Berg, 2009). This enzyme may interfere with fungal growth and development by hydrolyzing chitin, a constituent of the fungal cell wall (Collinge *et al*., 1993). Many studies have demonstrated the potential of chitinase against plant pathogenic *Colletotrichum spp.* (Mathivanan *et al*., 1998; Sandhya *et al*., 2005; Quecine *et al*., 2008). The only fungal isolate able to produce chitinase, *Fusarium* sp. (C6), showed greater control of the pathogen during the *in vitro* and *in vivo* assays.

Another important mechanism related to biological control is the production of low molecular weight VOCs. These compounds may inhibit fungal growth or even kill the fungus (Strobel *et al*., 2001). The authors described the fungus *Muscodor albus* as a producer of VOC mixtures. Those compounds inhibited the growth of *R. solani, Ustilago hordei, Sclerotinia sclerotiorium* and others. The authors observed that each organic class had some inhibitory effects, but none were lethal. However, collectively, the compounds acted synergistically to promote the death of plant-pathogenic fungi (Strobel *et al*., 2001). Minerdi *et al.* (2009) reported a non-pathogenic *F. oxysporum* capable of producing VOCs. This fungus was able to decrease mycelial growth of the pathogenic *F. oxysporum*, and the *Pestalotiopsis* spp. were also reported to be VOC producers capable of inhibiting different species of plant pathogens (Li *et al*., 2001; Strobel *et al*., 2001). Out of all the tested fungi isolated from the lesions, only *Fusarium* sp. C6 was able to produce chitinase; however, *Fusarium* sp. (C6 and C10) and *Pestalotiopsis* sp. (C3) were able to produce VOCs that inhibited the growth of *Colletotrichum* sp. Interestingly, *Fusarium* sp. (C6 and C10) and *Pestalotiopsis* sp. (C3) also inhibited the growth of *Fusarium* spp., which is pathogenic to sugarcane, pea and corn. Previously, *Pestalotiopsis* spp. were reported as potential biocontrol agents against *Fusarium* head blight of wheat and rotting in maize (Danielsen and Jensen, 1999). These data suggest that the isolates employ different mechanisms to control the pathogens, with VOC production as a key mechanism involved in the biological control.

Bogas *et al.* (2015) studied the bacterial community from guarana and its correlation with anthracnoses; however, this study was the first to describe the isolation of other fungi from anthracnose lesions on guarana leaves and the bioprospection of biocontrol agents to anthracnose. Some of the fungal genera were previously reported to be saprophytes in other plant species. Among our isolates, four were clearly able to inhibit the growth of the pathogen *Colletotrichum* sp *in vitro*. Three of them also inhibited the pathogen during *in vivo* assays. Our study strongly suggests that fungi may act alternatively as saprophytes-endophytes and have potential for the biological control of guarana anthracnose and other plant diseases. Moreover, the results of this study led us to conclude that VOCs produced by saprophytes-endophytes have a key role in controlling plant pathogenic fungi. The research may be improved with further evaluations of the control of anthracnose's under field conditions as well as the design of a fungi biocontrol formula for field application.

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