Isolation, identification and characterization of endophytic fungi of *Bambusa oldhamii* munro applied as antagonists to *Pyricularia oryzae*[1]

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ABSTRACT

Endophytic fungi, in addition to improving plant development, may produce antimicrobial substances that can inhibit pathogens. The objective of this study was to isolate and characterize endophytic fungi of *Bambusa oldhamii* and evaluate *in vitro* antagonism to *Pyricularia oryzae*. Fungal isolates of *B. oldhamii* shoots were purified and identified by nuclear ribosomal DNA (nrDNA) including internal transcribed spacer regions (ITS1 and ITS4). These isolates were then confirmed by phylogenetic analysis of the ITS1 and ITS2 sequences and morphological analysis. The isolates were evaluated in *in vitro* experiments of direct and indirect antagonism (volatile production). Five fungal isolates were identified and named as follows: isolate 27 (*Arthrinium* sp.); isolate 29 (*Acrocalymma* sp.); isolate 122 (*Botryobambusa fusicoccum*); isolate 711 (*Phoma* sp.) and isolate 712 (*Phoma sp.*). We found that the area of the *P. oryzae* colony was reduced by more than 80% by all endophytic isolates in this study, with emphasis on isolate 122, with a 96% reduction in area of the *P. oryzae*. These results were considered promising and will serve as a foundation for future studies on induction of resistance to *P. oryzae*.

Keywords: antagonism; antimicrobial activity; bamboo; bambusicolous fungi.

INTRODUCTION

Endophytic fungi are microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease. This relationship benefits the host in several ways, protecting it from biotic and abiotic stress (Selim *et al*. 2012). This relationship includes secretions of substances by endophyte, which are biologically active metabolites which promote antagonistic action against phytopathogens (Zhang *et al*. 2006; Sudha *et al*. 2016).

Bambusicolous fungi (formerly fungorum bambusicolorum) are species that have association with bamboo species (Dai *et al*. 2017). This group is estimated to include more than 1,450 species in approximately 115 genera around the world. They include ascomycetes, basiomycetes, coleomycetes, hyphomycetes and anamorphic fungi (Shukla *et al*. 2016; Dai *et al*. 2017). These fungi, are important because of the compounds they produce, such as metabolites with antioxidant, antimicrobial and antitumor properties (Oyetayo *et al*. 2009). Therefore, research involving the identification and characterization of species associated with bamboo is necessary in order to use these compounds in the biocontrol of pathogens in cultivated plants in the future (Oyetayo *et al*. 2009; Ebada *et al*. 2011; Li *et al*. 2012).

Currently, there is high demand for biological agents for disease control in various crops. Excessive use of pesticides for pathogen control in agriculture generates a series of environmental problems such as soil and groundwater contamination by heavy metals (Lu *et al*. 2015). Rice blast is the major disease in rice cultivation, caused by the fungus *Pyricularia oryzae* B. C. Couch [anamorph - *Magnaporthe oryzae* Cavara]. Under

favorable conditions, rice blast can cause crop losses of up to 100% (Prabhu *et al*. 1992; Prabhu & Filippi 2001; Prabhu *et al*. 2009). For disease control, in addition to the use of fungicides, integrated management by means of farming practices is also necessary (Prabhu & Filippi 2001).

P. oryzae has a cell wall composed of melanin, which is important for the mechanism of pathogen infection, which involves the formation and peg penetration of the appressorium. Melanin plays a structural part in strengthening the appressorium and forms an impermeable layer to prevent leakage of osmolytes, which generate the substantial internal turgor (Wilson & Talbot 2009). Thus, the loss or blockade of melanin in the colony also implies the loss of pathogenicity (Yan & Talbot 2016; Oses-Ruiz *et al*. 2017). Thus, the development of biological control techniques that provide blockade of melanin in *P. oryzae* colonies could provide improvements in rice production. Research involving antagonist relations between endophytes and pathogens is necessary for future search for novel natural products (Nisa *et al*. 2015), as well as the identification of bioactive compounds that could be used in research involving induction of resistance in plants (Busby *et al*. 2016).

When it comes to interactions involving plants and microorganisms, fungal endophytes are involved in multiple balanced antagonisms. *In planta*, endophytic fungi must deal with multiple organismal interactions. This relationship involves anti-fungal metabolites produced by endophytic fungi, which have the function of maintaining balances of antagonisms with microbial competitors, resulting in a compatible multipartite symbiosis (Schulz *et al*. 2015). Therefore, research involving identification of endophytic fungi and their antagonisms between *P. oryzae* are necessary in order to develop natural products in the future that may be inhibitors of pathogen growth under natural conditions, or even use these endophytes as growth resistance inducers. Thus, the objective of this study was to identify and morphologically characterize endophytic fungi of *Bambusa oldhamii* Munro and to evaluate possible antagonism to *P. oryzae*, for use in future studies on resistance and plant growth promotion.

MATERIALS AND METHODS

Isolation of endophytic fungi

Secondary shoots of *B. oldhamii* measuring 55 - 60 mm were collected in March 2016 at the Bamboo Collection of the Federal University of Goiás (Universidade Federal de Goiás), located in Goiânia, Goiás state, Brazil. The shoots were sectioned into 50 mm fragments, surface washed with neutral detergent solution (50 μL per 100 mL of water) and, after triple washing, immersed in sodium hypochlorite solution (0.0025% active chlorine) and stored

at 5 ºC for 24 h. In a laminar flow chamber, asepsis was performed with Tween 80® solution (50 μL per 100 mL) for 10 min, 70% ethanol for 5 min and sodium hypochlorite (2.5% active chlorine) for 15 min, followed by triple washing in deionized and autoclaved water after each step. After asepsis, the shoots were immersed in a 5% solution of Plant Preservative Mixture® (PPM) biocide for 24 h. Then, the samples were cut into 30 mm fragments and transferred to test tubes containing MS medium (Murashige & Skoog, 1962) supplemented with 30% sucrose, 0.45 μM thidiazuron (TDZ) (Lin *et al*. 2007), 0.2% PPM® and 103.5 μM kanamycin. The test tubes containing the shoots and culture medium remained in the dark for 15 days and then were incubated for 15 days in the growth room under cold white light with light intensity of 35 μmol m^{-2} s^{-1} and a photoperiod of 16 h light.

After 30 days, the fungal colonies grown in the ten test tubes were transferred twice consecutively to Petri dishes containing potato dextrose agar (PDA) medium for purification of the colonies. For this, the mycelium suspension was used. This suspension was spread on Petri dishes (containing 30 ml of PDA medium) with the aid of a glass stick. The color, texture and growth form of the mycelium were observed for purification, as well as the morphological characteristics in microscopic, in order to confirm the purification. In all, five distinct colonies were observed, and the dishes were stored in a growth room at 28 ± 1 ºC with a photoperiod of 12 h of light.

DNA extraction and isolate identification

The mycelium was scraped and the DNA was extracted from the fungal isolates (Dellaporta *et al*. 1983). The extraction quality was measured using a NanoDrop™ 2000 spectrophotometer and, after diluting the DNA to 20 ng/μl, the samples were amplified and sequenced by BPI Biotecnologia Pesquisa e Inovação. The DNA samples were amplified using primers for sequences from internal transcribed spacer genomic regions (ITS1 and ITS4) (White *et al*. 1990; Manter & Vivanco 2007). The amplified samples were evaluated on 2% agarose gels, stained with 0.03% (v/v) UniSafe Dye, visualized under UV light and quantified by fluorometry (Qubit, ThermoFisher). For the sequencing reaction, a BigDye® Terminator v3.1 and Cycle Sequencing Kit (Applied Biosystems) were used. For the precipitation reaction, ethanol/EDTA/sodium acetate were used, according to the protocol suggested by the manufacturer. Automated capillary electrophoresis sequencing was performed on a ABI3500 Genetic Analyzer (Applied Biosystems).

For molecular identification of the isolates, the resulting sequences were subjected to a BLAST search with the NCBI database (http://www.ncbi.nlm.nih.gov/) and were confirmed by phylogenetic analysis of the ITS1-

5.8S-ITS2 regions. These methods were combined with morphological analysis for isolates in which conidiogenesis was observed in the culture media (described in the next section) based on dichotomous keys and updated revisions (Barnett & Hunter 1998; Shukla *et al*. 2016; Dai *et al*. 2017). The ITS 1 and 2 sequences were aligned via ClustalW using the software MEGA version 7 (Kumar *et al*. 2016). The tree was constructed using the Maximum Likelihood method (Nei & Kumar 2000). Bootstrap analysis (10,000 replicates) was used as statistical support. The initial trees were obtained by applying the Neighbor-Join and BioNJ algorithms (Saitou & Nei 1987). Only sequences published in journals were used, and the sequences resulting from this study were deposited into GenBank.

Morphological characterization of colonies

A 5-mm mycelial disc of each purified fungal colony was transferred to Petri dishes (90 x 90 mm) containing 30 mL of different culture media to induce different morphologies, radial growth rates, and conidiogenesis. The media used were PDA [potato (200 g); dextrose (20 g); agar (20 g)], CMA [corn Meal (30 g); dextrose (10 g); agar (20 g)], OA [oats (40 g); dextrose (20 g); agar (20 g)]; and TSA [tomato (30 g); sucrose (20 g); agar (20 g)]. The dishes containing the culture media and segments of fungal colonies were sealed with PVC film and incubated in a growth room at 28 ± 1 °C with a photoperiod of 12 h of light.

The growth rate of the isolates (radial growth) was calculated as the mean value obtained with the equation: PRG – CRG/CRG, where PRG refers to the radial growth of the previous evaluation and CRG to the current radial growth. The evaluation was performed every 2 days, and the quantitative data were subjected to analysis of variance and the means were compared by the Tukey test at the 5% significance level.

The evaluation of colonies, color and the presence or absence of conidiogenesis was performed. In addition, the mycelium of isolates was observed under an optical microscope to characterize the hyphae and conidia. For the microscopic observation of the isolates, slides were prepared with fungal mycelium, 10 µL of deionized and distilled water and 1 µL of methylene blue at a concentration of 0.1% to stain hyaline hyphae. Observation was performed using an optical microscope coupled with a digital camera.

Antagonism between endophytic isolates of bamboo and the fungus Pyricularia oryzae

Mycelial discs of bamboo fungal isolates measuring 7 mm in diameter were placed in dishes containing 30 mL of PDA culture medium according to the circle method (Da Luz, 1990), in which discs of the two isolates are positioned equidistant from the dish edges. The dishes containing only *P. oryzae* mycelial discs placed in the center of the dish were used as controls. The dishes were incubated (28 ± 1 °C with a photoperiod of 12 h light) until full growth of the control was achieved (10 days). Next, the radial mycelium growth of *P. oryzae* on each dish was calculated (Cattelan 1999).

Mycelial discs measuring 5 mm in diameter of each endophytic isolate were placed in the center of Petri dishes (90 x 90 mm) containing 30 mL of PDA culture medium. *P. oryzae* mycelial discs grown in PDA culture medium were also placed in the same way in another Petri dish. The lids of all Petri dishes were removed, and the bases of all dishes containing endophytic isolates (antagonist) and the pathogen (*P. oryzae*) were combined, forming an experimental unit. Dishes containing only *P. oryzae* mycelial discs were used as controls. The dishes were incubated (28 ± 1 °C with a photoperiod of 12 h light) until full growth of the control was achieved. Next, the radial mycelium growth of *P. oryzae* on each dish was calculated (Dennis & Webster 1971; Cattelan 1999).

In both the direct and indirect antagonism experiments, the experimental design was completely randomized, with 7 treatments composed of 7 fungal isolates and 4 replicates. The data were subjected to Box-Cox transformation to meet the assumptions of the analysis of variance, and the means were compared by the Tukey test at the 5% significance level. The data were analyzed using R version 3.4.3 (R Core Team 2017).

The percentage of growth inhibition (PGI) in direct and indirect antagonism was calculated according to the equation (ND – ED)/ND x 100 (Shen *et al*. 2012), where ND refers to the diameter of the negative control, i.e., the *P. oryzae* isolate grown without the interference of any antagonist, and ED refers to the diameter of *P. oryzae* when subjected to the antagonism of a *B. oldhamii* endophytic isolate.

RESULTS

Morphological characterization

Morphological analysis of colonies in different culture media revealed several morphotype for isolates. (Table 1 and Figure 1). Isolate 27 showed conidiogenesis and chlamydospores in CMA medium (Figure 2). Morphological analysis of this isolate showed brown conidiogenous cells, aggregated or not, globose or lenticular subglobose, measuring 6.83 - 10.5 µm x 2.33 - 6.83 µm (width x length). Hyphae were hyaline, measuring 1.62 - 0.69 µm. This isolate showed clustered oblong chlamydospores measuring 2.00 - 6.93 µm x 4.02 - 1.33 µm (width x length).

Isolate 29 showed conidiogenesis in CMA medium and hyaline, oblong, conidiogenous cells, individual or clustered, measuring 1.62 - 1.78 µm x 1.40 - 1.71 µm. The hyphae were thin and hyaline, measuring 1.03 - 1.61 µm. Isolate 122 presented chlamydospores in OA culture medium. These presented oblong, black, multinucleate cells measuring 4.02 – 6.08 µm x 2.03 – 3.4 µm. The hyphae were black, septate, uni- or multinucleate, with diameters of 3.33 - 6.16 µm (Figure 2).

Conidiogenesis of isolate 711 was observed in CMA medium. The conidia were of the pycnidia type (Figure 2), measuring 31.87 - 100 µm x 81.25 - 100 µm (width x length), and had glabrous, globose, blackish brown ostioles (dimensions 12.5 - 87.26 µm) and a brown peridium. Hyphae were septate, uni- or multinucleate, with dimensions of 9.3 - 19.7 µm. The CMA culture medium also favored the formation of chlamydospores in isolate 712 (*Phoma* sp.). These measured 28.13 x 25.73 µm, had a red-brown catenulate peridium, and were glabrous, globose or subglobose, individual or clustered (Figure 2). The hyphae were septate, glabrous, uni- or multinucleate, measuring between 6.5 - 35.5 µm.

Table 1: Morphological characteristics of the colonies of the endophytic isolates of *Bambusa oldhamii* evaluated in different culture media

Isolate	Characteristics	PDA	OA	CMA	TSA
27	Color	White	White	Hyaline	Black/White
	Colony aspect	Cotton-like	Lumpy	Lumpy	Lumpy
	Growth	Aerial	Aerial	Sparse	Aerial
29	Color	White	Grey	Hyaline	White
	Colony aspect	Cotton-like	Cotton-like	Cotton-like	Cotton-like
	Growth	Sparse	Sparse	Sparse	Sparse
122	Color	Grey/Black	Gray	Brown	Black/White
	Colony aspect	Cotton-like	Lumpy	Lumpy	Lumpy
	Growth	Dense aerial	Sparce	Sparse	Aerial
711	Color	White - Straw yellow	White	Brown	Grey/White
	Colony aspect	Cotton-like	Cotton-like	Cotton-like	Lumpy
	Growth	Aerial	Aerial	Sparse	Aerial
712	Color	Pink/red	Pink/red	Black	Straw yellow/black
	Colony aspect	Lumpy	Lumpy	Lumpy	Lumpy
	Growth	Aerial	Sparse	Sparse	Sparse
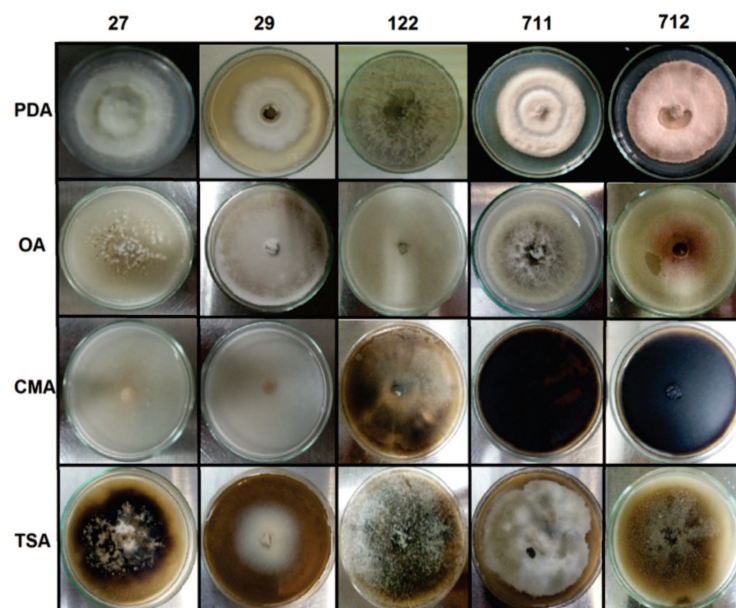
Figure 1: Morphological characterization of the endophytic isolates of *B. oldhamii*: *Arthrinium* sp. (27); *Acrocalymma* sp. (29); *Botryobambusa fusicoccum* (122); *Phoma* sp. (711); and *Phoma* sp. (712); submitted to different culture media: Potato dextrose agar (PDA), oatmeal agar (OA), corn meal agar (CMA), and tomato sucrose agar (TSA).

Among the different culture media evaluated, the CMA medium significantly favored the growth of isolates 711 and 712. However, no significant difference was observed between this culture medium and the PDA and TSA media regarding colonies of isolate 122. For isolates 27 and 29, all culture media favored colony growth, with no significant difference among them.

Isolate 122 presented faster growth than the other fungi (Table 2) and achieved full growth in the Petri dish (90 x 90 mm) after approximately 48 h in PDA medium. By contrast, isolate 29 (*Acrocalymma* sp.) presented the slowest mycelial growth in the culture evaluated media (Table 2).

Molecular identification

After purification of the mycelium, five isolates were obtained, which were called isolates 27, 29, 122, 711 and 712. Amplification of the ITS1-5.8S-ITS2 region with primers ITS1 and ITS4 produced fragments of approximately 580 bp for isolates 27 and 122; 510 bp for isolate 29; 500 bp for isolate 711; and 550 bp for isolate 712.

The ITS sequences obtained from the isolates were analyzed using NCBI BlastN from GenBank. There was 100% similarity for isolate 27 with *Arthrinium marii* (accession numbers CBS 200.57 and CPC 18902) and *A. sacchari* (accession number CBS:664.74). Isolate 29 showed 93% similarity with both *Acrocalymma vagum* (accession 167) and *A. medicaginis* (accession CPC 24340). Isolate 122 showed 99% similarity to *Botryobambusa fusicoccum* (accession MFLUCC 11 C0657) and 96.55% similarity to *Diploidia rosulata* (accession CBS 116470). Isolates 711 and 712 showed 100% and 99% similarity to *Phoma* sp. accessions EA-122 and CB-R-4, respectively.

Analysis of the ITS1-5.8S-ITS2 homologous sequences of each endophytic isolate was conducted, resulting in a phylogenetic tree with four families. The bootstrap percentage of the taxa is shown next to the branches (Figure 3). A separate branch was observed for isolate 29 (*Acrocalymma* sp.) within its respective taxon, indicating a previously unidentified species of *Acrocalymma* (Figure 3). The ITS1 and ITS2 sequence data of isolates were submitted in GenBank. The accession numbers for the nucleotide sequences were: MN381104 for isolate 27; MN381026 for isolate 29; MN381103 for isolate 122; MN381110 for isolate 711; and MN383185 for isolate 712.
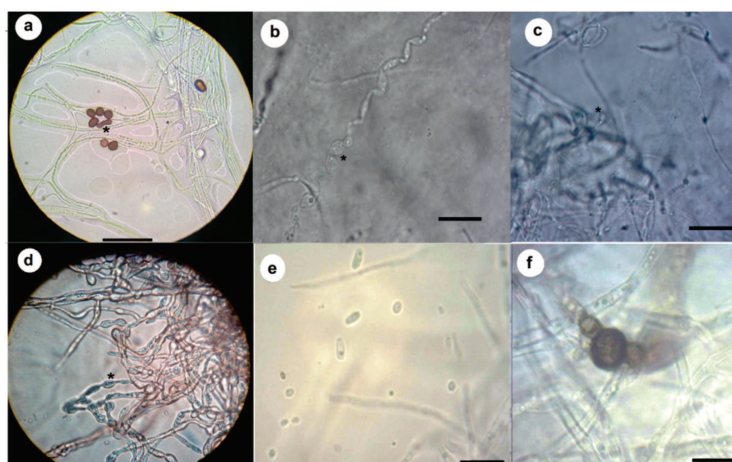
Figure 2: (a) and (b): Isolate 27 (*Arthrinium* sp.), with emphasis on conidiogenous cells (asterisk) (a), and the chlamydospores (asterisk) (b). (c): conidiogenous cells (asterisk) and hyphae of isolate 29 (*Acrocalymma* sp.). (d): Chlamydospores (asterisk) of isolate 122 (*Botryobambusa fusicoccum*); (e) Immature pycnidia of isolate 711 (*Phoma* sp.). Chlamydospores (f) of isolate 712 (*Phoma* sp.). Magnification of 40x in (a), (b), (c) and (d), and 100x in (e) and (f). Bar = 10 µm in (a), (b), (c) and (d) and 100 µm in (e) and (f).

Table 2: Growth rate (%) per day (mean ± standard error) of endophytic fungi of *Bambusa oldhamii* in different culture media: potato dextrose agar (PDA), oatmeal agar (OA), corn meal agar (CMA), and tomato sucrose agar (TSA)

Isolate	Genera	PDA	OA	CMA	TSA
27	*Arthrinium* sp.	22.43 ± 5.42bA	16.68 ± 1.44bA	37.77 ± 12.2abA	20.31 ± 0.6bA
29	*Acrocalymma* sp.	09.13 ± 0.59cA	10.10 ± 1.01bA	12.02 ±2.03 cA	11.34 ± 0.07 cA
122	*Botryobambusa fusicoccum*	49.22 ± 0.84aA	40.50 ± 0.07 aB	50.00 ± 0.01aA	50.00 ± 1.00aA
711	*Phoma* sp.	10.91 ± 1.30bcD	11.33 ± 1.27bCD	38.80 ± 1.90abA	29.76 ± 1.44bB
712	*Phoma sp.*	12.62 ± 2.77bcB	9.58 ± 2.40bB	36.57 ± 3.4abA	30.41 ± 3.97bAB

* means followed by the same lowercase letter vertically for analysis of isolates and uppercase letter horizontally for analysis of culture media are not significantly different by the Tukey test at a significance level of 5%.

Antagonism between endophytic fungi and *Pyricularia oryzae*

The mycelial growth of *P. oryzae* was reduced by all isolates in direct antagonism, with emphasis on isolate 122 (*B. fusicoccum*) (Figure 4), showing a probable competition-type interaction. Similarly to direct antagonism, the volatile components (Figure 5) produced by the isolates under study reduced the area of the *P. oryzae* colony, with reductions of over 80% (Table 3 and Figure 6), showing a probable inhibition-type interaction. The phenotypes of *P. oryzae* colonies were altered, with loss (total or partial) of the black color (melanin).

DISCUSSION

Identification of the genera of endophytes of *B. oldhamii* was successful. Isolate 27 (*Arthrinium* sp.) belongs to a genus that includes saprophytic species with sexual forms called Apiospora. This species is reported to be a pathogen of bamboo (Martínez-Cano *et al*. 1992), an endophyte (Ramos *et al*. 2010) and a lichen (He & Zhang 2012). Isolate

29 was identified as belonging to the genus *Acrocalymma*, which includes saprophytic and phytopathogenic species, and includes *A. medicaginis* (Alcorn & Irwin 1987). Isolate 122 was identified as belonging to the genus *Botryobambusa*, whose sole member is the saprophyte *B. fusicoccum*. This species is commonly associated with bamboo, especially of the genus *Bambusa* (Liu *et al*. 2012). Isolates 711 and 712 were identified to the genus *Phoma*, a diverse genus that includes, in addition to growth-promoting microorganisms (Hamayun *et al*. 2009), phytopathogens, entomopathogens, soil saprophytes, aquatic saprophytes and opportunistic human pathogens (Rai *et al*. 2009). Endophytic fungi of *B. oldhamii* were previously reported by Pasqualini *et al*. (2019), which report that research associated with the discovery of these isolates may increase the current knowledge about the diversity of fungi associated with bamboo. They report the isolation of phylogenetically similar isolates to those found in the present paper, which include: *Phoma herbarum, Arthrinium kogelbergense, Arthrinium arundinis, Fusarium graminearum, Wojnowiciella leptocarpi,* and *Fusarium* sp.
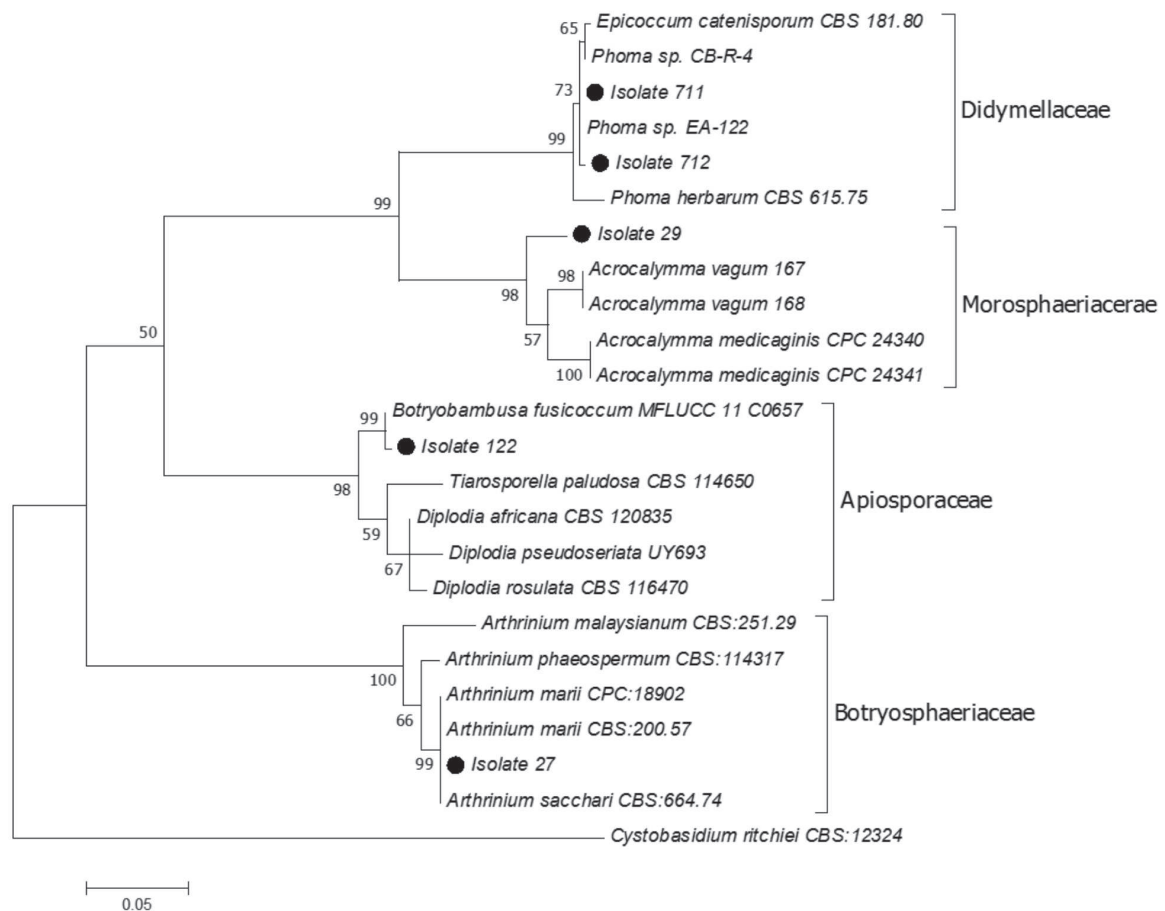

Figure 3: Phylogenetic tree of the ITS 1-5.8S-ITS2 homologous sequence from five endophytic isolates of *Bambusa oldhamii* (Log = -2149.26) and other nineteen nucleotide sequences. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Isolate 712: *Phoma* sp.; Isolate 711: *Phoma* sp.; 29: *Acrocalymma* sp.; 27: *Arthrinium* sp.; and 122: *Bobryobambusa fusicoccum*. *Cystobasidium ritchiei* (CBS:12324) is presented as an outgroup.

The phylogenetic position of isolates 711 and 712 (*Phoma* sp.) can still be investigated in the future. In fact, the taxonomic circumscription of the *Phoma* genus has been complex (Chen *et al*. 2015; Valenzuela-Lopez *et al*. 2018). *Phoma* is ambiguously defined and is subdivided into nine sections based on morphological characters. These sections are classified into four different families, with strains genetically similar to *P. herbarum* belonging to the family Didymellaceae, which includes the genus *Epicoccum* (Jayasiri 2017). For this reason, *Phoma* constitutes a polyphyletic group (Gruyter *et al*. 2009). Despite the efforts of researchers to characterize the group (Chen *et al*. 2015), there are still gaps in its systematics, both with regard to morphological and molecular characters because many species still remain in nonspecific clades (Aveskamp *et al*. 2008). Thus, sequencing of additional genomic regions and of isolates similar to isolates 711 and 712 are necessary to better understand the phylogenetic relationships and species boundaries.

The mycelial growth rate and conidiogenesis of the endophytic isolates were influenced by the different culture media evaluated. The nutrient composition of a given culture medium is an important factor that influences the growth rate, as it allows the fungi to grow without restrictions and express phenotypes (Meletiadis *et al*. 2001). Sharma and Pandey (2010) used different fungal isolates in different culture media and found that the best culture medium for conidiogenesis of the genera *Chaetomium*, *Fusarium*, and *Penicillium* was LCA medium (glucose, 1 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.2 g;
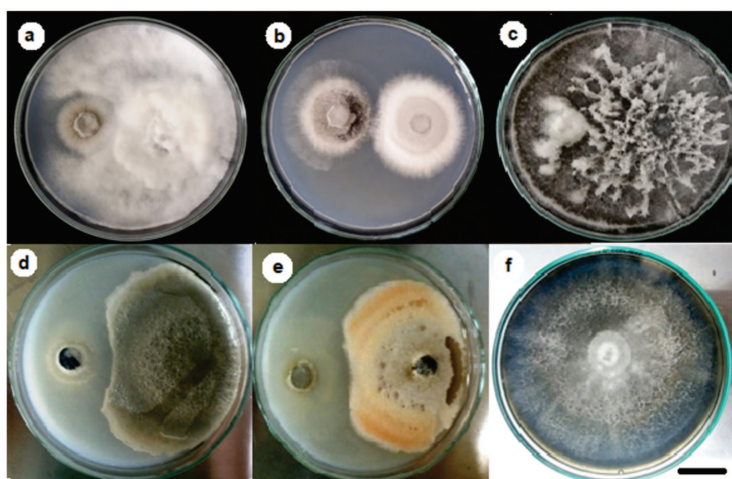
Figure 4: Direct antagonism between endophytic fungi of *Bambusa oldhamii* (colonies to the right of the dish) and *Pyricularia oryzae* (colonies to the left of the dish) at ten days of growth (a): 27 (*Arthrinium* sp.); (b): 29 (*Acrocalymma* sp.); (c): 122 (*Botryobambusa fusicoccum*); (d): 711 (*Phoma* sp.); (e): 712 (*Phoma* sp.); (f): Control without the antagonist. Bar = 20 mm
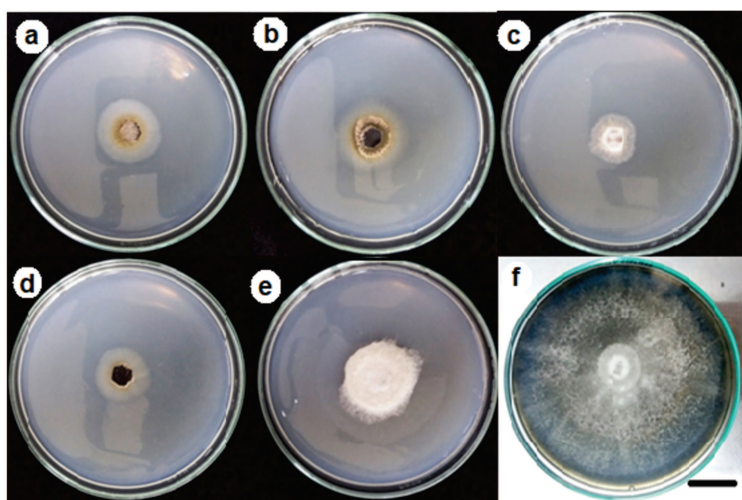
Figure 5: *Pyricularia oryzae* under indirect antagonism involving endophytic fungi of *Bambusa oldhamii* (volatile production) at ten days of growth. (a): 27 (*Arthrinium* sp.); (b): 29 (*Acrocalymma* sp.); (c): 122 (*Botryobambusa fusicoccum*); (d): 711 (*Phoma* sp.); (e): 712 (*Phoma* sp.); (f): Control without the antagonist. Bar = 20 mm.

KCl, 0.2 g; NaNO$_3$, 2 g; yeast extract, 0.2 g; agar, 13 g; distilled H$_2$O, 1 L) which is a culture medium with reduced amounts of nitrogen and carbohydrates, which may induce conidiogenesis (Miura & Kudo 1970). In the present study the CMA medium was evaluated, which contains significant amounts of carbohydrates, vitamins and salts. However, not sufficiently rich in amino acids, which are sources of nitrogen (Gernah *et al*. 2011). Thus, probably the conidiogenesis was influenced by the lack of nitrogen in the culture medium. However, further studies should be performed to substantiate this claim.

Using the direct antagonism assay, we found that the area of the *P. oryzae* colony was reduced by more than 80% by all endophytic isolates in this study. The interactions involved with biological control include competition, which comprises direct antagonism and is the result of physical contact between the biocontrol organism and the pathogen, in which there is a high degree of selectivity for the pathogen via the mechanism or mechanisms expressed by the biocontrol microorganisms (Harman *et al*. 2004). These mechanisms include the secretion of enzymes with antimicrobial activity, including PPOs, chitinases, and glucanases (Brzezinska & Jankiewicz 2012; Naznin *et al*. 2014). Probably, this metabolite

secretion inhibited the production of melanin by *P. oryzae* colonies, when they were submitted to treatments with endophytic isolates. Under natural conditions, *P. oryzae* needs melanin for activation of pathogenicity mechanisms (Carvalho *et al*. 2015; Oses-Ruiz *et al*. 2017). Therefore, further *in vivo* research should be performed to confirm the antagonistic potential of the isolates of the present study.

Using the indirect antagonism assay, we also observed high inhibition of the *P. oryzae* colony area by all isolates studied (above 80%). This result indicates that these endophytes are likely stimulators of defense mechanisms in plants, which can be clarified in future studies on resistance induction (Harman *et al*. 2004; Silva *et al*. 2004).

The antagonistic activity of endophytes of bamboo was reported by (Shen *et al*. 2012), who investigated the antibiosis of endophytes isolated from a *Phyllostachys* species (*Cladosporium* sp., *Curvularia* sp., *Penicillium* sp., and *Didymella* sp.) to *Pleospora herbarum* and *Botryotinia fuckeliana*, the latter considered a bamboo pathogen (Mohanan 1997). In addition to these, the authors also reported antibiosis of these endophytes with *Candida albicans* and *Staphylococcus aureus*, both clinical pathogens.

Table 3: Colony area of *Pyricularia oryzae* resulting from direct and indirect antagonism with endophytic isolates of *Bambusa oldhamii*

Isolate	Direct antagonism (mm)		Indirect antagonism (mm)	
	Mean	Standard error	Mean	Standard error
Control	8.100a	000.0	8.100a	000.0
27 (*Arthrinium* sp.)	1025bc	241.0	0.765b	042.9
29 (*Acrocalymma* sp.)	1.540b	265.0	1.082b	112.0
122(*Botryobambusa fusicoccum*)	0.320c	017.9	0.513b	045.0
711 (*Phoma* sp.)	0.960bc	219.0	0.599b	043.3
712 (*Phoma sp.*)	1319bc	272.0	1.401b	133.0

* means that do not share a letter are significantly different by the Tukey test at the 5% significance level.
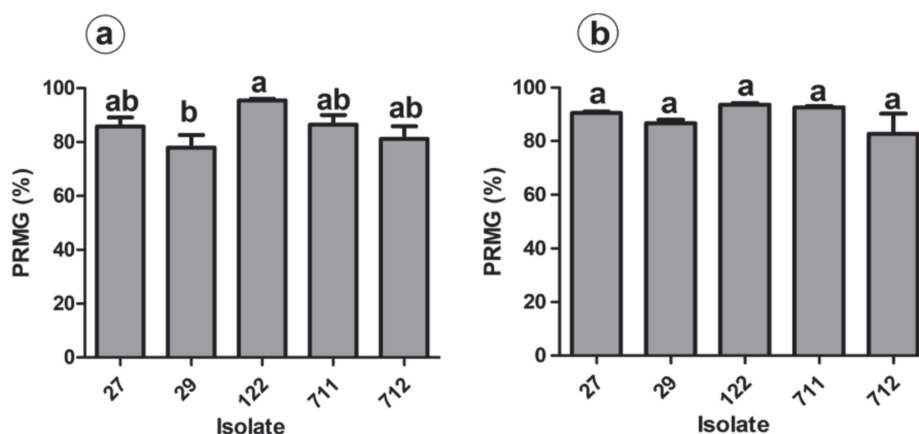
Figure 6: Percentage reduction of mycelial growth (PRMG) of *Pyricularia oryzae* colonies (mean ± standard error) by direct (a) and indirect (b) antagonism to endophytic isolates of *Bambusa oldhamii*. 27 (*Arthrinium* sp.), 29 (*Acrocalymma* sp.), 122 (*Botryobambusa fusicoccum*), 711 (*Phoma* sp.), and 712 (*Phoma sp.*).

No inhibition halo was observed in the direct antagonism assay between *P. oryzae* and isolate 27 (*Arthrinium* sp.) or isolates 29 (*Acrocalymma* sp.) and 122 (*B. fusicoccum*) (Figure 4). However, species of the genus *Arthrinium* have been reported to have antibacterial (Calvo *et al.* 1999; Miao *et al.* 2006), antifungal (Bloor 2008; Ramos *et al.* 2010) and antiviral (Wang *et al.* 2015) activity. For this reason, it is suggested that isolate 27 characterized in this study may be the object of future research with other plant species as well as in the interaction with other bacterial pathogens.

CONCLUSIONS

Five fungal endophytes of *B. oldhamii* were successfully identified and characterized, which were named as: Isolate 27 (*Arthrinium* sp.); isolate 29 (*Acrocalymma* sp.); isolate 122 (*B. fusicoccum*); isolate 711 (*Phoma* sp.) and isolate 712 (*Phoma* sp.).

The isolate 122 (*B. fusicoccum*) presented basic characteristics as an antagonist against *P. oryzae*. The other isolates were also identified as promising for antagonism, including isolate 711 (*Phoma sp.*) and 712 (*Phoma sp.*). However, further *in vivo* studies are required.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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