ASSESSMENT OF FUNCTIONAL TRAITS IN THE ASSEMBLAGE OF ENDOPHYTIC FUNGI OF *ANACARDIUM OTHONIANUM* RIZZINI

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Abstract

Plants maintain symbiotic relationships with microorganisms as a strategy to withstand adversities. From this exchange, organisms receive photoassimilates and provide benefits to the plant. *Anacardium othonianum* Rizzini, locally known as 'caju-de-árvore-do-cerrado' (tree cashew ofthe cerrado), is a tree species of the family Anacardiaceae nativeto the Midwest region of Brazil. The objective of this study was to characterize the culturable endophytic fungal community, its functional traits and its association with the roots of *A. othonianum*. The roots of *A. othonianum* were fragmented (1 cm) and inoculated in medium for the isolation of endophytic microorganisms. The molecular identification of the isolates was performed through the partial sequencing of the internal transcribed spacer (ITS). The endophytic isolates were tested for the synthesis of indole acetic acid (IAA) and phosphate solubilization through the colorimetric method.The root fragments were cleared, stained and examined under a microscope. Structures characteristic of endomycorrhizal and endophytic microorganisms were found on the slides analyzed. A total of 67 fungal strains were isolated and identified in 12 species: *Fusarium oxysporum, Bionectria ochroleuca, Periconia macrospinosa, Phomopsis lagerstroemiae, Penicillium kloeckeri, Eupenicillium shearii, Phomopsis asparagi, Penicillium pinophilum, Agaricomycetes* sp.*, Diaporthe* sp.*, Cladosporium cladosporioide s*and *Paecilomyces lilacinus*. All the genera found have been reported in the literature as endophytic species. It can be concluded that *A. othonianum* maintains associations with endomycorrhizal and endophytic fungi. Twelve endophytic strains were isolated from *A. othonianum* Rizzini, seven of which have potential for phosphate solubilization and IAA synthesis.

Key words: Endophytic, Endomycorrhizal, Fungi, *Anacardium othonianum*, Anacardiaceae,

Introduction

Microorganisms constitute a little-explored realm of the Cerrado biodiversity and possess major biological potential to be exploited, either for studies of biotechnological application or for the description of new species (Khan *et al*., 2012). Several fungal species obtain their nutrients from other organisms using different strategies. Many of these species are associated with the rhizosphere or the internal tissues of plants such as the endophytes (Khan *et al*., 2012a). In the symbiotic relationships between plants and microorganisms, the plant usually uses the association as a strategy to withstand biotic and abiotic stress (Piccoli *et al*., 2011; You *et al*., 2012).

The connection with the root systems of host plants is well documented in endomycorrhizal fungi, especially the arbuscular mycorrhizal fungi (AMF) belonging to the phylum *Glomeromycota* (Helgason & Fitter, 2009) because of their low host specificity and their ability to increase their nutrition and growth (Sharma & Jha, 2012). This connection can function as an information exchange channel and consequently influence seedling establishment, nutrient competition and the diversity and dynamics of plant communities, mainly due to helping the plants to absorb nutrients and water (Khan *et al*., 2011). The benefit of the association for the host arises from the increased absorption surface; in return, the fungus receives carbohydrates from the plant (Wu *et al*., 2009; Zhan *et al*., 2011).

The microorganisms that live inside the plants without causing visible damage or morphological changes in their hosts are known as endophytic microorganisms (Kang *et al*., 2012; Kogel *et al*., 2006; White & Bacon, 2012). In general, endophytic microorganisms penetrate the plant organs through natural openings such as stomata and hydathodes, through openings caused by insects or even by active penetration through the production of enzymes or structures that facilitate the process (Qin *et al*., 2012).

Endophytic microorganisms have the ability to provide benefits to the host, such as the solubilization of insoluble phosphate. This ability is due to the production of organic and inorganic acids and pH reduction, which leads to the dissociation of phosphate, thus making it available to plants (El-Azouni, 2008). In addition, microbes can aid in phytohormone synthesis and their supply to the plant. One phytohormone,indole acetic acid (IAA), can be synthesized through two pathways, one in which tryptophan is used as the precursor (Contreras *et*

al., 2010) and a tryptophan-independent pathway (Kochar & Srivastava, 2012). Currently, fungi endophytes are considered as unexplored source of bioactive natural compounds. They have been found to play a crucial role in the production of beneficial chemical compounds. (Khan *et al*., 2010; Korejo *et al*., 2014).

Anacardium othonianum Rizzini is an Anacardiaceae native to the Brazilian Cerrado, locally known as 'caju-de-árvore-do-cerrado' (tree cashewof the cerrado). It has great regional importancedue to its exploitation as a fruit and medicinal species (Caramori *et al*., 2004). The 'caju-de-árvore-do-cerrado' differentiates itself from the species *Anacardium humile*, *Anacardium nanum* and *Anacardium corymbosum* due its arboreal size, which can reach 6.0 m (Vieira *et al*., 2006). Its pseudofruit is consumed in the form of juices, jellies, ice cream and 'aguardente' and its high nutritional value is well established (Silva *et al*., 2008). However, the slow growth of its seedlings has restricted its commercial cultivation, making its use limited to small producers and to extractivism (Vieira *et al*., 2006).

However, studies on the microbial community associated with the roots of this species are lacking. Thus, the aim of this study was to characterize the assemblage of culturable endophytic fungi associated with the roots of *A. othonianum* Rizzini and determine their functional traits that are important for plant growth.

Materials and Methods

Plant material: Three 'caju-de-árvore-do-cerrado' seedlings measuring approximately 50 cm were collected in summer (March 2012) in the municipality of Montes Claros de Goiás, state of Goiás-GO (16°08' S latitude and 51º17' W longitude, 592 m altitude). The plants were fully removed from the soil using a manual digger, and they were carefully handled to prevent damage to the root and to preserve a large amount of rhizosphere soil. Subsequently, the samples were taken to the Laboratory of Agricultural Microbiology, Federal Institute of Goiás (Instituto Federal Goiano - IF Goiano) - Rio Verde Campus for processing.

Analysis of the association between fungi and 'caju-de-árvore-do-cerrado' roots: Root samples previously kept in 70% alcohol were cleared by a modified Koskey and Gemma method (1989). The roots were immersed in KOH (2%), autoclaved at 121°C for 20 minutes and transferred to a fresh KOH solution (2%) for 24 h at room temperature. They were then immersed in an alkaline solution of ammonium hydrogen peroxide (0.5% NH_4OH and 0.5% H_2O_2 0in water) for 1 h.

Root staining was performed with trypan blue (0.05%) in lactoglycerol (Phillips & Hayman, 1970), and 10 microscope slides were prepared with three root fragments each. This approach allowed the visualization of the structures under a Leica DM500 microscope with a Leica ICC 50 camera adapted to the LAZ EZ software,

version 1.8.0 to classify them according to the specialized literature (Petrini, 1986, Peterson *et al*., 2004).

Isolation, genetic characterization and molecular identification of the culturable endophytic fungal strains: The 'caju-de-árvore-do-cerrado' roots were washed in running water, immersed in water with neutral detergent and stirred for 5 minutes. Subsequently, the roots were rinsed with distilled water until the complete removal of all the detergent.

Under laminar flow, the roots were immersed in alcohol (70%) for one minute, sodium hypochlorite (2.5% active chlorine) for three minutes and alcohol (70%) for 30 seconds. They were subsequently rinsed three times with sterile distilled water, and the excess moisture was removed with sterile filter paper. As a control of the aseptic process, 500µL of water used in the final rinse of the samples was collected for inoculation into tubes containing culture medium.

Root fragments of approximately 1 cm each were cut off with tweezers and scissors, and groups of seven fragments were placed into 12 Petri dishes containing PDA medium (infusion of 200 g of potato, 20 g of dextrose and 15 g of agar in 1000 mL of water) supplemented with 100 mg L^{-1} of Chloramphenicol®. Fragments were also placed in nine Petri dishes with Nutrient Agar (NA) medium (3 g of beef extract, 5 g of peptone and 15 g of agar). In total, 84 fragments derived from each 'caju-de-árvore-do-cerrado' individual were analyzed on PDA medium, and 63 fragments were analyzed on NA medium.

The microorganisms that grew on the fragments were transferred to Petri dishes containing PDA, and the fungi were purified by plating the mycelium on dishes containing solid medium. The fungi were grouped by morphological characteristics including mycelium color, texture and the extent of mycelial growth, and they were then kept at 2 to 8°C in glass vials of PDA containing penicillin.

A representative of each morphotype was used for genomic DNA extraction, which was performed with a Miniprep kit following the manufacturer's recommendations (Axygen Biosciences, USA). The genetic characterization was performed with the use of molecular markers, IRAP-PCR (Inter-Retrotransposon Amplified Polymorphisms) and ISSR-PCR (Inter-Simple Sequence Repeat).

For the IRAP-PCR ("Inter-Retrotransposon Amplified Polymorphisms") marker, the primers CL IRAP 1 (5' –CGT ACG GAA CAC GCT ACA GA– 3') and CL IRAP 4 (5' –CTT TTG ACG AGG CCA TGC– 3') (Dos Santos *et al*., 2012) were used with a reaction volume of 25µL and the following amplification conditions: initial denaturation at 94°C for 4 minutes, 35 denaturation cycles at 94°C for 0.40 minutes, annealing at 44°C for 0.40 minutes, synthesis at 72°C for 2 minutes and final synthesis at 72°C for 10 minutes.

For the ISSR-PCR ("Inter-Simple Sequence Repeats" or inter-microsatellites) marker, the primer BH1 (5' – GTG GTG GTG GTG GTG – 3') was used (Smith *et al.*, 2003) in a reaction volume of 25μL and the following amplification conditions: initial denaturation at 94°C for 2 min, 35 denaturation cycles at 94°C for 1 min, annealing at 50°C for 2 minutes, synthesis at 72°C for 2 minutes and final synthesis at 72°C for 10 minutes.

The products of the IRAP-PCR and ISSR-PCR amplifications were subjected to electrophoresis on a 1.2% agarose gel. The sizes of the amplification products were determined with a 123bp DNA Ladder (Sigma-Aldrich, Inc.). The images were saved in a photo documentation system (Loccus Biotechnology, Brazil) for further analysis.

The diversity was assessed through visual inspection of the gel, taking all the visible bands into account. The amplification profile was transformed into a binary matrix with the values 0 for the absence and 1 for the presence of each amplicon. The similarity of the data was calculated through the Jaccard coefficient (Sneath & Sokal, 1973). The clustering, using the Unweighted Pair-Group Method with Arithmetical Average (UPGMA) algorithm, was obtained based on the similarity matrix with the NTSys v.2.1 software (Rohlf, 2001). A level of 75% minimum similarity between the isolates was considered for the definition of the Operational Taxonomic Unit (OTU) (Torres *et al.*, 2008).

The molecular identification of isolates was performed through the partial sequencing of the internal transcribed spacer (ITS) of the rDNA region of representatives of the 13 OTUs. The ITS 4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS 5 (5`-GGA AGT AAA AGT CGT AAC AAG G-3') oligonucleotides (White *et al.*, 1990) were used to amplify the intergenic region. The reaction volume was 25μL, and the amplification conditions were as follows: initial denaturation at 94°C for 4 minutes, followed by 35 cycles at 94°C for 40 seconds, 58°C for 35 seconds and 72°C for 1.20 minutes, and a final elongation step at 72°C for 10 minutes in an AMPLITHERM Thermal Cycler. The amplification products were purified (Dunn & Blattner, 1987) and quantified through electrophoresis on a 1.2% agarose gel.

The sequencing was performed by the Sanger method with the Big Dye kit in the ABI3100 Genetic Analyzer (Applied Biosystems). The sequences were compared with known sequences in the Genbank database (http://www.ncbi.nlm.nih.gov) through a similarity search via Blastn (Altschul *et al.*, 1990).

The relative frequencies of the species were calculated by dividing the number of isolates of a species by the total number of isolates. The Shannon's diversity index (H') was calculated according to Kumar & Hyde (2004).

Functional traits of the endophytic species: A total of 12 endophytic isolates of *A. othonianum* were used for the assessment of the synthesis of indole acetic acid (IAA) and the solubilization of calcium, iron and aluminum phosphates.

Solubilization of calcium, iron and aluminum phosphates: The fungal isolates were grown on PDA medium (infusion of 200 g of potato, 20 g of dextrose and 15 g of agar) for three days at 30ºC. Subsequently, 5mm mycelial discs were inoculated in triplicate in glass vials containing 9 mL basic GYP medium (glucose, yeast extract and peptone) and penicillin with three different sources of phosphate (5 g L^{-1} of calcium phosphate - $CaHPO_4$, 1 g L^{-1} of iron phosphate - $FePO_4$ or2 g L^{-1} of aluminum phosphate - $AlPO_4$). Then, they were kept under constant agitation with the aid of an orbital shaker (Nova Técnica NT 712®) at 90rpm for 72 h at 30°C. The phosphate solubilization was evaluated through the colorimetric method described by Braga & Felipo (1974).

The phosphate solubilization by the fungi was determined using the standard curve equation, and the means were compared using the Scott-Knott test (5%).

IAA synthesis: The fungal isolates were grown on PDA medium for three days at 30ºC. Subsequently, 5mm mycelial discs were inoculated in triplicate in glass vials of penicillin containing 5 mL of PD medium (infusion of 200 g potato, 20 g of dextrose and 1000 mL of water) supplemented with 100μg mL^{-1}oftryptophan.

The isolates were kept under constant agitation with an orbital shaker (Nova Técnica NT 712®) at 90rpm for 72 h at 30°C.The production of IAA was assessed through the colorimetric method described by Gordon and Mitchell (1951). The synthesis of IAA by the fungi was determined using the standard curve equation, and the means were compared using the Scott-Knott test (5%).

Results

Analysis of the association between fungiand'caju-de-árvore-do-cerrado' roots: Morphological changes or symptoms of disease in the roots of *A. othonianum* collected for this study were not observed in the microscopic analysis. Two groups of microorganisms colonizing the roots of this arboreal species were observed, confirming that the method of the preparation of the roots was sufficient for this species.

The first group was the endophytic fungi called *dark septate* (DSE) (Fig. 1). This classification was possible through the observation of brown-stained septate hyphae (Figs 1A, 1B, 1C and 1D) and of microsclerotia structures (Figs 1E and 1F), which are typical of this group.

The fungi have inter- and extracellular growth, and as an intercellular colonization strategy, the hyphae decreases its diameter when penetrating the cell wall. Consequently, the extracellular portion of the hyphae expands, thus allowing a determination of the exact site of colonization (Figs. 1C and 1D).

Different stages of microsclerotia development were observed. According to Wu *et al.* (2009), the microsclerotia may be a structure responsible for storing special substances or be a structure of propagules.
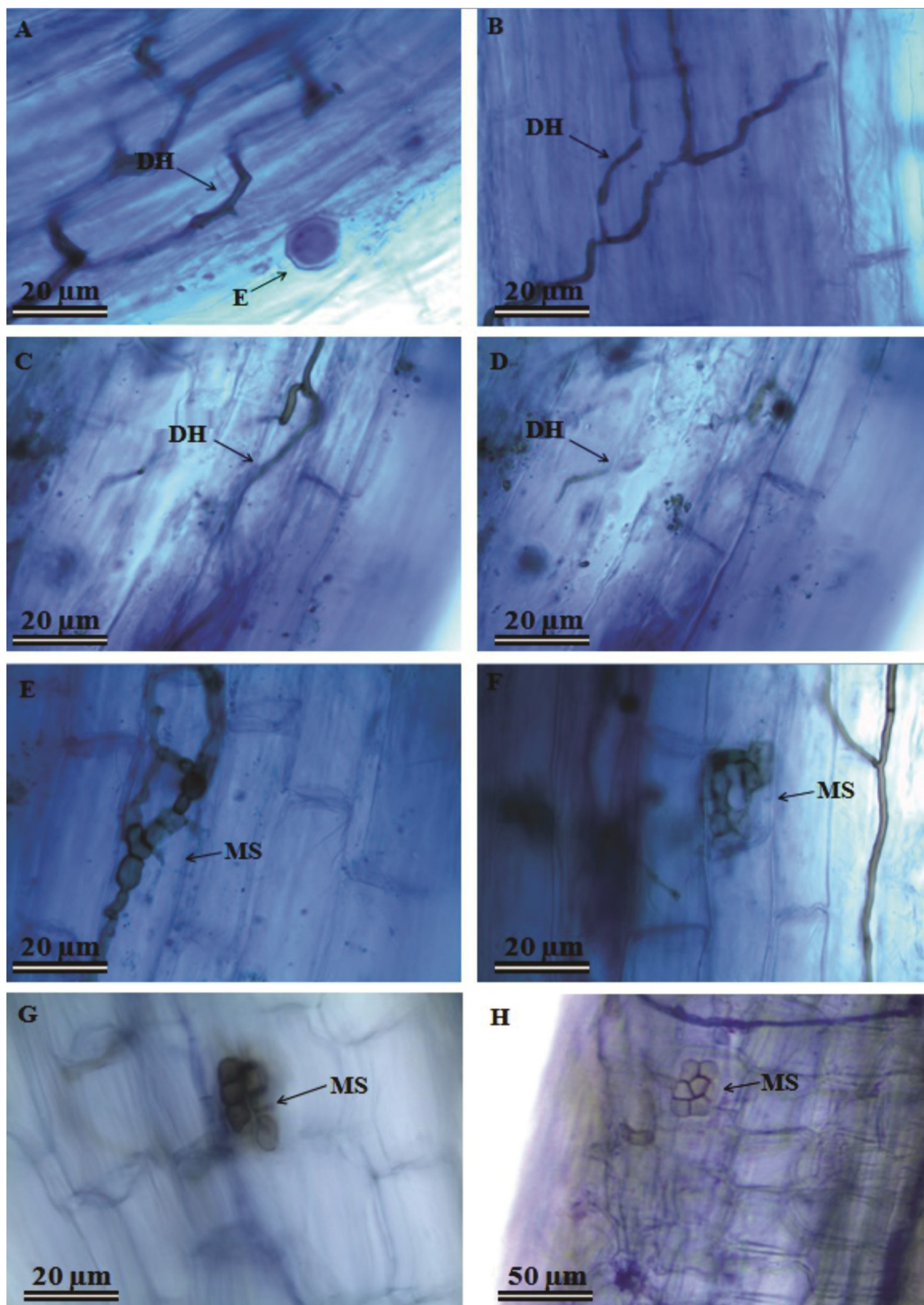
Fig. 1. Structures of endophytic fungi colonizing roots of *Anacardium othonianum* Rizzini collected in the municipality of Montes Claros de Goiás, GO. Legend: DH (dark septate hyphae) and MS (microsclerotia).
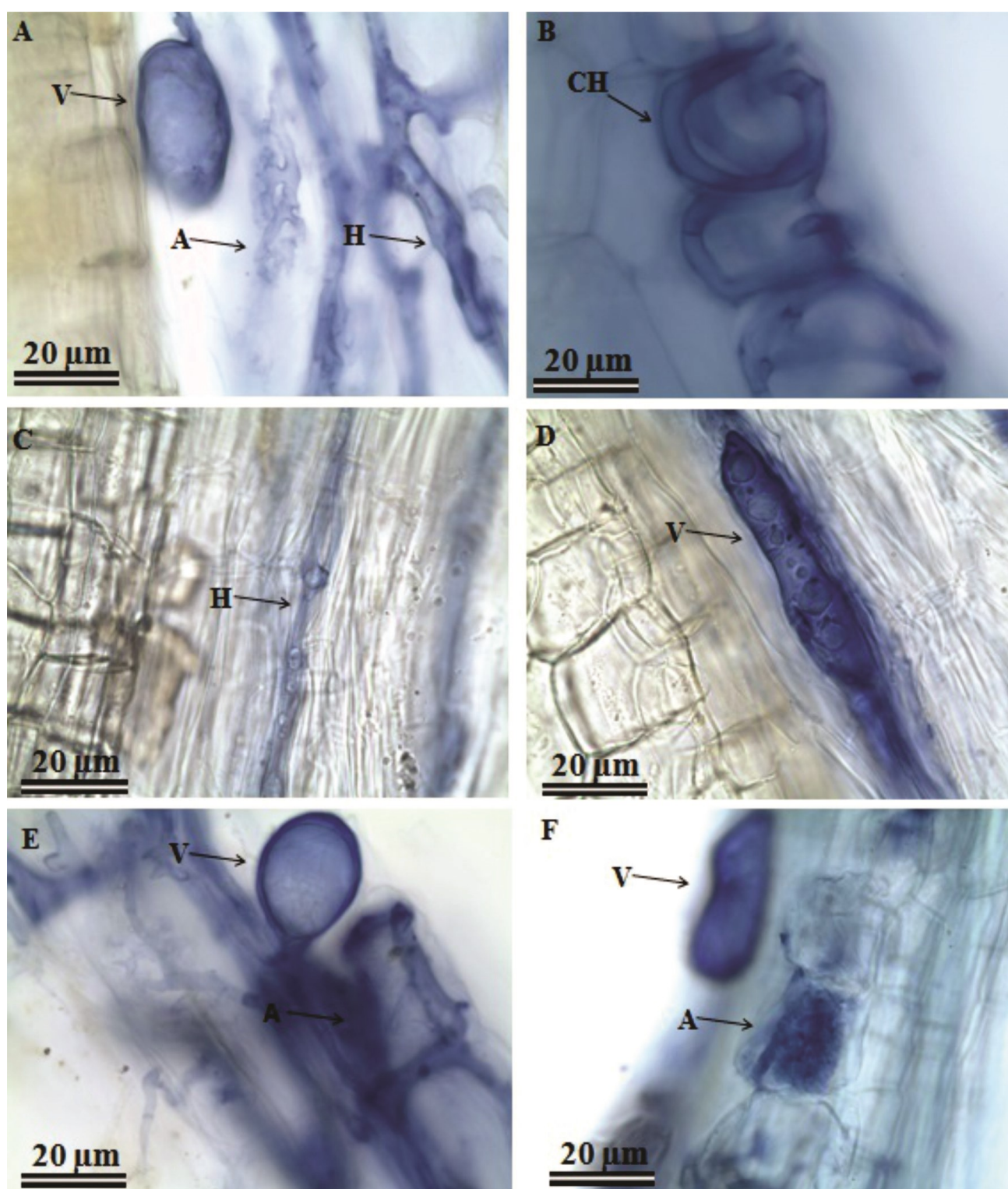
Fig. 2. Structures of mycorrhizal fungi colonizing roots of *Anacardium othonianum* Rizzini, collected in the municipality of Montes Claros de Goiás. Legend: A (arbuscules), V (vesicles), H (hyphae) and CH (coiled hyphae).

The second fungal group was the AMF (Fig. 2), which are characterized by aseptate hyphae and characteristic structures such as vesicles and the arbuscule (Fig. 2A). The hyphae of the AMF can be stained with trypan blue dye and grow intracellularly (Arum association) (Fig. 2) or between plant cells (Figs. 2B and 2C), allowing an association of the Paris type. Therefore, the morphology of endomycorrhiza in *A. othonianum* is intermediate.

The AMF found colonizing the 'caju-de-árvore-do-cerrado' rootscan store lipids within the hyphae (Figs. 2B and 2C) or in specialized structures called vesicles (Fig. 2D). Vesicles were found between the plant cells (Figs. 2B, 2D, 2E and 2F) and have variable size and shape.

The presence of arbuscules was confirmed through the observation of hyphae that branch out, forming a network of many smaller hyphae within the plant cells, thus allowing a constant communication between the fungus and the plant (Figs. 2A, 2E and 2F).

Isolation, genetic characterization and molecular identification of the culturable endophytic fungal strains: A total of 67 isolates of endophytic fungi of 'caju-de-árvore-do-cerrado' root fragments were obtained, 35 and 32 strains from the NA and PDA media, respectively. After the growth of the strains, they were separated into 20 morphological groups, which were differentiated through macroscopic (margin, type and mycelium color) and microscopic characteristics (differentiation of reproductive structures).

The analysis of the molecular markers showed differences between the markers used. A total of 13 OTUs were observed using the ISSR-PCR marker, nine by

unique profiles and four by groups of single isolates. In turn, 17 OTUs were detected using the IRAP-PCR marker, 15 formed by unique profiles and two formed by groups of isolates (Fig. 3).

The molecular marker systems based on long terminal repeat (LTR) retrotransposons (RTNs) explore the genomic DNA polymorphisms resulting from an insertion between two very close RTNs, in the case of the IRAP marker (Schulman *et al.*, 2004). This marker reflects the separation at the individual level and is efficient and very accurate. In this study, the IRAP marker allowed us to separate the isolates into 17 OTUs.
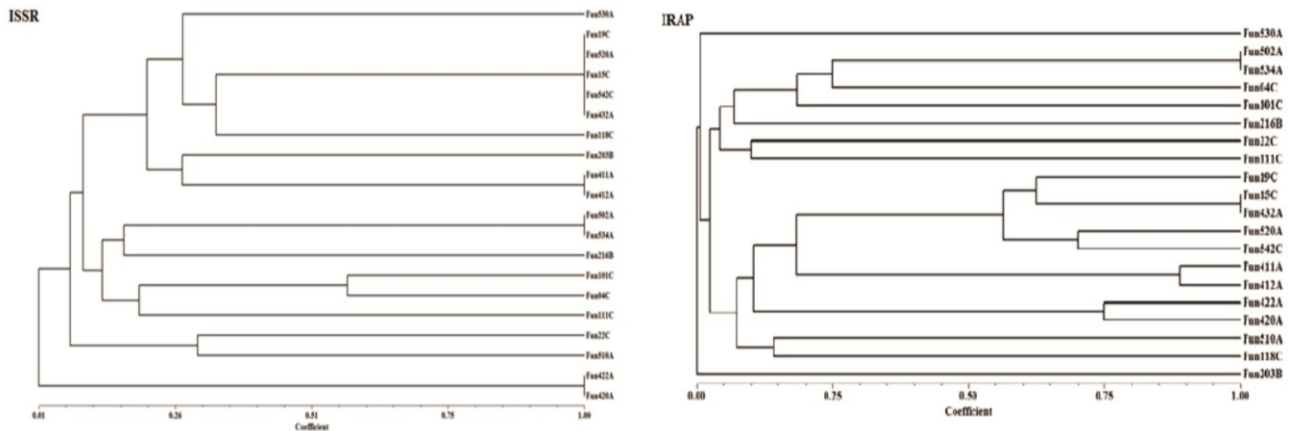

Fig. 3. Similarity between the morphotypes of fungi colonizing *Anacardium othonianum* Rizzini, collected in Montes Claros de Goiás, GO using the ISSR and IRAP markers. Rio Verde - GO, 2013.

The ISSR explores the ubiquity of microsatellites in the genomes and consists of the amplification of regions between two adjacent microsatellites, inversely oriented, thus making the markers *multilocus*. This marker yielded 13 OTUs, making it a less robust marker.

The molecular analysis revealed 11 fungal species: *Penicillium kloeckeri* (KF578130), *Eupenicillium shearii* (KF578127), *Paecilomyces lilacinus* (KF578135), *Fusarium oxysporum* (KF578128), *Periconia macrospinosa* (KF578132), *Cladosporium cladosporioides* (KF578125), *Bionectria ochroleuca* (KF578124), *Phomopsis lagerstroemiae* (KF578134), *Phomopsis asparagi* (KF578133), *Penicillium pinophilum* (KF578131), *Diaporthe* sp. (KF578126) and one class of *Agaricomycetes* sp. (KF578129).

Phomopsis lagerstroemiae Fun520A was the most frequent species (44.8%), followed by *Periconia macrospinosa* Fun502A (16.4%), *Agaricomycetes* sp. Fun04C (10.4%), *Cladosporium cladosporioides* Fun422A (7.5%) and *Phomopsis asparagi* Fun530A (6.0%). The other species were considered rare, having frequencies lower than 3% (Sun *et al.*, 2012) (Table 1).

Clustering by similarity between endophytic fungal species was observed through the analysis of maximum likelihood of the ITS sequence analysis (Fig. 4).

The first group is only composed of *Agaricomycetes* sp. Fun04C, representing the phylum Basidiomycota. The second group, classified as the phylum Ascomycota, yields two sub-groups, one composed of *Cladosporium cladosporioides* Fun420A representing the class Dothideomycetes. The species *Penicillium kloeckeri* Fun216B, *Eupenicillium shearii* Fun22C and *Penicillium*

pinophilum Fun118C are in the same group but in more derived conditions, composing the class Eurotiomycetes. The other group of the phylum Ascomycota comprises representatives of the class Sordariomycetes: *Phomopsis lagerstroemiae* Fun520A, *Phomopsis asparagine* Fun530A and *Diaporthe* sp. Fun510A comprise a subgroup, followed by *Periconia macrospinosa* Fun307B, *Paecilomyces lilacinus* Fun111C, *Fusarium oxysporum* Fun203 and *Bionectria ochroleuca* Fun411A.

Functional traits of endophytic species: The 12 fungal isolates tested were able to solubilize insoluble phosphate. Calcium phosphate was the more solubilized source, and *P. kloeckeri* Fun216B (4.1 mg L^{-1}), *E. shearii* Fun22C (3.9 mg L^{-1}) and *P. lilacinus* Fun111C (3.7 mg L^{-1}) were the isolates with greater potential for solubilization, followed by *F. oxysporum* Fun203B (3.2 mg L^{-1}), *P. macrospinosa* Fun307B (3.2 mg L^{-1}), *C. cladosporioides* Fun420A (3.2 mg L^{-1}) and *Agaricomycetes* sp. Fun04C (3.1 mg L^{-1}).

Regarding the iron phosphate, *Agaricomycetes* sp. Fun04C (2.2 mg L^{-1}) and *B. ochroleuca* Fun411A (2.0 mg L^{-1}), followed by *P. lilacinus* Fun111C (1.5 mg L^{-1}), *C. cladosporioides* Fun422A (1.5 mg L^{-1}), *F. oxysporum* Fun203B (1.4 mg L^{-1}), *Diaporthe* sp. Fun510A (1.4 mg L^{-1}) and *E. shearii* Fun22C (1.2 mg L^{-1}), had the highest responses.

P. pinophilum Fun118C had the lowest responses to calcium (1.4 mg L^{-1}) and iron (0.1 mg L^{-1}) phosphate solubilization. There were no differences between the microorganisms tested for solubilization of aluminum phosphate (Table 2).

Table 1. Identification of endophytic fungal isolates isolated from *Anacardium othonianum*, collected in the municipality of Montes Claros de Goiás, GO

Isolate (n)	GenBank	% ID	GenBank accession number	Relative frequency %
Fun530A (4)	*Phomopsis asparagi*	99	JX049385	5.970149254
Fun520A (31)	*Phomopsis lagerstroemiae*	98	AY622994	44.7761194
Fun118C (2)	*Penicillium pinophilum*	98	AB455516	2.985074627
Fun203B (1)	*Fusarium oxysporum*	99	HQ328030	1.492537313
Fun411A (2)	*Bionectria ochroleuca*	98	HQ607798	2.985074627
Fun111C (1)	*Paecilomyces lilacinus*	98	FJ765021	1.492537313
Fun307B (11)	*Periconia macrospinosa*	99	JN859364	16.41791045
Fun04C (7)	*Agaricomycetes sp.*	91	JF288547	10.44776119
Fun216B (1)	*Penicillium kloeckeri*	98	HM469393	1.492537313
Fun22C (1)	*Eupenicillium shearii*	99	JQ863221	1.492537313
Fun510A(2)	*Diaporthe sp.*	98	EF488448	2.985074627
Fun420A (5)	*Cladosporium cladosporioides*	99	JX230994	7.462686567

Table 2. *In vitro* solubilization of calcium ($CaHPO_4$), iron ($FePO_4$) and aluminum ($AlPO_4$) phosphate by endophytic fungi of 'caju-de-árvore-do-cerrado' (*Anacardium othonianum* Rizzini). Rio Verde - GO, 2013.

Species	Dissociated phosphate (mg L^{-1})		
	$CaHPO_4$	$FePO_4$	$AlPO_4$
Penicillium kloeckeri Fun216B	4.1 Aa	1.0 Cb	0.9 Ab
Eupenicillium shearii Fun22C	3.9 Aa	1.2 Bb	0.6 Ac
Paecilomyces lilacinus Fun111C	3.7 Aa	1.5 Bb	1.0 Ab
Fusarium oxysporum Fun203B	3.2 Ba	1.4 Bb	0.6 Ac
Periconia macrospinosa Fun502A	3.2 Ba	0.8 Cb	0.6 Ab
Cladosporium cladosporioides Fun422A	3.2 Ba	1.5 Bb	0.5 Ac
Agaricomycetes sp. Fun04C	3.1 Ba	2.2 Ab	1.2 Ac
Bionectria ochroleuca Fun411A	2.8 Ca	2.0 Ab	1.3 Ac
Phomopsis lagerstroemiae Fun520A	2.7 Ca	0.7 Cb	0.5 Ab
Diaporthe sp. Fun510A	2.5 Ca	1.4 Bb	1.0 Ab
Phomopsis asparagi Fun530A	2.3 Ca	0.9 Cb	0.8 Ab
Penicillium pinophilum Fun118C	1.4 Da	0.1 Db	0.5 Ab

Means followed by the same letter, uppercase in the column and lowercase in the row, do not differ by the Scott-Knott test (5%)

Most *A. othonianum* endophytic fungi synthetized IAA (Table 3). It is noteworthy that the fungi *F. oxysporum* Fun203B (1.7 µg mL^{-1}), *B. ochroleuca* Fun411A (0.3 µg mL^{-1}), *P. macrospinosa* Fun502A (0.3 µg mL^{-1}), *P. lagerstroemiae* Fun520A (0.3 µg mL^{-1}), *P. kloeckeri* Fun216B (0.2 µg mL^{-1}) and *E. Shearii* Fun22C (0.1 µg mL^{-1}) synthetized IAA.

Discussion

Association between fungus and 'caju-de-árvore-do-cerrado' roots: The Cerrado species generally have their growth limited by the edaphoclimatic conditions of the region, and various survival strategies are used, including the association with AMF (Zhang *et al*., 2011) or endophytes (Wu *et al*., 2009). This strategy provides advantages to the plant because the microorganisms compete with pathogens, increase the plant's absorption area of soil water and nutrients, such as P, and supply some phytohormones (Simard & Durall, 2004; You *et al*., 2012).

A group of endophytic fungi with brown hyphae that form microsclerotia is known as dark septate (DSE) and provides some benefits to plants; however, the benefit of the association with the DSE is not yet fully understood, and it is believed that such benefits would be similar to those of the AMF (You *et al*., 2012).

The dark color of dark septate fungi is related to the increased rigidity of the cell wall as well as the resistance to irradiation, high temperature and drying (Zhan *et al*., 2011) because these microorganisms have a high ability to colonize deserted or arid environments (Wu *et al*., 2009).

According to Dickson *et al*. (2007), the presence of typical arbuscules resulting from the intense branching within the cortex cells of the host is characteristic of Arum colonization, while the abundant presence of coiled hyphae inside the cortex cells is characteristic of the Paris colonization pattern. The simultaneous observation of the

two types of mycorrhizal colonization patterns, considering that the type of colonization is influenced by the identity of the symbiotic fungus, indicates that there may be more than one fungal species colonizing the roots.

Mycorrhizal symbiosis occurs due to the ability of fungi to increase the availability of nutrients and water, exploiting deeper regions than the roots themselves, which affects the establishment and survival of the species (Wu *et al.*, 2009). The soil in which the plant is established is another determining factor for this relationship because in soils with low fertility, especially regarding poorly soluble calcium (Ca), iron (Fe) and potassium (K) levels, symbiosis is stimulated (Uma *et al.*, 2012; Sharma & Jha, 2012).

The AMF can colonize woody or herbaceous species (Zang *et al.*, 2011; Vitorino *et al.*, 2012b), and studies show that they can colonize 64% of the root system of *Ficus* sp. (Uma *et al.*, 2012). Several families are already known to interact with AMF (Acanthaceae, Asclepidaceae, Amaranthaceae, Asteraceae, Caesalpinaceae, Chenopodiaceae, Commelinaceae, Cyperaceae, Labiatae, Nyctaginaceae, Poaceae, Rutaceae, Solanaceae, Umbellifereae, Verbanaceae, Lamiaceae, Phytolaccaceae, Fabaceae, Bignoniaceae, Moraceae, Myrtaceae, Rubiaceae, Melastomataceae and Myrsinaceae) (Detmann *et al.*, 2008; Sharma & Jha 2012; Vitorino *et al.*, 2012b).
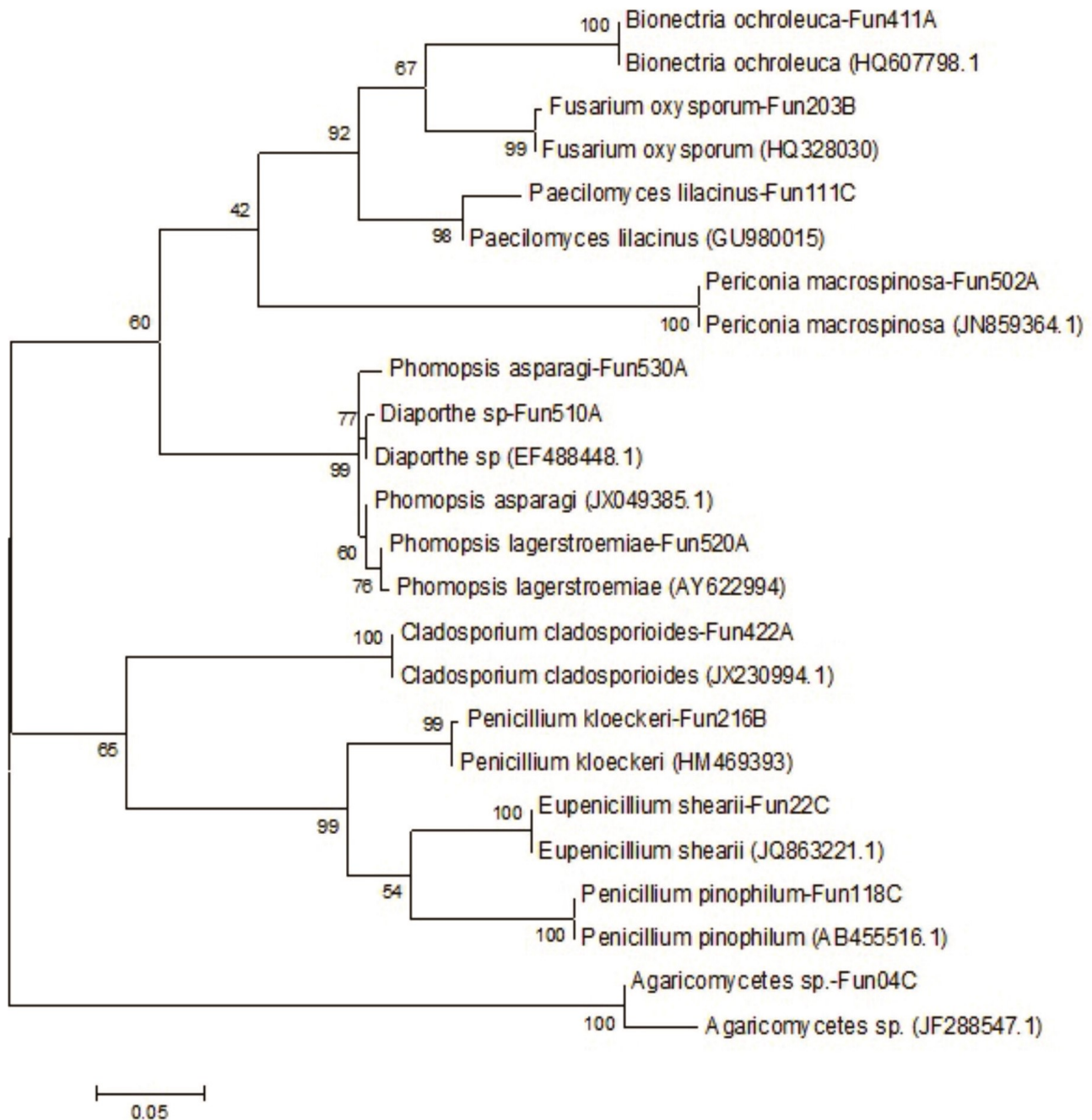

Fig. 4. Phylogenetic molecular analysis using the maximum likelihood method of endophytic fungal isolates of *Anacardium othonianum* Rizzini, collected in the municipality of Montes Claros de Goiás, Rio Verde - GO, 2013.

Table 3. *In vitro* production of indole acetic acid (IAA) by endophytic fungi of 'caju-de-árvore-do-cerrado' (*Anacardium othonianum* Rizzini). Rio Verde – GO, 2013.

Species	IAA µg mL^{-1}
Penicillium kloeckeri Fun216B	0.2 b
Eupenicillium shearii Fun22C	0.1 b
Paecilomyces lilacinus Fun111C	0 c
Fusarium oxysporum Fun203B	1.7 a
Periconia macrospinosa Fun502A	0.3 b
Cladosporium cladosporioides Fun422A	0 c
Agaricomycetes sp. Fun04C	0 c
Bionectria ochroleuca Fun411A	0.3 b
Phomopsis lagerstroemiae Fun520A	0.3 b
Diaporthe sp. Fun510A	0 c
Penicillium pinophilum Fun118C	0.1 c
Phomopsis asparagi Fun530A	0.1 c

Means followed by the same letter do not differ in the Scott-Knott test (5%).

When studying the characteristic morphology of AMF spores isolated from the rhizosphere of *A. occidentale* L., Proborini (2013) reported mycorrhizal colonization in the family Anacardiaceae. The double colonization of AMF and DSE in plants indicates the dynamics of the community that colonizes these roots, reinforcing the complexity of this interaction. The symbiosis with two fungal groups has been reported in the Cerrado species *Hyptis marrubioides, Alibertia edulis, Aeschynomene paniculata, Chamaecrista desvauxii, Chamaecrista nictitans, Coccocypselum* sp.*, Eugenia dysenterica, Miconia albicans, Myrsine guianensis, Palicuria* sp.and *Platypodium eleganspois* (Detmann *et al.*, 2008; Vitorino *et al.*, 2012b), as well as in *Centilla asiatica* and *Leucas plukenitti* (Sharma & Jha, 2012), species nativeto India.

A. othonianum is capable of being concomitantly colonized by DSE and AMF fungi. Further studies are needed to understand the dynamics of the relationships between these groups of microorganisms and the host.

Isolation, genetic characterization and molecular identification of culturable endophytic fungal strains: According to Wang *et al.* (2005), the phylum Basidiomycota is rarely isolated as endophyte. Seven strains (*Agaricomycetes* sp.) were isolated from *A. othonianum*. However, the phylum Ascomycota represented 90% of the total of individuals, with three classes being found: Sordariomycetes (85%), Dothideomycetes (8.4%) and Eurotiomycetes (6.6%). Hoffman & Arnold (2008) in the United States & Chaverri & Gazis (2010) in Peru also detected the classes Sordariomycetes, Dothideomycetes and Eurotiomycetes in conifers and in rubber trees, respectively, with the class Sordariomycetes being the most abundant.

The Shannon's diversity index H' found in populations of endophytic fungi of *A. othonianum* was 1.82. Sun *et al.*, (2012) obtained values for the Shannon index ranging from 0.29 to 4.78 when studying leaves and stems in desert areas in China. Bezerra *et al.* (2013) found total values of 2.99 while studying cactus (*Cereus jamacaru* DC) in the dry tropical forest of Brazil. Costa *et al.* (2012) found a Shannon Diversity Index of 1.035 in the dry season and a slightly lower value in the rainy season (H'=1.026) while studying endophytic isolates of mangrove in northeastern Brazil.

Although the morphological taxonomy greatly contributes to the elucidation of the diversity of endophytic fungi, molecular identification methods have proved to be excellent tools for the identification of morphospecies (Bensch *et al.*, 2012).

Sun *et al.* (2012) described the isolation of species of *Cladosporium*, *Fusarium* and *Penicillium* while studying the community of endophytic fungi from plants in the desert regions of China; the same genera were described in this study. This result may indicate that these species are common to hosts inhabiting places of intense light and subjected to water stress.

Penicillium species were described by Ge *et al.* (2008) and Wubsheta *et al.* (2013). *Penicillium pinophilum* is also known to be a saprophytic fungus that degrades cellulose (Maciel *et al.*, 2012); however, it is believed to have a synergistic effect with AMF, stimulating their establishment and sporulation (Quilliam & Jones, 2010).

Diaporthe is an endophytic genus known to produce enzymes that play a role in theinhibition of pathogen metabolism (Prada *et al.*, 2009; Anaya *et al.*, 2013). This process was observed by Sebastianes *et al.* (2013) in mango trees native to Brazil.

Although the species *Fusarium oxysporum* is traditionally recognized as phytopathogenic (Maciel *et al.*, 2012; Fantinel *et al.*, 2014), studies have shown its endophytic ability (Onofre *et al.*, 2013). This fungus has been reported to produce several enzymes such as proteases, cellulases and chitinases, which degrade the cell wall, as well as substances such as siderophores (Xing *et al.*, 2010; Zhao *et al.*, 2010; Vieira *et al.*, 2012).

Phomopsis is a genus of endophytic fungi that can promote plant growth, being able to produce compounds that accelerate the decomposition of residues, releasing nutrients into the soil and inducing the plant to improve its defense capacity (Chen *et al.*, 2013). Conversely, Fantinel *et al.* (2014) reported the existence of species of this genus that act as pathogens in yellowipê seeds (*Handroanthus chrysotrichus* (Mart. Ex DC) Mattos).

Cladosporium was described by Yang *et al.* (2013) as an endophytic species isolated from *Aconitum leucostomum* and reported as phytopathogenic in yellowipê seeds (Fantinel *et al.*, 2014). *Bionectria* was isolated from chili pepper in Korea (Paul *et al.*, 2013), *Eupenicillium* was isolated in China from *Murraya paniculata* leaves (Wang *et al.*, 2012). The class Agaricomycetes has been described by Brum *et al.* (2012) as an endophytic species that has the ability to control pathogenic species of fungi.

Paecilomyces lilacinus is a fungus widely commercially used as inoculant against nematodes (bioactive Bioact WG® - Prophyta) (Berg 2009). *Periconia macrospinosa* is an endophytic species described by Mandyam *et al.* (2012) that is found in association with prairie native grasses of eastern Kansas in the United States. This species is characterized by the presence of brown septate hyphae and structures of microsclerotia typical of the DSE group.

Functional traits of endophytic species: Phosphorus is considered a macronutrient essential to plant growth and development given its role in structural, functional and energy transfer processes (Ma *et al.,* 2011). Some microorganisms play an important role in the P cycle, being responsible for its hydrolysis and thus making it available to plants. These processes are mediated by enzymes and organic acids (Yang *et al.,* 2012).

Phosphate-solubilizing microorganisms act on insoluble phosphate through phosphatases, mainly the acid phosphatases, with the production of organic and inorganic acids, which reduce pH and make P available to plants (Ma *et al.,* 2011).

According to Vitorino *et al.* (2012a), endophytic fungi of *Hyptis marrubioides* Epling (Lamiaceae) showed no ability to solubilize $CaHPO_4$. The authors note that this Cerrado native species is adapted to acidic soils, in which P is found complexed to Fe^{3+} and Al^{3+}, making it possible that this functional trait of these fungi is related to the solubilization of $FePO_4$ or $AlPO_4$.

Endophytic *Trichoderma* isolates tested by Badawi *et al.* (2011) were able to produce IAA *In vitro*, and the use of the precursor L-tryptophan yielded a positive effect as an inducer for the synthesis of this phytohormone. According to Vitorino *et al.* (2012a), 52% of the endophytic isolates of *Hyptis marrubioides* Epling (Lamiaceae) were able to synthesize IAA *In vitro*.

Conclusions

The roots of *Anacardium othonianum* Rizzini are associated with arbuscular mycorrhizal and endophytic fungi.
- An association with groups of *dark septate* fungi was observed.
- Twelve endophytic species were isolated in*Anacardium othonianum* Rizzini, seven of which were able to solubilize phosphate and synthesize IAA.

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(Received for publication 12 March 2015)