



Molecular characterization by amplified ribosomal DNA restriction analysis and antimicrobial potential of endophytic fungi isolated from *Luehea divaricata* (Malvaceae) against plant pathogenic fungi and pathogenic bacteria

J. Bernardi-Wenzel¹, A. Garcia², J.L. Azevedo² and J.A. Pamphile²

¹Departamento de Biologia, Universidade Paranaense, Toledo, PR, Brasil

²Departamento de Biotecnologia, Genética e Biologia Celular, Universidade Estadual de Maringá, Maringá, PR, Brasil

Corresponding author: J.A. Pamphile

E-mail: prof.pamphile@gmail.com

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ABSTRACT. *Luehea divaricata* is an important plant in popular medicine; it is used for its depurative, anti-inflammatory, and other therapeutic activities. We evaluated the antimicrobial activity of endophytic fungi isolated from leaves of *L. divaricata* against phytopathogens and pathogenic bacteria, and characterized the isolates based on amplified ribosomal DNA restriction analysis (ARDRA). The *in vitro* antagonistic activity of these endophytes against the phytopathogen *Alternaria alternata* was assayed by dual culture technique. Based on this evaluation of antimicrobial activity, we extracted secondary metabolites from nine endophytic fungi by partitioning in ethyl acetate and methanol. These were tested against the phytopathogens *A. alternata*, *Colletotrichum* sp and

Moniliophthora perniciosa, and against the human pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*. Molecular characterization by ARDRA technique was used for phylogenetic analysis, based on comparison with sequences in GenBank. The endophytes had varied effects on *A. alternata*. One isolate produced an inhibition halo against *M. perniciosa* and against *E. coli*. This antibiosis activity indicates a role in the protection of the plant against microbial pathogens in nature, with potential for pharmaceutical and agricultural applications. Based on ARDRA, the 13 isolates were grouped. We found three different haplotypes of *Phomopsis* sp, showing interspecific variability. It appears that examination of the microbial community associated with medicinal plants of tropical regions has potential as a useful strategy to look for species with biotechnological applications.

Key words: Biocontrol; Biotechnology; Dual culture; Antagonism; Metabolic extracts

INTRODUCTION

Endophytic microorganisms are those that inhabit the interior of plants for at least one period of their life cycle without causing damage to their hosts (Carroll, 1986; Petrini, 1991; Azevedo et al., 2000).

Over the last thirty years, endophytic microorganisms have been recognized and studied due to the discovery of their capacity to protect their hosts from attack by insects, pathogens and domestic herbivores. They have the ability to produce physiological alterations in the plants that host them and are responsible for the production of medicinal compounds such as antibiotics and antitumor agents, besides promoting plant growth (Azevedo et al., 2000, 2002; Mucciarelli et al., 2003; Strobel, 2006; Gange et al., 2007).

Luehea divaricata Mart. (Tiliaceae) is a large tree reaching 15 to 25 m in height. It is popularly known as “açoita-cavalo” and can be found in Brazil from Rio Grande do Norte to Rio Grande do Sul, where it is used for medicinal purposes to treat dysentery, leucorrhoea, rheumatism, gonorrhoea, tumors, bronchitis and skin wounds, for grain cleaning, vaginal cleansing, decoction, and as anti-inflammatory, astringent, diuretic and anti-rheumatic (Lorenzi, 2000; Bighetti et al., 2004; Tanaka et al., 2005).

The presence of endophytic fungi in the hosts may benefit them. Because they colonize an ecological niche very similar to the one occupied by phytopathogens, inhabiting inter- and intracellular spaces, interacting intimately with their host, even in the species and races level, endophytes can be used in biological control of pathogens due to the direct action over them, antibiosis, induction of systemic resistance in the plant or due to competition for nutrients (Pamphile and Azevedo, 2002; Bernardi-Wenzel et al., 2010).

Several authors have already reported a large variety of chemicals produced by endophytic fungi, and many of them show biological activity, preventing microbial growth of pathogenic strains and controlling tumor cell growth, which indicates that endophytes are an important source of natural compounds with biotechnical uses (Strobel, 2006; Firáková et al., 2007; Gangadevi and Muthumary, 2008).

The discovery of new antimicrobial drugs, and their research mainly in countries with high biodiversity, such as Brazil, makes the prospects for the study of endophytes quite promising, especially with the possibility of discovering new antimicrobials, which may combat pests, as well as generate revenue for the country (Peixoto-Neto et al., 2002).

One of the strategies used to assess endophytic microbial diversity is based on the electrophoresis of fragments of ribosomal RNA gene (rDNA), generated by PCR, and subsequent digestions with restriction enzymes (ARDRA - amplified ribosomal DNA restriction analysis). The ARDRA technique has been used to quickly determine genotypic changes over time or between different sites, reflecting different environmental conditions. The method is based on the principle that the restriction sites existing in rDNA are relatively conserved and reflect phylogenetic patterns, thus evaluating differences between dominant phylogenetic groups in the community (Costa and Siqueira, 2004).

Considering the importance of endophytic fungi and their biotechnological application and relevance of further study of medicinal plants in the context of their interaction with the endophytic microbial community, this study aimed to determine the types of interactions between isolated endophytes from *L. divaricata* and pathogenic microorganisms. Antimicrobial activity against phytopathogens and pathogenic bacteria was assessed, and the endophytic isolates of *L. divaricata* were molecularly characterized using ARDRA.

MATERIAL AND METHODS

Isolation of endophytic fungi

The endophytic fungi used in this study were isolated by Bernardi-Wenzel et al. (2010) from leaves of two plants of *Luehea divaricata*. The isolates were named according to the group in which they were morphologically classified, isolate tree and sequence number of isolate (G1-A2-44: group 1, tree 2, fungal isolate 44).

Evaluation of antagonistic activity *in vitro* of the endophytic isolates against the phytopathogen *Alternaria alternata* and interaction in dual culture

The interactions between 46 endophytic fungi isolated from *L. divaricata* and the phytopathogen *A. alternata* were assessed, according to the paired culture method described by Campanile et al. (2007). The endophytic fungi and the phytopathogen were added separately to Petri dishes containing PDA medium (Smith and Onions, 1983) and incubated at $28 \pm 2^\circ\text{C}$ for seven days. Afterwards, fragments of 6 mm^2 were removed from each one of the endophytic fungi and from the pathogen and were added to a Petri dish at opposite poles, keeping a distance of 4 cm between the two test fungi.

The tests were performed in triplicate and a control was run containing the phytopathogen at the same position in the test plate. The plates were incubated at $28 \pm 2^\circ\text{C}$ for seven days. The method for analyzing endophyte-phytopathogen interactions was based on the scale of Badalyan et al. (2002), according to three types of interactions, A, B and C, and four subtypes (CA1, CA2, CB1 and CB2), where: A = deadlock with mycelial contact, B = deadlock at a distance, C = replacement, overgrowth without initial deadlock; CA1 and CA2 = partial and complete replacement after initial deadlock with mycelial contact; and CB1 and CB2 = partial and complete replacement after initial deadlock at a distance.

The following scores were assigned to each type of interaction: A = 1.0, B = 2.0, C = 3.0; CA1 = 3.5; CB1 = 4.0; CA2 = 4.5 and CB2 = 5.0. The antagonism index was assessed for each isolate according to the following formula: $AI = \sum N \times I$, where: N = the frequency of each type of interaction, and I = corresponding score.

AI was also calculated for the 46 endophytes of *L. divaricata* tested. The results of the interactions of the antagonistic test between the fungi were evaluated for significant differences by ANOVA, and the antagonistic effects were compared using the Dunnett test, with the aid of the Statistica software.

Evaluation of the production of secondary metabolites and antimicrobial activity

Secondary metabolites were isolated using the method of Li et al. (2005), with modifications. The fungi were incubated in BD liquid medium in Erlenmeyer flasks and incubated at $25^\circ \pm 2^\circ\text{C}$ for nine days on an orbital shaker (New Brunswick Scientific-Edison NJ, USA) at 160 rpm. The fermented medium was centrifuged at 3600 rpm for 10 min. The supernatant was then added to a separatory funnel along with an equal volume of ethyl acetate. The funnel was shaken and the phases were separated. The extraction was repeated twice. The resulting ethyl acetate from the extraction was 98% concentrated on a rotary evaporator at $50 \pm 2^\circ\text{C}$. The material resulting from evaporation was stored at $4 \pm 2^\circ\text{C}$. Metabolites extracted directly from the mycelium collected on the surface of the medium were also used. The mycelium was filtered and maintained for 24 h in methanol. Afterwards, it was centrifuged and the supernatant was collected and 98% concentrated on a Marconi AM 120 rotary evaporator at $50 \pm 2^\circ\text{C}$. The material resulting from evaporation was stored at $4 \pm 2^\circ\text{C}$.

Antimicrobial activity was tested using qualitative biological assays in triplicate. The microorganisms used in the test were the phytopathogenic fungi *Alternaria alternata*, *Colletotrichum* sp and *Moniliophthora perniciosa* and the human pathogenic bacteria *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). Tests with the bacteria were performed with the disk diffusion technique using the metabolite extract of endophytic isolates. The test bacteria were grown for 24 h in LB liquid medium (Sambrook and Russel, 2001), adjusted to a concentration of 10^8 cells/mL, using the antibiotic tetracycline (Sigma) ($50 \mu\text{g/mL}$ in absolute ethanol) as the positive control. The bacteria were added ($100 \mu\text{L}$) to Petri dishes containing LB medium and they were spread with Drigalsky spatula. Subsequently, four sterile 6-mm discs of Whatman filter paper No. 4, inoculated with $10 \mu\text{L}$ metabolite extract, were placed equidistant in the dishes.

The plates were incubated at $37 \pm 2^\circ\text{C}$ for 24 h. We evaluated the antimicrobial activity by the formation of an inhibition halo, according to Souza et al. (2004). For the analysis of inhibition of phytopathogen growth, the fungi were grown on PDA medium for seven days at $28 \pm 2^\circ\text{C}$. We then prepared a spore suspension (10^6 spores/mL). We added $100 \mu\text{L}$ of the spore suspension to Petri dishes. The positive control was the fungicide Derosal Plus[®] at a 1:10 dilution. Later, we added four sterile Whatman No. 4 filter paper disks (6 mm), inoculated with $10 \mu\text{L}$ metabolite extract and placed equidistant. The plates were incubated at $28 \pm 2^\circ\text{C}$ for seven days. We evaluated the antimicrobial activity by the formation and extent of the inhibition halo according to Li et al. (2005).

Analysis of genetic variability based on ARDRA

To analyze the genetic variability of the isolates, we used endophytic fungi isolated

from *Luehea divaricate*, previously identified by Bernardi-Wenzel et al. (2010), belonging to the more common genera *Diaporthe* and *Phomopsis*, where the latter is the asexual anamorphic form of *Diaporthe*. Accordingly, we used the ARDRA technique.

The method used for genomic DNA extraction was that of Pamphile and Azevedo (2002), with the modifications described by Bernardi-Wenzel et al. (2010). In the DNA amplification, 25- μ L reaction mixture contained 2.5 μ L buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 2.5 μ L dNTPs (2.5 mM), 1.5 μ L each one of the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCC TCCGCTTATTGATATGC) (10 μ M; Invitrogen), 1.0 μ L MgCl₂ (50 mM), 0.2 μ L Taq DNA polymerase (5 U/ μ L), 1 μ L DNA (10 ng/ μ L), and 14.8 μ L autoclaved distilled water.

The reaction mixture was placed in a thermocycler (TTC-100; MJ Research, Inc.) programmed to perform 35 cycles after an initial denaturation at 92°C for 4 min. Each amplification cycle consisted of three steps: denaturation (92°C, 40 s), annealing (55°C, 1 min 30 s) and extension (72°C, 2 min.). Final extension was performed at 72°C for 5 min.

Of a total of 25 μ L amplified product (approximately 600 bp), 5 μ L were digested using 10 U of the restriction enzymes *Mbo*I, *Cla*I, and *Hha*I (Invitrogen) diluted in 1.5 μ L buffer and 8 μ L sterile distilled water. The digestion products were separated on a 1.4% agarose gel containing 0.5 μ g/ml ethidium bromide, immersed in TBE buffer (Tris-borate-EDTA 1.0 mM, pH 8.0) at 70 V. The size of the amplified product was estimated by comparison with a 100-bp molecular marker (Gibco-BRL, Gaithersburg, MD, USA). The DNA bands were visualized under ultraviolet light and photographed with a photo documentation system, according to Oliveira and Costa (2002).

Genetic distance between the isolates analyzed was determined using MEGA software (Tamura et al., 2007) with grouping by the neighbor-joining method (Saitou and Nei, 1987), using “p-distance” for nucleotides with “pairwise gap deletion” and with 10,000 bootstrap replications. The evaluation was conducted using for comparison fungi already deposited in the NCBI database, which were similar to the isolates from *L. divaricata*.

RESULTS

***In vitro* antagonistic activity of endophytic isolates against the phytopathogen *Alternaria alternata* and interaction between fungi in dual culture**

Of the 46 endophytes analyzed regarding interactions with the phytopathogen *A. alternata*, 22 were previously identified by Bernardi-Wenzel et al. (2010) as being of the following species: G1 A2-44 and G2 A1-32 as *Alternaria alternata*, G3 A1-34 and G12 A2-39 as *Phomopsis phyllanthicola*, G5 A1-59 as *Epicoccum nigrum*, G6 A1-20 as *Cochliobolus intermedius*, G8 A1-01 as *Guignardia vaccinii*, G9 A1-40 as *Cochliobolus lunatus*, G15 A1-23, G26 A1-03 and G45 A2-42 as *Phomopsis* sp, G18 A2-24, G35 A2-18, G37 A1-65 and G46 A2-58 as *Phomopsis chiamonanthi*, G23 A1-62 and G44 A1-14 as *Diaporthe helianthi*, G24 A2-28 as *Phoma herbarum*, and G27 A2-41, G29 A2-48, G36 A1-22 and G39 A2-59 as *Diaporthe* sp.

The rates of antagonism varied from 3.7 to 62.7%. About 68% of the endophytic isolates of *L. divaricata* tested (Table 1) showed an *in vitro* antagonism rate over 40% inhibition of growth of *A. alternata* compared to the control.

Table 2 shows the types of interactions observed by the paired culture test, according

to the scale of Badalyan et al. (2002). Two types and a subtype of competitive interactions between endophytes of *A. alternata* were observed: A, B and CA1 (Figure 1).

Table 1. Effect of antagonism of endophytic fungi isolated from *Luehea divaricata*, in inhibiting growth of *Alternaria alternata*.

Fungal isolate	Mycelial growth of <i>A. alternata</i> (% reduction over control)*	Fungal isolate	Mycelial growth of <i>A. alternata</i> (% reduction over control)*
G1 A2-44	49.9 ^{***}	G26 A1-03	48.1 ^b
G2 A1-32	33.3 ^b	G27 A2-41	40.1 ^b
G4 A1-08	46.6 ^b	G28 A1-46	51.3 ^b
G5 A1-59	41.2 ^b	G29 A1-27	56.2 ^b
G6 A1-20	29.7 ^b	G30 A2-57	48.2 ^b
G7 A1-56	51.0 ^b	G31 A2-48	41.4 ^b
G8 A1-01	3.7 ^a	G31 A2-56	40.0 ^b
G9 A1-57	44.2 ^b	G32 A1-33	41.3 ^b
G10 A1-52	38.1 ^b	G33 A2-20	18.5 ^a
G11 A2-26	47.7 ^b	G34 A1-30	50.0 ^b
G12 A2-39	42.5 ^b	G35 A2-46	58.0 ^b
G13 A2-33	34.9 ^b	G36 A1-22	50.2 ^b
G14 A2-03	35.9 ^b	G37 A1-65	47.4 ^b
G15 A1-23	11.5 ^a	G38 A2-16	49.5 ^b
G16 A1-43	31.7 ^b	G39 A2-59	36.2 ^b
G17 A2-19	42.6 ^b	G39 A2-14	35.9 ^b
G17 A2-06	44.7 ^b	G40 A2-43	43.9 ^b
G18 A1-15	42.4 ^b	G41 A1-11	33.3 ^b
G18 A2-24	32.0 ^b	G42 A2-01	26.8 ^b
G19 A1-25	47.7 ^b	G42 A2-13	21.5 ^b
G20 A1-26	55.8 ^b	G43 A1-16	54.5 ^b
G23 A1-62	54.2 ^b	G44 A1-14	51.9 ^b
G24 A2-28	58.0 ^b	G45 A2-62	62.7 ^b
G25 A1-63	48.3 ^b	G46 A1-60	46.0 ^b
G26 A1-04	41.5 ^b	Control	0.0 ^a

*Average of triplicates. **Values followed by the same letter(s) are not statistically significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Table 2. Interactions and antagonism index between the endophytic fungi isolated from *Luehea divaricata*, and the phytopathogen *Alternaria alternata*.

Type/subtype interaction	Antagonism index (AI)	Fungal isolate
A	3.0	G1 A2-44, G4 A1-08, G9 A1-57, G11 A2-26, G13 A2-23, G14 A2-03, G16 A1-43, G17 A2-19, G17 A2-06, G18 A1-15, G18 A2-24, G25 A1-63, G26 A1-03, G28 A1-46, G30 A2-54, G30 A2-52, G30 A2-57, G31 A2-56, G32 A1-33, G33 A2-20, G34 A1-30, G36 A1-21, G37 A1-65, G39 A2-59, G38 A2-16, G39 A2-14, G42 A2-01, G42 A2-13, G43 A1-42, G45 A2-62, G46 A2-58, G46 A1-60
B	6.0	G2 A1-32, G5 A2-14, G6 A1-20, G7 A1-56, G8 A1-01, G9 A1-40, G10 A1-12, G10 A1-52, G11 A1-37, G13 A2-33, G12 A2-39, G15 A1-23, G19 A1-25, G20 A1-26, G23 A1-48, G23 A1-62, G24 A2-28, G25 A1-49, G26 A1-04, G27 A2-41, G28 A1-64, G29 A2-45, G29 A2-33, G29 A1-27, G29 A1-47, G29 A2-61, G31 A2-48, G35 A2-46, G36 A1-18, G41 A1-11, G43 A1-16, G43 A2-51, G46 A1-58, G45 A2-11, G45 A2-42, G44 A1-14
CA1	10.5	G29 A1-41, G40 A2-43

In G4 and G11, there was “deadlock” with mycelial contact, characterizing the type A interaction; in G5 growth inhibition observed with the formation of a “deadlock” at a distance barrier, characterizing the type B interaction; G40 showed overlapping growth of the endophyte on the phytopathogen after formation of an initial “deadlock” with mycelial contact, characterizing the interaction of type C, subtype CA1.

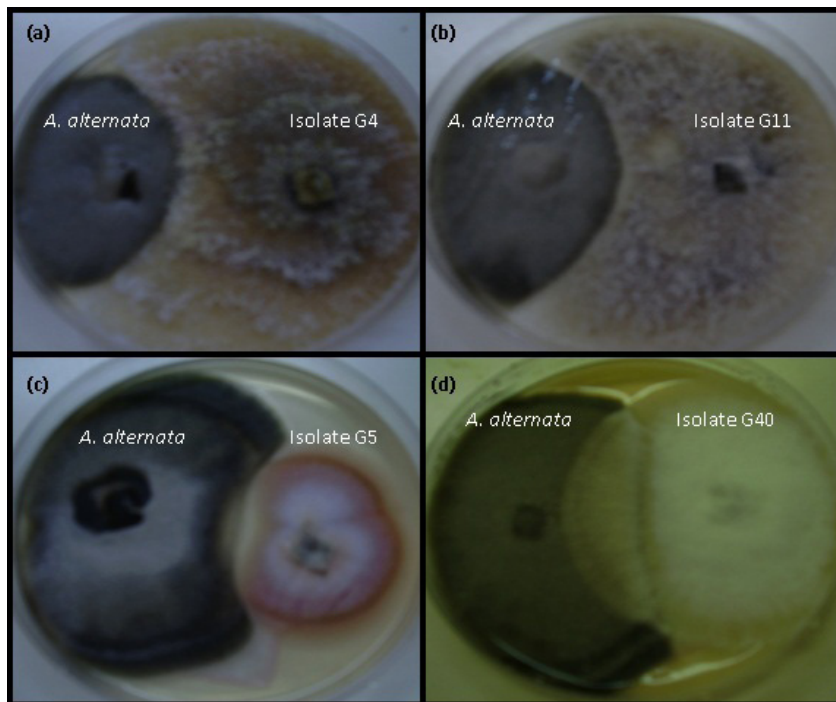


Figure 1. Interactions among different groups of endophytic fungi isolated from leaves of *Luehea divaricata* and *Alternaria alternata* after 7 days of incubation at 28°C. **a.** and **b.** Interaction of type A = deadlock with mycelia contact; **c.** interaction of type B = deadlock at a distance; **d.** interaction of type CA1 = partial replacement after initial deadlock with mycelia contact.

The antagonism index shown by the *L. divaricata* endophytes concerning *A. alternata* was observed in all the endophytic fungi tested, with values from 3.0 to 10.5.

Evaluation of antimicrobial activity

In the evaluation of antimicrobial activity, we tested some of the endophytic fungi isolated from *L. divaricata* selected in the antagonism test, that displayed the type B interaction. The fungi used were: G5 A2-14, G7 A1-56, G8 A1-01, G19 A1-25, G23 A1-62, G29 A2-33, G29 A1-27, G39 A2-59, and G43 A1-16, from which metabolites were extracted by fermentation and separation with ethyl acetate or by incubation of the mycelium with methanol. These metabolites were initially tested against pathogenic strains of the bacteria *E. coli* and *S. aureus*.

Among the metabolite extracts of the endophytic fungi tested, none of them showed antibacterial activity against *S. aureus*. However, against *E. coli*, there was the formation of discrete inhibition halos with the following isolates: G5 A2-14, G7 A1-56 (extracted with ethyl acetate), G19 A1-25, G23 A1-62, G29 A2-33, G29 A1-27, G39 A2-59, and G43 A1-16 (extracted with methanol).

Unlike isolate G23 - A1-62 (whose metabolite extract was obtained by incubation of the mycelium in methanol), which showed inhibition halos with the phytopathogen *M. perniciosa* (Figure 2) and pathogenic strain of *E. coli*, no other extract tested was able to inhibit the growth of the phytopathogens analyzed.

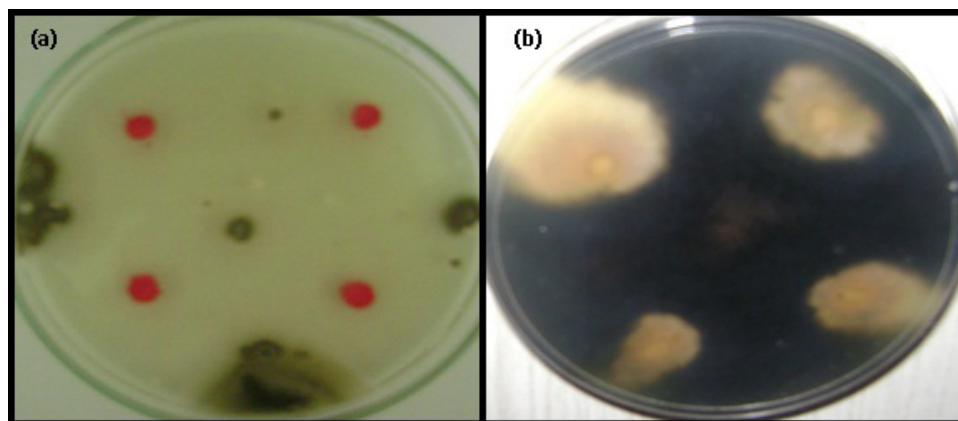


Figure 2. Inhibition halo produced against phytopathogen *Moniliophthora perniciosa* incubated at 28°C for 5 days. **a.** Positive control with Derosal Plus® fungicide; **b.** inhibition halo generated by the endophytic isolate G23 - A1-62.

Genetic variability analysis based on ARDRA

As can be seen in Figure 3, unlike the result of the *MboI* and *ClaI* enzymes, which did not show polymorphism, restriction analysis with the enzyme *HhaI* resulted in a polymorphic pattern among the isolates tested. The restriction profile of the ITS region of the rDNA allowed a separation of these isolates into three different ARDRA patterns (haplotypes).

The isolates G12 and G03 (*Phomopsis phyllanthicola*) were grouped into haplotype A, isolates G15, G26, G36, G37, G44, G45 (*Phomopsis* sp) and G39 (*Diaporthe* sp) in haplotype B, and isolates G18, G35 and G46 (*Diaporthe chiamonanthi*) in haplotype C. The DNA of isolate G18 had a partial restriction perhaps due to the DNA concentration, since a band appeared slightly above 500 bp, corresponding to whole rDNA. All these isolates were molecularly identified as belonging to the genus *Phomopsis* or *Diaporthe*. The genus *Phomopsis* is the asexual, anamorphic form of *Diaporthe*. By analyzing the sequences of rDNA with BLAST in the NCBI database, G44 and G39 had a higher similarity with the genus *Diaporthe*, and these two isolates were grouped by ARDRA in the same haplotype. The separation of the different endophytic isolates from *Phomopsis* into three different haplotypes indicated an interspecific variability.

Figure 4 shows the phylogenetic tree constructed on the basis of data obtained by sequencing the isolates of genera *Diaporthe* and *Phomopsis* belonging to haplotypes A, B and C, compared to isolates already deposited in the NCBI database, with their respective families, indicating the pattern of similarity of the isolates. It can be observed that although G46 was clustered in the same haplotype as G18 and G35, due to their belonging to the same species, this isolate showed greater distance regarding the isolates of its haplotype than regarding isolates of different haplotypes.



Figure 3. Restriction profiles of the ITS region of rDNA of some endophytic isolates from *Luehea divaricata* using the enzyme *Hha*I. PM = 1-kb ladder molecular weight DNA marker.

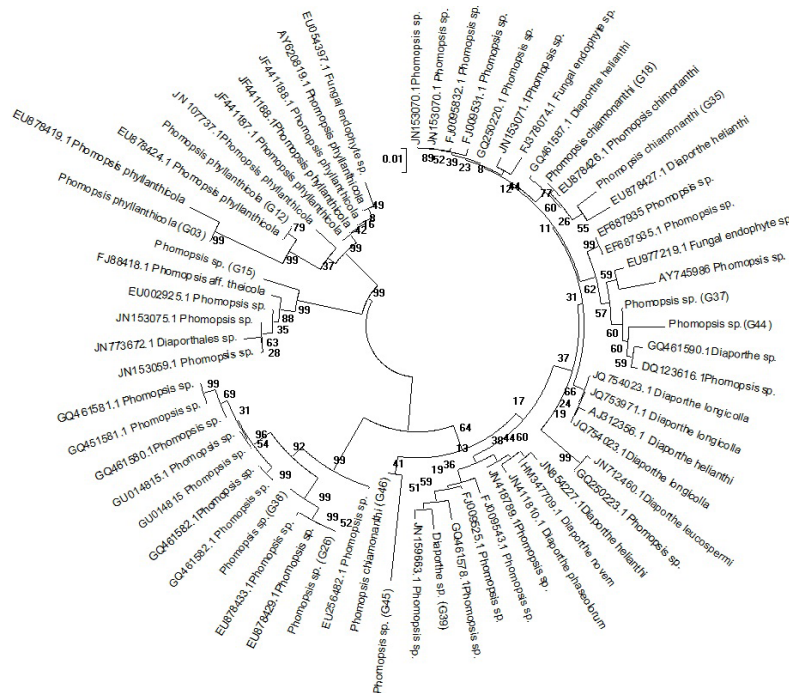


Figure 4. Phylogenetic tree constructed with sequences of endophytic isolates from leaves of *Luehea divaricata* of the genera *Phomopsis* and *Diaporthe* (containing codes on the right), belonging to the phylum Ascomycota, class Sordariomycetes, and order Diaporthales, and sequences from GenBank (indicated by the code on the left) using the method of neighbor-joining clustering using P distance for nucleotides with the option “pairwise deletion gap”. The numbers on the tree indicate the percentage of times the right group occurred in the same node during the evaluation of consensus (bootstrap with 10,000 repetitions).

DISCUSSION

The reduction in mycelial growth of *A. alternata* mediated by endophytic fungi isolated from *L. divaricata* showed that some endophytes are capable of considerable competition, reaching 62.7%. Other studies analyzing the interactions between endophytic isolates and different pathogens demonstrated a reduction in pathogens growth ranging from 28.5 to 78.8% (Gomes-Figueiredo et al., 2007; Campanile et al., 2007; Sempere and Santamarina, 2007; Bailey et al., 2008).

Crozier et al. (2006), also studying the phytopathogens *Moniliophthora roreri* and *M. perniciosa*, the causative agents of fresh green bean rot and witches' broom disease, respectively, used endophytic fungi to combat pathogens and showed that the endophytic fungi of this plant could prevent the initial colonization of the pathogens competing for the same ecological niche and invading them. Furthermore, the endophytic fungi were able to produce bioactive metabolites with antagonistic action against these pathogens.

A similar study evaluating the potential of endophytic fungi to combat phytopathogens was carried out by Rojo et al. (2007), in which the authors observed that isolates of the genus *Trichoderma* produced compounds that inhibited the activity of *Fusarium solani*, increasing the yield and vigor of the plants.

Pileggi et al. (2002) isolated an endophytic fungus from comfrey (*Symphytum officinale* L.), a medicinal plant widely used for its anti-inflammatory and anti-psoriasis properties, and this isolate showed antimicrobial activity against *S. aureus*, once again suggesting the possible medicinal action of metabolites produced by endophytic fungi from medicinal plants. The antagonism index displayed by the endophytes of *L. divaricata* in relation to *A. alternata* indicates that all were antagonistic toward the phytopathogen, at least creating a barrier "deadlock" (interaction type A), which prevented its growth after mycelial contact with the endophytic fungus.

The mild antibacterial activity of extracts of endophytic isolates from *L. divaricata* shown in this work against *E. coli* indicates the probable role of endophytes in the medicinal activity of *L. divaricata*, partially agreeing with de Souza et al. (2004), who demonstrated that *L. divaricata* produced slight inhibition of growth of *Micrococcus luteus*, but had no effect on *S. aureus*, *S. epidermidis*, *E. coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Candida albicans*.

Castillo et al. (2007) found that endophytic fungi of the genus *Streptomyces* isolated from plants of the genus *Nothofagus* and other plants found in southern Patagonia were effective in the control of several pathogenic fungi such as *Pythium*, *Sclerotinia*, *Rhizoctonia*, *Mycosphaerella* and *Phytophthora*. The antimicrobial activity of 13 strains of the fungus *Phomopsis* sp, endophytic isolates from medicinal plant leaves of *Aspidosperma tomentosum* and from petioles of the medicinal plant *Spondias mombin* was demonstrated by Corrado and Rodrigues (2004). Among the extracts tested, three were able to inhibit all strains tested, including bacteria, yeasts and filamentous fungi, showing the great potential of this fungus as a source of bioactive products.

The identification and effective selection of antagonism among microorganisms is the first step in biological control (Kamalakaran et al., 2004). The mechanism by which endophytic fungi caused growth inhibition of the phytopathogen at a distance is probably due to some metabolite released into the culture medium, which prevented the growth of *A. alternata* towards the endophytic fungus. Thus, antimicrobial activity was again demonstrated for the endophytic fungi, which could provide bioactive compounds of high commercial value.

Concerning the ARDRA results, they are consistent with those of Oliveira and Costa (2002), who used the enzyme *Hae* III and distinguished three groups among the isolates of the soy phytopathogen and the bean *Fusarium solani*. Although the isolates that were pathogenic to soybean or to bean were not differentiated from the ones that were pathogenic to both cultures, it was possible to differentiate the isolates of *F. solani* f. sp. *phaseoli* and *F. solani* f. sp. *glycines* through distinct restriction patterns. Procopio et al. (2009), using combined analysis of ARDRA and RAPD to analyze endophytic bacteria isolated from *Eucalyptus* spp, found that even though occurring in the same group by ARDRA, it was possible to determine sufficient variations to classify the isolates into different groups by RAPD based on the frequency of polymorphisms found, which indicates an adaptation of endophytes to different environmental conditions or different geographic areas.

The phylogenetic analysis based on sequencing data of 20 endophytic isolates from *L. divaricata* confirmed the ARDRA results when assigning the strains G3 and G12 to the same group (haplotype). It is also noted that these combined results showed an intraspecific variability in the genus *Phomopsis*.

The construction of phylogenetic trees based on 18S rDNA genes and also on the internal transcribed spacer (ITS) has been shown to be an important taxonomic tool for the grouping of endophytic fungi, demonstrating the presence of common genera and new endophytic genera associated with plants, allowing their correct identification and classification to later determine the biotechnological potential of these isolates (Orlandeli et al., 2012; Garcia et al., 2012; Rhoden et al., 2012).

The results presented in this study support the need to study the biotechnological potential of endophytes isolated from various medicinal plants, in view of the more than 60% antagonism of endophytes against the phytopathogen *A. alternata*. This antagonistic action may be of great importance in the maintenance of communities of endophytic fungi within host plants, in a mutualistic process, positively selected through a process of co-evolution between host and endophyte. Furthermore, we demonstrated the importance of phylogenetic analysis in revealing the interspecific variability of endophytic fungi, even when belonging to the same genus, which may show very close taxonomic relationships between endophyte and host.

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