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Diversity of foliar endophytic fungi from the medicinal plant *Sapindus saponaria* L . and their localization by scanning electron microscopy

Adriana García[1], Sandro A Rhoden[1], Celso J Rubin Filho[2], Celso V Nakamura[3], João A Pamphile[1]*

[1] Department of Cell Biology and Genetics - Universidade Estadual de Maringá; [2] Department of Biology - Universidade Estadual de Maringá; [3] Department of Microbiology - Universidade Estadual de Maringá.

ABSTRACT

Endophytic fungi inhabit vegetable tissues or organs, without causing them any harm. Endophytes can co-evolve with plant hosts and possess species-specific interactions. They can protect the plant from insect attacks and diseases, and are also able to produce substances of biotechnological interest. In folk medicine, the bark, roots and fruits of *Sapindus saponaria* is used to produce substances with anxiolytic, astringent, diuretic and expectorant properties, as well as tonics, blood depuratives and cough medicine. This study evaluated the diversity of endophytic fungi present in the leaves of *S. saponaria* L. and observed the colonization of host plants by endophytes, using light and scanning electron microscopy. We verified that these fungi are found in intercellular and intracellular spaces. The genera of some isolates of *S. saponaria* were identified mainly by sequencing of ITS region of rDNA and, when possible, also by their microscopic features, as follows: *Cochliobolus*, *Alternaria*, *Curvularia*, *Phomopsis*, *Diaporthe* and *Phoma*. Phylogenetic analysis showed the existence of genetic variability of the genera *Phomopsis* and *Diaporthe* and interspecific variation among the *Curvularia*, *Alternaria* and *Phoma*, belonging to family Pleosporaceae.

Key words: Endophyte, light microscopy, phylogenetic analysis, SEM.

INTRODUCTION

Endophytic fungi, or endophytes, are microorganisms that colonize healthy plant tissue, remaining there for at least one cycle of their lives without causing any damage to the host plant through a symbiotic relationship. Several fungal (Li et al., 2010; Qiu et al., 2010) and even bacterial (Andreote et al., 2009; Figueiredo et al., 2009) endophytes colonize plants. Endophytes can also protect the plant from many biotic and abiotic threats (Azevedo et al., 2000). Endophytes colonize intercellular spaces as well as the interior of xylem and phloem cells (Hallmann et al., 1997).

The endophyte-plant relationship may have begun with the growth of higher vegetables hundreds of millions years ago, resulting from coevolutive processes (Strobel et al., 1996; Strobel and Long, 1998). Around 100,000 fungal species have been described and nearly 10% of these obtain nutrients from associations with other organisms, such as plants and animals (Tunlid and Talbot, 2002).

There is increasing effort to characterize and identify endophytic fungi isolated from medicinal plants. Many studies have shown that some medicinal properties of plants may be related to endophytic fungi hosted by these plants (Azevedo et al., 2002).

Sapindus saponaria L. belongs to Sapindaceae family. In Brazil, it is popularly known as "sabão-de-soldado", among other names. It is found in South and Central America, from forests to "cerrado", a vast tropical savanna ecoregion. In Brazil, it is found from Para State to Rio Grande do Sul State (Albiero et al., 2001; Lorenzi, 2004).

The bark, roots and fruit of *S. saponaria* are used in folk medicine as anxiolytic, astringent, diuretic, expectorant, tonic, blood depurative, cough medicine and wound healer (Albiero et al., 2001). Fruit extracts present antifungical (Tsuzuki et al., 2007), larvicidal (Silva et al., 2004; Fernandes et al., 2005; Barreto et al., 2006), and bleeding neutralizer activities (Castro et al., 1999).

Many fungi have been identified by morphological markers; mainly reproductive structures. However, several fungi do not present these structures in artificial cultures, making classification and identification difficult. According to Magnani et al. (2005) the development of molecular biology for genetic differentiation of fungi species resulted in great advances in taxonomy, due to the sensitivity and specificity of these techniques.

Analysis of rDNA sequencing is used to estimate endophytic fungi diversity. Ribosomal genes are most often employed in fungal phylogenetic analysis. Within the rDNA locus, the ITS region has been particularly useful for analysis of closely related fungal species in many genera (Zervakis et al., 2004; Stefani and Berube 2006). The rRNA gene unit is composed of the internal transcribed spacer (ITS) and the sequences of the coding rRNAs.

According to Kuldau and Yates (2000), light and electron microscopy can reveal the precise internal locations of endophytic fungi. Generally, assessments are made to determine whether the fungal infection is intercellular or intracellular and to establish the plant cellular anatomy. These authors argue that assigning endophytic status to a fungus based on microbiological techniques would be much more conclusive, with microscopic evidence to confirm the location of the fungus.

Considering the importance of the medicinal plant *S. saponaria*, the aim of the present work was to determine foliar endophytic fungal diversity of *S. saponaria*. We also sought to determine if endophytic fungi of *S. saponaria* are inter- or intracellular.

*Author to correspondence - João A. Pamphile, Universidade Estadual de Maringá - Av. Colombo, n. 5790 - CEP 87020-900 – Maringá PR Brazil. e-mail: prof.pamphile@gmail.com Tel: +44-3011-4342/ Fax: +44-3011-4893.

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METHODS

Isolating endophytic fungi

The endophytic fungi strains used in this study were isolated from a 15 year-old *S. saponaria* tree (*Sapindacea*), located in Maringa (Parana-Brazil - 23° 24' W, 51°56' S and 518 m.a.s.l.). The collection was carried out in May 2007. The monthly temperature was between 18º and 31ºC and relative humidity was approximately 75%. Only apparently healthy leaves without any stains or other injuries were selected. The leaves were collected with the branch, kept in sealed plastic bags and immediately submitted to surface disinfection.

Fungi were isolated by the methodology of Pamphile and Azevedo (2002), with slight modifications. NaOCl at 5% was used for three minutes. Three hundred foliar fragments were used. The disinfected leaves were aseptically cut into 5 mm^2 fragments; five foliar fragments were placed in Petri dishes containing potato-dextrose agar (PDA), and 50 $\mu g/mL$ of tetracycline (Sigma) in order to avoid bacterial growth. After that, the material was incubated at 28ºC in B.O.D. for about 5 days.

As negative control, the bath water for the last leaves was used. An aliquot of 100 μL was put into Petri dishes containing PDA and incubated under the same conditions as the samples were.

Characterization and identification of endophytic fungi according to morphology

Macroscopic characterization was based on morphological observation, considering color, mycelial growth of colonies, and the presence and development of reproductive structures. This characterization permitted a preliminary identification. We employed the micro-cultivation technique described by Ribeiro and Soares (2002). After this procedure, microscope slides were observed under a light microscope, with a system of image capturing (Zeiss Axioskop 2 Plus). Macroscopic and cytological analysis employed identification keys (Ellis, 1971; Barnett and Hunter, 1972).

DNA extraction, rDNA amplification and sequencing

We used the methodology of Pamphile and Azevedo (2002) for genomic DNA extraction, with slight modifications, growing the endophytic fungi on a dialysis membrane in PDA medium for 5 days, at 28°C, instead of growing it in a totally liquid medium. The DNA concentration was estimated in agarose gel 1.0%. High DNA Mass Ladder (Invitrogen) was used as a molecular weight standard. The final concentration of DNA was adjusted to 10 ng. mL^{-1} for rDNA amplification.

The ITS1- 5.8S - ITS2 fragments were amplified with ITS1 and ITS4 primers (White et al., 1990). The amplification was based on the methodology described by Magnani et al. (2005). The amplified products at the rDNA regions ITS1- 5,8S- ITS2 of the isolated endophytic fungi were purified with GFX PCR DNA and Gel Band Purification (Amersham Biosciences), according to the manufacture's instructions.

The sequencing reaction of the ITS rDNA regions was under the same conditions as described by Magnani et al. (2005). The sequencing was carried out with a MegaBace TM 1000 sequencer (Amersham Biosciences). The injection and electrophoresis conditions were of 2 Kv/60 s and 6 Kv/230min, respectively. For the identification of endophytic lineages of *S. saponaria*, the rDNA sequences were compared to those from NCBI (National Center for Biotechnology Information) database using the BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The species identification was based on the best similarity rate obtained. The sequences determined were aligned and edited with the MEGA 3.1 software (Kumar et al., 2004), with grouping by the "neighbor-joining" method (Saitou and Nei, 1987), using "p-distance" for nucleotides with the option of "pair wise gap deletion", using bootstrap (BP) with 10,000 repetitions.

The rDNA sequences obtained in this study have been submitted to the GenBank and assigned accession numbers GQ 461566 to GQ 461579.

Sapindus saponaria anatomic analysis - Light Microscopy

Aiming at verifying the presence of endophytic fungi inside the leaves, the plant tissue was submitted to anatomic analysis. The methodology used was based on Guerrits (1991), with slight modifications by including material in historesin. The leaves, after the collection, were submitted to surface disinfection. After this, the leaves were transferred to amber flasks and fixed in bouin for 24 hours. Subsequently, the material was removed from the fixer and the leaves were kept in alcohol at 70% for 2 hours. This procedure was repeated for the same durations, substituting alcohol at 70% for alcohol at 80% and 90%, absolute alcohol, absolute ethanol with xylol (1:1) and finally xylol. The content was removed again and the xylol bath was repeated for 40 and 30 minutes, respectively.

The xylol was removed and the material was submitted to 3 paraffin baths. After that, the leaves were inserted into paper blocks with hot paraffin and kept in a vertical position until the paraffin solidified, so that the leaves could also stay in the same vertical position. Before making histological cuts, the material was kept in the mould for at least 24 hours.

The histological cuts were done in rotative microtome, using a 0.7 mm steel razor. Subsequently, the cuts were heated under a water bath and then microscope slides using a brush. The material was put into a greenhouse to dry and to melt the paraffin.

The cuts were submitted to a rehydration process. The slides were placed on glass slides and then submitted to three baths in xylol 30 minutes, twice in absolute alcohol for one minute, alcohol at 90% for one minute, alcohol at 70% for one minute, distilled water for one minute and then submitted to Trypan Blue (TB) staining, adapted from Barrow (2003). The slides were then washed with xylol, assembled with slides and observed in an optical microscope with an image capturing system.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was conducted according to Pamphile et al. (2008), with slight modifications, using alcohol gradient instead of acetone (30, 50, 70, 90 and 100%).

The leaves were collected with branches and submitted to surface disinfection. The fragments were dried in a critical point dryer, undergoing 7 cycles. The samples were assembled in stubs with vertical positioning of the fragments, with adhesive tape conductors of SEM. The fragments were covered

with a thin layer of gold (260 seg., 50mA, at 27ºC) for 3 cycles, in a metallic covering apparatus (sputtering). The fragments covered with gold were observed at a SEM emission field of 5 kV, from a distance of 7 mm.

To carrying out the SEM, a critical point BAL-TEC-CPD 030 device was used; for the metallic coverage, a SHIMADZU-IC 50 device and SHIMADZU-SS550 electron scanning microscope were used, made available by the Central Complex for Research Support (COMCAP-UEM).

RESULTS

Isolation of endophytic Sapindus microorganisms

Colonization was obtained 100% of the 300 sampled foliar fragments of *S. saponaria*. One hundred and two endophytic fungi were randomly selected for isolation in an axenic culture on PDA, for further analysis. The negative control shows the absence of microbiological growth.

Identification of endophytic isolates

A hundred and two *S. saponaria* isolates were initially grouped in 27 morpho-groups, according to morphological characteristics like color, mycelium growth in PDA and the presence of reproductive structures. The morphological and cytological observations were sufficient to identify strains G2-20 as *Cochliobolus*, G3-92 and G5-4A as *Alternaria* sp. However, other isolates did not develop reproductive structures in the artificial culture of PDA medium, making it difficult to identify them with micro-cultivation.

By using data from the sequencing of the ITS1-5,8S-ITS2 endophytic fungi region and the BLAST search of the NCBI database, it was possible to determine the genus and/or species of 13 isolates, corresponding to 13 morphotypes, from a total of 27. Among the endophytic fungi identified by rDNA-ITS region sequencing were *Cochliobolus*, *Alternaria*, *Curvularia*, *Phomopsis*, *Diaporthe* and *Phoma* (Table I). The results of the ITS-DNA sequencing blasting analysis were congruent with results of the morphological and cytological analysis.

Phylogenic analysis of foliar fungal endophytes of S. saponaria based on rDNA sequencing data

The phylogenetic analysis was based on the data from 14 endophytic *S. saponaria* isolates, together with those that presented similarity in the NCBI database, and others sequences belonging to differents families and orders, with the objective of confirming that the isolates are grouped with the families with the closest Blast identity. The phylogenetic analysis is presented in Figure 1. The rDNA sequences of *S. saponaria* isolates presented about 456 to 685 pb. The fungi lineages could be grouped into 3 main clades (A, B and C). Clade A groups fungi from th Dothideomycetes class with 54% bootstrapping (BP) analysis: *Cochliobolus, Curvularia, Alternaria, Phoma, Bipolaris* (Pleosporaceae family) and *Septoria* (Mycosphaerellaceae family) and the endophytic isolates G2-20, G6-32, G5-4A, G24-91 and G25-66. Clade B groups other Dothideomycetes belonging to more distant species than those in Clade A. *Curvularia/Alternaria* could be placed in one clade with 95% BP. Clade C groups the fungi genera from the sordariomycete class with 91% BP support

TABLE I

Endophytic isolates of S. saponaria leaves, identified according to their genera or species and the similarity percentage found in the NCBI (National Center for Biotechnology Information) database.

Endophytic Isolate	Fungal Genus/Species with the most related sequence	NCBI access number of the most related sequence	Identity (%)
G2-20	Cochliobolus intermedius	AF071327.1	97
G3-92	*Alternaria* sp.	FJ210489.1	90
G5-4A	*Alternaria alternata*	DQ093656.1	96
G6-32	*Curvularia* sp. *Curvularia affinis* *Curvularia affinis*	HM775181.1 AF071335.1 GU073105.1	95 95 95
G12-60	*Phomopsis* sp.	EU878436.1	88
G13-63	*Diaporthe* sp.	AB245446.1	96
G15-64	*Phomopsis* sp.	FN597586.1	97
G17-70	*Phomopsis* sp.	EF687936.1	96
G19-18	*Phomopsis chimonanthi* *Phomopsis micheliae* *Diaporthe helianthi*	AY622993.1 AY620820.1 AJ312356.1	95 95 95
G22-97	*Phomopsis* sp.	EF687936.1	97
G24-91	*Phoma* sp.	AY513965.1	98
G25-66	*Phoma* sp.	EF120407.1	97
G26-89	Ascomycete sp. Ascomycota sp.	AM08445.1 GU062291.1	91 97

values: *Phomopsis* and *Diaporthe* (Valsaceae family) and more distant species like *Beauveria*, *Paecilomyces*, *Cordyceps*, *Isaria*, *Metarhizium*, *Trichoderma*, *Ophiocordyceps*, *Fusarium*, *Paecilonyces* and *Gibberella*. The endophytic isolates G12-60, G13-63, G19-18, G15-64, G22-97 and G17-70 also belong to Clade C. These endophytes and the species *Phomopsis*/*Diaporthe* can be placed in one subclade with 99%BP.

Light microscopy

In the transversal cuts of *S. saponaria* leaves stained with Tripan Blue, it was possible to observe foliar mesophile colonization by fungi hyphae (Fig. 2 a). We also observed that the hyphae move between the palisade and the lacunous parenchyma, colonizing vascular tissue. It was possible to verify fungi colonization in foliar parenchymas (Fig. 2b). We stained transversal cuts of *S. saponaria* leaves with and without pre-incubation (incubation of leaves in BDA for 24 h) and observed strong staining after incubation, indicating the presence of endophytes, as found by Bernardi-Wenzel et al. (2010). We mainly considered observations of individual hyphae in the results and discussion sections.

Electron microscopy

S. saponaria leaves visualized with a scanning electron microscope presented fungi hyphae in intracellular and intercellular leaf spaces. It was also possible to visualize endophytes intensively colonizing interior tissue (Fig. 3).

DISCUSSION

Considering the isolation of endophytic fungi from leaves of *S. saponaria*, we observed a high occurrence of colonization, in which 100% of the fragments showed fungal growth from their edges. The present work analyzed fungal endophytes found in leaves, although the parts of the plant that are used in folk medicine are bark, roots and fruit. The leaves were selected for this study because, according to Arnold et al. (2000), they are especially rich and abundant. As well, most studies have focused on endophytes that colonize leaves. In their study, Souza et al. (2004) observed that the levels of endophytic fungal colonization are higher in leaves than in stems, decreasing even more in roots in the toxic plants of the Amazon *Palicourea longiflora* and *Strychnos cogens*.

One hundred and two endophytes were randomly selected from incubated fragments for isolation in an axenic culture on PDA. These endophytes were grouped into 27 morphogroups. Phongpaichit et al. (2006) isolated endophytic fungi from leaves and branches of many *Garcini* species, under surface disinfection conditions similar to those used for *S. saponaria*. The authors isolated one thousand nine hundred ninety-nine fungi, of which 377 were further analysed. In a similar study, Bernadi-Wenzel et al. (2010) isolated endophytic fungi from *Luhea divaricata*, also with 100% colonization. Sustaining our findings, Arnold et al. (2007), isolating *Pinus taeda* fungi, obtained an isolation frequency of 96.7% and 439 isolates in total.

The genera of endophytes observed in *S. saponaria* leaves were *Alternaria*, *Cochliobolus, Curvularia*, *Diaporthe*, *Phomopsis* and *Phoma*. Considering a safety margin of isolates at least 95% identical to sequences available in GenBank, 67% of *S. saponaria* isolates were identified at the species level. Fungi from *Alternaria* genus were also isolated as endophytes from *P. barbatum* by Yandry et al. (2006), using morphological techniques of identification. The genus *Cochliobolus* is a teleomorph of *Bipolaris*. It can be found as a pathogen (Tsukiboshi et al., 2005) or as an endophyte (Campos et al., 2008; Bernardi-Wenzel et al., 2010). The genus *Phomopsis*, represented by ascomycetous anamorphs, contains about 1000 species (Uecker, 1988). As endophytes, they are often prominent in their hosts (Murali et al., 2006). There are about 180 species described by their teleomorph, represented by the genus *Diaporthe* (Van Der Aa and Vanev 2002). The genus *Phoma* is composed of imperfect fungi, dematiaceous, and toxigenic saprophytic to plants and animals (Vishniac, 1996) and occasionally to humans (Taskinen et al., 1997). Otherwise, it can be found as an endophyte. Weber (2004) has isolated endophytic *Phoma medicaginis* as prevalent in the plants *Medicago sativa* and *M. lupulina,* observing the production of brefeldina in these species.

Magalhães et al. (2008), studying the diversity of endophytic fungi isolated from *Eremanthus erythropappus*, obtained variations in colonizations rates from different tissues (seeds, leaves and stem fragments). Using macro and microscopic identification, they observed that the genera *Xylaria* and *Phomopsis* in all the sampled tissues. In *Eremanthus erythropappus*, the genera with the highest specificity, *Alternaria* and *Fusarium,* were found in seeds, *Nigrospora* and *Aspergillus* in leaves and *Dothiorella* in stems.

The diversity of endophytic fungi in *Paris polyphylla* var., a traditional Chinese medicinal plant, was studied by Li et al. (2008). They isolated around 63 fungi; endophytic isolates were initially grouped according to morphological characteristics and the presence of reproductive structures. After that, they carried out molecular identification by analyzing the rDNA-ITS region. The isolates were identified as *Gliocladiopsis irregularis, Plectosphaerella cucumerina, Padospora* sp., *Gliomastix murorum* var. *murorum, Aspergillus fumigatus, Pichia guilliermondii, Neonectria radicicola* (anamorph: *Cylindrocarpon*), *Fusarium redolens, Fusarium oxysporum* and a mycorrhizal Ascomycete. According to the authors, the molecular identification of endophytic fungi was basically the same as their morphologic identification.

The morphological diversity reported in the studies involving isolation of fungi does show precisely the types of strains that inhabit plant species, since only isolates that can develop in a culture medium are selected. These methods offer only relative indications of the microbial population structure, because many organisms cannot evolve outside their natural habitat.

We used molecular techniques to identify the endophytes since many of the taxa isolated will not sporulate in culture and are mycelia sterilia (Lacap et al., 2003). At least one isolate from 13 different morphogroups was identified based in ITS1-5,8S-ITS2 sequencing analysis, where 11 had the identification of genus in BLAST search with the minimum 95% nucleotide identity. One isolate has 90% (G3-92) and another under 90% (G12-60).

The BLAST search resulted, in some cases, identifying several species with the same percentage of identity for a specific endophyte. For example, the endophyte G19-18 had 95% nucleotide identity with *Phomopsis chimonanthi*, *P. micheliae* and *Diaporthe helianthi*. The genus *Diaporthe* is a teleomorph of *Phomopsis*, and as we were not able to find a sexual stage

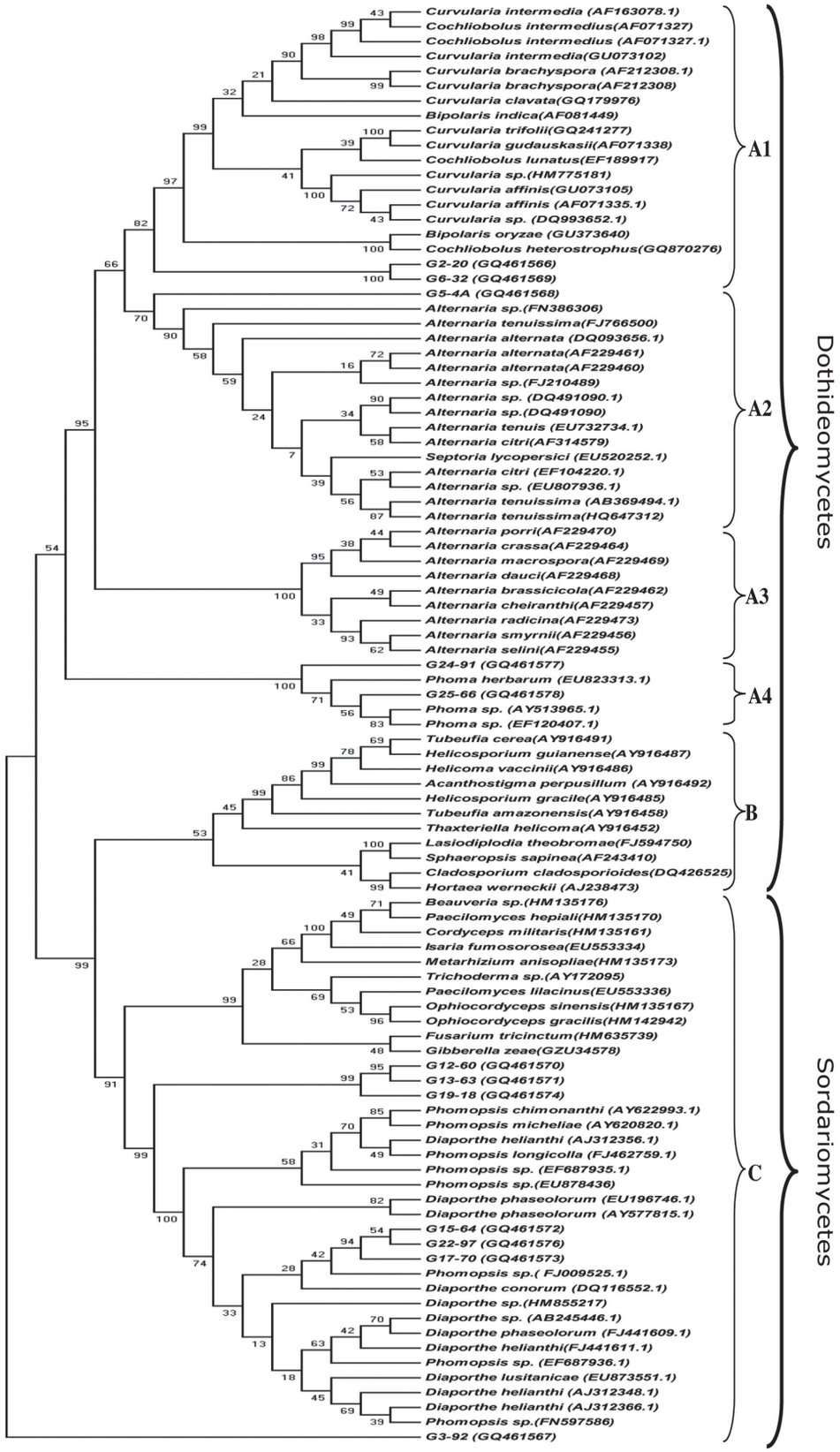

Figure-1. Phylogenetic tree of endophytic fungi isolated from *S. saponaria*, built with the "neighbor-joining" grouping method, using "p-distance" for nucleotides with "the pairwise gap deletion" option. The letter G followed by a number represents the group, the number represents the isolate and the tree numbers represent the percentage of times that the group on the right occurred at the same knot during the evaluation of agreement (bootstrap with 10.000 repetitions). A, B and C represent the clades.

of this endophyte, we could infer that it belongs to the genera *Phomopsis* sp.

Some isolates endophytics presented a low percentage of identity in BLAST analysis. The endophyte G12-60 with relatively low nucleotide identity (88%) with *Phomopsis* sp., were grouped with endophyte G13-63 (96% identity with *Diaporthe* AB245446.1 by BLAST) and G19-18 (95% identity with *Phomopsis chimonanthi* AY622993.1 by BLAST) with 99% BP, in phylogenetic analysis. Those endophytes were grouped with *Phomopsis*/*Diaporthe* sequences with 99% BP, including the *S. saponaria* endophytes G15-64 (97% identity with *Phomopsis* sp. FN597586.1 by BLAST), G22-97 (97% identity with *Phomopsis* sp. EF687936.1 by BLAST) and G17-70 (96% identity with *Phomopsis* sp. EF687936.1 by BLAST). The placement of the endophyte G12-60 in the genus *Phomopsis* (Teleomorph: *Diaporthe*) is supported by the phylogenetic analysis. The majority of foliar fungal endophytes of *S. saponaria* that were molecularly identified were *Phomopsis* sp. (Teleomorph: *Diaporthe*). The phylogenetic analysis carried out with the use of rDNA sequencing allowed us to verify the existence of genetic variability between isolates belonging to the genera *Phomopsis* and *Diaporthe*.

The molecular identification of endophyte G3-92 as *Alternaria* sp. with 90% of nucleotide identity by BLAST, was not supported by phylogenic analysis because it was placed as an outgroup. Similarly, Lin et al. (2010) found strain A23 was dissimilar to most fungi, being 71% similar to *Phialemonium* sp. (CBS 111658). They pointed out that low similarity indicates that strain A23 may be a new genus, or alternatively if already described, the genus has not yet been sequenced.

The endophytes of *S. saponaria* belonging to genus *Phomopsis* (Teleomorph: *Diaporthe*) were grouped with another strain from the same genus (99% BP). They were grouped separately from the others fungal strains obtained in GenBank belonging to Sordariomycetes, but from different genus and species (placed with 67% BP in the same subclade). These phylogenetic patterns demonstrate that most of the identifications of endophytes based on BLAST analysis were correct.

Sakayaroj et al. (2010), working with the tropical seagrass *Enhalus acoroides* in Thailand, identified 10 genera and two species of endophytes, while others were only identified at the family and order levels. In spite of this, Sakayaroj et al. (2010) considers that a major limitation for molecular identification of endophytes is the limited number of sequences, and lack of named sequences from the GenBank database for comparison. However, Albrectsen et al. (2010) considers that wherever ITS information is available in the database, it is more likely to provide a taxonomically correct match.

Bernardi-Wenzel et al. (2010) observed interspecific variability in the genera *Phomopsis* and *Diaphorte* using isolates from *Luhea divaricata* and phylogenetic analysis based on rDNA sequencing data. Our findings were also corroborated by Chareprasert et al. (2006), who observed that the genus *Phomopsis* was predominant in leaves of *Tectona grandis* L. and *Samanea saman* Merr.

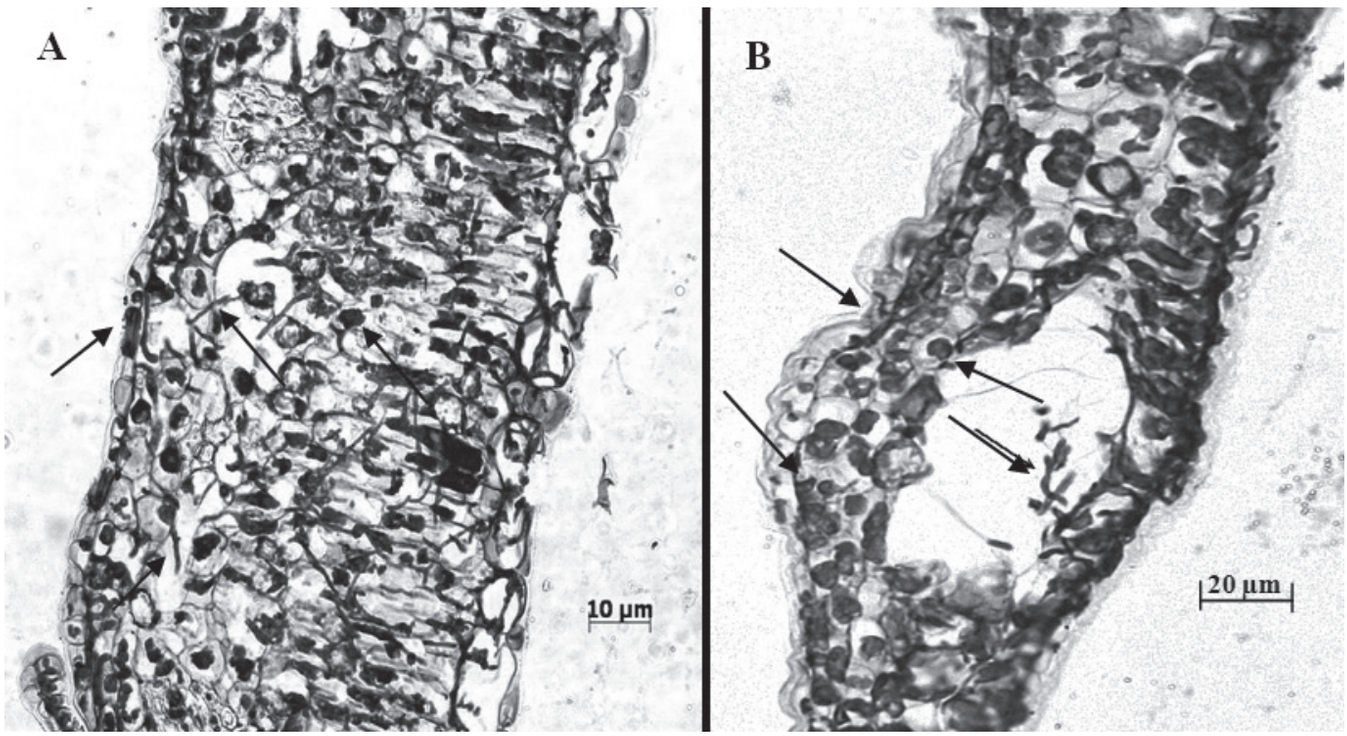

Figure 2: Light microscopy showing transversal cuts of Sapindus saponaria leaves colored with Tripan Blue. a: the arrows indicate the colonization of the foliar mesophile by hyphae and fungi; it is also possible to note the hyphae crossing the palisade parenchyma towards the lacunous parenchyma and the fungal colonization in conductive leaf vessels. b: mycelium colored in blue in inter and intra spaces of palisade and lacunous parenchymas tissues. The arrows indicate the presence of endophytes, colored in blue, in most intracellular spaces.

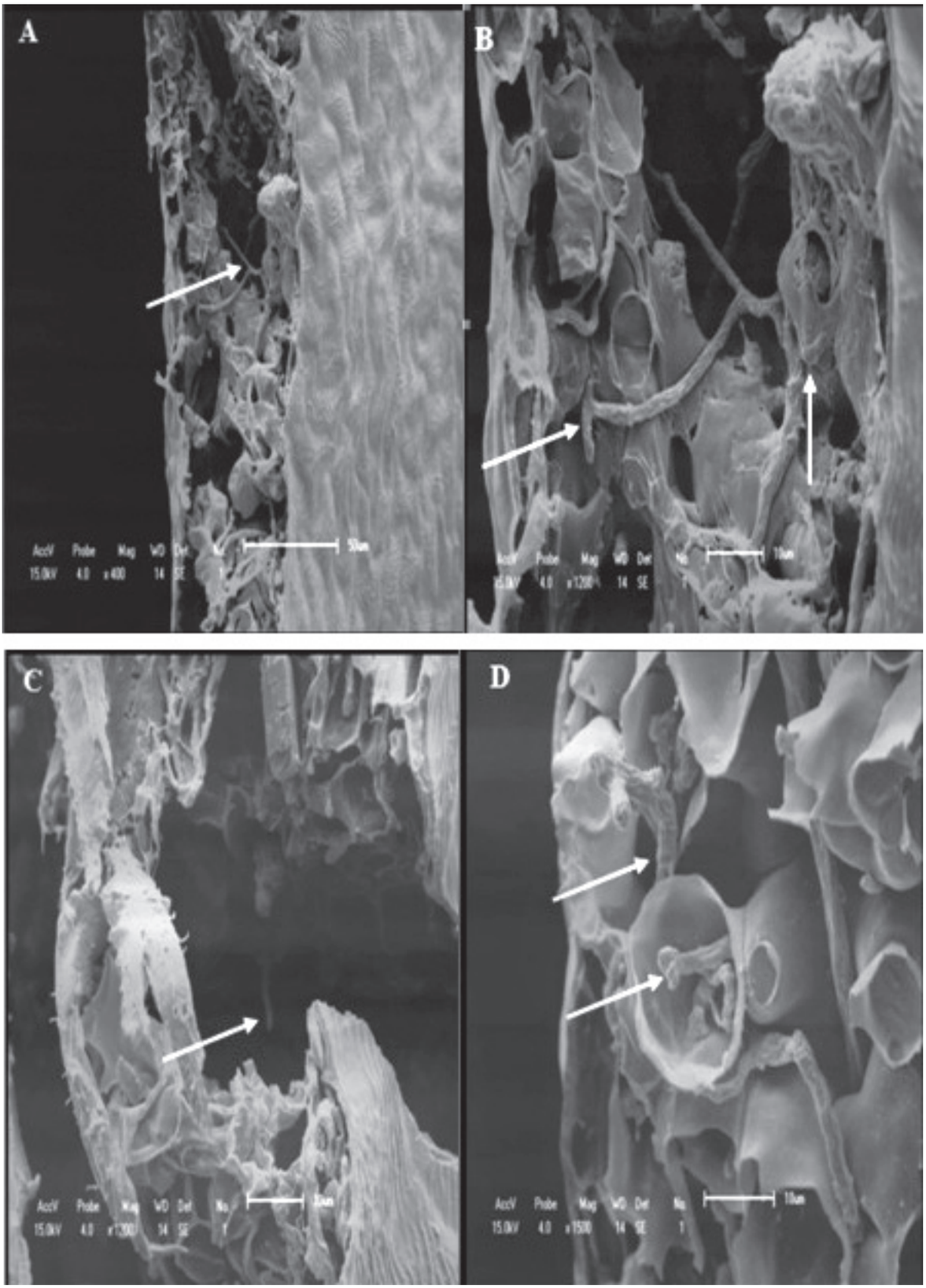

Figure 3: SEM of S. saponaria leaves, showing their colonization by endophytic fungi; a: visualization of parenchymal tissues presenting endophytes (bar 50 µm); b: hyphae of endophytic fungi in intracellular spaces (bar 10 µm); c and d: hyphae in intercellular spaces from the interior of the cells (bar 10 µm).

Similar to what was obtained in the present study with *S. saponaria,* where we identified endophytes belonging to 7 genera, Pimentel et al. (2006), studying the diversity of endophytic fungi in adult and young leaves of *Ilex paraguariensis* (yerba mate), observed 10 groups of fungi. These belong to the genera *Acremonium* sp.; *Aspergillus* sp.; *Colletotrichum* sp.; *Dendrophoma* sp.; *Fusarium* sp.; *Penicilium* sp.; *Rhizoctonia* sp.; *Scopulariopsis* sp.; *Trichoderma* sp. and *Verticillium* sp.

Agusta et al. (2006) isolated 6 groups of endophytic fungi from the medicinal plant *Camellia sinensis*, classified in 6 species belonging to the genera *Fusarium* sp., *Penicillium* sp., *Schizophyllum* sp., and *Diaporthe* sp. Phongpaichit et al. (2007) isolated 51 endophytic fungi from species of *Garcinia*. Based on DNA sequencing analysis (ITS1-5,8S-ITS2 region), 15 isolates were identified as *Aspergillus, Botryosphaeria, Curvularia, Fusicoccum, Guinardia, Muscodor, Penicillium, Pestalotiopsis* and *Phomopsis* spp. The phylogenetic analysis confirmed the rates found by BLAST (Higgins et al., 2007).

Studies confirm that additional information is needed to adapt and infer phylogenetic information for such a data set. Very concise, phylogenetics is the key for diagnosing the taxonomic affinity of unknown endophytes. It is also necessary to adapt the ecological address for these fungi. Further studies will be beneficial for multiplying current analytical methods, especially for linking traditional morphological concepts of identification of species to those based on molecular data groups (Hoffman and Arnold, 2008).

The endophytes of *S. saponaria* G2-20, G3-92 and G5-4A were identified by molecular analysis and by morphological and microscopics characteristics. Additionally, in the phylogenetic analysis, endophytes G2-20 (97% identity with *Cochliobolus intermedius* AF071327.1 by BLAST) and G6-32 (95% identity with *Curvularia* sp. HM775181.1 by BLAST) were clustered with 100% BP and grouped with several *Curvularia* (Teleomorph: *Cochliobolus*) species with 82% BP. Many *Alternaria* species have formed a clade with the endophyte G5-4A (96% identity with *Alternaria alternata* DQ093656.1 by BLAST) with 70% BP support.

Two endophytic strains: G24-91 (98% identity with *Phoma* sp. AY513965.1 by BLAST) and G25-66 (97% identity with *Phoma* sp. EF120407.1 by BLAST) were grouped in a subgroup comprising *Phoma* sp. with 100% BP support.

Steiner et al. (2006) carried out the phylogenetic analysis based on rDNA genes 18S and on the ITS of 12 Clavicipitaceous (ascomycetes) endophytic fungi and one epibiotic fungus (which grows on the plant surface and uses its nutrients) of dicotyledon plants (Convolvulaceae). They observed that between the epibiotic fungus and the endophytes, only the later belonged to the family Clavicipitaceae (Ascomicota), while none of the endophytes belonged to this family.

Nowadays, techniques of staining, light microscopy and scanning electron microscopy (SEM) detect the presence of fungal structures in hosts. This has also allowed for the study of the colonizing process. Light microscopy evidenced the presence of endophytic fungi in *S. saponaria* leaves. By means of light microscopy using Trypan –Blue, we visualized hyphae of endophytic isolates colonizing tissue of the host plant (Fig. 2). SEM confirmed the presence of endophytic fungi in the leaves, including spaces within cells (Fig. 3 d). The importance of detecting inter-or intracellular colonization by fungal endophytes is to better understand endophyte/host interaction. In that regard, we note that intracellular colonization of leaves by fungal endophytes indicates a high interaction between them, positively selected in a coevolutionary event. Kneip et al. (2007), discussing new models for symbiosis, compared the morphological, physiological and molecular characteristics of nitrogen fixing symbiotic associations of bacteria and their diverse hosts. Special features of the interaction, e.g. vertical transmission of symbionts, grade of dependency of partners and physiological modifications have been considered in terms of the extent of co-evolution and adaptation. In their view, some interactions reveal a more obligate status of co-evolution. The diverse degrees of symbioses, ranging from loose associations to highly specific intracellular interactions, might themselves reflect the range of potential evolutionary fates for symbiotic partnerships.

The intracellular space consists of a series of connections of regions that do not have living plant components, known as apoplast. The latter is distinct from the symplast, the contact region between the cells. The intercellular spaces are found in the cortical root region and parenchymal leaf tissue. These spaces are formed by the juxtaposition of three or four cells and the medium lamel dissolution, not significantly different in the leaves and in the branches if compared to the roots (Kuldau and Bacon, 2008).

The endophytic fungi benefit from intracellular spaces because these are protected niches where there is little competition from other organisms and because of the nutritional abundance they find there. According to Kuldau and Bacon (2008), the intracellular spaces actually have many organic and inorganic nutrients, able to support the concentration of endophytic fungi observed in gramineae.

Bernardi-Wenzel et al. (2010), using light microscopy and SEM, visualized endophytic fungi that inhabit inter and intracellular spaces in leaves of *Luhea divaricata*. It was possible to observe extensive and disseminated colonization along all leaf parts, in the palisade parenchyma, sclerenchyma, adaxial epidermis and conductive vessels, indicating close interaction among the endophytes in multiple structural and trophic sub-niches in the host. These results confirm the data presented in the microscopic study of *S. saponaria* colonization by endophytes.

Durán et al. (2005) visualized fungical hyphae stuck to surface cells of palisade parenchyma in leaves of *Citrus limon* with SEM. In *S. saponaria*, with light microscopy and Tripan-Blue staining, it was possible to identify the presence of hyphae and mycelial mass in the cells, in intercellular spaces and crossing from one cell to another. (Fig. 2 a and b).

By using light microscopy and Tripan-Blue staining, Gómez-Vidal et al. (2006), studying endophytic colonization of palm leaves (*Phoenix dactylifera* L.) by entomopathogenic fungi, observed the colonizing of *Beauveria bassiana* in the parenchyma, especially in intracellular spaces. Considering thirteen-day incubation, the authors observed a high density colonizing hyphae in the inter- and intracellular spaces of parenchyma.

The cytological and molecular characterizations allowed the identification of 6 endophytic fungi genera isolated from *S. saponaria*. The phylogenetic analysis showed intraspecific variability in the genera *Phomopsis* and *Diaporthe* and the interspecific variation among the genera *Curvularia*, *Phoma* and *Alternaria* from the Pleosporaceae family. In the phylogenetic

analysis, we observed the formation of specific sub-groupes formed by *S. saponaria* endophytes separately from the others species closely related. The use of sequences of strains belonging to distant genera not related to those found by BLAST analysis gave greater strength to the findings on the molecular identification of endophytic isolates of *S. saponaria*.

With light microscopy and SEM techniques, we observed endophytic fungi colonizing inter and intracellular spaces of leaves of *S. saponaria*. These results demonstrate the endophytic colonization of *S. saponaria* leaves, indicating a process of intimate interaction between the endophyte and host plant.

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