



Acta Biológica Colombiana
ISSN: 0120-548X
ISSN: 1900-1649
Universidad Nacional de Colombia, Facultad de
Ciencias, Departamento de Biología

ENZYMATIC AND ANTAGONIST ACTIVITY OF ENDOPHYTIC FUNGI FROM *Sapindus saponaria* L. (SAPINDACEAE)

SANTOS, Caroline Menicoze dos; RIBEIRO, Amanda da Silva; GARCIA, Adriana; POLLI, Andressa Domingos; POLONIO, Julio Cesar; AZEVEDO, João Lúcio; PAMPHILE, João Alencar
ENZYMATIC AND ANTAGONIST ACTIVITY OF ENDOPHYTIC FUNGI FROM *Sapindus saponaria* L.
(SAPINDACEAE)

Acta Biológica Colombiana, vol. 24, no. 2, 2019

Universidad Nacional de Colombia, Facultad de Ciencias, Departamento de Biología

Available in: <http://www.redalyc.org/articulo.oa?id=319060771012>

DOI: 10.15446/abc.v24n2.74717

ENZYMATIC AND ANTAGONIST ACTIVITY OF ENDOPHYTIC FUNGI FROM *Sapindus saponaria* L. (SAPINDACEAE)

Actividad enzimática y antagonista de los hongos endofíticos
de *Sapindus saponaria* L. (Sapindaceae)

Caroline Menicoze dos SANTOS¹

Universidade Estadual de Maringá, Brazil

Amanda da Silva RIBEIRO¹

Universidade Estadual de Maringá, Brazil

Adriana GARCIA²

Instituto Federal Catarinense, Brazil

Andressa Domingos POLLI¹

Universidade Estadual de Maringá, Brazil

Julio Cesar POLONIO¹

Universidade Estadual de Maringá, Brazil

João Lúcio AZEVEDO³

Universidade de São Paulo, Brazil

João Alencar PAMPHILE^{1*}

Universidade Estadual de Maringá, Brazil

Acta Biológica Colombiana, vol. 24, no.
2, 2019

Universidad Nacional de Colombia,
Facultad de Ciencias, Departamento de
Biología

Received: 05 September 2018
Revised document received: 31
December 2018
Accepted: 22 February 2019

DOI: 10.15446/abc.v24n2.74717

CC BY-NC-SA

ABSTRACT: Endophyte microorganisms have great biotechnological interest, with features applicable to different areas and are potentially useful in agriculture. The current study determines the biotechnological potential of endophytic fungi, isolated from leaves of *Sapindus saponaria*, to control phytopathogenic fungi and evaluate their enzyme production. Molecular taxonomy was performed by sequencing of the ITS1-5.8S-ITS2 ribosomal DNA region, identifying the genera *Phomopsis*, *Sordariomycetes*, *Diaporthe*, and *Colletotrichum*. In vitro antagonism against phytopathogens showed better results against *Fusarium solani* and provided inhibition indices between 41.8 % and 67.5 %. The endophytic strain SS81 (*Diaporthe citri*) presented the highest antagonism index against the pathogen. Against *Glomerella* sp. and *Moniliophthora perniciosa*, inhibition rates ranged between 18.7 % and 57.4 % and between 38.3 % and 64.8 %, respectively. Enzyme assays revealed that strain SS65 (*Diaporthe* sp.) produced 1.16 UI $\mu\text{mol}/\text{min}$ of amylase; strain SS77 (*Diaporthe* sp.) produced 2.74 UI $\mu\text{mol}/\text{min}$ of pectinase, and strain SS08 (*Diaporthe* sp.) produced 1.51 UI $\mu\text{mol}/\text{min}$ of cellulase. Thus, the current study shows evidence the importance of isolated endophytes with phytoprotective properties of plants with medicinal properties as alternatives for biological control and natural sources of products with biotechnological interest.

Keywords: Biological control, endophytes, enzymes, microbial antagonisms, molecular taxonomy.

RESUMEN: Los microorganismos endofíticos tienen gran interés biotecnológico, con características aplicables a diferentes áreas y potencialmente útiles en la agricultura. El presente estudio determinó el potencial biotecnológico de los hongos endofíticos,

aislados de las hojas de *Sapindus saponaria*, en el control de hongos fitopatógenos y evaluación de su producción de enzimática. La taxonomía molecular fue realizada por la secuencia de la región ITS1-5.8S-ITS2 del ADN ribosomal, identificando los géneros *Phomopsis*, *Sordariomycetes*, *Diaporthe* y *Colletotrichum*. El antagonismo in vitro contra fitopatógenos mostró mejores resultados contra *Fusarium solani* y proporcionó índices de inhibición de entre el 41,8 % y el 67,5 %. El linaje endofítico SS81 (*Diaporthe citri*) presentó el mayor índice de antagonismo contra los patógenos. Contra *Glomerella* sp. y *Moniliophthora perniciosa*, las tasas de inhibición variaron entre el 18,7 % y el 57,4 % y entre el 38,3 % y el 64,8 %, respectivamente. El ensayo enzimático reveló que el linaje SS65 (*Diaporthe* sp.) produjo 1,16 UI $\mu\text{mol} / \text{min}$ de amilasa; el linaje SS77 (*Diaporthe* sp.) produjo 2,74 UI $\mu\text{mol} / \text{min}$ de pectinasa; y el linaje SS08 (*Diaporthe* sp.) produjo 1,51 UI $\mu\text{mol} / \text{min}$ de celulasa. Así, el presente estudio evidencia la importancia de los endófitos aislados con propiedades fitoprotectoras como alternativas para el control biológico y como fuentes naturales de productos con interés biotecnológico.

Palabras clave: Antagonismo microbiano, control biológico, endofitos, enzimas, taxonomía molecular.

INTRODUCTION

Endophytic fungi are microorganisms that colonize plant tissues intercellularly and intracellularly throughout their life cycle, or part of it, and do not cause any disease in the host (Hyde and Soyong, 2008). They maintain stable relations with their host, since they live asymptotically with the plant (Pamphile and Azevedo, 2002). Azevedo and Araújo, (2007) define endophytes as any microorganism, cultivated or not, that colonizes the interior of the host plant without causing apparent damages or visible external structures. Mendes and Azevedo, (2008) extended the concept of endophytes and classified them into two main types: Type I, which do not produce structures external to their hosts, and Type II, which produce external structures such as mycorrhizal fungi and bacteria that produce nitrogen fixation nodules.

Endophytic microorganisms have great biotechnological interest. Their properties apply to different areas and are highly useful in agriculture in by plant growth promoting, biological pest control and increasing the resistance of plants to stress conditions (Ikram *et al.*, 2018; Li *et al.*, 2018). Several studies indicate that endophytes reduce the attack of insects and pathogenic fungi against the host plant (Landum *et al.*, 2016; Jaber and Ownley, 2018). Further, the interaction between endophytes and plants involves the production of several secondary metabolites or bioactive substances, with industrial interest for the in development of pathogen control systems or with diverse biological activities (Nisa *et al.*, 2015; Venugopalan and Srivastava, 2015).

Certain endophytes are uncultivable; they are difficult to maintain in a physiologically stable state without host cells, being more complex than other free-living fungi (Dissanayake *et al.*, 2018). However, a considerable number of endophytes are cultivable, and the production of bioactive compounds from host plants by these endophytes is important from ecological, molecular and biochemical points of view (Bungtongdee *et al.*, 2018). This is because it is easier to access microorganism cultivation conditions and optimize the process, thus reducing costs,

and increasing productivity and reproducibility, without any adverse impact on the environment (Kusari *et al.*, 2012). Therefore, endophytic micro-organisms are acknowledged sources of new natural products to be exploited by medicine, agriculture, and industry (Pimentel *et al.*, 2011; Nisa *et al.*, 2015). The application of endophytic microorganisms in the biological control of pests and insects has shown promising results; however, methods for the effective application on the field must be further developed. The interference of external factors, such as climatic conditions in different regions, interactions with other plants and microbial species, the number of endophytes to be applied, as well as the need to demonstrate the phytosanitary safety of the strains are factors that currently limit the application of endophytic microorganisms in biological control (Polonio *et al.*, 2015).

Enzymes are used in various industrial processes such as textiles, cosmetics, foods, and beverages. Microbial enzymes are not subject to production or supply limitations, and their diversity increases daily due to the discovery of new enzymes and applications (Corrêa *et al.*, 2014). The use of enzymes improves the quality of a product and makes it easier to obtain it. Moreover, each substance has specific enzymes that degrade it (Robinson, 2015).

Microorganism-produced enzymes have great interest in several areas, including food processing (amylases, pectinases, proteases and cellulases), the tissue industry (cellulases), the leather industry (proteases and lipases), the manufacture of detergents (proteases and lipases), pharmaceuticals, medical therapy and in the field of molecular biology (Nielsen, 1998; Corrêa *et al.*, 2014).

The Laboratory of Microbial Biotechnology of the State University of Maringá, Maringá PR Brazil, has isolated and studied endophytes from different plant hosts during the last decade, including *Sapindus saponaria* Linnaeus 1753, that is used in popular medicine as a tranquilizer, astringent, diuretic, and expectorant and the fruit extracts have activity as antifungal (Garcia *et al.*, 2012; Alberto *et al.*, 2016). Several endophytes have not yet been analyzed, albeit maintained under optimal laboratory conditions. Therefore, the current study objectives the bioprospection of several *S. saponaria* endophytes for the enzymatic production of amylase, cellulase, and pectinase, and the antagonistic activity against phytopathogens *Fusarium solani* (Mart.) Sacc. 1881, *Glomerella* sp. Spauld. & H. Schrenk 1903, and *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora 2006. Although these phytopathogens are not specifically associated with *S. saponaria*, they affect crops of agronomic importance such as soybean, grapevine, and cocoa, respectively.

MATERIALS AND METHODS

Microbial strains

Strains of endophytic fungi, used in the current assay (Table 1), were isolated from *S. saponaria* L. (Sapindaceae), retrieved from the campus

of the State University of Maringá, Maringá PR Brazil, by Garcia *et al.*, (2012), and are included in the stock of the Microbial Biotechnology Laboratory (LBIOMIC) of the Department of Biotechnology, Genetics and Cell Biology (DBC) of the State University of Maringá, Maringá PR Brazil. The pathogenic fungus strains used were *F. solani*, *M. pernicioso* (Laboratory of Genetics of Microorganisms, ESALQ-USP, São Paulo, Brazil) and *Glomeralla* sp. (CNPUV378, Embrapa Grape and Wine, Bento Gonçalves RS Brazil).

Table 1
Endophytes isolated from *Sapindus saponaria* and fungi with higher identity in NCBI.

Strains	% Identity	Fungi with higher identity (BLAST Genbank)	NCBI accession number	Taxonomical identification	NCBI deposit
SS08	99 %	<i>Phomopsis</i> sp.	JN153073.1	<i>Diaporthe</i> sp.	MH665254
SS11	99 %	<i>Sordariomyces</i> sp.	JQ761037.1	<i>Diaporthe</i> sp.	MH665242
SS13	100 %	<i>Phomopsis</i> sp.	KM979832.1	<i>Diaporthe</i> sp.	MH665243
SS38	100 %	<i>Phomopsis</i> sp.	GQ461582.1	<i>Diaporthe</i> sp.	MH665244
SS39	100 %	<i>Phomopsis</i> sp.	GQ461582.1	<i>Diaporthe</i> sp.	MH665245
SS40	100 %	<i>Phomopsis</i> sp.	GQ461582.1	<i>Diaporthe</i> sp.	MH665246
SS43	96 %	<i>Phomopsis</i> sp.	GQ461582.1	<i>Diaporthe</i> sp.	MH665247
SS45	100 %	<i>Diaporthe helianthi</i>	KM979834.1	<i>D. helianthi</i>	MH665248
SS55	100 %	<i>Phomopsis</i> sp.	GQ461582.1	<i>Diaporthe</i> sp.	MH665249
SS62	100 %	<i>Colletotrichum gloeosporioides</i>	KF171519.1	<i>Colletotrichum gloeosporioides</i>	MH665250
SS65	99 %	<i>Diaporthe</i> sp.	GQ461590.1	<i>Diaporthe</i> sp.	MH665251
SS77	99 %	<i>Diaporthe phaseolorum</i>	KC771507.1	<i>Diaporthe</i> sp.	MH665252
SS79	96 %	<i>Diaporthe</i> sp.	KM113379.1	<i>Diaporthe</i> sp.	MH665253
SS81	99 %	<i>Diaporthe citri</i>	KC343053.1	<i>Diaporthe citri</i>	MH665237
SS84	100 %	<i>Phomopsis</i> sp.	GQ461576.1	<i>Diaporthe</i> sp.	MH665238
SS86	99 %	<i>Phomopsis</i> sp.	JN153051.1	<i>Diaporthe</i> sp.	MH665239
SS93	98 %	<i>Diaporthe</i> sp.	KF928282.1	<i>Diaporthe</i> sp.	MH665240
SS99	99 %	<i>Phomopsis</i> sp.	GQ461576.1	<i>Diaporthe</i> sp.	MH665241

Molecular Taxonomy and Phylogenetic analysis

So that endophytes could be molecularly identified, DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad CA, USA), following the manufacturer's instructions. The amplification of ribosomal DNA region, ITS1-5.8S-ITS2, using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCCGCTTATTGATATGC-3'), were performed as described by White *et al.*, (1990).

The amplified regions of isolates were purified by enzyme shrimp alkaline phosphatase (SAP, Phosphatase, Alkaline shrimp - Sigma Aldrich, Saint Louis, USA) and exonuclease I (EXO, Exonuclease I, *E. coli*-BioLabs, New England, USA). Purification reactions were performed with 8 µL PCR product, 0.5 µL EXO (10 U/L) and 1 µL SAP (1 U/L) and incubated in a thermocycler for 1 h at 37 °C, followed by 15 min at 80 °C. Extracted DNA was then subjected to quantification on 1 %

agarose gel, and photographed. PCR products were sequenced using the ABI-PRISM 3100 Genetic Analyzer equipment (Applied Biosystems).

Nucleotide sequences were compared with those deposited in the NCBI database (National Center for Biotechnology Information) for taxonomic identification, initially determined by the best identity rate obtained. Phylogenetic analysis was performed as described by Garcia *et al.*, (2012). Sequences with greater NCBI similarity and other genetically distant sequences were used as external groups to force alignment.

In vitro antagonist activity of endophytic fungi

Antagonist activity against pathogenic fungus *F. solani*, *M. perniciosa* and *Glomeralla* sp. was evaluated by a dual-culture technique described by Campanile *et al.*, (2007), with modifications described by Polonio *et al.*, (2015).

The in vitro competition interactions between endophytes and pathogens were analyzed according to scale by Badalyan *et al.*, (2004), with modifications, based on three types of interactions: A, B, and C, with C being divided into subcategories. The interaction types are: A = the inhibition of mycelial growth with contact; B = inhibition from a distance; C = endophytic growth on the pathogen without initial inhibition; CA1 and CA2 = partial and complete endophytic growth, respectively, on the pathogen after initial inhibition with mycelial contact; CB1 and CB2 = partial and complete endophytic growth, respectively, on the pathogen after initial inhibition from a distance.

Analysis of enzymatic production

The enzymatic assay was performed as described by Ribeiro *et al.*, (2018) with modifications: In Mancini solution, 0.5 % of inductive substrate was added and pH was adjusted for each enzyme: amylase (starch soluble, pH 6.0, HiMedia, Mumbai, India); cellulase (Carboxymethylcellulose or CMC, pH 5.0, Sigma Aldrich, Saint Louis, USA) and pectinase (pectin, pH 5.0, Sigma Aldrich, Saint Louis, USA).

The culture media was modified for the detection of each enzyme: starch agar medium (18 g/L agar; 10 g/L starch; 0.1 M citrate-phosphate buffer, pH 5.0) for amylase; CMC-agar (18 g/L agar; 10 g/L CMC; 0.1 M sodium acetate buffer, pH 5.0) for cellulase; and pectin-agar (18 g/L agar; 10 g/L pectin; 0.1 M sodium acetate buffer, pH 5.0) for pectinase.

A positive control experiment was performed using a commercially available enzyme (cellulase from *Aspergillus niger*, α -amylase from porcine pancreas, pectinase from *Aspergillus*, Sigma Aldrich, Saint Louis, USA) at 1 mg/mL. Plates were incubated at 28 °C for 24 h and then stained with 0.1 % Congo red dye (cellulase), iodine solution 1 N (amylase) and hydrochloric acid solution 5 N (pectinase).

Determination of enzymatic activity

Determination of enzymatic activity was performed as described by Ribeiro *et al.*, (2018), with modifications: Induction solution (1 % w/v) was adjusted for each enzyme (CMC solution in sodium citrate buffer 50 mM, pH 4.8; starch solution in acetate buffer 0.2 M, pH 5.0; pectin solution in citrate buffer 0.1 M, pH 4.0).

Statistical analysis

The means obtained in antagonist activity and enzymatic production were compared by the Scott-Knott test ($p < 0.05$) using the statistical program Sisvar 5.5 (Ferreira, 2008).

RESULTS

Phylogenetic Analysis

BLAST and phylogenetic analyses based on the ITS1-5.8S-ITS2 sequencing data of 18 endophytic fungi isolated from *S. saponaria*, together with those with the highest identity in the NCBI database, resulted in the molecular taxonomy based on the identification of the genera *Phomopsis*, *Sordariomycetes*, *Diaporthe* and *Colletotrichum* (Table 1, Fig. 1). *Phomopsis* was the most prevalent genus endophytic isolates of the analyzed samples of our microbial collection.

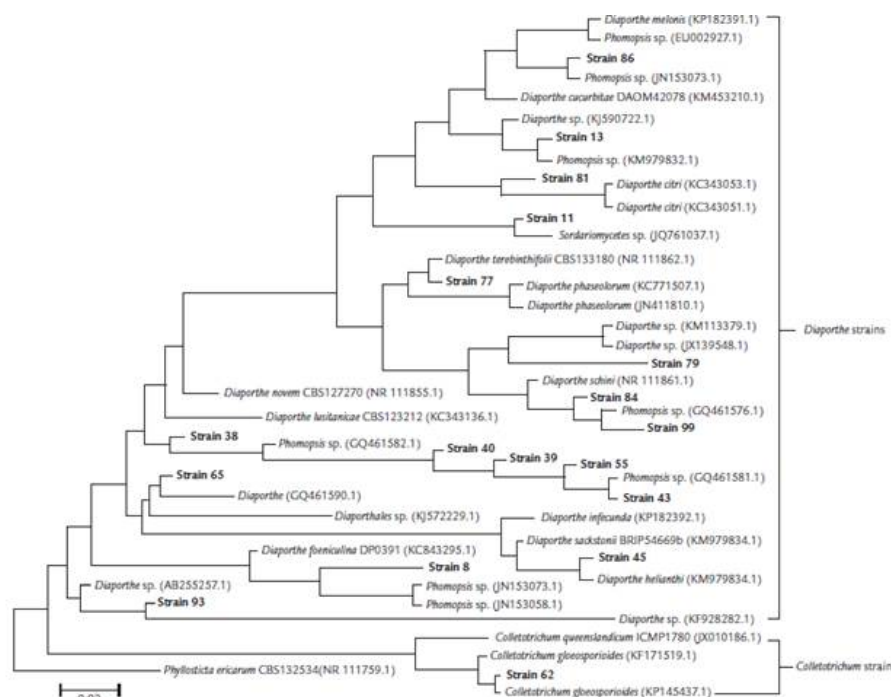


Figure 1

Phylogenetic tree of foliar endophytic fungi of *Sapindus saponaria* with other fungi obtained from the GenBank database, constructed by MEGA 6.05, with neighbor-joining method, using p-distance for nucleotides with the pairwise gap deletion. The numbers on the tree indicate the percentage of times the group on the right occurred on the same node during consensus evaluation (bootstrap with 10,000 replications). The strain *Phyllosticta ericarum* CBS 132534 (NR 111759.1) was used as an external group.

Antagonistic activity

Table 2 shows endophyte antagonistic activity against phytopathogens. The best performance was observed against *F. solani* with an inhibition rate (Im %) between 41.8 % and 67.5 %. The endophytic strain with the best result was SS81 (*Diaporthe citri*), with a 67.5 % inhibition rate. The best inhibition rate (57.4 %) against *Glomerella* sp. was obtained for endophyte SS79 (*Diaporthe* sp.) and against *M. perniciosa* for SS77 (*Diaporthe phaseolorum*) with 64.8 %. Most of the *in vitro* interactions were type A, the inhibition of mycelial growth with contact.

Table 2

Antagonism of endophytic fungi from *Sapindus saponaria* against pathogenic fungi.
Average plant pathogen area in cm² (A) and percentage inhibition rate (IP %).

Endophytes	Pathogenic fungi					
	Fusarium solani		Glomerella sp.		Moniliophthora perniciosa	
	A	IP%	A	IP%	A	IP%
SS08 (<i>Diaporthe</i>)	18.20 ^b	43.1%	21.60 ^d	22.6%	23.26 ^b	43.2%
SS11 (<i>Diaporthe</i>)	13.70 ^a	57.2%	17.50 ^b	37.3%	18.58 ^a	54.6%
SS13 (<i>Diaporthe</i>)	13.72 ^a	57.1%	22.71 ^d	18.7%	25.29 ^b	38.3%
SS38 (<i>Diaporthe</i>)	18.61 ^b	41.8%	20.13 ^c	27.9%	24.95 ^b	39.1%
SS39 (<i>Diaporthe</i>)	17.92 ^b	44.0%	18.41 ^b	34.1%	20.19 ^a	50.7%
SS40 (<i>Diaporthe</i>)	12.85 ^a	59.8%	19.20 ^c	31.2%	25.15 ^b	38.6%
SS43 (<i>Diaporthe</i>)	16.26 ^b	49.1%	19.75 ^c	29.3%	24.69 ^b	39.7%
SS45 (<i>Diaporthe</i>)	17.46 ^b	45.4%	22.70 ^d	18.7%	24.99 ^b	39.0%
SS55 (<i>Diaporthe</i>)	12.06 ^a	62.3%	16.51 ^b	40.9%	19.99 ^a	51.2%
SS62 (<i>Colletotrichum</i>)	11.96 ^a	62.6%	18.42 ^b	34.0%	23.43 ^b	42.8%
SS65 (<i>Diaporthe</i>)	13.58 ^a	57.5%	16.35 ^b	41.4%	23.01 ^b	43.8%
SS77 (<i>Diaporthe</i>)	15.94 ^b	50.1%	12.77 ^a	54.3%	14.43 ^a	64.8%
SS79 (<i>Diaporthe</i>)	14.80 ^a	53.7%	11.89 ^a	57.4%	16.45 ^a	59.8%
SS81 (<i>Diaporthe</i>)	10.38 ^a	67.5%	14.62 ^a	47.6%	17.69 ^a	56.8%
SS84 (<i>Diaporthe</i>)	12.25 ^a	61.7%	12.73 ^a	54.4%	16.92 ^a	58.7%
SS86 (<i>Diaporthe</i>)	16.79 ^b	47.5%	16.85 ^b	39.6%	24.11 ^b	41.1%
SS93 (<i>Diaporthe</i>)	14.38 ^a	55.0%	17.59 ^b	37.0%	19.33 ^a	52.8%
SS99 (<i>Diaporthe</i>)	12.86 ^a	59.8%	14.18 ^a	49.2%	17.91 ^a	56.3%
Control	31.98 ^c	-	27.92 ^c	-	40.97 ^c	-

Enzymatic activity

After the growth of *S. saponaria* endophytic fungi in a liquid medium and cup plate, halos measuring 15.53 mm for cellulase, 8.66 mm for amylase (only endophyte SS77 presented amylase results in this test) and 11.33 mm for pectinase were obtained (Table 3). The endophytes that produced pectinase comprised SS65 (*Diaporthe* sp.), SS77 (*Diaporthe phaseolorum*) and SS84 (*Phomopsis* sp.), while strains that produced cellulase were SS08 (*Phomopsis* sp.), SS13 (*Phomopsis* sp.), SS62 (*Colletotrichum gloeosporioides*), SS65 (*Diaporthe* sp.) and SS93 (*Diaporthe* sp.).

Table 3
Evaluation of enzymatic production by endophytic fungi isolated from *Sapindus saponaria* in cup plate test.

Endophytes	Amylase Halos (mm)	Pectinase Halos (mm)	Cellulase Halos (mm)
SS08 (<i>Diaporthe</i>)	-	-	15.53 ^a
SS13 (<i>Diaporthe</i>)	-	-	13.93 ^a
SS62 (<i>Colletotrichum</i>)	-	-	14.80 ^a
SS65 (<i>Diaporthe</i>)	-	9.86 ^c	15.53 ^a
SS77 (<i>Diaporthe</i>)	8.66 ^b	11.33 ^b	-
SS84 (<i>Diaporthe</i>)	-	10.26 ^c	-
SS93 (<i>Diaporthe</i>)	-	-	15.53 ^a
Control	15.00 ^a	31.00 ^a	22.33 ^a

Endophytes with positive results in enzyme production were evaluated by spectrophotometer. Several strains with no visible halos in the cup plate had positive results in spectrophotometric evaluation: three in the test for amylase and pectinase (SS65, SS77, and SS84), and five in the test for cellulase (SS08, SS13, SS62, SS65, SS93). The 3,5-dinitrosalicylic acid (DNS) assay is a robust colorimetric technique for the quantification of enzymatic activity based on the liberation of reducing sugars and is widely used in several papers (Sharma *et al.*, 2016; Salim *et al.*, 2017), facilitating comparison of the obtained results. Current analysis demonstrated that strain SS65 produced 1.16 UI $\mu\text{mol}/\text{min}$ of amylase, SS77 produced 2.74 UI $\mu\text{mol}/\text{min}$ of pectinase, and SS08 produced 1.51 UI $\mu\text{mol}/\text{min}$ of cellulase (Table 4).

Table 4
Spectrophotometric evaluation of enzymatic production of *Sapindus saponaria* endophytes for amylase, pectinase and cellulase.

Endophytes	Amylase UI $\mu\text{mol}/\text{min}$	Pectinase UI $\mu\text{mol}/\text{min}$	Cellulase UI $\mu\text{mol}/\text{min}$
SS08 (<i>Diaporthe</i>)	-	-	1.51 ^a
SS13 (<i>Diaporthe</i>)	-	-	1.27 ^a
SS62 (<i>Colletotrichum</i>)	-	-	1.24 ^a
SS65 (<i>Diaporthe</i>)	1.16 ^a	1.84 ^a	1.45 ^a
SS77 (<i>Diaporthe</i>)	1.37 ^a	2.74 ^a	-
SS84 (<i>Diaporthe</i>)	1.09 ^a	2.23 ^a	-
SS93 (<i>Diaporthe</i>)	-	-	1.50 ^a

DISCUSSION

The genus *Phomopsis* is represented by approximately 1000 species of anamorphic ascomycetes and its teleomorphic phase is represented by the genus *Diaporthe* (Pandey *et al.*, 2003; Murali *et al.*, 2006). In the current study, it was the most abundant genus. Similar results were found in other research works (Rhoden *et al.*, 2012; Sebastianes *et al.*, 2013;

Ribeiro *et al.*, 2018). Sebastianes *et al.*, (2013) analyzed the diversity of endophytic fungi isolated from the leaves and branches of *Rhizophora mangle*, *Avicennia schaueriana* and *Laguncularia racemosa* from two mangroves in the state of São Paulo. Molecular identification showed that the fungus community associated with these mangroves was composed of at least 34 different genera, with *Diaporthe* as the most frequent (22.67 %), coupled with *Colletotrichum* (19.19 %), *Fusarium* (11.34 %), *Trichoderma* (8.72 %) and *Xylaria* (7.56 %) (Sebastianes *et al.*, 2013).

The endophyte of the genus *Diaporthe* is commonly isolated from several plant families. Ribeiro *et al.*, (2018) demonstrated that the endophytic genus was the most abundant (78 %) in the foliar tissue of *Pachystachys lutea*. Rhoden *et al.*, (2012) also observed an 84.3 % abundance of *Diaporthe* sp. endophytic isolates in the leaves of *Trichilia elegans*. In crops with high economic value, such as cocoa, coffee, soybean, and common bean, this genus has also been isolated as an endophyte (Ribeiro *et al.*, 2018).

The current study also demonstrated the ability of *Diaporthe* to reduce the growth of phytopathogens *F. solani*, *Glomerella* sp. and *M. perniciosa*. Since the type of interaction between endophytes and pathogens was the inhibition of mycelial growth with contact, the above suggests that competition for space featured the inhibition mechanism (Gao *et al.*, 2010; Ribeiro *et al.*, 2018). Studies on endophytic *Diaporthe citri* isolated from guaco (*Mikania glomerata* Spreng.) obtained 60 % inhibition rates against *F. solani* and 66 % against *D. bryoniae*, with the same type of competitive interaction (Polonio *et al.*, 2015).

Agriculture has undergone severe production losses due to phytopathogens. Although decreasing the attack of phytopathogenic insects and microorganisms, xenobiotic pesticides represent high risks for humans, with deleterious environmental impacts (Jayaraj *et al.*, 2016). The natural and biological control of insect pests and diseases that affect crop plants has been focused during the last decades to reduce the use of pesticides in agriculture (Lacava *et al.*, 2014). Biocontrol has often been used in tropical countries, including Brazil (Lacava *et al.*, 2014). There has been increasing interest in endophytes as biological control agents because they inhibit plant pathogens (Bogner *et al.*, 2016; Bahroun *et al.*, 2017). Most interesting are observations which suggest that endophytes may play some role in the protection of the host plant from plant pathogens or other agents of destruction including insects and herbivores, these microorganisms produce substances (antibiotics and enzymes) that directly inhibit pathogens or induce systemic resistance in the host (Gao *et al.*, 2010; Strobel, 2018).

Landum *et al.*, (2016) also verified bio-control with endophytes isolated from *Olea europaea* against *Colletotrichum acutatum*. All fungal isolates showed some inhibitory action on the growth of the phytopathogen. The same authors reported that volatile substances produced by these isolates could play a significant role in reducing *C. acutatum*. Borgiono *et al.*, (2015) evaluated the endophytic fungi isolated from organic coffee against phytopathogens *Glomerella* sp. and

Colletotrichum sp. and obtained inhibition rate ranging between 46.21 % and 60.86 %. The biotechnological potential of endophytic fungi of the grapevine (*Vitis labrusca* L.) was tested *in vitro* for the control of phytopathogenic of vines, including *Alternaria* sp., *Sphaceloma* sp. and *Glomerella* sp., with more than 50 % inhibition (Felber *et al.*, 2015). It is important to point out that, although the genera *Diaporthe* and *Colletotrichum* could be found as phytopathogens, they were isolated in the endophytic condition in the present work, i.e., they do not cause any symptoms of diseases and could be used in the safely biotechnological process.

The data and results obtained in the current study confirmed that endophytic fungi are promising agents for biological control and may inhibit or reduce phytopathogen growth in several ways, including mycoparasitism, antibiosis, the production of metabolites, competition for nutrients, or resistance induction in plants (Terhonen *et al.*, 2016; Ribeiro *et al.*, 2018). The use of endophytes in crops presents advantages as these organisms can colonize and establish a long-lasting interaction with their hosts. Besides this, liquid formulations can be developed for foliar applications or seed treatments. However, there were some limitations, such as biotic and abiotic factors, as well as the preparation of farmers and the storage of formulations and the need for further developments to endophytic applications (Sessitsch *et al.*, in press).

Endophytic fungi from *S. saponaria* also produced amylase, pectinase, and cellulase, detected by the cup plate technique. The measurement of the halos formed after the addition of the dyes showed clearly defined zones, indicating enzymatic activity. The enzymatic production by endophytic fungi is variable since it is related to the specificity between the host plant and fungus (Tan and Zou, 2001). Enzyme production facilitates the penetration of endophytes in the host plant and is also involved in the competition with other microorganisms, such as pathogenic fungi (Fouda *et al.*, 2015; Ribeiro *et al.*, 2018). They are mutualists, but some may become pathogenic on occasion when environmental and physiological conditions of the plant may allow (Strobel, 2018). Endophytes are a new source of many interesting industrial enzymes, such as lipases, phytases, amylases, proteases and cellulases (Corrêa *et al.*, 2014).

One of the most studied models of cellulase production is *Trichoderma reesei*; the production by this species may vary between 0.2 and 137 $\mu\text{mol}/\text{min}/\text{mg}$ (Gutiérrez-Rojas *et al.*, 2015). In this study, strain SS08 (*Diaporthe* sp.) produced 1.51 U $\mu\text{mol}/\text{min}$ of cellulase. For amylase, the best production was 1.16 U $\mu\text{mol}/\text{min}$ for SS65 (*Diaporthe* sp.). Patil *et al.*, (2015) analyzed extracellular enzymatic activities of endophytic fungi isolated from various medicinal plants, and three endophytes were able to produce extracellular amylase ranging between 0.1 and 4.1 U/ml; the best result was produced by *Rhizoctonia* sp., with 2.74 U $\mu\text{mol}/\text{min}$ of pectinase being produced by SS77 (*Diaporthe* sp.). Hegde and Srinivas, (2017) evaluated the production of pectinase by endophytic fungi isolated from medicinal plants; the best result was 0.20 $\mu\text{mol}/\text{min}$

produced by *Talaomyces* sp. These results show that endophytic fungi from *S. saponaria* are promising for enzymatic production and may be optimized to increase efficiency.

CONCLUSIONS

The current study shows that endophytes from *S. saponaria* L. may be a biological control agent against all analyzed phytopathogens and useful for enzymatic production, reinforcing the need to evaluate the isolated endophytes of plant hosts and the importance of the conservation of these strains in optimal stock conditions.

ACKNOWLEDGMENT

The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the Master's Degree scholarship and CAPES/PNPD-UEM for the post-doctoral scholarship. Thanks are also due to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (307603/2017-2), and Secretaria de Estado da Ciência, Tecnologia e Ensino Superior/Unidade Gestora do Fundo do Paraná (SETI/UGF).

REFERENCES

- Alberto RN, Costa AT, Polonio JC, Santos MS, Rhoden SA, Azevedo JL, et al. Extracellular enzymatic profiles and taxonomic identification of endophytic fungi isolated from four plant species. *Genet Mol Res*. 2016;15(4). Doi: <https://doi.org/10.4238/gmr15049016>.
- Azevedo JL, Araújo W L. Diversity and applications of endophytic fungi isolated from tropical plant. In: Ganguli BN, Deshmukh SK, editor(s). *Fungi: Multifaceted Microbes*. New Delhi: Anamaya Publishers; 2007.p.189-207.
- Badalyan SM, Innocenti G, Garibyan NG. Interactions between xylotrophic mushrooms and mycoparasitic fungi in dual-culture experiments. *Phytopathol Mediterr*. 2004;43(1):44-48. Doi: http://dx.doi.org/10.14601/Phytopathol_Mediterr-1733
- Bahrour A, Jousset A, Mhamdi R, Mrabet M, Mhadhbi H. Anti-fungal activity of bacterial endophytes associated with legumes against *Fusarium solani*: Assessment of fungi soil suppressiveness and plant protection induction. *Appl Soil Ecol*. 2018;124:131-140. Doi: <https://doi.org/10.1016/j.apsoil.2017.10.025>
- Bogner CW, Kariuki GM, Elashry A, Sichtermann G, Buch AK, Mishra B, et al. Fungal root endophytes of tomato from Kenya and their nematode biocontrol potential. *Mycol Progress*. 2016;15(3):30. Doi: <https://doi.org/10.1007/s11557-016-1169-9>
- Bungtongdee N, Sopalan K, Laosripaiboon W, Iamtham S. The chemical composition, antifungal, antioxidant and antimutagenicity properties of bioactive compounds from fungal endophytes associated with Thai

- orchids. J Phytopathol. 2019;167(1):56-64. Doi: <https://doi.org/10.1111/jph.12773>.
- Campanile G, Ruscelli A, Luisi N. Antagonistic activity of endophytic fungi towards *Diplodia corticola* assessed by in vitro and in planta test. Eur J Plant Pathol. 2007;117(3):237-246. Doi: <https://doi.org/10.1007/s10658-006-9089-1>
- Corrêa RCG, Rhoden SA, Mota TR, Azevedo JL, Pamphile JA. Endophytic fungi: expanding the arsenal of industrial enzyme producers. J Ind Microbiol Biotechnol. 2014;41(10):1467-1478. Doi: <https://doi.org/10.1007/s10295-014-1496-2>.
- Dissanayake AJ, Purahong W, Wubet T, Hyde KD, Zhang W, Xu H, *et al.* Direct comparison of culture-dependent and culture-independent molecular approaches reveal the diversity of fungal endophytic communities in stems of grapevine (*Vitis vinifera*). Fungal Divers. 2018;90(1):85-107. Doi: <https://doi.org/10.1007/s13225-018-0399-3>
- Felber AC, Orlandelli RC, Rhoden SA, Garcia A, Costa AT, Azevedo JL, *et al.* Bioprospecting foliar endophytic fungi of *Vitis labrusca* Linnaeus, Bordô and Concord cv. Ann Microbiol. 2016;66(2):765-775. Doi: <https://doi.org/10.1007/s13213-015-1162-6>
- Ferreira DF. SISVAR: um programa para análise e ensino de estatística. Rev Científica Symp. 2008;6:36-41
- Fouda AH, Hassan SE, Eid AM, Ewais EE. Biotechnological applications of fungal endophytes associated with medicinal plant *Asclepias sinaica* (Bioss.). Ann Agric Sci. 2015;60(1):95-104. Doi: <https://doi.org/10.1016/j.aos.2015.04.001>
- Gao F, Dai C, Liu X. Mechanisms of fungal endophytes in plant protection against pathogens. Afr J Microbiol Res. 2010;4(13):1346-1351.
- Garcia A, Rhoden SA, Rubin Filho CJ, Nakamura CV, Pamphile JA. Diversity of foliar endophytic fungi from the medicinal plant *Sapindus saponaria* L. and their localization by scanning electron microscopy. Biol Res. 2012;45(2):139-148. Doi: <https://doi.org/10.4067/S0716-97602012000200006>.
- Gutiérrez-Rojas I, Moreno-Sarmiento N, Montoya D. Mecanismos y regulación de la hidrólisis enzimática de celulosa en hongos filamentosos: casos clásicos y nuevos modelos. Rev Iberoam Micol. 2015;32(1):1-12. Doi: <https://doi.org/10.1016/j.riam.2013.10.009>
- Hegde SV, Srinivas C. Evaluation and Optimization of Pectinase Production by Endophytic Fungi *Talaromyces* sp. isolated from *Calophyllum inophyllum*. IJETS. 2017;4:610-620.
- Hyde KD, Soyong K. The fungal endophyte dilemma. Fungal Divers. 2008;33:163-173.
- Ikram M, Ali N, Jan G, Jan FG, Rahman IU, Iqbal A, *et al.* IAA producing fungal endophyte *Penicillium roqueforti* Thom., enhances stress tolerance and nutrients uptake in wheat plants grown on heavy metal contaminated soils. PLoS One. 2018;13(11):e0208150. Doi: <https://doi.org/10.1371/journal.pone.0208150>.
- Kusari S, Hertweck C, Spiteller M. Chemical ecology of endophytic fungi: origins of secondary metabolites. Chem Biol. 2012;19(7):792-798. Doi: <https://doi.org/10.1016/j.chembiol.2012.06.004>.

- Jaber LR, Ownley BH. Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? Biol Control. 2018;116:36-45. Doi: <https://doi.org/10.1016/j.biocontrol.2017.01.018>
- Jayaraj R, Megha P, Sreedev P. Organochlorine pesticides, their toxic effects on living organisms and their fate in the environment. Interdiscip Toxicol. 2016;9(3-4):90-100. Doi: <http://www.doi.org/10.1515/intox-2016-0012>
- Lacava PT, Azevedo JL. Biological control of insect-pest and diseases by endophytes. In: Verma V, Gange A, editor(s). Advances in Endophytic Research. New Delhi: Springer, 2014. p. 231-256. Doi: https://doi.org/10.1007/978-81-322-1575-2_13
- Landum MC, Félix MR, Alho J, Garcia R, Cabrita MJ, Rei F, et al. Antagonistic activity of fungi of *Olea europaea* L. against *Colletotrichum acutatum*. Microbiol Res. 2016;183:100-108. Doi: <http://www.doi.org/10.1016/j.micres.2015.12.001>.
- Li H, Guan Y, Dong Y, Zhao L, Rong S1, Chen W, et al. Isolation and evaluation of endophytic *Bacillus tequilensis* GYLH001 with potential application for biological control of Magnaporthe oryzae. PLoS One. 2018;13(10):e0203505. Doi: <http://www.doi.org/10.1371/journal.pone.0203505>.
- Mendes R, Azevedo JL. Valor biotecnológico de fungos endofíticos isolados de plantas de interesse econômico. In: Maia LC, Malosso E, Yano-Melo AM, editors. Micologia: avanços no conhecimento. Recife, BR: Editora Universitária da UFPE, 2008. p.129-140.
- Murali TS, Suryanarayanan TS, Geeta R. Endophytic *Phomopsis* species: host range and implications for diversity estimates. Can J Microbiol. 2006;52(7):673-680. Doi: <http://www.doi.org/10.1139/w06-020>
- Nielsen RI, Oxenboll K. Enzymes from fungi: their technology and uses. Mycologist. 1998;12(2):69-71. Doi: [https://doi.org/10.1016/S0269-915X\(98\)80048-7](https://doi.org/10.1016/S0269-915X(98)80048-7)
- Nisa J, Kamili, AN, Nawchoo, IA, Shafi S, Shameem N, Bandh AS. Fungal endophytes as prolific source of phytochemicals and other bioactive natural products: A review. Microb Pathog. 2015;82:50-59. Doi: <http://www.doi.org/10.1016/j.micpath.2015.04.001>.
- Pamphile JA, Azevedo JL. Molecular characterization of endophytic strains of *Fusarium verticillioides* (*Fusarium moniliforme*) from maize (*Zea mays*. L). World J Microbiol Biotechnol. 2002;18(5):391-396. Doi: <https://doi.org/10.1023/A:1015507008786>.
- Pandey AK, Reddy MS, Suryanarayanan TS. ITS-RFLP and ITS sequence analysis of a foliar endophytic *Phyllosticta* from different tropical trees. Mycol Res. 2003;107(4):439-444. Doi: <https://doi.org/10.1017/S0953756203007494>.
- Patil JMG, Patil, APSN, Sidhu K. Extracellular Enzymatic Activities of Endophytic Fungi Isolated from Various Medicinal Plants. Int J Curr Microbiol App Sci. 2015;4(3):1035-1042.
- Pimentel MR, Molina G, Dionísio AP, Maróstica Junior MR, Pastore GM. The use of endophytes to obtain bioactive compounds and their application in biotransformation process. Biotechnol Res Int. 2011;2011:1-11. Doi: <http://dx.doi.org/10.4061/2011/576286>.

- Polonio JC, Almeida TT, Garcia A, Mariucci GEG, Azevedo JL, Rhoden SA, *et al.* Biotechnological prospecting of foliar endophytic fungi of guaco (*Mikania glomerata* Spreng.) with antibacterial and antagonistic activity against phytopathogens. Genet Mol Res. 2015;14(3):7297-7309. Doi: <http://www.doi.org/10.4238/2015.July.3.5>
- Ribeiro AS, Polonio JC, Costa AT, Santos CM, Rhoden SA, Azevedo JL, *et al.* Bioprospection of Culturable Endophytic Fungi Associated with the Ornamental Plant *Pachystachys lutea*. Curr Microbiol. 2018;75(5):588-596. Doi: <https://doi.org/10.1007/s00284-017-1421-9>.
- Rhoden SA, Garcia A, Rubin Filho CJ, Azevedo JL, Pamphile JA. Phylogenetic diversity of endophytic leaf fungus isolates from the medicinal tree *Trichilia elegans* (Meliaceae). Genet Mol Res. 2012;11(3): 2513-2522. Doi: <https://doi.org/10.4238/2012.June.15.8>
- Robinson PK. Enzymes: principles and biotechnological applications. Essays Biochem. 2015;59:1-41. Doi: <https://doi.org/10.1042/BSE0590001>
- Salim AA, Grbavac S, Sekuljica N, Stefanovic A, Tanaskovic SJ, Lukovic N, *et al.* Production of enzymes by a newly isolated *Bacillus* sp. TMF-1 in solid state fermentation on agricultural by-products: the evaluation of substrate pretreatment methods. Bioresour technol. 2017;228:193-200. Doi: <https://doi.org/10.1016/j.biortech.2016.12.081>
- Sharma A, Tewari R, Rana SS, Soni R, Soni SK. Cellulases: classification, methods of determination and industrial applications. Appl biochem biotechnol. 2016;179(8):1346-1380. Doi: <https://doi.org/10.1007/s12010-016-2070-3>
- Sebastianes FLS, Dumaresa ASR, Lacava PT, Harakava R, Azevedo JL, Melo IS, *et al.* Species diversity of culturable endophytic fungi from Brazilian mangrove Forest. Curr Genet. 2013;59(3):153-166. Doi: <https://doi.org/10.1007/s00294-013-0396-8>.
- Sessitsch A, Pfaffenbichler N, Mitter B. Microbiome applications from Lab to Field: Facing complexity. Trends in Plant Sci. 2019;24(3):194-198. Doi: <https://doi.org/10.1016/j.tplants.2018.12.004>
- Strobel G. The Emergence of Endophytic Microbes and Their Biological Promise. J Fungi. 2018;4(2). Doi: <https://doi.org/10.3390/jof4020057>.
- Tan RX, Zou WX. Endophytes: a rich source of functional metabolites. Nat Prod Rep. 2001;18(4):448-459. Doi: <https://doi.org/10.1039/B100918O>
- Terhonen E, Sipari N, Asiegbu FO. Inhibition of phytopathogens by fungal root endophytes of Norway spruce. Biol Control. 2016;99(8):53-63. Doi: <https://doi.org/10.1016/j.biocontrol.2016.04.006>
- Venugopalan A, Srivastava S. Endophytes as in vitro production platforms of high value plant secondary metabolites. Biotechnol Adv. 2015;33(6):873-887. Doi: <https://doi.org/10.1016/j.biotechadv.2015.07.004>.
- White TJ, Bruns TD, Lee S and Taylor JW. PCR Protocols: A Guide to Methods and Applications. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ, editor(s). Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. San Diego: Academic Press; 1990. p. 315-322.

Notes

Associate Editor: Juan F. González.

Citation/Citar este artículo como: Santos CM, Ribeiro AS, Garcia A, Polli AD, Polonio JC, Azevedo JL, Pamphile JA. Enzymatic and antagonist activity of endophytic fungi from *Sapindus saponaria* L. (Sapindaceae). Acta biol. Colomb. 2019;24(2):322-330. DOI: <http://dx.doi.org/10.15446/abc.v24n2.74717>

CONFLICT OF INTEREST The authors declare that there is no conflict of interest.

Author notes

Todo el contenido de esta revista, excepto dónde está identificado, está bajo una Licencia Creative Commons

*

For correspondence: japamphile@uem.br