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Full Length Research Paper

Phomopsis sp. as an endophyte of *Turnera subulata* L.: Isolation, identification and antimicrobial and antioxidant activity of their extracts

Giancarlo de Brito Lyra Santos[1], Luiz Carlos Caetano[2], Ariana Rafaela da Silva Nascimento[2], Roberto Ramos Sobrinho[2], Ricardo Manoel dos Santos Silva[2], João Manoel da Silva[3]*, Tania Marta Carvalho dos Santos[2] and Yamina Coentro Montaldo[2]

[1]Federal Institute of Alagoas, Maceió, Alagoas, Brazil
[2]Federal University of Alagoas, Chemical and Biochemical Department and Agricultural Sciences Center, Maceió, Alagoas, Brazil
[3*]Federal University of Sergipe, Department of Agronomy, São Cristóvão, Sergipe, Brazil,

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***Turnera subulata* L. is a plant that belongs to the Turneraceae family and is popularly known in Brazil as "Chanana"; it is used as an alternative medicine. Among all microorganisms, fungi are mostly associated with plants. The aim of this study is to isolate, identify and evaluate the antifungal and antioxidant activity of extracts of *Phomopsis* sp. isolated from *T. subulata*. From the leaf fragment obtained from *T. subulata,* the filamentous endophytic fungus *Phomopsis* sp was isolated. The fungal isolate had a higher growth in potato dextrose agar (PDA) and potato sucrose agar (PSA) culture medium, as well as in the presence of light. In the antagonism test of the endophytic *Phomopsis* sp. against human pathogens, there was inhibition zone against *Escherichia coli, Staphylococcus aureus, Candida albicans, C. tropicalis* and *C. glabrata*. Concerning the antioxidant activity, it was observed that the chloroform extract was more effective than hexane. On the other hand, all the extracts from the mycelium of *Phomopsis* sp. and its ethyl acetate extract from the cultured filtrate had low antimicrobial activity against strains of *E. coli, S. aureus, C. albicans, C. tropicalis* and *C. glabrata*. Therefore it was concluded that *Phomopsis* sp. may act as an endophyte of *T. subulata.* Extracts from *Phomopsis* sp. promoted inhibition zone of growth when tested against human pathogen. Its hexanic and chloroform extracts showed lower antioxidant activity.**

Key words: Biological control, endophytic fungus, secondary metabolites, filamentous fungus, fungal extract.

INTRODUCTION

Endophytic microorganisms are potentially useful to industries, particularly in food and pharmaceutical industries. Several selected species of endophytes are used in crop protection industries, besides being used as

*Corresponding author: E-mail: jm.agro@hotmail.com. Tel: +557921056600.

genetic vectors (Souza et al., 2004). These microorganisms are important genetic source for biotechnology; they have caught the attention of the scientific community because they produce secondary metabolites which are used in food industry and pharmaceuticals (Strobel, 2003).

Valsaceae mitosporico belongs to the genus, *Phomopsis;* phylumm Ascomycota; subphylum, Pezizomycotin; class, Sordariomycetes; subclass, Sordariomycetidae; order, Diaporthales; family, Valsaceae. *Diaporthe* is the telemorphic form of the genus, Valsaceae (Hanlin and Menezes, 1996). This genus has been isolated as an endophyte in several vegetable crops such as *Theobroma cacao* L. (Rubini et al., 2005), *Spondias mombin* L. (Rodrigues et al., 2000), *Aspidosperma tomentosum* (Corrado and Rodrigues, 2004), *Heterosmilax japonica* Kunth (Gao et al., 2005), among others.

Fungi are considered as promising sources of new drugs for therapeutic use and in fact, several medicines used in health centers are derived from fungal metabolites. In the past two decades, a group of microorganisms that stood out for the production of bioactive metabolites were endophytes, especially fungi. Endophytic microorganisms are found in many species of medicinal plants. Stierle et al. (1995) reported that the fungus *Taxomyces andreanae*, taxol-producing endophytic and other taxanos, that live in the interior of the medicinal plant, *T. brevifolia*, is used for cancer treatment.

According to Arbo (2004), the family Turneraceae has 10 genus and around 190 species with a wide distribution in tropical and subtropical regions of the world, with tropical America being the diversity Center of these species. The genus *Turnera* presents about 120 species scattered in the Americas and Africa, being the most representative of the family Turneraceae (Barbosa et al., 2007).

For every moment, more is known about the interaction between plants and endophytic microorganisms, as well as the new species found in symbiosis and the wealth of metabolites produced by them. Chen et al. (2014), in his review, discusses the evolution and discoveries of endophytic fungi. Malhadas et al. (2017) describe the antimicrobial activity of *Penicillium* species isolated from olive leaves, as well as the identification of their compounds. In view of the above, the goal of this study is to isolate and evaluate the antimicrobial potential of *Phomopsis* spp. isolated from *Turnera subulata* leaves.

MATERIALS AND METHODS

Isolation and purification of endophytic fungi from *T. subulata*

Leaves of *T. subulata* without symptoms of diseases or mechanical injuries were collected in the urban region of Maceio city and taken to Laboratory of Biotechnology of Plants and Endophytic Microorganisms where they were promptly washed with running water. All the materials were disinfected superficially through the following protocol: 1) the vegetal sample was washed in alcohol (70% v/v) for 1 min; 2) the material was washed in sodium hypochlorite (0.20% w/v) plus two drops of commercial detergent for 20 min and 3) triple washing in sterile distilled water.

After cleaning and disinfecting the *T. subulata* samples with 1 cm of diameter, they were inoculated on petri plates containing potato dextrose agar (PDA) medium, and incubated for five days at room temperature on mycelial growth. After incubation, each microorganism colony was taken as a sample of the vegetative mycelium and inoculated in PDA medium for six days under continuous light at temperature of 25°C (±3°C). After growth, the samples were checked on optical microscopy to verify the purity of the isolated fungal colonies.

Prior to the identification of the fungi isolated, micro culture methodology was done. The colony was scraped before it was grown in petri dishes containing PDA medium. Thereafter it was inoculated on slides with a drop of the same culture medium on an equilateral triangle and damp cotton with sterile water to grant moisture required for fungal growth. It was incubated for three days under continuous light.

After this procedure, slides were stained with bromophenol blue and observed by optical microscopy to determine the presence of the fungus *Phomopsis* spp. based on its morphologic characteristics.

DNA extraction from the endophytic fungus

The endophytic fungus isolated from *T. subulata* L. was grown in 100 ml of liquid medium potato dextrose (PD), at 23 to 25°C without agitation for four days. Such samples were forwarded to Forensic DNA Laboratory at the Natural History Museum of the Federal University of Alagoas for DNA extraction and subsequent sequencing of the prevalence of ITS2 regions (internal transcribed spaces 1 and 2, respectively) of rDNA.

The mycelium of *Phomopsis* sp. was isolated from the culture medium by centrifugation for 10 min at 7000 rpm (after the fourth day of growth) and subjected to DNA extraction using the CTAB protocol (Ferreira and Grattapaglia, 1995). After the extraction, DNA was stored at 4°C. The amplification of the rDNA was performed by PCR. Specific primers of Write et al. (1990) were used for the amplification of the prevalence of ITS1 and ITS2 regions.

The fragments produced were separated by capillary electrophoresis in automatic sequencer ABI Prism 310 Genetic Analyzer, using polymer POP6 (Applied Biosystems, Foster City, CA, USA). An analysis of the Electropherograms was made using the program SeqScape v2.5. The mixed sequence obtained was 515 nucleotides and was analyzed using the BLAST program of NCBI, which allowed for the comparison percentage of similarity found with the data available in NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov).

Chemical prospecting from the mycelium of *Phomopsis* sp.

Erlenmeyer, containing PD medium and inoculated with a fungal disk in a diameter of approximately 5 mm, was incubated in the dark without aeration at 25 (±3°C) for the production of biomass. After 30 days of cultivation, the biomass was filtered and taken to kiln drying at 45°C until dry mass was obtained. The dry biomass was removed through maceration with n-hexane (5 x 250 mL), chloroform (5 × 250 mL), ethyl acetate (5 x 250 mL) and methanol (5 x 250 mL).

Preparation of samples and determination of IC_{50}

The stock solutions of the samples (dry extracts; 40 mg/mL) were

prepared from the dissolution of each sample in methanol (HPLC grade). From these solutions were prepared dilutions in concentrations of 30, 20 and 10 mg/ml. Five experiments of dried extracts were performed with each of the solutions (10 to 40 mg/ml). Reaction mixtures consisting of 0.1 ml of the test solution and 0.9 ml of stock solution of DPPH 40 mg/ml (~100 Î¼mol/L) were prepared and the measures of absorbance of each were made at 515 nm, in triplicate every 15 min, for 1 h.

Antimicrobial activity of extracts of *Phomopsis* sp against human pathogenic microorganisms

Hexane, chloroform, ethyl acetate and partition of the mycelium and extract in ethyl acetate of the culture filtrate of *Phomopsis* sp were tested against *E. coli* (ATCC 8739), *S. aureus* (ATCC 6538), *Candida albicans* (URM 4126), *C. tropicalis* (URM 4977) and *C. glabrata* (CCIBM), through the agar diffusion method, described by Bauer et al. (1996). All strains are deposited in the culture bank of Microbiology Laboratory of the Institute of Biological Sciences and Health (ICBS).

The extracts were diluted in six different concentrations. Then 20 µL of each concentration was added to Whatman filter discs paper No. 5 (0.5 cm) that was properly sterilized and put in holes of approximately 5 mm with the aid of a puncher of agar. For inoculation, we used 100 µL of each bacterial suspension/yeast, depositing the same through agar nutrient in petri dishes. Then paper discs containing extracts were added. Each test was done in three repetitions in a completely randomized design.

RESULTS AND DISCUSSION

From the leaf fragments of *T. subulata, it* was possible to isolate a filamentous fungus that has been identified by classical methods of microscopy and molecular as the fungus *Phomopsis* sp.. Until now, this is the first report on the isolation of the fungus *Phomopsis* sp. as endophyte of *T. subulata* leaves.

The fragment from the region of *Phomopsis* sp. isolate obtained from *T. subulata* presented 439 pb (pairs of bases) arranged in the following way: 5'CCCTTTGTGAACTTATACCTATTGTTGCCTCGGCG AGGCCGGCCTCTTCACTGAGGCCCCCTGGAAACAG GGAGCAGCCCGCCGGCGGCCAACTAAACTCTTGTTT CTATAGTGAGTCTCTGAGTAAAAAACATAAATGAATC AAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA CATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGT TCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTG ATGGGGCACTGCCTTCTAGCGAGGGCAGGCCCTGA AATCTAGTGGCGAGCTCGCTAGGACCCCGAGCGTAG TAGTTATATCTCGTTCTGGAAGGCCCTGGCGGTGCC CTGCCG 3'

Using bioinformatics tool BLAST (Basic Local Alignment Search Tool) on the site http://blast.ncbi.nlm.nih.gov/ blast, this sequence was compared to the other existing ones in this region strings; it was recorded in the database of the program where the isolates of *Phomopsis* and *Diaporthe* were obtained. It was also possible, through this tool, to obtain a sequence dichotomous tree compared to sequences from the NCBI database (Figure 1).

After cultivating the isolate in a liquid medium for 30 days, with a total of approximately 16 g of dry biomass isolated from *Phomopsis* sp. (about 0.4 g per 100 ml of culture medium), the following quantities of extracts were obtained: n-hexane, 1.68 g; chloroform, 1.28 g; ethyl acetate, 0.72 g; and methanol, 1.92 g, showing an approximate sum of 35% extracts obtained from the total dry biomass (Table 1).

In the antimicrobial activity tested, the extracts, hexane, chloroform, ethyl acetate, mycelium of *Phomopsis* sp., and the extract in acetate filtered showed low antimicrobial activity against the tested micoorganisms: *E. coli* (ATCC 8739), *S. aureus* (ATCC 6538), *C. albicans* (URM 4126), *C. tropicalis* (URM 4977-) and *C. glabrata* (CCIBM). This contradicts the results obtained by Chareprasert et al. (2006), where *Phomopsis* sp., was inhibitory against *Bacillus subtilis, S. aureus*, *E. coli* and *C. albicans*.

However, the processes of cultivation and extraction can exert a high influence on the target microorganisms, as well as the metabolites produced by the fungus. In addition, the amount of metabolites varies, according to the host plant, environment in which the fungus is found, species and other factors, both environmental and genetic. Based on this, it is necessary to test other pathogens and fungal isolates, as well as to diversify the use of culture media, temperature, luminosity and carbon source. Genetic variations in the genus or even at species level are also capable of contributing to antimicrobial activity.

Corrado and Rodrigues (2004) observed the bactericidal activity of strains of the endophytic fungus *Phomopsis* sp. isolated from the leaves of *Aspidosperma tomentosum* and petioles of medicinal plant *Spondias mombin*. All the evaluated extracts inhibited the growth of bacteria, fungi and yeasts tested, showing the great potential of this fungus as a source of bioactive products.

The yeasts of the genus *Candida* have great importance because of the high frequency with which they colonize and infect the human host (Colombo and Guimarães, 2003). The appearance and increase in the incidence of several pathogens caused by species of a white-robed throng and other pathogens, and the resistance of the same therapies already existing led to the search for other active principles.

Therefore, the cellular extracts n-hexane and chloroform de *Phomopsis* sp., endophytic of *T. subulata* had low antioxidant activity; the extract in chloroform was slightly higher and got a maximum uptime of 20%.

Hexanics and chloroformic extracts, in ethyl acetate and methanolic extract of mycelium of *Phomopsis* sp. and filtered acetate extract showed low antimicrobial activity against the strains of *E. coli*, *S. aureus*, *C. albicans*, *C. tropicalis* and *Candida glabrata*. Other tests should be conducted with the purpose of increasing the

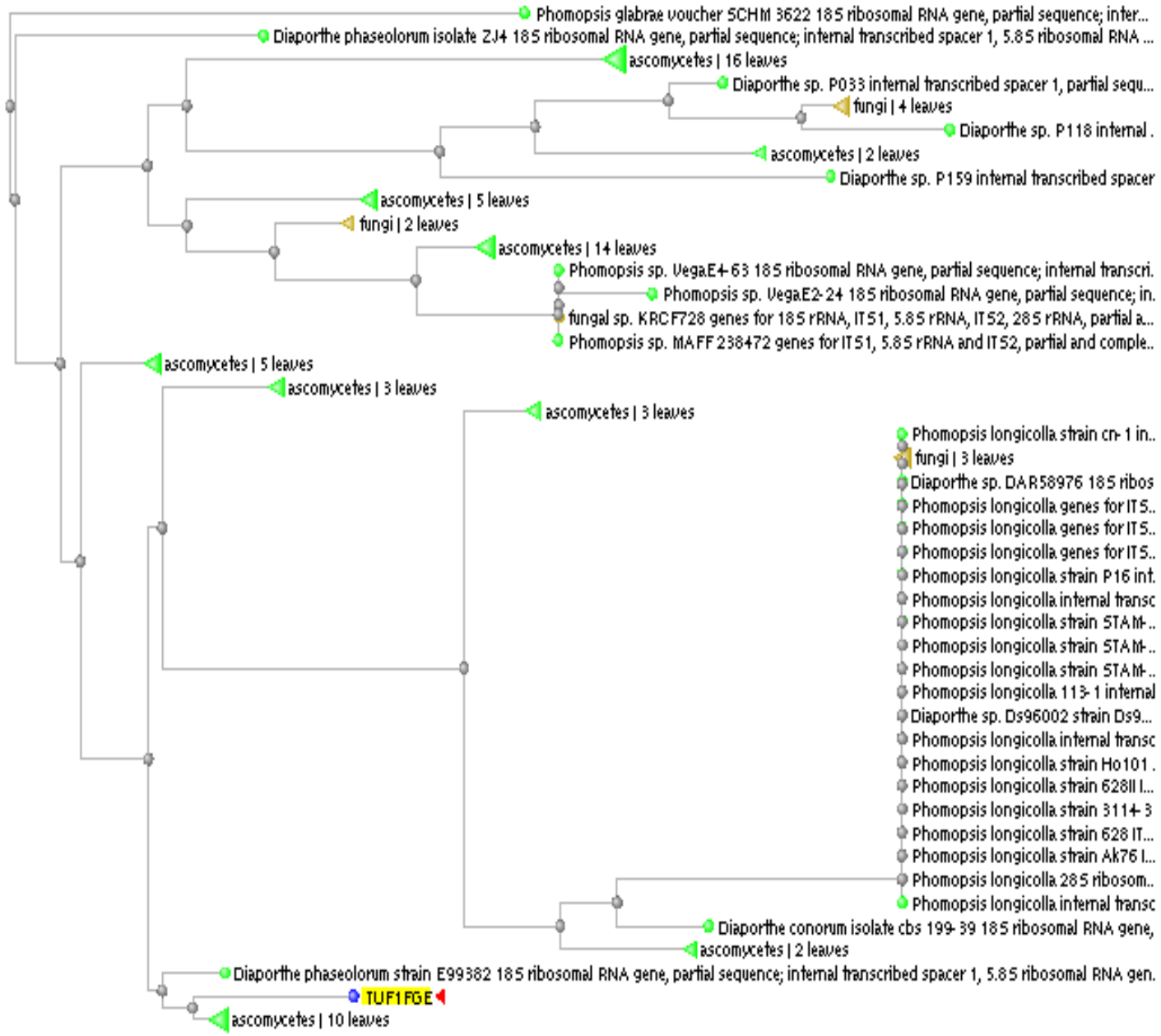

Figure 1. Neighbor joining phylogenetic tree obtained by the insertion of the sequence of bases of ITS region of rDNA from the fungal isolated from leaves of *T. subulata* in comparison with the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi#58396904). Identified as *Phomopsis* SP. (teleomorph phase: *Diaporthe* sp.).

Table 1. Yield of extracts of biomass of *Phomopsis* sp. (30 Days) obtained from *T. subulata* in dry biomass.

Isolate	Solvent	Dry weight (g)	Yield (%)
	n-hexane	1.68	10.5
	Chloroform	1.28	8.00
Phomopsis sp (16 g)	Ethyl acetate	0.72	4.5
	Methanol	1.92	12.0
	Total		**35.0**

action spectrum of *Phomopsis* sp. against other target microorganisms.

Conclusion

Phomopsis sp. fungus may be able to act as an endophyte of the plant *T. subulata*, as shown in this first report of its isolation in leaves of this plant. Although it has low antimicrobial activity, the same fungus must be tested with other target microorganisms, besides using other methodologies to identify its compounds.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

PDA, Potato dextrose agar; **ITS**, internal transcribed spaces; **PSA,** potato sucrose agar.

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