International Journal of Botany

ISSN: 1811-9700

International Journal of Botany 10 (1): 24-29, 2014
ISSN 1811-9700 / DOI: 10.3923/ijb.2014.24.29

Endophytic Mycobiota of Three Amazonian Medicinal Herbs: *Stachytarpheta cayennensis* (Verbenaceae), *Ayapana triplinervis* (Asteraceae) and *Costus spicatus* (Costaceae)

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Abstract: The interactions between plants and fungi result in metabolic modifications that lead to alternative sources of raw material for the production of bioactive molecules with potential for pharmaceutical, agronomical and chemical applications. In spite of this, not many endophytic fungi has been object of research. The aim of this study was to characterize the endophytic mycobiota of three medicinal plants, *Costus spicatus* (Jacq.) Sw., *Stachytarpheta cayennensis* (Rich.) Vahl and *Ayapana triplinervis* (M.Vahl) R.M. King and H. Rob. Samples from the vegetative parts were collected, disinfected and inoculated on agar plates to cultivate exclusively endophytic fungi. The rate of colonization (total plant) and the frequency of each fungal genus were calculated, contributing with relevant new knowledge about the fungal biodiversity inside these plant species. From a total count of 19 genera, 16 fungal isolates were from *C. spicatus*, 12 from *A. triplinervis* and 10 from *S. cayennensis*. The genera *Paecilomyces, Aspergillus, Fusarium, Trichoderma, Penicillium* and *Curvularia* were the most frequent isolated.

Key words: Endophytic fungi, medicinal plants, ecological interactions, secondary metabolism

INTRODUCTION

The products of secondary metabolism resulting from the interactions between endophytic fungi and plants are of great interest as they can provide a variety of raw materials with high biotechnological potential for industrial applications targeting human health pharmaceuticals, agronomics, environmental and food production. A great number of these substances have contributed to the release of antibiotics, chemotherapeutic drugs and pesticides, the latter being effective against plagues but pursuing low toxicity, thus generating less environmental impact (Montesinos, 2003; Kursar *et al*., 2007). These interactions may provide several advantages for the plant like increasing its resistance to environmental stresses, changes in the physiological properties and production of phytohormones (Azevedo *et al*., 2000; Souza *et al*., 2004).

The endophytic colonization is the establishment of micro-organisms in intra- and intercellular spaces of roots, stems and leaves, causing no apparent damage to the host (Zabalgogeazcoa, 2008). It was stated that these relationships are not well understood and may be classified as symbiotic, antagonistic or neutral. In the case of harmonic relationships, the nutrients donated by the host plant are used by mold in metabolic pathways and result in compounds with hormonal actions and/or a useful antibiotic to enhance the plant resistance against pathogens (Souza *et al*., 2004; Bérdy, 2005; Neelapu *et al*., 2009).

Several pharmacological properties of endophytes have been documented such as significant antifungal activity and the cytotoxic and antiproliferative effects against various carcinoma cells lines achieved with *Botryosphaeria rhodina* isolated from the stems of *Bidens pilosa* (Asteraceae) (Abdou *et al*., 2010). *Taxomyces andeanae* synthesizes taxol, a substance with action against cancer (Yuan *et al*., 2010). From *Edenia gomezpompae*, discovered and isolated from the leaves of *Callicarpa acuminata* (Verbenaceae), four organic

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compounds were described and revealed a potential biological activity against *Colletotrichum*, *Phomopsis*, *Guignardia manguifera*, *Oomycetes*, *Phythophtora capsici*, *Phythophtora parasitica* and *Fusarium oxysporum* (Macias-Rubalcava *et al*., 2008).

Among the properties of popular used medicinal herbs, *Costus spicatus* (known in Brazilian Amazon as “poor-oldman”) usage is listed as systemic cleanser, astringent and diuretic. More recently, a study has shown anti-inflammatory and anti-nociceptive activity of *C. spicatus* methanolic extracts by rodents (Quintans Jr. *et al*., 2010). *Stachytarpheta cayennensis* (also called “gervão”) is used against diarrhea, liver diseases, neuralgia, bleeding, as well as anti-rheumatic and against ulcers and stomach aches (Bueno *et al*., 2005; Penido *et al*., 2006). It shares with *Ayapana triplinervis* (popularly known as “japana”) the properties of being cardiotonic, diuretic, emetic, laxative and a good medicine against pneumonia (Rajasekaran *et al*., 2010). These therapeutic properties, in general, can be influenced by interactions with the associated mycobiota and may vary according to the environmental conditions.

The elucidation of the endophytic community can contribute to optimize the obtention of secondary metabolites already extracted from these plants. Another advantage is providing subsidies to respond to demands in the agronomical, biotechnological, pharmacological and chemical fields. Facing this challenge, this study aimed to characterize the endophytic fungi community of *Costus spicatus*, *Stachytarpheta cayennensis* and *Ayapana triplinervis* as a contribution for the knowledge of these three yet almost unknown species with high potential to be used in the pharmacological industry.

MATERIALS AND METHODS

Plant species and herborization: Ten specimens of each plant were collected as follows: *S. cayennensis* (Verbenaceae) and *C. spicatus* (Costaceae), Campus of the Federal University of Amazonas, UFAM; *A. triplinervis* (Asteraceae), Campus of the Federal Institute of Science and Technology of Amazonas, IFAM. Both sites are located in the urban zone of Manaus, capital state of Amazonas, Brazil. The exsiccates were deposited in the Herbarium Federal University of Amazonas, HUAM. They were recorded as *Stachytarpheta cayennensis* 8287; *Costus spicatus* 8288; *Ayapana triplinervis* 8684.

Sampling of endophytic fungi: Samples of stems, roots and leaves were obtained from 10 individuals of each species under study. Each sample was washed under water and neutral liquid soap, followed by air drying to lose any epiphytic colonies and dirty. The so cleaned samples were processed in a flow chamber for superficial disinfection by immersion for 3 min in ethanol (70%), 3 min in sodium hypochlorite (3%), 30 sec in ethanol (70%) and a final wash (3 times) in sterile distilled water. From the final flush, an aliquot of 50 μL was collected and inoculated for sterility control.

From each sample, under strict asepsis in the flow chamber, 5 fragments of 16 mm^2 were cut with sterile scissors and inoculated on potato dextrose agar (PDA + chloramphenicol) followed by incubation for 30 days at 28±2°C. A total of 300 agar plates were cultivated for each species (100 plates for each vegetative part: root, stalk and leaf samples). The experimental design was completely randomized with three treatments and ten repetitions for each vegetative part evaluated, each replicate represented by a Petri dish. The fungi growth was accompanied and the isolates maintained in PDA inclined tubes until the identification step.

Endophytes identification and colonization rate: The fungi strains were analyzed by macro and microscopic observation of vegetative and reproductive structures and compared to the identification keys of fungi available in the literature (Barnett and Hunter, 1972; Carmichael *et al*., 1980; Sutton, 1980). The frequency and rate of colonization of the fungi in a whole plant, respectively to the sampled vegetative parts were calculated followed the method described by Yuan *et al*. (2010).

RESULTS

A total of 19 fungal genera were identified in all three species. The major diversity of genera was detected by *C. spicatus* and each plant showed a different number and type of fungus colonizing roots, stalks and leaves, as summarized on Table 1. Analysis of variance of mean fungal colonization for each plant species revealed significant differences between *C. spicatus* and *A. triplinervis* (Table 2, 3).

DISCUSSION

Most of the available literature about the three plant species studied in this work focused on their

Table 1: Frequency (%) of endophytic fungi genera isolated from in *Costus spicatus*, *Stachytarpheta cayennensis* and *Ayapana triplinervis*

	Plants								
	C. spicatus			*S. cayennensis*			*A. triplinervis*		
Endophytic fungi	Root	Stalk	Leaf	Root	Stalk	Leaf	Root	Stalk	Leaf
Aspergillus	23.10	23.77	18.88	18.72	30.41	32.84	13.27	25.94	25.48
Beuveria	-	-	6.34	-	-	0.59	-	-	-
Fusarium	15.51	1.96	8.31	4.87	-	0.59	19.39	-	11.29
Paecilomyces	1.98	7.84	1.98	27.18	7.06	5.92	-	2.83	8.06
Penicillium	1.49	2.45	13.90	3.59	18.98	18.05	3.57	12.26	20.00
Pestalotia	-	-	2.87	-	-	-	-	-	4.52
Trichoderma	28.22	26.72	9.97	26.92	34.55	22.49	36.22	41.04	-
Colletotrichum	-	-	0.60	-	-	6.51	-	3.30	-
Scopulariopsis	-	-	-	-	-	1.48	-	-	-
Cladosporium	4.95	-	2.87	-	-	-	-	-	-
Aureobasidium	-	8.50	20.60	-	-	-	-	-	-
Nigrospora	3.63	-	-	-	-	-	-	-	-
Stachybotrys	2.15	-	-	-	-	-	-	-	-
Trichophyton	1.32	-	-	-	-	-	-	-	-
Botrytis	-	-	-	-	-	-	-	5.56	-
Curvularia	-	-	-	-	-	-	-	2.36	5.81
Acremonium	4.79	-	1.66	10.77	-	-	-	6.60	16.45
Memnoniella	3.30	7.11	-	-	-	-	9.18	-	-
Sterile micelia	9.57	21.57	7.55	7.95	9.00	11.54	18.37	-	8.39

Table 2: Analysis of variance of mean endophytic fungi present in species *Stachytarpheta cayennensis, Ayapana triplinervis* and *Costus spicatus*

		Square medios		
Source of variation	Degrees of freedom	*Stachytarpheta cayennensis*	*Ayapana triplinervis*	*Costus spicatus*
Vegetative part	2.00	7.09^{ns}	37.06*	181.11*
Residue	297.00	10.48	6.68	12.55
Total	299.00	17.57	43.74	193.66
Coeficient of variation (CV%)		83.67	83.67	62.93
Standard deviation (SD)		3.23	3.23	3.69
Overall average		3.87	3.87	5.63
Least significant difference (DMS)		1.08	1.08	1.18

[ns]Not Significant at F test (5% probability), *Significant at F test (5% probability)

Table 3: Comparison of means of endophytic fungi isolated from vegetative parts (leaf, stem and roots), *Stachytarpheta cayennensis*, *Ayapana triplinervis* and *Costus spicatus*

	Means[1]		
Vegetative part	Leaves	Stalks	Roots
Stachytarpheta cayennensis	3.59^b	4.12^a	3.90^b
Ayapana triplinervis	3.13^b	2.28^b	1.95^c
Costus spicatus	6.58^a	4.09^a	6.22^a

[1]Means followed by the same letter in the column do not differ by Tukey's test (5% probability)

pharmacological activity (Mesia-Vela *et al.*, 2004; Gauvin-Bialecki and Marodon, 2008). No information was available about the interactions of these plants with the microorganisms living within their structures until now.

A previous study showed that the association with symbiotic endophytes depends more on the availability of water than on the concentration of nutrients. Studying *Achnatherum sibiricum*, they found a lower level of infection by reducing water release, even when enough nutrients were available (Ren *et al.*, 2011). In the present study, a significant higher frequency of endophytic isolates was found by *C. spicatus* (Table 2), a species that typically grows in humid habitats, mostly near to water streams. However, the frequency and species composition of microbial communities are conditioned by the interactions between biotic and abiotic factors, inherent to its habitat, since the habitat associated with the plant is a dynamic environment (Costa-Neto, 2009).

The diversity of fungi found by *C. spicatus* may also be related to structural aspects of Costaceae. Its vegetative aerial parts exhibit simple filamentous trichomes, distributed along the epidermis. The roots are in form of rhizomes (Tomlinson, 1956; 1962; Paes *et al.*, 2013). Such features facilitate the uptake and adhesion of fungal spores over the whole body plant and favor the process of penetration and colonization by endophytes. A higher frequency of endophytic isolates was found by *Costus igneus* compared to three other medicinal species (Amirita *et al*., 2012). The genera *Aspergillus*, *Acremonium*, *Cladosporyum*, *Curvularia* and *Nigrospora* isolated from *C. igneus* were also found in the present study by *C. spicatus*. These findings could possibly indicate an intimate relationship between these fungi genera and the genus *Costus*.

The genera *Aspergillus* and *Penicillium* showed the highest frequency considering the three species studied. They occurred in all vegetative parts of *C. spicatus* and *S. cayennensis* showing no preference for a specific plant tissue. On the contrary, *Beuveria* sp. and *Pestalotia* sp. were isolated only from the leaves (Table 1).

For the species *C. spicatus* and *S. cayennensis* the fungal genera with the highest frequency were *Paecilomyces* and *Trichoderma*. Other studies about amazonian plants described the colonization by these species. Both endophytes were recovered from *Cladocolea micrantha* (Loranthaceae), a mistletoe growing over *Anarcardium occidentale* (Anacardiaceae). *Trichoderma pseudoviridae* was found in both parasitic and host plant (Guimarães *et al*., 2013). The diversity of endophytic fungi in stems of *Hevea brasiliensis* was evaluated obtaining results for *Trichoderma* that are similar to ours (Gazis and Chaverri, 2010).

The potentialities of the genus *Trichoderma*, include the production of xylanases from *Trichoderma harzianum* that help refining cellulose biobleaching and cellulose pulps (Kar *et al*., 2006) and biological control of plant pathogens by production of hydrolytic enzymes with antagonistic activity against *Crinipellis perniciosa*, the causal agent of witches' broom of cocoa (Marco *et al*., 2003). *Trichoderma* STC1 2.4, isolated from the stem of *Strychnos cogens* (Loganiaceae), showed antibiotic activity against *Escherichia coli* and was able to inhibit the growth of *Aspergillus flavus* (Souza *et al*., 2004).

The occurrence of other genera such as *Aspergillus*, *Fusarium*, *Pestalotia*, *Curvularia* as endophytes were also described in many studies (Azevedo *et al*., 2000; Gazis and Chaverri, 2010; Silva *et al*., 2006). The isolation of *Fusarium* sp. corroborates the results of other authors that indicated the endophytic colonization by this genus in *Cymbopogon citratus* (lemon grass) as well as the isolation of *Pestalotia* from *Vernonia condensata* (Mussi-Dias *et al*., 2012).

The presence of *Aspergillus* and *Fusarium* in these so called “medicinal plants” should be emphasized because of the production of mycotoxins and carcinogens that are indicative of health risk and by mostly stored foods can lead to death. Even by processing the plant material by dehydration and harvesting with the strictest care following the standards of quality, the presence of such fungi, even though invisible to the naked eye, can result in its further development and production of toxins (Bugno *et al*., 2006).

By *Ayapana triplinervis* (also known as *Eupatorium ayapana*) there was no reference to compare the diversity of fungal isolates or frequency of colonization. However, it was described by a correlated species, *Eupatorium arnottianum*, the presence of *Phomopsis* sp. which was able to produce phomopsidone, mellein and nectriapyrone (Meister *et al*., 2007).

Considering the overall fungi colonization on the three species by the analysis of variance (F Test), a significantly higher frequency of isolates was demonstrated by *C. spicatus* and *A. triplinervis*. To evaluate the colonization over different plant organs the Tukey’s Test (5% probability) was applied and a significantly higher endophytic colonization was found by leaves and roots of *C. spicatus*, followed by *A. triplinervis* stalks (Table 3). These findings are related to morphophysiological (presence of glandular trichomes and secretory canals) and environmental characteristics that differ among species. The differences between endophytic communities, both in species composition and abundance may occur due to the plant location, the tissue used for the isolation of endophytes and also the ecological environmental conditions around the plant (Gazis and Chaverri, 2010; Verma *et al*., 2007).

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