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Screening of *Piper hispidum* endophytic fungi that produce terpenes and antibacterial substances

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The plant species *Piper hispidum* has extensive economic potential due to the production of safrole, a component of its essential oil with proven antimicrobial and insecticidal activity. One strategy for obtaining bioactive compounds through extraction from plant species is by using endophytic microorganisms, since they can produce the same substances synthesized by the host. Therefore, this study aimed to isolate fungal endophytes from *P. hispidum* and verify their ability to produce terpenes and antimicrobial substances. Fifty-eight (58) endophytic fungi were investigated. In the metabolic media compounds with antimicrobial activity against *Staphylococcus aureus*, *Proteus vulgaris*, *Escherichia coli* and *Shigella sonnei* were detected; none of the fungi produced safrole. However, the results suggest the production of β-caryophyllene and terpinolene by three isolates. This study shows that investigation of fungal diversity associated with *P. hispidum* offers promising perspectives for biotechnology.

Key words: *Piperaceae*, Amazon fungi, high performance liquid chromatography (HPLC), essential oils, antibacterial activity.

INTRODUCTION

In plants, there are three major groups of secondary metabolites: phenolic compounds, alkaloids and terpenes. Terpenes are formed from mevalonic acid or from the reaction with pyruvate and glyceraldehyde 3-phosphate (Goodwin, 1964). In fungi, the induction for accumulation (or biosynthesis) of terpenes may occur through an alternative route to mevalonic acid, the methylerythritol phosphate pathway. This compound is derived from pyruvate and glyceradehyde 3-phosphate, originating from the degradation of glucose (Zhi-lin et al., 2007).

The components of essential oils, complex natural mixtures which can contain about 20 to 60 components at quite different concentrations, include two groups of distinct biosynthetical origin. The main group is composed of terpenes and terpenoids and the other of

aromatic and aliphatic constituents, all characterized by low molecular weight. These compounds have today an important role in several industries, since their use as raw materials has become indispensable for many products with high added value (Bakkali et al., 2008). These oils are used in cosmetics, perfumery and pharmacy, in the food industry, in disinfectants, soaps, plastics, paints, rubber and insecticides, among others (Robles and Garzino, 1998; Rozenbaum et al., 2006; Bakkali et al., 2008; Maia and Andrade, 2009). In particular, the antimicrobial activity has been considered the basis for various applications of essential oils including for food preservation and alternative pharmaceutical manufacturing (Bakkali et al., 2008).

The terpenes within the essential oils cover a wide variety of substances of vegetable origin and their ecological importance in the plant defense system is well established. They have also been widely reported to possess antimicrobial activity. The essential oil of *Satureja montana* L., rich in the monoterpene geraniol, has inhibited the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilis* (Cávar et al., 2008). Furthermore, many essential oils and com-pounds of these isolates have been recently recognized as powerful natural antioxidants, which could be used as replacements for synthetic antioxidants (Bozin et al., 2006). The monoterpene terpinolene, present in small amounts in the essential oils of *Pinus mugo*, *Manilla elemi*, *Nectandra elaiophora* and *Dacrydium colensoi*, among other species, effectively prevents the oxidation of low density lipoprotein (LDL) (Graßmann et al., 2005), shows antiviral activity, and acts against pathogenic fungi (Opdyke, 1976). The β-sesquiterpene caryophyllene has been associated with chemical communication between species. This volatile terpenoid is produced in maize roots when attacked by insects, attracting nematodes that parasitize the larvae of insects (Gershenzon and Dudareva, 2007). Moreover, β-caryophyllene has different interesting biological activities, including anticancer activity (Calixto, 2000), and has been found to be produced by microorganisms (Strobel et al., 2007; Strobel et al., 2011).

Piper hispidum L., or pepper jack, occurs naturally in the Amazon and has attracted great interest due to the production of essential oil rich in safrole, which has effective action in the control of pathogens such as fungi and bacteria, and proven anti-inflammatory and analgesic action with low toxicity (Maia and Andrade, 2009).

Over the last years, evidences have justified the interest on endophytic fungi, defined as those that live asymptomatically in the apoplastic spaces or within the plant cells, at least during a significant part of their life cycle (Petrini, 1991). These microorganisms can be ecologically important to their host, sometimes giving them support, and other times being the protagonists in fundamental processes of plant survival (Artursson et al.,

2006; Yue et al., 2000). Some endophytes can produce similar or identical biologically active constituents as the host (Kusari et al., 2014), such as taxol (Stierle et al. 1993). In addition, many fungal endophytes produce secondary metabolites and some of these compounds exhibit antibacterial activity that strongly inhibits the growth of other microorganisms (Gunatilaka, 2006, Tayung et al., 2012). Therefore, in this study endophytic fungi associated with *P. hispidum* were isolated and the metabolites produced by these microorganisms were analyzed, in order to identify fungi that may produce terpenes and compounds with antibacterial activity.

MATERIALS AND METHODS

Isolation of endophytes

The roots, stems and leaves of *P. hispidum* were collected in the urban area of Manaus (Specimen 1 was located at 3°3'4.42"S and 60°4'0.32"W; Specimen 2 was located at 3°6'17.31"S and 59°59'27.57"W), during the raining season, for the isolation of the endophytes. The surface of plant fragments of *P. hispidum* was disinfected in 70% ethanol for 2 min, 2% sodium hypochlorite for 2.5 min, 70% ethanol for 1 min, and finally washed with sterile distilled water for 3 min (Banhos et al., 2014). In this specific situation, the water was plated and incubated at 26°C as a control of sterilization procedure. The tissues were then cut into fragments of approximately 0.5 cm and transferred to dishes containing PDA (potato-dextrose-agar) media (Himedia, India) supplemented with tetracycline. The plates were incubated at 28°C for 7 days. The streaking purification technique was used to obtain isolated colonies. After allowing the isolates to grow, the frequency of isolation in the different tissues was calculated, expressed as colonization rate (CR), using the relation:

$$CR = \frac{number\ of\ plant\ fragments\ with\ fungal\ growth}{total\ number\ of\ plant\ fragments}$$

The endophytic fungi isolated from *P. hispidum* were stored using the method proposed by Castellani (1939). All isolated endophytic fungi were deposited into the Collection of the Laboratory of the School of Health Sciences, State University of Amazonas (ESA/UEA).

Obtainment of secondary metabolites

Five millimeter diameter discs of the PDA media (Himedia, India) containing fungi mycelia (previously grown on PDA for 7 days at 28°C) were transferred into 150 mL Erlenmeyer flasks with 50 mL of PD (potato-dextrose) liquid media supplemented with 0.2% yeast extract under sterile conditions, according to the method described by Banhos et al. (2014) with modifications. The flasks were incubated without agitation for 14 days at a temperature of 28°C. The culture broth was then vacuum filtered to separate the mycelia. The filtered metabolic broth was analyzed to verify the production of terpenes and antibacterial activity.

Evaluation of the production of essential oils

The metabolic broth was filtered through a 45 μm membrane (Millipore, Brazil) and analyzed by HPLC (Varian® ProStar 310)
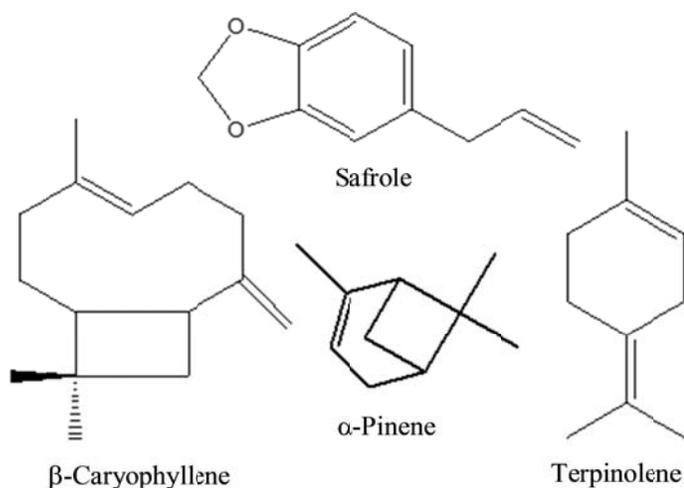
Figure 1. Chemical structure of safrole and the terpenes β-caryophyllene, α-pinene and terpinolene, present in the metabolites of endophytes from *Piper hispidum*.

Table 1. Antibiotic susceptibility presented by different bacterial strains used in this work.

Bacterial species	Antibiotic
Staphylococcus aureus	VAN (MIC < 4 mg/L)
Escherichia coli	CHL / CAR (MIC < 4 mg/L)
Shigella sonnei	STR (MIC < 4 mg/L)
Proteus vulgaris	CAR / STR (MIC < 4 mg/L)

VAN, vancomycin; CH, chloramphenicol; CAR, carbenicilin; STR, streptomycin; GEN, gentamicin; MIC, minimum inhibitory concentration.

using a C18 column (250 x 4.6 mm), UV-Vis detector at 257 nm and a acetonitrile/water gradient as the eluent (up to 5 min H_2O/ACN 50:50; from 5 to 35 min 100% ACN). The injections were performed in triplicate. The chromatograms obtained for the metabolic broths were compared with the chromatograms obtained for standard compounds: β-caryophyllene (98%, Aldrich, United States), α-pinene (98%, Fluka, Germany); safrole (97%, Sigma, United States) and terpinolene (85%, Fluka, Germany). These compounds have been reported as main constituents of the essential oil of *P. hispidum*, and its chemical structures are shown in Figure 1.

Antimicrobial activity assay

The metabolic broths were used for *in vitro* antibacterial tests against pathogenic bacteria. Strains of *Staphylococcus aureus*, *Escherichia coli* (O157: H7), *Proteus vulgaris* and *Shigella sonnei* were kindly provided by the Tropical Virology Laboratory of the National Institute for Amazonian Research - INPA. Bacterial cultures were kept in nutrient agar at 36°C. The bacterial strains were previously tested regarding its antibiotic susceptibility (NCCLS, 2007), as showed in Table 1.

A bacterial suspension was obtained in saline solution (6 $\times 10^8$ CFU/mL) and with the aid of a swab, the suspension was inoculated on Mueller Hinton agar (MHA, Himedia, Mumbai, India)

over the whole plaque. Small wells with 0.5 cm of diameter were made in each plate in order to add 100 µL of the metabolic broth. For the negative controls, the wells were filled with potato dextrose broth and tested against the bacterial strains. All assays were performed in triplicate, and results were considered consistent only when the three replicates presented the same result.

The plates were incubated aerobically for 18-24 h at a temperature of 35-37°C. The metabolic broths which indicated positivity for the inhibition of any of the test microorganisms were analyzed by high performance liquid chromatography (HPLC) in order to compare their chromatographic profile with that of a commercial antibiotic (Amoxicillin). A Varian ® ProStar 310 chromatograph was used, equipped with a C18 column (250 x 4.6 mm) and UV-Vis detector at 257 nm, and acetonitrile/sodium phosphate buffer 25 mM pH 3.0 (20:80) was employed as the eluent. The injections were performed in triplicate.

RESULTS AND DISCUSSION

Isolation of endophytes from *P. hispidum*

From the two specimens of *P. hispidum*, 120 endophytic fungi were isolated using 25 fragments of each plant tissue (roots, stems and leaves). After separation of the isolates into nine morphological groups (Barnett and Hunter, 1972), 58 isolates were selected for the subsequent stages, which represent the macro-morphological diversity of the endophytic fungi isolated from *P. hispidum*. The growth of a greater number of endophytes from the leaves of each *P. hispidum* specimen was noted, as shown in Table 2.

Wilson (1996) suggests that there are two major possibilities for fungal dissemination within the host plant: vertical transfer, when fungi are transmitted through the seeds, and horizontal transfer, when fungal colonies are passed from plant to plant, via spores. Considering the horizontal dissemination of fungi and the milder asepsis performed on leaves when compared with roots and stems, it is possible to understand the obtainment of a greater number of fungal isolates from the leaves of *P. hispidum*. Moreover, the stem and stalk usually present a greater resistance to changes in the natural plant habitat, thus offering a less favorable environment for the flux of microorganisms in these parts of the plant.

Colonization rates (CRs) were higher than 0.5 for all plant parts (Table 2), demonstrating that the technique used for the isolation of endophytic fungi was appropriate, enabling the acquirement of a significant number of fungal isolates from the two specimens of *P. hispidum*. Fungi were present in all cultivated fragments of specimen 2 leaves (CR = 1.00). For the stems and roots, the CR values remained close to those of specimen 1.

Specimens 1 and 2 had CR values of 0.81, and 0.79, respectively, indicating that all fragments inoculated from these specimens only 19 and 21%, respectively, showed no fungal growth. This finding may be related to the diversity of interactions of the specimens of *P. hispidum* with other plants in the region where they are grown,

Table 2. Number of isolated and selected fungi, and the colonization rate (CR) of *P. hispidum* specimens.

Sample	Code[a]	Isolated fungi	Selected fungi	Colonization rate
	PH-L	22	13	0.88
Specimen 1	PH-S	18	8	0.72
	PH-R	19	6	0.76
Total		59	27	0.79
	PH-L	25	14	1.00
Specimen 2	PH-S	21	10	0.84
	PH-R	15	7	0.60
Total		61	31	0.81

[a]Identification code for the isolates consists of the initials of the plant, PH, followed by the letter that represents the tissue from which it was removed (L - leaf, S - stem, and R - root).

Table 3. Retention times obtained for the major peaks of commercial standards analyzed by reverse phase HPLC.

Standard	Commercial source	Retention time[a] (min)
β-Caryophyllene	Aldrich, 98%	14.28
α-Pinene	Fluka, 98%	9.05
Safrole	Sigma, 97%	13.11
Terpinolene	Fluka, 85%	14.38

[a]Retention times obtained on a Varian® ProStar 310 chromatograph with C18 column (250 x 4.6 mm), UV-Vis detector at 257 nm and acetonitrile/water gradient as eluent (up to 5 min H_2O/ACN 50:50; from 5 to 35 min 100% ACN).

since vegetation type, plant age, and seasonal difference can change the diversity and abundance of endophytes (Yang and Dai, 2013).

The factors affecting endophyte community structure have been explored in many researches. Arnold and Lutzoni (2007) reported that the diversity of endophytes at both the individual and plant community levels increased with decreasing latitude (from poles to equator). Furthermore, they also found that endophytes isolated within a specific biogeographic zone (arctic, temperate or tropical) were often absent from other zones. According to Baynes et al. (2012), at the local level, other factors are operative, such as water availability, temperature, agricultural chemicals, and plant metabolites. Pimentel et al. (2006) found qualitative and quantitative differences in the type and number of soybean isolates obtained from greenhouse and field-grown plants, with more isolates being obtained from the latter. Saunders and Kohn (2009) demonstrated that production of plant defense compounds influenced the endophyte community within maize, and variable leaf chemistry can explained differences in endophyte communities among host species (Arnold and Herre, 2003).

In a recent study, Yang and Dai (2013) investigated interactions between endophytic fungi infecting the same host, *Atractylodes lancea*, an essential oil producer. The authors verified that the levels and components of essential oils affect the growth of endophytes, and high concentrations of these metabolites suppress the growth of endophytes, which have the capacity to degrade and biotransform essential oils.

Production of essential oil compounds by *P. hispidum* endophytes

In general, it could be noted that the chromatographic profiles obtained for the metabolic broths present two groups of molecules of different polarities, since reverse phase HPLC was used as analytical method. The first group consists of polar molecules, which have shorter retention times, (0 to 5 min). The second group contains less polar molecules, with retention times of between 10 and 20 min.

When submitted to chromatographic analysis, the commercial standards of safrole, β-caryophyllene, α-pinene and terpinolene presented major peaks with the
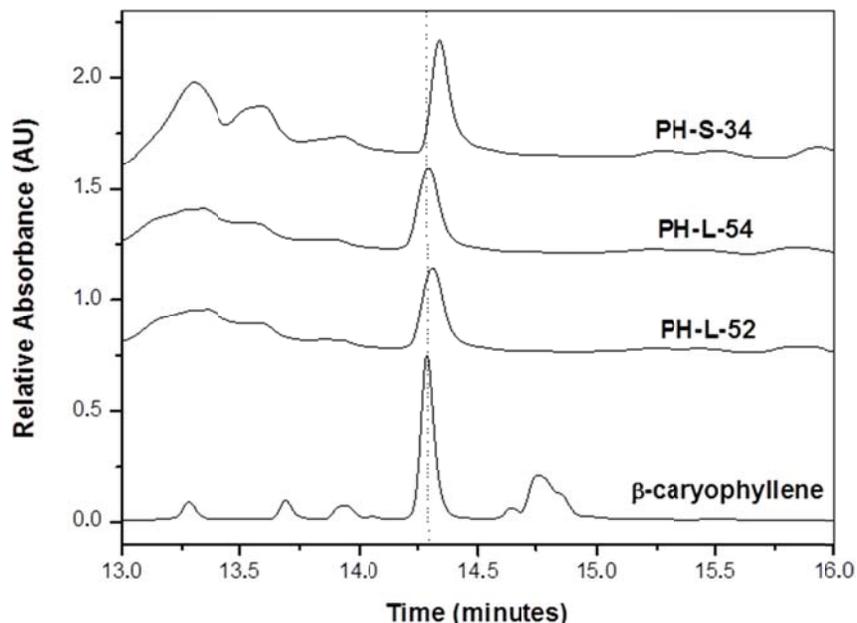
Figure 2. Chromatograms of β-caryophyllene, and the metabolic broths of endophytes PH-L 54, PH-L 52 and PH-S 34 isolated from *Piper hispidum.*

retention times shown in Table 3.

Safrole is a natural allylbenzene of wide distribution in the plant kingdom. The major component in the essential oil of *P. hispidum* (Wadt et al., 2004; Oliveira et al., 2007) had a characteristic peak at a retention time of 13.11 min. Surprisingly, the presence of safrole was not observed within the metabolic broth of the endophytic fungi selected in this study.

α-Pinene, found at a concentration of 10.2% in *P. hispidum* (Bottia et al. 2007), showed a major peak at 9.05 min. In the chromatogram obtained for the metabolites of the isolated endophytes, any peak with this retention time was not observed. Therefore, the isolates investigated in this study do not produce this terpene.

β-Caryophyllene is present at low concentrations in the essential oil of *P. hispidum* (Bottia et al., 2007). As can be seen in Figure 2, the terpene standard showed a major peak in the chromatogram at 14.28 min.

It can be noted in the chromatograms of Figure 2 that the metabolic media of the isolated endophytes PH-L 54, PH-L 52 and PH-S 34 show peaks centered at 14.29, 14.31 and 14.33 min, respectively. The retention times of all peaks are close to that observed for β-caryophyllene (14.28 min). These results suggest that there is the possibility that the isolated endophytes PH-L 54, PH-L 52 and PH-S 34 produce β-caryophyllene. However, due to the proximity of the retention times, PH-L 54 was identified as the prime candidate for the production of β-caryophyllene. The other fungal metabolic broth analyzed by HPLC showed no peaks with retention times similar to those of the spikes of β-caryophyllene.

Terpinolene has been consistently isolated from plants of the genus *Piper*. However, it is found in low concentrations (1.2%) in plants of the species *P. hispidum* (Bottia et al., 2007). The metabolic broth of the strain PH-L 52 showed a peak with a retention time similar to the major peak of terpinolene, as can be seen in the chromatograms of Figure 3. The presence of peaks centered at 14.29, 14.32 and 14.37 min can be noted in the chromatograms of the metabolic broths of PH-L 54, PH-L 52 and PH-S 34, respectively, which are very close to the major peak in the chromatogram of the terpinolene standard (14.38 min). Thus, there is the possibility that PH-L 54, PH-L 52 and PH-S 34 produce terpinolene. However, PH-S 34 was considered to be the most promising candidate for the production of terpene, since its metabolic broth shows a peak with a retention time closest to the peak of terpinolene. The other fungal extracts analyzed by HPLC showed no peaks with retention times similar to that of terpinolene. Thus, only these three strains could produce this terpene as a secondary metabolite.

The production of terpenes by endophytic fungi has been widely explored. Souza et al. (2011) reviewed the production of terpenoids by endophytic fungi and their biological activities, in the period of 2006 to 2010. Sixty five sesquiterpenes, 45 diterpenes, five meroterpenes and 12 other terpenes, amounting to 127 terpenoids were isolated from endophytic fungi.

Strobel et al. (2011) reported that a *Phoma* sp. isolated and characterized as endophytic and as a pathogen of *Larrea tridentata* produces a mixture of volatile organic compounds (VOCs), including a series of sesquiterpenoids.
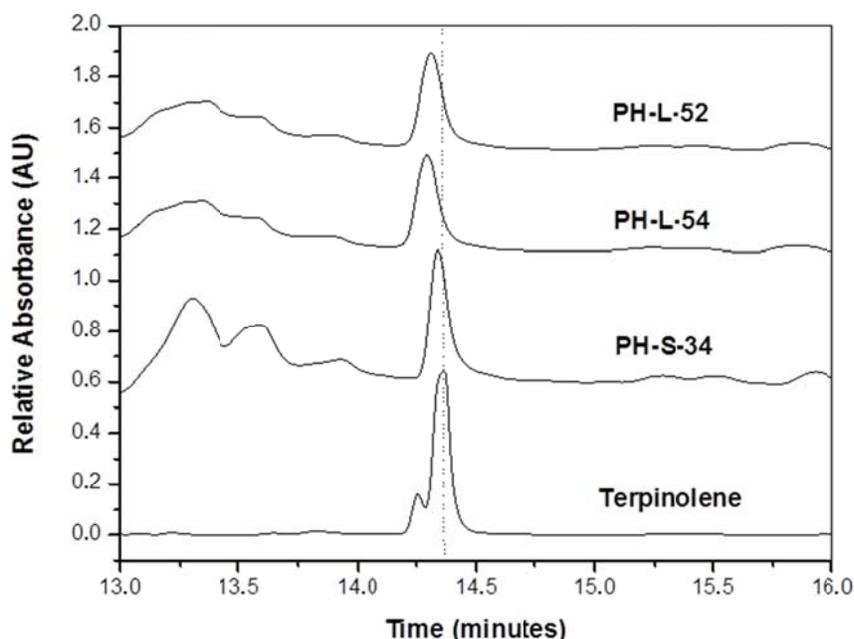
Figure 3. Chromatograms of terpinolene and the metabolic broths of endophytes PH-L 54, PH-L 52 and PH-S 34 isolated from *Piper hispidum.*

Trans-caryophyllene, a product in the fungal VOCs, was also noted in the VOCs of the host plant. Besides, the authors verified that the gases of *Phoma* sp. possess antifungal properties and is markedly similar to that of a methanolic extract of the host plant. The terpene caryophyllene was also produced by the fungus *Muscodor albus* E-6, an endophyte of *Guazuma ulmifolia* (Strobel et al., 2007).

The production of terpenes was also reported for an endophytic fungus isolated from *P. aduncum*. Silva et al. (2010) isolated two new presilphiperfolane sesquiterpenes from the ethyl acetate extract of *Xylaria* sp., obtained from the leaves of *P. aduncum*, along with two known eremophilane sesquiterpenes, phaseolinone and phomenone, which displayed cytotoxic and antifungal activities.

Production of antimicrobial compounds

The metabolic media of endophytic fungi isolated from *P. hispidum* were used in the tests for *in vitro* antagonism against pathogenic bacteria. Of the total number of metabolic media tested, 15 (25.9%) had antagonistic activity against one or more test bacteria, as can be observed in the data in Table 4.

The metabolic media of fungi PH-S 34 and PH-R 14, isolated from the stems and roots of *P. hispidum*, respectively, showed positive results against the test bacteria *S. aureus*. This Gram-positive bacterium belongs to the family *Staphylococcaceae* and is a multidrug-resistant

pathogen that not only causes a diverse array of human diseases, but also is able to survive in potentially dry and stressful environments, such as the human nose, on skin and on inanimate surfaces such as clothing and surfaces (Chaibenjawong and Foster, 2011). The fungal isolate PH-S 34, which produce compounds with activity against this pathogen, proved to be a potential producer of β-caryophyllene (Figure 2) and terpinolene (Figure 3).

The possibility that this endophyte isolate produces secondary metabolites similar to those synthesized by its host, as observed in this study, contributes to understanding the endophyte-host relationship, where the fungus, by producing compounds with antibacterial activity, may help to protect the plant against pathogens (Strobel et al., 2011).

Eight endophytic isolates of *P. hispidum* (PH-L 41, PH-L 44, PH-L 52, PH-L 54, PH-S 23, PH-S 24, PH-R 13 and PH-R 20) produced secondary metabolites that were active in the inhibition of *P. vulgaris*. This pathogen is a Gram-negative bacterium which inhabits the intestinal tract of humans and animals and can also be found in soil, water and fecal matter. Grouped with the *Enterobacteriaceae*, is an opportunistic bacterium of humans, known to cause urinary tract infections (Pelczar et al., 1993). Of the fungal isolates that produce compounds against this pathogen, four were isolated from the leaves, two from the stems and two from the roots. Among the *P. hispidum* leaf isolates which produced metabolites with activity against *P. vulgaris* are PH-L 52 and PH-L 54, possible producers of β-caryophyllene and terpinolene (Figures 2 and 3,

Table 4. Antibacterial activity of metabolic media from endophytic fungi isolated from *P. hispidum* against the bacteria pathogens. Experiments were performed in triplicate.

Endophytic isolates	Pathogenic bacteria			
	S. aureus	*P. vulgaris*	*E. coli*	*S. sonnei*
PH-L 41	-	+	-	-
PH-L 44	-	+	-	-
PH-L 51	-	-	+	-
PH-L 52	-	+	-	+
PH-L 54	-	+	-	+
PH-S 23	-	+	-	-
PH-S 24	-	+	-	+
PH-S 28	-	-	+	-
PH-S 30	-	-	-	+
PH-S 33	-	-	+	-
PH-S 34	+	-	-	+
PH-R 13	-	+	-	-
PH-R 14	+	-	-	+
PH-R 18	-	-	-	+
PH-R 20	-	+	-	-

(+) = Presence of a growth inhibition zone; (-) = no zone of inhibition of bacterial growth.

respectively). Similar results were observed in a study carried out by Cávar et al. (2008) who verified the antimicrobial activity against *S. aureus* of the essential oil of *Satureja montana*, composed of the terpenes geraniol (22.3%), carvacrol (10.6%), terpinen-4-ol (10.3%), caryophyllene oxide (5.2%), spatulenol (3.1%), β-caryophyllene (2.9%), among others. In 2006, Silva et al. reported the isolation of five cadinane sesquiterpene derivatives obtained from *Phomopsis cassiae*, an endophytic fungus isolated from the endemic Brazilian plant *Cassia spectabilis*. The authors found that these sesquiterpenes present remarkable antibacterial and antifungal activities. Therefore, as observed in different studies, terpenes have excellent potential for use as antimicrobial agents, whether produced by the plants themselves or by their endophytes.

There were three endophytic isolates from *P. hispidum* (PH-L 51, PH-S 28 and PH-S 33) that produced metabolites capable of inhibiting the growth of *E. coli*, a Gram-negative bacterium. This has been described as one of the oldest human symbiotic bacteria responsible for serious intestinal infections (Pelczar et al., 1993). Among the isolates that produced compounds with activity against *E. coli,* one was isolated from the leaves and two from the stems of *P. hispidum.* Xing et al. (2011) obtained similar results in a study where they isolated endophytic fungi from *Dendrobium devonianum* and *Dendrobium thyrsiflorum.* The authors verified that more

fungi isolated from the stems presented antimicrobial activity when compared with the roots. However, the isolates from roots were more effective against the pathogenic microorganisms. According to Xing et al. (2011), the reason for this contrast is the different environments within the parts of the plant, which influences the distributions and colonization of fungal endophytes.

The *in vitro* antagonist tests revealed that seven of the fungal isolates obtained from *P. hispidum* (PH-L 52, PH-L 54, PH-S 24, PH-S 30, PH-S 34, PH-R 14 and PH-R 18) produced compounds with inhibitory activity against the Gram-negative bacterium *S. sonnei*, the most common causative agent of shigellosis. This disease is spread by fecal-oral route, has high infectivity and is characterized by causing bloody diarrhea, often accompanied by abdominal pain (Pelczar et al., 1993). Knowledge of the epidemiology and molecular mechanisms of antimicrobial resistance in this important pathogen is essential for the implementation of intervention strategies. Ke et al. (2011) verified that in *Shigella* species, antimicrobial resistance is often associated with the presence of integrons that contain resistance gene cassettes. The structures and functions of integrons in *Shigella* species help to describe mechanisms that control integron-mediated events linked to antibiotic resistance. Among the isolates of *P. hispidum* which produced compounds with activity against *S. sonnei*, two were isolated from the leaves,
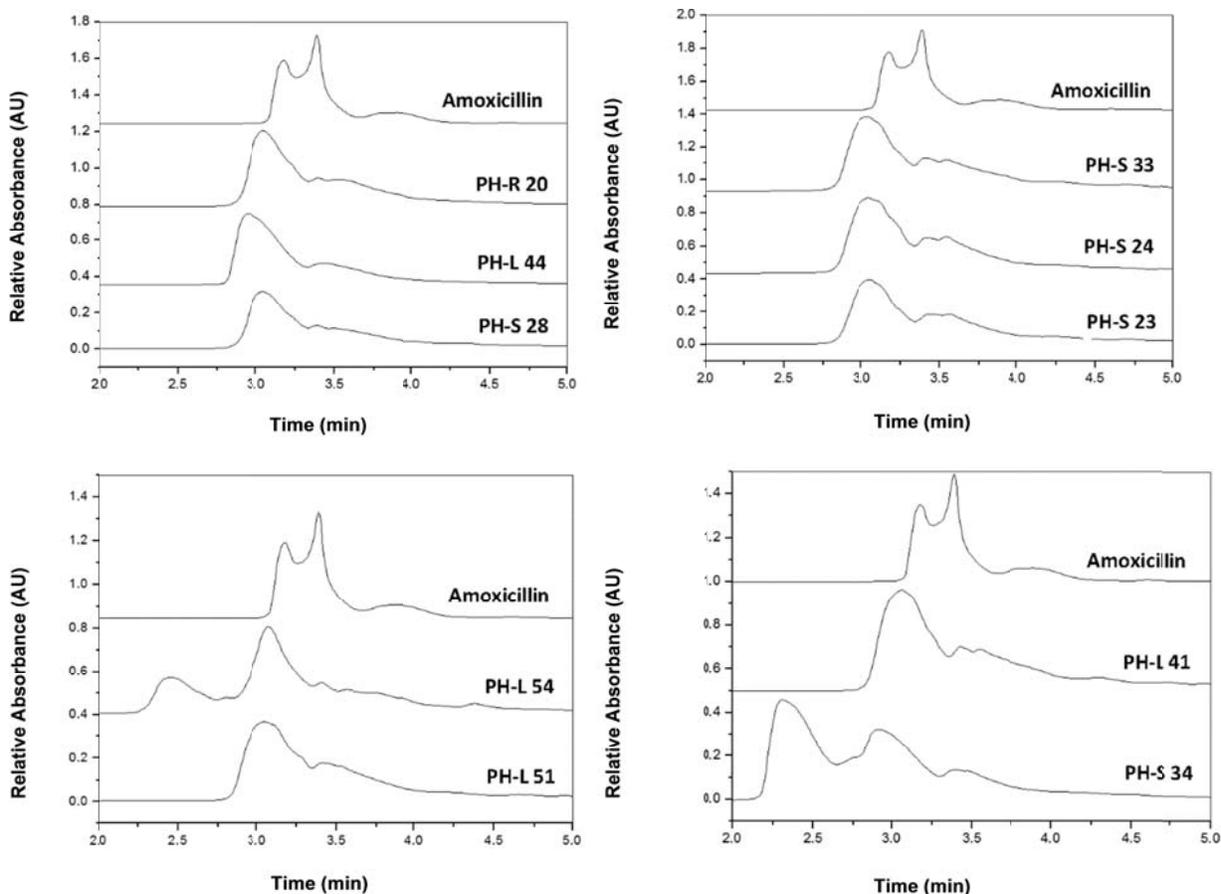
Figure 4. Chromatograms of commercial amoxicillin and metabolic broths of endophytic isolates from *Piper hispidum* that showed antibacterial activity.

three from the stems and two from the roots. Among the isolates obtained from the leaves, as was observed in tests against *P. vulgaris*, are the fungi PH-L 52 and PH-L 54, possible producers of β-caryophyllene and terpinolene (Figures 2 and 3, respectively). Among the isolates from the stem, it can be noted that once again the metabolites of fungus PH-S 34 showed antibacterial activity, as previously observed against the pathogen *S. aureus*. The fact that this endophyte produces compounds with activity against a Gram-positive bacterium and others against Gram-negative bacteria confirms its potential as a producer of secondary metabolites of commercial interest. The Gram-positive and negative bacteria do not differ only with regard to the structure of the cell wall, but also due to the presence of polysaccharides and lipoproteins in Gram-negative bacteria that form a barrier to hydrophobic compounds, which are fundamental aspects for antibiotic action (Mazutti et al., 2008).

From the biological point of view, the production of a particular metabolite can be related to different mechanisms involved in the interaction between a microorganism and its habitat, for example, competition for a niche when a microorganism occupies the same space as the other and competition for nutrients, or it may simply be a product of their metabolism (Li et al., 2007). These results reinforce the idea that the endophytes play a crucial role in the mechanisms of plant protection against pathogens.

Chromatographic profiles of metabolites of endophytic fungi

The 15 extracts of endophytic isolates of *P. hispidum* that provided positive results in the *in vitro* antagonism tests against pathogenic bacteria were analyzed by HPLC to compare their chromatographic profiles with that obtained for a commercial antibiotic (amoxicillin). Ten metabolic broths containing fungal metabolites produced by the isolates PH-L 41, PH-L 44, PH-L 51, PH-L 54, PH-S 23, PH-S 24, PH-S 28, PH-S 33, PH-S 34 and PH-R 20 presented molecules with similar chemical characteristics of the commercial amoxicillin, as can be seen in the chromatograms of Figure 4.

From the analysis of the chromatographic profiles of
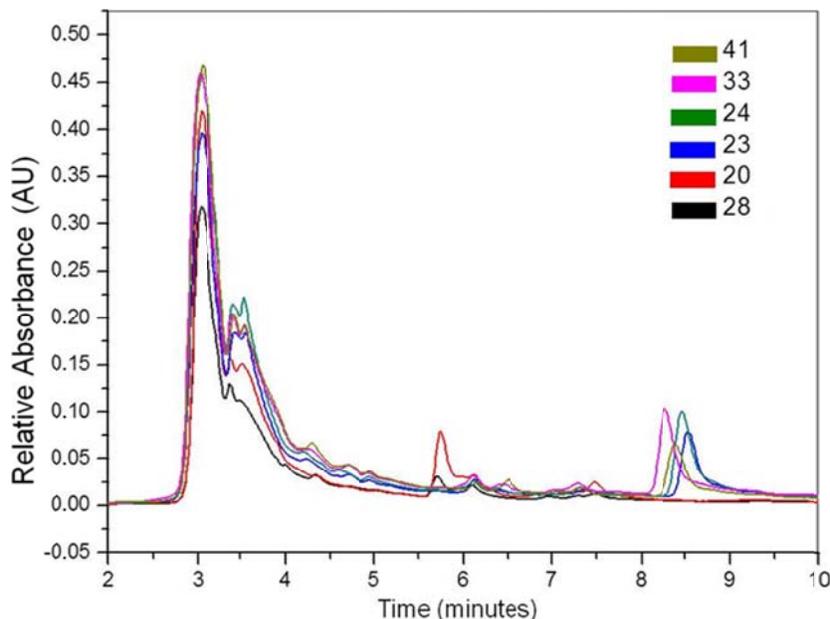
Figure 5. Overlaying chromatograms of the metabolic media of endophytes isolated from *Piper hispidum* that showed antibacterial activity.

Figure 4, it is possible to infer that these endophytic fungi isolated from *P. hispidum* produce compounds that are chemically similar to the commercial antibiotic, since the peaks in the chromatograms obtained appear in the same region. The similarity between the chemical compounds present in fungal metabolites with antibacterial activity and the commercial antibiotic suggests that the mechanism of action of substances produced by these endophytes could be the same as that of amoxicillin, that is, inhibition of the biosynthesis of cell wall proteins (Mikell et al., 2011). It can also be noted in Figure 4 that the chromatographic profile of metabolites from the isolate PH-S 34, which showed activity against Gram-negative and Gram-positive bacteria, indicates the presence of at least five compounds that may act synergistically, or specifically on the bacterial cell wall components.

On analyzing the chromatographic profiles of the metabolic broths of the 15 endophytic isolates from *P. hispidum* that showed antibacterial activity, the production of the same substance was found for the isolates PH-L 41, PH-S 23, PH-S 24, PH-S 28, PH-S 33 and PH-R 20.

The overlaying of the chromatograms of the isolate metabolites is shown in Figure 5. It can be noted that six metabolic broths have the same peak with a retention time of 3.05 min, in the same region of the chromatogram where the peaks were observed for the commercial amoxicillin.

Considering the results for the antibacterial activity of these isolates, and the chemical characteristics being similar to those of amoxicillin, it could be that this molecule

with a peak at 3.05 min may be the responsible for the antimicrobial activity of these isolates.

Conclusions

The endophytic fungi isolated from *P. hispidum* exhibit potential for the acquirement of interesting compounds. The HPLC results suggest that three fungal isolates may produce β-caryophyllene and terpinolene. However, it is necessary to obtain confirmation of the production of these molecules using specific techniques such as mass spectrometry and nuclear magnetic resonance.

Antagonist tests confirmed the production of antimicrobial compounds by endophytic fungi from *P. hispidum* with action against the tested pathogen strains (*S. aureus*, *S. sonnei*, *E. coli* and *P. vulgaris*). Comparison of the chromatographic profiles for the fungal metabolites with antimicrobial activity with that for the commercial antibiotic amoxicillin suggests that these fungi produce molecules with chemical structures similar to that of the drug.

Finally, these results indicate a complex interaction between endophytic microbes and their host, with the production of secondary metabolites with proven antibacterial activity, and that the endophyte may be helping the plant to defend itself against different pathogens.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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