Effects of inoculation by arbuscular mycorrhizal fungi on the composition of the essential oil, plant growth, and lipoxygenase activity of *Piper aduncum* L.

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Abstract

The aim of this study was to evaluate the changes in the production of secondary metabolites. *Piper aduncum* seedlings were inoculated by spores of the arbuscular mycorrhizal fungi (AMF) *Rhizophagus clarus* and *Claroideoglomus etunicatum*. *P. aduncum* seedlings were inoculated by spores of *R. clarus* and *C. etunicatum* and then, development parameters, root colonization, lipoxygenase (LOX) activity, and essential oil (OE) chemical composition were monitored at 30, 60 and 90 days’ post-inoculation (dpi). The inoculation had influenced the plant height and root length at 30 and 90 dpi and microscopic analysis of roots showed the presence of hyphae, arbuscules and vesicles in the inoculated plants. Phenylpropanoids and sesquiterpene hydrocarbons were the main compounds in the EO. In the leaves, the concentration of phenylpropanoids showed a decrease, mainly at 60 dpi, with increased sesquiterpene hydrocarbon production. The main compounds were dillapiole, myristicin, and germacrene D; the dillapiole concentration decreased in all treatments. LOX activity had an increase in the leaves and roots at 90 dpi. These results suggest that alterations in the secondary metabolites of *P. aduncum* can be induced by its mechanisms of resistance during AMF interaction.

Keywords: Arbuscular mycorrhizal fungi, Volatile compounds, Dillapiole, Lipoxygenase, Secondary metabolites

Introduction

*Piperaceae* have wide distributions in tropical and subtropical regions, and are known as a pantropical family with approximately 2700 species mainly of the genus *Piper* (The Plant List 2013). *Piper aduncum* L. is a bush native to tropical regions of the Americas, but it was introduced to Asia during the nineteenth century (Hartemink 2001; Yuncker 1972). In the Amazon region, it is commonly known as “pimenta-de-macaco”, and used in popular medicine to treat intestinal apathy and stomach problems (Sousa et al. 2008). In addition, the *P. aduncum* essential oil (EO) has demonstrated several biological properties, such as antifungal (Guerrini et al. 2009), antimicrobial (Kloucek et al. 2005), insecticidal (Misni et al. 2011), and larvicidal (Almeida et al. 2009) activities. These biological properties can be attributed to its main compound, dillapiole, a phenylpropanoid which presents in amounts of 31.5 to 91.1% (Maia et al. 1998).

The biosynthesis of secondary metabolites in medicinal and aromatic plants depends on genetic, physiological, and environmental factors (Freitas et al. 2004). Among these factors, the symbiotic association of plants by root colonization by arbuscular mycorrhizal fungi (AMF) can produce a difference in its biosynthesis of secondary metabolites (Carlsen et al. 2008). AMF belongs to the *Glomeromycota phylum* and the *Acaulospora, Entrophospora, Gigaspora, Glomus, Sclerocystis* and *Scutellispora* genera (Oehl et al. 2011). AMFs have shown associations...
with about 80% of ground plants in natural ecosystems and cultivated agroecosystems, varying the colonization level according to plant genotype (Bonfante and Genre 2010; Smith and Read 2008).

The plants colonized by AMFs are more tolerant to low availability of water in the soil, making more efficient use of the absorbed water. In addition, they improve the plant nutrition, development, and the content of the essential oils, due to changes in the biosynthesis of secondary metabolites (Al-karaki et al. 2004; Nell et al. 2010). Thus, the aim of this study was to evaluate the changes in the production of secondary metabolites during the association of *P. aduncum* with AMFs.

**Materials and methods**

**Plant material and cultivation**

*P. aduncum* was collected in Belém/PA, Brazil, and a voucher specimen was deposited under register MG 218522 in the Emílio Goeldi Museum herbarium, city of Belém, Pará, Brazil. Cuttings containing 1 to 2 nodes were propagated and conditioned in vermiculite expanded type B substrate (Urimuma Mineração Ltda, Santa Maria da Boa Vista, Brazil), and kept in a greenhouse under 70% shading. The commercial nutrient solution (Biofert Root) was applied to promote root development and reapplied after 15 days, and the cuttings were moistened daily. After 21 days, the roots had developed, and seedlings were transplanted into polypropylene bags of approximately 9 cm in diameter, on a commercial substrate containing a mixture of limestone, castor oil, bone meal, and expanded vermiculite type B.

**Multiplication of AMF spores and production of fungal inoculant**

AMF spores (*Rhizophagus clarus* and *Claroideoglomus etunicatum*) were obtained from rhizosphere soil samples from the southeast Pará State, Amazon region (Brazil). They were multiplied in a greenhouse in sterile sand, using *Brachiaria brizantha* as trap culture (Da Luz et al. 2016). The identification of species was realized by morphological comparison based in the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM 1992). Inoculates, with the proportion of 50% each fungal species, composed of a mixture of spores (density of 90 spores/g soil), hyphae, root fragments and sterile sand, were used during the inoculation. Holes with approximately 2 cm deep were opened and the 6 g of inoculum was surface-spread on the roots. Non-inoculated seedlings were used as the control group.

**Experimental design**

Experimental design was performed in completely randomized blocks. Each group was composed of 10 plants, which were labeled as control (non-inoculated) and AMF (inoculated by AMFs). Roots and leaves were collected at 30, 60 and 90 days post inoculation (dpi) to monitor the mycorrhizal colonization, plant development, secondary metabolites, and LOX activity. All analyzes were performed in biological triplicates.

**Mycorrhizal colonization in *P. aduncum* roots**

For the visualization of mycorrhizal colonization, usual techniques in plant anatomy were employed (Kraus and Arduin 1997). Root fragments of approximately 1 cm were fixed during 24 h in glutaraldehyde 1% in 0.1 M phosphate buffer, pH 7.2 (according to Karnovsky 1965, with modifications). Afterward, the samples were dehydrated with a series of butyl alcohol treatments and then encased in histological paraffin (Johansen 1940). Longitudinal sections (12 μm thick) were obtained using an automatic microtome (Leica® RM 2245, Nussloch, Germany), the sections were stained with safranine and astra blue (Gerlach 1969), and mounted in Entellan® synthetic resin (Merck, Darmstadt, Germany). Photomicrographs were obtained using in Canon digital camera (model A65015), coupled to a Zeiss microscope (model 426126.).

**Plant development evaluation**

The developmental parameters evaluated were: plant height (cm), number of leaves, plant basal stem (mm), number of nodes, root length (cm), and the fresh mass of leaves and roots (g) for each plant per replicate. The fresh leaf biomass production was based in the total weight per plant and the fresh root biomass in the total weight of the roots per plant.

**Extraction and analysis of the essential oils**

The essential oil fractions from fresh leaves and roots (2.0 g) of *P. aduncum* were obtained by simultaneous distillation–extraction process using a Likens-Nickerson apparatus for 2 h and *n*-pentane (3 mL) as solvent. After extraction, an aliquot (1.0 μL) of the organic phase was analyzed by gas chromatography. Qualitative analysis was carried out on a GC–MS (Shimadzu QP 2010 plus instrument) under the following conditions: Rtx-5MS silica capillary column (30 m × 0.25 mm × 0.25 mm film thickness); programmed temperature, 60–240 °C (3 °C/ min); injector temperature, 200 °C; carrier gas, helium, adjusted to a linear velocity of 1.2 mL/min; injection type, splitless; split flow was adjusted to yield a 20:1 ratio; septum sweep was a constant 10 mL/min; EIMS, electron energy, 70 eV; temperature of the ion source and connection parts, 200 °C. The retention index was calculated for all the volatile constituents using a homologous series of *n*-alkanes (C8–C32, Sigma-Aldrich) (Van Den Dool and Kratz 1966). The identification of compounds was
performed by comparison of mass spectrum and retention index with data present in the libraries of Adams (2007) and NIST (2011).

In vitro lipoygenase (LOX) activity
The substrate was prepared using 78 μL of linoleic acid (Sigma-Aldrich, USA) and 90 μL Tween 20 (Sigma-Aldrich), mixed with 10 mL of boiling water and a few drops of sodium hydroxide (0.5 N). The final volume was adjusted to 25 mL, resulting in a sodium linoleate solution (10 mM), which was stored at −20 °C. The LOX activity determination was carried out with 5 μL of crude leaf extract and 50 μL of sodium linoleate (10 mM), mixed with 1950 μL of sodium phosphate buffer (50 mM) at pH 6.5. The absorbance at 234 nm for the reaction was monitored for 60 s, using a UV–Visible spectrophotometer (Meireles et al. 2016).

Statistical analysis
All analyses were compared with the control group and the data were expressed as mean ± standard deviation. Analyses of variance were conducted using GraphPad 6.0, followed Bonferroni tests whenever appropriate. Differences at p < 0.05 were considered statistically significant.

Results
Monitoring of colonization of P. aduncum roots by AMFs
Histological sections of P. aduncum roots inoculated by AMF revealed evidence of the presence of mycorhizal structures such as hyphae, arbuscules, and vesicles, which were absent in the control plants (Fig. 1a). At 30 days post inoculation (dpi), the cortex was colonized, and the presence of penetration apparatus composed by hyphopodium and hyphae (Fig. 1b) were observed. At 60 dpi, an intense colonization was observed in the radicular hyphopodium and hyphae (Fig. 1b). At 90 dpi, the colonization showed completely established due to the presence of several hyphae, arbuscles and vesicles (Fig. 1d–f). In addition, hyphatic anastomosis was also observed (Fig. 1g).

Growth and development of inoculated and non-inoculated plants
Inoculation effects were evaluated on development of P. aduncum plants and the values for each parameter were compared to the control group (Table 1). Statistical differences were not observed for parameters such as basal stem diameter and leaf numbers between inoculated plants and control group (Table 1). Inoculated plants displayed a gradual increase in plant height at 30, and 90 dpi. The number of nodes in inoculated plants was higher at 90 dpi but had not displayed statistical differences at 30, and 60 dpi. These results indicate that inoculation benefits were demonstrated after 90 dpi. The increase of fresh mass was observed only in the roots at 30 dpi.

Variation of volatile compounds in the leaves and roots during the colonization by AMF
The GC–MS analysis of volatiles of P. aduncum leaves and roots resulted in the identification of 65 and 79 compounds, respectively. The most representative compound classes identified were phenylpropanoids and sesquiterpene hydrocarbons such as dillapiole, myristicin, germacrene D and elemicin. In the leaves, the phenylpropanoid concentrations displayed a difference between inoculated and non-inoculated plants at 60 dpi (Table 2). The main change was observed at 60 dpi, with a drastic decrease (87.94–52.58%) in inoculated plants. In the roots, the most representative classes were phenylpropanoids (≈95%) and sesquiterpene hydrocarbons (≈12%). The production of phenylpropanoid showed an increase only at 30 dpi. However, concentrations of sesquiterpene hydrocarbons were lower in the inoculated plants at 30 and 60 dpi (Table 2).

At 30 dpi, quantitative and qualitative changes were observed in the leaves. Quantitatively, a decrease in the dillapiole content (93.74–86.11%), and an increase of β-caryophyllene (0.27–2.62%) and germacrene D production (1.28-2.78%) was observed (Fig. 2). Qualitatively, the inoculated plants produced additional compounds not observed in the control plants, including (E)-β-ocimene (0.12%), terpinen-4-ol (0.11%), α-copaene (0.25%) and n-tetradeacanol (1.54%). At 60 dpi, there was an increase in the contents of β-caryophyllene (2.16–5.58%), germacrene D (3.19–5.49%), myristicin (2.71-4.70%), and a greater decrease of dillapiole (83.57–44.73%). Several monoterpenes and sesquiterpenes were produced only by inoculated plants, such as (Z)-β-ocimene (1.10%), (E)-β-ocimene (2.84%), and β-selinene (1.33%). At 90 dpi, only inoculated plants produced the monoterpene allo-ocimene (1.64%), also displaying a decrease in the concentrations of dillapiole (48.66–39.36%), myristicin (3.26–2.41%), and apiole (2.54–1.83%).

At 30 dpi, the hydrocarbon n-octane (2.43%) was identified only in the roots of inoculated plants (Table 3). Quantitatively, the production of dillapiole displayed an increase (71.65-84.65%) with a concomitant decrease in the concentrations of myristicin (17.45–10.52%) and elemicin (2.23–0.28%) (Fig. 2). In addition, there was a decrease in the amounts of α-copaene (1.20–0.30%), β-caryophyllene (1.50–0.53%), and germacrene D (1.16–0.37%).

At 60 dpi, important changes were observed: the inoculated plants produced 15 compounds which were absent in the control group. Dillapiole production showed a decrease (61.12–54.15%) and a slight increase in the
production of myristicin (18.38–19.93%) and elemicin (2.22–3.13%) was observed (Fig. 2). At 90 dpi, only inoculated plants produced detectible (> 0.1%) levels of the sesquiterpene trans-cadina-1(6),4-diene (1.34%). The phenylpropanoids myristicin (19.25–18.47%) and dillapiole (54.27–51.96%) showed a decrease as well as the sesquiterpene hydrocarbons δ-elemene (0.40–0.29%) and α-copaene (1.91–1.63%). The minor compounds produced in the roots showed a behavior different than leaves, with a decrease of monoterpenes (Z)-β-ocimene (0.86–0.68%) and (E)-β-ocimene (0.77–0.57%) in inoculated plants (Table 3).
Table 1 Developmental parameters of *P. aduncum* during inoculation by AMFs

| dpi | Treatments | Evaluation parameters* |
|-----|------------|------------------------|
|     |            | Basal stem (mm) | Leaves | Node | Height (cm) | Root (cm) | Fresh weight (leaves) | Fresh weight (root) |
| 30  | Control    | 3.9 ± 0.0         | 4.7 ± 0.6 | 43 ± 0.6 | 21.0 ± 1.7 | 37.7 ± 1.2 | 49 ± 0.9 | 43 ± 0.7 |
|     | AMF        | 3.8 ± 0.2         | 5.3 ± 0.6 | 53 ± 0.6 | 30.5 ± 2.2* | 44.0 ± 1.7 | 69 ± 1.1 | 75 ± 0.9* |
| 60  | Control    | 5.5 ± 0.9         | 7.3 ± 0.6 | 70 ± 1.0 | 37.7 ± 2.5 | 36.4 ± 1.1 | 22 ± 0.8 | 38 ± 0.9 |
|     | AMF        | 6.3 ± 0.4         | 6.7 ± 0.6 | 67 ± 0.6 | 41.3 ± 1.5 | 30.1 ± 7.0 | 45 ± 0.2 | 52 ± 1.2 |
| 90  | Control    | 6.1 ± 0.9         | 15.0 ± 1.0 | 160 ± 0.8 | 53.8 ± 0.9 | 53.3 ± 0.5 | 145 ± 1.9 | 78 ± 0.8 |
|     | AMF        | 7.2 ± 0.7         | 163 ± 2.1 | 183 ± 0.6* | 61.1 ± 4.3* | 57.0 ± 1.0 | 172 ± 1.5 | 88 ± 0.4 |

dpi: Days post inoculation; Control: *P. aduncum* non-inoculated with AMF; AMF: *P. aduncum* inoculated with AMF
* Statistical difference according to Bonferroni-test (*p* < 0.05)
* Mean ± standard deviation (n = 3)

Evaluation of lipoxygenase activity in *P. aduncum* during AMF inoculation

LOX activity was about 4 times greater in the leaves compared to the roots of *P. aduncum*. The leaves of inoculated plants showed an increase of LOX activity at 60 and 90 dpi (Fig. 3a). However, in the roots its increase was observed only at 30 and 90 dpi to inoculated plants (Fig. 3b).

Discussion

In the first stage of mycorrhizal colonization, the formation of the penetration apparatus (hyphopodium) occurred, presumably due to the recognition of signaling molecules of the plant by fungus and host (Gianinazzi-Pearson and Brechenmacher 2004; Requena et al. 2002). After the formation of the appressorium in the epidermis and the intracellular extension of the hyphae, the AMF was established between the cell walls of the plant until reaching the cortex (Kiriachek et al. 2009). *P. aduncum* roots showed a typical *Arum*-type colonization, which consists of an extension of the intracellular hyphae at the beginning of colonization, followed by penetration into the cells of the root cortex, forming terminal arbuscules that bind the hyphae through arbuscular trunks (Fig. 1f) (Smith and Smith 1997). *Arum*-type colonization has also been observed in roots of 22 plant species including *Piper nigrum*, inoculated with AMF from the genus *Acaulospora, Gigaspora, Glomus* and *Scutellospora* (Muthukumar and Tamilselvi 2010).

The presence of mycorrhizal structures into radicular cells indicates the colonization and exchange of nutrients between host plants and AMF, mostly in arbuscules, which are considered the key in this process, and present a development cycle until degeneration. In addition, water is absorbed by the external mycelium and moves through the hyphae, which favors the apoplastic flow in the root system of the plant (Bárzan et al. 2012). The vesicles are globular or elliptical structures, which store lipids and glycogen, serving as a reserve organ for the fungus, and their formation can occur within or between the cells of the root cortex (Smith and Read 2008). These fungal structures were also observed in roots of plants of *Poincianella pyramidalis* and *Cnidoscolus quercifolius* that were inoculated by *Acaulospora longula* and *C. etunicatum* (Frosi et al. 2016).

The developmental parameters of inoculated plants showed significant variation only in the height of plants at 30 and 90 dpi and node number at 90 dpi. Our results are distinct in comparison with *Piper longum* plants inoculated with *Glomus fasciculatum, Acaulospora foveata* and *Gigaspora margarita*, which showed an increase in leaf number. However, there was a decrease in root development, mostly for plants inoculated with *G. fasciculatum* (Seema and Garampalli 2015). The height variation in *P. aduncum* plants was similar to that observed in *basil* (*Ocimum basilicum*) and *rosemary* (*Rosmarinus officinalis*) inoculated with *G. clarum* spores, which showed an increase of 45.49 and 25.93%, respectively (Russomanno et al. 2008). AMF contributes to increasing photosynthesis rate, favoring plant growth (Tanaka and Fujita 1979). The increase in height, but not in the number of leaves, indicates a possible production of photoassimilates directed to the needs of the plant (Neumann et al. 2009). The AMF species *Gigaspora margarita, Acaulospora longula* and *C. etunicatum* were considered as promoters of growth and better biomass production in *P. longum* seedlings (Seema and Garampalli 2015).

The contribution of AMF to increases of nutrients and biomass can be important when nutrient availability in the soil is low, thereby promoting a higher efficiency through the benefits of photoassimilates produced in
Table 2: Comparison of volatile components produced in inoculated and non-inoculated leaves of *P. aduncum* (Mean standard deviation)

| Compound                  | Rf<sup>calc</sup> | Rf<sup>lit</sup> | 30 dpi<sup>a</sup> | 60 dpi<sup>a</sup> | 90 dpi<sup>a</sup> |
|---------------------------|-------------------|-----------------|-------------------|-------------------|-------------------|
|                           | Control AMF       | Control AMF     | Control AMF       | Control AMF       | Control AMF       |
| α-Thujene                 | 931               | 924             | 0.33±0.47         | 0.48±0.67         | 0.63±0.35         |
| β-Pinen                 | 976               | 974             | 0.21±0.30         | 0.15±0.21         | 0.22±0.23         |
| Sabinene                      | 977               | 969             | 0.24±0.34         | 0.79±0.53         | 0.84±0.03         |
| α-Phellandrene           | 996               | 1002            | 0.14±0.20         | 0.18±0.01         | 0.08±0.01         |
| δ-2-Carene                  | 1006              | 1001            | 0.06±0.08         | 0.00±0.00         | 0.00±0.00         |
| δ-3-Carene                  | 1015              | 1008            | 0.32±0.33         | 0.21±0.29         | 0.33±0.29         |
| Limonene                   | 1028              | 1024            | 0.02±0.00         | 1.10±0.21         | 2.07±0.33         |
| (Z)-β-Ocimene              | 1032              | 1032            | 0.12±0.00         | 2.84±0.01         | 6.58±2.03         |
| (E)-β-Ocimene              | 1043              | 1044            | 0.27±0.38         | 0.21±0.05         | 0.41±0.21         |
| γ-Terpinene                | 1046              | 1054            | 0.32±0.33         | 1.10±0.21         | 2.07±0.33         |
| Terpinolene                | 1084              | 1086            | 0.06±0.08         | 0.00±0.00         | 0.00±0.00         |
| αllo-Ocimene               | 1126              | 1128            | 0.02±0.00         | 1.10±0.21         | 2.07±0.33         |
| Terpinen-4-ol              | 1179              | 1174            | 0.11±0.24         | 0.36±0.30         | 0.32±0.24         |
| Piperitone                  | 1246              | 1249            | 0.04±0.05         | 0.43±0.92         | 1.40±1.00         |
| α-Terpinyl formate         | 1252              | 1306            | 0.14±0.08         | 0.35±0.16         | 1.92±0.36         |
| Saffrole                   | 1282              | 1285            | 0.01±0.01         | 0.94±0.07         | 3.19±1.73         |
| δ-Elemene                  | 1324              | 1335            | 0.08±0.06         | 0.28±0.28         | 1.46±0.71         |
| α-Cubebeene                | 1344              | 1345            | 0.25±0.17         | 0.73±0.01         | 1.71±0.04         |
| α-Ylangene                 | 1366              | 1373            | 0.04±0.05         | 0.32±0.33         | 1.12±0.21         |
| α-Copaene                  | 1368              | 1374            | 0.25±0.17         | 0.73±0.01         | 1.71±0.04         |
| β-Elemene                  | 1382              | 1389            | 0.14±0.08         | 0.35±0.16         | 1.92±0.36         |
| n-Tetradecane              | 1399              | 1400            | 0.05±0.01         | 0.94±0.07         | 3.19±1.73         |
| β-Caryophyllene            | 1412              | 1417            | 0.27±0.05         | 2.62±1.03         | 5.58±0.76         |
| γ-Elemene                  | 1422              | 1434            | 0.23±0.16         | 0.50±0.24         | 2.18±0.58         |
| β-Copaene                  | 1430              | 1430            | 0.42±0.59         | 0.05±0.01         | 0.03±0.04         |
| Aromadendrene              | 1435              | 1439            | 0.13±0.08         | 0.05±0.01         | 0.10±0.03         |
| 6,9-Guaiadiene             | 1438              | 1442            | 0.11±0.03         | 0.07±0.01         | 0.11±0.05         |
| Isogermacrene D            | 1441              | 1445            | 0.01±0.03         | 0.07±0.00         | 0.35±0.06         |
| α-Humulene                 | 1452              | 1452            | 0.05±0.07         | 0.62±0.47         | 2.38±1.11         |
| allo-Aromadendrene         | 1456              | 1458            | 0.01±0.03         | 0.29±0.11         | 1.13±0.27         |
| Dauc-5,8-diene              | 1469              | 1471            | 0.02±0.04         | 0.07±0.01         | 0.06±0.04         |
| γ-Murolene                 | 1472              | 1478            | 0.26±0.04         | 0.13±0.01         | 0.17±0.12         |
| Germacrene D               | 1474              | 1484            | 1.28±0.78         | 2.78±0.94         | 5.49±1.32         |
| β-Selinene                 | 1485              | 1489            | 0.02±0.04         | 0.13±0.13         | 0.70±0.10         |
| α-Selinene                 | 1485              | 1498            | 0.02±0.02         | 0.17±0.02         | 0.14±0.09         |
| Viridiflorene              | 1488              | 1496            | 0.23±0.18         | 0.36±0.40         | 2.15±0.34         |
| Bicyclomacerone            | 1492              | 1500            | 0.05±0.08         | 0.14±0.00         | 0.59±0.11         |
| α-Murolene                 | 1495              | 1500            | 0.07±0.29         | 2.28±0.46         | 1.66±1.07         |
| n-Pentadecane              | 1496              | 1500            | 0.05±0.11         | 0.07±0.04         | 0.50±0.18         |
| (E,E)-α-Farnesene          | 1502              | 1505            | 0.01±0.01         | 0.06±0.04         | 0.32±0.06         |
| γ-Cadinene                 | 1509              | 1513            | 0.19±0.02         | 0.85±0.05         | 0.53±0.14         |
| δ-Cadinene                 | 1514              | 1522            | 0.07±0.03         | 0.31±0.21         | 0.19±0.02         |
| Myristicin                 | 1517              | 1517            | 1.53±0.41         | 0.61±0.60         | 2.71±1.11         |
| 7-epi-α-Selinene           | 1526              | 1520            | 0.01±0.01         | 0.11±0.02         | 0.04±0.00         |
| trans-Cadin-1,4-diene       | 1529              | 1533            | 0.11±0.02         | 0.04±0.00         | 0.05±0.04         |
| α-Cadinene                 | 1533              | 1537            | 0.06±0.01         | 0.05±0.06         | 0.11±0.06         |
| α-Calacorene               | 1538              | 1544            | 0.01±0.03         | 0.22±0.13         | 0.05±0.06         |
Among the minor compounds, \((E)-\beta\)-ocimene and \(E\)-\(\beta\)-ocimene, of ocimene isomers made up of \((E)\)-\(\beta\)-ocimene and \((Z)\)-\(\beta\)-ocimene showed a gradual increase during inoculation by AMF in the leaves and a decrease in the roots. These compounds are emitted by plants in response to herbivore attack and changes in abiotic factors (Gouinguené and Turlings 2002).

Our observations showed a correlation with LOX activity in the leaves of inoculated plants; the increase of LOX activity indicating a possible defense mechanism of plant (Baysal and Demirdoven 2007). The regulation of the by-product of the LOX pathway, jasmonic acid, can promote changes in the colonization level by AMF in plants (Gutjahr et al. 2015). The activation of the LOX pathway enhances important functions in the primary and secondary metabolism in the plants (Morcillo et al. 2012).

LOX is involved in the production of volatile compounds in leaves and roots such as alcohols, ethers, and aldehydes, which are considered both signaling and defense compounds (Liavonchanka and Feussner 2006).
After biotic and abiotic stress, LOX activity is increased, and it depends mostly on inducing agents as well as the plant genotype and physiologic conditions (Silva et al. 2004). The activation of LOX activity was induced by inoculation in the roots of *Rhizophagus irregulars* in bean plants (*Phaseolus vulgaris* L.) (Mora-Romero et al. 2015). At 21 dpi, LOX activity was increased about 50% in *P. divaricatum* seedlings infected by *Fusarium solani* f. sp. *piperis* (Meireles et al. 2016).

*P. aduncum* presents many biological activities reported in the literature, which are attributed to the phenylpropanoid dillapiole (Bernard et al. 1995; Almeida et al. 2009; Souto et al. 2012). Alternatives to increase the production of dillapiole were investigated.
| Compound                  | Rf<sup>calc</sup> | Rf<sup>det</sup> | 30 dpi<sup>a</sup> | 60 dpi<sup>a</sup> | 90 dpi<sup>a</sup> |
|---------------------------|-------------------|------------------|---------------------|---------------------|---------------------|
|                           | Control AMF       | Control AMF      | Control AMF         | Control AMF         | Control AMF         |
Table 3 (continued)

| Compound                        | R1<sup>alc</sup> | R1<sup>bc</sup> | 30 dpi<sup>a</sup> | 60 dpi<sup>a</sup> | 90 dpi<sup>a</sup> |
|---------------------------------|------------------|-----------------|---------------------|------------------|------------------|
|                                 | Control          | AMF             | Control             | AMF             | Control          | AMF             |
| trans-Muurola-4(14),5-diene     | 1489             | 1493            | 0.05 ± 0.08         | 0.11 ± 0.06     | 0.05 ± 0.04     |
| α-Muurola-4,10(14)-dien-1-β-ol  | 1492             | 1498            | 0.03 ± 0.05         | 0.06 ± 0.10     |                 |
| α-Cadinene                     | 1500             | 1500            | 0.04 ± 0.06         | 0.19 ± 0.22     | 0.42 ± 0.27     | 0.22 ± 0.13     | 0.25 ± 0.02     |
| α-Cadinene                     | 1505             | 1505            | 0.02 ± 0.03         | 0.08 ± 0.07     | 0.08 ± 0.09     | 0.07 ± 0.02     | 0.13 ± 0.06     |
| α-Cadinene                     | 1507             | 1514            | 0.02 ± 0.03         | 0.06 ± 0.07     | 0.10 ± 0.06     | 0.03 ± 0.04     | 0.04 ± 0.03     |
| γ-Muurolene                     | 1510             | 1513            | 0.02 ± 0.04         | 0.05 ± 0.05     |                 |
| Caryophyllene oxide            | 1513             | 1514            | 0.18 ± 0.31         | 0.28 ± 0.39     |                 |
| α-Cadinene                     | 1515             | 1537            | 0.28 ± 0.12         | 0.05 ± 0.05     | 0.28 ± 0.48     | 0.39 ± 0.42     |
| Myristic                       | 1519             | 1517            | 17.45 ± 2.09        | 10.52 ± 2.95    | 18.38 ± 1.01    | 19.93 ± 1.13    | 19.25 ± 1.46    | 18.47 ± 0.91    |
| (E)-γ-Bisabolene                | 1524             | 1529            | 0.05 ± 0.05         | 0.03 ± 0.05     | 0.03 ± 0.04     | 0.02 ± 0.03     |
| trans-Cadina-1,4-diene          | 1529             | 1533            | 0.04 ± 0.04         | 0.10 ± 0.05     | 0.03 ± 0.04     | 0.05 ± 0.05     |
| α-Cadinene                     | 1535             | 1537            | 0.02 ± 0.03         |                 |                 |
| α-Copaen-11-ol                 | 1539             | 1539            | 0.03 ± 0.05         | 0.14 ± 0.14     | 0.28 ± 0.09     | 0.16 ± 0.11     | 0.20 ± 0.08     |
| Elemicin                       | 1545             | 1555            | 2.23 ± 1.16         | 0.28 ± 0.36     | 3.13 ± 0.43     | 3.56 ± 1.06     | 3.92 ± 0.75     |
| (E)-Nerolidol                  | 1559             | 1561            | 0.06 ± 0.06         | 0.13 ± 0.11     | 0.35 ± 0.22     | 0.17 ± 0.24     | 0.27 ± 0.10     |
| Spathuleneol                   | 1573             | 1577            | 0.08 ± 0.09         |                 |                 |
| Germacrene D-4-ol              | 1574             | 1574            | 0.05 ± 0.07         |                 |                 |
| Caryophyllene oxide            | 1575             | 1582            | 0.02 ± 0.03         | 0.06 ± 0.05     | 0.19 ± 0.12     | 0.08 ± 0.01     | 0.07 ± 0.02     |
| 6-Methoxyelemicin              | 1579             | 1595            | 0.02 ± 0.03         | 0.21 ± 0.14     | 0.18 ± 0.25     | 0.35 ± 0.18     |
| Viridiflorol                   | 1592             | 1592            | 0.06 ± 0.10         |                 |                 |
| Humulene epoxide II            | 1607             | 1608            | 0.02 ± 0.03         |                 |                 |
| Dillapiol                      | 1618             | 1620            | 71.65 ± 7.30        | 84.65 ± 2.66    | 61.12 ± 6.71    | 54.15 ± 6.56    | 54.27 ± 5.32    | 51.96 ± 5.88    |
| 1-epi-Cubenol                  | 1627             | 1627            | 0.13 ± 0.12         |                 |                 |
| Muurola-4,10(14)-dien-1-β-ol   | 1636             | 1630            | 0.15 ± 0.25         |                 |                 |
| α-epi-Muurol                   | 1649             | 1640            | 0.08 ± 0.09         |                 |                 |
| α-Cadinol                      | 1660             | 1652            | 0.01 ± 0.02         | 0.06 ± 0.07     | 0.02 ± 0.03     |
| Apiole                         | 1670             | 1677            | 0.64 ± 0.40         | 0.06 ± 0.11     | 0.85 ± 0.37     | 1.59 ± 0.78     | 1.78 ± 0.37     |
| Monoterpane hydrocarbons       | 0.74 ± 0.84      | 0.09 ± 0.16     | 2.35 ± 0.78         | 5.84 ± 1.70     | 6.45 ± 2.13     | 5.70 ± 3.34     |
| Oxygenated monoterpenes        | 0.63 ± 0.54      | 0.10 ± 0.10     | 0.75 ± 1.09         | 2.27 ± 1.17     | 2.11 ± 1.77     | 0.93 ± 0.34     |
| Sesquiterpene hydrocarbons     | 5.84 ± 3.18      | 1.63 ± 0.84     | 12.67 ± 5.73        | 10.35 ± 5.79    | 9.05 ± 2.51     | 10.41 ± 2.82    |
| Oxygenated sesquiterpenes      | 0.13 ± 0.17      | 0.52 ± 0.48     | 1.56 ± 1.51         | 0.77 ± 0.86     | 0.63 ± 0.24     |
| Phenylpropanoids               | 92.04 ± 11.02    | 95.51 ± 6.08    | 82.63 ± 8.47        | 79.24 ± 9.06    | 78.92 ± 8.88    | 76.70 ± 8.24    |
| Others                         | 0.21 ± 0.09      | 2.58 ± 1.30     | 0.41 ± 0.07         | 0.33 ± 0.20     | 0.22 ± 0.06     | 0.41 ± 0.10     |
| Total                          | 99.59 ± 15.84    | 99.91 ± 8.48    | 99.33 ± 18.62       | 99.59 ± 19.43   | 97.52 ± 17.21   | 94.78 ± 18.08   |

dpi: Days post inoculation; Control: P. aduncum non-inoculated with AMF; AMF: P. aduncum inoculated with AMF; R1: Retention index calculated; R1lit: Retention Index of Library

* Statistical difference according to Bonferroni test (p < 0.05)
* Microsoft ± standard deviation (n = 3)

through the inoculation by AMF in the roots. The results showed dillapiol production decreased in roots and leaves. However, several monoterpenes and sesquiterpenes increased in the leaves, and 15 components were produced in the roots of inoculated plants. These results indicate a metabolic activity was induced by the inoculation of AMF and can serve to contribute to future studies on plant-fungal interactions.
**Authors’ contributions**
JKRS and ARR participated in study designed; JSFO and LP conducted the experiments; ALFAL assisted the experiments of plant anatomy; EAA performed the GC–MS analyzes, AMH provided and identified the AMFs species; JSFO, ARR and JKRS wrote the manuscript; JGSM, WNS and JKRS edited and revised the manuscript. All authors read and approved the final manuscript.

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**Competing interests**
The authors declare that they have no competing interests.

**Availability of data and materials**
All necessary data supporting our finding can be found within the article.

**Consent for publication**
All the authors agreed to publish in the journal.

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**Fig. 3** Variation of LOX activity in inoculated and non-inoculated plants of *P. aduncum*. **a** Leaves; **b** Roots. *Statistical difference according to Bonferroni test (p < 0.05)*

**References**
Adams RP (2007) Identification of essential oil components by gas chromatography/mass spectrometry, 4th edn. Allured Publishing Corporation, Carol Stream.
Al-karaki G, McMichael B, Zak J (2004) Field response of wheat to arbuscular mycorrhizal fungi and drought stress. Mycorrhiza 14:263–269. https://doi.org/10.1007/s00572-003-0265-2
Almeida RP, Souto RNP, Silva MHL, Maia JGS (2009) Chemical variation in *Piper aduncum* and biological properties of its dillapiole-rich essential oil. Chem Biodiversity. 9:1427–1434. https://doi.org/10.1002/cbdv.200800212
Bárzan G, Aroca R, Paz JA, Chaumont F, Martinez-Ballesta MC, Carvajal M, Ruiz-Lozano JM (2012) Arbuscular mycorrhizal symbiosis increases relative apoplastic water flow in roots of the host plant under both well-watered and drought stress conditions. Ann Bot 109:1009–1017. https://doi.org/10.1093/aob/mcs007
Baysal T, Demirdoven A (2007) Lipoxygenase in fruits and vegetables: a review. Enzyme Microbl Technol. 40:491–496. https://doi.org/10.1016/j.enzmictec.2006.11.025
Bernard CB, Krishnamurthy HG, Durst DCT, Philogene BJR, Sanchez-Vindas P, Hasbun C, Poveda L, San Roman L, Arnason JT (1995) Insecticidal defenses of *Piperaceae* from the Neotropics. J Chem Ecol 21:801–814
Bonfante P, Genre A (2010) Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. Nat Commun 1:1–11. https://doi.org/10.1038/ncomms1046
Carlsten SCK, Understrup A, Fomsgaard IS, Mortensen AG, Ravnskov S (2008) Flavonoids in roots of white clover: interactions of arbuscular mycorrhizal fungi and a pathogenic fungus. Plant Soil 302:33–43. https://doi.org/10.1007/s11104-007-9452-9
Da Luz SMA, Reis LA, Lemos OF, Maia JGS, De Mello AH, Ramos AR, Da Silva JKR (2016) Effect of arbuscular mycorrhizal fungi on the essential oil composition and antioxidant activity of black pepper (*Piper nigrum* L). Int J Appl Res Nat Prod 9:10–17. http://www.ijarnp.org/index.php/ijarnp/article/view/355/pdf
Freitas MS, Martins MA, Vieira UC (2004) Yield and quality of essential oils of *Mentha arvensis* in response to inoculation with arbuscular mycorrhizal fungi. Pesq Agropec Bras 39:887–894. https://doi.org/10.1590/S0100-204X2004000900008
Frosi G, Barros VA, Oliveira MT, Cavalcante UMT, Maia LC, Santos MG (2016) Increase in biomass of two woody species from a seasonal dry tropical forest in association with AMF with different phosphorus levels. Appl Soil Ecol 102:426–52. https://doi.org/10.1016/j.apsoil.2016.02.009
Gerlach G (1969) Botanische Mikrotechnik. Georg Thieme Verlag, Stuttgart, p. 298.
Gianninzi-Pearson V, Brechenmacher L (2004) Functional genomics of arbuscular mycorrhiza: decoding the symbiotic cell programme. Can J Bot 82:1228–1234. https://doi.org/10.1139/b04-096
Godard K, White R, Bohlmann J (2008) Monoterpane-induced molecular responses in Arabidopsis thaliana. Phytochemistry 69:1838–1849. https://doi.org/10.1016/j.phytochem.2008.02.011

Gouinguené SP, Turlings TCJ (2002) The effects of abiotic factors on induced volatile emissions in corn plants. Plant Physiol 129:1206–1207. https://doi.org/10.1104/pp.011941

Guerrini A, Sacchetti G, Rossi D, Paganetto G, Muzzoli M, Andreotti E, Tognoloni M, Maldonado ME, Bruni R (2009) Bioactivities of Piper aduncum L. and Piper obliquum Ruiz & Pavon (Piperaceae) essential oils from Eastern Ecuador. Environ Toxicol Pharmacol 27:39–48. https://doi.org/10.1016/j.etap.2008.08.002

Gutjahr C, Siegler H, Haga K, Ino M, Paszkowski U (2015) Full establishment of arbuscular mycorrhizal symbiosis in rice occurs independently of enzymatic jasmonate biosynthesis. PLoS ONE 10:1–9. https://doi.org/10.1371/journal.pone.0123422

Hartemink AE (2001) Biomass and nutrient accumulation of Piper aduncum and Imperata cylindrica fallows in the humid lowlands of Papua New Guinea. For Ecol Manag 144:19–32. https://doi.org/10.1016/S0378-1127(00)00655-1

INVAM. International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi. 1992. http://invam.caf.wvu.edu/. Accessed 2 Mar 2018

Johansen DA (1940) Plant microtechnique. Mc Graw-Hill, New York, p 523

Karnovsky MJA (1965) Formaldehyde-gluutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27:137–138. https://doi.org/10.1083/jcb.27.1.137

Kiraichuk SG, Azevedo LCB, Peres LE, Lamba MR (2009) Regulação do desenvolvimento de micorrizas arbusculares. R Bras Ci Solo 33:1–16. https://doi.org/10.1590/S0103-65392009000100001

Klocek P, Polesny S, Zvobodova B, Vilova E, Kokoska L (2005) Antibacterial screening of some Peruvian medicinal plants used in Galleria District. J Ethno Pharmacol 99:309–312. https://doi.org/10.1016/j.jep.2005.01.062

Kraus JE, Arduin M (1997) Manual básico de métodos em morfologia vegetal. 1989. DPUR

Livanhancha A, Feusnier I (2006) Lipoxigenases: occurrence, functions and catalysis. J Plant Physiol 163:348–357. https://doi.org/10.1016/j.jplph.2005.11.006

Maia JGS, Zohbhi MGB, Andrade EHA, Santos AS, Da Silva MHL, Luz AIR, Bastos CN (1998) Constituents of the essential oil of Piper aduncum L. growing wild in the Amazon region. Flavour Fragr J 13:269–272. https://doi.org/10.1002/(SICI)1099-1026(19980701)13:4<269::AID-FFJ744>3.0.CO;2-A

Meireles EM, Xavier LP, Ramos AR, Maia JGS, Setzer WN, Da Silva JKR (2016) Phenylpropanoids produced by Piper divaricatum, a resistant species to infection by Fusarium solani f. sp. piperis, the pathogenic agent of Fusariose in Black Pepper. J Plant Pathol Microbiol 7:1–6. https://doi.org/10.4172/2157-7471.1000333

Misni N, Othman H, Sulaiman S (2011) The effect of Piper aduncum Linn. (Family:Piperaceae) essential oil as aerosol spray against Aedes aegypti (L.) and Aedes albopictus Skuse. Trop Biomed 28:249–258

Mora-Romero GA, Cervantes-Gámez RG, Galindo-Flores H, González-Ortiz MA, Félix-Gastélum R, Maldonado-Mendonza IE, Salinas Perez R, León-Félix J, Martínez-Valenzuela MC, López-Meyer M (2015) Mycorrhiza-induced protection against pathogens is both genotype-specific and graft-transmissible. Mycorrhiza 25:145–238. https://eprints.lib.hokudai.ac.jp/dspace/handle/2115/12192

The Plant List (2013) Version 1.1. http://www.theplantlist.org/. Accessed 02 Mar 2018

Van Den Dool H, Kratz PDA (1966) A Generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J Chromatogr A 1:146–466. https://doi.org/10.1016/0021-9673(01)80947-X

Xie X, Weng B, Cai B, Dong Y, Yan C (2014) Effects of arbustural mycorrhizal inoculation and phosphorus supply on the growth and nutrient uptake of Kandelia obovata (Sheue: Iiu & Yong) seedlings in autoclaved soil. Appl Soil Ecol 75:162–171. https://doi.org/10.1016/j.apsoil.2013.11.009

Yuncker TG (1972) The Piperaceae of Brazil. Hoehnea. 2:19–366. http://www.scielo.br/scielo.php?script=sci_nlm&pid=S0021-89060090003000400218&lng=en