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Arbuscular mycorrhizal fungi increase gallic acid production in leaves of field grown *Libidibia ferrea* (Mart. *ex* Tul.) L. P. Queiroz

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Because arbuscular mycorrhizal fungi (AMF) has been shown to induce concentration increases of pharmaceutically useful phytochemicals in some Brazilian semi-arid native plants, the current study examined whether mycorrhizal inoculation increased the production of bioactive compounds, especially gallic acid, in field grown *Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz plants. Seedlings were inoculated with *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler*, Acaulospora longula* Spain & N. C. Schenck*, Gigaspora albida* N. C. Schenck & G. S. Sm. or non-inoculated (control) and, seven months after transplanting, examined for growth parameters, chlorophyll, phenols, tannins and gallic acid concentrations, mycorrhizal colonization and rhizosphere AMF spore density. Plants inoculated with *C. etunicatum* had 21% higher gallic acid concentrations than control plants while those inoculated with *G. albida* had higher total chlorophyll concentrations. Mycorrhizal technology employing *C. etunicatum* can constitute an alternative to increase gallic acid production in field grown *L. ferrea* plants.

Key words: Glomeromycota, secondary compounds, ironwood, *pau ferro*, Caatinga, semi-arid.

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF), which belong to the phylum Glomeromycota, associate in a symbiotic way to the majority of plant species. The association benefits the plants because the AMF increase nutrient absorption in poor soils, in particular phosphorus (Yao et al., 2008), and alleviate the stress caused by biotic and abiotic

factors (Smith and Read, 2008). AMF has been shown to stimulate growth of seedlings of economically important species (Tristão et al., 2006), including medicinal species native of the Brazilian semi-arid region (Oliveira et al., 2013), which can accumulate high concentrations of primary and secondary metabolites (Mandal et al., 2013; Pedone-Bonfim et al., 2013).

Mycorrhizal biotechnology can constitute an alternative to increase production of secondary compounds in some species: essential oils in *Anethum graveolens* L., *Ocimum basilicum* L. and *Salvia officinalis* L. (Copetta et al., 2006; Geneva et al., 2010; Kapoor et al., 2002), caffeic acid in *O. basilicum* (Toussaint et al., 2007); artemisinin in *Artemisia annua* L. (Chaudary et al., 2008); and alkaloids in *Castanospermum australe* A. cunn. & C. Fraser and *Catharanthus roseus* L.G. Don (Abu-Zeyad et al., 1999; Ratti et al., 2010). However, there are no reports on AMF effect on gallic acid concentrations, and this is an important compound that belongs to the group of phenols and possesses antibacterial and antioxidant properties (Broinizi et al., 2007; Chanwitheesuk et al., 2007).

In the Brazilian semi-arid region, inhabitants benefit from various medicinal plant species with therapeutic properties (Agra et al., 2007). Among these, *Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz (*pau ferro* or ironwood), a species of the Fabaceae family, which has proven to be mycotrophic (Gattai et al., 2011), possesses therapeutic characteristics with antiulcerogenic, anti-inflammatory, analgesic, hypoglycemic, anti-cancerogenic, anti-histaminic, antimicrobial, anticoagulant, larvicidal against *Aedes aegypti*, and cicatrizing activities (Bacchi et al., 1995; Carvalho et al., 1996; Cavalheiro et al., 2009; Gonzalez, 2005; Oliveira et al., 2010; Sampaio et al., 2009). Such characteristics are related to the presence of secondary compounds (Gonzalez, 2005; Nozaki et al., 2007; Souza et al., 2006; Ueda et al., 2001), especially gallic acid (Nakamura et al., 2002). Therefore, alternatives to increase the production of gallic acid and other compounds in ironwood leaves are of interest to the phytotherapeutic industry.

There are no registers of cultivation of mycorrhized ironwood plants under field conditions with the objective of increasing the production of secondary compounds, especially gallic acid. Therefore, in this study hypothesis that inoculation of seedlings established in the field with AMF increases the growth of the plants and the production of gallic acid were tested, but these increases vary according to the AMF species used. The objective of this study was to determine whether mycorrhizal inoculation increases plant growth and production of gallic acid in mycorrhizal ironwood plants established in the field.

MATERIALS AND METHODS

Experimental field

An experiment was set up at the Experimental Field of the University of Pernambuco at Petrolina Campus, Northeast Brazil. The region has a climate of the Bswh type, according to the Köppen classification. During the period, the experiment was carried out (February to September, 2013). Averages of the maximum and minimum temperature were 33.2 and 21.7°C, respectively, relative humidity was 55.2% and total rainfall was 9.5 mm. The experiment consisted of four treatments, in a random block design, with six replicates: inoculation of *L. ferrea* seedlings with the arbuscular mycorrhizal fungi *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler (UFPE 06), *Gigaspora albida* N. C. Schenck & G. S. Sm. (UFPE 01) or *Acaulospora longula* Spain & N. C. Schenck (UFPE 21) and a non-inoculated control.

Ironwood seeds were collected from healthy plants in the Caatinga region of Juazeiro municipality, Bahia State. The seeds were treated with concentrated sulfuric acid for 20 min to break dormancy (Biruel et al., 2007), washed in running and distilled water, and were placed to germinate in 50 ml recipients containing vermiculite (medium granulation). When the plantlets had four definite leaves, they were transferred to polyethylene bags containing 1.2 kg of substrate composed of soil (not sterilized) + 5% vermicompost and inoculate or not with soil-inoculate containing 200 spores of each AMF.

The soil was collected from a native Caatinga area in a perpendicular path starting in KM 152 of the BR 428 road that departs from Petrolina. The collected soil had a density of 96 glomerospores in 100 g of soil and the presence of the following AMF species: *Scutellospora* species 1, *Glomus macrocarpum* Tul. & Tul., *Ambispora appendicula* (Spain, Sieverd. & N.C. Schenck) C. Walker, *Acaulospora* species 1, *Glomus* species 1 and *Acaulospora scrobiculata* Trappe (Lima, 2014). The soil that was mixed with vermicompost had the following chemical characteristics: P, 12.68 mg dm^{-3}; K, 0.26 $cmol_c\ dm^{-3}$; Ca, 2.7 $cmol_c\ dm^{-3}$; Mg, 1.8 $cmol_c\ dm^{-3}$; Na, 0.49 $cmol_c\ dm^{-3}$; Al, 0.05 $cmol_c\ dm^{-3}$; organic matter, 3.21 g kg^{-1}; and $pH_{H2O\ (1:2,5)}$ 5.2.

The inocula of *C. etunicatum*, *G. albida* and *A. longula* were obtained from the UFPE, multiplied in soil + 10% vermicompost, using millet (*Panicum miliaceum* L.) as host and stored for 22 months at 4°C until the moment of use.

After 225 days under protected roofing, the seedlings were transplanted to the experimental field. On this occasion, they had, on average, 72 cm of height, 5.1 mm of stem diameter, 14 leaves, and a foliar concentration of total chlorophyll of 50.07 FCI (Falker Chlorophyll Index). In the field, the seedlings were planted in holes of 0.4 × 0.4 × 0.4 m, with a spacing of 5 m of one to the next, in rows with four plants, with a distance of 5 m between the rows, with four rows per experimental plot, resulting in a density equivalent to 400 plants ha^{-1}. Since each of the four treatments was replicated in six plots and each plot had 4 plants, the total number of transplanted seedlings was 96.

Before transplanting, 10 points were marked zigzagging in the experimental area, from which soil samples were collected, in the layers from 0 to 20 and 20 to 40 cm depth. The samples were mixed to form two composite samples that were analyzed for their

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Table 1. Chemical characteristics of the soil of the experimental area at depths of 0 to 20 and 20 to 40 cm.

Soil depth	P (mg dm^{-3})	$cmol_c$ dm^{-3}					O.M. (g kg^{-1})	pH (H_2O[1:2,5])	C.E. (mS cm^{-1})
		K	Ca	Mg	Na	Al			
0-20	10.38	0.24	1.4	0.5	0.03	0.00	0.41	6.2	0.21
20-40	9.61	0.14	0.7	0.3	0.01	0.00	0.23	5.6	0.21

O.M.: Organic matter; E.C.: electric conductivity.

chemical characteristics (Table 1). The average density of the AMF spores in the samples was 54 spores per 100 g of soil. The area was plowed, disked and holes were made in each of which 5 L of vermicompost and 150 g of simple superphosphate were applied. The vermicompost had the following characteristics: P, 4.8 g kg^{-1}; C, 279 g kg^{-1}; N, 32.3 g kg^{-1}; K, 0.8 g kg^{-1}; Ca, 34.2 g kg^{-1}; $pH_{(H2O-1:2,5)}$ 6.65**;** Mg, 4.14g kg^{-1}; Cu, 36 mg kg^{-1}; and Mn, 823 mg kg^{-1}.

After transplanting, the area was irrigated with a semi-automatic drip system for 15 min on alternate days (8.4 L $plant^{-1}$ h^{-1}). The plants received daily care such as weeding and manual picking of herbivorous insects until evaluations were done seven months after transplanting. The following characteristics were evaluated: height, stem diameter, number of leaves, concentrations of chlorophyll, phenols, tannins and gallic acid in the leaves; number of glomerospores in the soil; and proportion of mycorrhizal colonization in the roots. Measurements were taken from the two central plants of each plot.

Phytochemicals analysis

Chlorophyll concentrations were measured with a clorofiLOG CFL1030 electronic meter. The total phenols and tannins were quantified by the Fiolin-Ciocalteu method and that of casein precipitation, respectively (Monteiro et al., 2006), using a foliar ethanol extract obtained by maceration (Oliveira et al., 2013).

Quantitative analysis by high performance liquid chromatography-photodiode array (HPLC-PDA)

Sample preparation

The herbal drug of leaves from *L. ferrea* was prepared by reflux in water bath (85 to 90°C) and using water as solvent. The extraction was performed in the round-bottom flask containing 0.5 g of drug and 15 ml of water by 30 min. The extractive was cooled to room temperature (25°C), filtered through cotton and transferred to 25 ml volumetric flask and the volume was adjusted with the same solvent. An aliquot of 5 ml of that solution was diluted in a 10 ml volumetric flask and the volume was completed with distilled water. The samples were filtered through membrane of polyvinylidene fluoride (PVDF) with a diameter of 25 mm and a pore size of 0.45 µm and transferred to vials.

Experimental conditions

The analyses was performed in HPLC-PDA (Ultimate 3000, Dionex®). Optimum separation was achieved with a column Dionex® (C_{18}, 250 × 4.6 mm, 5 µm) and pre-column (C_{18}; 4 mm; 3.9 µm). The mobile phase consisted of phase A: water ultrapure and phase B: metanol HPLC grade, both acidified with trifluoroacetic acid (0.05 %) and degasified in an ultrasound bath (Ultracleaner®). The phases were filtered through a membrane with 0.45 µm pore size before use. A gradient elution was carried out as follows: 0 to 10 min, 25% phase B; 10 to 11 min, 40% phase B and 11 to 12 min, 25% phase B; and at flow rate of 0.8 ml/min. The sample injection volume was 20 µl and the column was kept under controlled room temperature (24°C). The eluent was monitored by a PDA, and the detection wavelength was set at 280 nm. Data were analyzed using Chromeleon® software. A gallic acid standard solution was used as reference (>96%, Sigma Aldrich®). The gallic acid contents were expressed in gram percent, calculated in accordance to a standard calibration curve.

AMF analysis

The rizospheric soil was collected from the surface layer at a depth of 0 to 20 cm in three equidistant points from the stem and the glomerospores were quantified according to the methodologies of Gerdemann and Nicolson (1963) and Jenkins (1964). The roots were separated from the soil, diaphanized with KOH (10%, w/v) and H_2O_2 (10%, v/v), stained with Trypan blue in lactoglycerol, at 0.05% (Phillips and Hayman, 1970), and the mycorrhizal colonization was determined using the intersection of quadrants by the gridline intersecting method, according to Giovannetti and Mosse (1980).

Data analysis

The data were submitted to analysis of variance (ANOVA) and the means compared by the Tukey test (5%), using the Assistat 7.6 program (Assistat, 2011).

RESULTS AND DISCUSSION

The ironwood plants associated with *C. etunicatum* as well as with *A. longula*, were significantly taller than those of the non-inoculated control, while those inoculated with *G. albida* did not differ from the control (Table 2). The stem diameters, in spite of being 13% larger in plants inoculated with *C. etunicatum* and 10% larger in those associated with *A. longula*, did not differ significantly from the diameter of the control plants, nor did the number of total leaves differ significantly. Benefits of inoculation with AMF are not always observed in arboreal species transplanted to the field since the response differs according to the measured parameter and the species in question (Smith and Read, 2008). Vandresen et al. (2007), for example, did not observe any benefits of

Table 2. Height, number of leaves, stem diameter, colonization and spore density in *Libidibia ferrea* plants, inoculated or non-inoculated with arbuscular mycorrhizal fungi (AMF), seven months after transplanting to the field, in Petrolina, PE.

Variable	Inoculation treatment			
	Control	*Gigaspora albida*	*Claroideoglomus etunicatum*	*Acaulospora longula*
Height (m)	1.49[b]	1.52[b]	1.74[a]	1.65[a]
Number of leaves	45.75[a]	42.70[a]	41.42[a]	39.67[a]
Stem diameter (mm)	16.16[a]	16.55[a]	18.22[a]	17.76[a]
Colonization (%)	64.43[ab]	53.58[b]	73.97[a]	65.28[ab]
Spore density (100 g solo^{-1})	38.80[a]	44.33[a]	34.25[a]	34.25[a]

Averages (n= 6) on the line followed by the same letter do not differ according to the Tukey test (5%).

Table 3. Concentrations of chlorophyll, total phenols and tannins and gallic acid in *Libidibia ferrea* plants, inoculated or non-inoculated with arbuscular mycorrhizal fungi (AMF), seven months after transplanting to the field, in Petrolina, PE.

Variable	Inoculation treatment			
	Control	*Gigaspora albida*	*Claroideoglomus etunicatum*	*Acaulospora longula*
Chlorophyll (Falker Index)	65.3[b]	77.5[a]	66.7[b]	67.8[b]
Phenols (mg g planta^{-1})	2.11[ab]	2.16[a]	2.13[ab]	2.08[b]
Tannins (mg g planta^{-1})	2.02[ab]	2.09[a]	2.08[a]	1.99[b]
Gallic acid (g %)	0.038[b]	0.027[d]	0.046[a]	0.033[c]

Averages (n=6) on the line followed by the same letter do not differ from the Tukey test (5%).

inoculation in the height of five native tree species that were transplanted to the field in Paraná State, South of Brazil.

Although the plants that were inoculated with *G. albida* did not grow more than those of the control, the inoculation favored the accumulation of total chlorophyll (19%) in relation to the control (Table 3). Therefore, the symbiosis with the inoculated species or the change in composition of the symbiosis caused by the inoculation was efficient in increasing the levels of photosynthesizing pigments, which are important to the plant anabolism. Reports on the effects of AMF inoculation on leaf chlorophyll contents of Caatinga species were not found, but increased contents have been observed in inoculated species of other regions, such as those of *Gloriosa superba* L. inoculated with *Acaulospora laevis* (Yadav et al., 2013).

The density of glomerospores in the rizosphere and mycorrhizal colonization in plants of the inoculated treatments did not differ significantly from the density and colonization of the control treatment (Table 2). Positive responses have been reported by other researchers, like Gupta et al. (2002) who registered a higher rate of mycorrhizal colonization in plants of *Mentha arvensis* L. that were inoculated and kept in the field than in the control treatment. It is likely that the duration of our experiment was not sufficient to provoke detectable alterations in AMF colonization and reproduction. Higher colonization is usually reported following inoculation in pot experiments with sterilized substrates since the native AMF species are eliminated or substantially decreased in the control treatment (Oliveira et al., 2013).

There were no increases in the concentrations of total phenols and tannins in the inoculated plants in relation to the control (Table 3). Although no other measurements were found with ironwood plants, increases were registered in the contents of total phenols in *Myracrodruon urundeuva* (Engl.) Fr. All. (Oliveira et al., 2013), *C. roseus* L.G. Don (Rosa-Mera et al., 2011), *Cynara cardunculus* L. (Ceccarelli et al., 2010), *Echinacea purpurea* L. (Araim et al., 2009) and also in the contents of tannins in *Wedilla chinensis* (Osbeck) Merril. (Nisha and Rajeshkumar, 2010) and *Plectranthus amboinicus* (Lour) Spreng (Rajeshkumar et al., 2008). However, these patterns of increase are not always found and Geneva et al. (2010) and Nell et al. (2009), for example, also did not observe any increase in the contents of total phenols in *S. officinalis* L. High levels of photosynthetic pigments are proposed mechanisms to explain the influence of AMF on the increased concentrations of secondary compounds in medicinal plants (Dave and Tarafdar, 2011), but such a mechanism does not seem to have influenced the concentration of secondary compounds in ironwood plants that were inoculated with AMF.

Although the inoculation of AMF did not significantly

increase the concentration of total phenols, inoculation with *C. etunicatum* increased the production of gallic acid (21%) in relation to the non-inoculated control treatment and also in relation to the other inoculation treatments (Table 3). A proposed mechanism to explain the AMF influence on the increases of secondary compound concentrations is the activation of metabolic pathways (Zimare et al., 2013). Among these, the shikimic acid pathway, a precursor of phenolic compounds (Heldt, 2005) is one of the relevant ones and could justify the increase in foliar gallic acid concentration in plants inoculated with *C. etunicatum*. Furthermore, this increase was dependent on the AMF used (Copetta et al., 2006), which confirms the initial working hypothesis. Experiments carried out in the field with other species validate the application of mycorrhizal fungi with the objective of increasing the biosynthesis of secondary compounds with therapeutic characteristics as was observed by Ceccarelli et al. (2010) in plants of *C. cardunculus*, with an increased production of total phenols, and by Gupta et al. (2002), in cultivars of *M. arvensis*, with a high concentration of essential oils.

Leaves of ironwood are used by the local population because of their medicinal properties (Albuquerque et al., 2007). Mycorrhizal technology, applying *C. etunicatum*, can aggregate value to the phytomass of ironwood commercialized for the phytotherapeutic industry, considering the production of gallic acid. Farmers of the São Francisco Valley, where the study was performed, could cultivate ironwood and sell the leaves to the pharmaceutical industry, contributing to a reduction in the extractivism of this species, which is not abundant in the native caatinga vegetation.

Future studies must analyze the effect of mycorrhizal inoculation on the production of other foliar compounds of pharmaceutical importance in ironwood such as ellagic acid and pauferrol A.

Conclusion

Mycorrhizal inoculation can favor the growth of *L. ferrea* established under field conditions and increase the production of gallic acid, with the benefits varying according to the inoculated AMF. Cultivation of inoculated *L. ferrea* could be an alternative, instead of collecting from the native vegetation, in order to supply raw materials with a better quality to the phytotherapeutic industry.

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Conflict of Interest

Authors have not declare any conflict of interest.

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