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Biotechnical application of arbuscular mycorrhizal fungi used in the production of foliar biomolecules in ironwood seedlings [*Libidibia ferrea* (Mart. ex Tul.) L.P.Queiroz var*. ferrea*]

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***Libidibia ferrea*, popularly known as ironwood is a medicinal plant of the Caatinga, in semi-arid Brazil. It is used for traditional medicine because it has bioactive compounds. Arbuscular mycorrhizal fungi (AMF) have been shown to improve the production of biomolecules in some plants. The objective of this study was to select efficient AMF for optimizing the growth and production of bioactive compounds in *L. ferrea* seedlings. Mycorrhization, mainly with *Gigaspora albida,* was efficient in improving the production of *L. ferrea* seedlings. It resulted in a larger stem diameter, higher chlorophyll *a* leaf content, higher amount of total proteins and flavonoids compared to that of non- mycorrhized seedlings. The biotechnological system using *G. albida* is an alternative for the production of *L. ferrea* seedlings, with increased levels of foliar flavonoids.**

Key words: Glomeromycota, *Libidibia ferrea*, flavonoids, Leguminosae, semi-arid.

INTRODUCTION

A native tree of the Caatinga, *Libidibia ferrea* (Mart. ex Tul.) L.P.Queiroz var. *ferrea (*Fabaceae), also known as ironwood has various uses: it restores degraded forest areas, used for soil recovery and medicine (Maia, 2004). Parts of the plant are used in traditional medicine to treat various diseases due to its antiulcerogenic, anti-inflammatory, anti-cancerogenic, anti-histaminic, anti-microbial, anti-coagulant and cicatrizing properties (Bacchi et al., 1995; Carvalho et al., 1996; Nakamura et al., 2002; Gonzalez, 2005; Sampaio et al., 2009; Cavalheiro

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et al., 2009; Oliveira et al., 2010). These therapeutic characteristics are related to the presence of secondary compounds such as flavonoids, phenols, tannins, saponins, coumarins, anthraderivatives, quinines (Gonzalez, 2005; Almeida et al., 2005) present in the leaves, fruits, skin and roots of the plant (Maia, 2004).

Arbuscular mycorrhizal fungi (AMF) form a mutualistic symbiosis with the majority of the plants, where nutrients are exchanged between the partners. Mycorrhization is beneficial to the host plant as it stimulates the growth of the seedlings (Machineski et al., 2009) and accumulates nutrients in the aerial parts (Ngwene et al., 2010), besides protecting the plant from pathogens (Elsen et al., 2008). The association between AMF and *L. ferrea* has been documented (Carneiro et al., 1998; Gattai et al., 2011), but now there is no information on the effects of mycorrhization on the production of secondary compounds. Recent studies have shown that mycorrhizal symbiosis, with its medicinal potential which can be an alternative for maximizing the production of chemical compounds (Ratti et al., 2010; Oliveira et al., 2013), with the produced phytomass having a higher concentration of active principles (Toussaint et al., 2007; Chaudary et al., 2008; Ratti et al., 2010; Ceccarelli et al., 2010; Dave and Tarafdar, 2011; Karagiannidis et al., 2011).

In this study, we tested the hypothesis that inoculation with AMF increases the concentration of foliar phytochemicals in *L. ferrea.* Therefore, the objective of this study is to evaluate the production of foliar bioactive compounds in ironwood seedlings based on inoculation with AMF.

MATERIALS AND METHODS

The experiment was carried out at the Experimental Protected Cropping Unit of the University of Pernambuco – *Campus,* Petrolina, located in the municipality of Petrolina – PE (Northeastern Brazil), between August, 2011 and March 2012. According to Köeppen classification, the climate in the region is Bswh [Semi-arid, with high temperatures (> 22°C) and scarce rainfall in winter (< 250 mm)].

Experimental design

The experimental design was completely randomized with four inoculation treatments: (1) non-inoculated control and inoculated with: (2) *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schubler (UFPE 06), (3) *Gigaspora albida* N. C. Schenck & G. S. Sm. (UFPE 01) and (4) *Acaulospora longula* Spain & N. C. Schenck (UFPE 21), with five replicates (one plant per pot). Ironwood seeds were chemically scarred with sulfuric acid for 20 min to break dormancy (Biruel et al., 2007). They were washed with running water and subsequently with distilled water and allowed to germinate in recipients (50 ml) containing medium-sized granulated vermiculite. Non-disinfected latosoil was collected in the native Caatinga area (Km 152), Petrolina-PE and mixed with 5% vermicompost. The soil was characterized as follows: organic material, 3.21 g kg^{-1}; pH, 5.2; electric conductivity, 3.53 ds m^{-1}; 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}. Plantlets with two definite leaves were transferred to pots with 1.2 kg substrate and inoculated with soil-inoculum containing 200 glomerospores + hyphae + colonized roots of each tested AMF. The experiment was maintained in a protected cropping unit under environmental conditions of temperature ($T_{max.}$ 38.57°C and $T_{min.}$ 24.97°C) and relative humidity of the air ($RH_{max.}$ 84.50 % and $RH_{min.}$ 33.09%). The plants were irrigated daily. After 225 days, the experiment was evaluated: height, number of leaves, stem diameter and fresh and dry mass of the aerial part and of the roots and mycorrhizal colonization were determined. To determine the dry mass, the plant material was maintained in a drying oven (45°C) to constant weight. The dry matter of the aerial and subterranean part was determined separately. The chlorophyll content was estimated *in vivo* using a CFL1030 – electronic measuring device clorofiLOG and expressed in ICF (Falker® chlorophyll index) (Falker Automatação Agrícola, Brazil). The mycorrhizal colonization was determined by the gridline intersect method (Giovannetti and Mosse, 1980) in clear (100 g L^{-1} KOH and 100 ml L^{-1} H_2O_2) and 0.05 g 100 ml^{-1} Trypan blue stained roots (Phillips and Hayman, 1970).

Reagents

The following chemical products were used: glacial acetic acid, sulfuric acid, ethyl alcohol, methyl alcohol and sodium carbonate from F Maia (Cotia, SP, Brazil); bovine serum albumin and rutin hydrate from Sigma-Aldrich (São Paulo, SP, Brazil); tannic acid, casein, aluminum chlorate, Coomassie blue G-250, glucose, phosphoric acid, phenol, pyridine from Vetec (Duque de Caxias, RJ, Brazil); Folin–Ciocalteu's reagent from Merck (Rio de Janeiro, RJ, Brazil).

Preparation of plant extract

After drying, 500 mg of leaves was perforated and transferred to amber glass bottles and 20 ml of ethanol (950 ml L^{-1}) was added. After 12 days of maceration (20°C), which was protected from light, the extract was filtered in gauze and re-filtered with qualitative paper filter and stocked in an amber flask (-4°C) (Brito et al., 2008). In the extract, total proteins, soluble carbohydrates, phenols, flavonoids and total tannins were quantified as follows.

Total proteins

Total proteins were determined by the Bradford method (1976), modified. In a test tube, 50 µl of the ethanol extract and 2.5 ml of the Bradford reagent were added, after which they were stirred in a vortex mixer (Vision Scientific Co., Ltd., Korea). After five minutes, the readings were taken with a photo-spectrometer (595 nm) (Spectrum SP 2000 UV, Castelnuovo, Italy), using the BSA (bovine serum albumin) as the standard.

Soluble carbohydrates

The quantification was done based on the modified method of Dubois et al. (1956); 50 µl of the ethanol extract, 95 µl of distilled water, 50 µl of phenol (800 g L^{-1}) were put in a test tube with a screw

Table 1. Significance levels (levels of *p*).

Variable	Significance level
Concentration of total proteins	*
Concentration of soluble carbohydrates	*
Concentration of total phenolics	Ns
Concentration of total flavonoids	*
Concentration of total tannins	Ns
Total Chlorophyll	*
Chlorophyll *a*	*
Chlorophyll *b*	*
Leaf number	Ns
Stem diameter	**
Fresh matter of the aerial part	Ns
Dry matter of the aerial part	Ns
Fresh matter of the of the subterranean part	Ns
Dry matter of the of the subterranean part	Ns
Height	Ns
Mycorrhizal colonization	**

*(*p*<0.05); **(*p*<0,01); Ns (non-significant).

screw cap, followed by intense shaking in a vortex mixer. Afterwards, 2 ml of sulfuric acid was added and after incubation (10 min/22°C), photo-spectrometric readings were taken (490 nm). Glucose was used for the standard curve.

Total phenols

Total phenols were determined following the method of Monteiro et al. (2006). 2 ml of the extract, 5 ml of Folin-Ciocalteu's reagent (100 ml L^{-1}), 10 ml of sodium carbonate (75 g L^{-1}) and volume of 100 ml completed with distilled water were put in a volumetric flask (100 ml). After 30 min at rest, the absorption rates were taken (760 nm). Tannic acid was used as a standard.

Total tannins

The casein precipitation method as described by Monteiro et al. (2006) was used to quantify total tannins. Three ml of the extract and 0.5 g of casein powder were added to an amber glass bottle, which was subsequently agitated (160 rpm). Afterwards, the samples were filtered with a completed volume of 25 ml in a volumetric flask and the quantification was carried using the Folin-Ciocalteu's method. The concentration of tannins was obtained by the difference between the value found for this reading and the one obtained for the quantification of total phenols.

Total flavonoids

The levels of flavonoids were estimated as described by Araújo et al. (2008) (modified): 1 ml of the ethanolic extract, 0.6 ml of glacial acetic acid, 10 ml of pyridine: methanol solution (200 ml L^{-1}) and 2.5 ml of an methanolic solution of aluminum chlorate (50 g L^{-1}), with a final volume of 25 ml completed with distilled water were added to a volumetric flask. After 30 min at rest, the absorption was measured (420 nm), using rutin for the preparation of the standard curve.

Data analysis

Analysis of variance (ANOVA) was used to analyze the data and means were compared by the Tukey test (5%) using the Assistat 7.6 program.

RESULTS

The treatments with inoculation affected ($p < 0.05$) the following variables: concentration of soluble carbohydrates, total proteins, total flavonoids, chlorophyll (total *a* and *b*), stem diameter and mycorrhizal colonization (Table 1). Benefits of mycorrhization for the growth of *L. ferrea* were found only for stem diameter, when the seedlings were associated with *G. albida* (Table 2). The effect of the mycorrhizal inoculation was more apparent for other parameters such as the level of chlorophyll and the accumulation of biomolecules (Table 3). The colonization of the ironwood roots did not differ between the inoculation treatments, but was statistically higher than the non-inoculated plants (Table 3). The inoculation with *G. albida* increased the production of photosynthetic pigments in the leaves of *L. ferrea;* there was an increase in the concentration of total proteins, compared to the non-inoculated treatment (Table 3). However, the concentration of soluble carbohydrates was not optimized by the inoculation (Table 3). The concentration

Table 2. Height, fresh matter (FMAP) and dry matter (DMAP) of the aerial part, fresh matter (FMSP) and dry matter (DMSP) of the subterranean part, number of leaves, diameter of the stem of ironwood seedlings, associated or not with inoculated arbuscular mycorrhizal fungi (AMF), 225 days after the inoculation, in an experimental protected cropping unit.

Variable	Inoculation treatment			
	Control	*Gigaspora albida*	*Claroideoglomus etunicatum*	*Acaulospora longula*
Height (cm)	31.98[a]	32.36[a]	27.86[a]	32.46[a]
FMAP (g)	4.27[a]	3.99[a]	3.49[a]	4.23[a]
DMAP (g)	2.17[a]	2.28[a]	1.95[a]	2.33[a]
FMSP (g)	4.82[a]	4.28[a]	4.04[a]	3.65[a]
DMSP (g)	2.36[a]	1.89[a]	2.03[a]	1.89[a]
Leaf number	10.00[a]	10.80[a]	8.80[a]	10.80[a]
Stem diameter (mm)	3.42[b]	3.81[a]	3.39[b]	3.57[ab]

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

Table 3. Concentration of foliar biomolecules in ironwood seedlings, associated or not with inoculated arbuscular mycorrhizal fungi (AMF), 225 days after inoculation in experimental protected cropping.

Variable	Inoculation treatment			
	Control	*Gigaspora albida*	*Claroideoglomus etunicatum*	*Acaulospora longula*
Total proteins*	66.39[b]	100.33[a]	66.04[b]	86.24[ab]
Soluble carbohydrates	0.22[ab]	0.32[a]	0.21[b]	0.23[ab]
Total phenolics*	2.74[a]	3.41[a]	2.74[a]	2.98[a]
Total flavonoids*	0.92[b]	1.27[a]	0.99[b]	1.11[ab]
Total tannins*	2.70[a]	2.98[a]	2.71[a]	2.94[a]
Total Chlorophyll**	25.37[b]	44.21[ab]	39.27[ab]	45.35[a]
Chlorophyll *a***	3.42[b]	3.81[a]	3.39[b]	3.57[ab]
Chlorophyll *b***	9.93[ab]	12.52[a]	7.84[b]	10.31[ab]
Mycorrhizal colonization (%)	6.20[b]	41.99[a]	53.45[a]	39.68[a]

*(mg g plant^{-1}); **(FCI – Falker Chlorophyll Index). Averages followed by the same letter on the line do not differ from the Tukey test (5 %).

concentration of phenols and tannins in leaves of ironwood did not vary among the inoculation treatments (Table 3), but the association with *G. albida* increased the production of total foliar flavonoids in the seedlings (Table 3).

DISCUSSION

Benefits of mycorrhization for classic growth parameters are well documented (Cavalcante et al., 2001; Copetta et al., 2006) for some leguminosae but not for all species, as shown in *Copaifera martii* (Caldeira et al., 1997). To define the mycorrhizal efficiency, it is important to consider variables that represent the physiology of the photobiont (Huang et al., 2011; Ratti et al., 2010; Zubek et al., 2010). The mycorrhizal colonization was higher in inoculated seedlings than in the control. With similar results, Caldeira et al. (1997) observed that *Gigaspora margarita* produced more structures into roots of *C. martii* seedlings than the native fungi. Conversely, Zubek et al. (2010) observed that native fungi produced 90% of colonization in roots of *Inula ensifolia*, a value which was higher than that of the inoculated treatments.

The seedlings of *L. ferrea* inoculated with *G. albida* increased pigment concentration and protein in the leaves (Table 3). Similarly, in *Catharanthus roseus*, inoculation with *Glomus mosseae* promoted an increase in the concentration of total chlorophyll and chlorophyll *a*, while *Glomus fasciculatum* increased the concentration of chlorophyll *b* in relation to the control (Ratti et al., 2010). The efficiency of the FMA in increasing the protein

concentration was also documented in other medicinal plants such as *C. roseus* (Ratti et al., 2010) and *Echinacea purpurea* (Araim et al., 2009). Such improvement is generally the result of the activity of the arbuscules that optimize the biosynthetic pathways of mitocondrias, specifically the Krebs cycle (Lohse et al., 2005), increase the photosynthetic rate (Wright et al., 1998) and promote the vegetative anabolism.

The biosynthesis of phenolic compounds in medicinal plants can be improved by biotic factors, especially the AMF (Ceccarelli et al., 2010; Toussaint et al., 2007; Khaosaad et al., 2008) but the concentration of foliar phenols and tannins in ironwood do not vary among the inoculation treatments. Thus, the determination of mycorrhizal efficiency should be carried out before recommending mycorrhizal technology to increase the production of phenolic compounds in each plant species.

The inoculation with *G. albida* increased the production of total foliar flavonoids in ironwood seedlings, as also observed in red clover (Khaosaad et al., 2008). In the treatment with *G. albida*, 1.27 mg of flavonoids g plant^{-1} was quantified. In mycorrhized *Salvia officinalis,* Geneva et al. (2009) registered 0.022 mg flavonoids g plant^{-1}. These results indicate the potential of using leaves of mycorrhized ironwood as source of flavonoids. These are important compounds in the pharmaceutical industry due to their therapeutic properties (Zuanazzi and Montanha, 2003). The benefits of foliar flavonoids in ironwood in production can be attributed to the nutritional state of the plants, defense reactions and changes in the hormonal profile (Toussaint et al., 2007; Larose et al., 2002; Coppeta et al., 2006; Kapoor et al., 2002), besides the major activity of the routes of the shikimic acid, of acetate via malonic acid and precursor routes in the biosynthesis of these compounds (Zuanazzi and Montanha, 2003). Based on the obtained results, the initial working hypothesis has been proven, whereby inoculation interferes in the production of compounds with medicinal potential, in which responses vary according to the inoculated AMF.

The inoculation with AMF increases growth and optimizes the physiology of *L. ferrea s*eedlings but the benefits depend on the tested isolate. The biotechnological system using *G. albida* is more efficient, providing an alternative for the production of seedlings with increased levels of bioactive compounds, such as flavonoids. The use of mycorrhizal technology can be a promising alternative for the cultivation of *L. ferrea,* which leads to a major increase in the production of bioactive compounds, with medicinal properties, especially in the case of total flavonoids and total foliar proteins. Cultivars of this species can be established and also serve as an alternative culture for small local producers, leading to the avoidance of extractive use. Field tests and high performance liquid chromatography (HPLC) analysis should be carried out to validate the technology and characterization of flavonoids produced by mycorrhized *L. ferrea*.

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