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Efficiency of the AMF in increasing the production of foliar bioactive compounds in A. cearensis seedlings

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Amburana cearensis **(Allemao) A.C. Smith is a widely used legume by the population due to its medicinal properties. This species establish symbiosis with the arbuscular mycorrhizal fungi (AMF) that can increase the production of secondary metabolites, a fact which has not been clarified for this plant. Therefore, the aim of this study was to examine the contribution of the AMF in the production increase of foliar bioactive compounds in *A. cearensis* seedlings. The experiment which under-goes protected roofing was carried out using four inoculation treatments: non-inoculated control treatment, inoculated with *Gigaspora albida*, inoculated with *Claroideoglomus etunicatum* and inoculated with *Acaulospora longula.* After 160 days, the following was examined: dry matter of the aerial part, chlorophylls *a*, *b* and total, soluble carbohydrates, total proteins, total phenols, total flavonoids and total tannins. *A. cearensis* seedlings inoculate with *C. etunicatum* accumulated more dry matter of the aerial part (78.38%), total chlorophylls (24.28%) and chlorophylls *b* (53.63%), total phenols (47.82%), total flavonoids (32.28%) and total tannins (61.58%) in relation to the control treatment. Mycorrhizal technology using the *C. etunicatum* fungus is an alternative to increase the levels of foliar bioactive compounds in *A. cearensis* seedlings.**

Key words: Caatinga, phenolic compounds, arbuscular mycorrhizal fungi (AMF).

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are inhabitants of the soil and belong to the Phylum Glomeromycota (Schubler et al., 2001). Such organisms are obligatory symbionts because they complete their life-cycle only in the presence of a host plant (Souza et al., 2008). After the fungus has established on the root, the AMF absorb

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water and nutrients from the soil and in exchange the phytobiont makes about 20% of carbon available for the development of the fungus (Smith and Read, 2008).

Various studies relate the benefits of mycorrhizal association with legumes and point out the increased vegetable growth and optimized production of primary (Manoharan et al., 2010) and secondary metabolites (Silva et al., 2014a; Nisha and Rajeshkumar, 2010; Kapoor et al., 2004).

The increase in the production of secondary compounds in plants associated with AMF may be due to the increased nutritional supply (Toussaint et al., 2007), hormonal changes, enzymatic activation (Zhang et al., 2013) and increased activity of plastidial and mitochondrial pathways (Lohse et al., 2005), however, the effects seem to be somatory and multifactorial (Toussaint et al., 2007).

The Caatinga is a biome that is rich in leguminous species with medicinal properties that are widely used by the local population as phytotherapeutic drugs (Agra et al., 2007, 2008). *Amburana cearensis* is found among the medicinal plants of the Caatinga, a legume that is used by the local population. Parts of this plant, such as the stem, the seeds and bark are used in the production of pastilles, syrups and teas for the treatment of various diseases due to their antioxidant (Leal et al., 2003), anti-inflammatory (Leal et al., 2008), antifungal (Santos et al., 2009), antibacterial (Figueiredo et al., 2013) and antineoplastic (Costa-Lotufo et al., 2003) properties. Such therapeutic benefits have been attributed to the presence of secondary compounds, especially phenolic compounds (Canuto and Silveira, 2006; Bravo et al., 1999). However, it is unknown in mycorrhizal that inoculation influences the increase in the production of secondary metabolites in *A. cearensis* seedlings. Therefore, the following hypothesis was tested: inoculation with AMF increases the production of bio-active compounds in *A. cearensis* with the benefits de-pending on the fungus that was tested. The aim of this study was to examine the efficiency of the AMF in increasing the production of foliar bioactive compounds in
A. cearensis seedlings.

MATERIALS AND METHODS

Plant, AMF and experimental implementation

A. cearensis seeds were disinfected with 20% of NaClO (2% of active chlorine) for 2 min, washed in distilled water and put to germinate in plastic pots containing sterilized soil (autoclave at 121°C/30 min/2 consecutive days).

Three AMF isolates were tested: *Acaulospora longula* Spain & N.C. Schenck (UFPE 21), *Claroideoglomus etunicatum* (W. N. Becker & Gerdemann) C. Walker & A. Schussler (UFPE 06) and *Gigaspora albida* N.C. Schenck & G.S. Sm. (UFPE 01). The inoculums were supplied by the Department of Mycology from the Federal University of Pernambuco, Brazil, multiplied on millet (*Panicum miliaceum* L.) and stocked at 4°C, for 26 months, until the moment of inoculation.

Black polyethylene pots were filled with non-sterilized soil, which was collected from the Caatinga region and showed the following chemical characteristics: organic material, 3.21 g kg^{-1}; pH, 5.2; electric conductivity, 3.53 dSm^{-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}. The following AMF were identified in this soil: *Appendicispora appendicula* Spain, Sieverd. & Shenck, *Acaulospora scrobiculata* Trappe, *Acaulospora* sp.1, *Glomus macrocarpum* Tul. & Tul., *Glomus* sp.1 and *Scutellospora* sp.1 (Lima, 2014).

Plantlets with two definite leaves were transferred to the pots and inoculated at the root region with soil-inoculum of the tested AMF (200 glomerospores + colonized roots + hyphae). *A. cearensis* seedlings remained under experimental roofing for 160 days at the University of Pernambuco – Campus Petrolina, Brazil, under ambient temperature conditions (minimum: 21.7°C and maximum: 29.7°C), relative air humidity (42%) and an average global radiation (461.8 ly/day).

Evaluation of the experiment and preparation of the extract

The experiment was evaluated 160 days after inoculation. Chlorophylls (total, *a* and *b*) were tested *in vivo*, using the CFL1030 – an electronic chlorophyll level meter ClorofiLOG (Silva et al., 2014a). After examining chlorophyll, the aerial part was separated from the roots and dried (45°C) for 3 consecutive days to determine the dry matter of the aerial part. The subterranean part was removed from the substrate and the fine roots were separated from the stylopodium, washed and preserved in ethanol (50%) until examination.

Aliquots (100 mg) of the leaves were punctured and put in amber flasks containing 20 ml of ethanol (95% v/v) and maceration lasted 12 days at 25°C. After this period, the extract was filtered with gauze and refiltered with qualitative paper filter and stocked in amber flasks (- 4°C) (Brito et al., 2008). The extract was used to quantify the biomolecules.

Analysis of soluble carbohydrates and total proteins

Total proteins were quantified by a modification of the Bradford (1976): 50 µl of the extract was added to 2.5 ml of Bradford reagent and readings were taken with a spectrophotometer (595 nm) with a standard BSA curve (Bovine Serum Albumin). Total soluble carbohydrates were determined by a modification of the Dubois et al. (1956) method. The following was added to a test tube: 20 µl of the plant extract, 95 µl of distilled water, 50 µl of 80% phenol (w/v) and 2 ml of sulfuric acid. Readings were taken with a spectrophotometer (490 nm) and glucose was used to prepare the standard curve.

Analysis of phenols, flavonoids and total tannins

Total phenols were determined by a modification of the Folin-Ciocalteu method (Monteiro et al., 2006). The following was added to 100 ml volumetric balloons: 1 ml of the plant extract, 5 ml of the Folin-Ciocalteu reagent (10%, w/v) and 10 ml of sodium carbonate solution (7.5%, w/v) and the volume was completed with distilled water. Readings were taken with a spectrophotometer (760 nm) and tannic acid was used to prepare the standard curve.

Total flavonoids were quantified by a modification of the Araújo et al. (2008) method. The following was added to 25 ml flasks: 1 ml of the plant extract, 0.6 ml of glacial acetic acid, 10 ml of pyridine-methanol solution (2:8, v/v) and 2.5 ml of aluminum chlorate (5% w/v, in absolute methanol) and the volume was completed with distilled water. Readings were taken with a spectrophotometer

Table 1. Analysis of variance for the studied variables.

Variable	Effect
Dry matter of the aerial part	**
Total chlorophyll	*
Chlorophyll *a*	ns
Chlorophyll *b*	**
Mycorrhizal colonization	**
Concentration of total proteins	ns
Content of total proteins	*
Concentration of soluble carbohydrates	ns
Content of soluble carbohydrates	ns
Concentration of total phenols	*
Content of total phenols	**
Concentration of total flavonoids	**
Content of total flavonoids	**
Concentration of total tannins	*
Content of total tannins	**

*p ≤ 0.05; **p ≤ 0.01; ns: Non-significant.

(420 nm) and rutin was used to prepare the standard curve.

Analysis of total tannins was carried out with a modification of the Monteiro et al. (2006) method: 3 ml of the plant extract was mixed with 0.5 g of casein and the mixture was kept under agitation for 3 h at 25°C (160 rpm). After this period, the material was filtered with qualitative paper filter and the resulting volume was transferred to 25 ml volumetric balloons and completed with distilled water. Analysis of the remaining phenols was carried out by the Folin-Ciocalteu method and the concentration of total tannins corresponded to the difference between the levels found in this analysis and those found during quantification of total phenols.

Mycorrhizal colonization

For examination, the roots were bleached with KOH (10%, w/v, for 22 h), hydrogen peroxide (H_2O_2 10% v/v, for 20 min), acidified (HCl 1% v/v, for 5 min) and stained with Trypan blue (0.05% in lactoglycerol w/v, for 22 h) (Phillips and Hayman, 1970) . A physical examination is carried out using the intersection of quadrants method (Giovannetti and Mosse, 1980).

Reagents and equipment used

The following reagents were used: glacial acetic acid, sulfuric acid, ethyl alcohol, methyl alcohol, sodium carbonate, glycerin and hydrogen peroxide (F Maia, Cotia, Brazil); bovine serum albumin and rutin hydrate (Sigma-Aldrich, São Paulo, Brazil); lactic acid, tannic acid, casein, aluminum chloride, Coomassie blue G-250, Trypan blue, glucose, phosphoric acid, phenol, pyridine (Vetec, Duque de Caxias, Brazil) and Folin-Ciocalteu reagent (Merck, Rio de Janeiro, Brazil).

The following equipment was used: a vortex shaker (Vision Scientific, Korea), a magnetic stirrer with heating (Quimis, Diadema, Brazil), a vertical autoclave (Phoenix, Araraquara, Brazil), semi-analytic scales (Bel Engineering, Italy), a digital spectrophotometer (Biospectro, Curitiba, Brazil), a drying oven (Biopar, Porto Alegre, RS, Brazil), an electronic chlorophyll content meter – ClorofiLOG -CFL 1030 (Falker, Porto Alegre, Brazil) and an orbital (Marconi, Piracicaba, Brazil).

Experimental outline and statistical analysis

The experimental outline was entirely randomized with four inoculation treatments (AMF control, inoculated with *G. albida*, inoculated with *A. longula* or inoculated with *C. etunicatum*), with five repetitions, totaling 20 experimental units. The data were submitted for analysis of variance (ANOVA) and the means were compared by the Tukey test (5%) using the Assistat program (2013).

RESULTS AND DISCUSSION

The mycorrhizal treatments had no effect on the chlorophyll *a* content, on the concentration of total proteins and on the concentration and content of total carbohydrates (Table 1).

The dry matter of the aerial part (DMAP) increased when the seedlings were colonized by *G. albida* (52.70%) and *C. etunicatum* (78.37%), in relation to the non-inoculated control treatment (Table 2), which means that the mycorrhization with *G. albida* and *C. etunicatum* was beneficial for the growth of *A. cearensis*. Similar results were found by Araim et al. (2009), Baslam et al. (2011) and Toussaint et al. (2007), for *Echinacea purpurea*, in varieties of *Lactuta sativa* and in *Ocimum basilicum*, respectively.

Increased values of mycorrhizal colonization were found in the roots of inoculated plants in relation to the control (Table 2), which supports the results obtained for other Leguminosae, such as *Libidibia ferrea* (Silva et al., 2014a).

Table 2. Dry matter of the aerial part (DMAP), total chlorophyll *a* and *b* and mycorrhizal colonization (MC) in *Amburana cearensis* seedlings, inoculated or non-inoculated with arbuscular mycorrhizal fungi, 160 days after inoculation under experimental roofing.

Inoculation treatment	DMAP (g)	Total (FCI)	Chlorophyll *a* (FCI)	Chlorophyll *b* (FCI)	MC (%)
Control	0.74^{b}	36.98^{b}	29.26^{a}	7.72^{b}	7.40^{b}
Acaulospora longula	0.69^{b}	44.02^{ab}	31.82^{a}	10.40^{ab}	32.60^{a}
Gigaspora albida	1.13^{a}	45.88^{a}	34.16^{a}	1.72^{a}	36.78^{a}
Claroideoglomus etunicatum	1.32^{a}	45.96^{a}	34.18^{a}	11.86^{a}	33.68^{a}

Means followed by the same letter do not differ from the Tukey test (5 %). FCI: Falker chlorophyll index.

Table 3. Concentration and content of total proteins and foliar soluble carbohydrates in *Amburana cearensis* seedlings, inoculated or non-inoculated with arbuscular mycorrhizal fungi, 160 days after inoculation under experimental roofing.

Inoculation treatment	Total proteins		Soluble carbohydrates	
	Concentration (mg g plant^{-1})	Content (mg plant^{-1})	Concentration (mg g plant^{-1})	Content (mg plant^{-1})
Control	67.80^{a}	47.35^{ab}	150.79^{a}	112.03^{a}
Acaulospora longula	39.50^{a}	29.10^{b}	255.49^{a}	171.64^{a}
Gigaspora albida	73.90^{a}	84.08^{a}	168.38^{a}	198.13^{a}
Claroideoglomus etunicatum	65.70^{a}	68.63^{ab}	453.19^{a}	407.91^{a}

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

Inoculation with *G. albida* and *C. etunicatum* increased by 24.06 and 24.28% the concentration of total chlorophyll in relation to the non-inoculated control, respectively. Similar results were obtained for chlorophyll *b* (Table 2). On the other hand, the benefits of inoculation for chlorophyll *a* (Table 2) were not documented. As was suggested by Singh et al. (2012), the increase in chlorophyll content may be related to the increased nutrient absorption, taking into consideration that various studies indicate maximization in the production of photosynthetic pigments in terms of mycorrhization, which leads to an improvement of the nutritional status of the host (Selvaraj et al., 2009; Singh et al., 2012).

Mycorrhization did not alter the concentration and the total foliar protein content and soluble carbohydrates in *A. cearenis* (Table 3); on the other hand, there are situations in which inoculation with AMF favors the accumulation of proteins and plant sugars, as was documented by Ratti et al. (2010) and Baslam et al. (2011). There are situations in which the increase in the content of primary metabolites directs the synthesis of secondary compounds (Oliveira et al., 2013), a fact that has not been documented in this study (Tables 2, 3 and 4).

Mycorrhization with *C. etunicatum* increased in relation to the non-inoculated control, the production of total foliar phenolic compounds in the *A. cearensis* seedlings, both in content (198.92%) and concentration (47.82%) (Table 4). Levels of phenolic compounds also varied because of mycorrhizal inoculation, as was documented by Araim et al. (2009), Ceccarelli et al. (2010) and Singh et al. (2012), which makes the use of mycorrhizal technology an alternative to increase the production of such compounds with pharmacological importance.

The use of *C. etunicatum* maximized the content of total foliar flavonoids in relation to the non-inoculated control (Table 4). Possibly, mycorrhization lead to an increased absorption of nutrients, increasing the synthesis of production precursors of such compounds, such as the enzyme Chalcone synthase (*Chs*), which regulates the biosynthesis of this group of phenols (Zhang et al., 2013). An increase in the production of this group of phenolic compounds was also found in other situations (Antunes et al., 2006; Larose et al., 2002), as well as in other Leguminosae species in the Caatinga (Pedone-Bonfim et al., 2013).

Inoculation increased the production of total tannins when *C. etunicatum* was used (Table 5). Nisha and Rajeshkumar (2010) also observed an increase in the biosynthesis of tannins in *Wedilla chinensis* seedlings when inoculated with *Glomus aggregatum*. It is probable that intermediaries of biosynthetic pathways of the tannins, such as gallic acid have optimized the production through mycorrhization, as has been recently documented for the Leguminosae *L. ferrea* (Silva et al., 2014b).

Various mechanisms, nutritional and non-nutritional, have been suggested to explain the effects of mycorrhization on the increase in the biosynthesis of secondary compounds (Mandal et al., 2013; Zhang et al., 2013). Taking into consideration that mycorrhization did not alter the production of primary metabolites (Table 3), it is probable that the mechanisms that are involved in the

Table 4. Concentration of total foliar content of phenols and flavonoids in *Amburana cearensis* seedlings, inoculated or non-inoculated with arbuscular mycorrhizal fungi, 160 days after inoculation under experimental roofing.

Inoculation treatment	Total phenols		Total flavonoids	
	Concentration (mg g plant^{-1})	Content (mg plant^{-1})	Concentration (µg g plant^{-1})	Content (µg plant^{-1})
Control	7.11^{b}	4.67^{b}	627.14^{b}	393.92^{b}
Acaulospora longula	8.99^{ab}	6.23^{b}	843.75^{a}	582.76^{b}
Gigaspora albida	7.53^{b}	8.50^{b}	522.08^{b}	594.59^{b}
Claroideoglomus etunicatum	10.51^{a}	13.96^{a}	829.59^{a}	1098.08^{a}

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

Table 5. Concentration of total foliar content of tannins in *Amburana cearensis* seedlings, inoculated or non-inoculated with arbuscular mycorrhizal fungi, 160 days after inoculation under experimental roofing.

Inoculation treatment	Total tannins	
	Concentration (mg g plant^{-1})	Content (mg plant^{-1})
Control	6.09^{b}	4.06^{b}
Acaulospora longula	8.33^{ab}	5.90^{b}
Gigaspora albida	6.61^{b}	7.51^{b}
Claroideoglomus etunicatum	9.84^{a}	12.05^{a}

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

foliar phenols increase in *A. cearensis* are non-nutritional as was suggested by Toussaint et al. (2007). Such mechanisms involve an increase in the enzymatic activity, increase in the gene expression, maximized activation of the metabolic pathways and optimized biosynthesis of signaling in mycorrhizal plants (Walter et al., 2000; Lohse et al., 2005; Zhang et al., 2013). Furthermore, it is probable that the inoculated AMF increased the absorption of P, a fact that is well documented for mycorrhizal plants (Smith and Read, 2008), which is an important requirement for the biosynthetic pathways of phenolic compounds (Heldt, 2005).

Benefits of the mycorrhizal technology for the production of bioactive compounds were found for other plants from the Caatinga, as was referred to by Pedone-Bonfim et al. (2013), Oliveira et al. (2013) and Silva et al. (2014a), for *Anadenanthera colubrina, Myracrodruon urundeuva* and *L. ferrea*, respectively. Such benefits were also observed for the first time in *A. cearensis,* which confirms the initial working hypothesis.

The mycorrhizal technology, employing selected AMF, favored the production of the phytomass of *A. cearensis* with an elevated concentration of bioactive compounds, which possess various therapeutic properties. Therefore, the fungus *C. etunicatum* is recommended as a biotechnological alternative to maximize the production of foliar bioactive compounds in *A. cearensis* seedlings. This way, a low cost biotechnological protocol was established to maximize the production of plant biomolecules that are important to the phytotherapeutic industry. Other experiments have to be carried out to elucidate the benefits under field conditions and to determine whether there is a specific increase of molecules that are of industrial interest, such as vanillic acid.

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Conflict of interest

The authors do not have any conflicts of interest.

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