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Use of arbuscular mycorrhizal fungi and phosphorus for increase in the concentration of compounds with antioxidant activity in *Libidibia ferrea*

Uso de fósforo e fungos micorrízicos arbusculares para aumentar a concentração de compostos com atividade antioxidante em *Libidibia ferrea*

Uso de fósforo y hongos micorrizales arbusculares para aumentar la concentración de compuestos con actividad antioxidante en *Libidibia ferrea*

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Abstract

The increase of the concentration of secondary compounds in medicinal plants can be influenced by the association with arbuscular mycorrhizal fungi (AMF). Pharmacological studies have shown that secondary compounds, found ing *Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz, confer phytotherapeutic potential to the species due to antidiabetic, antibiotic and anticancer activity. Therefore, the aim of this work was to verify if the presence of AMF associated or not with phosphate fertilization has an effect on the concentration of foliar phenolic compounds with antioxidant activity in *L. ferrea* seedlings. The seedlings were transferred to pots with 1.2 kg of soil with phosphate fertilization (P_2O_5) or not. In the roots was deposited soil-inoculum, containing 300 spores {100 spores of each AMF species: *Claroideoglomus etunicatum*, *Gigaspora albida* and *Acaulospora longula Acaulospora longula*. The plants were maintained in greenhouse for seven months. AMF favored an increase in shoot dry matter production, accumulation of flavonoids and greater total antioxidant activity, dispensing fertilization of the soil. The mycorrhizal inoculation associated with phosphate fertilization maximized the biosynthesis of total chlorophyll and soluble carbohydrates. AMF inoculation presents as a possible biotechnological alternative to increase antioxidant activity and foliar flavonoid production in *L. ferrea* seedlings, avoiding expenses with agricultural inputs, such as phosphate fertilization, making phytomass more attractive for the production of phytotherapics.
Keywords: AMF; Leguminosae; Phosphate nutrition; Pau-ferro.

Resumo

O aumento da concentração dos compostos secundários em plantas medicinais pode estar influenciado pela associação com fungos micorrízicos arbusculares (FMA). Os estudos farmacológicos tem demonstrado que os compostos secundários, encontrados em *Libidibia ferr*ea (Mart. Ex Tul.) L. P. Queiroz, conferem potencial fitorerápico à espécie devido a sua atividade anitdiabética, antibiótica e anticancerígena. Portanto, o objetivo deste trabalho foi verificar se a presença de FMA associada ou não à fertilização com fosfato tem efeito sobre a concentração de compostos fenólicos foliares com atividade antioxidante em mudas de *L. ferrea*. As plântulas foram tranferidas para potes com 1,2 Kg de solo com fertilização fosfatada (P_2O_5) ou não. Nas raízes foi depositado solo-inóculo, contendo 300 esporos (100 esporos de cada espécie de FMA: *Claroideoglomus etunicatum*, *Gigaspora albida* e *Acaulospora longula*. As plantas foram mantidas em telado experimental durante sete meses. Os FMA favoreceram o aumento da produção de matéria seca da parte aérea, acúmulo de flavonoides e maior atividade antioxidante total, dispensando a fertilização do solo. A inoculação micorrízica associada com a fertilização com fosfato maximizou a biosíntese de clorofila total e carboidratos solúveis. A inoculação de FMA se apresenta como uma possível alternativa biotecnológica para

incrementar a atividade antioxidante e a produção de flavonoides foliares em mudas de *L. fer*rea, evitando gastos com insumos agrícolas, como a fertilização fosfatada, tornando mais atrativa a fitomassa para a produção de fitoterápicos.
Palavras-chave: FMA; Leguminosas; Nutrição fosfatada; Pau-ferro.

Resumen
El aumento de la concentración de compuestos secundarios en plantas medicinales puede estar influenciado por la asociación con hongos micorrízicos arbusculares (HMA). Los estudios farmacológicos han demostrado que los compuestos secundarios, encontrados en *Libidibia ferr*ea (Mart. Ex Tul.) L. P. Queiroz, confieren potencial fitoterapéutico a la especie debido a su actividad antidiabética, antibiótica y anticancerígena. Por tanto, el objetivo de este trabajo fue verificar si la presencia de HMA asociada o no a la fertilización con fosfato tiene efecto sobre la concentración de compuestos fenólicos foliares con actividad antioxidante en plántulas de *L. ferrea*. Las plántulas se trasladaron a macetas con 1,2 kg de suelo con fertilización fosfatada (P_2O_5) o no. En las raíces se depositó suelo-inóculo, que contenía 300 esporas {100 esporas de cada especie de HMA: *Claroideoglomus etunicatum*, *Gigaspora albida* y *Acaulospora longula*. Las plantas se mantuvieron en invernadero durante siete meses. La HMA favoreció un aumento en la producción de materia seca de los brotes, acumulación de flavonoides y una mayor actividad antioxidante total, dispensando la fertilización del suelo. La inoculación de micorrizas asociada con la fertilización con fosfato maximizó la biosíntesis de clorofila total y carbohidratos solubles. La inoculación de HMA se presenta como una posible alternativa biotecnológica para incrementar la actividad antioxidante y la producción de flavonoides foliares en plántulas de *L. fer*rea, evitando gastos con insumos agrícolas, como la fertilización fosfatada, haciendo más atractiva la fitomasa para la producción de fitoterápicos.
Palabras clave: HMA; Leguminosas; Nutrición fosfatada; Pau-ferro.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are abundant soil organisms and associate symbiotically with the roots of the majority of plants species. The association increases the growth of the host plants due to enhanced soil nutrient absorption, especially that of phosphorus (Smith & Read, 2008). In low P soils, plants associated with AMF benefit from the exploration by the AMF external mycelium of soil volumes beyond the limits reached by their roots to enhanced soil nutrient absorption, especially that of phosphorus (Moreira & Siqueira, 2002). On the other hand, high soil P concentrations are harmful to the association (Carneiro, Siqueira, & Davide, 2004) and may suppress the benefits obtained by the host plants.

The legume *Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz, plants from the Caatinga vegetation, commonly known as ironwood, stands out due to its high medicinal potential associated (Santos et al., 2021) with the presence of secondary compounds with a medicinal potential, such as flavonoids, phenols and tannins (Santos, Silva, & Silva, 2017)

One of the technological tools to increase the production of important pharmacological compounds is the inoculation of plants with efficient AMF (Santos, Lins, & Silva, 2021). The application of selected AMF favors the accumulation of primary and secondary compounds in medicinal plants (Mandal, Evelin, Giri, & Singh, 2013; Singh et al. 2012; Urcoviche, Gazim, Dragunski, Barcellos, & Alberton, 2015) and the availability of P in the soil can modulate the action of the AMF on the biosynthesis of these compounds (Pedone-Bonfim et al. 2013). However, the results are conflicting (Kapoor, Girin, & Mukerji. 2002; Toussaint, Smith & Smith, 2007; Zhang, Zhu, Zhao, & Yao, 2013). Which makes further studies necessary to define the role of symbiosis in maximizing the synthesis of bioactive compounds. In the case of *L. ferrea*, it is of interest to establish low-cost biotechnological protocols for plant production with increased concentrations of phytochemicals on leaves and, therefore, more attractive to the phytotherapy industry. *L. ferrea* is an experimental model of relevance, giving the fact that it forms symbiosis with AMF (Gattai, Pereira, Costa, Lima, & Maia, 2011).

Considering the above reasons, this study tested the hypothesis that the benefits obtained by mycorrhization can be maximized by phosphate fertilization, leading to increased production of bioactive foliar compounds. Therefore, the objective of this study was to determine the effects of the combined mycorrhizal inoculation and phosphate fertilization on the production of foliar biomolecules in *L. ferrea* seedlings.

2. Material and methods

2.1 Execution of the experiment

The experiment was set up as a completely randomized design with a factorial arrangement of 2 x 2, corresponding to two levels of P (fertilized and non-fertilized with simple superphosphate) and two inoculated treatments (non-inoculated treatment and inoculated with a mixture of AMF), with five replicates. Soil from the superficial layer (0 to 20 cm) of a Latosol was collected in a Caatinga area. Soil analysis showed the following characteristics: organic matter, 2,48 g kg^{-1}; pH $_{(H2O-1:2,5)}$, 4,9; electric conductivity, 3,16 ds m^{-1}; P, 5,5 mg dm^{-3}; K ,0,18 $cmol_c$ dm^{-3}; Ca, 2,5 $cmol_c$ dm^{-3}; Mg, 3,5 $cmol_c$ dm^{-3}; Na, 0,47 $cmol_c$ dm^{-3}; and Al, 0,05 $cmol_c$ dm^{-3}. Part of the soil was fertilized with simple superphosphate, in a dosage of 13 mg of P_2O_5 per kg of soil; analysis of a sample after mixing the fertilizer indicated the presence of 7,4 mg dm^{-3} of P extractable by the Mehlich method. Fertilized and non-fertilized soil portions of 1.2 kg, both non-sterilized, were added to pots, to which *L. ferrea* seedlings with two definitive leaves were transplanted. The seedlings had been previously grown from seeds obtained from selected native, healthy plants. Seed dormancy was broken by chemical scarification with sulphuric acid, for 20 minutes, followed by washing with running water and distilled water (Biruel, Aguiar & Paula, 2007) um granulated vermiculite.

Soil-inoculant, containing a mixture of 300 glomerospores (1:1:1) of *Claroideoglomus etunicatum* (Becker & Gerd.) C. Walker e A. Schüßler (UFPE 06), *Gigaspora albida* N. C. Schenck e G. S. Sm. (UFPE 01) and *Acaulospora longula* Spain e N. C. Schenck (UFPE 21) was placed at the root region of the seedlings.

The seedlings were harvested after seven months under experimental roofing. During this period the temperature under the screen varied between 21.0 °C and 32.6 °C and the relative air humidity between 26.5% and 76.6%. Watering was done when necessary.

Immediately before harvesting the level of chlorophyll in the leaves was examined, *in vivo*, with the aid of an Electronic Chlorophyll Level Meter, the ClorofilLOG CFL1030, and the data were expressed in the Falker Chlorophyll Index (FCI). Subsequently, the aerial part of each plant was collected and oven dried for determination of the dry biomass. The roots were washed, bleached (KOH 10 %, w/v and H_2O_2 10 %, w/w) and stained with Trypan blue (0.05 %, w/v) (Phillips & Hayman, 1970), and the percentage of colonization was estimated by the gridline intersect method (Giovannetti & Mosse, 1980).

2.2 Biochemical and phytochemical examination

An extract was prepared macerating 500 mg of dried leaves in amber flasks (80 mL), with 20 mL of ethanol for 12 days at 20 ºC, maintained under dark conditions. At the end of this period the extract was filtered with gauze, refiltered with quality filter paper r and stored in amber flasks at - 4 ºC (Brito, Noronha, França, Brito, & Prado, 2008). In the ethanol extract the following determinations were made: Total proteins: 50 µL of the ethanol extract was agitated in a test tube with 2.5 mL of the Bradford reagent, taking a spectrophotometric reading (595 nm) after five minutes rest. Bovine serum albumin (BSA) was used as the standard (Bradford, 1976).

Soluble carbohydrates**:** the quantification was carried out in 50 µL of the ethanol extract, 95 µL of distilled water, 50 µL of phenol (80%, w/v), intensely stirred in a vortex. After stirring 2 mL of sulphuric acid were added, leaving it to rest for 10 minutes at 22 ºC, at which point a photometric reading was taken at 490 nm. The standard curve was prepared by using glucose (Dubois, Guiles, Hamilton, Rebers, & Smith, 1956).

Total phenols: to 2 mL of the extract were added 5 mL of the Folin-Ciocalteau reagent (10 %, w/w) and 10 mL of sodium carbonate (7.5 % w/v) in a volumetric balloon and the volume was completed to 100 mL with distilled water. After 30 minutes of rest, an absorbance reading was made (760 nm) with tannic acid as the standard (Monteiro et al., 2006).

Total tannins: 3 mL of the extract and 0.5 g of casein powder were mixed in an amber flask and the material was kept under constant stirring for 3 hours) (160 rpm and 25 ºC). Subsequently, the material was filtered, the volume was completed to 25 mL in a volumetric balloon and the quantification was done by the Folin-Ciocalteu method. The difference between the readings by this procedure and those obtained in the quantification of total phenols were considered as representing the concentration of tannins (Monteiro et al., 2006).

Total flavonoids: The following was added to a volumetric balloon: 1 mL of the ethanol extract, 0.6 mL of glacial acetic acid and 10 mL of pyridine solution: methanol (2:8, w/w) and 2.5 mL of ethanol aluminum chloride solution (5 %, w/v). The volume was completed to 25 mL with distilled water and after 30 minutes of rest, the absorbance was measured (420 nm) by using rutin to prepare the standard curve (Araújo, Alencar, Amorim, & Albuquerque, 2008).

Antioxidant activity (AAT)**:** 0.1 mL of the extract and 3.9 mL of radical DPPH (0.6 mM) were added to a threaded test tube and homogenized in a vortex. To prepare the control treatment, 0.1 mL of the control solution (methyl alcohol, acetone and water) was added to a test tube together 3.9 mL of the radical DPPH. The methyl alcohol was used as whitening agent and the readings were taken in a photospectrometer (515 nm) after 30 minutes of rest (Rufino et al., 2007).

The following reagents were used: phenol, Coomassie blue G-250, phosphorus acid, pyridine, aluminum chloride, casein, glycose and tannic acid, obtained from Vetec® (Duque de Caxias, RJ, Brazil); sulphuric acid, ethyl alcohol, methyl alcohol, sodium carbonate, glacial acetic acid, obtained from F. Maia® (Cotia, SP, Brazil); Folin-Ciocalteu reagent, obtained from Merck ® (Rio de Janeiro, RJ, Brazil); BSA – bovine albumin serum, rutin hydrate and DPPH (2.2-diphenyl-picryhydrazyl) obtained from Sigma-Aldrich® (São Paulo, SP, Brazil).

2.3 Statistical analysis

The data were submitted to ANOVA and the averages compared by the Tukey test (5 %) on software Assistat (7.6).

3. Results and Discussion

Biomass production was larger in the inoculated seedlings and in the fertilized ones than in the seedlings of the control treatment but there was no positive interaction of the two factors (Table 1). Mycorrhizal inoculation in the non-fertilized soil resulted in a 17% increase in relation to the non-inoculated control treatment while fertilization resulted in a 13% increase. Growth benefits of mycorrhization of species without phosphate fertilization have been documented in *Anadenanthera colubrina* (Vell.) Brenan (Pedone-Bonfim et al., 2013).

In comparison to the control treatment, the content of soluble carbohydrates and the level of chlorophyll were higher for both the inoculated and the fertilized treatments and the two factors had a synergic effect (Table 1). Pedone-Bonfim et al., (2013) also reported increased accumulation of soluble carbohydrates in mycorrhizal seedlings of the legume *A. colubrina*. When inoculation and fertilization occurred jointly, the content of carbohydrates was 20% larger and the level of chlorophyll 11% larger than those of the control. Participation of the mechanisms linked by P and by the AMF in the increased cytoplasmic accumulation of carbohydrates was documented in relation to stevioside production in *Stevia rebaudiana* Bertoni (Mandal et al., 2013).

Table 1. Soluble carbohydrates, total chlorophyll, total phenolics, total tannins, total flavonoids contents in the aerial part of the plant, antioxidant activity, biomass and colonization of the roots by arbuscular mycorrhizal fungi in *Libidibia ferrea* seedlings grown under protected roofing, in pots non-fertilized or fertilized with P and inoculated or non-inoculated with arbuscular mycorrhizal fungi.

Phosphorus	**Control**	**Inoculated**
	Biomass (g pote^{-1})	
Control	1.60 bB	1.87 aA
Fertilized	1.81 aA	1.85 aA
	Soluble carbohydrates (mg g plant^{-1})	
Control	0.28 bB	0.33 bA
Fertilized	0.35 aB	0.42 aA
	Total chlorophyll (ICF*)	
Control	49.02 bB	54.15 bA
Fertilized	53.00 aB	58.64 aA
	Total phenolics (mg g plant^{-1})	
Control	7.48 aA	6.64 aA
Fertilized	6.14 bA	5.35 bA
	Total tannins (mg g plant^{-1})	
Control	6.5 aA	6.2 aA
Fertilized	5.9 aA	5.2 aA
	Total flavonoids (µg g plant^{-1})	
Control	113.6 aB	133.5 aA
Fertilized	109.6 aB	134.0 aA
	Total Antioxidant activity (mg plant mg DPPH^{-1})	
Control	0.59 aB	0.68 aA
Fertilized	0.45 bA	0.34 bB
	Colonization (%)	
Control	56.2 aB	66.3 aA
Fertilized	46.2 bB	64.6 aA

Averages (n=5) followed by the same small letter in the column for each variable and by the same capital letter on the line, do not differ by the Tukey test (5 %). * FCI = Falker Chlorophyll Index. Source: Authors.

Similar results with regard to chlorophyll increase were obtained by Singh et al., (2012) in mycorrhized plants of *Punica granatum* L. Increased production of chlorophyll (Table 1) is related to the glycosidic anabolism of the plant (Heldt, 2005). According to Baslam and Goicoechea (2012), mycorrhizal association increased the concentration of total chlorophylls, which resulted in increase of photosynthetic levels, benefitting the growth of the host plant, the development and the functionality of the symbiosis and directing the biosynthesis of carbohydrates (Shing, Pandey, Kumar, & Palni, 2010), as indicated in the present study.

The content of total flavonoids was larger with inoculation, without significant effects of fertilization (Table 1). Tests with other species and higher soil P levels have shown positive responses to P fertilization, such as an increase in flavonoids of *A. colubrina*, in soil with 50 mg dm^{-3} of P (Pedone-Bonfim et al., 2013), well over the 7.4 mg dm^{-3} present in the fertilized soil of the present study. Inoculation with AMF promoted an increase of 17% in the flavonoid content in relation to the non-inoculated treatment. Therefore, the sole use of AMF can be sufficient to optimize the biosynthesis of this compound in ironwood seedlings, which renders phosphate fertilization unnecessary. However, inoculation and fertilization may produce conflicting results, such as the increase in essential oil contents of *Mentha crispa* L. inoculated with *Claroideoglomus etunicatum* (Becker & Gerd.) Walker and Schüßler, what did not occur during association with *Rhizophagus clarus* (Nicolson & Schenck) Walker & Schüßler, and the suppression of response to *C. etunicatum* when the soil was fertilized with 200 mg P dm^{-3} of soil (Urcoviche et al. 2015).

It is likely that the production of flavonoids in ironwood seedlings is associated with a non-nutritional mechanism, as was suggested by Mandal et al. (2013) in relation to plants of *S. rebaudiana.* Recently, Zhang et al. (2013) found that increase in the production of flavonoids in mycorrhized plants of *Trifolium repens* L. was associated with the optimized activity of chalcone synthase (*Chs*).

The total antioxidant activity and the mycorrhizal colonization were also larger with inoculation but fertilization had a negative effect when it was not applied together with the inoculation. In the non-fertilized, inoculated seedlings, antioxidant activity increased 15.2 % in relation to the non-inoculated control treatment. Similar results were obtained by Lambais, Rios-Ruiz, and Andrade (2003). The negative effect of fertilization occurred in spite of the fact that the anabolic pathways for synthesis of compounds with a potential for capturing radicals are in need of P (Heldt 2005). This effect seems to indicate that the mechanisms involved in the antioxidant activity are both, nutritional and non-nutritional, therefore multi-factorial (Mandal et al. 2013), contrary to what was found for flavonoids. This further confirms the hypothesis that the mechanisms involved in the synthesis of bioactive compounds and the related antioxidant activity depend on the compound in question (Oliveira, Albuquerque, Campos, & Silva, 2013). The mycorrhizal inoculation had no effect, nor had the addition of P, on the content of total phenols, total tannins and total proteins.

The inoculated AMF were more competitive than the native fungi in colonizing the roots of the ironwood seedlings (Table 1) and this was translated into an increased growth and accumulation of biomolecules of the primary and secondary metabolism of the plant (Table 1). Similar results were reported by Cavalcante, Maia, Costa, Cavalcante and Santos (2002), who found that the inoculated fungi were more effective in colonizing seedlings of *Passiflora edulis* Sims than the native fungi, in soil non-sterilized and fertilized with P. The improved nutrition in mycorrhized plants may be one of the mechanisms to increase the biosynthesis of molecules (Toussaint et al. 2007; Pedone-Bonfim et al. 2013). However, there are controversies about the influence of phosphate fertilization in increasing the production of bioactive compounds in medicinal plants. Kapoor et al. (2002) showed the P as a possible inducer of production and the quality of essential oils in *Anethum graveolens* L. and *Trachyspermum ammi* (Linn.) Sprague inoculated with *Glomus macrocarpum* Tul and Tul. and *Glomus fasciculatum* (Thaxt.) Gerd. and Trappe. Toussaint et al. (2007) attributed the production of rosmarinic acid and caffeic acid in *Ocimum basilicum* L. to mycorrhizal inoculation. In the present study mechanisms were related only to the mycorrhizal inoculation for flavonoids and AAT (Table 1) and the action of both P and the AMF for the biosynthesis of chlorophylls and soluble carbohydrates (Table 1), which partially proves the initial hypothesis. However, the influence of P on the anabolism of mycorrhizal plants depends on the biomolecule considered, whereby generalizations should be avoided. Accumulation of soluble carbohydrates and total foliar chlorophyll in ironwood is dependent on soil P concentration and on mycorrhizal inoculation.

4. Conclusion

Inoculation with AMF increments growth, the production of total flavonoids and total foliar antioxidant activity in ironwood seedlings. Future studies must include chromatographic analysis of foliar extracts, validation of the results under field conditions and include analysis of other radicals, such as TPTZ (2.4.6-Tris (2pyridil)-s-triazine) and the ABTS (2.2 Azino Bis (3-ethylbenzo thiazoline 6 sulfonic acid).

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