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A MIXTURE OF ARBUSCULAR MYCORRHIZAL FUNGI FAVORS BRAZILIAN PEPPER SEEDLINGS UNDER AN INTERMEDIATE LEVEL OF SOIL PHOSPHORUS[1]

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ABSTRACT - Several tropical woody species are highly responsive to arbuscular mycorrhizal fungi (AMF) and depend on their symbiosis for nutrition and successful establishment in the field. The objective of this study was to evaluate the response of *Schinus terebinthifolius*, a native species of the Brazilian Atlantic Forest and Caatinga, to inoculation with three AMF species (*Dentiscutata heterogama*, *Gigaspora margarita*, and *Rhizophagus clarus*), either individually or mixed, at different levels of phosphorus fertilization (0, 71, 213, and 650 mg kg^{-1} of P). We conducted the experiment in 1 kg pots, following a completely randomized design with six replicates per treatment, to evaluate morphological and nutritional traits after 116 days, including stem diameter, plant height, plant biomass, and shoot P content. Our results showed that *S. terebinthifolius* was highly dependent on mycorrhizae and presented different responses depending on the AMF species. The greatest total biomass accumulation occurred when a mixture of the three AMF species was used, which indicated synergism between the fungi. The highest overall positive response to inoculation were observed at an intermediate P level (213 mg kg^{-1}), and although shoot biomass was reduced at the intermediate P doses, an increase in root biomass compensated for this. These results indicate complementarity as opposed to functional redundancy of the AMF species and highlight the importance of using a mixed inoculum in seedling production of *S. terebinthifolius* for revegetation programs.

Keywords: Mycorrhizal inoculation. Phosphorus nutrition. *Schinus terebinthifolius*. Symbiosis.

UMA MISTURA DE FUNGOS MICORRÍZICOS ARBUSCULARES FAVORECE MUDAS DE AROEIRA-VERMELHA EM NÍVEL INTERMEDIÁRIO DE FÓSFORO DO SOLO

RESUMO - Várias espécies lenhosas tropicais são altamente responsivas a fungos micorrízicos arbusculares (FMA) e dependem da simbiose para nutrição e sucesso no estabelecimento em campo. O objetivo deste estudo foi avaliar a resposta de *Schinus terebinthifolius*, uma espécie nativa da Mata Atlântica e Caatinga, à inoculação com três espécies de FMA (*Dentiscutata heterogama*, *Gigaspora margarita* e *Rhizophagus clarus*), individualmente ou misturadas, em diferentes níveis de adubação fosfatada (0, 71, 213 e 650 mg kg^{-1} de P). O experimento foi conduzido em vasos de 1 kg, seguindo um delineamento inteiramente casualizado com seis repetições por tratamento, para avaliar as características morfológicas e nutricionais, após 116 dias, incluindo diâmetro do colo, altura, biomassa e teor de P na parte aérea da planta. Nossos resultados evidenciaram que *S. terebinthifolius* foi altamente dependente de micorrizas e apresentou respostas diferentes dependendo da espécie de FMA. O maior acúmulo de biomassa total ocorreu quando foi utilizada uma mistura das três espécies de FMA, o que indicou sinergismo entre os fungos. De uma forma geral, a maior resposta positiva à inoculação foi observada em nível intermediário de P (213 mg kg^{-1}), e embora a biomassa da parte aérea tenha sido reduzida nas doses intermediárias de P, foi observada uma compensação com um aumento na biomassa radicular. Esses resultados indicam complementaridade em oposição à redundância funcional das espécies de FMA e destacam a importância do uso de inóculo misto na produção de mudas de *S. terebinthifolius* para programas de revegetação.

Palavras-chave: Inoculação micorrízica. Nutrição de fósforo. *Schinus terebinthifolius*. Simbiose.

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INTRODUCTION

Schinus terebinthifolius Raddi, commonly called Brazilian pepper, is a native South American pioneer evergreen tree species, which produces a large fruit biomass (SOUZA et al., 2013) that supplies food to frugivorous bird species (BALDIVIEZO; PASSOS; AZEVEDO, 2019). This species is naturally found in the Atlantic Forest (ARATO et al., 2017) and Caatinga regions (COSTA et al., 2015), and presents high decomposition rates of leaf litter (ARATO et al., 2017) as well as leaf structural aspects of xerophilic plants, such as multiple epidermal layers, a thick cuticle, and an abundance of hair and stomata, which prevent excessive drying (AZEVEDO; QUIRINO; BRUNO et al., 2015). These factors have stimulated seedling production experiments for the regrowth of native deforested areas in Brazil (SILVA et al., 2019), where *S. terebinthifolius* has shown high survival rates (RESENDE et al., 2015).

S. terebinthifolius seedlings inoculated with arbuscular mycorrhizal fungi (AMF) presented greater heights, stem diameters, and shoot and root dry biomasses (SCHOEN, AUMOND; STÜRMER, 2016), as well higher survival rates than uninoculated seedlings (GOETTEN; MORETTO; STÜRMER, 2016). Therefore, seedling inoculation with AMF is relevant for successful field survival and the establishment of forest species (MANAUT et al., 2015), which is particularly important in low-fertility soils (NOVAIS et al., 2014). Inoculation with AMF has also been shown to reduce seedling transplant time, as was verified for *Malpighia emarginata* D.C. (PINHEIRO et al., 2019).

Plants are known to exhibit different degrees of mycorrhizal dependence, and even in soils with high levels of available phosphorus, the species that benefit from the mycorrhizal association are considered highly dependent on this symbiosis (OLIVEIRA JÚNIOR et al., 2017). Different AMF species can distinctly affect the efficiency of this relationship, and some studies have shown that the symbiotic efficiency was higher in seedlings that were inoculated with a mixture of AMF species than those that received an isolated fungus (NOVAIS et al., 2014; OLIVEIRA JÚNIOR et al., 2017; SILVA et al., 2020). These studies show the importance of investigating the effect of the inoculation of forest species seedlings with different AMF symbionts, either alone or in a mixture.

We hypothesized that *S. terebinthifolius* responds differently to various combinations of AMF species and P levels. Therefore, our goal was to evaluate the effects of different AMF inoculation treatments on *S. terebinthifolius* under different P levels by evaluating plant growth, mycorrhizal colonization, and plant P content.

MATERIAL AND METHODS

Experimental design

We conducted the experiment following a completely randomized design with six replicates per treatment and 20 treatments, which were established by combining different levels of AMF inoculation and P fertilization. Five AMF inoculation levels were included: no inoculation, inoculation of each of the three AMF species individually, and inoculation of a mixture of the three species. Phosphorus was supplied at doses of 0, 71, 213, and 650 mg kg^{-1} to cover a low to high P concentration range, based on the solution equilibrium P (remaining P) (ALVAREZ et al., 2000).

Inoculum

The following AMF strains were used as inoculants: *Dentiscutata heterogama* CNPAB 02 (= BRM 033298), *Gigaspora margarita* CNPAB 001 (= BRM 033261), and *Rhizophagus clarus* CNPAB 005 (= BRM 033301). These fungi were multiplied separately in pots containing sterile soil as the substrate and *Uruchloa* sp. as the host species. The pots were sampled, and the substrates were used as inocula. The spore densities of *D. heterogama*, *G. margarita*, and *R. clarus* in the substrates were 17, 35, and 26 spores g^{-1}, respectively. To prepare the mixed inoculum, equivalent amounts of spores from the three AMF species were combined before inoculation. The substrate also contained hyphae and colonized roots, which were considered fungal propagules. The strains were kindly provided by the Johanna Döbereiner Resource Center (Embrapa Agrobiologia, Rio de Janeiro).

Substrate

The B horizon of an Inceptisol (Cambissolo Háplico Distrófico típico) with the following attributes was used as the substrate: pH in water (1:2.5 soil:water), 4.9; P and K extracted using Mehlich-1, 1.89 and 64 mg dm^{-3}, respectively; and Al, Ca, and exchangeable Mg (1 mol L^{-1} KCl), 1.21, 0.46, and 0.21 $cmol_c$ dm^{-3}, respectively. Liming of the substrate was conducted by applying 943 g $soil^{-1}$ of Minercal (relative neutralizing value = 91%, neutralizing index = 102%/CaO + MgO = 52%/CaO 39% and MgO 13%) prior to phosphate fertilization, following the recommendations based on the chemical analysis of the substrate. The soil was fertilized with a KH_2PO_4 solution at previously established doses, and the K concentration was balanced by applying a KCl solution. The substrate was then incubated for 60 days. Soil available P was determined using a Mehlich-1 extractor, and a high and significant correlation (F-test, $p < 0.01$, $\hat{y} = 29.28 + 0.44x$, $R^2 = 0.92$) was observed between the applied and available P.

Experimentation

We performed the experiment in pots containing 1 kg of the fertilized substrate. The pots consisted of 280 mL plastic tubes attached to the bottom of 700 mL disposable plastic cups (OLIVEIRA JÚNIOR et al., 2017). Each pot received three pre-germinated seeds, which were thinned to one seedling per pot after germination. The seeds were disinfected for 2 min with a 30% H_2O_2 solution and germinated under constant light at 28 °C for 5 days in Petri dishes containing sterile paper and cotton. The plants were inoculated with one gram of soil containing equivalent amounts of spores of each species to a total of 30 spores of AMF per treatment, as well as hyphae and colonized roots, according to the specific protocols. We added 10 mL of a filtrate solution from the mixed inoculum (all the three AMF species), which was free of propagules, to all pots to standardize their inoculum microbiota.

The following nutrient solution was applied monthly: $CaCl_2(H_2O)_2$, 2 mmol L^{-1}; $MgSO_4(H_2O)_7$, 1 mmol L^{-1}; KCl, 3 mmol L^{-1}; $ZnSO_4(H_2O)_7$, 0.9 µmol L^{-1}; H_3BO_3, 4 µmol L^{-1}; $CuSO_4(H_2O)_5$, 1 µmol L^{-1}; $MnSO_4H_2O$, 6 µmol L^{-1}; $NaMoO_4(H_2O)_2$, 0.1 µmol L^{-1}; and Fe EDTA, 1.66% (BERTRAND et al., 2000). We also applied 150 mg N per plant as NH_4NO_3. Plants were irrigated daily using an automatic sprinkler system, and the soil water content was maintained at 70% of the field capacity.

The following variables were measured after 116 days: shoot dry weight, root dry weight, shoot P content, and root colonization percentage. Shoots and roots were oven-dried at 65 °C for 72 h and weighed to three decimal places using an analytical balance. Shoots were then ground and subjected to sulfur digestion for P extraction and determination by colorimetry (SILVA, 1999). Root colonization was evaluated with thin, freshly collected roots (0.5 g) stored in 50% ethanol. These were then bleached and stained (KOSKE; GEMMA, 1989; GRACE; STRIBLEY, 1991), and the percentage of root colonization by AMF was evaluated using the crossline method (GIOVANNETTI; MOSSE, 1980) that was adapted from Newman's method (NEWMAN, 1966).

Data analysis

The effects of P and mycorrhizal colonization, and the symbiotic efficiency, were calculated by polynomial regression based on total plant biomass and the P dose-response curves (OLIVEIRA JÚNIOR et al., 2017). All data were transformed (Box-Cox) to achieve normality (ZAR, 1996), and then subjected to analysis of variance using SISVAR version 5.0 (FERREIRA, 2003). Regression analysis was applied to the quantitative factors (OLIVEIRA JÚNIOR et al., 2017). We performed principal component analysis to identify associations among the treatments and the attributes of the seedlings, and hierarchical clustering analysis using the Gower index, to identify dissimilarities among the treatments. These multivariate analyses were performed with version 2.17c of the Paleontological Statistics (PAST) program.

RESULTS AND DISCUSSION

The adjusted models for all the analyzed attributes presented determination coefficients ranging from 0.76 to 0.99, with the majority > 0.80 (Figure 1). We did not connect the points for treatments with no curve adjustments, because the points overlapped, and the visualization of the figures would be impaired.

We observed a significant and linear decrease in root colonization by *R. clarus* and *G. margarita* with increasing doses of P (Figure 1A). This trend also occurred in plants inoculated with *D. heterogama* or the mixed inoculum. Root colonization was extremely reduced at the highest dose of P (650 mg kg^{-1}), not exceeding 5% in all treatments. We expected this pattern, as it has been widely reported for other forest species, such as *Toona ciliata* (LIMA et al., 2015), *Eugenia uniflora* (DALANHOL et al., 2016), *Apuleia leiocarpa* (OLIVEIRA JÚNIOR et al., 2017), and *Colubrina glandulosa* (CAMARA et al., 2017; SILVA et al., 2020), as well as for plants of agronomic interest, such as *Zea mays* L. (NOVAIS et al., 2014) and coffee (MOREIRA et al., 2019).

The highest root colonization rates occurred in the absence of P, and we verified higher values for plants inoculated with the mixed inoculum (62%) and *R. clarus* (57%), followed by *D. heterogama* (43%) and *G. margarita* (36%). *R. clarus* appeared to have a greater capacity for root colonization, since this species also provided higher values of this attribute (44%) in vine seedlings (*Vitis berlandieri* Planchon × *Vitis rupestris* Scheele), than the inoculation with *D. heterogama*, *G. margarita*, *Acaulospora morrowiae* Spain & N. C. Schenck, or *Acaulospora colombiana* (Spain & N. C. Schenck) Kaonongbua, J. B. Morton & Bever, which returned values ranging from 25 to 35% under greenhouse conditions (AMBROSINI et al., 2015).

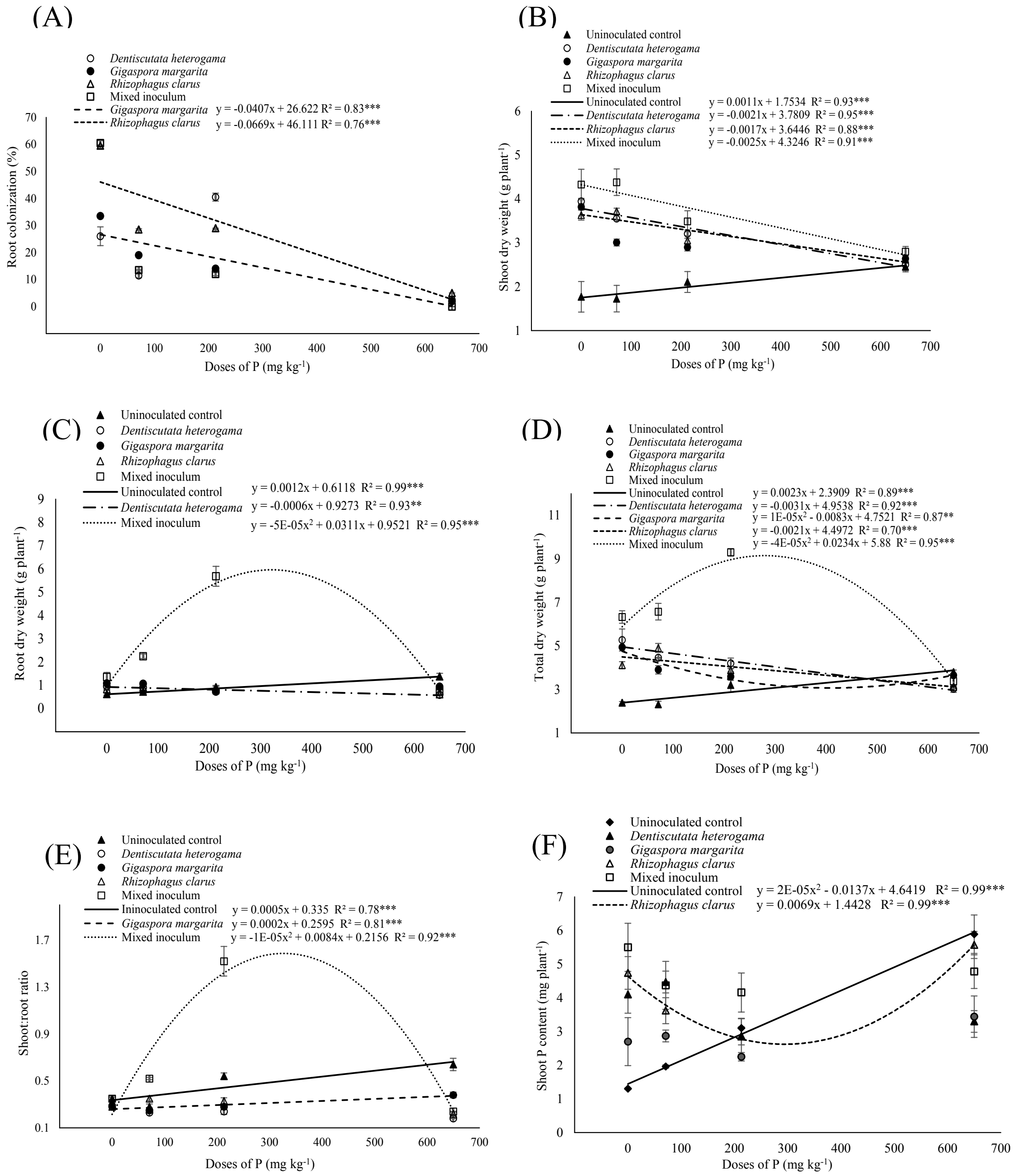

Figure 1. Root colonization (A), shoot (B), root (C), and total (D) dry weight, shoot/root ratio (E) and shoot P contents (F) of *Schinus terebinthifolius* inoculated with three species of arbuscular mycorrhizal fungi, either individually or mixed, in a soil fertilized with 0, 71, 213, and 650 mg kg^{-1} of P. Plants were sampled 116 days after sowing. (*) $p \leq 0.05$; (**) $p \leq 0.01$ (***); $p \leq 0.001$. Mixed inoculum = *Dentiscutata heterogama, Gigaspora margarita*, and *Rhizophagus clarus.*

The accumulation of shoot dry weight followed negative linear models in all inoculated treatments (Figure 1B), demonstrating that shoot biomass accumulation decreased with increasing P levels in the substrate. Inoculated plants accumulated the highest estimated values for shoot dry weight with a P dose that approached zero (1.89 mg kg^{-1}), particularly with the mixed inoculant. Plants inoculated with the mixed inoculum, at doses of 0 and 71 mg kg^{-1} of P, accumulated 145% and 153% more shoot biomass than uninoculated plants, respectively.

The uninoculated plants responded to increasing doses of P by showing a slight linear increase in the accumulation of shoot, root, and total dry weight (Figures 1B, 1C, and 1D, respectively). Shoot biomass accumulation was the greatest at 650 mg kg^{-1} of P. The comparison with other treatments revealed that these plants did not reach the maximum growth of shoot, root, and total dry weight.

The accumulation of root dry weight in uninoculated plants and those inoculated with *D. heterogama* presented positive and negative linear models, respectively, in response to increasing P levels (Figure 1C). These models indicated only slight variations in root biomass accumulation. A quadratic model presented the best fit for plants inoculated with the mixed inoculum and demonstrated an expressive response to inoculation at intermediate P levels (213 mg kg^{-1}). The estimated dose for maximum technical efficiency for this treatment was 311 mg kg^{-1} of P, yielding an estimated root dry weight of 5.78 mg $plant^{-1}$, and the root biomass accumulation was 832% and 328% higher than that of uninoculated plants or those inoculated with the mixed inoculum in the absence of P fertilization, respectively. The plants that received the mixed inoculum reached a maximum value of root biomass at intermediate P levels (Figure 1C), and this effect was sufficiently expressive to influence total biomass accumulation (Figure 1D). Root dry weights of plants inoculated with *D. heterogama*, *G. margarita*, or *R. clarus* were not significantly different from those of uninoculated plants at all P levels.

The accumulation of total dry weight presented negative linear models for *D. heterogama* and *R. clarus*, and quadratic models for *G. margarita* and the mixed inoculum (Figure 1D). The total dry weight of plants inoculated with *D. heterogama*, *R. clarus*, or *G. margarita* decreased with increasing doses of P. Inoculation with the mixed inoculum yielded the highest total biomass, ranging from 0 to 213 mg kg^{-1} of P, with markedly lower values in the treatment with the highest dose of P (650 mg kg^{-1} of P). The estimated dose of maximum technical efficiency with the mixed inocula was 292.5 mg kg^{-1} of P, yielding an estimated total biomass of 9.30 mg $plant^{-1}$. This total biomass accumulation was 289% and 47% higher than that of uninoculated plants or plants administered the mixed inoculum in the absence of P fertilization, respectively.

In general, plants receiving the mixed inoculum reached the highest values of root colonization and biomass increments compared to the control (uninoculated plants). A positive correlation exists between root colonization and the benefits conferred to plants by AMF; therefore, plant growth and nutrient availability increases with greater root colonization (SOARES et al., 2012; NOVAIS et al., 2014; AMBROSINI et al., 2015).

The root/shoot ratio of plants inoculated with the mixed inoculum exhibited a trend similar to that observed for the total dry weight (Figure 1E). The estimated dose of P for maximum technical efficiency and the estimated root/shoot ratio were 420 mg kg^{-1} and 1.97, respectively. The ratio increased linearly in uninoculated plants and those inoculated with *G. margarita*, with moderate variation. Plants administered *D. heterogama* and *R. clarus* presented similar root/shoot ratios at all P levels.

The shoot P content fit a positive linear regression model for uninoculated plants and a quadratic regression model for plants inoculated with *R. clarus* (Figure 1F). The shoot P content of uninoculated plants was the greatest at 650 mg kg^{-1} of P, and similar levels of were reached by plants inoculated with the mixed inoculum at the lowest dose. AMF inoculation increased P content significantly in treatments with 0 and 71 mg kg^{-1} of P. *D. heterogama*, *R. clarus*, and the mixed inoculum yielded increases of 215%, 265%, and 323% in the shoot P content, respectively, when compared to the uninoculated plants at the lowest P dose.

The response of *S. terebinthifolius* to the mixed inoculum was noticeably higher with the intermediate dose of P (213 mg kg^{-1}), indicating its strong responsiveness to mycorrhizae. This result demonstrated that the influence of mycorrhizae on *S. terebinthifolius* may have been underestimated if only a single AMF species have been used for inoculation. For a given host plant species, P, the AMF species, and different isolates of the same AMF species can influence plant growth and nutritional status because of variations in symbiotic efficiency (NOVAIS et al., 2014; OLIVEIRA JÚNIOR et al., 2017).

Instead of decreasing linearly as observed with individual inoculations, the total biomass increased at intermediate doses with the mixed inoculation, indicating synergism between the fungal species (OLIVEIRA JÚNIOR et al., 2017). Although shoot biomass was reduced at intermediate doses of P, this reduction was compensated by an increase in root biomass that reached its maximum at intermediate P doses, and this influenced the maximum total biomass at the same doses. This resulted in a more balanced biomass distribution between the root and shoot, which was corroborated by the maximum root/shoot ratio at intermediate doses. These results suggest a complementarity between the fungi, as opposed to a functional redundancy, and highlight the importance of using mixed inocula to produce *S. terebinthifolius* seedlings in revegetation programs. Therefore, it is important that studies on fungi-plant interactions consider inoculation with different fungal species (LIMA et al., 2015; OLIVEIRA JÚNIOR et al., 2017). Similar results were obtained for *Apuleia leiocarpa*, which responded differently to inoculation with three individual AMF species, but exhibited a synergy in its response to the mixed inoculum (OLIVEIRA JÚNIOR et al., 2017).

Uninoculated plants showed increasing biomass accumulation with higher doses of P, with maximum biomass accumulation and shoot P content at the highest P concentration (650 mg kg^{-1} of P), which suggests that higher P doses could yield increased values for these variables. However, shoot growth (stem diameter and biomass accumulation) and P content of uninoculated plants were not as significant as those of plants affiliated with AMF, in the absence of P fertilization or with supplementation at the same level, indicating the benefits conferred by mycorrhizal colonization, even at the highest dose of P.

This demonstrates that *S. terebinthifolius* can be classified as greatly dependent on the mycorrhizal association (OLIVEIRA JÚNIOR et al., 2017) and highly responsive to AMF inoculation, which is required to obtain better quality seedlings of this species. Several areas of tropical forests exhibit nutrient-deficient soils; thus, mycorrhizae play an essential role in the absorption of nutrients, particularly P, by various plant species. AMF are also present in the litter, which allows plants to readily absorb nutrients released from litter decomposition (POSADA; MADRIÑAN; RIVERA, 2012). Furthermore, our results indicate that *S. terebinthifolius seedlings* should be administered a mixed AMF inoculum and fertilized with intermediate doses of P *(*213 mg kg^{-1}) to maximize their establishment in the nursery or field. The use of different AMF species, and more importantly, a mixed inoculum, should be considered when determining the mycorrhizal dependency and response of a given plant species.

The principal component analysis revealed that, in general, the treatments with no AMF inoculation, with AMF regardless of the dose of P, or with inoculation combined with the highest doses of P (213 and 650 mg kg^{-1}) were positioned on the left of principal component 1 (eigenvectors with positive values) (Figure 2A). In contrast, the treatments with inoculation in the absence of the P supplementation or with the lowest dose of P (doses 0 and 71, respectively), were positioned on the right of the principal component (eigenvectors with positive values). Thus, the relationship between the principal components allowed for individualization among the treatments. Principal component 1 explained the majority of the data variability compared to principal component 2 (approximately 53% and 29%, respectively). The relationship between principal components 1 and 2 explained approximately 82% of the data variability.

(A) (B)

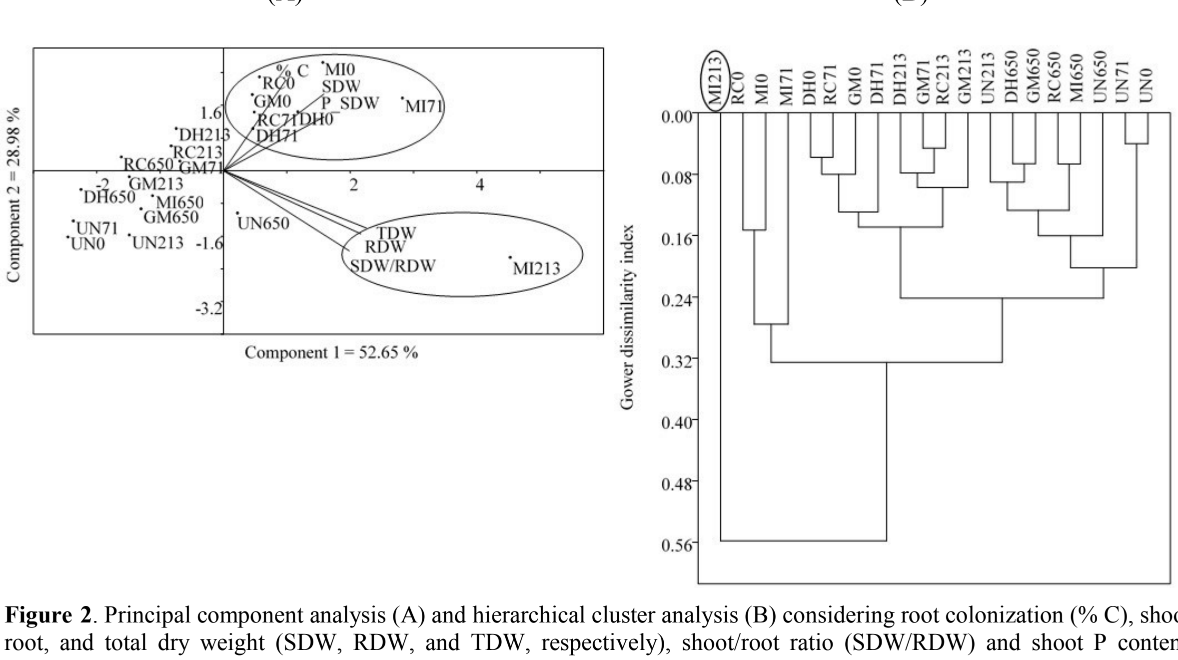

Figure 2. Principal component analysis (A) and hierarchical cluster analysis (B) considering root colonization (% C), shoot, root, and total dry weight (SDW, RDW, and TDW, respectively), shoot/root ratio (SDW/RDW) and shoot P contents (P_SDW) of *Schinus terebinthifolius* inoculated with three species of arbuscular mycorrhizal fungi (AMF), either individually (DH: Dentiscutata heterogama, GM: Gigaspora margarita, RC: Rhizophagus clarus), mixed (MI), or uninoculated with AMF (UN) in a soil fertilized with 0, 71, 213, and 650 mg kg^{-1} of P. Plants were sampled 116 days after sowing.

The seedlings of S. *terebinthifolius* that were inoculated in the absence of phosphate fertilization (dose 0 of P), regardless of the type of inoculum, or those that received *D. heterogama, R. clarus*, or the mixed AMF in the presence of the lowest dose of P (71 mg kg^{-1}), were associated with higher values of root colonization, shoot dry weight, and shoot P content (Figure 2A). In contrast, seedlings inoculated with the mixed inoculum at the intermediate dose of P (213 mg kg^{-1}) were associated with higher values of root and total dry weight, and shoot/root ratios of the seedlings. Seedlings not inoculated and, in the presence, or absence of phosphate fertilization, as well as those inoculated with *G. margarita*, regardless of the applied P dose (71, 213, or 650 mg kg^{-1}) were not associated with any attribute, nor were *D. heterogama* or *R. clarus* at the two higher doses of P (213, 650 mg kg^{-1}), or the mixed inoculum in the presence of the highest dose of P (650 mg kg^{-1}).

Shoot, root, and total dry mass, as well as the shoot dry mass/root dry mass ratio, showed high correlation coefficients (> 0.70) with principal component 1 (0.72, 0.83, 0.98, and 0.75, respectively), while the root colonization percentage had a high correlation coefficient with principal component 2 (0.79). Only the shoot dry mass P content did not show a high correlation coefficient with either principal component 1 or 2 (0.48 and 0.29, respectively). The hierarchical cluster analysis reinforced the pattern indicated by the principal components analysis, due to the individualization of the mixed inoculum treatment at the intermediate dose of P (213 mg kg^{-1}). This treatment was isolated from the remaining by a Gower dissimilarity index of approximately 0.56 (Figure 2B).

CONCLUSIONS

S. terebinthifolius is highly responsive to mycorrhizae and the magnitude of its response depends on the associated fungal species. The inoculation of *S. terebinthifolius* seedlings with a mixed inoculum (spores of *R. clarus*, *D. heterogama*, and *G. margarita*) at an intermediate P concentration (213 mg kg^{-1}) provided the highest biomass accumulation. The maximum P technical efficiency for production of root and total biomass and the root/ shoot ratio with the mixed inoculum were 311, 292.5, and 420 mg kg^{-1} of P, respectively.

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