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Effect of Phosphorus Fertilization on Arbuscular Mycorrhizal Colonization of *Zeyheria tuberculosa* a Native Species in Brazil's Forest

[1]*Marcela Claudia Pagano and* [2]*Maria Rita Scotti*

[1]Institute of Biological Sciences, Federal University of Minas Gerais,
Av. Antônio Carlos, 6627, Pampulha, 31270-901, Belo Horizonte, MG, Brazil
[2]Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

Abstract: A greenhouse experiment was done to assess the mycotrophy in native soil of *Zeyheria tuberculosa*, a tree with potential use in recuperation of degraded tropical lands throughout agroforestry and agrosilvopastoral systems. The effects of triple superphosphate fertilization on the growth and colonization of this species were evaluated. The fertilized seedlings presented higher height and dry matter. *Acaulospora*, *Gigaspora*, *Glomus*, *Scutellospora* and *Racocetra* were recorded at both conditions (fertilized and unfertilized); however, the rhizosphere of *Z. tuberculosa* was found to be dominated by *Glomus*. The higher percentage of root colonization as well as the presence of arbuscules and other arbuscular mycorrhizal (AM) structures confirmed that *Z. tuberculosa* is an AM plant.

Key words: Arbuscular mycorrhizal fungi · Tropical tree species · Phosphorus fertilization · *Zeyheria tuberculosa* · Agroforestry

INTRODUCTION

Arbuscular mycorrhizas (AM) occur in most vascular plants; however, there are species less susceptible to AM colonization or that are not dependent of symbioses for growth [1]. For the more susceptible species, the low nutrient availability found in tropical soils favors these associations, which are frequently necessary for their optimal growth [2-4].

Phosphorus (P) is a major limiting nutrient for plant productivity, mainly because of its low mobility in soil. Phosphorus might be present in large amounts in soil, but the preferred form for assimilation, orthophosphate, is usually depleted owing to adsorption to soil particles or conversion into organic complexes [5]. In tropical areas, soil P deficiency is one of the limiting factors for plant growth because of slow diffusion through the soil [6] and plants have evolved various strategies for obtaining and recycling P. Different plant genotypes express these strategies in varying degrees, one such strategy being the association with AM [5].

Mycorrhizas benefit both partners and the main effect of AM colonization is an increase in phosphorus uptake by the host plant under P limiting conditions. The extraradical AM hyphae can extend beyond root P depletion zones, thereby improving P uptake by mycorrhizal plants [7]. Moreover, AM increase the uptake of other nutrients, the tolerance to drought and the resistance to transplantation stresses of seedlings [8].

Nowadays, there is a need to optimize nursery practices to meet the demand for high quality seedlings for outplanting and to understand the effects of interactions between AM species [1]. In nursery experiments non-pasteurized soil has been less used [9], as the common nursery practice of soil sterilization may be detrimental because it eliminates beneficial AM; besides, species not native to the site may not survive because they may not be adapted to the local pathogens [10].

Fertilization and nutrient supply imbalance, especially higher levels of nitrogen (N) and P, are important factors influencing AM sporulation, which could be reduced under those conditions [1]. Furthermore, different AM species can show different responses to fertilization. For example, the relative abundance of *Glomus intraradices* increased in response to fertilization, while it decreased for *Gigaspora* and *Scutellospora* species [11].

Corresponding Author: Marcela Claudia Pagano, Department of Biology, Federal University of Ceará,
Campus do Pici, Bloco 902, 60455-760, Fortaleza, Ceará, Brazil
Mobile: 0055 85 81064060, E-mail: pagano@netuno.lcc.ufmg.br, marpagano@gmail.com.

Moreover, Bhadalung *et al*. [12] showed that some spores of *Glomus* were classified as slightly sensitive to fertilization and others, as highly sensitive. Due to the difficulty in making specific fertilization recommendations, the study of native plants needs to be fostered. Although reports of plant species from Brazil have been recently increasing because of their importance for reforestation and restoration purposes, further research is needed to investigate the differential colonization of different plant successional groups by AM fungi [13].

Zeyheria tuberculosa Bureau ("bolsa de pastor", "ipê-felpudo") is a semi-deciduous full-sun plant, endemic to Brazil and threatened by habitat loss. Mostly used as a timber and urban tree, this plant can be intercropped with other species in the agroforestry systems in Brazil [14]. *Zeyheria tuberculosa* is a pioneer tree with relatively fast growth (mean annual increment reaches up to $24m^3$ ha^{-1} a year [14] and potential use in recuperation of degraded lands. This is an invasive species in grasslands and degraded areas (pioneer anthropic), which forms natural, pure populations. This species presents monopodial growth (forming straight trunk up to 2/3 of the total tree height) and natural pruning; it can be included, in monoculture or mixed with other tolerant species, in agroforestry as well as in agrosilvopastoral systems [15]. Highly positive features, such as sprouting of the coppice after successive cutting at different ages, broad leaves (50 to 90 cm length in seedlings and 30 to 40 cm in trees) and caducifoly (in July to September, when produces their fruits), may contribute towards more sustainable agroecosystems [16]. Thus, *Z. tuberculosa* could be considered as a good soil protector because it has standing biomass along most of the year, is broad-leaved and could benefit by AMF, characteristics desired for restoration purposes.

Z. tuberculosa's very hard wood (density 0.75 to 0.80 g/cm^3) is good for manufacturing furniture, heavy construction, parquet flooring, wheels and for use in farm activities (posts, pillars, fences, wooden handle tools, etc.). Its wood density is similar to that presented by commercial "ipês" (*Tabebuia* and *Paratecoma*) [17].

Little is known about the nutritional requirements for this plant species and its AM status has been previously showed in nursery conditions by Carneiro *et al*. [18], who observed low root colonization (1 to 19%). However, the authors attributed the low colonization to the fact that soil fumigation (methyl bromide) eliminates propagules of AMF and that there was later a contamination of seedlings, which were cultivated in bags on the soil. The authors used bags containing 1 kg of substrate charred rice husk, tanning curral manure and 20 mg P/kg substrate, as single superphosphate (SSP).

On the other hand, Zangaro *et al*. [19] reported *Z. tuberculosa* as a non-mycorrhizal (NM) plant and, consequently, in their review, Wang and Qiu [20] mention this species as NM. The experiment by Zangaro *et al*. [19] was carried out in a greenhouse in plastic bags filled with a mix of subsoil and sand (85:15), inoculated or not with spores of native AM fungi. These were obtained from rhizosphere soil of different native tree species in an area with natural vegetation dominated by woody pioneer species, in the southern Brazilian State of Paraná. They found 0% of colonization at both inoculated and non-inoculated treatments.

One of the purposes of the present study was, therefore, to clarify the *Z. tuberculosa* – AM association; this involved an experiment in plastic bags. As plant interactions with soil microbes vary with abiotic factors, a fertilization treatment was also included.

We evaluated the mycorrhizal colonization of *Z. tuberculosa* in a natural soil. An additional aim in our study was to indicate the potential AMF species as inoculants for *Z. tuberculosa,* a native species which presents ecological and environmental interest for Brazil.

MATERIALS AND METHODS

Soil Properties: A clay "cerrado" soil extracted from a secondary forest area in the surroundings of the city of Belo Horizonte, Brazil, was used as substrate. The soil was passed through a 2 mm sieve before being packed into bags of 1.7 dm^3 capacity. The soil was analyzed at the Institute of Agronomy of Minas Gerais- Agropecuary Chemical Laboratory, Brazil. Organic matter and P were determined. Potassium (K), calcium (Ca) and magnesium (Mg) were determined by atomic-absorption spectrometry using 1 N ammonium acetate as extracting solution. Soil texture was determined by the hydrometer method and the pH of the soil was measured in H_2O. Exchangeable aluminum (Al) was extracted with 1 M KCl solution and determined by titration with NaOH.

Plant Material and Treatment: Seeds of *Zeyheria tuberculosa* Bureau (Bignoniaceae) were collected from mature plants (forest fragments) located in Taquaraçú de Minas (19° 40'S 43°41'W) – State of Minas Gerais, Brazil. After sterilization with mercury bichloride ($HgCl_2$) 10% for 1 min, the seeds were placed in sterilized sand and were watered with sterilized water for 7 days. Ten pre-germinated seeds of *Z. tuberculosa* were sown into each 24 bags.

Fertilization of triple superphosphate (TSP) (P_2O_5) was applied based on nutrient tests for native trees. Phosphorus addition was to levels equivalent to statistically analyzed using MINITAB version 13.2 for 60 Kg ha^{-1} of P_2O_5. These treatments with 12 replicates involved bags, containing 1.25 kg of dry soil with one seedling each. Bags were arranged on glasshouse benches in a completely randomized design. The applied treatments were fertilized native soil and un-fertilized native soil (control). Twelve bags as replicates were used for each particular treatment.

The experiment was conducted from 15 February 2007 to 15 July 2007 in a greenhouse at the Botany Department of the Federal University of Minas Gerais, Brazil, under natural conditions of temperature, light and humidity. The maximum and minimum averages of temperature were: 38.6°C and 19.6°C. Bags were watered as needed and, twice a month, 20 ml of 1/4-strength Hoagland nutrient solution without P was added to the bags during the entire course of the experiment.

Plants were harvested at 5 months, measured, dried to constant weight at 75°C and weighed. Aerial plant biomass was determined. The root system was also weighed. One-way ANOVA using Tukey test ($p< 0.05\%$) was performed to verify the differences between treatments.

Spore Numbers and Species Richness of AM Fungi: Rhizosphere soils of *Z. tuberculosa* were collected from each treatment for analysis of mycorrhizal spores. AMF spores were recovered from soil samples (100g dry soil) of each treatment (5 samples for the native soil and 5 samples for the fertilized one), separated from soil by wet sieving and decanting and sucrose centrifugation after Pagano and Scotti [21]. The analyzed data were expressed as number of spores 100g-1 dry soil. Healthy spores (intact, healthy-looking spores) were picked under the dissecting microscope and counted. Each spore type was mounted sequentially in PVLG (polyvinyl alcohol-lactic acid-glycerol) and a mixture of PVLG and Melzer's reagent for identification and for obtaining permanent slide specimens.

The morphological properties and subcellular structures were observed under a light microscope at 100x to 1000x magnifications. Identification was based on spore colour, size, surface ornamentation and wall structure, with reference to the descriptions, International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM, West Virginia, USA), Arbuscular Mycorrhizal Fungi (Glomeromycota), Endogone Complexipes species deposited in the Department of Plant Pathology, University of Agriculture in Szczecin, Poland and the original species descriptions. Total spore numbers were logarithmically transformed and statistically analyzed using MINITAB version 13.2 for Windows and means were compared by Fisher's least significant difference (LSD) test ($p< 0.05$) [22]. The isolation frequency was calculated as the percentage of samples (n=5) from which a particular species was isolated. Spore morphology and differential staining were observed. Photos were taken with a Microscopic camera Olympus BH-2.

Root Colonization by AM Fungi: Roots of *Z. tuberculosa* were collected from each plant and were fixed in FAA solution [21] until samples could be processed. Roots were stained and assessed for mycorrhizal infection as follows: fine roots were taken from FAA, washed several times in tap water and bleached in 10% (w/v) KOH overnight and then heated to approximately 90°C in a water bath for 1 h. The cooled root samples were washed and stained with 0.05 % Trypan blue. Roots were cut into 1 cm segments and thirty one-cm-root fragments were examined per sample for their AM status under a compound microscope. If at least one root segment was found to contain fungal mycelia, arbuscules or vesicles, then the sample was considered as an AM plant, recorded as ''+''. Plants were recorded as non-mycorrhizal (''-'') when neither arbuscules/vesicles nor fungal mycelia were detected in their root cortical cells. Quantification of mycorrhiza colonization was according to Pagano and Scotti [21] and results were expressed as percentage of colonized segments (number of colonized segments /total analyzed segments). External hyphae (%) and auxiliary cells (AC) (%) were calculated as number of colonized segments by this structure/ total analyzed segments.

Intensity of AMF colonization was assessed as explained in Pagano and Scotti [21], in which %M indicates the intensity of mycorrhization according to an arbitrary scale of 1 to 5 (1 - trace of AM colonization; 5= >90% of the root cortex colonized). Then %M is calculated as the proportion of root centimeters colonized by AM, but weighted by the intensity of the colonization: $\%M = (95n5 + 70n4 + 30n3 + 5n2 + n1) \div N$, where $n5$, $n4$,...$n1$ indicate the number of root centimeters with an intensity 5, 4,…1 and N is the number of fine root centimeters observed.

These data were arcsin $(x/100)^{1/2}$ transformed. The data were subjected to one-way ANOVA using MINITAB version 13.2 and means were compared by Fisher's least significant difference (LSD) test ($p< 0.05$). Photos were taken with a microscope using an Olympus BH-2 camera.

RESULTS

The soil used in this study was nutrient-poor and acidic. Its analysis revealed (Table 1) 170 mgN kg-1, as calculated N from organic matter, 3.5 mg P kg-1 and 29 mg K kg-1; the pH was 4.9 (in water). This means that the soil contained moderate amounts of basic nutrient N and lower concentrations of K, Ca and Mg. The pH was strongly acid. There was a high C and extremely low available P content.

Several tested morphological parameters were significantly affected by the TSP fertilization, with the exception of the length of the main root, shoot and root fresh matter and root dry matter (Table 2).

Table 1: Properties of the cultivation soil

Soil property[a]	
pH (H_2O)	4.9
Soil organic matter (%)	3.47
C (%)	2.01
N (mg 100g-1)	170
Avail. P (mg dm^3)	3.5
Avail. K (mg dm^3)	29
Exchang. Al^{3+} (mmol kg-1)	1.87
Exchang. Ca^{2+} (mmol kg-1)	0.45
Exchang. Mg^{2+} (mmol kg-1)	0.11
CEC (mmol kg-1)	8.25
Base saturation (%)	7.71
Texture (%)[b]	
Coarse sand	6.4
Fine sand	19.1
Clay	60.72
Silt	13.78

[a]Mean of two measures from one composite sample. [b]Particle size distribution: coarse sand 2-0.2 mm, fine sand 0.2-0.02 mm, silt 0.02-0.002 mm and clay < 0.002 mm. mg L^{-1} = milligram per liter, CEC = cation exchange capacity

Plants without TSP grew significantly less than the fertilized ones (increase 11% in height). Fertilized plants showed increased shoot (54%) and total dry matter (38%) in relation to the native soil treatment (Table 2). Enhanced shoot growth resulted in significantly lower root/shoot ratios for fertilized plants.

Gigaspora margarita, *Scutellospora cerradensis* and *Racocetra gregaria*, were present in low spore numbers; whereas, *Glomus* sp. 1 presented a higher spore number. In the present study, increasing TSP fertilization decreased (with no significance) AMF spore number consistently by 37% (39.9 to 25 spores 100g soil-1) (Table 3). The same AMF species were found in both rhizospheric soils evaluated; however, the isolation frequency (F) of *A. scrobiculata* in the rhizosphere of *Z. tuberculosa* decreased with TSP fertilization (Table 3).

Table 2: Growth of *Z. tuberculosa* seedlings after 5 months in greenhouse

Growth	Native soil	Fertilized native soil[a]	F ratio/ significance level
Height (cm)	8.5[a] b	9.9 a	8.84*
Length of the main root (cm)	17.1	19 ns	
Shoot dry matter[#]	0.65 b	1.43 a	7.94*
Shoot Fresh matter[#]	2.48	2.48 ns	
H/ Shoot dry matter	13.07 a	6.92 b	7.73*
Root fresh matter[#]	2	2.4 ns	
Root dry matter[#]	0.53	0.45 ns	
Total dry matter[#]	1.18 b	1.89 a	27.8*
R/S	0.82 a	0.32 b	10.82**

[a]Fertilized= 6gTSP kg-1 soil; [a]Mean height of 5 plants; [#](g/plant); R/S: Root:shoot ratio. Means followed by the same letters are not significantly ($p < 0.05$) different within different treatments according to Fisher's least significant difference (LSD) test. *P < 0.05 **P < 0.01, ns: not significantly different

Table 3: AM species isolated from *Z. tuberculosa* rhizosphere at greenhouse conditions

	Native soil		Fertilized native soil	
AM Species	SN	F	SN	F
Gigasporaceae				
Gigaspora margarita Becker & Hall	1	100	3	100
Gigasporaceae				
Scutellospora cerradensis (Spain & J.Miranda) Sieverd, F.A.Souza & Oehl	3	100	3	100
Racocetraceae				
Racocetra gregaria (N.C. Schenck & T.H. Nicolson) Oehl, F.A. Souza & Sieverd.)	0.6	66.6	1	66.6
Acaulosporaceae				
Acaulospora scrobiculata Trappe	2.7	100	1.5	66.6
Glomeraceae				
Glomus sp. 1	32.5	100	16.5	100
Total spore number (N)	39.9ns		25 ns	
Species Richness[♦]	5		5	

SN: spore number; F: Isolation frequency (%). ns: not significantly different.

Table 4: AM root colonization levels of *Z. tuberculosa* at greenhouse conditions

Treatment	AM hyphal colonization†	AM vesicles‡	Arbuscules (%)	External hyphae (%)	Auxiliary cells (%)	Intensity of mycorrhization (%)
Native soil	85 a	82 a	10.7ns	3.5 a	3.5 a	5.4 a
Fertilized Native soil	54 b	45.8 b	16.6	0 b	0.04 b	1.8 b

†% AM hyphae in roots; ‡ % AM vesicles in roots; Means with different letters (column) are significantly different according to Fisher's least significant difference (LSD) test ($p < 0.05$); ns: not significantly different.

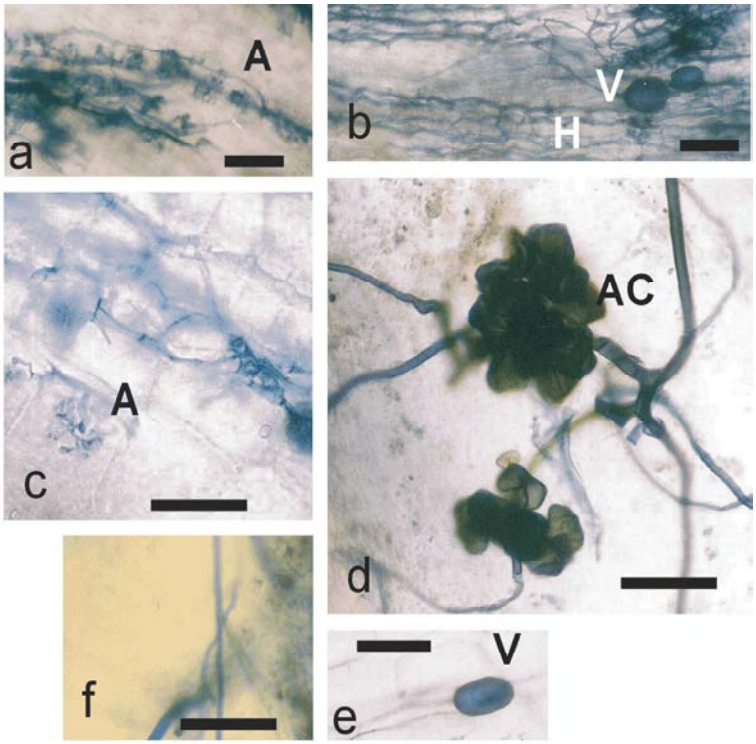

Fig. 1: AM structures observed in the roots of Z. tuberculosa. a-c Intra radical hyphae on Z. tuberculosa roots (a), bearing vesicles (b), arbuscules (c). Bar = 100µm. (d) auxiliary cells with shallow swellings. (e) Terminal vesicle on an Z. tuberculosa root. (f) auxiliary cells with narrow projections. Bar = 50µm.

Results regarding AM colonization showed that TSP fertilization affected colonization in native soils. Fertilized plants had lower levels of colonization by AMF in comparison to the non-fertilized ones, which shows a very high colonization and a higher intensity of mycorrhization (Table 4). Moreover, the treatment without TSP fertilization also showed the highest root length colonization (hyphae and vesicles), external hyphae percent colonization and AC (Table 4).

Figure 1 shows the mycorrhizal status of *Z. tuberculosa* under greenhouse conditions. This species presented hyphae, arbuscules (Fig. 1 a,c) and vesicles (Fig. 1 b,e) in their roots and also AC (Fig.1 d,f). The brown AC with shallow swellings (Fig. 1,d) were in line with *Scutellospora* and *Racocetra.* Other types of AC present in *Z. tuberculosa* roots have narrow projections (Fig. 1,f) similarly to *Gigaspora*'s auxiliary cells.

DISCUSSION

As fertilized plants presented higher shoot growth and significantly lower root/shoot ratios than non-fertilized ones, this study corroborates height measurements to estimate the quality of seedlings as an efficient morphological parameter for use in nurseries, since it is a nondestructive method.

In the present study, we observed that TSP fertilization consistently lowered (with no significance) the total number of spores, which are in general correlated with the proportion of colonized roots. Five AMF species associated with *Z. tuberculosa*. *Glomus* was the dominant genus within Glomeraceae, as is commonly found [3] and *S. cerradensis* was the dominant genus within Gigasporaceae, as is commonly found in Brazilian savanna soils De Souza *et al*. [23].

Fertilization can make AMF total spore numbers decrease and can cause variation in species diversity [12]. The consistent decrease in spore number of *Glomus* (49.3%) in the presence of fertilization is in line with the findings of Bhadalung *et al*. [12], who showed that some *Glomus* species were highly sensitive to fertilization (TSP). The same authors classified *Scutellospora fulgida* as a species slightly sensitive to fertilization. In our study species of *Gigaspora*, *Scutellospora* and *Racocetra* did not show a decrease in numbers, suggesting that they can be slightly sensitive to fertilization (TSP).

With respect to root colonization by indigenous AMF, there were significant differences for unfertilized and fertilized seedlings. The lower colonization by AMF in fertilized plants is in line with studies showing that increased concentrations of phosphate decrease AMF colonization in roots [24-26]. It is known that for Brazilian trees, which colonization is favored by an intermediate P level and moderately inhibited at higher P levels [4].

Contrary to the findings by Zangaro *et al*. [19], we found *Z. tuberculosa* highly colonized in native soil. Carneiro *et al*. [18] found a low colonization but have not reported on the structures found.

In the present study the different AMF's structures present in the roots, corresponding to Glomeraceae (presence of dark stained vesicles) and Gigasporaceae (presence of auxiliary cells), reflect colonization by different AMF. Jansa *et al*. [27] showed the functional complementarily among species within the AMF community colonizing a single root system; however, the mechanisms of competition among AMF need further elucidation. Therefore, a mix of Glomeraceae and Gigasporaceae or Dentiscutataceae could be an efficient inoculant for *Z. tuberculosa*. AMF species that are non-sensitive to fertilization and that are able to rapidly colonize seedling roots in pot culture are of utmost interest. In this study the presence of AMF spores not belonging to *Glomus*, which tolerate TSP fertilization, shows the importance of including these AMF in greenhouse trials.

In our study, without P addition, the soil was considered P-deficient and the change in AMF species that colonize *Z. tuberculosa* roots according to P availability suggest that *Gigaspora*, *Scutellospora* and *Racocetra* can play a role in acquiring P, as morphological characteristics unique to these genera were present in roots from unfertilized soil.

Carneiro *et al*. [18] attributed the low colonization of *Z. tuberculosa* to the soil fumigation and posterior contamination of seedlings, which were cultivated in bags on the soil. They also used manure and 20 mg SSP kg^{-1} substrate; however, SSP cannot inhibit the AMF symbioses [28] but it will depend on their quantity as well as other factors. Chu [29] reports that application of 1g of SSP kg^{-1} soil and low P content of soil (7 mg P kg^{-1}) plus AMF inoculation increased growth of *Euterpe oleracea* seedlings.

The presence of external hyphae in the non-fertilized soil could be due to Acaulosporaceae colonization, which is characterized by the presence of a highly infective extra-radical mycelium [25]. However, *Acaulospora* presented low spore numbers, which could be accounted for by the fact that not all AM species may be sporulating at the time of sampling.

The presence of vesicles, considered lipid storage structures as well as fungal propagules [24] in the roots of *Z. tuberculosa* implies that this plant species might rely on these structures for nutrient storage. The higher percent of vesicles in unfertilized native soil treatment suggests that this plant could store phospholipids as an insurance against future deficits and this is in agreement with the general remark that the AM symbiosis develops most readily under low-phosphate conditions. On the other hand, the lower percent of vesicles in roots of fertilized plants may be due to the fertilization [1].

It is known that co-existence of different AMF species leads to varying states of compatibility between host and fungus depending on the developmental state of the plant and that of the colonizing fungus [30]. Species of *Gigasporaceae*, *Dentiscutataceae* and *Racocetraceae* do not form vesicles but instead form AC on the extraradical mycelium. In this study, the presence of brown AC with shallow swellings (*Scutellospora* like) and ornamented cells with narrow projections (*Gigaspora* like), in the treatment without TSP, reveals an important role for these genera in the root colonization by AMF.

Although vesicles were the most common AM structure in all the samples, the presence of AC was noticeable. Our findings are in agreement with previous observations that in *Scutellospora* the pigmented AC formed initially on pigmented hyphae [30]. Needless to say, according to the recent revision of *Scutellospora* [31] the spores isolated in the present study (*D. cerradensis* and *R. gregaria*) were previously included in *Scutellospora*.

The observed presence of arbuscules in our study, which are the most probable site of release of P into the interfacial apoplast and then to the plant [32], confirms the functional mycotrophy of *Z. tuberculosa*.

From a practical point of view, an addition of TSP to soil was sufficient to produce plants with a higher height and dry matter. Differences in growth-enhancing effects by fertilization are documented for various Brazilian tropical trees [33] and this study adds to this knowledge focusing on *Z. tuberculosa*. The fertilization was beneficial, due to improved total seedling growth, which is in line with reports that pioneer species have a more elevated nutrient requirement than climax ones, indicating greater fertilizer demand [33]. Moreover, these plants presented a medium AM colonization with TSP fertilization, suggesting that lower levels of TSP must be tested in order to guarantee more mycorrhizal benefits.

CONCLUSION

It could be conclude that *Z. tuberculosa* seedlings presented mycotrophy and exhibited better growth when fertilized with TSP. Moreover, different AM fungal types colonized the roots of this plant species. A reduced fertilization and AM inoculation (when these fungi were not present in the soil) could be a better technology than that of sole fertilization and may be preferred for the management and nutrient recovery from the degraded areas and agroforesty systems. Moreover, these plants presented a medium AM colonization with TSP fertilization, suggesting that lower levels of TSP must be tested in order to guarantee more benefits from mycorrhizas. Thus, we believe that this study is an important starting point for further investigations, which can confirm additional benefits from AM.

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