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Digging deeper to study the distribution of mycorrhizal arbuscular fungi along the soil profile in pure and mixed *Eucalyptus grandis* and *Acacia mangium* plantations

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ABSTRACT

The presence of Arbuscular mycorrhizal fungi (AMF) in pure and mixed *Eucalyptus grandis* and *Acacia mangium* plantations has not been clearly characterised, especially regarding their vertical distribution along a deep soil profile. Our aim was to study the AMF community in layers from 0 to 800 cm deep, comparing pure and mixed *E. grandis* and *A. mangium* plantations. We investigated whether AMF are able to colonize roots and produce spores down to 800 cm of depth, and whether intercropping of *A. mangium* increases AMF abundance and diversity in *E. grandis* plantations at the surface and subsurface layers of soil. AMF spore abundance and identification were evaluated morphologically, while the quantification of root colonization following the methodology of root clarification, dyeing and determination of the percentage of root length colonized by AMF. Total root DNA was extracted, and specific PCR was applied, which resulted in clones of the 18S rRNA small-subunit (SSU) gene region of AMF. Results show that AMF spores and some root colonization were present even in the deepest soil layers. Spore evaluation identified 16 species across six AMF genera (*Acaulospora*, *Gigaspora*, *Glomus*, *Intraornatospora*, *Scutellospora* and *Racocetra*). The genus *Glomus* was the most abundant and found in all treatments and depths. *E. grandis* in the mixed plantation with *A. mangium* presented a significant increase in root colonization in the 0–20 and 20–50 cm layers, indicating a possible stimulation at superficial soil layers of the symbiosis in *E. grandis* roots when in consortium. Amplification of the 18S region of AMF revealed specific predominant groups at each soil depth. We found that the deep layers, although presenting comparatively low root colonization, still harbour a considerable range of AMF.

1. Introduction

The genus *Eucalyptus* is one of the most cultivated in the world covering an estimated area of 22 million hectares. Brazil is the world's largest producer with 7 million hectares (Abraf, 2013). However, most of *Eucalyptus* plantations are monospecific, which may in the long run cause nutritional imbalances in the soil and increased demand for mineral fertilizers, raising both economic input and risk of environmental pollution (Laclau et al., 2008; Rachid et al., 2013). To minimize these effects, there has been a surge of proposals over the last decade to implement forest stands to contain *Eucalyptus* and *Acacia* trees in a mixed system (Bini et al., 2013; Laclau et al., 2008; Pereira et al., 2017). *Acacia* is a legume of the family Fabaceae (native of Australia, New Guinea and Indonesia), and the diazotrophic bacteria associated with its roots are known to efficiently fix N_2 (Bouillet et al., 2008; Fonseca et al., 2017). This management practice aims at supplying the nutritional demand for N fertilization in *Eucalyptus* plantations (Bouillet et al., 2008; Voigtlaender et al., 2012). Several studies demonstrated the improvements in soil fertility, acceleration of nutrient cycling, increase in the organic matter quality, greater effectiveness in water and light use and increase in wood productivity generated by this association (Bini et al., 2013; Bouillet et al., 2008; Laclau et al., 2008; Pereira et al., 2018).

AMF are classified in the phylum *Glomeromycota* (or *Glomeromycotina*) and form a mutual symbiosis with over 80% of terrestrial plants (Schüßler et al., 2001; Spatafora et al., 2016). This association can provide innumerable benefits to the host plant through its interconnection with numerous and extensive networks of hyphae in the soil (Friese and Allen, 1991; Cardoso et al., 2013). The great mass of extra-radicular hyphae promotes the expansion of the root system and,

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consequently, facilitates the translocation of mineral nutrients (Wu et al., 2012) and water (Finlay and Read, 1986) to the plant, besides providing protection against pathogens (Borowicz, 2001).

Both *Acacia* and *Eucalyptus* genera associate with AMF (Steffen et al., 2010; Bini et al., 2018). Bini et al. (2018) published possibly the first evaluation of AMF effects on a mixed cropping system. Through a study of the dynamics of AMF during the first 20 months after planting of pure and mixed stands of *E. grandis* and *A. mangium*, Bini et al. (2018) reported that colonization by AMF in *E. grandis* roots was significantly greater when the trees were grown in mixed systems with *A. mangium,* and also identified a strong correlation between AMF colonization rates and acid and alkaline phosphatase activities in soil, which is produced in greater quantity by *A. mangium*.

It is generally assumed that AMF colonization is restricted to the surface soil layer, and hence little is known about deeper regions of the soil profile. None of the few published reports on AMF in deeper soil layers relates to *Eucalyptus*, and only describe the greatest spore density and diversity at the soil surface layer (Muleta et al., 2008; Oehl et al., 2005; Shukla et al., 2013; Virginia et al., 1986).

Virginia et al. (1986) evaluated soil layers up to 4 m deep and found that AMF and other symbionts in a *Prosopis glandulosa* system were substantially more numerous in deep layers. Oehl et al. (2005) investigated the soil profile of grassland, vineyard and maize systems from 0 to 70 cm and revealed a great diversity of spores even in the deepest layers (50–70 cm), indicating that the AMF communities in deep layers can be diverse and different from the soil surface. In an agroforestry system, consisting of coffee plantations, *Acacia abyssinica*, *Albizia gummifera*, *Ficus sur* and *Ficus vast*, Muleta et al. (2008) collected soil samples from 0 to 50 cm deep and found that coffee trees intercropped with leguminous trees resulted in effective numbers of AMF even in the deepest assessed layers. In a *Withania somnifera* and *Ocimum sanctum* cultivation, Shukla et al. (2013) sampled soil layers between 0 and 40 cm and showed that in deep layers, where plants present fewer roots, fewer mycorrhizae and fewer AMF propagules, the distribution of AMF is determined by soil pH and moisture. As a result, they postulate that management practices that improve the chemical and physical soil conditions in deep layers can contribute to a more balanced distribution of AMF in the soil.

However, as a rule AMF studies in cropping systems are based exclusively on spore morphology and root colonization rates. To find out more about AMF diversity levels in the roots, a possible approach is to clone and sequence fragments of the 18S region of *Glomeromycota* in colonized roots. However, this methodology may also act on other fungi affiliated to *Basidiomycota* and *Ascomycota*. The lack of specificity of the set of primers used in cloning of the18S region is one of the main problems of molecular studies with AMF. Since AMF are obligate biotrophic fungi, cloning approaches must necessarily amplify samples extracted from inside the roots. Primers such as AM1 (Helgason et al., 1999) do not amplify all known AMF groups (Redecker, 2000) and may present some degree of non-specificity. However, their use is reliable when one intends to study AMF colonizing the internal tissues of the roots since they do not amplify plant DNA (Bonfim et al., 2016; Redecker, 2000).

In plants that exhibit a root system capable of exploring deep layers of the soil, microbial activity can occur at depths well below those usually studied, enriching the soil and promoting the functioning of symbiotic organisms (Pereira et al., 2017; Virginia et al., 1986). Moreover, microbes may reveal a certain degree of specificity towards certain AMF and host plants, which may be crucial for water and nutrient absorption during periods of prolonged water deficit in the soil (Oehl et al., 2005; Virginia et al., 1986). Additionally, Oehl et al. (2005) emphasize that AMF sampling should include deep layers of soil to obtain a complete picture of their distribution.

Here we present a study of pure and mixed *E. grandis* and *A. mangium* plantations based on soil trenches which reached down to 800 cm of depth with the aim of identifying changes in AMF abundance and diversity through a combination of traditional and molecular approaches. Our main questions were: (i) is it possible for tree roots at great depth (down to 800 cm) to associate with AMF?; and (ii) can intercropping of *A. mangium* increase AMF abundance and diversity in *E. grandis* plantations at the surface and subsurface layers of soil?

2. Material and methods

2.1. Experimental site

The study was performed at the Experimental Station of Forest Sciences of Itatinga (23°02′ S 48°38′ W, 860 m a.s.l.), Luiz de Queiroz College of Agriculture, University of São Paulo, Brazil. The experiment implemented three treatments: pure stands of *Eucalyptus grandis* (E) and *Acacia mangium* (A), in addition to mixed stands of *E. grandis* and *A. mangium* (A + E). In the mixed plantations, we sampled roots and soil at the base of *E. grandis* E(A + E) and at the base of *A. mangium* A(A + E). During sampling, we followed the roots up to their origin at the plant to ensure that they belonged to a specific tree. When this procedure was impossible, we distinguished the roots of *E. grandis* and *A. mangium* by evaluating the presence of nodules and species-specific morphological characteristics such as color and thickness among others.

The experimental design was randomized complete blocks, containing three blocks and three treatments, totalling nine experimental plots. The plots measured 36 × 36 m, but only the inner areas of each plot measuring 24 × 24 m were considered in the analyses in order to eliminate any possible edge effects. The mixed stands were planted with a 1:1 ratio of *E. grandis* and *A. mangium* trees (Laclau et al., 2008). Samples were collected in October 2013, when trees were four years old and 12 m tall on average. The soil was Ferralsol (FAO classification) with medium-sandy characteristics (~85% sand), low cation-exchange capacity and low nutrient concentrations (Laclau et al., 2008; Pereira et al., 2017). The concentration of macro- and micronutrients in the soil (in each layer of the trenches) was determined following the methodology proposed by Raij et al. (2001) and can be found for consultation in Table S1.

2.2. Soil and fine roots sampling

We dug deep trenches to sample fine roots and soil at different layers along their whole extension. Trenches measured 0.6 × 1.65 m and were from 0 to 800 cm deep (Pereira et al., 2017). One trench was built in each monoculture plot (E and A) and two trenches in the mixed stand plots (A + E), one at the base of each plant species [(E(A + E) and A(A + E)]. In both cases, trenches were opened at the centre of the plots. We sampled soil and roots at 10 soil layers with increasing depths along the soil profile: 0–20 cm; 20–50 cm; 50–100 cm; 100–200 cm; 200–300 cm; 300–400 cm; 400–500 cm; 500–600 cm; 600–700 cm and 700–800 cm (Pereira et al., 2017). From each soil layer, we collected and homogenized four subsamples to create composite samples (Pereira et al., 2017). Before sampling, about 25 cm of the outer borders of the trenches were discarded in each layer, ensuring that the collected samples did not suffer cross-contamination by any soil particles or root fragments from the higher parts of the trench. Thus, 120 soil and root samples were collected, representing the four treatments E, A, (E (A + E) and A(A + E), the three replicates (blocks), and the 10 soil depths.

2.3. AMF morphological identification

Spore extraction was carried out by wet sieving and decantation of 50 g of soil (Gerdemann and Nicolson, 1963). Different sizes of sieves 0.71 mm and 0.045 mm were used in the process. Spores collected in the 0.045 mm sieve were centrifuged in sucrose solution (70%) for 3 min at 3500 rpm (Bonfim et al., 2016). The supernatant was rinsed with water, sieved at 0.045 mm and the retained spores were stored at