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Intercropping *Acacia mangium* stimulates AMF colonization and soil phosphatase activity in *Eucalyptus grandis*

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ABSTRACT: Arbuscular mycorrhizal fungi (AMF) are very important to plant nutrition, mostly in terms of acquisition of P and micronutrients. While *Acacia mangium* is closely associated with AMF throughout the whole cycle, *Eucalyptus grandis* presents this symbiosis primarily at the seedling stage. The aim of this study was to evaluate the dynamics of AMF in these two tree species in both pure and mixed plantations during the first 20 months after planting. We evaluated the abundance, richness and diversity of AMF spores, the rate of AMF mycorrhizal root colonization, enzymatic activity and soil and litter C, N and P. There was an increase in AMF root colonization of *E. grandis* when intercropped with *A. mangium* as well as an increase in the activity of acid and alkaline phosphatase in the presence of leguminous trees. AMF colonization and phosphatase activities were both involved in improvements in P cycling and P nutrition in soil. In addition, P cycling was favored in the intercropped plantation, which showed negative correlation with litter C/N and C/P ratios and positive correlation with soil acid phosphatase activity and soil N and P concentrations. Intercropping *A. mangium* and *E. grandis* maximized AMF root colonization of *E. grandis* and phosphatase activity in the soil, both of which accelerate P cycling and forest performance.

Keywords: AMF spores, mycorrhiza, mixed species, soil enzymes, phosphorus

Introduction

Many studies have been conducted in intercropped plantations of *Eucalyptus* and leguminous trees to verify and validate the sustainability of these systems. Positive results of *Eucalyptus* intercropped with leguminous trees (e.g. *Acacia mangium* and *A. mearnsii*) have been reported to stimulate the biogeochemical cycles in soil, plant biomass productivity, soil fertility, and more (Bachega et al., 2016; Binkley et al., 1992; Bini et al., 2013a; Bouillet et al., 2013; Forrester et al., 2005; Khanna, 1997; Paula et al., 2015; Voigtlaender et al., 2012). It is known that legumes require more P than non-leguminous plants because maintaining the biological N_2 fixation process in root nodules is highly dependent on P (Binkley, 1992). In this case, associations with arbuscular mycorrhizal fungi (AMF) may positively influence plant P nutrition and, indirectly, the biological nitrogen fixation process (Cardoso et al., 2013).

Though both species, *E. grandis* and *A. mangium*, can associate either with AMF or with ectomycorrhizal fungi (Mendes-Filho et al., 2009; Pagano et al., 2008), our studies were restricted to the first group, since our observations refer to very young plants, beginning at the seedling stage, a period in which there is a predominance of AMF colonization in the roots of both plants (Adams et al., 2006; Pagano and Scotti, 2008). However, very little is known about the association between AMF and *Eucalyptus*, especially when intercropped with *A. mangium*, which is characterized by forming symbioses with N-fixing bacteria and AMF (Mendes-Filho et al., 2009). Intercropped systems generally show greater diversity and abundance of AMF than pure stands (Pagano et al., 2009).

High diversity and high inoculum potential of AMF in soils, even under low P availability, generally lead to a more efficient use of P and other nutrients (Cardoso et al., 2013; Ma and Rengel, 2008), owing to an increase in the root absorptive surface and to the external fungal hyphae mining the surrounding soil (Hinsinger, 2001). A decrease in available P in soil cultivated with intercropped trees was attributed to higher P uptake by mycorrhizal plants (Koutika et al., 2014). In addition, decreases in the litter C/P and C/N ratios in mixed plantations is a factor that favors litter decomposition by soil microorganisms and stimulates nutrient cycling (Bini et al., 2013a), mainly because of enzyme activities. Soil enzymes such as cellulase and phosphatases are good indicators of soil quality and mineralization rates of organic matter (Cardoso et al., 2013). Phosphatases are enzymes produced by plants and/or microorganisms, which are able to hydrolyze organic P into mineral phosphates and, thus, represent the mineralizing of organic P (Krämer and Green, 2000). The general aim of this manuscript was to evaluate the dynamics of AMF and soil enzyme activity in *A. mangium* and *E. grandis* plantations, and in mixed plantations during the first 20 months after planting.

Materials and Methods

Experimental area

The experiment was conducted in the municipality of Itatinga, SP (23°02′01″ S and 48°37′30″ W), with an average altitude of 830 m. The soil of the area is a Rhodic Ferralsol (FAO classification), with approximately the following constituents: 23 g kg^{-1} of organic mat-

ter, 11 mmol$_c$ kg^{-1} of Al^{3+} and 52 mmol$_c$ kg^{-1} of cation exchange capacity (CEC), 3.8 of pH (CaCl$_2$) and 5.3 µg g^{-1} of available P (Bini et al., 2013a).

Prior to the experiment's installation, the experimental area had been occupied by a very old plantation of *E. saligna* (1940 to 1998) that had never been fertilized. After clear cutting, a plantation of *E. grandis* with low fertilization (300 kg ha^{-1} 10:20:10 Nitrogen:Phosphorus:Potassium - NPK) was established, and the trees were harvested in Dec 2002. Later, in 2003, a first rotation of pure or intercropped *E. grandis* and *A. mangium* were planted and maintained until May 2009.

Experimental design and treatments

The experiment was installed in Nov 2009 by means of the implantation of a second rotation or a second cropping cycle of pure and intercropped *E. grandis* and *A. mangium* plantations, consisting of an array of three randomized blocks with tree treatments: (E) *E. grandis* monoculture (100E:0A); (A) *A. mangium* monoculture (0E:100A); and intercropped plantation (50A:50E) of *E. grandis* (EMS) and *A. mangium* (AMS), all trees installed within lines, with the same experimental design and minimum tillage as had been used in the first rotation. Each plot measured 30 m × 30 m, in which only an inner plot of 18 m × 18 m (36 trees) was considered for sampling, leaving two buffer rows. Trees were spaced 3 m × 3 m in every treatment.

Soil, roots and litter sampling

Soil and roots (0-10 cm depth) as well as litter (0.25 m^2 on the surface) were sampled at three intervals: 7 months after planting (a.p.) (winter – June 2010), 14 months a.p. (summer – Jan 2011) and 20 months a.p. (winter – July 2011). In Brazil's tropical climate we can differentiate only between winter (colder temperature and less rainfall, an average of 16 °C and 45 mm, respectively, from June to Aug) and summer (higher temperature and rainfall, average of 24 °C and 360 mm, respectively, from Dec to Feb). Spring and autumn are intermediary in their characteristics. The first AMF spore counting was performed 2 months after transplanting (Jan 2010), followed by the other three as previously described.

For sampling, nine representative trees were randomly selected in each of the three blocks with monocultures for each species. In intercropped areas, 18 trees were chosen, nine *A. mangium* and nine *E. grandis* seedlings, as explained above. Three soil, root and litter samples were taken from around each tree (at a distance of 1.5 m from the stem, between lines) and pooled to get three composite samples per block in monocultures and six composite samples in the intercropped plantation, three around *A. mangium* and three around *E. grandis* trees. Although considering that there might have been a certain degree of intermingled root growth from both tree species between lines, we made this separation dur-

ing sampling just to learn about the most expressive influences of each particular root system on the surrounding AMF community.

The soil samples were sieved (2 mm) and stored at 4 °C up to 5 days for microbiological analysis. Air-dried soil was used for chemical analysis. Litter was sampled and consisted of twigs, branches and leaves, that were dried to constant weight at 45 °C.

The available soil P was extracted using ion-exchange resin (Van Raij et al., 2001) and soil pH was determined in a suspension with 0.01 mol L^{-1} CaCl$_2$ in a 1:2.5 soil: water ratio. The total P in litter was quantified following Murphy and Riley (1962), whereas C and N in litter and in soil were measured in an elemental analyzer. Further information on the chemical analyses (C, N and P in soil and litter) can be found in Bini et al. (2013a).

Activity of soil enzymes in soil samples

Acid and alkaline phosphatases were assessed by spectrophotometry (420 nm). One gram of fresh soil was incubated at 37 °C for 1 h, with 0.2 mL of toluene, 4 mL buffer (pH 6 for acid phosphatase and pH 11 for alkaline phosphatase) and 1 mL of *p*-nitrophenyl phosphate (0.05 mol L^{-1}). After incubation, the filtered extract was treated with 1 mL of CaCl$_2$ (0.5 mol L^{-1}) and 5 mL of NaOH (0.5 mol L^{-1}) and used for determining enzymatic activities, based on the hydrolysis of *p*-nitrophenyl and the determination of the *p*-nitrophenol generated (Tabatabai and Bremner, 1969). Cellulase activity was evaluated as described by Schinner and von Mersi (1990), using 10 g of fresh soil incubated at 50 °C for 24 h in phosphate buffer (pH 5.5). Carboxy-methylcellulose was used as substrate and the reducing sugars formed were quantified with a spectrophotometer (690 nm) according to the Prussian blue method.

Mycorrhizal root colonization

To obtain the percentage of AMF root colonization, roots were first cleared with 10 % KOH overnight followed by 10 min in 10 % H$_2$O$_2$. The use of H$_2$O$_2$ was necessary because these tree roots presented a pigmentation that was quite dark and needed bleaching for greater transparency of the roots and their visualization under microscope.

Subsequently, roots were stained with blue ink and submerged in glycerol (Vierheilig et al., 1998). Stained roots were analyzed under a dissecting microscope at 40x magnification. Mycorrhizal colonization was estimated accounting for the presence or absence of fungal structures such as hyphae, arbuscules and vesicles inside the roots, using the grid-line intersection method (Giovannetti and Mosse, 1980).

Quantification and identification of AMF spores

Spores were extracted from 50 g soil samples according to Gerdemann and Nicolson (1963). The soil samples were wet-sieved through mesh sequences (from 0.71 to 0.045 mm). Subsequently, sequential centrifuga-

tions were performed with up to 70 % sucrose solutions to separate the spores from the soil particles. The extracted spores were counted, separated into groups of similar morphology, transferred and mounted on semi-permanent slides with glycerol and polyvinyl alcohol resin and Melzer's reagent. Spores were identified at species level under a light microscope, using Schenck and Pérez's Manual (1990) and descriptions provided by the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM http:// invam.wvu. edu). After identifying the AMF species, we determined the number of spores in each species together with the total number of species (richness), Simpson's dominance index (Is), and Shannon's diversity index (H).

Statistical analyses

Data were subjected to analysis of variance (ANOVA) and Duncan's test for comparison of means ($p \leq 0.05$), and Pearson's correlation analysis using SAS 9.2 software. The environmental and biological variables were tested by multivariate principal components analysis (PCA) to identify correlations between biological data (AMF, acid phosphatase, alkaline phosphatase and cellulase) and environmental data (N, P and C/N and C/P ratios of the soil and litter), for each sampling. The PCA was conducted using the Canoco 4.5 software program.

Results

There was a great fluctuation in spore numbers from one period to the next, sometimes increasing in one treatment and decreasing in another. Generalizing from all treatments, it would be possible to say that there was a quite low spore number at 2 months, with a tendency to decrease even more at 7 and 14 months after planting in a number of cases, followed by a more pronounced tendency to increase at 20 months or, at least, to recuperate equivalent values to those presented at 2 months (Table 1). The total number of AMF species found was 17, five species belonging to the genus *Acaulospora*, one to *Ambispora*, three to *Gigaspora*, three to *Glomus*, two to *Racocetra*, two to *Scutellospora*, and one unidentified specimen. *Gigaspora decipiens* and *Racocetra intraornata* stood out for being present in all samplings and all treatments. *Acaulospora mellea* showed higher numbers of spores at 2 and 20 months after planting. Shannon's index was higher at 2 months for pure *Eucalyptus* and at 20 months for three treatments (AMS, EMS and A). Simpson's index was always lowest at 20 months, and highest for three treatments at 7 months and, for pure *Acacia*, highest at 14 months (Table 1).

Overall, there was a high degree of AMF root colonization in all treatments and samplings, with roots showing up to 40 % colonization or even more, especially at 14 months (Figure 1). Pure *Acacia* presented the highest mycorrhizal colonization in all samplings. In contrast, there was overall, lower root colonization in the pure *Eucalyptus*. However, the *E. grandis* root coloni-

Table 1 – Number of spores (in 50 g soil) and ecological indices of AMF (Arbuscular mycorrhizal fungi) at 2, 7, 14 and 20 months after planting. A (pure *Acacia*); E (pure *Eucalyptus*); AMS (*Acacia* mixed stand); EMS (*Eucalyptus* mixed stand). Values followed by different lowercase letters differ within each evaluation period. Values followed by different uppercase letters show differences between the evaluation periods within each treatment (Duncan $p < 0.05$).

|	2 months				7 months				14 months				20 months			
	A	E	AMS	EMS	A	E	AMS	EMS	A	E	AMS	EMS	A	E	AMS	EMS
Acaulospora mellea (Spain; Schenck)	3	-	24	-	5	-	-	-	-	-	-	-	4	-	4	5
Ac. foveata (Trappe; Janos)	-	[a]-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
Ac. scrobiculata (Trappe)	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-
Ac. sp1	-	-	2	12	-	-	-	-	-	-	-	-	1	1	-	-
Ambispora leptoticha (Spain, Sieverd and N.C. Schenck)	-	-	-	3	-	1	4	4	-	-	-	-	1	-	2	3
Gigaspora decipiens (Hall; Abbott)	3	3	-	3	5	-	-	-	2	-	1	1	11	9	5	7
Gigaspora sp1	-	1	-	-	-	-	-	-	-	-	-	-	3	-	-	-
Gigaspora sp2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Glomus clarum (Nicol.; Schenck)	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Glomus sp1	-	2	1	1	-	-	-	-	-	-	-	-	-	-	1	-
Glomus sp2	-	3	2	-	-	-	-	-	-	-	-	-	-	-	-	-
Racocetra intraornata (Goto, Maia and Oehl)	-	1	-	-	-	-	1	1	1	2	2	2	2	3	1	4
R. verrucosa (Koske; Walker)	-	2	-	-	2	-	-	-	-	-	-	-	2	-	-	-
Scutellospora biornata (Spain, Sieverding and Toro)	-	1	1	-	-	-	-	-	6	-	3	1	-	3	-	-
S. pellucida (Nicol.; Schenck)	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1
Not identified	-	-	-	3	-	-	-	-	-	-	-	-	-	2	-	2
Abundance	6 cB	13 bcA	31 aA	22 abA	12 aB	3 bB	5 bB	6 abB	9 aB	2 bB	6 abB	5 abB	24 aA	18 bA	19 abAB	23 abA
Richness	2	7	6	5	3	2	2	3	3	3	3	4	7	5	5	7
Shannon	0.3 aB	0.8 aA	0.4 abAB	0.6 abAB	0.4 aAB	0.3 aAB	0.2 aB	0.4 aB	0.4 aAB	0.0 bB	0.4 aAB	0.6 aAB	0.7 abA	0.6 abAB	0.5 bA	0.8 aA
Simpson	0.5 abAB	0.2 bAB	0.6 aAB	0.4 abAB	0.4 bAB	0.6 aA	0.7 aA	0.5 bA	0.5 abA	1.0 aB	0.4 bAB	0.3 bAB	0.3 aB	0.3 aAB	0.1 aB	0.2 aB

[a]zero counting.
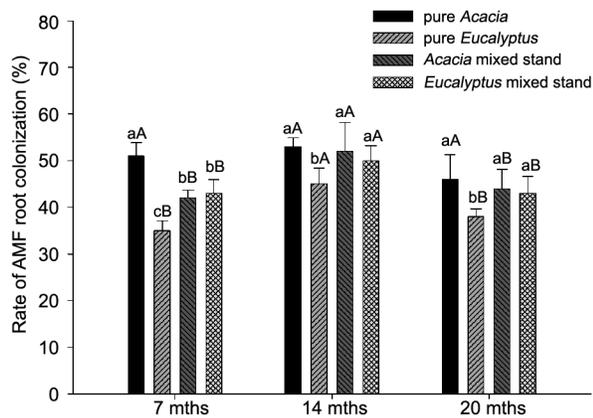
Figure 1 – Arbuscular mycorrhizal fungi (AMF) root colonization at 7, 14 and 20 months (mths) after planting; values (with standard deviation) followed by different lowercase letters differ within each evaluation period (7, 14 or 20 months); values followed by different uppercase letters show differences between the evaluation periods within each treatment (Duncan $p < 0.05$).

Table 2 – Pearson's correlation between litter or soil chemical data and AMF (Arbuscular mycorrhizal fungi) root colonization at 7, 14 and 20 months after planting.

	7 months	14 months	20 months
	% AMF colonization		
Soil moisture	ns	ns	ns
Litter C	-0.62***	ns	ns
Litter N	ns	0.42*	0.52**
Litter P	ns	ns	0.52**
Litter C/N	-0.68***	-0.45*	-0.63***
Litter C/P	ns	ns	-0.40*
Soil C	0.42*	ns	ns
Soil N	ns	ns	ns
Soil C/N	ns	ns	ns
pH	ns	ns	-0.33*
Available P	ns	ns	0.46*
Alkaline phosphatase	ns	ns	ns
Acid phosphatase	0.65***	ns	0.40*
Cellulase	-0.46**	-0.58**	-0.53**

***$p < 0.0001$; **$p < 0.001$; *$p < 0.05$; ns = not significant.

zation in the intercropped plantation was similar to that of pure *Acacia* and *Acacia* in mixed stands (AMS), at 14 and 20 months (Figure 1).

Mixed plantations stimulated the activity of acid and/or alkaline phosphatase (Figures 2A, B, C and 3A, B and C). Acid phosphatase was more active in pure *Acacia* at 14 and 20 months (Figure 2A), although the intercropped plantation showed similar values at 7 months. On the other hand, alkaline phosphatase had higher activity in the intercropped plantation at all samplings, in the following order: AMS and EMS > pure *Eucalyptus* and pure *Acacia* (Figure 2B). In general, cellulase showed the highest activity in treatment E, mainly at 14 and 20 months, since it had little variation in the other treatments over the 20 month period (Figure 2C).

At 7 months, mycorrhizal root colonization correlated positively with soil total C and acid phosphatase activity. For the same 7 months, there was negative correlation between root colonization and cellulase activity, litter C content and litter C/N ratio, the latter persisting at all samplings (Table 2). There was positive correlation between AMF root colonization and litter N at 14 and 20 months, negative correlation between AMF root colonization and litter C/P ratio, and positive correlation between root colonization and litter P concentration and acid phosphatase activity was also recorded at 20 months (Table 2).

For the PCA (Figure 3A, B and C) there was great similarity between samples originated from around *Acacia* or *Eucalyptus* trees in the mixed plantation, and this pattern was repeated at all samplings. In addition, pure *Eucalyptus* always appeared opposite pure *Acacia* in the figure. Furthermore, during the whole study period, we found a very close association between alkaline phosphatase and the intercropped plantation, in both the *Eucalyptus* or *Acacia* root systems (Figure 3A, B and

C). Acid phosphatase activity was closer to pure *Acacia* at 20 months, while AMF colonization was equidistant from pure *Acacia* and the mixed plantation at 14 and 20 months. However, pure *Eucalyptus* was associated only with cellulase and pH, while pure *Acacia* was associated with AMF colonization, litter P, litter N, acid phosphatase, available P, and soil N. At 20 months (Figure 3C) there was closer association between alkaline phosphatase and the mixed plantation. At this sampling there was greater separation between the three treatments: axis 1 separated pure *Acacia* from pure *Eucalyptus*, while axis 2 separated the mixed plantation (represented by both plant species) from both monocultures.

Discussion

AMF sporulation dynamics at the initial stages after planting

High root colonization rates starting at 7 months after planting, when it was possible to get the first root samples, are probably due to an inoculum potential left by previous plantations of *Eucalyptus* grown for more than 50 years, and also by intercropped stands from the first rotation. However, this inoculum potential may have been due mostly to other AMF propagules, different from spores, perhaps as fungal external hyphae or colonized root pieces. At early successional stages in pioneer or initial secondary species, tree development shows greater dependence on AMF than at the more advanced stages (climax). In fact, this evidence can be found in both, in initial secondary forests (Zangaro et al., 2003; Zangaro et al., 2007) and renewed *Eucalyptus* plantations (Adams et al., 2006). Initial plant development depends mostly on soil nutrients, and early AMF root colonization can be an advantage for the plant. At
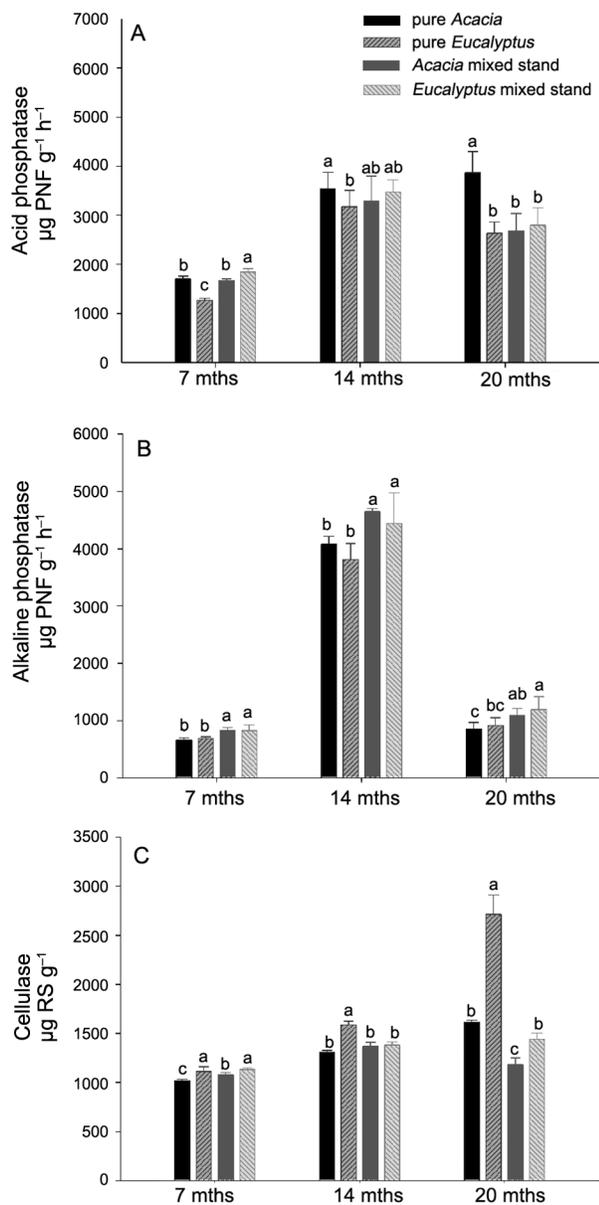
Figure 2 – Acid phosphatase (A), alkaline phosphatase (B) and cellulase (C) activities under pure and intercropped plantation of *E. grandis* and *A. mangium* at 7, 14 and 20 months (mths) after planting; RS = reducing sugars; PNF = *p*-nitrophenol phosphate. Values (with standard deviation) followed by different lowercase letters differ within each treatment (Duncan $p < 0.05$).

advanced growth stages, the plants become more efficient in recycling nutrients (biochemical cycling) which can decrease their demand for soil nutrients and fungal associations (Dighton and Mason, 1985). This has been verified generally in forest environments. Sometimes for certain plants this can also be said for ectomycorrhizae (Adams et al., 2006; Cheng et al., 2000). No ectomycorrhizal structures were found on the roots during our
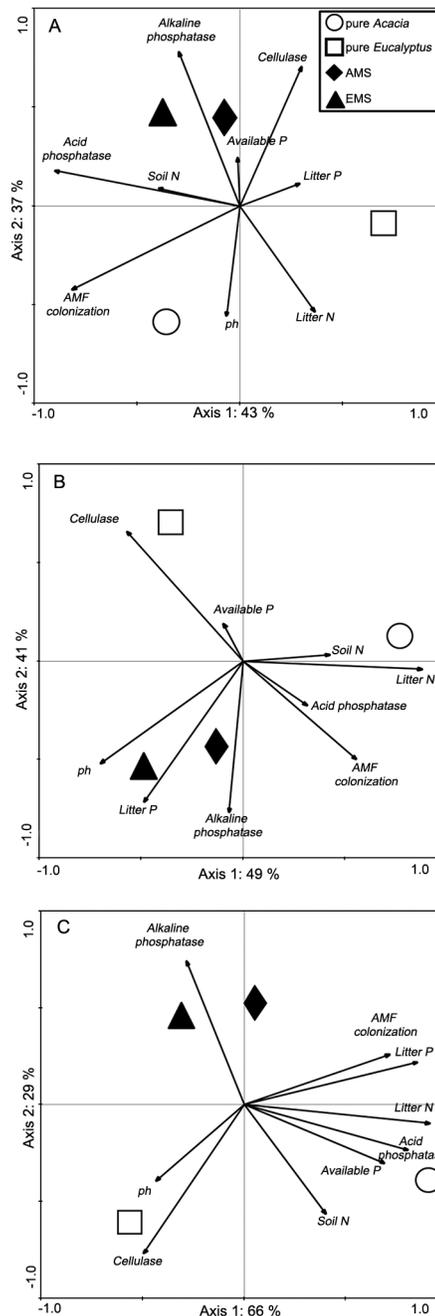
Figure 3 – Principal Components Analysis (PCA) of the environmental and biological variables at 7 months (A), 14 months (B), 20 months (C). Geometrical forms (triangles, squares, diamonds and circles) represent the centroid of each treatment, from nine subsamples. AMS = *Acacia* mixed stand; EMS = *Eucalyptus* mixed stand; AMF = Arbuscular mycorrhizal fungi.

study. It is known that ectomycorrhizae may predominate in *Eucalyptus* at later stages, after two to three years of growth, and are also frequent in certain leguminous trees (Pagano and Scotti, 2008).

Another interesting form of behavior of early plantings refers to the dynamics of mycorrhizal fungi in terms of spore density, which was similar for all treatments. Although the ecological indices showed high variation, we found low diversity, abundance and richness of spores at 7 and 14 months, which may be related to the spore germination process in response to the presence of tree roots and the release of chemical precursors that stimulate germination and root infection (Smith and Read, 1997). Mycelial growth and root colonization can be prioritized at a number of developmental stages of AMF instead of spore multiplication (Moreira-Souza et al., 2003; Stürmer and Siqueira, 2011), as probably occurred at 7 and 14 months, corroborated by high rates of root colonization. After 20 months of planting, there might have been a change in strategy, leading to emphasis on reproduction, triggered by several factors, such as seasonal weather changes or even the developmental phase of the host plant (Moreira-Souza et al., 2003; Pereira et al., 2014). According to Pereira et al. (2014), instable areas, different host species, soil preparation, and crop rotation among others, may induce AMF sporulation to ensure their survival. The increase in diversity, abundance and richness of AMF spores in all areas at 20 months may be due to the reproduction of AMF associated with the host roots. Pagano et al. (2009) studied AMF in plantations of *E. camaldulensis* and *Tabebuia heptaphylla* and found greater spore diversity in intercropped plantations. In our experiment, this probably also occured, since by two months after planting the intercropped plantation had already presented greater diversity and richness of AMF spores (Table 1).

AMF colonization

In addition to the successional stage of forests, other factors can affect the degree of mycorrhizal root colonization, such as host specificity, seasonality, soil pH and texture, and other physical or chemical attributes, as well as available P (Zangaro et al., 2007). Higher root colonization observed during summer, at 14 months, regardless of the treatment, contrasted with lower root colonization in winter, displaying a seasonal effect. Regardless of the treatment, the low P availability (5.4 µg g^{-1}) (see Bini et al., 2013a) in the soil may have stimulated the colonization rate even more than other factors (Smith and Read, 1997).

Ignoring seasonal effects, AMF colonization was always higher in *A. mangium* monoculture and intercropped plantations (AMS and EMS), since this is a highly mycotrophic species (Mendes-Filho et al., 2009). According to Ghosh and Verma (2006), *Acacia* seedlings inoculated with AMF (*Glomus mosseae*, *G. occultum* and *G. aggregatum*) show an increase in shoot dry matter, root diameter, leaf area, chlorophyll content and biomass of *A. mangium*, when compared with the non-inoculated control plants. The greater mycorrhizal dependency of *A. mangium* and, consequently, maintenance of a higher inoculum potential in the surrounding soil,

may have been the main reason for the higher rate of mycorrhizal colonization in the intercropped *E. grandis* at 20 months of transplanting, as reported by Pagano et al. (2009). Our results are corroborated by Adams et al. (2006), for whom the presence of understory may increase AMF colonization in monocultures of *E. grandis*. Thus, the presence of AMF colonized *Acacia* roots seems to stimulate AMF colonization in *E. grandis*. Many authors have reported an increase in AMF diversity and density in soils under mixed vegetation, in contrast to monocultures (Bainard et al., 2011; Dobo et al., 2016).

It is generally assumed that *Eucalyptus* trees form arbuscular mycorrhiza only at the seedling or plantlet stages (Adams et al., 2006; Chen et al., 2000), while there is increased association with ectomycorrhizal fungi in older plants (Adams et al., 2006; Santos et al., 2001), although it is not yet clear if this phenomenon always prevails. We showed an average of 37 % and 43 % AMF colonization at 20 months after planting in E and EMS, respectively, which is almost quadruple the colonization reported by Adams et al. (2005). Knowledge of the association between AMF and *Eucalyptus* and the dynamics of the mycorrhizal symbiosis in different management systems and soil types is precarious. According to Bainard et al. (2016), soil management can play an important role in maintaining the association between *E. grandis* and AMF. Therefore, a management system that enables the non-disruption of the network of underground hyphae, and a greater abundance of AMF spores in predominantly low-P soils, would have two highly desirable features that may result in higher AMF colonization.

Intercropping, AMF and nutrient cycling

The presence of leguminous species and root colonization increases the diversity and quality of litter in intercropped plantations and, therefore, decreases cellulase activity. Cellulase decomposes the recalcitrant and hard to break down cellulose, which is dominant in plant species such as *Eucalyptus* and presents a high C/N ratio. In all samplings cellulase activity was positively correlated with C concentrations of litter and high C/N and C/P ratios, and negatively with litter N concentrations and AMF colonization (Table 1; Figure 3A, B and C). Higher activity of cellulase was also found in soil that received plant residues with high C/N from *Pinus*, in comparison to residues from *Araucaria* from either native or secondary forest (Bini et al., 2013b). On the other hand, the intercropped system produces higher quality litter (Bini et al., 2013a), resulting in changes in soil enzymatic activity. The presence of *A. mangium* in the intercropped plantation with *E. grandis* offers a less recalcitrant mixture of plant litter with higher N concentration, thereby suppressing cellulase activity, especially in the rhizosphere. There are many reports on *Eucalyptus* intercropped with leguminous trees favoring N and P cycling, mostly due to a higher biological N fixation of the legume and higher mycorrhizal colonization of both plant species (Forrester et al., 2005; Khanna, 1997). Ac-

cording to Treseder (2013), high AMF colonization levels correlate with increased P concentration and plant growth. In our study, the increase in plant P concentration and P cycling in soil seem to be consequences of mycorrhizal colonization and phosphatase activities. This also explains the negative correlations between root colonization and litter C/N (in all samplings) as well as litter C/P (at 20 months) (Table 1), which indicate that systems with higher root colonization may generate litter of better quality, with narrower C/N and C/P ratios. We also found negative correlation between AMF and litter C/N ratios at all samplings, suggesting that AMF may be involved in the transference of soil N to plants, mainly for EMS, or that AMF are responsible for greater P acquisition which is fundamental to biological N fixation in legumes (Binkley, 1992; Hodge and Fitter, 2010). However, we observed only negative correlation between AMF colonization and litter C/P at 20 months, since the cycling of P is slower than that of N (Forrester et al., 2005). Furthermore, P cycling and phosphatase activity were maximized by N cycling in our study and AMF are possibly involved, especially with regard to EMS. According to Koutika et al. (2014), high translocation of P from soil to tree may explain the lower soil P availability in intercropped plantations of *A. mangium* and a first generation of the cross between *E. urophylla* × *E. grandis.* In fact, Tang et al. (2013) have shown the growth stimulation of *E. camaldulensis* intercropped with legumes caused by the return of nutrients to the soil via litter. Therefore, mycorrhizal transfer of nutrients such as P to plants promotes the enrichment of the deposited litter, decreasing the C/P ratio. Furthermore, certain mycorrhizal plants acquire nutrients through an extensive hyphal network connecting one plant to another of the same or different species (Simard et al., 2003). Thus plantations of *E. grandis* and *A. mangium* would help in the N nutrition of *Eucalyptus* via N transported from *Acacia* by AMF.

The increased AMF colonization may have additional positive effects, such as promoting the activity of acid phosphatase and alkaline phosphatase in intercropped plantations and pure plantations of *A. mangium*. In our study, conducted in soils with low available P, the dependency of plants on phosphatase activity (acid and alkaline) for mineralization of organic P may have been crucial for improving soil health and plant growth (Cardoso et al., 2013; Hinsinger, 2001; Vance et al., 2003). In intercropped or pure *A. mangium* we found maximum P cycling, also inferred by positive correlation between AMF colonization and acid phosphatase at 7 and 20 months. In general, the production of acid phosphatase is assigned mainly to plant roots and fungi (Dakora and Phillips, 2002) and there might have been additional stimulation for acquisition of organic P through the release of phosphatases by AMF. In addition, alkaline phosphatase activity was stimulated by intercropped plantations. This enzyme is produced mostly by bacterial groups (Dakora and Phillips,

2002). In this case, AMF may affect the diversity and structure of bacterial communities in the rhizosphere and hyphosphere (Toljander et al., 2006; Andrade et al., 1997). Hyphosphere refers only to the soil zone surrounding individual fungal hyphae (Andrade et al., 1997), which may stimulate phosphatase activity (Taktek et al., 2015; Zhang et al., 2014). Thus, we suggest that in either pure or intercropped *A. mangium* a double strategy for P acquisition occurs, i.e., via mycorrhizal hyphae and stimulation of phosphatase activity. This result is a clear demonstration that, during the first 20 months of growth in the mixed plantation there were no relevant differences between the root regions of the intercropped species, at least for the parameters that were evaluated in our study.

Acknowledgements

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