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Research Article

Enzymatic stoichiometry in tropical soil under pure and mixed plantations of eucalyptus with N_2-fixing trees

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ABSTRACT: Soil enzymes play a fundamental role in nutrient cycling in forest systems. The stoichiometry of C, N, and P–acquiring enzymes has been used to indicate nutrient limitation in the soil. However, the enzymatic stoichiometry remains poorly understood in pure and mixed eucalypt plantations. Thus, this study aims to assess the activity of enzymes in the soil to address the hypothesis that the introduction of N_2-fixing trees could influence the enzymatic stoichiometry on C, N, and P cycling. The activity of β-glucosidase (BG), urease (U), and acid phosphatase (AP) was assessed in soil (0-20 cm depth) of pure *Eucalyptus grandis* without (E) and with N fertilization (E+N), and a mixed system with *E. grandis* and *Acacia mangium* (E+A), and a pure *A. mangium* (A) plantation at 27 and 39 months after planting. The activities of BG/U, BG/AP, and U/AP were used to calculate the enzyme C/N, C/P, and N/P ratios, respectively. Rates of N–acquiring enzymes were higher in E and E+N, while soil microorganisms invested in P–acquiring enzymes in A and E+A. The vector length and angle demonstrated that C demand by microorganisms does not change in relation to N and P, regardless of the treatment. However, N demand decreased in relation to P in A and E+A (mainly at 27 months). Our results suggest that enzymes activity in pure eucalypt systems is limited for their soil-litter nutrient contents. At the same time while acacia and mixed plantation seem to invest in P–acquiring enzymes to improve biological N_2 fixation promoted by diazotrophic bacteria associated to acacia.

Keywords: microbial metabolism, forest soils, nutrient cycling, mixed forest systems

Introduction

Eucalyptus grandis is an exotic plant species cultivated in Brazil due to its high adaptability to edaphoclimatic fluctuations (Araujo et al., 2010). However, *E. grandis* cultivated under a monocrop system presents low environmental sustainability, mainly because of its potential effects on soil properties (Gonçalves et al., 2013; Pereira et al., 2018). For instance, *E. grandis* requires high extraction of nutrients and exerts a long-term negative balance in the soil N–pool (Bouillet et al., 2008; Pulito et al., 2015).

Regarding soil microorganisms, studies have reported positive (Mendhama et al., 2002), negative (Behera and Sahani, 2003), and even transitional effects (Araujo et al., 2010) on soil microbial biomass and activity after the planting of *E. grandis*. A recent report demonstrated that pure *E. grandis* plantations deposit nutrient-poor litter into the soil (high C/N ratio); thereby reducing the nutrient cycling by microbiome (Pereira et al., 2018). However, introducing an N_2-fixing plant species intercropped with *E. grandis* has been suggested to improve the soil microbial properties (Koutika et al., 2021). In this context, *Acacia mangium* has been recommended as an intercropping plant species, mainly due to its capacity of fixing N_2 from the atmosphere, which increases N availability in the soil (Paula et al., 2015), improves P dynamics (Pereira et al., 2021) and mycorrhizal associations (Bini et al., 2013; Pereira et al., 2018).

The microbial activity drives soil nutrient dynamics (Koutika et al., 2020; Liu et al., 2020). Thus, assessing the stoichiometry of β-glucosidase, urease, and acid phosphatase allows determining soil nutrient availability (Moorhead et al., 2016). In addition, these enzymes indicate nutrient turnover (Das and Varma, 2010) and provide a measurement of ecosystem metabolism (Hill et al., 2012), showing potential nutrient limitations for soil microorganisms.

No studies have investigated enzyme stoichiometry in pure and mixed eucalypt and acacia plantations, especially in tropical soils. This is particularly important since tropical soils are limited in terms of organic matter (OM) and *Acacia mangium* presents the potential to improve soil fertility and ecosystem services (Cardoso et al., 2020; Pereira et al., 2020). These conditions can shift the enzymatic stoichiometry of nutrients, mainly C, N, and P. Thus, in this study we hypothesized that introducing the N_2-fixing *Acacia mangium* could influence the enzymatic stoichiometry, mainly those enzymes related to nutrient cycling.

Materials and Methods

Experimental site, treatments, and sampling

This study was carried out at the municipally of Itatinga (23°03′47″ S, 48°37′16″ W, altitude 830 m), São Paulo State, Brazil. The climate in the region is Cfa (Köppen

system), with an annual rainfall of 1,350 mm (Alvares et al., 2013). The soil of the region is a Rhodic Ferralsol (FAO classification), with medium texture (~83 % sand) (Laclau et al., 2008).

The area with *E. grandis* was planted in Dec 2013, under a random block design with four treatments: a) pure *E. grandis* without N fertilization (E); b) *E. grandis* with N fertilization (E+N); c) pure *A. mangium* (A); and d) mixed system with *E. grandis* and *A. mangium* (E+A). The trees were planted spaced 3 m × 3 m, resulting in a total area of 1,296 m^2 (36 m × 36 m) in each plot. The mixed system (E+A) was installed with double rows (i.e., two *A. mangium* and two *E. grandis* lines, successively) (Pereira et al., 2018). The treatment E+N was fertilized in Dec 2013 and 2014, using 10 and 90 kg^{-1} of N ha^{-1} as ammonium sulphate, respectively.

Soil and litter sampling

We sampled soil and litter at the 27th and 39th months after tree plantation, corresponding to Mar 2016 and 2017, respectively, to assess differences between the beginning and the maximum litterfall period. The soil was sampled at 0-20 cm following the Voronoi polygon procedure (Honda, 1978; Saint-André et al., 2005; Pereira et al., 2018). In this case, six trees in each plot were chosen, and soil samples were homogenized in a sterile bag, obtaining a composite sample (Santana et al., 2021). The sampled litter followed the same standardization procedure. However, we used a template (25 cm × 25 cm) placed on the soil surface and sampled all organic material underneath. Thus, we sampled 16 soil samples (from four treatments and four blocks) in two sampling periods, totaling 64 samples, 32 for each soil and litter layer.

Soil and litter characterization

Before analyzing the enzyme activities, soil samples were sieved (2 mm) and stored at 4 °C. For chemical and physical analyses soil samples were also sieved (2 mm) and air-dried for 72 h. Litter was oven-dried at 60 °C for 24 h and ground (1 mm) for the chemical analyses.

The soil pH was determined in 0.01 mol L^{-1} $CaCl_2$ solution (Raij, 2001). Soil organic matter (SOM) was physically fractionated from 20 g using the granulometric method (Brandani et al., 2016; Christensen, 2001). This process separated four fractions; however, we used only the most labile organic fraction (OF – 2,000 – 75 µm) because no differences were observed between treatments for the other fractions. Total organic carbon (TOC), on full mineral soil, as well as the C, N and P contents in the soil labile OF fraction (i.e., labile-C, labile-N and labile-P) were determined via dry combustion with an elemental analyzer (Nelson and Sommers, 1983; Christensen, 2001). Dry combustion in the elemental analyzer determined Total-C, Total-N, and Total-P in the litter (Pereira et al., 2018). The relationships between C, N, and P were calculated (i.e., C/N and C/P ratios, respectively). The soil-litter properties in pure and mixed *E. grandis* and *A. mangium* plantations are shown in Table 1.

Measurement of soil enzyme activities

Enzyme activities, that is, β-glucosidase (BG), urease (U), and acid phosphatase (AP), were determined according to standard methods. Briefly, the β-glucosidase (EC 3.2.1.21) activity was measured using ρ-nitrophenyl β-glucopyranoside as substrate under incubation (1 h, 37 °C) in a modified buffer

Table 1 – Soil and litter properties in pure and mixed *E. grandis* and *A. mangium* plantations. E = *E. grandis*, E + N = *E. grandis* with N fertilization, E + A = mixed *E. grandis* and *A. mangium* and A = *A. mangium* plantation at 27 and 39 months after planting.

		E	E+N	A	E+A	E	E+N	A	E+A
		27 months				39 months			
Litter	-	-	-	-	-	-	-	-	-
C	g kg^{-1}	521^{ns}	526^{ns}	514^{ns}	527^{ns}	518^{ns}	519^{ns}	517^{ns}	519^{ns}
N	g kg^{-1}	8.48^{Ca}	8.52^{Cb}	14.09^{Ab}	11.50^{Bb}	9.10^{Ca}	10.33^{Ca}	16.60^{Aa}	13.79^{Ba}
P	g kg^{-1}	3.50^{Ba}	3.11^{Ba}	4.16^{Aa}	4.60^{Aa}	3.30^{Bb}	3.38^{Bb}	4.01^{Ab}	4.82^{Ab}
C/N	-	61.65^{Aa}	61.99^{Aa}	36.56^{Ca}	46.16^{Ba}	57.41^{Aa}	50.93^{Bb}	31.02^{Cb}	38.12^{Bb}
C/P	-	146^{Aa}	167^{Aa}	140.2^{Ba}	123.7^{Ba}	155.4^{Aa}	152^{Aa}	128^{Ba}	105^{Ba}
Soil	-	-	-	-	-	-	-	-	-
pH	-	4.03^{ns}*	3.58^{ns}	4.10^{ns}	4.40^{ns}	3.78^{ns}	3.70^{ns}	4.23^{ns}	4.45^{ns}
Total C	g kg^{-1}	41.7^{Aa}	35.8^{Ba}	44.7^{Aa}	43.77^{Aa}	27.6^{Bb}	28.5^{Bb}	29.07^{Bb}	44.15^{Aa}
Total N	g kg^{-1}	1.85^{Aa}	1.60^{Aa}	1.95^{Aa}	1.89^{Aa}	1.31^{Bb}	1.31^{Bb}	1.27^{Bb}	1.93^{Aa}
Labile C	g kg^{-1}	4.82^{Ba}	5.27^{Ba}	10.02^{Aa}	9.97^{Aa}	6.00^{Ba}	6.60^{Ba}	9.80^{Aa}	10.44^{Aa}
Labile N	g kg^{-1}	0.16^{Ba}	0.17^{Ba}	0.35^{Aa}	0.34^{Aa}	0.20^{Ba}	0.22^{Ba}	0.35^{Aa}	0.36^{Aa}
Labile P	mg dm^{-3}	2.20^{Bb}	2.11^{Bb}	4.63^{Aa}	4.58^{Aa}	1.19^{Bb}	1.53^{Bb}	5.01^{Aa}	4.74^{Aa}

*Means followed by the same letter do not differ (Tukey's test at > = 5 %). Capital letters in the row compare treatments within each period and lowercase letter in the row compare periods within each treatment (n = 4). ns, not significant. Total C, Total Organic Carbon; Total N, Total Nitrogen; Labile C, N and P, carbon, nitrogen, and phosphorus determined in the labile soil organic fraction (2,000–75 µm); C, N and P, total content of carbon, nitrogen, and phosphorus in litter and C/N and C/P their ratios.

adjusted to pH 6.0. The ρ-nitrophenol form was determined spectrophotometrically at 410 nm (Eivazi and Tabatabai, 1988). The acid phosphatase (EC 3.1.3.2) activity was measured using disodium ρ-nitrophenyl phosphate as substrate under incubation (1 h, 37 °C) in a modified universal buffer adjusted to pH 6.5. The amount of ρ-nitrophenol formed was measured spectrophotometrically at 420 nm (Tabatabai and Bremner, 1969). The urease (EC 3.5.1.5) activity was determined using the method of Kandeler and Gerber (1988) with urea as substrate under incubation (1 h; 37 °C). The amount of ammonium produced was determined using the Kjeldahl method (Pereira et al., 2018). All results of enzyme activities were expressed in nmol g^{-1} h^{-1}. After obtaining the results for the C, N, and P–acquiring enzymes, they were used to calculate the enzymes C/N, C/P, and N/P ratios, respectively.

Enzymatic stoichiometry

The enzymatic stoichiometry was estimated according to the vector method proposed by Moorhead et al. (2016), as follows:

$$\text{Vector L (unitless)} = \sqrt{X^2 + Y^2} \quad (1)$$

$$\text{Vector A (degree)} = \text{Degrees (Atan2 (X, Y))} \quad (2)$$

where:

$$X = \frac{Enzyme\ C}{Enzyme\ C + Enzyme\ P}, Y = \frac{Enzyme\ C}{Enzyme\ C + Enzyme\ N}$$

Statistical analyses

The homogeneity and normality of variance were examined by the Levene and Shapiro-Wilks test, respectively. The dataset was analyzed using the ANOVA and the Tukey test compared significant attributes at 5 %. A Principal Components Analysis (PCA) biplot was used to compare the soil properties between the different pure and mixed plantations, including soil and litter chemical properties, enzyme activity and stoichiometry. The data analyses were performed in the R software (version 3.6.3) and Canoco® software for Windows (v. 4.5).

Results

The activity of N–, C– and P–acquiring enzymes varied according to treatments and periods (Figures 1A-C). The highest N–acquiring enzyme values were observed in E and E + N at both 27 and 39 months after planting (Figure 1A). However, N–acquiring enzymes had the highest values at 39 months compared to 27 months. In contrast, the highest values of the P–acquiring enzyme were observed in A and E + A, but the values did not vary between periods (Figure 1C). C–acquiring enzyme did not generally vary between treatments in both periods (Figure 1B).

The enzymatic stoichiometry varied between treatments and periods (Figure 2). In general, the values of enzyme C/N ratio were higher at 27 months than 39 months. The highest values of the enzyme C/N ratio were observed in A and E + A, at 27 months, while no differences were observed at 39 months. In contrast, the highest values of enzyme C/P and enzyme N/P were observed in E and E + N. In addition, the values of enzyme C/P and enzyme N/P were highest at 39 months than 27 months. The vector L (length) did not vary between treatments in both periods, while vector A (angle) increased in A and E + A at 27 months and did not vary at 39 months (Table 2).

The PC1 and PC2 explained 45.6 % and 23.0 % of the total variation, respectively, and clustered the values of the C–, N–, and P–acquiring enzymes and their stoichiometry with soil and litter properties (Figure 3). In general, four clusters were found, being E and E + N

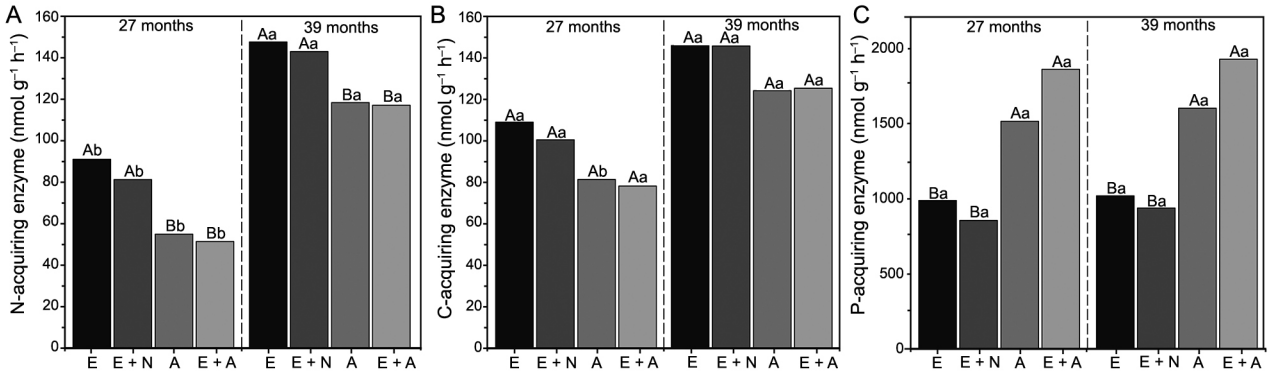

Figure 1 – Potential activity of soil N, C, and P acquiring enzymes [(A), (B) and (C), respectively], in pure and mixed *E. grandis* and *A. mangium* plantations. E = *E. grandis*, E + N = *E. grandis* with N fertilization, E + A = mixed *E. grandis* and *A. mangium* and A = *A. mangium* plantation at 27 and 39 months after planting. Means followed by the same letter are not different at the 5 % probability level using the Tukey's test (n = 4). Uppercase letters compare treatments within each period and lowercase letters compare periods within each treatment.

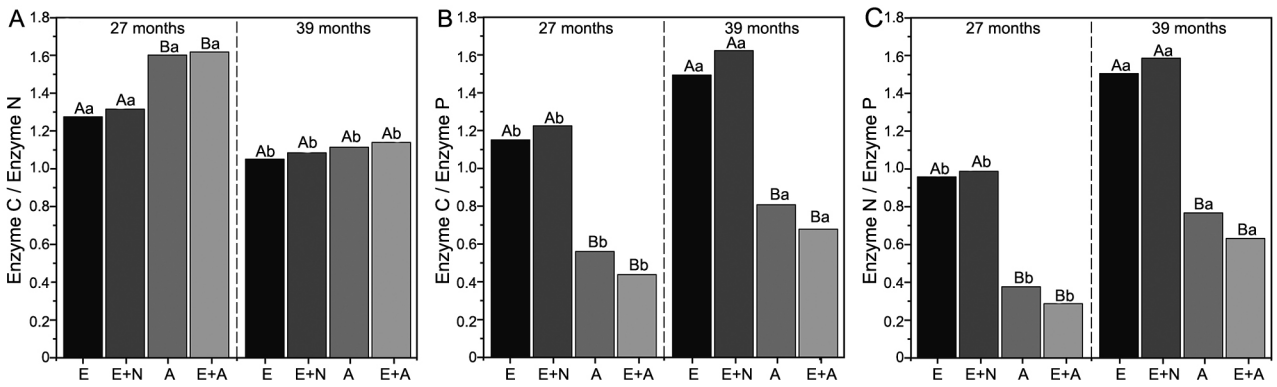

Figure 2 – Soil enzymatic stoichiometry in pure and mixed *E. grandis* and *A. mangium* plantations. E = *E. grandis*, E + N = *E. grandis* with N fertilization, E + A = mixed *E. grandis* and *A. mangium* and A = *A. mangium* plantation at 27 and 39 months after planting. Means followed by the same letter are not different at the 5 % probability level by the Tukey's test (n = 4). Uppercase letters compare treatments within each period and lowercase letters compare periods within each treatment.

Table 2 – Vector analysis between pure and mixed *E. grandis* and *A. mangium* plantations. E = *E. grandis*, E + N = *E. grandis* with N fertilization, E + A = mixed *E. grandis* and *A. mangium* and A = *A. mangium* plantation at 27 and 39 months after planting.

	Vector L (Length)		Vector A (Angle)	
	27 months	39 months	27 months	39 months
E	0.68^{ns}	0.66^{ns}	1.00^{b}	1.02^{ns}
E + N	0.66^{ns}	0.67^{ns}	1.01^{b}	1.01^{ns}
A	0.65^{ns}	0.64^{ns}	1.15^{a}	1.02^{ns}
E + A	0.64^{ns}	0.64^{ns}	1.17^{a}	1.03^{ns}

(27 months) correlated to vector L and influenced by C/P and C/N ratios of litter, while E and E+N (39 months) correlated with the C- and N-acquiring enzymes, and enzymes N/P and C/P ratios. On the other hand, A and E+A (27 months) correlated with vector A and the enzyme C/N ratio, and with total-C, total-N, and litter-P. Finally, A and E+A (39 months) correlated with the P-acquiring enzymes, litter-N, labile-N, labile-C, labile-P, and the pH.

Discussion

In this study, we assessed the stoichiometry of C-, N-, and P-acquiring enzymes in the soil covered by pure and mixed eucalypt with acacia plantations under tropical conditions. We investigated the hypothesis that the introduction of *A. mangium*, an N_2-fixing tree species, could influence the enzymatic stoichiometry in soils. Enzymes play a fundamental role in soil functioning, acting on biochemical processes related to organic matter transformations. The C-, N-, and P-acquiring enzymes catalyze several reactions in organic wastes, promoting nutrient cycling in a forest system. In particular, urease, and other amidohydrolasesare responsible for the breakdown of protein-related compounds, generating NH_4^+ as final product. In this study, the values of the N-acquiring enzymes were

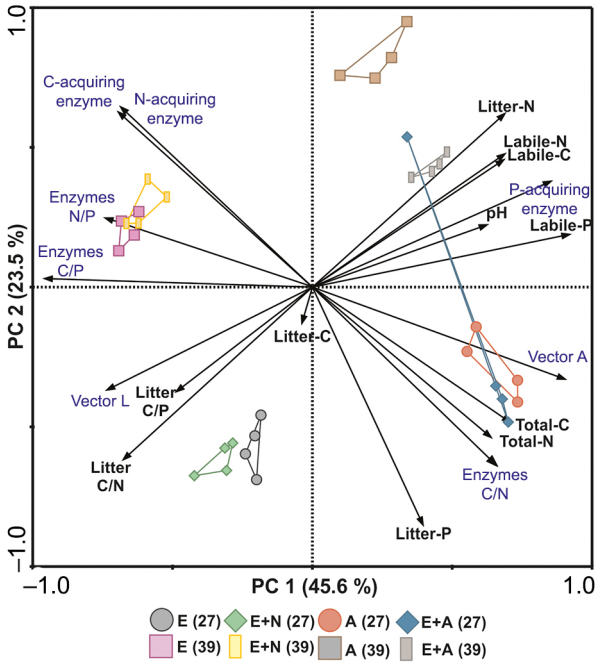

Figure 3 – The Principal Component Analysis (PCA) between soil-litter properties, microbial diversity, enzymes activities and stoichiometry in pure and mixed *E. grandis* and *A. mangium* plantations. E = *E. grandis*, E + N = *E. grandis* with N fertilization, E + A = mixed *E. grandis* and *A. mangium* and A = *A. mangium* plantation at 27 and 39 months after planting (n = 4).

highest after 39 months (Figure 1A), suggesting a depletion of organic N from the litter that stimulated the higher activity of the N-acquiring enzymes (Ma et al., 2014). When considering treatments, the N-acquiring enzymes showed higher activities in E and E+N, suggesting a microbial community delivering a significant content of N-acquiring enzymes to promote litter degradation. Interestingly, as the pure eucalypt

plantation presented a nutrient-limited litter (Table 1), with a high C/N ratio (Pereira et al., 2018) and numerous recalcitrant compounds (Bini et al., 2013), the increase in the N-acquiring enzyme activity did not reflect in differences in total N soil N contents in pure eucalypt, regardless of the sampling period (Table 1). Thus, as eucalypt is a non–N_2–fixing species, we expect that the microbial community might utilize resources to acquire N in the litter. In contrast, A and E + A have observed the highest P–acquiring enzyme values in both periods (Figure 1C). In A and E + A the values of organic labile P were higher (Table 1), which could explain the higher activity of the P–acquiring enzyme. Recently, Hummel et al. (2021) showed that the activity of phosphatase was higher in soil containing more organic P. Another possible explanation could be that, since A and E + A presented the lowest C/N and C/P ratios in the litter (Table 1), favoring the microbial-induced transformations and thus improving the P availability to plants. Naturally, N_2–fixing tree species require a significant amount of available P to efficiently promote the biological nitrogen fixation. Therefore, microbes probably associated with A and E + A plantations can overcome P limitation by synthesizing phosphatases enzymes to acquire organic P from soil and/or litter. In this study, no differences were observed to C–acquiring enzymes between treatments (Figure 1B) and these enzymes did not correlate with any differences between the C content in the litter (Table 1). In addition, different types of litter, such as those found in mixed plantations, do not seem to stimulate specifically the activity of β-glucosidase, since other enzymes can play significant roles in this condition (e.g., cellulase) (Leroy et al., 2018).

The results for enzymatic stoichiometry showed that in A and E + A plantations, the soil microorganisms invested less N (Figure 2), while P–acquiring enzymes increased (Figure 2). Once again, it suggests that in A and E + A, an N_2-fixing plant species, i.e., *A. mangium*, could increase soil N-pools (Paula et al., 2018; Pereira et al., 2021), therefore decreasing the N–acquiring enzymes. Regarding E and E + N, the results could suggest that in soil with the contribution of a high C/N ratio from the litter (Table 1), the P–acquiring enzyme (phosphatase) decreases (Mooshammer et al., 2012), thereby impacting nutrient dynamics.

The analysis of the vectors allows estimating the relative microbial investments in C versus nutrient acquisition (vector length) and P versus N acquisition (vector angle) (Moorhead et al., 2016). Vector L showed no differences between treatments and periods (Table 2), meaning that probably the C demand does not change in relation to N and P. On the other hand, the vector angle increased in A and E + A at 27 months, suggesting decreased demands for N in relation to P (Fanin et al., 2016). As discussed above, *A. mangium* contributes to increasing N in the soil thus, decreasing the N demand for microbial communities.

The PCA demonstrated that soil or litter properties clustered pure and mixed treatments differently (Figure 3). Greater litter C/N ratios positively correlated with E and E + N, especially 27 months after plantation. Litter decomposition can be limited by N, partly explaining the positive correlation of N–acquiring enzymes in later periods (39 months) and perhaps in E and E + N in subsequent years. It demonstrates that the activity of soil enzymes increased towards a great litter C/N ratio, suggesting N limitation for decomposers (Güsewell and Freeman, 2005). On the other hand, labile soil elements (C, N and, P), as well as the total content of C, N, and P of litter positively correlated with pure A and E + A, both at 27 and 39 months after planting (Figure 3). Recently, Zhou et al. (2018) demonstrated that N addition via biological fixation stimulated the soil microbial functions, especially the enzyme activities in the initial stage of litter decomposition, increasing soil fertility.

Similar forest management demonstrated an increase in alkaline and acid phosphatase in the presence of *A. mangium* under a mixed plantation with *E. grandis* (Bini et al., 2013). More importantly, the ratio of arbuscular mycorrhizal colonization was strongly correlated with soil phosphatase activity. Thus, these interactions present important biological indicators to plant nutrition under mixed forest systems. In addition, our results could integrate future studies to introduce enzymes as a win–win combination for soil health and functions in forest plantations, as it is currently documented for cereal crops in Brazil (Lopes et al., 2013; Mendes et al., 2021; Lopes et al., 2021).

Conclusions

This study demonstrated that pure and mixed eucalypt plantations changed the stoichiometry of the C–, N–, and P–acquiring enzymes in the soil; thereby, confirming our initial hypotheses. The rates of N–acquiring enzymes were higher in pure eucalypt plantation, regardless of N fertilization, especially at 27 months after planting; however, this increase did not result in enhancement in N–pools in the soil or litter. Conversely, soil microorganisms invested in P–acquiring enzymes in pure *A. mangium* and mixed plantation, resulting in more significant amounts of labile soil elements and possibly increasing the energy flow to biological N fixation promoted by *A. mangium*. The vector analyses (L and A) demonstrated that C demand by microorganisms does not change in relation to N and P, regardless of the treatment. However, N demand decreased in A and E + A (mainly at 27 months) in relation to P. These results suggest that short-term mixed plantations affect soil fertility and microbial functions in mixed eucalypt plantations, while the nutrient availability in pure systems is closely limited for their soil-litter nutrient contents.

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Author's Contributions

Conceptualization: Pereira, A.P.A.; Araujo, A.S.F.; Verma, J.P.; Cardoso, E.J.B.N. **Data acquisition:** Pereira, A.P.A.; Santana, M.C.; Araujo, V.L.V.P. **Data analysis:** Pereira, A.P.A.; Araujo, A.S.F.; Verma, J.P.; Cardoso, E.J.B.N.; Santana, M.C.; Araujo, V.L.V.P.; Lima, A.Y.V. **Writing and editing:** Pereira, A.P.A.; Araujo, A.S.F.; Verma, J.P.; Cardoso, E.J.B.N.; Santana, M.C.; Araujo, V.L.V.P.; Lima, A.Y.V.

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