Organic phosphorus immobilization in microbial biomass controls how N2-fixing trees affect phosphorus bioavailability in two tropical soils

Kittima Waithaisong, Agnès Robin, Valentin l'Huillery, Josiane Abadie, François-Xavier Sauvage, Patrick Chemardin, Louis Mareschal, Jean-Pierre Bouillet, Jean-Paul Laclau, José Leonardo Moraes Gonçalves, et al.

Environmental Advances 8 (2022) 100247

Contents lists available at ScienceDirect

Environmental Advances

journal homepage: www.sciencedirect.com/journal/environmental-advances

Organic phosphorus immobilization in microbial biomass controls how N_2-fixing trees affect phosphorus bioavailability in two tropical soils

Kittima Waithaisong [a,g,$], Agnès Robin [a,b,c,$], Valentin L'Huillery [a], Josiane Abadie [a], François-Xavier Sauvage [d], Patrick Chemardin [d], Louis Mareschal [a,b,e], Jean-Pierre Bouillet [a,b,c], Jean-Paul Laclau [a,b,f], José Leonardo Moraes Gonçalves [c], Claude Plassard [a,*]

[a] Eco&Sols, Univ Montpellier, CIRAD, INRAE, IRD, Institut Agro, Montpellier, France
[b] CIRAD, UMR Eco&Sols, F-34398 Montpellier, France
[c] ESALQ, University São Paulo, 13418-900 Piracicaba SP, Brazil
[d] SPO, Univ Montpellier, CIRAD, INRAE, Institut Agro, Montpellier, France
[e] CRDPI, 1291 Pointe Noire, Congo
[f] UNESP-São Paulo State University, School of Agricultural Sciences, Botucatu, São Paulo 18610-307, Brazil
[g] Department of Microbiology, Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand

ARTICLE INFO

Keywords:
Enzyme-reactive Po
Bioassay
Acacia mangium
Eucalyptus
Plant P accumulation

ABSTRACT

Eucalyptus is the tree most widely planted in tropical countries to satisfy growing demand for wood products, but high yields require high fertilizer inputs. Introducing N_2-fixing trees (NFT), such as *Acacia mangium,* has been proposed to improve soil fertility and aboveground tree biomass in *Eucalyptus* plantations. In addition to N inputs, NFT species may increase plant P nutrition through increased rates of organic P (Po) cycling. However, the positive effect of acacia on soil P availability and plant P nutrition was found to vary substantially between sites. The ability of acacia to improve P bioavailability might mainly depend on Po sequestration in microbial biomass, preventing Po mineralization by phosphatases and efficient Po recycling. This hypothesis was tested at two tropical sites, Itatinga (Brazil) and Kissoko (Congo) by measuring inorganic phosphate (Pi), Po and enzyme-labile Po in bicarbonate extracts from the topsoil collected from plots with *Eucalyptus*, acacias, or native vegetation. We used bicarbonate enzyme-labile Po after soil autoclaving as an indicator of microbial Po, and a *Eucalyptus* bioassay to measure the actual P bioavailability for *Eucalyptus* seedlings. At Itatinga, bicarbonate-Pi was very low, while Po was the main P form. Enzyme-labile Po was very weak in intact soils and high in autoclaved soils, indicating high immobilization in microbial biomass. At Kissoko, Po was highly enzyme-labile in both intact and autoclaved soils, especially from acacia plots, suggesting very low Po immobilization in microbial biomass. Growth and P accumulations in *Eucalyptus* seedlings were low in all soils at Itatinga and were the highest in *Eucalyptus* plants grown in acacia soils at Kissoko. Our results highlight the potential of acacia trees for improving P bioavailability for other tree species if labile Po enrichment in the soil provided by this N_2-fixing tree is not locked into the microbial biomass.

1. Introduction

In order to satisfy growing demand for wood products, monoculture plantations with fast-growing species have been encouraged, especially in tropical countries (Cossalter and Pye-Smith, 2003). *Eucalyptus* is the tropical tree genus most widely planted, but high yields require high fertilizer inputs (Gonçalves et al., 2013). Introducing nitrogen-fixing tree (NFT) species in monoculture plantations of non-nitrogen-fixing tree (non-NFT) species has been proposed to improve soil fertility and aboveground tree biomass (Forrester et al., 2006; Gonçalves et al., 2013; Laclau et al., 2008; Richards et al., 2010). Hence, to gain insight into how mixing NFT trees, such as *Albizia* or *Acacia,* with non-NFT trees, such as *Eucalyptus*, affects the biogeochemical cycles of nutrients, as well as non-NFT productivity, several mixed plantation trials were set up under contrasting soil and climate conditions. The trials generally revealed a positive impact of NFT tree introduction on soil N availability

* Corresponding author at: Ecologie Fonctionnelle et Biogéochimie des Sols, 2 place Pierre Viala, 34060 Montpellier Cedex 01, France
E-mail address: claude.plassard@inrae.fr (C. Plassard).
$ The two authors contributed equally to the work

https://doi.org/10.1016/j.envadv.2022.100247
Received 20 March 2022; Received in revised form 12 May 2022; Accepted 18 May 2022
Available online 19 May 2022

and N cycling due to fixation of atmospheric N, with a possible increase in total *Eucalyptus* wood production through the establishment of belowground facilitation processes between the species (Binkley et al., 2003a; Forrester et al., 2004; Koutika and Mareschal, 2017; Oliveira et al., 2021; Paula et al., 2018; Santos et al., 2017). The benefits of mixed plantations with NFT trees for *Eucalyptus* productivity have mainly been seen at N-poor sites (Bouillet et al., 2013; DeBell et al., 1997; Epron et al., 2013; Koutika and Mareschal, 2017; Tchichelle et al., 2017), because of (i) high N_2 fixation favored by N-limiting conditions and (ii) greater enhancement of *Eucalyptus* N nutrition in N-poor soils than in N-rich soils.

In contrast to N, changes in P availability in mixed plantations compared to monocultures are still poorly documented. Improving the understanding of P cycling is a major issue for tropical regions, where soils can be particularly poor in available P (Bouillet et al., 2013, Du et al., 2020) due to its high fixation on soil oxyhydroxides (Fujii et al., 2018). Greater accumulation of P has been reported in the biomass of NFTs than in that of non-NFTs, when grown as monocultures (Le Cadre et al., 2018), suggesting that NFTs may be able to modify the biogeochemical processes leading to soil P bioavailability (Aleixo et al., 2020). So far, the effect of NFTs on P cycling has mainly been addressed by measuring inorganic and organic P concentrations in soils from either monospecific or mixed plots of non-NFT and NFT species. As it is assumed that plants are only able to take up orthophosphate ions (Pi) free in the solution (Hinsinger, 2001), it might be expected that easily extractable Pi levels will be higher in soil sampled around NFT species than around non-NFT species. Surprisingly, bicarbonate-Pi extractions (Binkley et al., 2000; Koutika et al., 2016; Cabreira et al., 2022) or anionic traps in the field (Binkley et al., 2000; Le Cadre et al., 2018) revealed lower concentrations of labile Pi in NFT soils than in non-NFT soils. In contrast to Pi, several studies showed that NFT species increased the concentrations of organic P (Po) in comparison with non-NFT species (Aleixo et al., 2020; Cabreira et al., 2022; Waithaisong et al., 2020). These results suggest that labile organic P cycling may supply free Pi, which is then taken up very efficiently by NFT roots, thus explaining their greater P uptake and the lower Pi concentrations measured in soil compared to non-NFT species.

Hence, accessing the actual bioavailability of Po in soil is a key factor for estimating Po cycling and its contribution to plant P nutrition. In addition to measuring chemical P availability, other methods could be used to estimate Po bioavailability, such as measuring enzyme-labile P fractions and carrying out bioassays. Indeed, the first step of Po cycling is the mineralization of organic P through the activity of enzymes, namely phosphatases. Such enzymes are able to release free orthophosphate from phospho-monoesters (P-O-C bond) and condensed phosphate (P-O-P bond) accounting for the main forms of Po compounds in soil (George et al., 2018). Hence, adding phosphatases to soil extracts has been proposed as a way of estimating the proportion of enzyme-labile Po (Bünemann, 2008). So far, the enzymes commonly used have been acid phosphatase from *Solanum tuberosum* (Hallama et al., 2021; He et al., 2004; Jarosch et al., 2015; Jarosch et al., 2019; Wyngaard et al., 2016). However, in forests, tree roots are associated with mycorrhizal fungi capable of releasing huge amounts of acid phosphatases into the environment (Plassard & Dell, 2010; Plassard et al., 2011; Ali et al., 2009, 2014). In their work, Louche et al. (2010) used cation-exchange chromatography to separate four fractions displaying acid phosphatase activity secreted by the ectomycorrhizal fungus *Hebeloma cylindrosporum* grown in a pure culture. When measured *in vitro*, each fraction showed very broad substrate specificity, indicating that the enzymes secreted by this fungal species are good candidates for measuring the proportion of hydrolysable Po in a soil extract. Using such fungal enzymes to measure the production of Pi in soil extracts could be a good strategy for approaching the *in situ* recycling of P in forest soils.

The efficiency of Po cycling depends on enzyme quantities and substrate availability in the soil. Jarosch et al. (2019) addressed this issue in ten soils with different properties, origins and land uses and concluded that the enzymatic hydrolysis of organic P compounds appears to be limited by the substrate rather than by enzyme availability. This situation could be due to Po sequestration in microbial biomass, making the substrate unavailable to enzymes. Hence, determining the share of Po locked into microbial biomass is a key factor when assessing the potential importance of Po cycling in a given soil. To date, soil microbial P has been determined by assaying inorganic P released by microbial cells after fumigation (Brookes et al., 1982; Jenkinson and Powlson, 1976), or after adding hexanol to soil samples (McLaughlin et al., 1986; Solaiman, 2007). In both cases, the microbial P content is calculated from the difference between bicarbonate-extracted Pi concentrations measured before and after soil treatment. However, the actual contribution made by Po compounds to microbial P using these chemical extractions has yet to be investigated. To address this issue in podzol soils, Louche et al. (2010) compared Pi released by the four fungal acid phosphatases from *H. cylindrosporum* into bicarbonate-extracted P from intact or autoclaved soils. These authors found that soil autoclaving greatly enhanced the levels of enzyme-labile P and concluded that the increase originated from microbial Po that was made available for enzyme hydrolysis after soil autoclaving. Hence, this method could help to determine the amount of microbial Po in soil, with results similar to those obtained by fumigation, the most frequently used method (Sinegani and Hosseinpur, 2010).

Another way of measuring the possible contribution of Po cycling in soil is by bioassays, growing a target species in soil samples of interest. Recently, bioassays were used to estimate soil productivity and fertility following crop diversification through the introduction of legumes (Sauvadet et al., 2021). This method has also been used to estimate growth and actual P and N bioavailability for non-tropical forest species (Ali et al., 2014; Ali et al., 2009), or tropical tree species (Binkley, 1997; Binkley et al., 2003b; Graciano et al., 2006; Tng et al., 2014; Wan Juliana et al., 2009). In particular, it was possible to measure the effect of an NFT species (*Albizia*) on the growth and mineral nutrition of a non-NFT species (*Eucalyptus*) and vice versa (Binkley, 1997). Although bioassays do not replace field experiments, they enable researchers to access all plant compartments, especially the whole root system. They provide valuable insights into the ability of a given species (especially NFT species) to improve soil fertility by altering the bioavailability of P and N for itself or for another species (Binkley 1997).

Despite the potential merits of NFT introduction in tropical non-NFT monoculture plantations, either in mixed plantations or in rotation, the effectiveness of NFTs in improving P bioavailability remains variable depending on the site. We hypothesized that this variability in the ability of NFTs to increase P bioavailability could be due to different Po cycling in soil, because Po sequestration in microbial biomass would prevent Po mineralization by phosphatases and therefore efficient Po recycling. To check this hypothesis, we addressed three research questions, namely (i) Is the enhancement of plant growth associated with the increase of enzyme-labile Po by NT? (ii) Does the size of the enzyme-labile Po pool depend on the immobilization of P in microbial biomass? (iii) Does this immobilized fraction of microbial P depend on soil P status, in particular on the availability of easily labile Pi?

We carried out our investigations at two tropical sites, one in Brazil and one in Congo, both planted with *Acacia mangium* (as NFT) and *Eucalyptus* (as non-NFT). As shown recently (Waithaisong et al., 2020), these two sites have contrasting Pi levels, but similar Po levels. Moreover, at both sites, acacia grown for one rotation after several rotations of *Eucalyptus* was able to increase soil Po concentrations measured in NaOH extracts compared to *Eucalyptus*, confirming the effect of acacia on the biogeochemistry of P. In this study, we extracted the labile fractions of Pi and Po with bicarbonate and evaluated the potential enzymatic lability of the Po fraction under different types of vegetation (monospecific acacia, monospecific *Eucalyptus* and native vegetation) at these two sites by using an acid phosphatase released by the basidiomycete ectomycorrhizal fungus *H. cylindrosporum*. This enzyme was expected to illustrate what happens *in situ*, as roots of acacia and

Eucalyptus have been found to be highly ectomycorrhizal (Robin et al., 2019, Pereira et al., 2020). To estimate the proportion of Po potentially immobilized in microbial biomass, we carried out enzyme assays in bicarbonate solutions extracted from autoclaved soil (Louche et al., 2010). Lastly, we used the bioassay technique (Binkley, 1997) to grow young *Eucalyptus* seedlings in soils taken from under the three vegetation types at each site. By measuring plant growth, as well as P accumulation within plants, we were able to quantify the effect of the three types of vegetation (monospecific acacia, monospecific *Eucalyptus* and native vegetation) on P bioavailability of each soil for *Eucalyptus*.

2. Material and Methods

2.1. Site description

The two sites used in this study were the same as those described in Waithaisong et al. (2020). Briefly, the Itatinga site was located in Brazil, in São Paulo state, and the Kissoko site in the Congo, on the Atlantic coast of Pointe-Noire. Annual rainfall figures were similar at the two sites (1370 mm at Itatinga and 1430 mm at Kissoko), while the mean annual temperature was around 5°C lower at Itatinga (20°C) than at Kissoko (25°C). Afforestation with *Eucalyptus* began in 1940 at Itatinga, with *E. grandis* W. Hill ex Maiden, and in 1984 at Kissoko, with a hybrid between *E. grandis* and *E. urophylla* S.T. Blake (*E. urophylla x grandis)*. *A. mangium* was introduced in May 2003 at Itatinga and in May 2004 at Kissoko, in order to compare wood production under different silviculture practices (Bouillet et al., 2013). The native ecosystems before afforestation were tropical savannas dominated by trees and shrubs at Itatinga (Maquere, 2008) and grasses at Kissoko, such as *Loudetia arundinacea* (Hochst.) (Epron et al., 2013), *Loudetia simplex* and *Hyparrhenia diplandra* (Versini, 2012). Some legume species, such as *Eriosema psoraleoides,* are common at Kissoko (Versini, 2012).

The soils used were Ferralsols at Itatinga and Ferralic arenosols at Kissoko (FAO-UNESCO, 1989). The texture of the 0-10 cm soil layer is dominated by sand (84% and 93%), with a low proportion of silt (4% and 1% at Itatinga) and clay (11% and 6% at Kissoko). The two soils are acidic (pH of 5.5 at Itatinga and 3.9 at Kissoko) with low concentrations in exchangeable elements and a low Cation Exchange Capacity (1.76 $cmol_c.kg^{-1}$ at Itatinga and 0.82 $cmol_c.kg^{-1}$ at Kissoko). Total C content was higher at Itatinga (13.8 g kg^{-1}) than at Kissoko (10.8 g kg^{-1}). Soils in planted plots exhibited large differences in P contents, as the total NaOH-extractable P concentrations at Itatinga (47 mg kg^{-1} dry soil) were almost 4 times lower than at Kissoko (180 mg kg^{-1} dry soil). In addition, at Itatinga, the soil was dominated by NaOH-Po, which accounted for 85% of the total NaOH-extractable P, whereas the Kissoko site was dominated by NaOH-Pi, which accounted for 60% of the total NaOH-extractable P (Waithaisong et al., 2020). The soil characteristics of the two sites are presented in detail in Waithaisong et al. (2020).

The field trials used in this study were set up in May 2003 at Itatinga (Bouillet et al., 2008; Laclau et al., 2008) and in May 2004 at Kissoko (Epron et al., 2013), consisting of monospecific stands of *Euucalyptus* or acacias. Plantation densities were 1111 (Itatinga) and 800 (Kissoko) trees per hectare. At planting, *Eucalyptus* and acacias received starter fertilization applied within a radius of 50 cm around each tree (Bouillet et al., 2013). The starter fertilization varied with the site, with P (40 kg ha^{-1} as superphosphate), K (75 kg ha^{-1} as KCl) applied at Itatinga (Bouillet et al., 2013) and only N as ammonium nitrate (43 kg ha^{-1}) at Kissoko (Epron et al., 2013).

2.2. Soil sampling

Soils were collected at each site on two occasions. The first was for P measurements and the second to set up the pot experiment. At each site, there were three replicates for each treatment, consisting of monospecific acacias (Ac), monospecific *Eucalyptus* (Euc), and nearby native vegetation on the same soil type (Nat). The soil samples used for P measurements were of the same provenance as those used in Waithaisong et al. (2020). Samples of topsoil (0-10 cm), consisting of mineral soil without organic layer nor leaves, were collected at the end of the rainy season at both sites (in February 2012 at Itatinga and May 2011 at Kissoko). These periods corresponded to the end of the second 8-year rotation (Itatinga) and the end of the first 7-year rotation (Kissoko) of the acacia trees. The soil samples were air dried, sieved at 2 mm and stored at room temperature pending analysis.

The soils for the bioassay experiment were collected in November 2013 at both sites. Cores were used to collect the topsoil in the same monospecific acacia or *Eucalyptus* plots or native vegetation ones as those used for P measurements. In planted forests plots (Euc or Ac), three soil cores were collected around one tree, whereas in the native vegetation plots the soil cores were collected along a 10-m transect, a distance chosen to take into account most of the spatial heterogeneity of the area. A composite sample was then made up from all intact soil cores from a given treatment before filling each pot with 300 g of wet soil.

2.3. Bioassays

Bioassays were carried out at the Itatinga station nursery and at the Kissoko experimental station. Seedlings were obtained from seeds of *E. grandis* at Itatinga (provided by the Suzano Company, Brazil, (http://ri.suzano.com.br/) and from cuttings of *E. urophylla x grandis* (clone 18-147) at Kissoko. The bioassay experiment was launched by transferring one 1-2 cm tall seedling to a pot containing topsoil collected from Euc, Ac or Nat plots. There were six plants per soil origin at Itatinga and 10 at Kissoko. Pots were watered with tap water regularly over the growth period. Plants were harvested 7 (Itatinga) and 6 (Kissoko) months after planting. They were separated into roots, stems and leaves before drying (60°C) and weighing. The dried plant material was stored in plastic bags at room temperature pending P measurements carried out in France.

2.4. Soil extractions and chemical assays

Soil labile P was extracted with 0.5 M $NaHCO_3$, pH 8.5, according to Olsen et al. (1954). Briefly, the soil–bicarbonate solution mixture (1/10, w/v) was shaken end-over-end for 30 min at room temperature. After centrifugation (2683 RCF, 10 min), the supernatant was filtered through a 0.22 μm cellulose membrane filter. Labile P was extracted from intact or autoclaved soil, as autoclaving has been proposed to release the microbial Po pool (Louche et al., 2010). Dry soil was rewetted with ultrapure water to field capacity and then autoclaved twice 24 h apart (110°C, 60 min) (Louche et al., 2010). Total labile P was obtained by adding 12N HCl to bicarbonate extracts (v/v) and digestion of the mixtures at 110°C for 16 h (Ali et al., 2009). Organic labile P was estimated by subtracting free Pi from total P.

To measure total P in the plants, roots, stems and leaves were finely ground and re-dried overnight at 50°C prior to acid digestion following a slightly modified McDonald method (1978). In brief, around 10 mg of dry material was placed in a Pyrex glass tube (15 mm in diameter and 160 mm in height) with 0.2 ml of 36 N H_2SO_4 and salicylic acid (5%, w/v). The tubes were placed in a heating block under a fume hood and the temperature was gradually increased to 330°C. After 10 min at 330°C, the heating block was turned off and the tubes were allowed to cool outside the block before adding 0.2 ml of ultrapure H_2O_2 (110 volumes, non-stabilized with phosphate) to each tube, in order to oxidize organic compounds. The glass tubes were heated again for 5 min at 330°C and this procedure was repeated until the solution became transparent. The resulting H_2SO_4 (36 N) solution was diluted to 0.1 N before P measurements. The malachite green method (Ohno and Zibilske, 1991) was used to measure orthophosphate P in the different assays (0.1 N H_2SO_4 plant digest, labile P as free Pi and total P and soil enzyme-reactive P).

2.5. Production of the acid phosphatase from H. cylindrosporum and substrate specificity

Soil enzyme-reactive organic P was measured using an acid phosphatase released by the ectomycorrhizal fungus *H. cylindrosporum* whose complete cDNA sequence was retrieved from a cDNA library available for this species (Lambilliotte et al., 2004). The protein was named HcPhoA (genbank accession number FR692330, Louche et al., unpublished). Using antibodies raised against the protein and Dot-blot analysis, HcPhoA was detected mainly in the first of the four acid phosphatase fractions separated from the P-depleted culture medium of the fungus grown *in vitro* (Louche et al., unpublished). The HcPhoA protein was produced using recombinant technology and the full procedure, from obtaining the full-length gene from the *H. cylindrosporum* cDNA library to producing the recombinant enzyme by *Pichia pastoris,* is described in Supporting Information S1. The enzyme was obtained after 7 days of methanol induction (Ragon et al., 2008) in a 3-liter fermenter. After centrifugation of the culture medium, the supernatant was concentrated by dialysis to obtain an enzyme solution (7 mg protein ml^{-1}) stored at -20°C. The substrate specificity of the recombinant enzyme was determined against 12 Po compounds purchased from Sigma, namely *p*NPP (4-Nitrophenyl phosphate disodium salt hexahydrate, product N4645), PrP (Sodium pyrophosphate dibasic, product P8135), TripolyP (Sodium triphosphate pentabasic, product 72061), glucose 1-P (α-D-Glucose 1-phosphate disodium salt hydrate (x H_2O), product G7000), glucose 6-P (D-Glucose 6-phosphate disodium salt hydrate (x H_2O), product G7250), glycero-P (ß-Glycerophosphate disodium salt hydrate, product G9422), Fructose 6-P (D-Fructose 6-phosphate dipotassium salt, product F1502), Fructose 1,6-bis P (D-Fructose 1,6-bisphosphate trisodium salt hydrate (x H_2O), product F6803), AMP (Adenosine 5′-monophosphate disodium salt, product 01930), ADP (Adenosine 5′-diphosphate monopotassium salt dihydrate, product A5285), ATP (Adenosine 5′-triphosphate disodium salt hydrate (x H_2O), product A2383) and phytate (myo-Inositol hexakis (dihydrogen phosphate) (x Na), product 68388). The conditions for measurement were as follows: 50 µl of enzyme solution previously diluted 1000 times, equivalent to 0.35 µg of HcPhoA protein, was added to 200 µl of a 0.2 M acetate buffer, pH 5, containing the substrate at 10 mM. The reaction was incubated at 28°C and stopped with 1 ml of 0.2 N HCl after 30 min for all substrates except phytate, which was stopped after 60 min. Substrate hydrolysis was measured by the release of Pi into the solution (Ohno and Zibilske, 1991) and expressed in µmole Pi sec^{-1} mg $protein^{-1}$.

2.6. Measurement of soil enzyme-reactive organic P

For soils, the assay was carried out on bicarbonate extract from intact or autoclaved soil (110°C for 60 min, twice 24 h apart) previously buffered at pH 5 following the protocol of Louche et al. (2010). Briefly, 3 ml of the bicarbonate extract was mixed with 6 ml of acetate buffer (1 M, pH 5) and 0.25 ml HCl 6N. The enzyme assay consisted of 100 µl of buffered bicarbonate extract, 50 µl of ultrapure water and 50 µl of enzyme containing 0.35 µg of HcPhoA protein distributed in a 96-well microplate with a volume of 300 µl per well. The reaction was incubated at 28°C and stopped with 40 µl of the first reagent of the malachite green assay. Preliminary tests were carried out to determine the linearity of the reaction from 0 to 2h of reaction. P release into the extracts from intact soil sampled at Itatinga was linear over the reaction time, so Pi concentrations were measured after 0, 60, 90 and 120 min of reaction. For the other samples (autoclaved soils from both sites and intact soil from Kissoko), Pi release was fast and Pi concentrations were measured after 0, 10, 20 and 30 min of reaction. For each sample, Pi concentrations as a function of incubation time were plotted to obtain the slope of the linear regression, its value corresponding to acid phosphatase activity. Enzyme activity was expressed as pmol Pi released per sec and per g of fresh soil. We also calculated the share of Po present in the sample that had been hydrolyzed after 30 minutes of reaction, i.e., the percentage of enzyme-reactive Po, using the equation:

$$Percentage of enzyme - reactive Po = [(a * t) / (Po sample)] * 100$$

Where "a" is the slope of Pi production per second for each sample, "t" is the enzyme reaction time (here 1800 sec), "Po sample" is the total amount of bicarbonate-Po in the sample at t0. It was calculated by multiplying the Po concentration of each sample by the volume of bicarbonate extract used in the reaction (here 100 µl).

The auto-hydrolysis of Po in soil extracts was checked using the same conditions as above, by replacing 50 µl of enzyme solution with 50 µl of ultrapure water in the mixture reaction. The effect of bicarbonate soil extracts on phosphatase activity was determined by replacing 50 µl of water with 50 µl of 10 mM glucose-6-P.

2.7. Data analysis

All analyses were performed using R Statistical Software (v4.1.2; R Core Team 2021). Values expressed as a percentage (substrate specificity, percentage of Po hydrolysis) were transformed ($\arcsin\sqrt{x}$) as described in Legendre & Legendre (1998) before statistical analysis. The effect of the treatments on the quantity of Po and Pi contained in the soil extracts (whether autoclaved or not) and Pi released after incubation with the acid phosphatase was analyzed with a two-way ANOVA, while the effect of soil origin on plant biomass and P accumulation was analyzed with a one-way ANOVA. The homogeneity of the variances was verified with the Levene test and the normality of the ANOVA residuals with the Shapiro-White test. If these tests were significant ($p < 0.05$), the data were log-transformed before analysis. The differences between means were analyzed using the Tukey HSD post-hoc test and the predictmeans package (version 1.0.6) (https://CRAN.R-project.org/package=predictmeans).

3. Results

3.1. Labile P concentrations in soils

Both labile (bicarbonate-extractable) Pi and Po concentrations were lower at Itatinga site) than at Kissoko, whatever the plant cover in the sampled plots, except in the native vegetation for Po (Table 1). At Itatinga, the two-way ANOVA indicated that labile Pi concentrations varied with the original vegetation combined with soil autoclaving, due to an increase in Pi concentrations extracted from autoclaved soils in native plots (Nat soil) compared to all other soil samples. At Kissoko, bicarbonate-extractable Pi concentrations did not change with the original vegetation or autoclaving. However, the values were 10 times higher at Kissoko than at Itatinga in soils from afforested plots and 3-5 times higher in native plots, whatever the autoclaving treatment.

For bicarbonate-extractable Po, only the concentrations measured in soils sampled at Itatinga varied significantly depending on the original vegetation and soil autoclaving, without any significant interaction between these two factors. Whatever the autoclaving treatment, the highest Po concentrations were twice as high in soil from native vegetation as in soil from afforested plots (Table 1). Po concentrations were lower at Itatinga than at Kissoko, but only in soils from afforested plots, and differences between the two sites were much lower for Po than for Pi, with a factor ranging between only 1.85 and 1.38 (Table 1).

3.2. Enzyme-reactive Po in soils

Enzyme-reactive Po was measured by using a recombinant purified acid phosphatase produced from *H. cylindrosporum*. First, we tested the substrate specificity of this recombinant phosphatase under controlled conditions (Fig. 1). We used Po compounds that were phosphomonoesters (P-O-C bond) and condensed phosphate (P-O-P bond), which are the main P-molecules detected in soil extracts. Among the P-

Table 1
Concentrations of bicarbonate-extractable P forms (mg P kg^{-1} dry soil) as free phosphate (Pi) and organic P (Po) from the topsoil (0-10 cm) sampled at Itatinga or Kissoko in plots with acacia (Ac), *Eucalyptus* (Euc) and native vegetation (Nat), used either intact or autoclaved. Each value is the mean (n = 6) with standard deviation between brackets. For each P form at a site, the effects of the original vegetation (Veg) and soil autoclaving (Autoclav), and their interaction (Veg x Autoclav), were analyzed with a two-way ANOVA followed by a pairwise comparison of means (Tukey-HSD post-hoc test), with means with different letters being significantly different ($p < 0.05$). For a given original vegetation, the effect of the site on a given form of P (Pi or Po) was analyzed using a Student-t test. The significance of the associated *p*-value of the two statistics is reported as follows: *NS* ($p>0.05$), * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

		Soil autoclaving[a] and original vegetation[b]								
		Intact soil (mg P kg^{-1} dry soil)			Autoclaved soil (mg P kg^{-1} dry soil)			Two-way ANOVA		
Site	P form	Ac	Euc	Nat	Ac	Euc	Nat	Veg	Autoclav	Veg x Autoclav
Itatinga	Pi	2.88b (0.39)	2.53b (0.9)	3.02b (0.59)	2.42b (0.24)	2.65b (0.44)	4.27a (1.06)	***	*NS*	*
Kissoko	Pi	22.76 (7.57)	22.55 (3.8)	17.87 (9.98)	18.74 (4.17)	19.58 (4.05)	13.40 (8.07)	*NS*	*NS*	*NS*
Between-site *p*		***	***	**	***	***	*			
Itatinga	Po	4.31c (0.96)	4.88c (0.46)	10.39ab (4.03)	6.46bc (0.91)	6.85bc (1.67)	14.6a (5.31)	***	***	*NS*
Kissoko	Po	7.99 (0.53)	7.23 (1.13)	6.35 (2.89)	10.02 (2.58)	9.48 (1.87)	9.65 (4.39)	*NS*	*NS*	*NS*
Between-site *p*		***	**	*NS*	*	*	*NS*			

[a] Soil autoclaving (Autoclav: intact or autoclaved)
[b] Original vegetation (Veg: Ac, Euc, Nat)

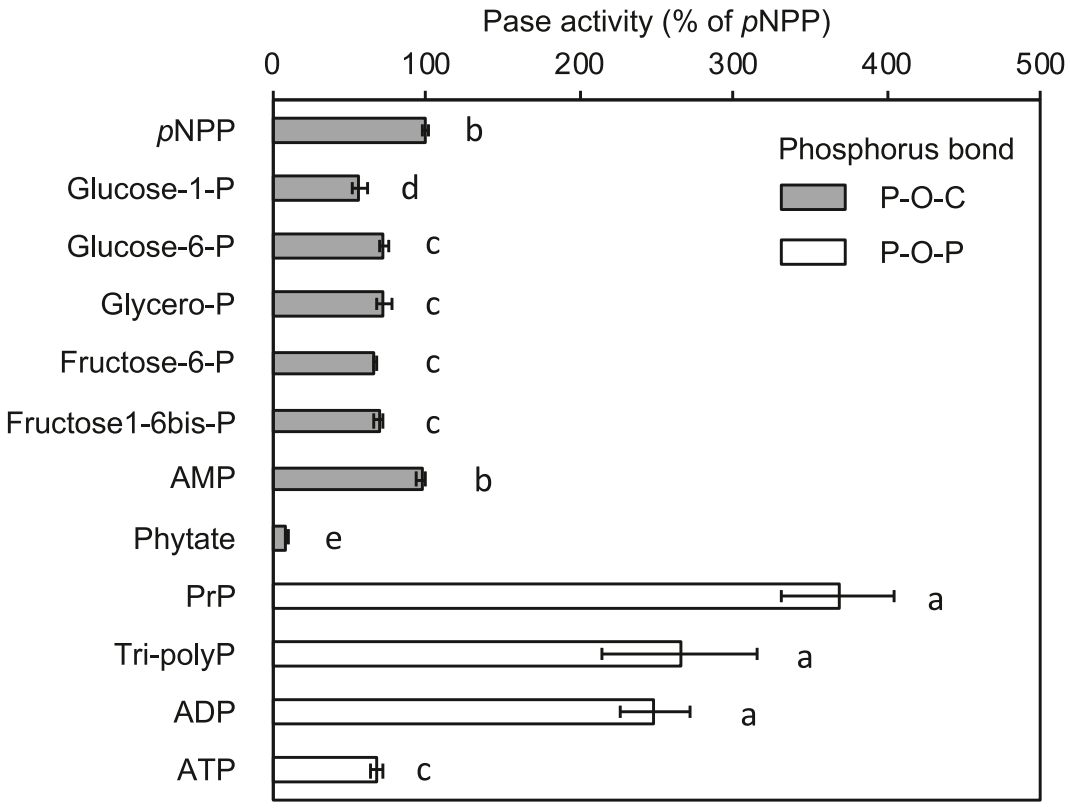

Figure 1. Substrate specificity of the recombinant acid phosphatase from *H. cylindrosporum* against 12 organic P compounds with two types of phosphorus bond, either monoester (P-O-C bond, gray bar) or phosphoric acid anhydride (P-O-P bond, white bar). Release of free phosphate (Pi) from para-nitrophenylphosphate (*p*NPP) was used as a control and set at 100%. 100% of activity for pNPP was equal to 74.3 pmoles of Pi released sec^{-1} $sample^{-1}$, corresponding to 0.21 μmoles of Pi released sec^{-1} mg^{-1} of protein. Bars are means ± standard deviation (n=4) and different letters indicate significantly different means (one way-ANOVA on $\arcsin\sqrt{x}$ transformed values and Tukey HSD post hoc test).

esters, *para*-nitrophenylphosphate (*p*NPP) is widely used as an artificial substrate to measure phosphatase activities (Tabatabai and Bremner, 1969). HcPhoA activity with *p*NPP was equal to 0.21 μmoles of Pi released sec^{-1} mg^{-1} of protein and was used as a control and set at 100% (Fig. 1). At first glance, Pi was released from all the compounds tested, except phytate. Phosphate esters (P-O-C bond) were hydrolyzed at rates significantly lower (≈ 80%) than those measured on *p*NPP, except AMP (≈ 100%). In contrast, the ability to hydrolyze condensed phosphate (P-O-P) was much greater than the other P-esters, suggesting that the enzyme was able to hydrolyze all the P bonds of the molecules. However, this observation did not apply for ATP, whose degradation rate was only around 80% of *p*NPP (Fig. 1). We also compared Pi release from glucose-6-P in enzyme assays carried out with or without each soil extract from Itatinga and Kissoko, and the values did not differ (data not shown), indicating that the bicarbonate soil extracts did not inhibit the enzyme, allowing us to use it to measure enzyme-reactive Po in our soil samples. The absence of Pi release in each soil extract in the absence of enzyme confirmed that no auto-hydrolysis was occurring in our samples (data not shown).

Pi release rates from bicarbonate-extracted Po following enzyme addition differed considerably between sites, especially in intact soils (Fig. 2). At Itatinga, enzyme-reactive Po was significantly affected by the original vegetation and soil autoclaving. The values measured in intact soil samples were very low, whatever the original vegetation. In

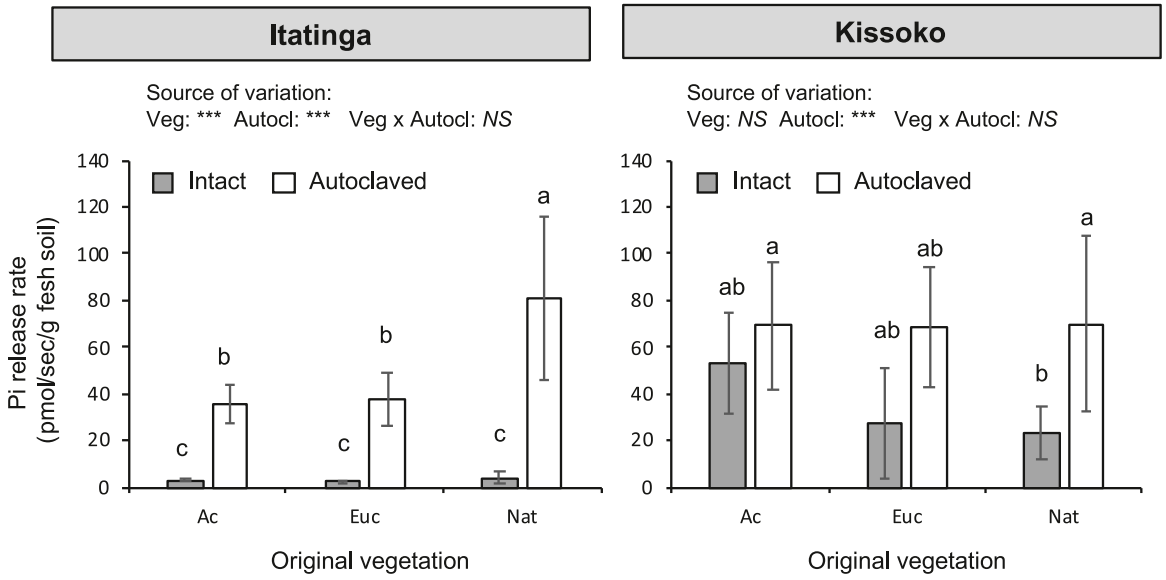

Figure 2. Enzyme-reactive Po measured by the release of free Pi following addition of the recombinant acid phosphatase from *H. cylindrosporum* to bicarbonate extracts of soils from Itatinga and Kissoko. Topsoils were collected under different original vegetations (Veg), namely acacia (Ac), eucalyptus (Euc) and native vegetation (Nat), and were used either intact (gray bars) or after autoclaving (white bars). Each bar is the mean (n=6) with standard deviation. The effects of the original vegetation (Veg), soil autoclaving (Autocl) and their interaction (Veg x Autoclav) were analyzed with a two-way ANOVA followed by a pairwise comparison of means (Tukey-HSD post-hoc test), with means with different letters being significantly different ($p < 0.05$). The significance of the associated *p*-value of the two-way ANOVA is reported as follows: *NS* ($p>0.05$), *** ($p<0.001$).

contrast, bicarbonate-extracted Po from autoclaved soils was much more reactive to the phosphatase, by a factor of around 10 in Ac and Euc soils, and 20 in Nat soils. In addition, Po extracted from autoclaved soil under native vegetation was around twice as enzyme-labile as when extracted from afforested plots. At Kissoko, intact soil samples displayed much higher values of enzyme-reactive Po than at Itatinga, with factors ranging from 17 in Ac soils, 11 in Euc soils to 8 in Nat soils (Fig. 2). Despite a significant effect of autoclaving given by the two-way ANOVA, only soil collected under native vegetation displayed significant differences after autoclaving, with values of Pi released in autoclaved soils multiplied by 3 compared to intact soils. However, this effect was much lower for soils collected at Kissoko than at Itatinga.

The percentage of enzyme-reactive Po after 30 minutes with the enzyme confirmed the extremely low reactivity of bicarbonate-extractable Po from intact soils at Itatinga, with values under 5% in all soils (Table 2). In addition, the values calculated in soils from native vegetation were significantly lower than those in Ac soils. Autoclaving greatly increased enzyme-reactive Po in soils from Itatinga, by a factor ranging from 7.5 to 11, whereas it had no effect on soils from Kissoko.

3.3. Growth of Eucalyptus seedlings and P accumulation

Bioassays carried out by growing eucalypt seedlings in intact soil samples showed that the original vegetation induced contrasting effects on plant growth depending on the site (Fig. 3). At Itatinga (Fig. 3A), seedling total dry weight was the highest in Nat soil and the lowest in Euc soil, whereas the biomass of seedlings grown in Ac soil was intermediate. The decrease in seedling biomass in Euc soil was mainly due to lower root growth than in the other two soils. Seedlings grown in Nat soil displayed greater stem and leaf biomass than in the other two soils.

At Kissoko (Fig. 3B), the total biomass of *Eucalyptus* plants was significantly lower in the soil collected in the native vegetation than in the soil sampled in Ac and Euc planted plots.. For each plant organ (roots, stems, or leaves), the dry weight values varied in the same order Ac > Euc > Nat. The total amounts of biomass were 3.5 (Ac soils) and 1.7 (Euc soils) times higher than in Nat soil. The same trends were found in individual organs, but stem biomass was the most affected by the soil origin, being increased by a factor of 3.9 in Ac compared to Nat soil.

In contrast to total biomass, total P accumulation in plants grown in

Table 2
Percentage of bicarbonate-extractable enzyme-reactive Po[a] accessible to the recombinant acid phosphatase from *H. cylindrosporum*. Po was extracted with sodium bicarbonate from soils used either intact or after autoclaving. Topsoils were collected under different original vegetation, namely acacia (Ac), *Eucalyptus* (Euc) trees and native vegetation (Nat) at Itatinga or at Kissoko. Each value is the mean (n = 6) with standard deviation between brackets. For a given site, the effects of the original vegetation (Veg: Ac, Euc, Nat), soil autoclaving (Autoclav: intact, autoclaved), and their interaction (Veg x Autoclav), were analyzed with a two-way ANOVA followed by a pairwise comparison of means (Tukey-HSD post-hoc test), with means with different letters being significantly different ($p < 0.05$). For a given original vegetation and soil autoclaving, the effect of the site was analyzed using a Student-t test. The significance of the associated *p*-value of the two statistics is reported as follows: *NS* ($p>0.05$), *** ($p<0.001$).

	Soil autoclaving and original vegetation								
	Enzyme-reactive Po (%) intact soil			Enzyme-reactive Po (%) autoclaved soil[b]			Two-way ANOVA		
Site	Ac	Euc	Nat	Ac	Euc	Nat	Veg	Autoclav	Veg x Autoclav
Itatinga	4.33b (1.20)	2.71bc (0.78)	2.46c (1.64)	36.94a (15.12)	20.33a (14.96)	28.51a (17.78)	*NS*	***	*NS*
Kissoko	30.95 (5.35)	33.24 (12.59)	30.40 (2.76)	43.77 (28.40)	41.49 (15.36)	43.22 (23.59)	*NS*	*NS*	*NS*
Between-site *p*	***	***	***	*NS*	*NS*	*NS*			

[a] The percentage of enzyme-reactive Po in 100 µl of bicarbonate extract was calculated after 30 minutes of reaction (pH 5.0, 28°C) using the equation: [Percentage of enzyme-reactive Po = [(a * t) / (Po sample)] * 100], where "a" is the slope of Pi production per sec in the sample, "t" is the enzyme reaction time (here 30 min), "Po sample" is the total amount of bicarbonate-Po in the sample at t0.

[b] Autoclaving was carried out twice (110°C, 60 min).

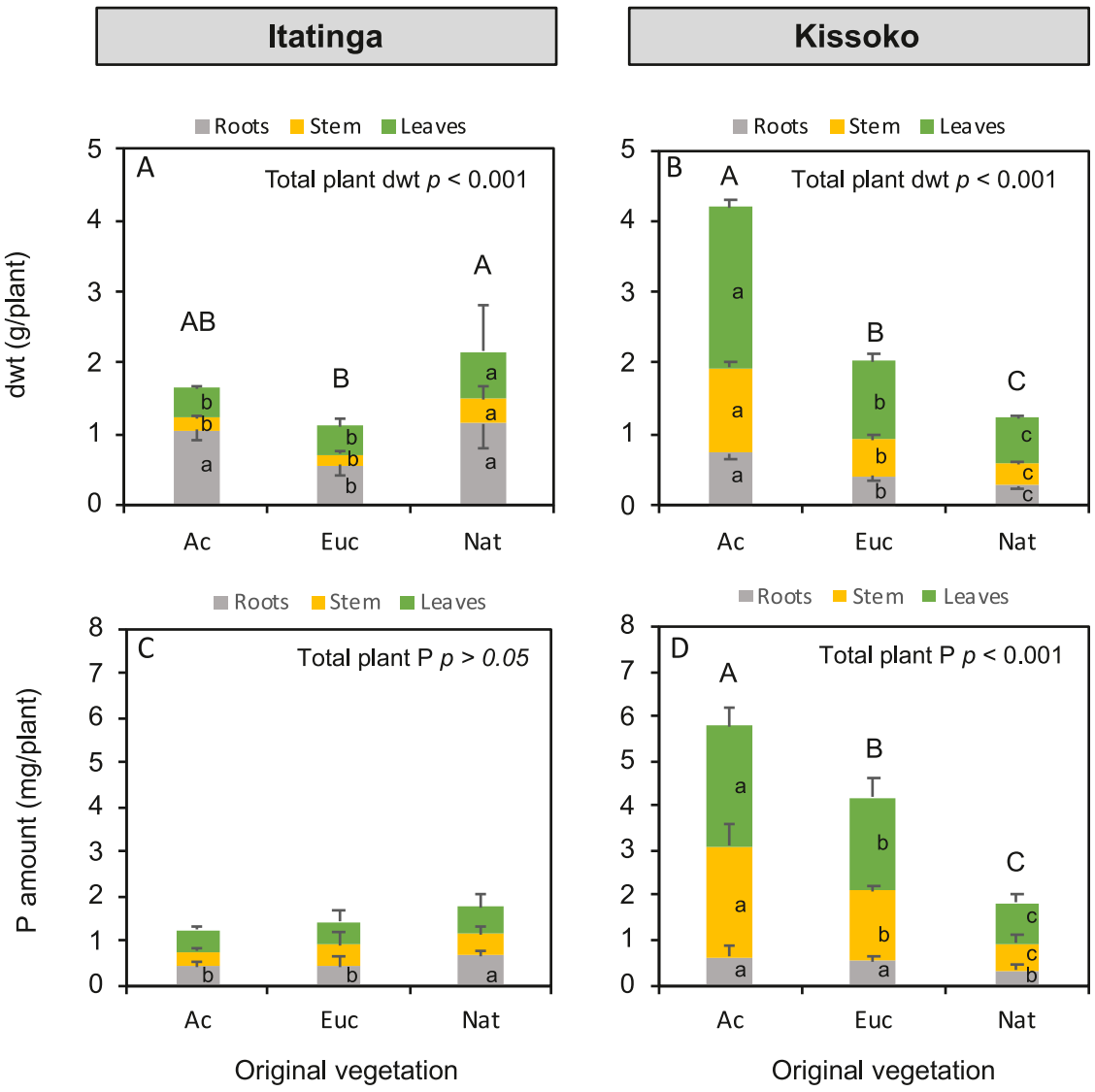

Figure 3. Growth and P accumulation of eucalypt seedlings in bioassay on intact topsoils collected under different original vegetation (Veg), namely acacia (Ac), *Eucalyptus* (Euc) and native vegetation (Nat) at Itatinga) and at Kissoko). Each bar is the mean (n= 6 at Itatinga, n=10 at Kissoko) with standard deviation. A, B: biomass (g dry weight/plant) in roots, stem and leaves; C, D: total P accumulation (mg P/plant) in roots, stem and leaves. Within each site, different lowercase letters inside the bars denote significant differences between original vegetation for individual organs and uppercase letters above the bars indicate different total dry weight and total P amount according to the original vegetation. For each variable within a site, the effects of the original vegetation were analyzed with a one-way ANOVA followed by a TukeyHSD post-hoc test.

soil collected at Itatinga did not vary significantly with soil provenance (Fig. 3A and 3C). While this was also true for P accumulation in stems and leaves, the roots of *Eucalyptus* grown in Nat soil accumulated approximately 50% more P compared to Ac or Euc soils. The concentrations of P (Table S1) ranged from 0.04 to 0.06% in roots, from 0.17 to 0.3% in stems, and averaged 0.12% in leaves. Here, only P concentrations in leaves were not affected by the soil origin, whereas those of roots varied in the order Ac < Euc = Nat and those in stems in the order Ac = Nat < Euc.

Total P accumulation in *Eucalyptus* seedlings grown in soil collected at Kissoko followed the same trend (Ac > Euc > Nat) as biomass (Fig. 3B and Fig. 3D). Significant differences were found between soils in aboveground biomass. Leaves accumulated ≈ 3 and ≈ 2.3 times more P in Ac and Euc soils than in Nat soil. Also stems accumulated ≈ 4.2 and ≈ 2.6 times more P in Ac and Euc soils than in Nat soil. Roots accumulated the same amounts of P in Ac and Euc soils, being ≈ 1.6 times higher than in Nat soil (Fig. 3D). P concentrations (Table S1) ranged from 0.08 to 0.12% in roots, from 0.19 to 0.29% in stems and from 0.12 to 0.14% in leaves. P concentrations in each organ were significantly affected by soil origin, with the concentrations observed in Euc soil generally being higher than in Ac and Nat soils.

4. Discussion

Nitrogen-fixing tree species (NFT) generally increase the concentrations of soil Po in comparison to non-nitrogen fixing tree species (Aleixo et al., 2020; Cabreira et al., 2022; Waithaisong et al., 2020), leading often, but not always, to enhanced P bioavailability for plants in mixed plantations of NFT and non-NFT species relative to monospecific non-NFT stands (Richards et al., 2010; Le Cadre et al., 2018). In this study, we put forward the hypothesis that Po immobilization in microbial biomass might explain variable effects of NFT species on P cycling from soil Po fractions. We used chemical, biochemical and bioassay methods to elucidate whether an increase in easily-extractable Po concentrations in soil is sufficient to enhance growth and soil P bioavailability for *Eucalyptus*, a non-NFT species, as influenced by the level of

available Pi concentrations in soil.

4.1. Relationship between enzyme-labile Po, Po immobilization in microbial biomass and labile Pi content in soil

First, we used chemical methods to estimate easily available Pi and Po in our intact soil samples. As reported previously (Bouillet et al., 2013; Waithaisong et al., 2020), our data indicated that soils at Itatinga had low Pi availability and those at Kissoko had high Pi availability with no influence of the different vegetations. Bicarbonate-extracted Po concentrations were of the same order of magnitude at both sites and were more variable at Itatinga, with the highest values measured in soils under native vegetation. We then used a biochemical method to estimate potential mineralization of bicarbonate-Po in the soil following Pi release by the acid phosphatase secreted by the ectomycorrhizal species *H. cylindrosporum* (enzyme-labile Po). This methodology has the advantage of being more representative of bioavailable P as the Po measured is the Po accessible by enzymes in soil (Louche et al., 2010). It is worth noting that the enzymatic degradability of the bicarbonate-extractable Po pools from intact soils was very different between sites, being very low at Itatinga (< 5%), even in Nat soil, and high at Kissoko (> 30%). These differences in enzyme-labile Po might be explained by greater Po immobilization in microbial biomass at Itatinga than at Kissoko. We used soil autoclaving to check this hypothesis as this method is able to release microbial P (Sinegani and Hosseinpur, 2010) and a new pool of bicarbonate-extractable Po (Anderson & Magdoff, 2005). This new pool of Po becomes available to phosphatase activity and was attributed to microbial Po (Louche et al., 2010). However, although Sinegani and Hosseinpur (2010) showed that autoclaving led to results similar to those obtained with the reference method for measuring microbial biomass (chloroform fumigation), autoclaving has potential biases. It will destroy organic material (litter, plants, roots, soil animals etc.), potentially increasing the release of Po not originating from microbial populations. In our study, we used soil previously sieved at 2 mm, thus without litter and plant debris as well as soil animals belonging to the macrofauna and a part of the mesofauna, suggesting that this new pool of Po originates mainly from microbial populations. Also, soil autoclaving was shown to decrease Pi sorption (Serrasolses et al., 2008), indicating that we cannot exclude an overestimation of available Pi. So far, this method has been applied in sandy soils (Louche et al., 2010 and our study) and cannot be generalized to all soils before assessing its effects on P fixation rates, C and SOM levels and textures. However, as expected, the values for enzyme-reactive Po in autoclaved soil increased dramatically at Itatinga, with the greatest effect on soils from native vegetation. We previously showed that these soils sampled under native vegetation contained more pyrophosphate (PrP) than the other soils from planted forests (Waithaisong et al., 2020). As the enzyme was much more active on this substrate than on the other forms of Po, the enhanced Pi release from native vegetation soil extracts may have come from the hydrolysis of PrP released during soil autoclaving. In contrast to Itatinga, soil autoclaving had no significant effect at Kissoko. Hence, in our study, Pi and enzyme-labile Po concentrations extracted in bicarbonate had contrasting values between sites. Specifically, the effect of autoclaving suggested high (at Itatinga) and low (at Kissoko) Po immobilization in the microbial fraction. Overall, our results in two tropical soils tallied with those reported in Australian forest soils by Serrasolses et al. (2008), showing that the ratio of microbial P to organic P varied inversely with soil total P, suggesting that low soil P contents promote P immobilization by soil microorganisms.. Also, in temperate beech forests, the proportion of microbial P decreased exponentially with water-extracted Pi concentrations (Joergensen et al., 1995) and labile Pi was rapidly incorporated into the microbial Po, especially when available Pi was low (Pistocchi et al., 2018). Hence, our results suggest that autoclaving could be used as an easy and quick way of accessing immobilized microbial P, as shown by Sinegani and Hosseinpur (2010) but further studies are needed to evaluate in which soil types this method can be used.

4.2. Effects of acacia trees on soil nutrient bioavailability for Eucalyptus cultivation

In our work, we used a bioassay to assess how the vegetation type, and in particular soils from acacia plantations, affected the growth and mineral nutrition of *Eucalyptus* at each site. This method was previously used with success to recognize whether or not plant diversification in a given ecosystem can modify the soil fertility (Binkley, 1997; Binkley et al., 2003b; Sauvadet et al., 2021). Regarding biomass production, we found in our bioassay that the total biomass of *Eucalyptus* seedlings grown in Euc and Ac soils was around twice as high at Kissoko as at Itatinga. Based on Bouillet et al. (2013), we calculated that *Eucalyptus* grown for 72 (Itatinga) and 77 (Kissoko) months (the entire rotation) produced 98 and 61 kg of stem/tree in monospecific *Eucalyptus* plots, and 131 and 104 kg of stem/tree in mixed plots (*Eucalyptus* /*A. mangium*, with 50 % of each species). A comparison of these data with the stem growth of the seedlings showed that bioassays underestimated *Eucalyptus* growth at Itatinga when compared to Kissoko. However, the increase in stem biomass of *Eucalyptus* in monospecific and mixed plots in the field were thus +33% at Itatinga and + 70% at Kissoko, indicating a strong positive effect of *A. mangium* introduction on *Eucalyptus* stem growth at Kissoko. In contrast with total biomass, these field data were more in line with the increase in stem biomass of the seedlings in bioassays, that were +12% at Itatinga and +135% at Kissoko in Ac soils compared to Euc soils.. Besides biomass measurements, bioassays can also be used to measure plant nutrient accumulation. Our study also revealed site-specific differences in plant P supply depending on the previous vegetation. While native vegetation soil had the highest P bioavailability at Itatinga compared to Ac and Euc soils, P bioavailabity in native vegetation soil at Kissoko was the lowest. Soil total P (Waithaisong et al., 2020) or labile Pi contents, which varied very little between native soil and afforested soil with *Eucalyptus*, were therefore not directly linked to P bioavailability for the plant, especially at Kissoko. Overall, as highlighted by Binkley (1997), bioassays can really help in forecasting the effect of different strategies of forest management, including the introduction of a new species, but require a comparison of several treatments to estimate trends rather than absolute values.

4.3. The positive effect of acacia on plant P bioavailability depends on P locked in microbial biomass

The positive effect of acacia soil on the P nutrition of *Eucalyptus* seedlings depended on the site as it was only observed at Kissoko, not at Itatinga. This better P bioavailability may have been due to levels of bicarbonate-extracted Pi that were systematically higher at Kissoko than at Itatinga, in all soils. However, this first explanation was not supported by the soils collected under native vegetation, which provided similar plant P bioavailability for the two sites, despite the bicarbonate-extractable Pi concentrations being 5 times higher at Kissoko than at Itatinga. Alternatively, bicarbonate-extracted Po may also contribute to plant P bioavailability, requiring easily bicarbonate-extractable Po to be available for mineralization by phosphatase enzymes to contribute to plant P nutrition. Hence, measuring enzyme-reactive Po extracted by bicarbonate could reflect potential Po cycling *in situ*. In our study, we measured this enzyme-reactive Po by using the acid phosphatase secreted by *H. cylindrosporum*. Although we know that this fungal species is not found in *Eucalyptus* plantations, *Eucalyptus* roots are associated with many ectomycorrhizal species (Robin et al., 2019; Pereira et al, 2021). We hypothesized that the acid phosphatases released by ectomycorrhizal fungal species share similar properties such as substrate specificity, enabling us to use the phosphatase from *H. cylindrosporum* as a representative enzyme of organic P hydrolysis in forest soil, even if we are aware of possible limitations of this approach. Interestingly, at Kissoko, the amount of enzyme-reactive Po was maximum in intact Ac soil

and minimum in Nat soil. This trend in enzymatic activity parallels that in total biomass and amounts of P measured in seedlings, suggesting that Po easily accessible by enzymatic hydrolysis plays a major role in the bioavailability of P for plants. Our observations are consistent with the results of Waithaisong et al. (2020), who suggested that the P cycle under acacia is dominated by organic P mineralization at Kissoko. At Itatinga, all the soils used had very low levels of bicarbonate-extractable Pi and, in this case, the previous cultivation of acacia was not able to improve P bioavailability for *Eucalyptus*. However, unlike the Ac soil at Kissoko, the proportion of Po easily hydrolyzed by the fungal phosphatase was very low for Itatinga, leading to very low plant P accumulation and growth, indicating very low P bioavailability in this soil. However, the N:P ratio for soil microbial biomass is quite stable and has been found to be around 7:1 (Cleveland and Liptzin, 2007). Hence, increasing soil N inputs, for example trough the introduction of acacia trees in *Eucalyptus* plantations, would result in an increased microbial demand for inorganic P which would become immobilized in microbial biomass as organic P, depending on the C availability in the soil (Bünemann et al., 2008). The lower carbon contents in the soil at Kissoko than at Itatinga could explain the lower immobilization in the microbial biomass at Kissoko due to stoichiometric constraints. However, the immobilized P could be released to increase the stock of available P during the microbial biomass turnover, which is not considered in this study. Altogether our results thus demonstrate that actual plant P bioavailability depends mainly on Po status in the soil, and the more it is locked into the microbial biomass, the less it is available to plants.

5. Conclusion

In this study, we addressed three research questions to explain the variable effects of introducing a NFT species (*A. mangium*) on P recycling and subsequent P bioavailability for a non-NFT species (*Eucalyptus*) at two tropical sites, one at Itatinga (Brazil) and the other at Kissoko (Congo). These sites were chosen because of their contrasting levels of labile Pi and the opposite effects of *A. mangium* on wood production of *Eucalyptus* in mixed plantations, which were either not visible at Itatinga or strongly positive at Kissoko. Although our results are not generalizable as they were obtained by comparing two sites only and used one NFT species, our results showed that acacia trees must increase enzyme-labile Po to enhance plant growth and plant P nutrition (first question) because we found the largest proportion of enzyme-labile Po in soil sampled under acacias, together with the highest growth and P accumulation of *Eucalyptus* seedlings at Kissoko. Conversely, at Itatinga, acacia soil showed no increase in enzyme-labile Po and no increase in seedling growth and P accumulation compared to other soil samples. We also showed that the very low levels of enzyme-labile Po at Itatinga increased dramatically with soil autoclaving, becoming similar to those measured at Kissoko, which were not greatly influenced by autoclaving. These results demonstrate that most of the Po at Itatinga was immobilized in the microbial biomass, contrary to the Kissoko soils, thus answering our second question, namely the higher the microbial Po is, the lower the enzyme-labile Po is in the easily extractable Po fraction. Lastly, by using soils from two sites, exhibiting contrasting levels of available Pi, we demonstrated that Po immobilized in the microbial fraction depends on labile-Pi contents, as soils with the lowest level of bicarbonate-extracted Pi had the highest level of microbial Po, and vice-versa (third question).

Overall, our results highlighted the potential of *A. mangium* for improving growth and P uptake of *Eucalyptus* seedlings resulting from increased Po cycling in the soil. However, the comparison of processes involved in two tropical soils showed that P mobilization by acacia for the growth and P nutrition of *Eucalyptus* is more complex than it seems at first glance. The differences between the two sites clearly showed the merits of undertaking more multi-site studies to gain a better understanding of processes that can be complex, in order to increase P bioavailability for plants. These results are important for forest management, by reinforcing the merits of mixed N-fixing species, such as acacia, but also by recommending rotations with a beneficial effect of soils from acacia plantations on the growth and accumulation of P, together with N, for *Eucalyptus*. Nevertheless, it should be kept in mind that the Po fraction locked into microbial biomass appears to be the main driver for explaining plant P bioavailability increases or decreases after growing N_2-fixing tree species. It would be interesting to estimate microbial P at other sites and we propose comparing the proportion of enzyme-reactive Po to total bicarbonate-extractable Po in intact and autoclaved soils, as this method makes it easy to assess Po immobilization in microbial biomass.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by the French National Research Agency through the “Intens&Fix” project (ANR-10-STRA-004-04). K.W. was supported by a scholarship from the Ministry of Science and Technology, Thailand (2556001073) and Silpakorn University for her PhD project. The work was also supported by the ‘Investments for the future’ program (ANR-10- LABX-04-01) through the use of the Ecotrop platform from CeMEB labEx. The two sites belong to the SOERE F-ORE-T network, which is supported annually by ECOFOR, AllEnvi and the French National Research Infrastructure ANAEE (http://www.anaeefrance.fr). We warmly thank the reviewers and the scientific managing editor for their comments that helped us to improve the manuscript. We should like to thank Lourival Fermiano and Rildo Moreira of the Itatinga Experimental Station (ESALQ-USP), and Tiburce Matsoumbou of the Pointe-Noire Experimental Station (CRDPI) for their technical support for bioassay experiments. We also thank Eder Araujo da Silva (Floragro) and Manon Villeneuve (Eco&Sols) for their technical support for soil sampling. We thank Siobhan Staunton (Eco&Sols) for abundant advice and documents in enzyme research, Jean Trap (Eco&Sols) for his advice for data analysis. We thank Peter Biggins for English editing.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2022.100247.

References

Aleixo, S., Gama-Rodrigues, A.C., Gama-Rodrigues, E.F., Campello, E.F.C., Silva, E.C., Schripsema, J., 2020. Can soil phosphorus availability in tropical forest systems be increased by nitrogen-fixing leguminous trees? Sci. Total Environ. 712, 136405 https://doi.org/10.1016/j.scitotenv.2019.136405.

Ali, M.A., Louche, J., Legname, E., Duchemin, M., Plassard, C., 2009. Pinus pinaster seedlings and their fungal symbionts show high plasticity in phosphorus acquisition in acidic soils. Tree Physiol 29, 1587–1597. https://doi.org/10.1093/treephys/tpp088.

Ali, M.A., Louche, J., Duchemin, M., Plassard, C., 2014. Positive growth response of Pinus pinaster seedlings in soils previously subjected to fertilization and irrigation. For. Ecol. Manage. 318, 62–70. https://doi.org/10.1016/j.foreco.2014.01.006.

Anderson, B.H., Magdoff, F.R., 2005. Autoclaving Soil Samples Affects Algal-Available Phosphorus. J. Environ. Qual. 34, 1958–1963. https://doi.org/10.2134/jeq2005.0024.

Binkley, D., 1997. Bioassays of the influence of *Eucalyptus saligna* and *Albizia falcataria* on soil nutrient supply and limitation. For. Ecol. Manage. 91, 229–234. https://doi.org/10.1016/S0378-1127(96)03861-3.

Binkley, D., Giardina, C., Bashkin, M.A., 2000. Soil phosphorus pools and supply under the influence of *Eucalyptus saligna* and nitrogen-fixing *Albizia facaltaria.* For. Ecol. Manage. 128, 241–247. https://doi.org/10.1016/S0378-1127(99)00138-3.

Binkley, D., Senock, R., Bird, S., Cole, T.G., 2003a. Twenty years of stand development in pure and mixed stands of *Eucalyptus saligna* and nitrogen-fixing *Facaltaria moluccana.* For. Ecol. Manage. 182, 93–102. https://doi.org/10.1016/S0378-1127(03)00028-8.

Binkley, D., Senock, R., Cromack, K., 2003b. Phosphorus limitation on nitrogen fixation by *Facaltaria* seedlings. For. Ecol. Manage. 186, 171–176. https://doi.org/10.1016/S0378-1127(03)00240-8.

Bouillet, J.P., Laclau, J.P., Gonçalves, J.L.M., Moreira, M.Z., Trivelin, P.C.O., Jourdan, C., Silva, E.V, Piccolo, M.C., Tsai, S.M., Galiana, A., 2008. Mixed-species plantations of *Acacia mangium* and *Eucalyptus grandis* in Brazil. 2: Nitrogen accumulation in the stands and biological N_2 fixation. For. Ecol. Manage. 255, 3918–3930. https://doi.org/10.1016/j.foreco.2007.10.050.

Bouillet, J.-P., Laclau, J.-P., Gonçalves, J.L., de, M., Voigtlaender, M., Gava, J.L., Leite, F. P., Hakamada, R., Mareschal, L., Mabiala, A., Tardy, F., Levillain, J., Deleporte, P., Epron, D., Nouvellon, Y., 2013. *Eucalyptus* and Acacia tree growth over entire rotation in single- and mixed-species plantations across five sites in Brazil and Congo. For. Ecol. Manage. 301, 89–101. https://doi.org/10.1016/j.foreco.2012.09.019.

Brookes, P.C., Powlson, D.S., Jenkinson, D.S., 1982. Measurement of microbial biomass phosphorus in soil. Soil Biol. Biochem. 14, 319–329. https://doi.org/10.1016/0038-0717(82)90001-3.

Bünemann, E.K., 2008. Enzyme additions as a tool to assess the potential bioavailability of organically bound nutrients. Soil Biol. Biochem. 40, 2116–2129. https://doi.org/10.1016/j.soilbio.2008.03.001.

Bünemann, E.K., Smernik, R.J., Marschner, P., McNeill, A.M., 2008. Microbial synthesis of organic and condensed forms of phosphorus in acid and calcareous soils. Soil Biol. Biochem. 40, 932–946. https://doi.org/10.1016/j.soilbio.2007.11.012.

Cabreira, W.V., Pereira, M.G., de Carvalho Balieiro, F., Chaer, G., dos Santos, R.N., dos Santos, F.M., da Rocha, P.V., 2022. Soil organic phosphorus (P) increases in mixed plantations of *Acacia mangium* and *Eucalyptus urograndis* in Planosol from Brazil.

Cleveland, C.C., Liptzin, D., 2007. C:N:P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass? Biogeochemistry 85, 235–252. https://doi.org/10.1007/s10533-007-9132-0.

Cossalter, C., Pye-Smith, C., 2003. Fast-wood forestry. Myth. Realities CIFOR, Jakarta, Indones.

DeBell, D., Cole, T., Whitesell, C., 1997. Growth, development, and yield in pure and mixed stands of. Eucalyptus and Albizia. For. Sci. 43, 286–298. https://doi.org/10.1093/forestscience/43.2.286.

Du, E., Terrer, C., Pellegrini, A.F.A., Ahlström, A., van Lissa, C.J., Zhao, X., Xia, N., Wu, X., Jackson, R.B., 2020. Global patterns of terrestrial nitrogen and phosphorus limitation. Nat. Geosci. 13, 221–226. https://doi.org/10.1038/s41561-019-0530-4.

Epron, D., Nouvellon, Y., Mareschal, L., Moreira, R.M.e, Koutika, L.-S., Geneste, B., Delgado-Rojas, J.S., Laclau, J.-P., Sola, G., Gonçalves, J.L., de, M., Bouillet, J.-P., 2013. Partitioning of net primary production in Eucalyptus and Acacia stands and in mixed-species plantations: Two case-studies in contrasting tropical environments. For. Ecol. Manage. 301, 102–111. https://doi.org/10.1016/j.foreco.2012.10.034.

FAO-UNESCO, 1989. Soil Map of the World at 1: 5 000 000, 2nd edition. Food and Agriculture Organization, Rome, Italy, 5000000.

Forrester, D.I., Bauhus, J., Cowie, A.L., Vanclay, J.K., 2006. Mixed-species plantations of Eucalyptus with nitrogen-fixing trees: A review. For. Ecol. Manage. 233, 211–230. https://doi.org/10.1016/j.foreco.2006.05.012.

Forrester, D.I., Bauhus, J., Khanna, P.K., 2004. Growth dynamics in a mixed-species plantation of *Eucalyptus globulus* and *Acacia mearnsii*. For. Ecol. Manage. 193, 81–95. https://doi.org/10.1016/j.foreco.2004.01.024.

Fujii, K., Shibata, M., Kitajima, K., Ichie, T., Kitayama, K., Turner, B.L., 2018. Plant–soil interactions maintain biodiversity and functions of tropical forest ecosystems. Ecol. Res. 33, 149–160. https://doi.org/10.1007/s11284-017-1511-y.

George, T.S., Giles, C.D., Menezes-Blackburn, D., Condron, L.M., Gama-Rodrigues, A.C., Jaisi, D., Lang, F., Neal, A.L., Stutter, M.I., Almeida, D.S., Bol, R., Cabugao, K.G., Celi, L., Cotner, J.B., Feng, G., Goll, D.S., Hallama, M., Krueger, J., Plassard, C., Rosling, A., Darch, T., Fraser, T., Giesler, R., Richardson, A.E., Tamburini, F., Shand, C.A., Lumsdon, D.G., Zhang, H., Blackwell, M.S.A., Wearing, C., Mezeli, M. M., Almås, Å.R., Audette, Y., Bertrand, I., Beyhaut, E., Boitt, G., Bradshaw, N., Brearley, C.A., Bruulsema, T.W., Ciais, P., Cozzolino, V., Duran, P.C., Mora, M.L., de Menezes, A.B., Dodd, R.J., Dunfield, K., Engl, C., Frazão, J.J., Garland, G., González Jiménez, J.L., Graca, J., Granger, S.J., Harrison, A.F., Heuck, C., Hou, E.Q., Johnes, P.J., Kaiser, K., Kjær, H.A., Klumpp, E., Lamb, A.L., Macintosh, K.A., Mackay, E.B., McGrath, J., McIntyre, C., McLaren, T., Mészáros, E., Missong, A., Mooshammer, M., Negrón, C.P., Nelson, L.A., Pfahler, V., Poblete-Grant, P., Randall, M., Seguel, A., Seth, K., Smith, A.C., Smits, M.M., Sobarzo, J.A., Spohn, M., Tawaraya, K., Tibbett, M., Voroney, P., Wallander, H., Wang, L., Wasaki, J., Haygarth, P.M., 2018. Organic phosphorus in the terrestrial environment: a perspective on the state of the art and future priorities. Plant Soil 427, 191–208. https://doi.org/10.1007/s11104-017-3391-x.

Gonçalves, J.L., de, M., Alvares, C.A., Higa, A.R., Silva, L.D., Alfenas, A.C., Stahl, J., Ferraz, S.F., de, B., Lima, W., de, P., Brancalion, P.H.S., Hubner, A., Bouillet, J.-P.D., Laclau, J.-P., Nouvellon, Y., Epron, D., 2013. Integrating genetic and silvicultural strategies to minimize abiotic and biotic constraints in Brazilian eucalypt plantations. For. Ecol. Manage. 301, 6–27. https://doi.org/10.1016/j.foreco.2012.12.030.

Graciano, C., Goya, J.F., Frangi, J.L., Guiamet, J.J., 2006. Fertilization with phosphorus increases soil nitrogen absorption in young plants of Eucalyptus grandis. For. Ecol. Manage. 236, 202–210. https://doi.org/10.1016/j.foreco.2006.09.005.

Hallama, M., Pekrun, C., Pilz, S., Jarosch, K.A., Frąc, M., Uksa, M., Marhan, S., Kandeler, E., 2021. Interactions between cover crops and soil microorganisms increase phosphorus availability in conservation agriculture. Plant Soil 463, 307–328. https://doi.org/10.1007/s11104-021-04897-x.

He, Z., Griffin, T.S., Honeycutt, C.W., 2004. Enzymatic Hydrolysis of Organic Phosphorus in Swine Manure and Soil. J. Environ. Qual. 33, 367–372. https://doi.org/10.2134/jeq2004.3670.

Hinsinger, P., 2001. Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. Plant Soil 237, 173–195. https://doi.org/10.1023/A:1013351617532.

Jarosch, K.A., Kandeler, E., Frossard, E., Bünemann, E.K., 2019. Is the enzymatic hydrolysis of soil organic phosphorus compounds limited by enzyme or substrate availability? Soil Biol. Biochem. 139, 107628 https://doi.org/10.1016/j.soilbio.2019.107628.

Jarosch, K.A., Doolette, A.L., Smernik, R.J., Tamburini, F., Frossard, E., Bünemann, E.K., 2015. Characterisation of soil organic phosphorus in NaOH-EDTA extracts: A comparison of ^{31}P NMR spectroscopy and enzyme addition assays. Soil Biol. Biochem. 91, 298–309. https://doi.org/10.1016/j.soilbio.2015.09.010.

Jenkinson, D.S., Powlson, D.S., 1976. The effects of biocidal treatments on metabolism in soil—I. Fumigation with chloroform. Soil Biol. Biochem. 8, 167–177. https://doi.org/10.1016/0038-0717(76)90001-8.

Joergensen, R.G., Kübler, H., Meyer, B., Wolters, V., 1995. Microbial biomass phosphorus in soils of beech (Fagus sylvatica L.) forests. Biol. Fertil. Soils 19, 215–219. https://doi.org/10.1007/BF00336162.

Koutika, L.-S., Mareschal, L., 2017. Acacia and eucalypt change P, N and C concentrations in POM of Arenosols in the Congolese coastal plains. Geoderma Reg 11, 37–43. https://doi.org/10.1016/j.geodrs.2017.07.009.

Koutika, L.-S., Mareschal, L., Epron, D., 2016. Soil P availability under eucalypt and acacia on Ferralic Arenosols, republic of the Congo. Geoderma Reg 7, 153–158. https://doi.org/10.1016/j.geodrs.2016.03.001.

Laclau, J.P., Bouillet, J.P., Gonçalves, J.L.M., Silva, E.V, Jourdan, C., Cunha, M.C.S., Moreira, M.R., Saint-André, L., Maquère, V., Nouvellon, Y., Ranger, J., 2008. Mixed-species plantations of Acacia mangium and Eucalyptus grandis in Brazil. 1. Growth dynamics and aboveground net primary production. For. Ecol. Manage. 255, 3905–3917. https://doi.org/10.1016/j.foreco.2007.10.049.

Lambilliotte, R., Cooke, R., Samson, D., Fizames, C., Gaymard, F., Plassard, C., Tatry, M. V, Berger, C., Laudie, M., Legeai, F., Karsenty, E., Delseny, M., Zimmermann, S., Sentenac, H., 2004. Large-scale identification of genes in the fungus *Hebeloma cylindrosporum* paves the way to molecular analyses of ectomycorrhizal symbiosis. NEW Phytol 164, 505–513. https://doi.org/10.1111/j.1469-8137.2004.01185.x.

Le Cadre, E.Le, Kinkondi, M., Koutika, L.-S., Epron, D., Mareschal, L., 2018. Anionic exchange membranes, a promising tool to measure distribution of soil nutrients in tropical multispecific plantations. Ecol. Indic. 94, 254–256. https://doi.org/10.1016/j.ecolind.2018.06.041.

Legendre, P., Legendre, L., 1998. Numerical Ecology (2nd English Edn. Elsevier Science BV, Amsterdam, The Netherlands.

Louche, J., Ali, M.A., Cloutier-Hurteau, B., Sauvage, F.-X., Quiquampoix, H., Plassard, C., 2010. Efficiency of acid phosphatases secreted from the ectomycorrhizal fungus *Hebeloma cylindrosporum* to hydrolyse organic phosphorus in podzols. FEMS Microbiol. Ecol. 73, 323–335. https://doi.org/10.1111/j.1574-6941.2010.00899.x.

Maquere., V., 2008. Dynamics of mineral elements under a fast-growing eucalyptus plantation in Brazil. Implications for soil sustainability. Thesis, Silviculture, forestry, AgroParisTech. pp. 375.

McDonald, M.S., 1978. A Simple and Improved Method for the Determination of Microgram Quantities of Nitrogen in Plant Material. Ann. Bot. 42, 363–366. https://doi.org/10.1093/oxfordjournals.aob.a085468.

McLaughlin, M.J., Alston, A.M., Martin, J.K., 1986. Measurement of phosphorus in the soil microbial biomass: A modified procedure for field soils. Soil Biol. Biochem. 18, 437–443. https://doi.org/10.1016/0038-0717(86)90050-7.

Ohno, T., Zibilske, L.M., 1991. Determination of low concentrations of phosphorus in soil extracts using malachite green. Soil Sci. Soc. Am. J. 55, 892–895. https://doi.org/10.2136/sssaj1991.03615995005500030046x.

Oliveira, I.R., Bordron, B., Laclau, J.P., Paula, R.R., Ferraz, A.V, Gonçalves, J.L.M., le Maire, G., Bouillet, J.P., 2021. Nutrient deficiency enhances the rate of short-term belowground transfer of nitrogen from *Acacia mangium* to *Eucalyptus* trees in mixed-species plantations. For. Ecol. Manage. 491, 119192 https://doi.org/10.1016/j.foreco.2021.119192.

Olsen, S.R., Cole, C., Watanabe, F.S., Dean, L.A., 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. U.S. Dept. of Agriculture, Washington,D.C.

Paula, R.R., Bouillet, J.-P., Goncalves, J.L.de M., Trivelin, P.C.O., Balieiro, F.de C., Nouvellon, Y., Oliveira, J.de C., de Deus Junior, J.C., Bordron, B., Laclau, J.-P., 2018. Nitrogen fixation rate of *Acacia mangium* Wild at mid rotation in Brazil is higher in mixed plantations with *Eucalyptus grandis* Hill ex Maiden than in monocultures. Ann. For. Sci. 75 https://doi.org/10.1007/s13595-018-0695-9.

Pereira, A.P., de, A., Santana, M.C., Zagatto, M.R.G., Brandani, C.B., Wang, J.-T., Verma, J.P., Singh, B.K., Cardoso, E.J.B.N., 2021. Nitrogen-fixing trees in mixed forest systems regulate the ecology of fungal community and phosphorus cycling. Sci. Total Environ. 758, 143711 https://doi.org/10.1016/j.scitotenv.2020.143711.

Pistocchi, C., Mészáros, É., Tamburini, F., Frossard, E., Bünemann, E.K., 2018. Biological processes dominate phosphorus dynamics under low phosphorus availability in organic horizons of temperate forest soils. Soil Biol. Biochem. 126, 64–75. https://doi.org/10.1016/j.soilbio.2018.08.013.

Plassard, C., Dell, B., 2010. Phosphorus nutrition of mycorrhizal trees. Tree Physiol 30, 1129–1139. https://doi.org/10.1093/treephys/tpq063.

Plassard, C., Louche, J., Ali, M.A., Duchemin, M., Legname, E., Cloutier-Hurteau, B., 2011. Diversity in phosphorus mobilisation and uptake in ectomycorrhizal fungi. Ann. For. Sci. 68, 33–43. https://doi.org/10.1007/s13595-010-0005-7.

Ragon, M., Neugnot-Roux, V., Chemardin, P., Moulin, G., Boze, H., 2008. Molecular gene cloning and overexpression of the phytase from *Debaryomyces castellii* CBS 2923. Protein Expr. Purif. 58, 275–283. https://doi.org/10.1016/j.pep.2007.12.003.

R Core Team, 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL. https://www.R-project.org/.

Richards, A.E., Forrester, D.I., Bauhus, J., Scherer-Lorenzen, M., 2010. The influence of mixed tree plantations on the nutrition of individual species: a review. Tree Physiol 30, 1192–1208. https://doi.org/10.1093/treephys/tpq035.

Robin, A., Pradier, C., Sanguin, H., Mahé, F., Lambais, G.R., de Araujo Pereira, A.P., Germon, A., Santana, M.C., Tisseyre, P., Pablo, A.-L., Heuillard, P., Sauvadet, M., Bouillet, J.-P., Andreote, F.D., Plassard, C., de Moraes Gonçalves, J.L., Cardoso, E.J. B.N., Laclau, J.-P., Hinsinger, P., Jourdan, C., 2019. How deep can ectomycorrhizas go? A case study on Pisolithus down to 4 meters in a Brazilian eucalypt plantation. Mycorrhiza 29, 637–648. https://doi.org/10.1007/s00572-019-00917-y.

Santos, F.M., Chaer, G.M., Diniz, A.R., Balieiro, F., de, C., 2017. Nutrient cycling over five years of mixed-species plantations of Eucalyptus and Acacia on a sandy tropical soil. For. Ecol. Manage. 384, 110–121. https://doi.org/10.1016/j.foreco.2016.10.041.

Sauvadet, M., Trap, J., Damour, G., Plassard, C., Van den Meersche, K., Achard, R., Allinne, C., Autfray, P., Bertrand, I., Blanchart, E., Deberdt, P., Enock, S., Essobo, J.-D., Freschet, G.T., Hedde, M., de Melo, Virginio, Filho, E., Rabary, B., Rakotoarivelo, M., Randriamanantsoa, R., Rhino, B., Ripoche, A., Rosalie, E., Saj, S., Becquer, T., Tixier, P., Harmand, J.-M., 2021. Agroecosystem diversification with legumes or non-legumes improves differently soil fertility according to soil type. Sci. Total Environ. 795, 148934 https://doi.org/10.1016/j.scitotenv.2021.148934.

Serrasolses, I., Romanyà, J., Khanna, P.K., 2008. Effects of heating and autoclaving on sorption and desorption of phosphorus in some forest soils. Biol. Fertil. Soils 44, 1063–1072. https://doi.org/10.1007/s00374-008-0301-7.

Sinegani, A.A.S., Hosseinpur, A., 2010. Evaluation of effect of different sterilization methods on soil biomass phosphorus extracted with $NaHCO_3$. Plant, Soil Environ. 56, 156–162.

Solaiman, Z., 2007. Measurement of Microbial Biomass and Activity in Soil. Adv. Tech. Soil Microbiol. 201–211. https://doi.org/10.1007/978-3-540-70865-0_13.

Tabatabai, M.A., Bremner, J.M., 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1, 301–307. https://doi.org/10.1016/0038-0717(69)90012-1.

Tchichelle, S.V., Mareschal, L., Koutika, L.-S., Epron, D., 2017. Biomass production, nitrogen accumulation and symbiotic nitrogen fixation in a mixed-species plantation of eucalypt and acacia on a nutrient-poor tropical soil. For. Ecol. Manage. 403, 103–111. https://doi.org/10.1016/j.foreco.2017.07.041.

Tng, D.Y.P., Janos, D.P., Jordan, G.J., Weber, E., Bowman, D.M.J.S., 2014. Phosphorus limits *Eucalyptus grandis* seedling growth in an unburnt rain forest soil. Front. Plant Sci. https://doi.org/10.3389/fpls.2014.00527 https://www.frontiersin.org/article/.

Versini, A., 2012. Effet de la manipulation des litières aériennes sur les cycles du C et de N dans les sols en plantation d'Eucalyptus au Congo. Thesis Docteur de l'université de Lorraine En Géosciences 221. CIRAD.

Waithaisong, K., Robin, A., Mareschal, L., Bouillet, J.-P., Laclau, J.-P., Deleporte, P., Gonçalves, J.L.M., Harmand, J.-M., Plassard, C., 2020. Introducing N2-fixing trees (Acacia mangium) in eucalypt plantations rapidly modifies the pools of organic P and low molecular weight organic acids in tropical soils. Sci. Total Environ. 140535 https://doi.org/10.1016/j.scitotenv.2020.140535.

Wan Juliana, W.A., Burslem, D.F.R.P., Swaine, M.D., 2009. Nutrient limitation of seedling growth on contrasting soils from Pasoh Forest Reserve, Peninsular Malaysia. J. Trop. For. Sci. 316–327.

Wyngaard, N., Cabrera, M.L., Jarosch, K.A., Bünemann, E.K., 2016. Phosphorus in the coarse soil fraction is related to soil organic phosphorus mineralization measured by isotopic dilution. Soil Biol. Biochem. 96, 107–118. https://doi.org/10.1016/j.soilbio.2016.01.022.

Supplementary data

Table S1: P concentrations (mg P/100 mg dwt) measured in roots, stems and leaves of eucalypts grown in pots containing intact soils collected from topsoil under different original vegetation, namely acacia (Ac), *Eucalyptus* (Euc) and native vegetation (Nat) at Itatinga or at Kissoko. Each value is the mean (n = 6 at Itatinga, n = 10 at Kissoko) with standard deviation. For each organ and each parameter, significant differences between vegetation origins were analyzed with a one-way ANOVA, followed by a Tukey-HSD post-hoc test, with means with different letters being significantly different ($p < 0.05$). The significance of the associated *p*-value of the one-way ANOVA is reported as follows: *NS* ($p>0.05$), ** ($p<0.01$), *** ($p<0.001$).

		Original vegetation			
Site	Organ	Ac	Euc	Nat	*p*
Itatinga	Roots	$0.041^{b} \pm 0.005$	$0.073^{a} \pm 0.006$	$0.063^{a} \pm 0.015$	***
	Stem	$0.168^{b} \pm 0.078$	$0.302^{a} \pm 0.067$	$0.158^{b} \pm 0.071$	**
	Leaves	$0.0117^{a} \pm 0.023$	$0.0127^{a} \pm 0.015$	$0.108^{a} \pm 0.028$	*NS*
Kissoko	Roots	$0.083^{b} \pm 0.024$	$0.119^{a} \pm 0.022$	$0.123^{a} \pm 0.034$	***
	Stem	$0.209^{b} \pm 0.049$	$0.294^{a} \pm 0.030$	$0.195^{b} \pm 0.071$	***
	Leaves	$0.119^{b} \pm 0.017$	$0.197^{a} \pm 0.060$	$0.138^{b} \pm 0.031$	***

Supporting Information S1

Production of the recombinant acid phosphatase HcPhoA from the ectomycorrhizal fungus *Hebeloma cylindrosporum*

Chemicals, enzymes and vectors for molecular biology

All products used to obtain the recombinant HcPhoA enzyme were from Invitrogen™ and were purchased from Thermofisher Scientific. Those products were the Platinum® taq DNA polymerase high fidelity, the TA Cloning™ Kit containing the ExpressLink™ T4 DNA ligase, the pCR™2.1 Vector and One Shot™ TOP10F' Chemically Competent *E. coli* used to perform the initial cloning of the full length *HcPhoA* cDNA. Then, the transformation of *Pichia pastoris*, strain GS115 (his4) that was provided by UMR IATE (Montpellier, France) was carried out using the expression vector pPIC9K. Transformants were first screened using the antibiotic geneticin® (G418 Sulfate). The media for regeneration (regeneration dextrose base, RDB) and selection of the best candidates for HcPhoA production (buffered minimal glycerol, BMG and buffered minimal methanol, BMM) were prepared according to the manual of the Pichia Expression kit (Invitrogen™).

Cloning of *HcPhoA* full-length cDNA

The complete cDNA of the *HcPhoA* gene (genbank accession number FR692330) was cloned from *H. cylindrosporum* cDNA library (Lambilliotte et al., 2004). Alignment of predicted amino-acid sequences of genes encoding AcPases secreted by various organisms (Bernard et al., 2002) was used to identify conserved regions among these polypeptides. We chose the region DYRVHNPL that is located roughly in the central part of the predicted acid phosphatase from *Aspergillus fumigatus* to design degenerated primers. They were named ACPmid forward (5'-GAYTAYGTNMCNAARCAYAAYCC-3') and ACPmid reverse (5'GGRTTRTGYTTICKIACRTARTC-3'). Specific primers from the yeast expression vector pFL61 used to construct the cDNA library (Lambilliotte et al., 2004) were designed (L1: 5'-CTTCTAACCAAGGGGTGGTTTAGTTTAG-3' and R1: 5'-CTGCATAAAGCCATTAAA AAGA GCG-3'). The amplification of 5' (L1/ACPmid rev) and 3' (R1/ACPmid forward) parts of *HcphoA* was performed with the Platinum taq DNA polymerase on 10 ng of cDNA library in a final PCR volume of 20 µl using a 30-cycle amplification program of 94°C for 30 sec, 56°C for 45 sec, and 72°C for 1.3 min, with a first denaturation step at 94°C for 10 min

and a final polyadenylation step at 72°C for 10 min. The amplified fragments were then cloned into the pCR™2.1 Vector and sequenced by genewiz company (https://www.genewiz.com). A new primer corresponding to the 3' extremity of the gene was designed (PhoA1169rev: 5'-GTTCTTCCACAGGGGTGTTGAAT-3') and used to amplify the complete cDNA using L1/PhoA1169rev primers as described above. The new cDNA sequence was verified and analyzed with the SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/) which predicted a peptide signal of 19 amino acids.

Construction of the pPIC9K-*HcPhoA* vector and recombinant enzyme production

The sequence encoding the mature HcPhoA without its peptide signal was then amplified by PCR using a forward primer (5'-ACATACGTAGGGAATAATGAAGATAATGGTC-3') starting from amino acid 20 and containing a SnaBI site and a reverse primer (5'-AAAGCG GCCGCTCAACACCCCTGTGGAAG-3') containing the *HcPhoA* stop codon immediately followed by a NotI site from the complete cDNA of the *HcPhoA* gene. The PCR products were cloned into the pCR™2.1 Vector and verified by sequencing as previously. The plasmid pCR™2.1-*HcPhoA* was digested with SnaBI and NotI to recover the 1.115-bp fragment that was ligated to the pPIC9K vector digested with the same enzymes. The vector and the insert were purified after electrophoresis run on 1.5 % agarose gel and ligated together using the T4-DNA ligase (https://www.thermofisher.com) following the manufacturer's instructions. The sequence of the new pPIC9K-*HcPhoA* expression vector was verified before its digestion with SacI. The SacI-digested pPIC9K-*HcPhoA* DNA was used to transform the host strain GS 115 (his4) by electroporation as described in Ragon et al. (2008). Transformants were selected on agar plates lacking histidine (RDB medium) to reveal the integration of pPIC9K-HcPhoA (His selection). Among the positive clones, 95 were randomly selected to screen those exhibiting the greatest tolerance to geneticin®, supplied at concentrations ranging from 0.25 to 3.0 mg ml^{-1}. Three clones able to grow at the highest concentration of geneticin® were chosen randomly to measure their release of acid Pase into the culture medium after methanol induction according to the manual of the *Pichia* Expression kit. Finally, the best transformant was chosen for the production of the recombinant acid phosphatase from *H. cylindrosporum* from methanol induction according to the method described in Ragon et al. (2008). After 7 days of methanol induction, we obtained around 170 ml of dialyzed filtrated and concentrated enzyme solution with a protein concentration of 7 mg l^{-1}.

References

Lambilliotte, R., Cooke, R., Samson, D., Fizames, C., Gaymard, F., Plassard, C., Tatry, M. V, Berger, C., Laudie, M., Legeai, F., Karsenty, E., Delseny, M., Zimmermann, S., Sentenac, H., 2004. Large-scale identification of genes in the fungus *Hebeloma cylindrosporum* paves the way to molecular analyses of ectomycorrhizal symbiosis. NEW Phytol. 164, 505–513. https://doi.org/10.1111/j.1469-8137.2004.01185.x

Ragon, M., Neugnot-Roux, V., Chemardin, P., Moulin, G., Boze, H., 2008. Molecular gene cloning and overexpression of the phytase from *Debaryomyees castellii* CBS 2923. Protein Expr. Purif. 58, 275–283. https://doi.org/10.1016/j.pep.2007.12.003