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Responses of C-, N- and P-acquiring hydrolases to P and N fertilizers in a subtropical Chinese fir plantation depend on soil depth

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ABSTRACT

Large amounts of carbon (C), nitrogen (N) and phosphorus (P) in soil are stored in forms that are not bioavailable unless they are modified by hydrolytic enzymes. The activity of hydrolases is affected directly and indirectly by fertilizers treated, but the effects depend on soil depth. We collected soil core (0–80 cm depth) from fertilized and unfertilized (control) plots in a subtropical Chinese fir plantation. The soil with fertilizers had been treated for five years with either N (50 kg N ha^{-1} yr^{-1}), P (50 kg P ha^{-1} yr^{-1}) or N and P together. The kinetics of three hydrolases (β-1,4-glucosidase (βGluc), β-1,4-*N*-acetylglucosaminidase (NAG) and acid phosphatase (Phos)) were measured, and their potential activities (V_{max}), half-saturation constants (K_m), and enzyme efficiencies (V_{max}/K_m) were calculated for 0–10 cm, 10–20 cm, 20–40 cm, 40–60 cm, and 60–80 cm soil depth increments. The effect of depth on enzyme kinetics was greater than fertilizers treated. Smaller soil organic carbon (SOC) contents were related to the stable efficiencies of NAG and Phos from 0 to 60 cm in the control plots. In general, decreases in SOC content with depth triggered the production of enzymes with low K_m (i.e. high substrate affinity). The minimum and maximum decreases in the K_m of βGluc, NAG and Phos were 0%, 22% and 64% in the top 20 cm and 30%, 54% and 79% at 80 cm depth, respectively, compared to 0–10 cm soil depth. Nitrogen fertilizer downregulated NAG production without changing K_m with soil depth, leading to decreased V_{max} and V_{max}/K_m compared to the control. Responses of Phos to P and NP fertilizers depended on soil organic matter (SOM) and available P contents. Relative to the control, the Phos V_{max} decreased in the top 10 cm, increased between 10 and 40 cm, and lack of difference between 40 and 80 cm in the P- and NP-treated soils. The V_{max} and V_{max}/K_m of βGluc increased by P fertilizers between 60 and 80 cm compared to the control, indicating microbial mobilization of P from SOM to meet energy and C requirements in nutrient-poor subsoil. We conclude that limitations in microbial activity caused by decreases in SOM with depth with N and P fertilizers Chinese fir plantations are offset by the production of more efficient enzymes. However, longer term management to build SOM in tree plantations is required to prevent further soil degradation and loss of productivity.

1. Introduction

The biological availability of carbon (C), nitrogen (N) and phosphorus (P) stocks in subsoils is limited and largely controlled by the activities of hydrolytic enzymes (Dungait et al., 2012; Kautz et al., 2013; Stone et al., 2014; Xu et al., 2013). Potential enzyme activities

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(V_{max}) generally decrease with soil depth in parallel to microbial biomass, soil organic carbon (SOC) and nutrient contents (Parvin et al., 2018; Stone et al., 2014). The application of fertilizers (N and P) affects the activities of hydrolases directly and indirectly, and the effects vary with soil depth (Liu and Greaver, 2010; Loeppmann et al., 2016; Parvin et al., 2018; Xu et al., 2013). It is proposed that N or P fertilizers offsets the limited availability of SOC and nutrients to microorganisms in subsoils, and decreases microbial production of enzymes, especially under P-deficient conditions in tropical ecosystems (Camenzind et al., 2018).

In tropical forest soils, microorganisms and plants are most often limited by P (Maranguit et al., 2017). Soil microbes and roots synthesize extracellular phosphatase to acquire P from soil organic matter (SOM) if the N supply increases (Marklein and Houlton, 2012), but this activity may decrease in tropical forest soils if P is added (Zhang et al., 2018). Phosphorous limitations are intensified in soils suffering from acidification caused by profligate N fertilizers (Deng et al., 2017; Li et al., 2016; Zamanian et al., 2018; Zamanian and Kuzyakov, 2019). In lateritic tropical and sub-tropical soils aluminium (Al^{3+}) and ferrous/ferric ($Fe^{2+/3+}$) cations released by acidification bind strongly to phosphate anions (PO_4^{3-}) further constraining microbial activity already challenged by low pH (Deng et al., 2017; Tian and Niu, 2015; Vitousek et al., 2010; Zhang et al., 2017; Zhou et al., 2018).

Enzymes regulate SOM decomposition both by their activities and their substrate affinity (Alvarez et al., 2018). The enzyme potential activity (V_{max}) and its half-saturation constant (K_m) can be calculated using the Michaelis-Menten equation, $V = (V_{max} \cdot [S]) / (K_m + [S])$, where [S] is the substrate concentration, V_{max} represents the maximum potential activities, and K_m is the substrate concentration at 1/2 V_{max} (Stone et al., 2012). Value of K_m is a constant that describes an individual enzyme and represents the quality of the enzyme pools (Alvarez et al., 2018; Stone et al., 2012). The ratio of V_{max} to K_m (V_{max}/K_m) is defined as the catalytic efficiency, high values of which indicate higher enzyme performance (Razavi et al., 2016; Stone and Plante, 2014). In tropical forest soils, the V_{max}/K_m of acid phosphatase (Phos) is thought to remain stable with depth because of increases in substrate affinity and decreases in activity. We therefore predict that the catalytic efficiency should also remain stable as nutrients decrease with depth in the soil profile.

In general, the V_{max} responses to N or P fertilizers are consistent with the microbial economic theory (Jian et al., 2016; Marklein and Houlton, 2012; Xiao et al., 2018), which states that microorganisms preferentially allocate resource-procuring enzymes to the most limited nutrients (Burns et al., 2013; Sinsabaugh et al., 2008). However, the responses in tropical and temperate forest soils have not always been consistent with expectations. For example, the V_{max}/K_m of Phos remained stable in temperate and subtropical forest N-treated soils because of corresponding increases in the V_{max} and K_m (Zhang et al., 2018). Furthermore, the Phos kinetic parameters in temperate and subtropical soils responded differently to applications of P and both N and P; i.e., the V_{max}/K_m remained stable in a temperate forest soil, but decreased in a subtropical soil, when P and both N and P were added (Zhang et al., 2018). The V_{max} of β-1,4-*N*-acetylglucosaminidase (NAG) in an N-limited temperate forest soil increased when N was added, and the V_{max}/K_m of C-acquiring enzymes also increased as the V_{max} increased and the K_m decreased (Saiya-Cork et al., 2002). The V_{max}/K_m of NAG in temperate forest soils increased when N was added because of decreases in the K_m (Stone et al., 2012). Similarly, the V_{max} of Phos was stimulated when N or P were added to a P-limited topsoil in a subtropical forest (Camenzind et al., 2018; Dong et al., 2015). These results show that it is difficult to make reliable predictions of how fertilizers treated might affect resource-limited soils, as the hydrolase activities may increase unexpectedly (Blagodatskaya and Kuzyakov, 2008).

Timber production in Chinese fir (*Cunninghamia lanceolate*) plantations on the lateritic soils in subtropical regions has been progressively limited by poor management driving the loss of soil fertility with subsequent recommendations for remedial fertilizers (N and P) treated (Zhang et al., 2017). This has driven the requirement for the understanding of the effect of fertilizer management on biogeochemical cycling in deep soils pertinent to the root depths of trees, previously described for subsoils (< 1.0 m) in temperate grasslands and arable cropping systems (Beniston et al., 2014; Gregory et al., 2016). However, to date, there have been very few studies about how long-term N and P fertilizers regulate the activities of C-, N- or P-acquiring enzymes at different depths in tropical and subtropical forest soils.

In this study, we aim to explore how the enzyme kinetics of Phos, β-1,4-glucosidase (βGluc), and NAG (V_{max}, K_m and V_{max}/K_m) in topsoil and subsoil changed in response to N and P fertilizers. This investigation used an experimental area in a subtropical forest that had been treated with fertilizers (N, P or N and P) for 5 years. We hypothesized that (1) the V_{max}/K_m of C-, N- and P-acquiring hydrolases would remain stable with soil depth because of corresponding decreases in the V_{max} and K_m; and, (2) in agreement with the microbial economic theory, that microorganisms would allocate resources to produce efficient hydrolase to meet the demand for limiting nutrients.

2. Materials and methods

2.1. Study site

The experimental site was established in 2012 in the Shixi Chinese fir plantation (115°04′13″E, 26°44′52″N) in Taihe County, Jiangxi Province, China. The plantation is in a hilly region and the trees were planted in 2000. The area has a subtropical monsoon climate and has a mean annual temperature of 17.9 °C and a mean annual precipitation of 1475 mm (Wen et al., 2010). The soil is classified as a Dystrudepts Udepts Inceptisol, according to the US Soil Taxonomy (Soil Survey Staff, 2010). The soil is naturally acidic and has a pH of 4.6. The soil is P-limited, and the total P content in the top 10 cm is about 0.1 g P kg^{-1} (Dong et al., 2015).

2.2. Experimental design

Twelve plots, comprising 4 treatments with 3 replicates, were established in March 2012 using a completely randomized block design. The treatments were N (50 kg N ha^{-1} yr^{-1}), P (50 kg P ha^{-1} yr^{-1}), NP (50 kg N ha^{-1} yr^{-1} + 50 kg P ha^{-1} yr^{-1}), and control (no N or P). The plots measured 20 × 20 m and were separated by buffers of no < 10 m. Nitrogen and P were added as NH_4NO_3 and NaH_2PO_4 and the total annual application was split into four, with 30%, 30%, 20%, and 20% added in March, June, September, and December, respectively. The understory plants were manually removed every year to ensure they had no effect on the Chinese fir growth.

2.3. Soil sampling and analysis

Soil samples were collected from depths of 0–10 cm, 10–20 cm, 20–40 cm, 40–60 cm and 60–80 cm in April 2016. Five soil cores to a depth of 80 cm were collected in each plot and divided into depth increments. The different depth increments from each plot were mixed to provide one composite sample for each depth for each plot, providing a total of 60 soil samples for analysis. The samples were immediately transferred to the laboratory, where they were sieved through a 2-mm mesh and stored at 4 °C. The soil pH was measured using a digital pH meter (at a ratio of 10 g soil to 25 ml CO_2-free distilled water). The soil ammonium (NH_4^+) and nitrate (NO_3^-) contents were measured by continuous flow analysis (Auto Analyzer 3, Bran Lubbe, Germany) after extraction with 1 mol L^{-1} KCl (1:10 g/g). Soil dissolved organic carbon (DOC) was determined with an element analyzer (Liquid TOC *II*, Elementar, Germany) after extraction with distilled water (1:5 g/g). Soil available P and total P were measured by continuous flow analysis (Auto Analyzer 3, Bran Luebbe, Germany) after extraction with

0.03 mol L^{-1} NH_4F and digestion with 0.025 mol L^{-1} HCl and H_2SO_4–$HClO_4$, respectively. Soil total N and organic C (SOC) were determined using a CN analyzer (Vario $_{MAX}$, Elementar, Germany).

2.4. Enzyme kinetics assays

Acid phosphatase, βGluc, and NAG, were measured following the methods of Saiya-Cork et al. (2002) with 4-MUB-β-D-glucoside, 4-MUB-*N*-acetyl-β-D-glucosaminide, and 4-MUB-phosphate as the substrates. Briefly, fresh soil (1 g) was mixed evenly with 125 ml of 50 mmol·L^{-1} sodium acetate buffer (pH = 4.5). Homogenate (200 μl) and substrate (50 μl) were added to 96-well black microplates and incubated at 20 °C for 4 h in the dark. Fluorescence values were read by a microplate fluorometer (Synergy H4, BioTek) with excitation and emission filters at 365 and 450 nm, respectively. Each sample had 8 replicates. We calculated the enzyme activities as the rate of substrate decomposition (nmol g^{-1} h^{-1}).

Each enzyme was measured under 8 substrate concentrations and the V_{max} and K_m of βGluc, NAG, and Phos were calculated by fitting to the Michaelis-Menten equation, $V = (V_{max}\cdot[S]) / (K_m + [S])$, using non-linear regression, where [S] is the substrate concentration, V_{max} represents the potential activities under saturated substrate concentrations, and K_m is the substrate concentration at 1/2 V_{max} and represents the affinity of the enzyme to the substrate. The substrate concentration gradients for βGluc, NAG, and Phos were in the order of 5, 10, 25, 50, 100, 150 and 200 μmol L^{-1}. We also calculated the enzyme efficiency, the ratio of V_{max} to K_m (Stone et al., 2012).

2.5. Statistical analyses

Data for the soil properties and enzyme kinetics followed the normal distribution at each individual depth. The differences between the effects of the fertilizers treated or depths and their interactions on enzyme kinetic parameters were tested by two factor analysis of variance (ANOVA) and Turkeys Test. The relationships between the enzyme kinetic parameters, soil properties, and the net changes in the Phos V_{max} were determined by fitting exponential and linear equations in SigmaPlot 10.0 software. All statistical analyses were performed in SPSS Statistics 19.0. The figures were drawn in SigmaPlot. The values in the figures are the means ± standard errors. We applied a significance level of $p < 0.05$.

3. Results

3.1. Soil chemical properties

Soil total and available C, N and P contents (but not the NO_3^- content) decreased with depth in all treatments (Fig. 1 and Table S1). The total C, N and P contents were not affected by fertilizers treated ($p > 0.05$, Table S1). The NO_3^- contents were higher in the N, P and NP soils than in the control ($p < 0.05$) and were between 33% to 203% higher throughout the profile. The NH_4^+ contents were also between 4% and 104% higher between 10 and 80 cm in the N and NP soils than in the control. The available P contents in the top 10 cm were higher in the P (46 mg kg^{-1}) and NP (99 mg kg^{-1}) soils than in the control but were about 1.5 and 2.4 mg kg^{-1} higher between 20 and 80 cm, which indicates that the majority of P was immobilized in the topsoil (Fig. 1). The DOC contents were between 15% and 54% higher from 0 to 40 cm in the N, P or combined N with P soils than in the control. The pH in the control soils ranged from 4.2 to 4.5 and increased by 0.1 and 0.3 units in the soil with P and N fertilizers (Fig. 1).

3.2. Enzyme kinetic parameters

In the control soils, the V_{max}/K_m of NAG and Phos did not vary significantly between 0 and 60 cm because both the V_{max} and K_m decreased with depth (Fig. 2a, b). The V_{max}/K_m of βGluc was lower between 10 and 80 cm because the decrease in the V_{max} was greater than the decrease in the K_m (Fig. 2b). The V_{max} and K_m of Phos from 0 to 10 cm were higher in the N soils, and lower in the P and NP soils than in the control. The V_{max}/K_m of Phos from 0 to 80 cm in the N and/or P soils did not vary significantly because of the synchronous change of V_{max} and K_m (Fig. 2a). The V_{max}/K_m of βGluc increased in the N and NP soils from 10 to 80 cm deep, reflecting an increase in the V_{max} and a decrease in the K_m (Fig. 2b). The V_{max}/K_m of NAG decreased in the N and NP soils with depths between 10 and 80 cm where the V_{max} decreased and the K_m did not vary significantly (Fig. 2c). Relative to the control soils, the V_{max}/K_m of βGluc and NAG did not vary significantly in the 0–10 cm layer in the P soils, reflecting increases in both the V_{max} and K_m (Fig. 2b, c).

3.3. Relationships between the soil chemical properties and enzyme kinetic parameters

The V_{max} and K_m of βGluc, Phos and NAG increased with increases in the soil pH, and the total and available C, N and P contents (Table S2). In the control soils, the V_{max}/K_m of Phos, βGluc and NAG increased as the SOC increased (Fig. 3, $p < 0.05$). In the N and P soils, the lnV_{max}/K_m of βGluc decreased as the lnSOC decreased, and the lnV_{max}/K_m of Phos fitted to a saturation curve with lnSOC at all depths ($p < 0.01$, Fig. 4a). The lnV_{max} and lnV_{max}/K_m of NAG increased as the $ln(NH_4^+ + NO_3^-)$ increased (Fig. 4b). Between 40 and 80 cm deep, the V_{max} of βGluc increased as the available P contents increased (Fig. 4b). The net change in the Phos V_{max} was decreased with increase in available P ($p < 0.01$, Fig. 5).

4. Discussion

Soil depth had a greater influence on enzyme activities than N and/or P fertilizers treated which suggests that the microbial enzyme production was controlled by resource availability (Fig. 1), also indicated by the positive relationships between SOC and the V_{max}/K_m of all three enzymes (Fig. 3, 4a). Enzyme activities are determined by the size of the enzyme pool and the enzyme quality (low K_m, Alvarez et al., 2018). The K_m of all three enzymes decreased with depth in the control plots (Fig. 2a, b, c), consistent with our first hypothesis, that high quality (low K_m) enzymes were produced in the subsoil due to the nutrient stress imposed on microbial communities by a change from eutrophic to oligotrophic conditions (Eilers et al., 2012; Sinsabaugh et al., 2014).

The net changes in the Phos response were related to the available P ($p < 0.01$, Fig. 5). Although the microbial response to fertilizers treated may have been obscured by mineral-related protection of enzymes and organics (Nannipieri et al., 2018), the close relationships imply that shifts in the microbial community were responsible for the enzymes' response to N and P fertilizers with depth. In the control soils, the V_{max}/K_m of NAG and Phos did not vary significantly up to 60 cm depth. Consequently, microbes appear to have maintained catalytic efficiency across the resource gradient, and the observed V_{max} decreases with depth of all three enzymes were caused by the smaller microbial biomass in the subsoil (Agnelli et al., 2004; Xu et al., 2013).

Based on the nutrient contents and enzymes' distribution, we defined two soil layers as either (1) nutrient-rich but stoichiometrically imbalanced topsoil (0 to 40 cm), which is P-limited, and (2) oligotrophic subsoil (40 to 80 cm). The responses of all three enzymes to N and P fertilizers were depth-specific. For example, the available N increased at all depths after N fertilizers treated because of NO_3^- leaching (Fig. 1). In agreement with our second hypothesis and microbial economic theory, the V_{max} of βGluc increased (Fig. 2b), but that of NAG decreased, in the soil when N was leached between 0 and 80 cm (Fig. 2c). However, N fertilizers caused a similar NAG quality to the control, improved that of βGluc. Consequently, N fertilizers increased the catalytic efficiency of βGluc, but decreased that of NAG. As C is an

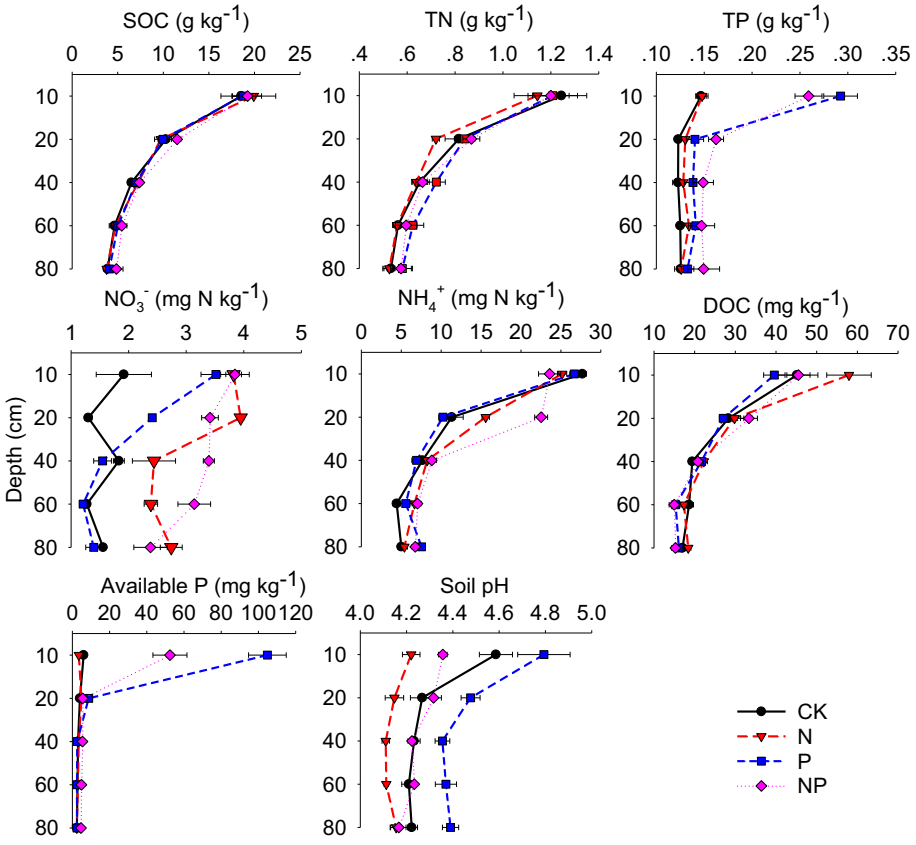

Fig. 1. Effects of N and P fertilization (N, P or NP) on total and available nutrient contents, dissolved organic C (DOC) and pH depending on soil depths. SOC: soil organic carbon; TN: total nitrogen; TP: total phosphorus; NO_3^-: nitrate-N contents; NH_4^+: ammonium-N; available P: available phosphorus; soil pH: soil acidity. The same abbreviations are used in Figs. 3–5.

energy resource and N is an essential element for enzymes, more energy was allocated to meet the microbial demand for C in resource-poor subsoils (Hobbie and Hobbie, 2013). Because the proportion of N in the soil increased with depth, the N-acquiring microbial communities produced low quality N-acquiring enzymes. Nitrogen fertilizers lowered the soil pH approximately 0.5 units than the control (Fig. 1) as a results of acidification caused by N fertilizer (Zamanian et al., 2018) that inhibited enzyme activities, presumably due in part to general toxic effects of solubilized Al^{3+} (Tian and Niu, 2015) and binding of PO_4^{3-} anions to Al^{3+} and Fe^{3+} mentioned previously (Vitousek et al., 2010).

In agreement with microbial economic theory and the results of similar studies, the V_{max} and K_m of Phos decreased by the P and the NP fertilizers in the top 10 cm compared to control because the available P contents were correspondingly increased (Figs. 1 & 2a, Jian et al., 2016; Marklein and Houlton, 2012; Xiao et al., 2018). The P or NP fertilizers decreased the nutrient imbalance in the top 10 cm, especially the P limitation. Consequently, after P fertilizers over five years sufficient P was available in the top 10 cm and resulted in decreases of Phos activities. Although the available P concentrations were between 2 and 3 times higher in the P and NP plots than in the control (Fig. 1), an ~1.5 to 2.4 mg kg^{-1} of net increase was not sufficient to eliminate the P limitation below 10 cm. Whereas even a slight increase in available P caused an increase in the V_{max} of Phos between 10 and 40 cm (Fig. 2a). Those results consisted with our earlier study, which P fertilizers increased the V_{max} of Phos in the second year compared to the control in the top 10 cm (Dong et al., 2015), meaning P availability increase triggered more Phos activities when P element was limited to microbial enzyme productions relative to C and N.

Because NP increased the available P compared to the control between 40 and 80 cm depth, the V_{max} increased and the K_m decreased, causing an increase in the V_{max}/K_m of βGluc in the NP soils. Oligotrophic subsoils (between 40 and 80 cm depth in our study) are mainly constrained by energy (Hobbie and Hobbie, 2013). Microbes mineralized P from SOM to meet the C demand, which is consistent with reports that multiple resources will be assimilated to meet the microbial demand for energy (Hobbie and Hobbie, 2013; Spohn and Kuzyakov, 2013; Tischer et al., 2015). This is also supported by the decrease in DOC in the NP soils and the decrease in the Phos activity with increases in available P (Figs. 2, 5).

The V_{max} of NAG was lower in the P soils compared to the control between 10 and 20 cm. Phosphorus promotes ammonia oxidizers (Tang et al., 2016), with the result that P fertilizers increased the NO_3^- contents than the control (Fig. 1). This may be linked to the decrease in the activities of N-acquiring enzymes caused by applications of N. Available P contents were lower in NP than in P soils, which was attributed to higher P immobilization by ammonia oxidizers in NP than P soil (Tang et al., 2016). This indirect increase of NO_3^- after P fertilizers treated contributes to an increase in P-acquiring enzyme activities (Zhang et al., 2018).

5. Conclusions

A decrease in substrate contents and availability for microorganisms with soil depth induced the production of high quality, efficient (low K_m) enzymes to maintain activity. The enzyme kinetics in soil with N and P fertilizers for 5 years were depth-specific. The soil depth was grouped as (1) nutrient-rich but stoichiometrically imbalanced topsoil from 0 to 40 cm and (2) oligotrophic subsoil below 40 cm. Consistent with the microbial economic theory, the substrate affinity of βGluc increased when N fertilizers were added (i.e. decreased the K_m), but that of NAG did not vary significantly when comparing to control treatments within the same depth. Hence, N fertilizers increased the efficiency of βGluc above 40 cm but decreased that of NAG below 10 cm when compared to control. The V_{max} of Phos decreased in the top 10 cm after P and NP fertilizers, but did not vary significantly or increased between 10 and 40 cm. This suggests that the nutrient

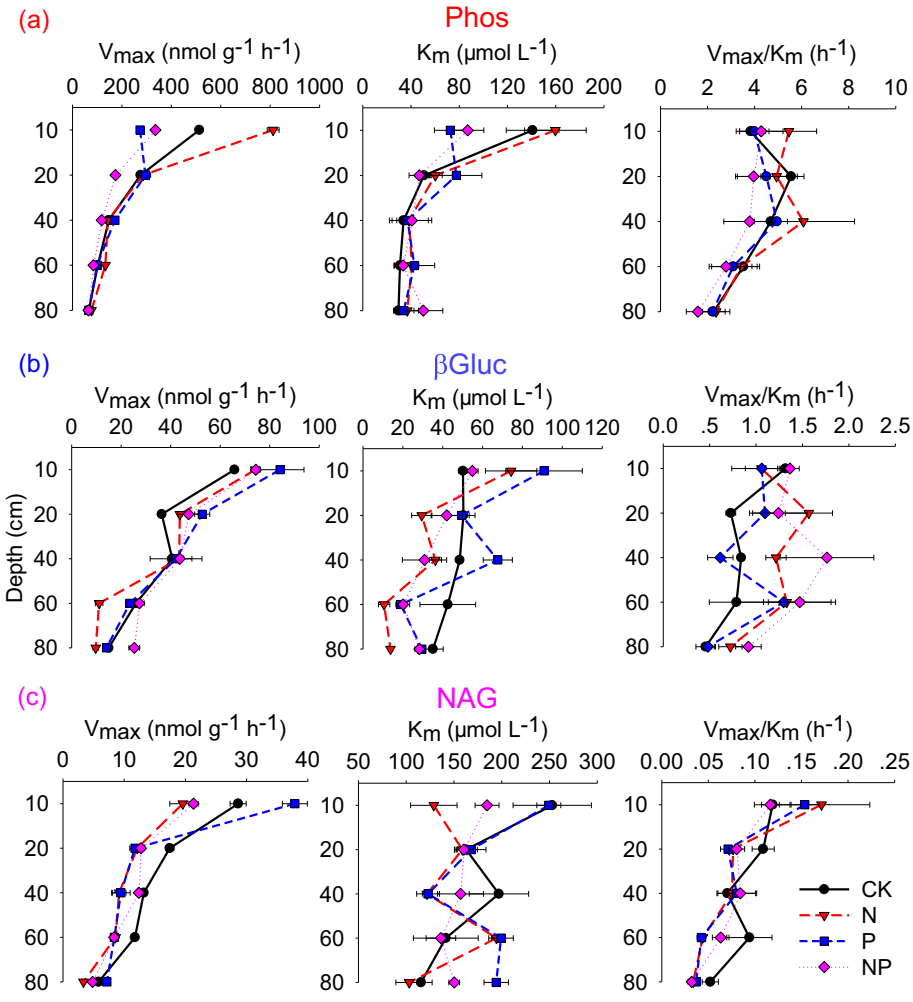

Fig. 2. Effects of N and P fertilization (N, P, N + P) on the kinetic parameters of acid phosphatase (Phos, a), β-1,4-glucosidase (βGluc, b), and β-1,4-*N*-acetylglucosaminidase (NAG, c) depending on soil depths. The same abbreviations in Fig. 3–5.

stoichiometry controls the V_{max} of Phos when C and N meet microbial demands. In the subsoils (below 40 cm), the activities (V_{max}) and affinity (low K_m) of βGluc increased with P fertilizers. Consequently, microorganisms mineralized P from SOM to meet the demand for C in oligotrophic conditions. We conclude that whilst fertilizers treated may ameliorate a decrease in soil fertility in Chinese fir plantations, the microbial requirement for energy will lead to further loss of SOM down the soil profile with wider consequences for soil degradation.

Declarration of competing interest

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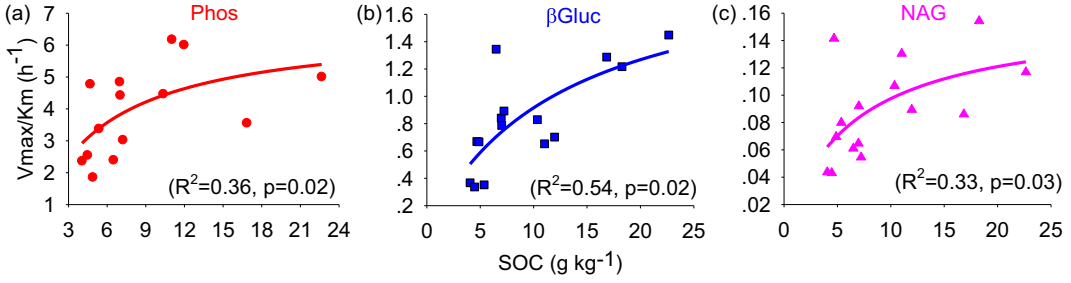

Fig. 3. The enzyme efficiency (V_{max}/K_m) of (a) Phos, (b) βGluc, and (c) NAG in relation to SOC in the unfertilized control soil.

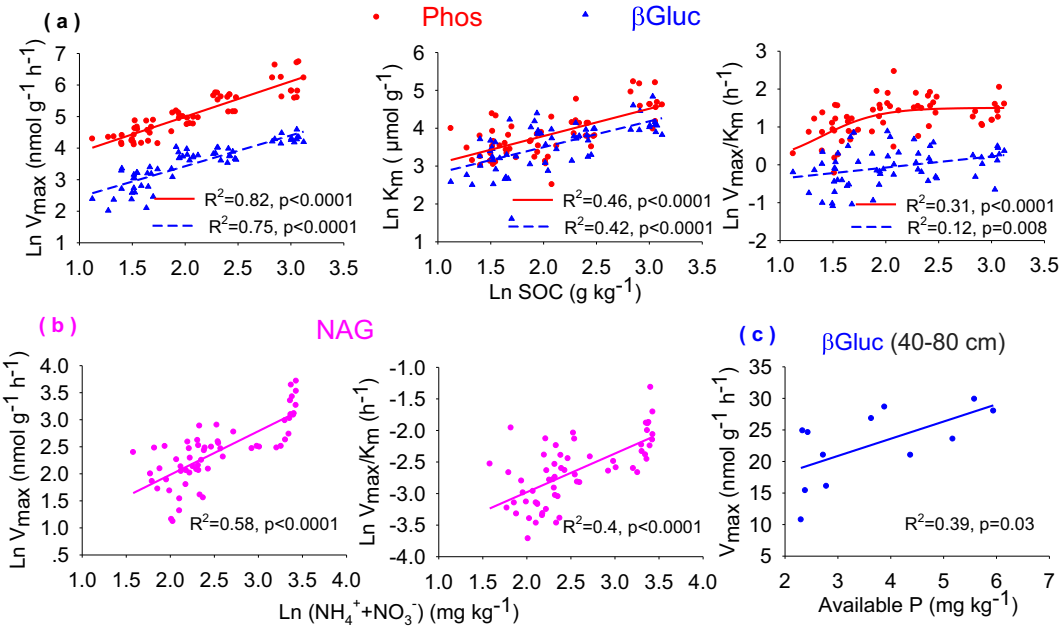

Fig. 4. Linear regression lines between enzyme kinetic parameters and (a) soil organic carbon (SOC) contents; (b) available mineral nitrogen (sum of NH_4^+-N and NO_3^--N) contents; (c) available P contents across all soil depths.

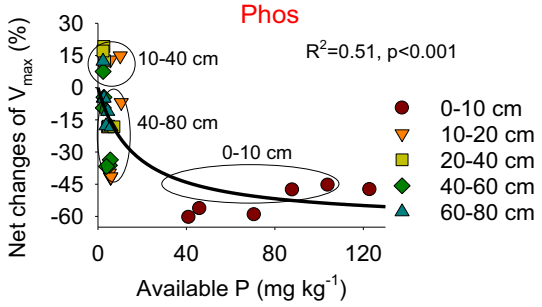

Fig. 5. Available P in relation to net changes of acid phosphatase V_{max} in P and NP fertilized soils comparing to unfertilized control.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2019.103465.

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