Effects of phosphorus addition on soil microbial biomass and community composition in a subalpine spruce plantation

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ARTICLE INFO

Article history:
Received 6 July 2015
Received in revised form 20 December 2015
Accepted 14 December 2015
Available online 24 December 2015

Keywords:
P addition
Soil microbial biomass
Soil microbial community composition
Carbon availability

ABSTRACT

Phosphorus (P) availability is expected to affect soil microbial community. However, our knowledge about the responses of soil microbial biomass and community composition to P availability is still limited. In this study, we established field plots with addition of 0, 5 (LP), 15 (MP) and 30 (HP) g P m⁻² yr⁻¹ in a subalpine spruce plantation and investigated the responses of soil microbes. Chloroform fumigation-extraction and phospholipid fatty acids (PLFA) analysis were used to determine soil microbial biomass and community composition, respectively. After two growing seasons of P addition, the HP treatment significantly increased soil microbial biomass carbon, nitrogen and P. The P addition exerted the specific influences on soil microbial community composition. The abundance of most groups of soil microbial community (bacteria, fungi, and arbuscular mycorrhizal fungi) increased in the HP treatment and the ratio of fungi to bacteria decreased in the LP and MP treatment, whereas the nonmetric multidimensional scaling ordination revealed no significant difference in the PLFA pattern between the P treatments and the control. Although soil P availability increased in all P treatments compared to the control, the dissolved organic carbon, indicative of soil carbon availability, was promoted only by the HP treatment. Besides, soil microbial biomass was positively correlated to soil carbon availability and pH. These results indicate that soil microbes are insensitive to the elevated P availability in this spruce plantation and P addition increases soil microbial biomass mainly through improving carbon availability and pH. © 2015 Elsevier Masson SAS. All rights reserved.

1. Introduction

Soil microbes play a critical role in regulating decomposition and thereby nutrient cycling in forest ecosystems [1]. In turn, soil nutrient availability, especially nitrogen (N) and phosphorus (P), can also affect the microbial biomass and community composition [2,3]. Treseder [2] reviewed the available studies addressing the effects of N on soil microbes and proposed that N addition reduced soil microbial biomass. Compared with microbial response to N availability, only a few studies focus on the effects of P availability on soil microbes. Furthermore, the limited experimental data is rather controversial, and there is no general agreement on the effects of P availability on soil microbial biomass and community composition. DeForest et al. [4] found no significant changes in soil microbial biomass after P addition in either unglaciated or glaciated soil of temperate deciduous forests, while Li et al. [5] reported that P addition increased soil microbial biomass and fungi:bacteria (F:B) ratio and altered the community composition in P-deficient tropical forests. Other scholars found positive, neutral even negative effects of P on soil microbes in temperate forests [6–8]. Additionally, Liu et al. [3] found that responses of soil microbial biomass and community composition to P addition varied in three forest types in tropical China. These inconsistencies imply that the effects of P addition on soil microbial biomass and community composition may vary.
with the forest biome at global scale and the forest type at local scale, and that the exact relationship between P availability and soil microbial community is poorly understood [4,5]. Given the distinct discrepancy in climate, vegetation and soil types across forest biomes and among forest types, more studies from specific forest ecosystems are indispensable to conclude the effects of P availability on soil microbes.

To date, multiple mechanisms have been proposed to explicate the P effects on soil microbes [e.g., 3, 5, 7, 9] (Fig. 1). For instance, P addition can increase litter fall and fine root biomass, resulting in increased C availability to soil microbes, and eventually improved soil microbial biomass and altered the community composition [3,5]. Besides, the relieved P constraints and changes in soil pH and osmotic potential after P addition can also influence the microbial growth [5,7,10]. Nevertheless, the uncertainties of mechanisms for P effects on soil microbial community exist extensively. Therefore, it is necessary to explore how soil microbial biomass and community composition respond to P addition in a specific forest ecosystem.

As a unique group of cold-temperate coniferous forests, the subalpine coniferous forests of China, concentrating in areas of high mountains, deep valleys and plateaus in western China and dominated by Picea and Abies species, differ from the boreal forests of the cold-temperate zone and montane coniferous forests of the temperate zone mainly in the species composition and soil condition [11]. However, little is known about the relationship between soil microbes and P availability in this region, which hinders our accurate estimation of the decomposition and nutrient cycling because of the important role of soil microbes in regulating the two processes in forest ecosystems.

The objective of this study was to investigate the changes in soil microbial biomass and community composition after two growing seasons of P addition in a subalpine spruce plantation in the eastern Tibetan Plateau of China. We focused on two questions: (1) How soil microbial biomass and community composition respond to different doses of P addition? (2) Which soil parameters are correlated with the microbial community after P addition? We hypothesized that soil microbes were more sensitive to changes in soil pH and C availability after P addition than to the elevated P availability in this subalpine spruce plantation.

2. Materials and methods

2.1. Study site description

This study was conducted in a spruce (Picea asperata) plantation at Ma'er Kang County in the northwest of Sichuan province, China (102°16′38.400″E, 31°43′11.142″N). This study site has an altitude of about 3724 m and a slope of 15° with a montane temperate climate with mean annual precipitation of 750 mm falling mainly from May to September. The mean annual temperature at the site is 4.9 °C with the mean maximum monthly temperature of 13.2 °C in July and the mean minimum monthly temperature of −4.7 °C in January [12].

The spruce plantation in our study site, one of the typical forest types in this region, was established on cutovers in 1992 by reforestation with 4-year-old spruce seedlings. The plantation did not receive any management after planting, but it has been slightly disturbed by the activity of yaks. In 2012, the tree density of the plantation was 2750 stems ha⁻¹. The tree canopy coverage was 88%.

The trees had a mean height of 8.3 m and a mean diameter at breast height was 11.8 cm. The understory coverage was approximately 10%, dominated by some shade-tolerant species, and the rest was covered by litter. The litter layer was about 5.2 cm in depth and 3.5 kg m⁻² in storage. The soil at the experimental site, which derived from metamorphic rocks including phyllite, slate and schist, was classified as Haplic Luvisol [12]. Before the P addition in November 2012, the mineral soil 0–10 cm had a pH of 5.18, organic C concentration of 82.9 g kg⁻¹, total N concentration of 6.2 g kg⁻¹, and total P concentration of 0.9 g kg⁻¹.

2.2. Experimental design

The fertilized experimental plots were established in October 2012 in a 5 ha spruce plantation, fenced by a wire netting against the disturbance of big animals (e.g., yaks). Experimental plots were designed in a randomized complete block. The plot size was 5 m x 5 m, and a 2 m wide buffer strip was maintained between each plot. Four treatments included in this study were control (CK), low P input (5 g P m⁻² yr⁻¹, LP), medium P input (15 g P m⁻² yr⁻¹, MP) and high P input (30 g P m⁻² yr⁻¹, HP). Each treatment was replicated three times.

The P was added as NaH₂PO₄·2H₂O. The fertilizer was mixed with 3.75 L of water and uniformly sprayed on the forest floor in two monthly portions since December 2012 and continued through June 2014. Each control plot received the same water (3.75 L) with no fertilizer.

2.3. Soil sampling and processing

The soil sampling was conducted in August 2014, after two growing seasons of P addition. Five soil cores with 5 cm in diameter were taken from each plot to a depth of 10 cm and mixed into one composite sample. After removing stones and coarse roots, the fresh soil sample was sieved to pass a 2 mm sieve. One subsample of the sieved fresh soil was kept in a refrigerator at 4 °C and analyzed within ten days after sampling for soil phospholipid fatty acids (PLFAs), microbial biomass C (MBC), microbial biomass N (MBN), microbial biomass P (MBP), soil dissolved organic C (DOC), and mineral N. Another subsample of the sieved soil was air-dried at the room temperature and homogenized for analysis of pH, soil organic carbon (SOC), total N, total P and available P (AP). All soil parameters values were presented in oven dried soil.
2.4. Laboratory analysis

Soil PLFAs were analyzed by using the method described by Frostegård et al. [13]. Lipids were extracted with a mixture of chloroform, methanol and phosphate buffer (1:2:0.8, v/v/v). The extracted lipids were subjected to mild alkaline methanolysis to transform the fatty acids into free methyl esters after successive elution with chloroform, acetone, and methanol on silica columns. Then, the PLFA methyl esters were separated and identified by gas chromatography (6890N, Agilent, USA) with a flame ionization detector (19091B–102, Agilent, USA). The abundance of individual fatty acid was expressed as nmol per g dry soil and classified according to standard nomenclature [14]. The sum of all peaks identified <20.5C atoms long were considered to be representative of the total PLFAs of soil microbial community [15]. In addition, PLFAs were divided into different microbial taxonomic groups based on previously published PLFA biomarker data in Table A.1. Total bacterial PLFAs were calculated as the sum of Gram-negative (GN), Gram-positive (GP) and general bacteria (GB). The ratio of fungal to total bacterial PLFAs (F:B) was used to estimate the ratio of fungal to bacterial biomass in soils and the ratio of GP to GN bacterial PLFAs (GP:GN) was used to estimate the ratio of GP to GN bacterial biomass.

Soil microbial biomass (MB) was estimated by chloroform fumigation-extraction method [16] with a slight modification. In brief, two portions of about 20 g subsample of each moist soil sample were weighed to measure MBC and MBN, and 8 g to measure MBP. One portion was fumigated for 24 h at room air temperature with ethanol-free CHCl₃ and the other portion was kept in room-air temperature as is. The subsamples for MBC and MBN were then extracted with 80 ml 0.5 M K₂SO₄ for 30 min by reciprocating shaker and filtered. The subsamples for MBP were then extracted with 100 ml 0.5 M NaHCO₃ at a pH of 8.5 for 60 min by reciprocating shaker and filtered. Organic C and total N in the extracts was determined with TOC/TN analyzer (Multi N/C®2100(S), Analytik Jena AG, Germany). The extracted P was determined by colorimetry according to the method from Olsen and Sommers [17]. The MB was calculated as follows: MB = E/ke, where E is the difference between C, N or P extracted from fumigated and non-fumigated soils and ke = 0.45 for MBC and MBN and ke = 0.4 for MBP [18]. All soil parameters values were presented in oven dried soil.

Concentration of DOC was determined by the methods described in Jones and Willett [19]. Briefly, soil DOC was extracted from 5 g moist soil with an addition of 25 ml distilled water. The mixture was shaken for 1 h at 250 rpm on a reciprocal shaker, and then centrifuged for 15 min at 8000 rpm. The supernatant liquid was filtered through 0.45 μm membrane filter. The concentration of the water-soluble C was determined using a TOC/TN analyzer (Multi N/C®2100(S), Analytik Jena AG, Germany). Ammonium (NH₄⁺) and nitrate (NO₃⁻) nitrogen were extracted with a 2 M KCl solution and then measured by colorimetry on an AutoAnalyser III (SEAL Analytical, Germany). The NO₃⁻ in the extracts was found to be below instrument detection level. The AP was determined by colorimetry after extraction with 0.5 M NaHCO₃ at a pH 8.5 [17]. Soil pH was measured in a 1:2.5 soil–water solution using a glass electrode. The SOC and soil TN were measured with an elementary analyzer (Vario MACRO cube, Elementar, Germany). The total P was determined with ICP-OES 8300 (Perkin Elmer, USA) after digestion with H₂SO₄–HClO₄.

2.5. Statistical analysis

The Shapiro–Wilk test and Levene’s test showed all data were normally distributed and homogenous. Since there’s no block effect (Table A.2), one-way ANOVA was used to test the effects of P treatments on soil chemical properties, PLFAs, MBC, MBN and MBP with SPSS 19.0 for Windows (SPSS Inc.). The mean values of three replicate plots were compared using Duncan’s multiple range test when a significant F-value was obtained (P < 0.05). Correlations between the microbial biomass/groups and soil properties (pH, DOC, NH₄⁺ and AP) were determined using Pearson correlation analysis. We also used simple linear regression analysis to evaluate relationships between the relative abundance of those PLFAs with mean % mole abundance >1% and soil properties across the 12 plots, and for simplicity, only those with significant correlations were shown in figures. All statistical significance was determined at P < 0.05.

We further tested patterns in PLFA biomarkers among treatments with the nonmetric multidimensional scaling (NMDS) ordination using R 3.1.1 (R Core Team 2014). The NMDS is an unconstrained ordination technique representing multivariate community data in a reduced set of dimensions. Function “metaMDS” in the vegan package (R package version 2.10–10) was used to perform NMDS on PLFAs % nmol abundance data with the following options: MDS engine = “monoMDS”, dimensionality = 2; dissimilarity measure = “Bray–Curtis”; data transformed by Wisconsin double standardization; and maximum number of random starts = 100. Treatment effects based on plot scores extracted from NMDS were tested using ANOVA. In addition, we created a biplot using soil properties (pH, DOC, NH₄⁺ and AP) as environmental variables with function “envfit”. As suggested by Weand et al. [11], only those PLFAs with mean % mole abundance > 1% were used in the ordination analyses (20 PLFAs out of 85 met this criteria) to avoid bias from rare and low abundance PLFAs.

3. Results

3.1. Soil chemical characteristics and microbial biomass

No significant differences in SOC and soil TN concentration and pH were found between three P treatments and the control (Table 1). However, soil total P and AP concentrations increased with the increasing level of P addition (P < 0.001). The HP treatment showed significantly higher soil DOC concentration compared to the control. The LP treatment had obviously lower concentrations of NH₄⁺ and DOC than control.

The P addition had significant influences on soil microbial biomass (P = 0.047, P = 0.013 and P = 0.013 for Soil MBC, MBN and MBP, respectively) (Fig. 2a–c). Soil microbial biomass was obviously higher in the HP treatment than the control and LP treatment. The MP treatment additionally showed higher MBP than the control and LP treatment. There were no significant differences in C:N, C:P and N:P ratios in the soil microbial biomass between three P treatments and the control (Fig. 2d–f).

3.2. Soil microbial community composition

In total, 46 fatty acid biomarkers were included in the calculation of total PLFAs. With P addition, total PLFAs and abundance of most specific taxonomic groups (bacteria, fungi, and arbuscular mycorrhizal fungi [AMF]) were significantly higher in the HP treatment than the control (Fig. 3). Generally, there was little difference in total PLFAs and those of microbial individual groups between the control and MP or LP treatment. The LP and MP treatment had significantly lower soil F:B ratio than the control (Fig. 3i). The P addition had no effects on the GP:GN ratio (Fig. 3j) and the relative abundance of all groups of soil microbial community with exception of the lower relative abundance of fungi in the MP treatment (Table A.3).
The 20 PLFAs with >1% nmol abundance were used in a NMDS ordination (Fig. 4). The NMDS solutions reached a final stress of 0.11 after 39 iterations. The PLFA patterns of HP treatment were the most distinct from other treatments. The NMDS ordination analysis revealed well separation between the HP and LP and between the HP and MP treatment along NMDS 1, whereas the control and the P treatments showed poor separation.

3.3. The relationships between soil chemical and microbial parameters

Pearson correlation analysis revealed significantly positive correlations between soil microbial biomass (MBC, MBN and MBP) and pH, DOC and AP, whereas NH$_4^+$ was weakly correlated to soil microbial biomass (Table A.4). Total PLFA, AMF, GN, GP and total bacteria had a significantly positive relationship with AP but not with pH, DOC or NH$_4^+$. Besides, fungi, F:B ratio or GP:GN ratio showed no significant relationship with soil properties (pH, DOC, NH$_4^+$ and AP). Simple linear regression analysis showed that the relative abundance of i14:0 and cy17:0 increased linearly with soil pH and DOC, respectively, while the relative abundance of i16:0 and a17:0 showed negative linear correlation with DOC (Fig. 5). The biplot of PLFAs and soil property patterns suggested no significant relationship between microbial community composition and soil properties in this study (Fig. 4a).

4. Discussion

4.1. Effects of P addition on soil microbial biomass

One interesting finding was that soil microbial biomass was significantly increased by the HP treatment (Fig. 2a–c). Another important finding was that the increased soil microbial biomass in the HP treatment was more closely correlated to the improved C availability than P availability, which supports our hypothesis. Soil available P was clearly higher in the MP and LP treatments.
compared to the control, but no significant increase in soil microbial biomass was observed in these two P addition treatments. Furthermore, the DOC, which well represented the labile soil carbon, acted as the primary energy source for microbes [20] and was significantly correlated to soil microbial biomass, increased in the HP treatment. Regardless of the relationship between soil microbial biomass and P availability, our results indicate that P availability is not a limiting factor for soil microbial growth in this spruce plantation, which consists with the relatively high P availability in the control (over 40 mg kg$^{-1}$). Concerning the significantly positive correlation between soil microbial biomass and C availability, it is not surprising. After all, C availability is usually considered to be the main limiting resource for soil microbial growth [6]. Fine root and floor litter have been found to be important sources of soil DOC [21]. Our field data showed that fine root biomass increased by 39% in the HP treatment compared to the control (Fig. A.1). In addition, the increased litter fall [3] and enhanced litter decomposition [22] were also reported in P addition trials. So the increased soil DOC in the HP treatment can be due to increases in fine root biomass, litter fall and its decomposition. Sporn and Kuyakov [23] also found the connection between soil C and P cycle that microbial P mineralization can be driven by microbial C acquisition from which plants potentially can benefit, and in turn, plants may invest more C input to soil microbes. It is necessary to note that this result is based on a single sampling, and microbial community can have a temporal variation. Therefore, more studies from forests in this region are still needed to confirm whether the responses of soil microbial biomass to P addition are related to C availability.

Fig. 3. Total PLFA abundance (a), abundance of different soil microbial groups (b–h), and F:B (i) and GP:GN (j) ratios at the 0–10 cm mineral soil depth of various P treatments in a spruce plantation. T-PLFAs: total PLFAs; Act: actinomycete; AMF: arbuscular mycorrhizal fungi; GN: gram-negative bacteria; GP: gram-positive bacteria; GB: general bacteria; T-Bacteria: total bacteria; F: fungi; B: bacteria. Columns followed by the same letter are not significant different by Duncan test ($P < 0.05$). Bar represents stand error ($n = 3$).
Our studies also showed that soil microbial biomass was positively correlated to soil pH (Table A.4). This confirmed the significantly positive relationship between microbial biomass and pH [24]. In addition, Aciego Pietri and Brookes [24] concluded that soil pH had marked effects on microbial biomass through affecting the availability of biologically toxic Al and organic C solubility. In our study, the pH in the HP treatment was increased by 0.43 unit (from 4.92 to 5.35) compared to the control, which was in line with previous studies suggesting P addition increased soil base cations via stimulation on litter nutrient input and eventually elevated soil pH [9,10]. All in all, these results imply that soil pH may be another factor affecting soil microbial biomass in the HP treatment.

4.2. Effects of P addition on soil microbial community composition

Our results showed the specific influences of P addition on soil microbial community composition. On one hand, the abundance of most groups of soil microbial community (bacteria, fungi, and AMF) was only increased by the HP treatment and the F:B ratio was suppressed by the LP and MP treatment (Fig. 3). On the other hand, P addition had no effects on the GP:GN ratio and the relative abundance of nearly all groups of soil microbial community with exception of the lower relative abundance of fungi in the MP treatment (Table A.3). Besides, the NMDS ordination revealed no significant difference in the PLFA pattern between the P treatments and the control.

As with the improved soil microbial biomass, we think the changes in the abundance of the community in the HP treatment is not directly due to the elevated P availability. Soil C availability and pH are important factors affecting soil microbial community [25]. Our results also showed significant relationships between soil mi-

![Image](image_url)

**Fig. 4.** The NMDS ordination biplot of microbial phospholipid fatty acids (PLFAs) at the 0–10 cm mineral soil depth of various P treatments in a spruce plantation. NMDS solutions reached a final stress of 0.11 after 39 iterations. (a) Includes plot scores and correlational vectors of soil properties. (b) Includes weighted average of PLFAs scores. For simplicity and due to differing scales on the x- and y-axis between the two panels, vector lengths of soil properties were increased by 15% in b.

![Image](image_url)

**Fig. 5.** Relationships between relative abundance of individual PLFA markers and soil pH and dissolved organic carbon (DOC) across all the plots (n = 12).
microbial community and pH and C availability (Fig. 5). Soil C availability was negatively correlated to some GP bacteria (i16:0 and a17:0) and positively correlated to some GN bacteria (cy17:0), which consisted with previous studies suggesting that GP bacteria were associated with oligotrophic communities and the utilization of more calcitrant C resource [26]. The AMF are expected to decrease with nutrient input [27]. On the contrary, we found an increase in the abundance of AMF in the HP treatment, which may be induced by the higher C availability deriving from the increased fine root biomass and litter fall as discussed above. The increased abundance of AMF has been also associated with the improved soil pH [10]. Our results showed that soil pH was increased by 0.43 units by the HP treatment. Therefore, the increased abundance of AMF in the HP treatment maybe resulted from the increased soil C availability and pH.

Unlike the increased abundance of microbial community in the HP treatment, we attributed the decreased F:B ratio in the LP and MP treatment to the elevated P availability. As the major decomposer in soil, fungal:bacterial (F:B) dominance indicates both its response to environmental change and its impact on ecosystem function [28]. The hyphal growth form makes fungi more accessible to nutrients and resources because of its capacity for translocation of nutrients and resources from abundant microsites to limiting sites [28]. Broadcast P addition to soil may negate this advantage of fungi. So greater nutrient availability favors the bacterial-based energy channel. Wardle et al. [29] also reported reduced F:B ratio after P addition in a subalpine tundra ecosystem. However, we found no obvious difference in F:B ratio between the HP treatment and the control. This may depend on the higher C availability in the HP treatment. Additionally, an increase in F:B ratio induced by P addition was observed in tropical forests [3,5]. This can be due to the facts that P availability in the tropical forests is always low and microbial utilization of C is constrained by P [5]. Thus, the response of F:B ratio to P addition seems to be related to the status of soil P availability.

5. Conclusions

Our results show that soil microbes were more sensitive to changes in soil pH and C availability after P addition than to the elevated P availability in the subalpine spruce plantation. We observed significantly higher soil microbial biomass and abundance of most groups of soil microbial community (bacteria, fungi, and AMF) in the HP treatment, which was due to the increased soil pH and C availability rather than the elevated P availability. These results suggest that the effects of P addition on soil microbes are mainly through its indirect influence on soil C cycling and chemical characters (e.g., pH).

Acknowledgments

This study was funded by the National Natural Science Foundation of China (No. 31270492), the Strategic Priority Research Program of the CAS (No. XDA05070306) and the National Science & Technology Pillar Program in 12th Five-year Plan of China (No. 2011BAC09B04-02). We are grateful to Yuanyuan Shu and Fengxian Zhong for their assistance in laboratory work. We thank two anonymous reviewers and the editor for their constructive comments on a previous version of this manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejsobi.2015.12.007.

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