Soil phosphorus composition determined by $^{31}$P NMR spectroscopy and relative phosphatase activities influenced by land use

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1. Introduction

Phosphorus is an essential and common limiting element for plants. Inorganic orthophosphate, including $\text{HPO}_4^{2-}$ and $\text{H}_2\text{PO}_4^-$ in a soil solution, is the primary source of P for plants and microorganisms. However, organic P was reported to make an important contribution to P nutrition in plants [4] and usually accounted for 20%–80% of total soil P [15].

To be available to plants, soil organic P needs to be mineralized first. Phosphatases, such as acid phosphomonoesterase (AcP), alkaline phosphomonoesterase (AlP), phosphodiesterase (PD) and pyrophosphatase (PY), play critical roles in hydrolyzing soil organic P and condensing inorganic P. Land use can influence phosphatase activities. Sandoval–Pérez et al. [21] found that AcP activities were lower in pasture soils than in primary and secondary forest soils. Acosta–Martínez et al. [1] found that agricultural soils had much lower values of AcP than pasture and forest soils. Similar results were found by Guo et al. [11], who reported that AlP and AcP activities were lower in intensive agricultural soils than in native soils. In agricultural soils, no–till and residue input treatment increased soil AlP and PY activities [31].

Previous studies also found that the type of land use could influence soil P forms and concentrations [17,22,26]. Compared to uncultivated soil, arable soil applied with mineral fertilizer and manure generally increased soil P contents, including total P, inorganic P and organic P in grassland, forest or native soils [26]. For different organic P forms, Guggenberger et al. [10] found that monoester contents in pasture and woodland soils were higher than those in arable soils. Condron et al. [6] also reported that native prairie soils without the application of manure or fertilizer contained more monoesters than cultivated soils. McDowell and Stewart [17] found that pasture soils had more orthophosphate than forest soils, as detected by $^{31}$P NMR spectroscopy, while Chiu et al. [5] found that orthophosphate was higher in grassland soils than in forest soils. McDowell and Stewart [17] reported that pasture soils had more monoesters than forest soils did, and a similar result was found by Chiu et al. [5]. Chiu et al. [5] also found that forest soils contain more diesters than grassland soils, while opposite results were found by Guggenberger et al. [10].

However, few studies have focused on soil P speciation by utilizing $^{31}$P NMR and soil AcP, AlP, PD, and PY activities under various land uses, including cultivated and uncultivated soils in the...
same region. Additionally, few studies have investigated the relationships between P compounds and soil phosphatase activities. In this study, two cultivated land uses (Maize and Paddy) and two uncultivated land uses (Woodland and Fallow) with similar climatic conditions and parent materials were chosen in the National Field Research Station of Shenyang Agroecosystems. This study aimed to determine 1) soil P composition by solution $^{31}$P NMR spectroscopy and 2) soil phosphatase activities and their relationships with different P composition under four land uses.

2. Materials and methods

2.1. Site description and soil sampling

Soils were sampled from the National Field Research Station of Shenyang Agroecosystems (41°32′ N 122°23′ E), Chinese Academy of Sciences, which is located in the Sujialun District of Shenyang, in northeastern China. The annual mean temperature is 7°C–8°C, the active accumulated temperature $>10^\circ$C ranges between 3300°C and 3400°C, the annual mean precipitation is 650–700 mm, and the non-frost period lasts 147–164 days.

Four land- use types, Maize (Zea mays L), Paddy (Oryza sativa L), Woodland (Populus cannadensis) and Fallow field, were initiated in 1990. Before the establishment of the research station, the area was uniformly cultivated as Paddy fields. The soil was classified as Haplud–Udi–Luvisol according to the World Reference Base for Soil Resources [8]. In the Maize and Paddy fields, urea was applied as N fertilizer, and diammonium orthophosphate was applied as P fertilizer. Maize plots received approximately 172 kg N ha$^{-1}$ and 35 kg P ha$^{-1}$ annually, and Paddy plots received 242 kg N ha$^{-1}$ and 47 kg P ha$^{-1}$ annually. Woodland and Fallow plots received no fertilizers.

Samples of up to 20 cm of soil were collected by a soil auger on October 30, 2009, after crop harvest. There were four replications per land use type, and each replication was composed of 10 soil sample cores. Fresh samples were sieved (<2 mm) after removing debris and stored at 4°C for phosphatase assay. Subsamples were air-dried at the ambient temperature for at least one week and stored for the analysis of soil properties and P speciation.

2.2. Soil properties analysis

Soil pH was measured using a glass electrode with a soil to water ratio of 1:2.5. Soil organic matter (SOM) and total N were determined by an automatic element analyzer (Vario EL, Elementar, Germany). Soil total P was detected by a digestion procedure with perchloric acid. Available P (Olsen–P) was extracted by sodium bicarbonate at pH 8.5 [18]. All produced PO$_4^{3-}$ was detected by molybdate colorimetry.

2.3. Soil P composition by $^{31}$P NMR measurement

Phosphorus was extracted by shaking 5 g of air-dried soil with 100 ml of a solution containing 0.25 mol l$^{-1}$ NaOH and 0.05 mol l$^{-1}$ Na$_2$EDTA for 16 h at 20°C [3]. The extracts were centrifuged at 10,000×g for 30 min. Total P contents were determined in diluted extracts (1:2.5) to prevent interference from EDTA during analysis [29] by ICP–OES. To reduce interference from paramagnetic ions such as Fe and Mn in the $^{31}$P NMR measurement, 5% (v/v) of bicarbonate buffered dithionite (BD) solution (0.11 mol l$^{-1}$ NaHCO$_3$ + 0.11 mol l$^{-1}$ Na$_2$S$_2$O$_4$) was added to the extracts before freeze-drying.

Freeze-dried NaOH–EDTA extracts (200 mg) were re-dissolved in 0.1 ml 10 mol l$^{-1}$ NaOH and 0.5 ml D$_2$O (for signal lock) and transferred to 5-mm NMR tubes. Solution $^{31}$P NMR spectra were obtained using a JOEL ECA 600 spectrometer operating at 243 MHz with a 5.2 μs pulse (45°), a delay time of 1.0 s, and an acquisition time of 1,077 s. Approximately 26,000 scans were performed for all samples. Peaks and chemical shifts of signals were assigned based on previous research [10,28,29]. The spectra were plotted with 16-Hz line broadening and processed with NMR Utility Transform Software (NUTS) for Windows (Acorn NMR, Livermore, CA).

2.4. Phosphatase activities assay

Four phosphatase activities involved in P cycles were determined. (a) acid and alkaline phosphomonoesters (EC 3.1.3.2 and EC 3.1.3.1, AcP and AlP) were assayed with p-nitrophenyl phosphate as substrates with modified universal buffer pH values of 6.5 and 11, respectively [23], (b) phosphodiesterase (EC 3.1.4.1, PD) was assayed with bis-p-nitrophenyl phosphate as substrate with a buffer pH of 8 [2]. (c) inorganic pyrophosphatase (EC 3.6.1.1, PY) was assayed with sodium pyrophosphate decahydrate as the substrate [23]. The activities of AcP, AlP and PD were expressed as mg p-nitrophenol kg$^{-1}$ soil (dry weight) h$^{-1}$, and PY activity was expressed as mg PO$_4^{3-}$ – P kg$^{-1}$ soil (dry weight) h$^{-1}$.

2.5. Statistical analysis

All soil results were reported based on their oven-dry (105°C) weight. Data shown were the means (±standard deviation) of four replications for each land use type. Comparisons among means were evaluated using a one-way ANOVA with Duncan test at the P = 0.05 level. Linear relationships between variables were based on Pearson correlations. The statistical analysis mentioned above was performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Principal components analysis (PCA) was applied to identify the effects of land use and soil properties on soil phosphatase activities and P composition using Canoco Software 4.5 (Microcomputer Power, USA).

3. Results

3.1. Soil properties

Soil pH was significantly lower in Maize soil than in soil from the other three land use types. Soil organic matter contents were significantly higher in Paddy and Fallow soils but were lowest in Maize soil. Total N concentrations were lower in cultivated soils (Maize and Paddy) than in uncultivated soils (Woodland and Fallow). Higher Olsen-P concentrations were found in Paddy soils compared with other land-use types. Soil total P contents were highest in Paddy soil and lowest in Woodland soil (Table 1).

3.2. Phosphorus composition in NaOH–EDTA extracts determined by solution $^{31}$P NMR spectroscopy

3.2.1. Inorganic P forms

Inorganic P detected in NaOH–EDTA extracts included inorganic orthophosphate, pyrophosphate and polyphosphates (Table 2, Fig. 1). Inorganic orthophosphates, averaging at 6.25 ppm chemical shift, ranged between 105 and 195 mg P kg$^{-1}$ soil (57%–82% of extractable P) and were the major fractions of P in all tested soils. Pyrophosphate, averaging at 3.52 ppm chemical shift, ranged between 0.6 and 8.3 mg P kg$^{-1}$ soil and only accounted for 0.2%–4.0% of extracted P. Polyphosphates only existed in Maize (2.8 mg P kg$^{-1}$ soil) and Fallow (2.3 mg P kg$^{-1}$ soil) soils (Table 2), but a terminal P group of polyphosphates was detected at ~3.08 ppm chemical shift in Maize soil.
2.3 mg P kg$^{-1}$ in uncultivated soils than in cultivated soils, ranged between 1.7 and 4.7 mg P kg$^{-1}$.

2.3.2. Organic P forms

Organic P detected by $^{31}$P NMR included orthophosphate monoesters and diesters. Dominant orthophosphate monoesters ranged between 40 and 77 mg P kg$^{-1}$ soil, equivalent to 17%–37% of total extracted P, and were higher in uncultivated soils than cultivated soils. Myo-inositol hexakisphosphate was not confirmed, as no distinct C-2 peaks were found because of poor spectral resolution (Fig. 1).

Orthophosphate diester concentrations were higher in uncultivated soils than in cultivated soils, ranging between 1.7 and 2.3 mg P kg$^{-1}$ soil, and comprised 0.8%–1.2% of the total extracted P (Table 2). Concentrations of phospholipids presented a trend similar to that of total diesters but ranged between 1.2 and 2.3 mg P kg$^{-1}$ soil. Trace amounts of DNA were detected at approximately 0.21 ppm chemical shift in Maize (0.3 mg P kg$^{-1}$ soil) and Paddy (0.8 mg P kg$^{-1}$ soil) soils. Phosphonates were only detected in Maize and Fallow soils at 19.19 ppm chemical shift with concentrations of 0.8 and 0.7 mg P kg$^{-1}$ soil, respectively.

3.3. Soil phosphatase activities

Generally, soil AcP, AlP, PD and PY activities were higher in uncultivated soils than in cultivated soils (Fig. 2). Soil AcP activity found in Paddy soils was lower than the soil AcP activity found in the other three land–use types. The highest AlP activity was in Woodland soil, whereas the lowest AlP activity was in Maize soil. For soil PD activities, values in Maize soil were lower than those in the other three land–use types. Remarkable differences in soil PY activity were found among the land uses (Fig. 2), with decreasing activity in the following order: Fallow, Woodland, Maize and Paddy (Fig. 2).

3.4. PCA of soil properties, P composition and phosphatase activities

The PCA, based on soil properties, phosphatase activities and major P composition determined by $^{31}$P NMR spectroscopy in the various soils, explained 87.7% of the data variation (Fig. 3). Factor 1 explained 52.5% of the total variability and was related to total P and Olsen–P. This factor distinguished fertilized (Paddy with 47 kg P ha$^{-1}$ and Maize with 35 kg P ha$^{-1}$ annually) soils from unfertilized (Woodland and Fallow) soils. Factor 2 explained 35.2% of the total variability and was related to SOM, pH, AlP and PD activities. This factor distinguished the Maize soils from the soils of the other three land–use types (Fig. 3).

The PCA showed that Woodland and Fallow had more similarity and that there were higher values observed for soil total N, monoesters, diesters and PY activity in uncultivated soils (Woodland and Fallow). The PCA also showed a decrease in the values of soil pyrophosphate and AcP activity and an increase in orthophosphates, which reflect the effect of submerged conditions on Paddy soil.

3.5. Relationships among land uses, soil properties, soil phosphatase activities and P composition

Pearson correlations between soil phosphatase activities and soil properties, as well as between soil phosphatase activities and P composition using $^{31}$P NMR, were analyzed (Table 3). For soil pH, significant and positive relationships were found with both soil AlP and PD activities. Soil organic matter correlated positively with PD activities, whereas total N correlated positively with most soil phosphatase activities (all except AcP). No relationship between soil phosphatase activities and total P was found in this study, but soil AcP and PY activities were significantly and negatively correlated with soil Olsen–P. Soil AcP and PY activities were both significantly and positively correlated with soil pyrophosphate and monoesters. Negative relationships were found between soil orthophosphate and AcP and PY activities. No relationship was found between soil phosphatases and total diesters. Neither soil AlP nor PD had a significant relationship with soil P composition.

4. Discussion

4.1. Soil P composition

Paddy soil contained more total P and Olsen–P (Table 1), which might have resulted from higher input of P fertilizers [26] and lower output of P by plant biomass compared to maize soil. In Woodland soil and Fallow soil, lower concentrations of Olsen–P may be attributed to the lack of P input compared to cultivated soils.

Orthophosphate was the major extractable P fraction in all kinds of land use types [27,29,30], and greater concentrations of orthophosphate in Paddy soil (Table 2) corresponded to the higher soil Olsen–P content (Table 1) because of higher P fertilizer input. Pyrophosphate and long–chain polyphosphates both originated from microbial activity in soils [9]. However, as pyrophosphates degrade more rapidly than long-chain polyphosphates,
pyrophosphate could have remained for many months through adsorption in soils [29], resulting in the detection of pyrophosphates in all land-use types. In contrast, polyphosphates were only found in Fallow soil and Maize soil. Although the hydrolysis of pyrophosphates was enhanced in submerged soils, the lower PY activity in Paddy soil indicates that the lowest concentrations of pyrophosphates in Paddy soil in this study resulted from a decreased formation of pyrophosphates rather than faster hydrolysis.

Orthophosphate monoesters dominated organic P because they are stabilized in soils by association with amorphous metal oxides and can also be produced by alkaline degradation of RNA and phospholipids [28]. The orthophosphate monoester and diester P contents in cultivated soils decreased compared with uncultivated soils (Table 2; Fig. 3). Cultivation could greatly decrease orthophosphate monoester and diester P contents because of soil disturbance [6,17]. Orthophosphate diesters, including compounds such as DNA and phospholipids with only one covalent bond to a C–moiety [17], were more readily degraded by microbes and enzymes than monoesters. Monoesters were more easily protected by sorption to soil sesquioxide or cations, organic matter and clay [24]. In alkaline extractions, diesters could be hydrolyzed and degraded [13], which was another reason why there were low contents in each land use type (Table 2). Trace amounts of DNA were only detected in Maize and Paddy soils, possibly because of lower soil pH in Maize soil and wet conditions in Paddy soil. Makarov et al. [14] reported that DNA tended to accumulate in cold, wet and acidic soils. Phosphonates were only detected in extracts of Maize and Fallow soils at 19.19 ppm chemical shift, which were assigned to phosphonolipids.

### 4.2. Soil phosphatase activities

In both cultivated soils and uncultivated soils, the hydrolysis of inorganic or organic P forms was largely the result of biochemical processes. This makes soil phosphatase activities good indicators for assessing the hydrolysis of soil P. Previous studies also showed that enzyme activities were lower in cultivated soils than in uncultivated or less–disturbed soils [1,19]. However, non-disturbed Woodland and Fallow soils had similar AcP activities as Maize soil but higher AcP activities than Paddy soil, probably because of the lower and more suitable soil pH in Maize field soil (Table 1). Dick et al. [7] noted that AcP activities were higher in acid soils and that the optimum pH was 4.0. Soil AcP mainly originated from plant roots, microbial organisms, soil animals, etc., whereas soil AlP only originated from soil microbial organisms [25], which could indicate higher microbial activity in Woodland soil. Maize soil had the lowest PD activity, which may have resulted from lower soil pH and lower SOM in the soil (Table 1). The intimated relationships between PD activities and SOM and soil pH found in our study (Table 3, Fig. 3) support this theory.

![Fig. 2. Soil phosphatases activities under four land–uses. Soil acid phosphomonoesterase (AcP), alkaline phosphomonoesterase (AIP), phosphodiesterase (PD) activities were expressed as mg para–nitrophenol kg⁻¹ soil h⁻¹ while inorganic pyrophosphatase (PY) activities expressed as mg PO₄⁻₃ kg⁻¹ soil h⁻¹. Values with the same letter are not significant at 5% level (Duncan) and error bars around means are shown as standard deviations.](image)

![Fig. 3. Principal components analysis (PCA) of soil basic chemical characters, phosphatases activities and main phosphorus composition determined by ³¹P NMR spectroscopy with different land–uses. P1, orthophosphate; P2, pyrophosphate; P3, monoesters; P4, diesters; AcP, acid phosphomonoesterase; AIP, alkaline phosphomonoesterase; PD, phosphodiesterase; PI, inorganic pyrophosphatase; *, maize; ●, Paddy; ▲, woodland; ▼, Fallow.](image)

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<tr>
<th>Total P (mg P kg⁻¹ soil)</th>
<th>Inorganic P (mg P kg⁻¹ soil)</th>
<th>Organic P (mg P kg⁻¹ soil)</th>
<th>Orthophosphateb</th>
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<td>Total P (mg P kg⁻¹ soil)</td>
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<td>Maize</td>
<td>172 (42)</td>
<td>116 (67)</td>
<td>5.3 (3.1)</td>
<td>2.8 (1.6)</td>
<td>45 (26)</td>
<td>1.7 (1.0)</td>
<td>0.3 (0.2)</td>
<td>1.4 (0.8)</td>
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<td>Paddy</td>
<td>238 (53)</td>
<td>195 (82)</td>
<td>0.6 (0.2)</td>
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<td>40 (17)</td>
<td>2.0 (0.8)</td>
<td>0.8 (0.3)</td>
<td>1.2 (0.5)</td>
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<tr>
<td>Woodland</td>
<td>176 (47)</td>
<td>105 (60)</td>
<td>3.5 (2.0)</td>
<td>nd</td>
<td>64 (37)</td>
<td>2.1 (1.2)</td>
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<tr>
<td>Fallow</td>
<td>208 (50)</td>
<td>118 (57)</td>
<td>8.3 (4.0)</td>
<td>2.3 (1.1)</td>
<td>77 (37)</td>
<td>2.3 (1.1)</td>
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**Table 2** Phosphorus concentrations determined in NaOH–EDTA extracts of various land–used soils by solution ³¹P NMR spectroscopy.

*Values in parentheses are the proportion (%) of the total soil P.*

*Values in parentheses are the proportion (%) of the total extracted P in NaOH–EDTA. nd, not detected.*

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4.3. Relationships among land uses, soil properties, soil phosphatase activities and P composition

The statistically significant positive relationships of ACP and PD activities with soil pH (Table 3, Fig. 3) could be explained by the high optimum soil pH of these two enzymes. Wang et al. [31] and Saha et al. [20] found significant relationships between phosphatase activities and soil organic C. In the present study, positive relationships were only detected between PD activities and SOM. Phosphatase activities, except AcP activity, had positive relationships with total N, which could demonstrate the influence of N on soil phosphatase activities [12]. When N availability is low, the soil biota may be able to increase production of enzymes to enhance the supply of N and P. Significant negative correlations were found between Olsen-P and the activities of AcP and PY because less available P can increase phosphatase activities [16].

A significant and negative relationship of AcP activities with orthophosphate was also found in this study, which indicates that low available inorganic P could induce an increase in soil phosphatase activities. Strong positive relationships between orthophosphate monoesters and ACP activities and between pyrophosphate and PY activities indicated that the activities of phosphatases were affected by their substrates. However, a relationship between PD activity and total diesters was not found, which may be because of the alkalinity of the solutions [13] and because microbes and enzymes degrade and hydrolyze the diesters [12].

5. Conclusions

Land use systems such as cultivated Maize and Paddy soils caused an increase of inorganic P and a decline of organic P including monoesters, diesters compared with uncultivated Woodland and Fallow soils. Generally, soil phosphatase activities under uncultivated soils were higher than cultivated soils. Soil AcP and PY had a positive relationship with monoester and pyrophosphate, respectively. Olsen-P and Orthophosphate are not related to the total AcP and PY activities, which may be because of low available inorganic P could induce an increase in soil phosphatase activities and phosphorus monoesters and AcP activities and between pyrophosphate and PY activities indicated that the activities of phosphatases were affected by their substrates. However, a relationship between PD activity and total diesters was not found, which may be because of the alkalinity of the solutions [13] and because microbes and enzymes degrade and hydrolyze the diesters [12].

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