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Depletion of organic phosphorus from Oxisols in relation to phosphatase activities in the rhizosphere

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Summary

Phosphorus (P) deficiency is a major limitation to agricultural production in many parts of the world. It is therefore desirable to identify plants with enhanced abilities to utilize P more efficiently. Exudation of phosphatase from roots may improve P availability, yet there is little direct evidence for this. Here we report the dynamics of organic P in the rhizosphere of plants that have enhanced rhizosphere phosphatase activity. Agroforestry species and transgenic subterranean clover (engineered to produce phytase) were compared with crop and wild-type plant controls, respectively. Depletion of organic P was measured in pools defined by chemical extraction, solution ^{31}P NMR spectroscopy, and microbial immobilization of radio-isotopic P. Plants that had greater extracellular phosphatase activity depleted more organic P from P-deficient Oxisols than control plants. Depleted organic P forms were primarily phosphate monoesters. Plants with enhanced extracellular phosphatase activity also had access to a pool of soil P that was less isotopically exchangeable. Transgenic subterranean clover that expresses a microbial phytase gene appeared to have greater access to recently immobilized P, whereas plants expressing endogenous phosphatases utilized the unlabelled portion of soil organic P to a greater extent. Collectively, these results indicate that the enhancement of phosphatase activity in the rhizosphere of plants is implicated in the depletion of organic P forms from soils, most notably orthophosphate monoesters, whilst also suggesting that there is some exclusivity to the pools of organic P utilized by plants and microorganisms.

Introduction

Phosphorus (P) is an essential plant nutrient, a shortage of which limits agricultural production on a global scale. Mineral P fertilizers are scarce throughout much of the world, but are essential to increase and maintain crop yields. However, when applied to soils they transform rapidly into inorganic and organic forms that are of limited availability to plants. Applied P is especially strongly fixed in highly weathered Oxisols and Ultisols, which account for up to 70% of the world's P-deficient soils (Fairhurst *et al*., 1999). In such soils, the cycling of P from organic pools, rather than equilibration of the soil solution with bound inorganic P, is of particular significance. Synthesis and mineralization of organic P by soil microorganisms is integral to this process (Seeling & Zasoski, 1993).

Plants have developed various mechanisms to enhance the acquisition of P from soil. These include the production of phosphatases, which importantly catalyse the hydrolysis of P from organic forms of P, predominant in many soils (Richardson *et al*., 2005). The release of extracellular phosphatases by roots in response to P deficiency has thus been implicated in improved plant nutrition (Chen *et al*., 2002). Phytases, which initiate the catalysis of inositol hexa*kis*phosphates are of particular interest, as metal (Fe, Al, Ca) salts of this compound (phytate) generally constitute a major proportion of the total organic P found in soil (Turner *et al*., 2002).

Phosphatases have consequently been the focus of several approaches to improve the availability of phosphate to agricultural plants. The agroforestry species *Tithonia diversifolia* (Hemsley A. Gray) and *Crotalaria grahmiana* (Wight and Arn) have been investigated for soil fertility replenishment in east Africa (Rao *et al*., 1998). Importantly, these species have enhanced activities of acid phosphatase in the rhizosphere and can accumulate large concentrations of P in their biomass, even when grown in P-deficient soils (George *et al*., 2001, 2002a). Alternatively, transgenic plants (e.g. *Trifolium subterraneum* L.) that express fungal phytase genes and release extracellular phytase have been developed (Richardson *et al*., 2001a; George *et al*., 2004, 2005a). These plants have unique

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abilities to utilize P when it is supplied as phytate in culture and, in some instances, show improved P nutrition when grown in soils (George *et al.*, 2005a). Although there is evidence that plants that exhibit enhanced phosphatase activities in their rhizosphere have improved access to soil organic P, little is known about the forms or origin of the P depleted from the rhizosphere.

Here we investigate depletion of the organic P in the rhizosphere of plants that have enhanced activity of extracellular phosphatases. We use chemical extraction, solution ^{31}P NMR spectroscopy and ^{32}P and ^{33}P isotopic labelling to assess the forms of P depleted by the plants when grown in two P-deficient Oxisols.

Materials and methods

Soil sampling and characterization

Samples of topsoil (0–10 cm depth) were collected from two sites: Robertson in New South Wales, Australia, and Khwisero in the Maseno District, Kenya (Table 1). Both soils are Oxisols with similar textural class and mineralogy, but were under contrasting management systems. The Robertson soil was from the pasture phase of a rotation with potatoes, which had a history of P fertilization, whereas the Khwisero soil was from the bare fallow phase of a rotation with maize and had no previous record of P fertilization. In addition, the Kenyan soil was from higher altitude and received greater annual rainfall. The soil samples were air-dried, mixed and sieved (<2-mm) to remove coarse material and vegetative matter. They were then stored air-dried for up to 2 years prior to use in experiments.

Table 1 Site and general soil characteristics of the Robertson and Khwisero soils

	Robertson	Khwisero
Country	Australia	Kenya
Longitude and latitude	150°36 ′E, 34°34′ S	34°33′ E, 00°08′ N
Altitude/m above sea level	130	1450
Mean annual rainfall/mm	986	1750
Land use	Pasture and potatoes	Subsistence maize
Soil type (USDA)	Oxisol	Oxisol
Soil texture	Clay loam	Clay loam
Dominant mineralogy	Kaolinite (haematite)	Kaolinite
pH/in H_2O	5.7	4.8
pH/in $CaCl_2$	4.8	4.3
Organic matter content/mg g^{-1}	163	92
Resin extractable P/μg g^{-1}	1.5	1.2
Total P/μg g^{-1}	2450	757

Plant characteristics and growth

Wild-type *Trifolium subterraneum* L. (subterranean clover, cv. Dalkeith) and those transformed with a constitutively expressed *Aspergillus* phytase gene (*ex::phyA*) were grown in the Robertson soil. These transgenic clovers release extracellular phytase and were described previously (subterranean clover line E, George *et al.*, 2004). *Tithonia diversifolia* (tithonia), *Crotalaria grahamiana* (crotalaria) and *Zea mays* L. (maize, cv Botaris) were grown in the Khwisero soil.

In both experiments, five replicate pots were sown with five surface-sterilized seeds of uniform size and protruding radicles. The plants were subsequently thinned to three plants per pot approximately 1 week after planting. The soils were maintained at a water content of *c.* 80% field capacity and were supplied with all nutrients, except P, on a weekly basis by addition of a modified Hoaglands solution (Hoagland & Arnon, 1950). The pots were arranged in a randomized design in glasshouses and rotated regularly to reduce effects of environmental gradients. Subterranean clover was grown at 14–22°C in approximately 16 hours of daylight. Tithonia, crotalaria and maize were grown at 20–30°C in approximately 14 hours of daylight. All plants were harvested after 56 days.

Rhizosphere sampling

Samples of rhizosphere soils from subterranean clover were obtained using a system similar to that described by Chen *et al.* (2002) and as outlined by George *et al.* (2005b). The rhizosphere of the agroforestry species and maize were sampled as described by George *et al.* (2002a). In both systems the root mats were separated from rhizosphere soils by a 24-μm nylon mesh, through which only root hairs, mycorrhizal hyphae and root exudates could pass. A key difference between the two systems was that the agroforestry species and maize roots were in contact with the rhizosphere soils for 56 days, whereas subterranean clover root mats were first developed for 28 days and then placed in contact with a 1-mm thick section of soil contained on filter paper for a further 28 days. In addition, the system used for tithonia, crotalaria and maize allowed sampling of the rhizosphere at various distances (up to 30 mm) from the root mat.

Analysis of soil phosphorus and extracellular phosphatase activity

Two-gram samples of soil were extracted with 20 ml of 0.1 M NaOH (pH 8.5) on a reciprocal shaker for 16 hours and the supernatants were collected after centrifuging at 5500 *g* for 10 minutes. Aliquots were analysed for both inorganic P (NaOH-Pi) and organic P (NaOH-Po). For NaOH-Pi, extracts were acidified with 0.12 parts of 1.8 M H_2SO_4 and centrifuged to remove precipitated humic material. Total P (NaOH-Pt) was determined on individual aliquots that were digested (3:1

by volume) with three parts of a digest mixture (1.8 M H_2SO_4 and 0.4 M $(NH_4)_2S_2O_8$). Phosphate was determined after reaction with malachite green (Irving & McLaughlin, 1990) and NaOH-Po was derived as the difference between NaOH-Pt and NaOH-Pi even though this procedure may overestimate the organic P concentration (Turner *et al*., 2003b).

The component of NaOH extracts that was amenable to hydrolysis by a commercially available *Aspergillus* phytase (Sigma phytase; Sigma-Aldrich, St Louis, MI, USA) was also determined for samples of the Robertson soil. This phytase was active not only against *myo*-inositol hexa*kis*phosphate (InsP6) but also other monoester and some diester forms of organic P (Hayes *et al*., 2000a). The fraction of P quantified by this procedure is therefore referred to as phosphatase labile (NaOH-Pphos). For NaOH-Pphos assays, 100 µl of NaOH extract was made up to 300 µl with buffer (15 mM MES, 1 mM EDTA, pH 5.5; final concentration) and phytase added to a final activity of 0.5 nKat ml^{-1}. Reactions were incubated at 37°C and terminated with an equal volume of 10% trichloroacetic acid at either time zero or after 6 hours. Phosphate released during the assay was determined by reaction with malachite green.

Soil acid phosphatase activities in rhizosphere soils collected from tithonia, crotalaria and maize were measured in soil suspensions by reaction with methylumberlliferyl-phosphate (MUP) as described by George *et al*. (2002b). Phytase, phosphomonoesterase and phosphodiesterase activities in root exudates of subterranean clover lines were measured against InsP6, *p*-nitrophenyl phosphate (*p*NPP) and bis-*p*-nitrophenyl phosphate (bis-*p*NPP) (Sigma-Aldrich) as described previously (George *et al*., 2004).

Solution ^{31}P-NMR analysis of soil extracts

Air-dried samples of rhizosphere soils and unplanted controls (1 g) were extracted in triplicate with 20 ml of a solution containing 0.25 M NaOH and 0.05 M EDTA for 16 hours at 21°C (Cade-Menun & Preston, 1996). Samples were centrifuged at 10 000 *g* for 30 minutes and the supernatants of replicate extracts were combined. Total P in the extracts was measured as described previously, and the remainder of the extracts was frozen at − 80°C and lyophilized. The extracts were then analysed by solution ^{31}P NMR spectroscopy using parameters described in Turner *et al*. (2003a) for the Robertson soil and Turner *et al*. (2003b) for the Khwisero soil. Phosphorus functional groups on NMR spectra were assigned according to Turner *et al*. (2003c) and are expressed as a percentage of the total signal area and as a concentration (µg g^{-1} soil) in the soil extracted.

Isotopic labelling of soil and determination of L-values

The Khwisero and Robertson soils were labelled with ^{32}P at 30.8 kBq g^{-1} soil and with ^{33}P at 6.2 kBq g^{-1} soil, respectively, added as carrier-free solutions. Isotopes were added as solutions while soils were mixed and bought up to *c*. 80% field capacity. Soils were then incubated for 28 days at 30°C, and water contents maintained by weight on a daily basis.

After incubation, or following plant growth, samples of soils were extracted sequentially with anion exchange resin charged with HCO_3, followed by 0.1 M NaOH (Saggar *et al*., 1990; Tiessen & Moir, 1993). Phosphorus content (Pt, Pi and Po) of the extracts was determined as previously outlined. Phosphatase-labile P was also determined in extracts of the Robertson soil. Microbial P was measured by a procedure modified from McLaughlin & Alston (1986), whereby soils were extracted during fumigation with anion exchange resin charged with HCO_3, rather than after fumigation with 0.5 M $NaHCO_3$. Briefly, replicate 2-g samples of soil were extracted for 16 hours in 20 ml of deionized H_2O with anion exchange resin strips either with or without addition of 0.8 ml hexanol. Phosphorus was eluted from the resin strips for 1 hour with 0.1 M HCl, and the phosphate concentration was determined by reaction with malachite green. Microbial P was calculated by the difference between samples with and without hexanol. A correction factor to account for sorption of P during extraction was determined from samples spiked with 2 µg P ml^{-1}. However, correction for the re-equilibration of isotopic P was not performed as the length of incubation between addition of isotope and sampling was 28 days and any measurable equilibration of specific activity between the microbial and soil solution specific activities was assumed to have already occurred (Oehl *et al*., 2001).

The activity of ^{32}P in soil extracts was measured by Cherenkov counting, while the equivalent ^{33}P activity was determined with the aid of a scintillant (ReadySafe™, Beckman Coulter, Fullerton, CA, USA) at a ratio of 1:4 (sample:scintillant). Where necessary, free phosphate was removed from isotopically labelled organic and colloidal P, extracted from solutions, by isobutanol and acidified molybdate (Jayachandran *et al*., 1992). Activities were corrected to the date of labelling and calculated as either Bq g^{-1} soil or Bq μg^{-1} P (specific activity). Differences in specific activity due to plant growth were determined by comparison of soil samples from planted treatments with those from unplanted controls.

The use of labelled P by plants was determined by the *L*-value (Larsen, 1952), which measures the specific activity of isotopic P accumulated in shoots. This measure is considered to be equivalent to the specific activity of the plant-available pool in soils (Bühler *et al*., 2003) and gives an indication of the plant's ability to acquire P from less isotopically exchangeable pools. Individual plants were grown in triplicate for 28 days in 450 g of isotope-labelled soil that had been allowed to incubate for 28 days prior to planting. The phosphorus content and radioactivity of shoots were determined on a representative sample of 0.1 g of oven-dried (70°C for 48 hours) shoot material that was digested in 5 ml of 18 M H_2SO_4 at 360°C for

20 minutes in the presence of an excess of 30% H_2O_2 (Heffernan, 1985). Total phosphate content of the digests was determined by reaction with malachite green, and radioactivity was measured by scintillation counting. The *L*-values (μg P g^{-1} soil) were derived from parameters set out for strong P-fixing soils by Bühler *et al.* (2003), which account for initial seed P content.

Statistical analysis

All data are presented as the means of between 3 and 12 replicates, and error bars represent one standard error either side of the mean. Significant differences were established by analysis of variance (ANOVA) and treatment means were compared by least significant differences at $P = 0.05$. Skewed data (all NMR data, any percentages, phosphatase activity in subterranean clover exudates and change in NaOH-Pi in the rhizosphere of subterranean clover) were transformed to natural logarithms before analysis.

Results

Characterization of soil organic phosphorus

Both soils were acidic and contained little plant available P (1.2–1.5 μg P g^{-1} soil) as determined by anion-exchange resin (Table 1). Extraction with 0.1 M NaOH recovered 540.6 and 166.5 μg total P g^{-1} soil, respectively, from the Robertson and Khwisero soils (NaOH-Pt; Table 2). The greater P content in the Robertson soil was indicative of its P-fertilizer history. Extracts of both soils were dominated by organic P (485 and 146.5 μg P g^{-1} soil, respectively), which accounted for at least 90% of the P extracted by 0.1 M NaOH.

Rhizosphere phosphatase activity and depletion of NaOH-Po

Soil acid phosphatase activity was significantly greater (130.7 and 218.8 pKat g^{-1} soil, $P < 0.05$) in the rhizosphere of tithonia and crotalaria, respectively, compared with unplanted control soil (Figure 1A). In contrast, phosphatase activity in the rhizosphere of maize was not different from that in the unplanted control.

Coincident with enhanced acid phosphatase activity, NaOH-Po in rhizosphere soil from the agroforestry species decreased ($P < 0.05$) by between 22.8 and 35.5 μg P g^{-1} soil relative to unplanted soil (Figure 1B). The NaOH-Po concentration in rhizosphere soil from maize was not significantly different to the unplanted control.

Transgenic subterranean clover expressing the *ex::phyA* gene exuded 60-fold more phytase per day to the rhizosphere

Table 2 Total, inorganic and organic P (Pt, Pi and Po) content in 0.1 M NaOH and 0.25 M NaOH + 0.05 M EDTA extracts from Robertson and Khwsiero soils

Extract	P fraction	Robertson/μg P g^{-1} soil	Khwisero/μg P g^{-1} soil
NaOH	Pi	55.6 (1.2)	20.0 (1.4)
	Po	485.0 (12.2)	146.5 (3.3)
	Pt	540.6 (13.2)	166.5 (4.4)
NaOH-EDTA	Pi	191.6 (2.7)	39.9 (1.1)
	Po	691.5 (25.8)	50.9 (10.7)
	Pt	883.0 (27.5)	90.8 (11.6)

Values are the mean of three replicates and values in parenthesis indicate one standard error.

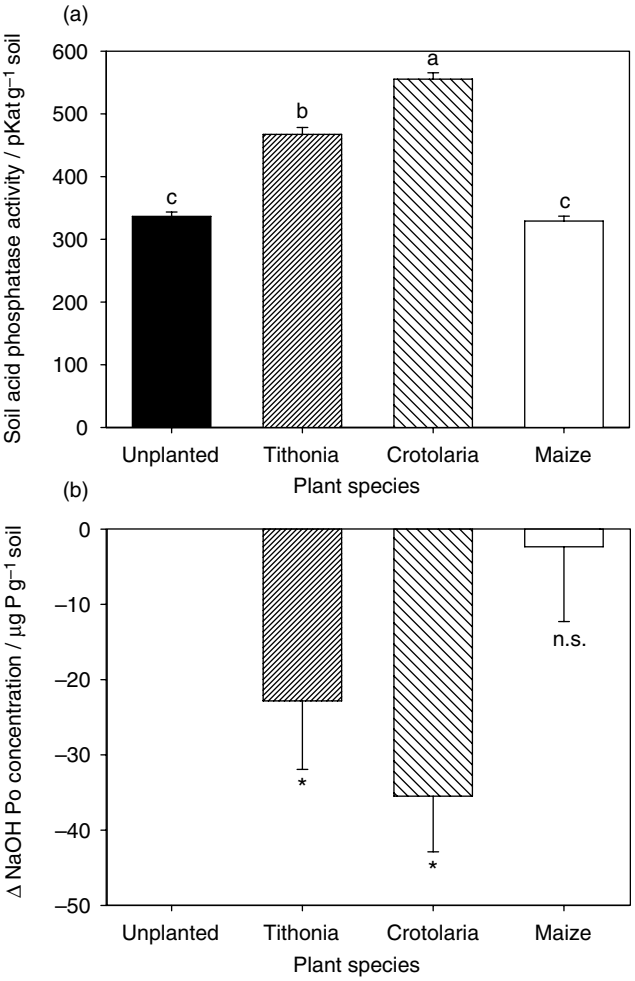

Figure 1 (A) Soil acid phosphatase (pKat g^{-1} soil) and (B) change in NaOH-Po concentration (μg P g^{-1} soil) of rhizosphere soil (0–1 mm samples) for tithonia, crotolaria and maize when grown in the Khwisero Oxisol. Values for ΔNaOH-Po are shown relative to the unplanted control, which had a NaOH-Po content of 155 μg P g^{-1} soil. Data are the mean of five replicates and error bars show one standard error. For panel A, columns with different letters are significantly different (LSD, $P < 0.05$), and for panel B, * indicates significant difference ($P < 0.05$), compared with the unplanted control, and n.s. indicates no significant difference.

than the wild-type control and up to sixfold more phytase than either monoesterase or diesterase (Figure 2A). Exudation of phytase by wild-type plants was negligible (less than 1 nKat g^{-1} dry root day^{-1}) and was significantly less ($P < 0.05$) than the other two phosphatase activities. The exudation of both monoesterase and diesterase activities by the transgenic clover (6–8 nKat g^{-1} dry root day^{-1}), was not affected by the expression of the *ex::phyA* gene.

Transgenic *ex::phyA* plants depleted three times more NaOH-Po from the rhizosphere than the wild-type control, which was significantly different ($P < 0.05$) to the unplanted control (Figure 2B). However, the depletion of P (24.2 μg P g^{-1} soil) from the NaOH-Po fraction was comparable with an equivalent increase ($P < 0.05$) in the NaOH-Pi.

By comparison, no significant changes occurred in either the NaOH-Pi or NaOH-Po pools in rhizosphere soil from the wild-type control (Figure 2B). In addition, both the *ex::phyA* transgenic and wild-type plants produced negligible changes in the fraction of NaOH-Po that was hydrolysable by phosphatase, although this pool of P was relatively small (7.8 μg P m^{-1} root g^{-1} dry soil), and for either plant line was not significantly different from the unplanted control.

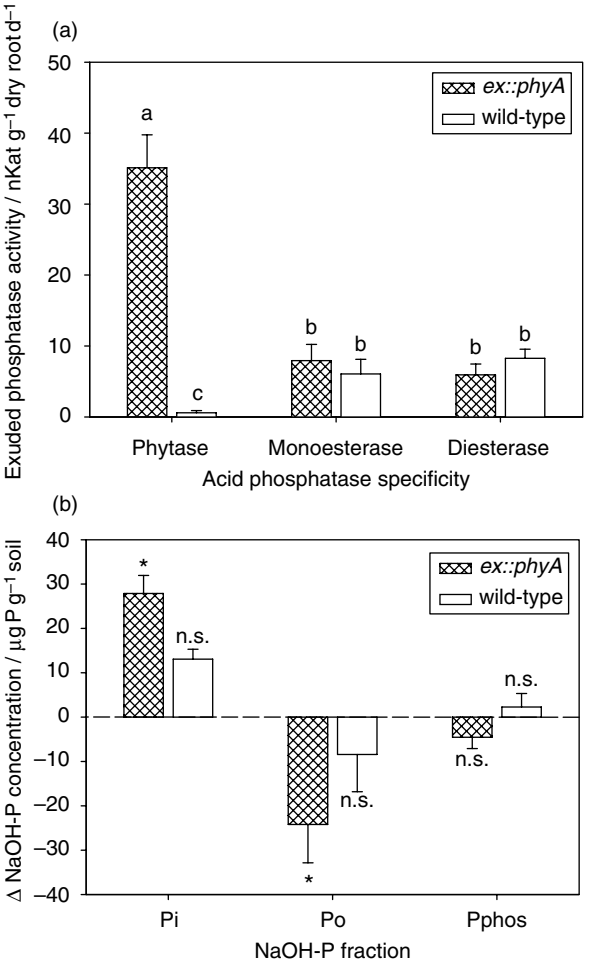

Figure 2 (A) Exuded phosphatase activities (nKat g^{-1} dry root day^{-1}) measured as phytase, monoesterase and diesterase and (B) change in NaOH-P concentration (μg P g^{-1} soil) of rhizosphere soil (0–1 mm sample) for transgenic subterranean clover expressing a fungal phytase gene (*ex::phyA*) and wild-type plants grown in the Robertson Oxisol. Values for ΔNaOH-P are shown relative to an unplanted control, which contained 61.4 (NaOH-Pi), 504.9 (NaOH-Po) and 7.8 (NaOH-Pphos) μg P g^{-1} soil. Data are the means of five replicates and error bars show one standard error. For panel A, columns with different letters are significantly different (LSD, $P < 0.05$), and for panel B the * indicates significant difference ($P < 0.05$), compared with the unplanted control, and n.s. indicates no significant difference.

Changes in rhizosphere phosphorus fractions determined by solution ^{31}P NMR spectroscopy

The presence of EDTA in a stronger NaOH extract (0.25 M) resulted in a lower proportion of organic P being removed (56–78%, Table 2) than with 0.1 M NaOH. Furthermore, the relatively small proportion of total soil P extracted by this procedure is unusual given that the NaOH-EDTA is reported to be a quantitative extractant for organic P. Despite this, in both soils the NaOH-EDTA extractable P was dominated by phosphate monoesters (Table 3), which accounted for 46% and 56% of the extracted P in the Robertson and Khwisero soils, respectively. Orthophosphate also constituted a large proportion of the P extracted from both soils, whereas phosphate diesters contributed less than 8% of the total extractable P. These three groups of compounds accounted for more than 90% of the P extracted, while there were only trace amounts of pyrophosphate measured.

Changes in the concentrations of the three major groups of P compounds in the rhizosphere of tithonia and maize grown in the Khwisero soil are shown in Figure 3. A decline in the concentration of both orthophosphate and phosphate monoesters relative to bulk soil was evident in the rhizosphere of tithonia, with a maximum decrease of 6.0 and 10.2 μg P g^{-1} soil, respectively, occurring at a distance of 2 mm from the root mat (Figure 3). However, directly adjacent to the plant root mats (0.5 mm away) concentrations of monoesters

Table 3 Concentration (μg P g^{-1} soil) and percentage of total P in pools measured by solution ^{31}P NMR spectroscopy of NaOH-EDTA extracts of Robertson and Khwisero soils

	Robertson		Khwisero	
Functional group	μg P g^{-1} soil	% total P	μg P g^{-1} soil	% total P
Orthophosphate	364.7 (11.3)	41.3	30.0 (2.1)	33.0
Monoester	403.1 (12.5)	45.7	51.8 (3.5)	56.1
Diester	65.3 (2.0)	7.4	4.0 (0.3)	4.4

Data are the mean of three replicates and data in parenthesis represent one standard error.

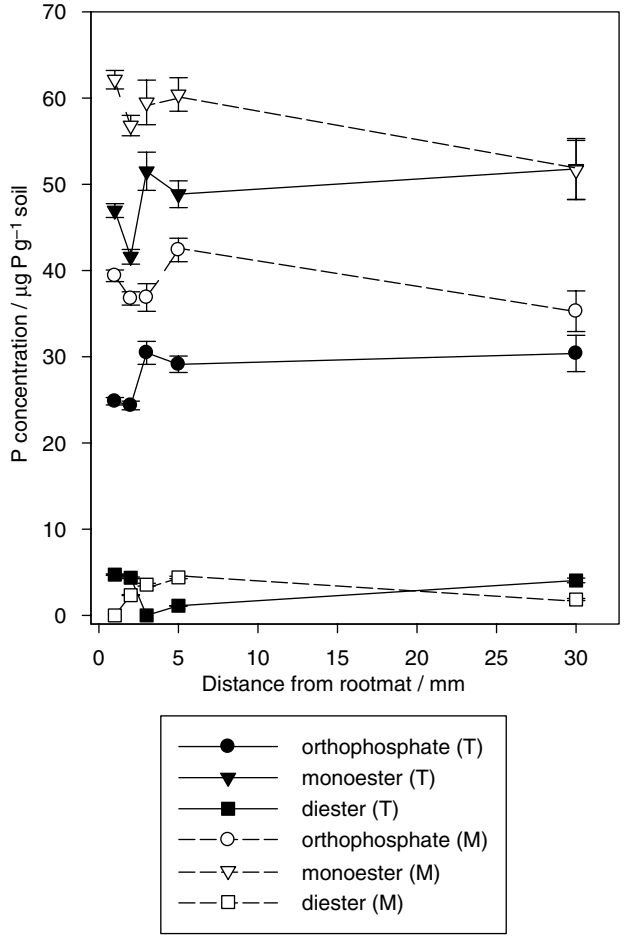

Figure 3 Orthophosphate, monoester and diester P concentration (µg P g^{-1} soil) in NaOH-EDTA extracts of the Khwisero Oxisol measured by ^{31}P-NMR. Shown are P concentrations of pools in the rhizosphere of tithonia (T) and maize (M) sampled at various distances (0.5–5 mm) from the root mat compared with bulk soil (30 mm sample). Data are the mean of three replicates and error bars show one standard error either side of the mean.

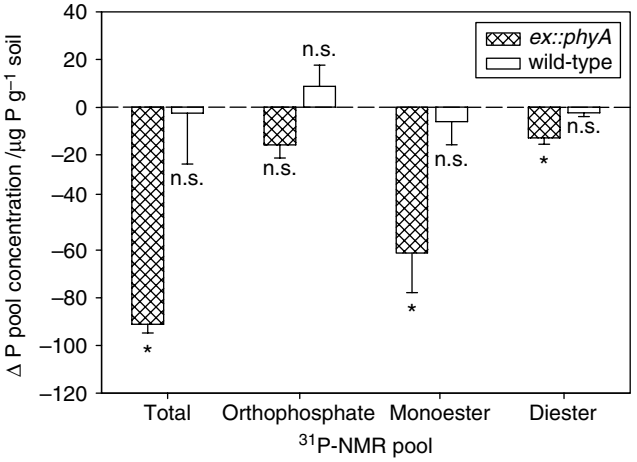

Figure 4 Changes in P concentration of NaOH-EDTA extracts of the Robertson Oxisol, with plant growth, measured by ^{31}P-NMR. Values of ΔP (µg P g^{-1} soil) are shown for transgenic subterranean clover expressing a fungal phytase gene (*ex::phyA*) and wild-type plants relative to an unplanted control. The control soil contained 883 (total-P), 365 (orthophosphate-P), 403 (monoester-P) and 65 (diester-P) µg P g^{-1} soil. Data are the mean of three replicates, and error bars show one standard error. Significant differences ($P < 0.05$) compared with the unplanted control are indicated by *, and n.s. indicates no significant difference.

increased. There were no similar decreases of orthophosphate and monoesters for maize and there was no major change in the concentration of phosphate diesters for either species (Figure 3).

Growth of wild-type subterranean clover in the Robertson soil did not change the concentration of any of the three major groups of P compounds in the rhizosphere (Figure 4). In contrast, significant declines ($P < 0.05$) in the total P extracted by NaOH-EDTA were observed for plants that expressed *ex::phyA*. This change in soil P concentration was associated with a significant depletion of both phosphate monoester and diester compounds. No differences in orthophosphate concentration were evident in the rhizosphere of either transgenic or wild-type clover plants and these were not different from the unplanted control (Figure 4).

Dynamics of isotopic phosphorus in the Khwisero and Robertson soils

Between 19% and 28% of added isotope was recovered from the soils, 28 days after addition, by sequential extraction with anion resin strips followed by NaOH. Of the labelled P added, less than 2% was found in the resin pool in each soil, and significantly greater amounts (11–12 times more, $P < 0.05$) were present in the NaOH extractable fraction (Table 4). In the Khwisero soil, the labelled P recovered was associated predominantly with the NaOH-Pi fraction (55%, $P < 0.05$), whereas in the Robertson soil 76% ($P < 0.05$) of the label was found in the NaOH-Po fraction (Table 4). Importantly, 40% of the label in the NaOH-Po fraction (7.2% of the total) of the Robertson soil was amenable to hydrolysis by phosphatase (Table 4). Furthermore, in this soil microbial-P accounted for 2% of the isotope added, which was equivalent to that found in the resin-extractable P.

Despite the small recovery of radioactivity in the resin pool for both soils, the specific activity of the P within these pools was significantly greater ($P < 0.05$) than that in NaOH-extractable fractions. Greater specific activities in all fractions for the Khwisero soil (at least 10-fold, Table 4) were attributable to the difference in activity of the isotopes added (^{32}P and ^{33}P) to the two soils (Oberson *et al.*, 2001) and to the large difference in extractable P contents between the two soils. In the Robertson soil, specific activity in the NaOH-extractable P was similar in both the Pi and Po fractions, but was

Table 4 Activity (Bq g^{-1} soil), specific activity (Bq μg^{-1} P) and recovery (% of ^{32}P or ^{33}P added) in different inorganic, organic, phosphatase-labile or total P fractions (Pi, Po, Pphos, Pt, respectively), 28 days after addition of ^{33}P or ^{32}P as carrier free solutions to the Robertson and Khwisero soils, respectively

	Robertson			Khwisero		
P fraction	Activity/ Bq g^{-1}soil	Specific activity/ Bq μg^{-1} P	Recovery/ % ^{33}P added	Activity/ Bq g^{-1}soil	Specific activity/ Bq μg^{-1} P	Recovery/ % ^{32}P added
Resin-Pi	122.9 (14.1)	37.4 (3.5)	2.0 (0.2)	491.2 (8.5)	358.0 (14.1)	1.6 (0.0)
Microbial-P	124.9 (13.1)	14.5 (2.0)	2.0 (0.1)	n.d.	n.d.	n.d.
NaOH-Pi	313.7 (29.7)	3.0 (0.3)	5.1 (0.5)	2926.8 (18.0)	121.2 (0.9)	9.5 (0.1)
NaOH-Po	1115.0 (65.6)	3.0 (0.2)	18.1 (1.1)	2440.5 (33.0)	31.8 (0.5)	7.9 (0.1)
NaOH-Pphos	441.6 (30.7)	4.0 (0.5)	7.2 (0.5)	n.d.	n.d.	n.d
NaOH-Pt	1428.7 (93.8)	3.0 (0.2)	23.7 (1.5)	5367.3 (41.4)	53.1 (0.6)	17.4 (0.1)
LSD ($P < 0.05$)	162.3	4.7	2.6	78.7	19.8	0.3

Data are the means of 12 replicates with standard errors in parenthesis. For each parameter, the value of the least significant difference LSD ($P < 0.05$) is also shown. n.d. = not determined.

significantly greater ($P < 0.05$) in the pool of Po that was amenable to hydrolysis by phosphatase (Table 4). Specific activity was also significantly greater ($P < 0.05$) in the microbial-P. For the Khwisero soil, specific activity in the NaOH extractable P was initially greatest ($P < 0.05$) in the Pi fraction (Table 4). However, the specific activities of the NaOH extractable fractions in the Khwisero soil changed with further incubation (from 28 to 56 days), whereby the specific activity of the NaOH-Pi declined with a corresponding increase in NaOH-Po (Table 5). In contrast, specific activity in the NaOH-Pi and NaOH-Po fractions of the Robertson soil did not change over this time period (Table 5).

L-values and changes in specific activity of NaOH-P fractions after plant growth

The amount of P potentially accessed by plants grown in the two soils was estimated by *L*-values. Growth of tithonia produced a significantly greater *L*-value ($P < 0.05$) than either crotalaria or maize in the Khwisero soil, being estimated to have access to between 216 and 263 μg more P g^{-1} soil (Table 5). Likewise, when grown in labelled Robertson soil, transgenic subterranean clover that expressed *ex::phyA* had a greater *L*-value ($P < 0.05$) than wild-type plants, with an estimated access to an additional 49 μg P g^{-1} soil (Table 5).

Table 5 *L*-value (μg P g^{-1} soil) and specific activity of inorganic, organic and phosphatase-labile P fractions (Pi, Po and Pphos, respectively) extracted with NaOH after 28 days plant growth (56 days after labelling) in isotope-labelled Khwisero (^{32}P) and Robertson (^{33}P) soils

		NaOH-Pi		NaOH-Po		NaOH-Pphos	
Plant species	*L*-value/ μg P g^{-1} soil	Specific activity/ Bq μg^{-1} P	% change from no plant control	Specific activity/ Bq μg^{-1} P	% change from no plant control	Specific activity/ Bq μg^{-1} P	% change from no plant control
Khwisero							
No plant	–	87.7 (1.2)		90.9 (3.3)		n.d.	
Tithonia	619.3 (54.1)	90.1 (1.8)	3	202.2 (31.0)	123	n.d.	–
Crotalaria	402.9 (60.1)	91.5 (1.5)	5	146.6 (4.5)	61	n.d.	–
Maize	356.4 (35.7)	97.0 (2.7)	11	155.3 (6.0)	71	n.d.	–
LSD ($P < 0.05$)	109.9	5.4	12	47.2	61		
Robertson							
No plant	–	3.0 (0.3)		3.0 (0.2)		4.0 (0.5)	
Clover (*ex::phyA*)	173.1 (15.6)	3.3 (0.1)	12	2.8 (0.2)	−10	2.8 (0.6)	−30
Clover (wild-type)	124.1 (12.6)	3.1 (0.2)	7	4.1 (0.5)	44	3.7 (0.5)	−7
LSD ($P < 0.05$)	40.0	0.6	12	1.0	20	1.6	20

Data are the means of 12 replicates with standard errors in parenthesis. For each parameter value of least significant difference LSD ($P < 0.05$) are also shown. n.d. = not determined.

With the exception of soil from the rhizosphere of transgenic clover, the specific activity of the NaOH-Po fraction was increased by between 44 and 123% ($P < 0.05$) in both soils in the presence of plants (Table 5). This suggests that the plants either hastened immobilization of the isotope into organic forms (compared with unplanted soil) or, consistent with their general ability to deplete NaOH-Po (Figures. 1 and 2), accessed non-labelled forms of P from this pool. In contrast, in the rhizosphere of transgenic subterranean clover that released phytase, the specific activity of NaOH-Po remained unchanged (Table 5). Moreover, the specific activity of the portion of NaOH-Po hydrolysable by phosphatase (NaOH-Pphos) was reduced by 30% ($P < 0.05$) in the Robertson soil by growth of transgenic plants.

Discussion

Phosphatase activity and depletion of organic P

Organic P extracted by NaOH is a potentially important form of available P in unfertilized soil (Oberson *et al*., 1999). Several studies have shown that depletion of organic P in the rhizosphere of plants (including NaOH-Po) is associated with enhanced phosphatase activity (Chen *et al*., 2002; George *et al*., 2002a). However, the contribution of rhizosphere phosphatases to improved P-nutrition of plants remains poorly understood. In particular, the importance of specific phosphatases in relation to the utilization of defined forms of organic P in soil is largely unknown (Richardson *et al*., 2005). Here we provide further evidence that enhanced phosphatase activity in the rhizosphere is coincident with a depletion of NaOH-extractable organic P. However, such declines may not be indicative of improved P nutrition of plants. For example, the potential of transgenic plants that exude phytase has been shown to be compromized when grown in soils (George *et al*., 2004). Although this may be associated with a number of confounding factors (George *et al*., 2005b), reactions of mineralized phosphate with soil constituents cannot be discounted. Rapid sorption of inorganic P and small concentrations of Pi in soil solution are characteristic of Oxisols (Sen-Tran *et al*., 1988). Indeed, in the strong P-sorbing Oxisols studied here, the depletion of NaOH-Po in the rhizosphere of transgenic plants was matched by an increase in NaOH-Pi, which might not be a readily available form of P for plant uptake.

Depletion of phosphate monoesters

Analysis by solution ^{31}P NMR spectroscopy showed that phosphate monoesters were the predominant form of organic P extracted by NaOH-EDTA in both soils, whereas phosphate diesters constituted only a small proportion of the extracted P, which is typical for Oxisols and other strongly weathered soils (Adams & Byrne, 1989). Although degradation of diesters to monoesters can occur during NaOH-EDTA extraction (Turner *et al*., 2003c), it is apparent that phosphate monoesters, if made fully available, could be a major source of organic P for plant nutrition in these soils. Indeed, phosphate monoesters were specifically depleted in the rhizosphere of tithonia and transgenic subterranean clover, both of which had enhanced phosphatase activities. In contrast, control plants did not produce a significant depletion in any of the P forms identified by ^{31}P NMR. Specific depletion of phosphate monoesters in the rhizosphere of other plant species has similarly been observed (Chen *et al*., 2004). Phosphate monoesters primarily occur as high-order inositol phosphates with lesser amounts of sugar phosphates and mononucleotides (Turner *et al*., 2003b). Significantly, Chen *et al*. (2004) demonstrated that a large proportion of the organic P depleted by radiata pine roots was as phytate, and attributed this to enhanced production of acid phosphatases by ecto-mycorrhizae. It may therefore be speculated that the depletion of phosphate monoesters observed here, particularly by transgenic phytase exuding subterranean clover, indicates mineralization of phytate, although further work to confirm this is required.

Consistent with increased depletion of phosphate monoesters from the rhizosphere of the agroforestry species and transgenic subterranean clover, *L*-values were greater for tithonia and the transgenic clover, relative to maize and wild-type clover, respectively. This suggests that plants with enhanced phosphatase activities, also had access to a larger pool of soil P. However, an exception to this was crotalaria, which did not have a significantly larger *L*-value than maize, but did have enhanced rhizosphere phosphatase activity. The difference between the transgenic subterranean clover that expressed *ex::phyA* and wild type plants is of particular significance, as these plants essentially differ by only one gene, suggesting that the ability to exude phytase to the rhizosphere gave the plants access to a significantly larger pool of P.

Immobilization of phosphorus into organic forms and differential depletion by plants

Most of the radioactive P added to the soils was not accounted for by the extraction procedures used, indicating a large flux of P into undefined forms. Magid *et al*. (1996) suggested that approximately 50% of organic P remains un-extractable, and might not be biologically unavailable. Of the recovered radioactive P, *c*. 90% (*c*. 19–26% of that added) was found in NaOH-extractable P fractions after 28 days incubation. Moreover, between 42 and 65% of the P recovered was found in organic P forms. Other studies also demonstrated rapid immobilization of labelled-P into organic P fractions in a range of soils. For example, *c*. 10% of radioactive P was found in organic pools within 2 days by Friesen & Blair (1988) and Oberson *et al*. (2001), while up to 40% of added P occurred in organic forms after 28 days in studies by Blair *et al*. (1976). Whilst accumulation of P into organic forms may, to some extent, be attributed to adsorption of orthophosphate to

organic matter (Harter, 1969), or due to incomplete separation during isobutynol extraction (Jayachandran *et al.*, 1992), these results indicate that a significant proportion of the P added to the soils was immobilized into organic fractions by soil microorganisms. Immobilization of P by microorganisms in the Robertson soil is verified by the presence of labelled P within the microbial-P pool (7% of the P recovered), and the significant proportion (40%) of the NaOH-Po that was hydrolysable by phosphatase, validating that the P was associated with ester bonds. Furthermore, the quantity of labelled-P found in the NaOH-Po fraction compared with that in microbial-P indicates that the flux of P through the microbial biomass in the Robertson soil was rapid, and that complete turnover might have occurred several times.

The role of soil microorganisms in immobilization of P and rapid turnover of microbial P has been demonstrated previously (Oehl *et al.*, 2001). Addition of labelled-P to the soils at the time of rewetting would also account for a large flux of P into organic forms, as this would coincide with a significant flush in microbial activity (Magid *et al.*, 1996). Large amounts of isotopic P (up to 70% of that added) have been found in the biomass after microbial activity was stimulated by wetting and drying of soil and in the presence of carbon and nitrogen (Oehl *et al.*, 2001; Bünemann *et al.*, 2004). Moreover, microbial immobilization of inorganic P has been implicated in the increase in organic P content of soils following fertilization, which is particularly apparent in soils under pasture (Condron & Goh, 1989; Tiessen *et al.*, 1992).

The specific activity of the NaOH-extractable Po was, with the exception of transgenic subterranean clover, increased (44–124%) in soils with plant growth as compared with unplanted controls. This indicates that either the presence of the plants accelerated the incorporation of labelled-P into organic fractions or the plants preferentially accessed endogenous (un-labelled) forms of organic P, as compared with recently immobilized organic P. This was particularly evident for tithonia and crotalaria, which also simultaneously depleted the NaOH-Po fraction. It also suggests that the plants have a limited ability to deplete P that was recently immobilized by the microbial biomass. Interestingly, transgenic subterranean clover, which expressed a fungal phytase gene, was the only plant that did not increase the specific activity of the NaOH-Po fraction. This suggests that plants that express microbial genes have access to recently immobilized P and that at least a portion of the recently immobilized P occurs as phytate. Synthesis of *myo*-inositol hexaphosphate in soil by microorganisms has been reported by Caldwell & Black (1958) and different isomers of phytate (e.g. *scyllo*-inositol hexaphosphate) are thought to occur exclusively in soil (Turner & Richardson, 2004). It is presently unknown whether these isomers are a direct product of microbial synthesis or are a result of epimerization reactions (Cosgrove, 1976). However, given that plants have limited capacity to utilize P from phytate directly (Hayes *et al.*, 2000b, Richardson *et al.*, 2001b), active synthesis of phytate by microorganisms in soil may provide them with a competitive advantage over plants with regard to utilization of immobilized organic P. This may be substantiated by the range of soil microorganisms that promote plant growth and P nutrition that have been identified and shown to possess phytase activity (Koide & Kabir, 2000; Richardson *et al.*, 2001b; Idriss *et al.*, 2002; Yadav & Tarafdar, 2003), while there is also evidence that microbial phosphatases are more efficient at hydrolysing soil organic P forms, including phytate, than equivalent plant enzymes (Tarafdar *et al.*, 2001).

Conclusions

Depletion of NaOH Po occurred in two P-deficient Oxisols and was associated with enhanced extracellular acid phosphatase activity in the rhizosphere of different plants. Depletion of organic P was most evident for tithonia and a transgenic clover that released extracellular phytase. Analysis by solution ^{31}P NMR spectroscopy of NaOH-EDTA extracts indicated that organic P depleted from the rhizosphere was primarily from the phosphate monoester pool. Plants with enhanced extracellular phosphatase activity also appeared to have access to a larger proportion of P from the soils as assessed by increased *L*-values over control plants. Radioactive P added to the soils was rapidly immobilized into the NaOH-Po fraction, and the specific activity of this fraction, with the exception of the transgenic clover, was greater in the presence of plants than in unplanted controls. Transgenic subterranean clover that expressed a microbial phytase gene appeared to have greater access to recently immobilized P, whereas plants expressing endogenous phosphatases utilized un-labelled soil organic P to a greater extent. Collectively, these results indicate that the enhancement of phosphatase activity in the rhizosphere of plants is implicated in the depletion of organic soil P, most notably orthophosphate monoesters. The data also provide some evidence that there is exclusivity in the pools of organic P utilized by plants and microorganisms, which has implications for competition for P in the rhizosphere.

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