

# Organic Anions Facilitate the Mobilization of Soil Organic Phosphorus and Enhances Its Subsequent Lability To Phosphatases

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## Research Article

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# Abstract

## Purpose

Organic anions commonly released from plant roots are widely reported to mobilize soil phosphorus (P). We characterized soil organic P that was mobilized by organic anions and assessed its amenability to hydrolysis by phosphatase enzymes.

## Methods

Six soils differing in organic P content were extracted with citrate, malate or oxalate solutions and incubated with preparations of phosphomonoesterase, phosphodiesterase, or phytase. Organic P compounds present in these extracts were putatively identified and quantified with solution  $^{31}\text{P}$  NMR spectroscopy and the enzyme-labile P fractions were assessed by changes in molybdate reactive P (MRP) concentration.

## Results

Organic P mobilization varied markedly among the organic anions. Extraction with 10 mM citrate was most effective and extracted 7.8-fold more total P than the water controls across all soils. Approximately 95% of the extracted P was non-MRP. The organic anions increased both the amount of P extracted and the proportion of the total extracted P that was phosphatase labile. Phytase was generally the most effective enzyme with up to 60% of the total non-MRP being amenable to hydrolysis by phytase across all extracts. The presence of inositol hexakisphosphates in the extracts, as well as other forms of organic P including nucleic acids and phospholipids, was verified by  $^{31}\text{P}$  NMR with concentrations dependent on both organic anion and soil type.

## Conclusion

The combination of organic anions and phosphatases represents a key mechanism by which plants and microorganisms can enhance the bioavailability of soil P. This has important implications for understanding P dynamics in natural and managed ecosystems and for ongoing efforts to improve the P-use efficiency of agricultural plants.

## Introduction

Phosphorus (P) is essential for plant growth and function and is often present in soils at concentrations that are deficient for plant growth. Not only is P important for driving plant productivity and plant diversity in natural systems (Lambers et al. 2015; Turner et al. 2018; Wassen et al. 2005; Zemunik et al, 2015), it is often a limiting nutrient for productivity in many managed agricultural systems. This is particularly true for the ancient and highly weathered soils in Australia, which are among the most naturally P-deficient in the world. The adaptation of plants to soils with low levels of plant-available P has been studied extensively across a wide range of ecosystems. Indeed, research conducted in the Lambers' laboratory at the University of Western Australia has been instrumental in helping to understand how native plants and crops adapt and respond to P-limited environments, and especially the role of root exudates in mobilizing soil P (Lambers et al. 2002; Lambers et al. 2008; Lambers et al. 2011; Lambers et al. 2015). The pivotal articles and reviews published in *Plant and Soil*, including many by Lambers and his team, have revealed much about

the chemistry of soil P (McLaughlin et al. 2011; Weaver and Wong 2011) and led to potential strategies for managing fertilizer use in agriculture (Richardson et al. 2011a; Simpson et al. 2011; Mclvor et al. 2011).

In addition to the physiological and morphological changes to roots (Lynch and Brown 2001; Richardson et al. 2009), P deficiency is often associated with an increased release of organic anions (also referred to as carboxylates) from roots into the rhizosphere. Organic anions increase the availability of P in soil for plant uptake, especially under conditions of P deficiency. The increased rates of organic anion release in response to P deficiency have been described in many native Australian plant species that develop cluster roots (e.g., the Proteacea: Roelofs et al. 2001; Lambers et al. 2002; Lambers et al. 2015) as well as in a wide range of agricultural species with and without cluster roots, including pasture legumes and grasses, grain legumes and cereals (Nuruzzaman et al. 2006; Pearse et al. 2006; Wang et al. 2013; Wouterlood et al. 2004; Kidd et al. 2018). The release of citrate and malate from the specialized cluster roots of white lupin (*Lupinus albus* L.) has been studied in greatest detail (e.g., Veneklass et al. 2003; Shane et al. 2008; Wang et al. 2013). In this species, citrate concentrations of 5 to 50  $\mu\text{mol g}^{-1}$  soil (corresponding to expected soil solution concentrations of 1 to 10 mM) have been reported around the cluster roots (Dinkelaker et al 1989; Gerke et al. 1994).

Various studies using soil-grown plants have shown that organic anions released from roots can increase the concentration of the plant-available P (as orthophosphate) in the soil solution and many examples (e.g., as outlined in references above) have been reported. The effectiveness of different organic anions in mobilizing P, however, is highly dependent on soil type and the form of P either present in soil or provided to the plants. Plant species and genotype is also important because it determines the type and amount of each organic anion released and the dynamics of that release (Wang and Lambers 2020). More direct examples of P mobilization by organic anions have extracted soils *in vitro* with different organic anion solutions (Khademi et al. 2009; Ryan et al. 2014). For example, using seven contrasting Australian soils differing in pH and P content, Ryan et al. (2014) showed that both 1 mM and 10 mM citrate increased the concentration of orthophosphate, with greater mobilization occurring in acidic soils than for alkaline-calcareous soils. Citrate was able to mobilize P from both the inorganic and organic P fractions and this varied markedly between soils. Organic anions are proposed to mobilize P from sparingly-available pools of soil P through a number of processes including: (i) competition for sorption sites (i.e., desorption of orthophosphate), (ii) ligand-promoted mineral dissolution or exchange reactions with cations of iron ( $\text{Fe}^{2+/3+}$ ), aluminum ( $\text{Al}^{3+}$ ) or calcium ( $\text{Ca}^{2+}$ ), or (iii) interactions with soil microorganisms. For the latter process, microorganisms may directly release organic anions themselves, or mobilize P by promoting root growth or through biomass turnover (Richardson et al. 2011a; Richardson et al. 2011b; Wang and Lambers, 2020). Organic anion release is also commonly associated with release of protons ( $\text{H}^+$ ) which can induce local regions of acidification that further influence the desorption and diffusion of P in soil (Barrow et al. 2017). Local acidification may also promote the solubilization of precipitated pools of Ca-P prevalent in alkaline soils (Jones 1998).

Organic P in soil typically accounts for at least 50% of the total soil P and forms of organic P can be identified and quantified using solution  $^{31}\text{P}$  NMR spectroscopy (Turner et al. 2002; McLaren et al. 2019a). Most of the organic P in soil occurs as phosphomonoesters (e.g., inositol hexakisphosphates, lower order inositol phosphates and other sugar-phosphates), phosphodiester (e.g., phospholipids and nucleic acids, primarily as DNA) and a large pool of poorly characterized monoester compounds (McLaren et al. 2019a). Of these, the *myo* and *scyllo* stereoisomers of inositol hexakisphosphate are the most prevalent in many soils (Turner 2007). Similar to orthophosphate anions, inositol hexakisphosphates are readily adsorbed in soils and can also be precipitated with  $\text{Al}^{3+}$ ,  $\text{Fe}^{2+/3+}$  and  $\text{Ca}^{2+}$  (Celi and Barberis 2005; Jackman and Black 1951; Tang et al. 2006). Inositol phosphates are furthermore complexed within high molecular weight soil organic matter as structurally complex, supra- and macro-molecular monoester material that otherwise remains poorly characterized (Hong and Yamane 1981; McLaren et al. 2019b). Whilst various studies have demonstrated that organic anion extractions can increase the concentration of organic P (Otani and Ae 1999; Hayes et

al. 2000; Wei et al. 2010, Ryan et al. 2014), the identity of the organic P compounds released and their contribution to plant nutrition remains to be further investigated (Richardson et al., 2005; George et al. 2018).

The nature of organic P in soil has been characterized by the lability of extracted P to dephosphorylation by various phosphatase enzymes, which show differing specificity toward mono-ester and di-ester forms of organic P (Hayes et al. 2000; Bünemann 2008; Darch et al. 2016; Jarosch et al. 2019). A meta-analysis by Bünemann (2008) showed that up to 60% of organic P was typically amendable to dephosphorylation across a wide range of soil extracts and water samples, with phytases (i.e., inositol hexakisphosphate phosphohydrolyases) generally showing the greatest release of orthophosphate. Significantly, using two Australian pasture soils, Hayes et al. (2000) showed that up to 40% of the total P extracted by 50 mM citric acid was hydrolyzed by a highly purified phytase, whereas up to 79% was hydrolyzed by a commercially available phytase preparation that exhibited a wider substrate specificity. By contrast, lesser quantities of organic P (<17% and <9%) were dephosphorylated by the commercial phytase in water and 0.5 M sodium bicarbonate extracts, respectively, even though bicarbonate itself extracted three to four times more organic P (Hayes et al. 2000). This indicates a potentially strong interaction between organic anions in the mobilization of organic P substrates and their subsequent lability to phosphatases to release bioavailable P as orthophosphate.

In this study we sought to further investigate the interaction between organic anions and phosphatases in the mobilization of soil organic P and lability of the extracted P to dephosphorylation. We hypothesized that different combinations of organic anions and phosphatases would differentially influence P mobilization across a range of contrasting soil types, and that that greater release of P would occur when specific combinations of organic anion and phosphatase were present as potential functional components of root exudates. To test these hypotheses, we used a range of citrate, malate and oxalate concentrations in combination with commercially available preparations of acid-phosphomonoesterase (PME), phosphodiesterase (PDE) and phytase (PHY) to examine P release from six contrasting agricultural soils. Solution  $^{31}\text{P}$  NMR spectroscopy was used to identify and quantify the presence of phosphomonoester and phosphodiester forms of organic P directly in the extracts. Our findings demonstrate that organic anions both mobilize organic P from soil and render it more labile to dephosphorylation by phosphatases. These results have important implications for the P nutrition of plants and the dynamics of P in soils.

## Materials And Methods

### Soil characterization and analysis

Diverse soils differing in total P and organic P content were collected from the 0 to 10 cm layer of the profile from six sites across central and southern New South Wales and the Australian Capital Territory in 2001 (Table 1). The soils were representative of pasture and crop systems and were classified according to the Australian classification system (Isbell 1996). Large organic matter fragments were first removed from the samples which were then thoroughly mixed, air dried and passed through a 2 mm sieve prior to storage. Subsamples were also pulverized with a puck mill (Labtechnics, Model LM1, Australia).

Table 1  
Classification and properties of soils (0 to 10 cm) used in the study.

Soil	Robertson	Grenfell	Wallaroo	Camden	Greenthrope	Berthong
Location	Southern Highlands NSW	Central West NSW	Hall ACT	Sydney NSW	Central West NSW	Central West NSW
Soil type <sup>a</sup>	Red Ferrosol	Brown Kandosol	Yellow Chromosol	Black Vertosol	Brown Kandosol	Red Kandosol
Land-use	pasture	cropping	pasture	pasture	cropping	cropping
Soil pH (water)	5.58	5.37	5.59	5.61	6.03	6.25
Soil pH (CaCl <sub>2</sub> )	4.90	4.41	4.42	4.87	5.42	5.58
Organic matter <sup>b</sup> (%)	19.9	nd	5.7	9.2	3.3	6.8
Total C <sup>c</sup> (% soil mass)	5.98	2.03	1.73	3.49	1.12	2.28
Total N <sup>c</sup> (% soil mass)	0.54	0.17	0.14	0.31	0.10	0.19
Total P <sup>d</sup> (mg kg <sup>-1</sup> )	2664 (22) <sup>e</sup>	305 (6)	375 (12)	633 (19)	332 (12)	602 (12)
<i>Total P (ignition-extraction) <sup>f</sup></i>						
Total P (mg kg <sup>-1</sup> )	1540.6 (11.7)	175.2 (0.4)	194.7 (1.3)	557.0 (2.5)	238.1 (1.3)	407.5 (3.2)
Inorganic P (mg kg <sup>-1</sup> )	478.7 (2.1)	65.0 (1.6)	68.7 (1.8)	195.2 (3.4)	127.8 (0.7)	218.2 (4.5)
Organic P (%)	68.9	62.9	64.7	65.0	46.3	46.4
<i>Available P (extraction) <sup>g</sup></i>						
Total P (mg kg <sup>-1</sup> )	36.8 (0.8)	31.4 (0.9)	23.3 (0.2)	42.7 (0.6)	27.1 (0.7)	35.1 (0.5)
Inorganic P (mg kg <sup>-1</sup> )	6.3 (0.1)	6.6 (0.1)	3.4 (0.1)	11.0 (0.3)	15.3 (0.2)	20.1 (0.3)
Organic P (%)	82.9	79.0	85.4	74.2	43.5	42.7

<sup>a</sup> Based on Australian Soil Classification system (Isbell, 1996).

<sup>b</sup> Organic matter estimated by loss of ignition (nd = not determined).

Soil	Robertson	Grenfell	Wallaroo	Camden	Greenthrope	Berthong
<sup>c</sup> Total C and N as measured by mass spectrometry.						
<sup>d</sup> Total P as measured by x-ray spectrophotometry ( $n = 2$ ).						
<sup>e</sup> Values in parenthesis (where shown) show 1 standard deviation.						
<sup>f</sup> Ignition and $H_2SO_4$ extraction for measure of total P and inorganic P (Saunders and William, 1955), and by difference organic P ( $n = 3$ ).						
<sup>g</sup> Available P determined by $NaHCO_3$ extraction (Olsen et al., 1954) of total P and inorganic P (as measured by molybdate reactive P) in the extracts and by difference organic P (as being the molybdate un-reactive component) ( $n = 2$ ).						

Samples of sieved soil were analyzed for pH in water and in 0.01M  $CaCl_2$  using a 1:5 soil to solution suspension (Rayment and Lyons 2011). Loss on ignition (LOI) as an indicator of organic matter content was determined on milled samples by loss of mass from whole soil after drying the soils at 105°C and then ignition in a muffle furnace at 550°C for 16 hours. Total C and N content on milled samples was determined by mass-spectrometry gas chromatography (Europa Scientific, Model 20-20). Total P was determined by both X-ray fluorescence (XRF) spectrophotometry and on replicated samples by the ignition extraction procedure of Saunders and Williams as outlined by Olsen and Sommers (1982). Using this procedure total organic P content was determined by difference of ignited (total P) and un-ignited (total inorganic P) samples extracted with 0.5 M  $H_2SO_4$ . Soils were also extracted with 0.5 M  $NaHCO_3$  for 30 mins as a measure of 'plant-available P' (Olsen et al. 1954), which both inorganic (as a measure of 'available' P) and total P measured in the extracts. Total P was determined by acid-persulfate digestion following autoclaving (120 kPa, 121°C; 40 min) of a 1 ml subsample in the presence of 0.6 M  $H_2SO_4$  and 3.3% ammonium persulphate (Schoenau and Huang 1991). In all cases molybdate reactive P (MRP), as a measure of orthophosphate, was determined by direct assay of extracts with malachite green reagent using orthophosphate P standards (Irving and McLaughlin 1990). Molybdate un-reactive P (MUP) was determined by difference of the direct extract MRP with the total P as determined by acid-persulfate digestion. All samples were routinely measured using a 96 well microplate procedure with 80  $\mu$ l of reagent and 200  $\mu$ l of sample at a wavelength of 620 nm.

## Extraction of soils with organic anions.

Soils were extracted with organic anions using a range of concentrations of citrate, oxalate and malate prior to analysis of MRP and MUP (by difference to the total P). Initially the Robertson and Grenfell soils (i.e., as representatives of high and low total soil P content; Table 1), were extracted with water (as control) and 0.4, 1.0, 4.0, 10.0 and 40.0  $\mu$ mol per gram of soil of each organic anion. In each case, seven grams of air-dried soil was equilibrated with a final volume of 28.0 ml of organic anion solution that had been pre-adjusted with potassium hydroxide to the water-pH of the soil (i.e., pH 5.6 and pH 5.4 for the Robertson and Grenfell soils, respectively; Table 1). Organic anion solutions were prepared from stock solutions and added to soil ( $\mu$ mol  $g^{-1}$ ) by dilution to achieve final soil solution extraction concentrations of 0, 0.1, 0.25, 1.0, 2.5 and 10.0 mM. All six soils were similarly extracted with water and 1.0 and 10 mM of citrate, oxalate and malate, pre-adjusted in each case to the soil-water pH (Table 1). All extractions were performed in triplicate using 50 ml plastic screw-cap centrifuge tubes. Samples were extracted for 30 min at room temperature ( $\sim 22^\circ C$ ) on an end-over-end shaker at  $\sim 30$  rpm. The samples were subsequently centrifuged for 10 min ( $\sim 8000 g / 5,000$  rpm) and the supernatant solutions recovered. Subsamples of the supernatant were withdrawn with a syringe and immediately passed through 0.22  $\mu$ m filters (MF-Millipore). Samples were then analyzed by the malachite green procedure for

determination of MRP and MUP by difference with total P as previously outlined. Checks were conducted using orthophosphate standards to verify that the presence of the organic anions did not interfere with the colorimetric-based assay. Data was analyzed by one-way analysis of variance (ANOVA; Genstat, Release 20.1, VSN International, Hemel Hempstead, UK) with differences between means being determined by least significant difference (LSD) at  $P = 0.05$ . Percentage data was also analyzed by arcsine transformation ( $\%/100$ ) to confirm differences were consistent with untransformed data.

## Enzyme lability of extracted phosphorus

The bioavailability of the MUP in the organic anion extracts for the six soils was evaluated by incubating the extracts with each of three preparations of phosphatases based on combinations of commercially available enzymes (Sigma Chemical Company).

1. Acid phosphomonoesterase (PME) from wheat germ; orthophosphoric-monoester phosphohydrolase Type 1, EC 3.2.3.2 (product P3627).
2. Phosphodiesterase (PDE) from *Crotalus atrox* (crude dried venom); phosphodiesterase I Type IV, EC 3.1.4.1 (product P4506).
3. Phytase (PHY) from *Aspergillus ficuum* (crude); *myo*-inositol-hexakisphosphate 3-phosphohydrolase (i.e, 3-phytase), EC 3.1.3.8 (product P9792).

PME, PDE and PHY were specified by the supplier as 0.8, 0.022 and 3.5 Units of activity  $\text{mg}^{-1}$  solid, respectively. Each enzyme was dissolved in 50 mM MES (2-N-morpholino ethanesulfonic acid) buffer adjusted to pH 5.5 containing 5 mM ethylenediaminetetraacetate (EDTA) and 2 mM  $\text{MgCl}_2$ . The PHY solution was centrifuged at 1500 g for 10 min to remove particulate material and all preparations were then filter-sterilized by passage through a 0.22  $\mu\text{m}$  filter and stored at 4°C for prior to use. Previously we have shown that the PME preparation from Sigma had wide substrate specificity across a range of monoester (and diester) substrates including *myo*-inositol hexakisphosphate, glucose 1-phosphate, ribonucleic acid, adenosine triphosphate, phosphoglyceric acid and the model substrates *p*-nitrophenyl and *bis p*-nitrophenyl phosphate (Hayes et al. 2000). The Sigma PHY likewise was similarly active across these substrates, but importantly showed 47-times greater specific activity (per mg of protein) toward *myo*-inositol hexakisphosphate than PME, and also had greater specific activity toward diesters. As suggested by Hayes et al. (2000), the commercially available PHY preparation likely contained acid phosphatase activity as a contaminant. On this basis, the enzyme incubations in the present work were set up as PME alone, PME+PDE (to ensure that di-esterase activity was not limited) and PHY alone.

Enzyme assays were designed to run to completion with an excess of enzyme being added (Hayes et al. 2000). Each assay consisted of 4.5 ml of water or organic anion extract (from above), 0.25 ml of 0.1 M sodium azide ( $\text{NaN}_3$ , 5 mM final concentration) to prevent microbial interference and 0.25 mL of 40x MES-EDTA incubation buffer and enzyme mixture (PME, PME+PDE or PHY) that provided 0.1 U of activity  $\text{ml}^{-1}$  of final assay (based on the enzyme activities specified by Sigma Chemical Company). This was equivalent to 1.67 nkat of total activity, or 1.5 nkat  $\text{g}^{-1}$  soil, where 1 nkat is defined by capacity to release 1 nmole of substrate  $\text{sec}^{-1}$ . Assays were conducted at a final buffer concentration of 50 mM MES, 5 mM EDTA, 2 mM  $\text{MgCl}_2$  and were incubated for 16 h at 37°C in 25 ml plastic centrifuge tubes.

The concentrations of MRP following enzyme incubation were determined on completion of the reactions using the malachite green assay. The presence of enzymes at the concentrations used did not interfere with MRP detection as confirmed by running orthophosphate standards containing enzyme blanks, and there was no evidence of acid-induced hydrolysis during P detection by molybdate complexation. The amount of MUP hydrolyzed in each soil extract by PME, PME+PDE or PHY was determined by subtracting initial MRP concentrations, with correction for enzyme-free blanks and

any trace amounts of MRP present in the enzyme preparations. The amount of MRP released was also determined as a percentage of the total MUP prior to incubation. The data was analyzed by two-way ANOVA with differences between means being determined by LSD of the interaction (Extract x Enzyme) at  $P = 0.05$ . Percentage data was also analyzed by arcsine transformation to confirm differences.

## Solution $^{31}\text{P}$ NMR spectroscopy

Phosphorus was extracted from 10 g of the Robertson, Grenfell and Camden soils with 40 ml of 10 mM citrate, oxalate or malate (adjusted to pH 5.6 for the Robertson and Grenfell soils, and pH 5.5 for the Camden soil) in 50 ml plastic tubes by end-over-end shaking for 1 hour at room temperature. The extractions were centrifuged for 30 min (8000 *g*) and supernatants filtered through 0.22  $\mu\text{m}$  membrane filters (MF-Millipore). A subsample was taken for determination of total P content following acid persulfate digestion. Extracts were then frozen ( $-20^\circ\text{C}$ ) and freeze-dried (lyophilized) under vacuum at  $-50^\circ\text{C}$ . Separate samples of the Robertson and Grenfell soils were also extracted for NMR analysis with 0.25 M NaOH and 50 mM  $\text{Na}_2\text{EDTA}$  (sodium ethylenediaminetetraacetate) for 16 h at  $22^\circ\text{C}$  (Bowman and Moir 1993).

For NMR spectroscopy, lyophilized extracts ( $\sim 100$  mg) were dissolved in 0.1 ml of deuterium oxide and 0.9 ml of a solution containing 1.0 M NaOH and 100 mM  $\text{Na}_2\text{EDTA}$ , and then transferred to a 5 mm NMR tube. The inclusion of EDTA was essential to yield acceptable spectral resolution. Solution  $^{31}\text{P}$  NMR spectra were obtained using a Bruker Avance DRX 500 MHz spectrometer (Bruker, Germany) operating at 202.456 MHz for  $^{31}\text{P}$ . Samples were analyzed using a 6  $\mu\text{sec}$  pulse ( $45^\circ$ ), a delay time of 2.0 sec and an acquisition time of 0.4 sec with broadband proton decoupling. Approximately 30,000 scans were acquired for each sample. Spectra were plotted with a line broadening of 5 Hz and chemical shifts of signals were determined in parts per million (ppm) relative to an external standard of 85%  $\text{H}_3\text{PO}_4$ . Signals were assigned to P compounds based on literature reports of model compounds spiked in NaOH-EDTA soil extracts (Turner et al. 2003). Signal peaks were assigned following spectral deconvolution and integration of the peak areas was conducted to allow expression as a proportion (%) of the total extracted P. Spectral processing was performed using NMR Utility Transform Software (NUTS) for Windows (Acorn NMR Inc., Livermore, CA).

## Results

### Soil properties, P content and extractable P

The six soils used in the study were all moderately or strongly acidic (i.e., pH 6.3 to pH 5.4 in water) and showed contrasting properties in terms of soil organic matter content, C and N content and P characteristics with respect to total P, percentage organic P and levels of extractable and available P (Table 1).

The pasture soil from Robertson had the largest P content with four to nine times more total P (by XRF) than the other soils and the cropping soil from Grenfell had the lowest P content (Table 1). This was consistent with the ranking of the soils for P content as determined by the ignition-extraction procedure (175.2 to 1540.6 mg P  $\text{kg}^{-1}$  soil), where total P ranged from 57 to 87% of that measured by XRF. Across all six soils, organic P accounted for 46-69% of the total P content (110 to 1062 mg organic P  $\text{kg}^{-1}$  soil) as determined by extraction difference (ignited verses unignited) and was typically greatest as a percentage of total P ( $>65\%$ ) in the three pasture soils (Table 1). The organic P concentration of the Robertson soil was  $\sim 10$  times greater than the Grenfell soil. Organic P content of the soils was also generally consistent with differences in organic matter content and soil C (Table 1). Whilst the C:N ratio of the soils was relatively consistent (11.1 to 12.5 across all soils), the C:P and C:organic P ratios (based on extracted P) were more variable ranging between 39 to 116 for C:P, and 56 to 184 for C:organic P, for the Robertson and Grenfell soils, respectively. These large differences in C:P ratios are likely related to soil type and management practices (including fertilizer history) that differentiate pasture from cropping soils.

The 'availability of P' as determined by bicarbonate extraction (Table 1) and water extraction (Table 2) similarly differed across the six soils. Between 2.4–17.9% of the total P (for Robertson and Grenfell, respectively) was extracted with bicarbonate (ranging from 27.1 to 36.8 mg P kg<sup>-1</sup> soil). Importantly, and despite the similarity in concentration of the extractable P across the soil, the proportion of the total P that was 'available' as inorganic P (identified as MRP) was highly variable, ranging from 15% for the Wallaroo pasture soil, to 57% for the Greenthorpe and Berthong cropping soils. By contrast, the level of 'unavailable' P in the bicarbonate extracts across the soils (identified as MUP) was large and greater than 43% of the total (Table 1). Water similarly extracted a small amount of the total soil P (<1% across all soils, concentration ranging from 1.03 to 5.23 mg P g<sup>-1</sup> soil) with between 40% (Greenthorpe) and 97% (Robertson) of the P present in the water extracts being molybdate unreactive (MUP; Table 2)

Table 2

Organic anion extraction of phosphorus (P) from six Australian soils. Shown are the concentrations (mg P kg soil<sup>-1</sup>) of molybdate-reactive P (MRP) and molybdate-unreactive P (MUP), and the proportion (%) of the total extracted P measured as MUP. Soils were extracted by water and by citrate, oxalate, and malate each at two concentrations (1 and 10 mM).

Soil	F Fraction	Water		Citrate		Oxalate		Malate		LSD ( <i>P</i> = 0.05)
		1 mM	10 mM	1 mM	10 mM	1 mM	10 mM			
(mg P kg <sup>-1</sup> )										
Robertson	MRP	0.05 <sub>a</sub>	<b>0.13</b>	<b>0.79</b>	<b>0.10</b>	<b>0.46</b>	<b>0.09</b>	<b>0.77</b>	<i>0.02</i>	
	MUP	1.89	<b>2.49</b>	<b>11.51</b>	<b>2.10</b>	<b>4.54</b>	<b>2.32</b>	<b>5.77</b>	<i>0.15</i>	
	MUP (%)	97.2	95.0	93.6	95.2	90.7	96.1	88.3	<i>0.5</i>	
Grenfell	MRP	0.26	<b>0.89</b>	<b>2.03</b>	<b>0.61</b>	<b>2.09</b>	<b>0.60</b>	<b>1.52</b>	<i>0.04</i>	
	MUP	0.77	<b>2.20</b>	<b>4.27</b>	<b>1.08</b>	<b>4.04</b>	<b>1.19</b>	<b>2.95</b>	<i>0.14</i>	
	MUP (%)	74.8	71.1	67.7	64.0	65.9	66.6	66.0	<i>1.3</i>	
Wallaroo	MRP	0.22	<b>0.64</b>	<b>1.55</b>	<b>0.33</b>	<b>0.88</b>	<b>0.39</b>	<b>1.03</b>	<i>0.05</i>	
	MUP	0.92	<b>1.97</b>	<b>4.07</b>	1.10	<b>2.50</b>	<b>1.23</b>	<b>2.73</b>	<i>0.20</i>	
	MUP (%)	80.3	75.5	72.4	76.8	74.0	75.7	72.6	<i>2.3</i>	
Camden	MRP	1.58	<b>3.07</b>	<b>6.96</b>	<b>2.15</b>	<b>4.19</b>	<b>1.88</b>	<b>4.07</b>	<i>0.17</i>	
	MUP	3.65	<b>5.45</b>	<b>6.84</b>	3.78	<b>7.31</b>	<b>4.28</b>	<b>5.39</b>	<i>0.34</i>	
	MUP (%)	69.8	64.0	49.5	63.8	63.6	69.2	57.0	<i>1.3</i>	
Greenthorpe	MRP	1.19	<b>3.13</b>	<b>6.06</b>	<b>2.93</b>	<b>5.56</b>	<b>1.63</b>	<b>2.85</b>	<i>0.23</i>	
	MUP	0.80	<b>1.90</b>	<b>3.48</b>	<b>1.26</b>	<b>3.28</b>	0.90	<b>1.35</b>	<i>0.24</i>	
	MUP (%)	40.4	37.7	36.4	30.1	37.1	35.5	32.2	<i>3.1</i>	
Berthong	MRP	0.84	<b>1.73</b>	<b>4.55</b>	<b>1.53</b>	<b>3.61</b>	<b>1.05</b>	<b>1.78</b>	<i>0.12</i>	
	MUP	1.38	<b>1.94</b>	<b>2.94</b>	<b>1.86</b>	<b>3.25</b>	1.57	<b>1.84</b>	<i>0.23</i>	
	MUP (%)	62.2	52.8	39.2	54.9	47.3	59.9	50.9	<i>2.8</i>	

<sup>a</sup> Values are the mean of 3 replicates (*n*=3) and for each row the least significant difference (LSD) across the extraction treatments is shown. MRP and MUP extraction values shown in bold are significantly different (*P* = 0.05) to the water control.

## Extraction of soil P by organic anions.

The capacity of organic anions to extract MRP and MUP from soil was assessed by first extracting the Robertson and Grenfell soils with increasing concentrations of citrate, malate and oxalate up to 40.0  $\mu\text{mol g}^{-1}$  soil (equivalent to 10 mM solution concentration, Figure 1). Then all six soils with the organic anions at 1 mM and 10 mM (Table 2), concentrations that were expected to be relevant to that found for cluster roots of white lupin.

The three organic anions showed a concentration dependency for extraction of both MRP and MUP as compared to water as control (Figure 1). All three organic anions extracted less MRP from the Robertson soil than from the Grenfell soil in terms of both absolute concentrations ( $<0.75 \text{ mg P g}^{-1}$  soil in all cases) and as a percentage of the total P extracted (average of 2.7%). The MUP extracted from the Robertson soil by citrate, oxalate and malate was considerably greater than the MRP and accounted for 93 to 97%, 91 to 97% and 88 to 98% of the total P extracted, respectively. There was strong linear correlation ( $R^2 > 0.996$ ) between organic anion concentration and the MRP and MUP extracted in the Robertson soil with citrate being most effective at mobilizing MUP (Figure 1). By contrast, the extraction of MRP and MUP in the Grenfell soil showed more of a saturating relationship and the amounts of P extracted were comparable at the highest concentration of each organic anion. The proportion of the total P extracted as MUP in the Grenfell soil was less than the Robertson and ranged from 64 to 76% across the three organic anions.

The relative effectiveness of organic anions in mobilizing MRP and MUP was also assessed at two concentrations (1 mM and 10 mM) for each organic anion across the six soils. The concentration of MRP in extracts was increased significantly ( $P < 0.05$ ) by all three organic anions (Table 2). Across the soils, the average increase in MRP extracted at 10 mM (relative to water extract) was 7.3-fold for citrate and 5.3-fold for both oxalate and malate. At 1 mM, the comparable increase in MRP across the three organic anions was 1.6, 1.9 and 2.6-fold for oxalate, malate and citrate respectively. For all soils, a large proportion of the extracted P occurred as MUP and accounted for 62 to 94% of the total P for the three pasture soils and 36 to 68% for the three cropping soils (Table 2). In most cases, the MUP extracted by organic anions was significantly greater (1.3 to 4.1-fold) than the MUP extracted by water. The exceptions were 1 mM oxalate in the Wallaroo and Camden pasture soils and 1 mM malate in the Berthong cropping soil.

## **Phosphatase enzyme lability of extracted phosphorus**

Incubation of the organic anion extracts with different combinations of phosphatase enzymes resulted in increased levels of MRP through the hydrolysis of MUP. In most cases, the release of MRP was significantly greater than the water control (Table 3). In the water extracts, addition of the PME, PME+PDE and PHY phosphatase preparations liberated 10 to 31%, 8 to 27% and 19 to 38%, respectively, of the MUP across the six soils (Figure 2). In the organic anion extracts, both the MRP concentration (Table 2) and the percentage of the MUP released (Figure 2) was increased, with strong interactions occurring between the enzyme preparation and the type and concentration of organic anion.

Table 3

Concentrations of enzyme-labile phosphorus (P) following extraction of soil with organic anions and incubation with phosphatase enzymes. Shown is the concentration of molybdate-reactive P (MRP) following incubation of soil extracts with phosphomonoesterase (PME), PME in combination with phosphodiesterase (PME + PDE), or with phytase (PHY). The values represent the increase in MRP from levels prior to incubation. Soils were extracted by water and by citrate, oxalate, and malate each at two concentrations (1 and 10 mM).

Soil	Enzyme	Water	Citrate		Oxalate		Malate		LSD ( $P = 0.05$ )
			1 mM	10 mM	1 mM	10 mM	1 mM	10 mM	
(mg P kg <sup>-1</sup> )									
Robertson	PME	0.19 <sup>a</sup>	<b>0.44</b>	<b>3.46</b>	0.24	<b>0.89</b>	<b>0.34</b>	<b>1.74</b>	
	PME+PDE	0.15	<b>0.49</b>	<b>4.20</b>	0.16	<b>0.73</b>	<b>0.34</b>	<b>1.95</b>	<i>0.14</i>
	PHY	0.38	<b>0.53</b>	<b>6.75</b>	<b>0.61</b>	<b>1.65</b>	<b>0.55</b>	<b>2.59</b>	
Grenfell	PME	0.13	<b>0.49</b>	<b>0.49</b>	<b>0.21</b>	<b>0.47</b>	<b>0.37</b>	<b>0.68</b>	
	PME+PDE	0.13	<b>0.67</b>	<b>0.80</b>	<b>0.22</b>	<b>0.52</b>	<b>0.40</b>	<b>0.97</b>	<i>0.05</i>
	PHY	0.18	<b>1.40</b>	<b>2.24</b>	<b>0.32</b>	<b>1.57</b>	<b>0.44</b>	<b>1.67</b>	
Wallaroo	PME	0.28	<b>0.77</b>	<b>1.10</b>	<b>0.43</b>	<b>0.66</b>	<b>0.55</b>	<b>0.96</b>	
	PME+PDE	0.25	<b>0.84</b>	<b>1.60</b>	<b>0.36</b>	<b>0.70</b>	<b>0.58</b>	<b>1.42</b>	<i>0.09</i>
	PHY	0.35	<b>1.22</b>	<b>2.52</b>	<b>0.59</b>	<b>1.40</b>	<b>0.60</b>	<b>1.71</b>	
Camden	PME	0.53	<b>1.86</b>	<b>2.18</b>	0.58	<b>1.09</b>	<b>0.97</b>	<b>1.74</b>	
	PME+PDE	0.62	<b>2.01</b>	<b>2.41</b>	0.63	<b>1.35</b>	<b>1.43</b>	<b>2.22</b>	<i>0.19</i>
	PHY	0.81	<b>2.60</b>	<b>3.45</b>	0.91	<b>2.25</b>	<b>1.42</b>	<b>2.45</b>	
Greenthorpe	PME	0.21	<b>0.48</b>	<b>0.88</b>	0.25	<b>0.43</b>	<b>0.44</b>	<b>0.79</b>	
	PME+PDE	0.18	<b>0.55</b>	<b>1.05</b>	<b>0.31</b>	<b>0.80</b>	<b>0.32</b>	<b>0.71</b>	<i>0.10</i>
	PHY	0.18	<b>0.49</b>	<b>1.06</b>	0.27	<b>0.97</b>	0.23	<b>0.55</b>	
Berthong	PME	0.26	<b>0.44</b>	<b>0.63</b>	0.21	<b>0.45</b>	<b>0.43</b>	<b>0.50</b>	
	PME+PDE	0.23	<b>0.51</b>	<b>0.84</b>	0.32	<b>0.68</b>	0.32	<b>0.47</b>	<i>0.13</i>
	PHY	0.27	<b>0.48</b>	<b>0.74</b>	0.35	<b>0.95</b>	0.27	<b>0.47</b>	

<sup>a</sup> Values are the mean of 3 replicates ( $n=3$ ) and for each soil the least significant difference (LSD) for the Enzyme x Extract interaction ( $P = 0.05$ ) is shown, with values shown in bold for each of the enzymes being significantly different when compared to the water control for that enzyme. The main effect of Extract was significant ( $P < 0.001$ ) for all soils, and for Enzyme; PME < PME+PDE < PHY ( $P < 0.001$ ) for Robertson, Grenfell, Wallaroo and Camden soils; PME < PHY, and PME+PDE = both PME and PHY ( $P = 0.01$ ) for Greenthorpe soil and PME = PME+PDE = PHY for Berthong soil.

In terms of the observed increases in MRP concentrations presented in Table 3, all extractants (water and the three organic anions) and enzyme preparations (PME, PME+PDE and PHY) had significant effects. The 10 mM organic anion

treatments in combination with all enzyme preparations increased the MRP concentration, with citrate being the most effective across all soils. The next most effective on the three pasture soils and Grenfell soil was malate followed by oxalate, whereas on the Greenthorpe and Berthong cropping soils, with their lower total organic P contents (Table 1) and smaller proportions of organic P (with all organic anion extracts, Table 2), oxalate was more effective than malate. The 10 mM citrate extraction increased MRP release by 7.8-fold (on average compared with the water controls) across all soils with a range from 2.9-fold, in the Berthong soil, to 20.1-fold in the Robertson soil. By comparison, the average MRP released with 10 mM oxalate across all soils was 3.8-fold and 5.0-fold for 10 mM malate. At the lower 1 mM organic anion treatments, the average MRP released was 3.2, 1.4 and 1.9-fold for citrate, oxalate and malate respectively. Interestingly, 1 mM oxalate was generally ineffective on all soils except for Grenfell and Wallaroo, and 1 mM malate treatment showed strong interactions with the enzyme preparations on the Greenthorpe and Berthong soils. By contrast, 1 mM citrate increased MRP on all soils compared with water controls and with each of the enzyme preparations (Table 3).

The relative effectiveness of the different phosphatases in releasing MRP varied with soil type and showed interactions with the extractants. Across the three enzymes, the PHY preparation resulted in significantly greater increase in MRP than PME in all soils except for Berthong. Incubation of extracts with PHY enzyme was also more effective than PME+PDE in all soils except for the Greenthorpe and Berthong soils. Across the enzyme preparations the PME, PME+PDE and PHY increased the concentrations of MRP by an average of 3.2-fold (range 1.7 to 6.3), 4.3-fold (range 2.3 to 8.8) and 4.1-fold (2.0 to 7.2), respectively, relative to that observed for the water controls (Table 3).

In addition to the increases in MRP concentration mediated by the phosphatases in the organic anion extracts, the proportion of the MUP hydrolyzed by the phosphatases (i.e., enzyme-lability) was also significantly increased (Figure 2). When averaged across all the organic anion extracts, the lability of the MUP was increased by 1.4, 1.7 and 1.6-fold for the PME, PME+PDE and PHY preparations, respectively (relative to the combined average in water). Once again, clear interactions were evident between the different enzyme preparations and soil types (Figure 2). The PHY preparation was significantly more effective than the PME in the Robertson, Grenfell, Wallaroo and Camden soils, but was less effective than PME in the Greenthorpe soil. The PME+PDE preparation was more effective than PME only in the Grenfell, Wallaroo and Camden soils but equivalent to PME in the Robertson and Greenthorpe soils. There was no difference between the three phosphatase enzymes in the Berthong soil, where MUP lability increased by an average of 1.3-fold across the enzyme and extractant combinations. In all cases, however, the effect of the organic anion extractant was significant ( $P < 0.001$ ) with citrate generally being most effective (1.6 and 1.8-fold at 1 and 10 mM across all soils). Citrate was effective in most soils (especially in combination with PHY), with the exceptions being the two cropping soils (Greenthorpe and Berthong) with their lower organic P contents. Enzyme-lability in the malate and oxalate extracts showed strong interactions with soil type, extractant concentration and phosphatase preparation. Interestingly, malate was more effective than citrate or oxalate in the Greenthorpe soil (especially for PME and PME+PDE), and relatively more effective than oxalate for all three enzymes across the other five soils (Figure 2).

## **Solution $^{31}\text{P}$ NMR spectroscopy of organic anion extracts**

Solution  $^{31}\text{P}$  NMR spectroscopy was used to identify the P compounds extracted from the Robertson, Grenfell and Camden soils with 10 mM citrate, oxalate and malate. Whilst the quality of the spectra (as compared to NaOH-EDTA) varied markedly across extractants and soils (Figure 3), orthophosphate and phosphomonesters were clearly identified as the major forms of extracted P (Table 4).

Table 4

Phosphorus (P) composition of soil extracts determined by solution  $^{31}\text{P}$  NMR spectroscopy. Soils were extracted with 10 mM organic anions (citrate, oxalate or malate) or NaOH-EDTA (not determined for Camden) and the proportion of P within recognizable classes of P compounds expressed as a percentage of the total P extracted.

Soil	Extract	Total P extracted	Ortho-phosphate	Phospho-monoester	Phospho-lipid	DNA	Pyro-phosphate	Other
		(mg P kg <sup>-1</sup> )	(% of total P extracted) <sup>a</sup>					
Robertson	Citrate (10 mM)	12.5	19.0	48.5	6.8	4.9	20.9	
	Oxalate (10 mM)	5.2	14.5	46.8	12.9	17.7	8.1	tr
	Malate (10 mM)	6.3	15.3	47.5	11.7	tr	25.6	tr
	NaOH-EDTA	1013.2	47.5	36.7	nd	2.2	13.6	
Grenfell	Citrate (10 mM)	5.7	47.4	52.6	nd	nd	nd	
	Oxalate (10 mM)	5.9	44.0	49.7	tr	6.3	nd	
	Malate (10 mM)	4.0	39.5	53.5	tr	tr	7.0	tr
	NaOH-EDTA	153.2	48.0	50.8	nd	1.2	nd	
Camden	Citrate (10 mM)	13.8	40.4	50.4	tr	tr	9.2	tr
	Oxalate (10 mM)	12.7	34.4	32.5	5.3	17.2	6.6	4.0
	Malate (10 mM)	9.6	41.5	31.6	7.9	tr	14.5	4.6

<sup>a</sup> based on identifiable peaks from NMR spectra as indicated in Figure 3 (a to e for each class of P compound, respectively), or tr = trace, nd = not detected.

Across the three organic anion extracts, phosphomonesters constituted 46.8 to 48.5% of the P in the Robertson soil, 49.7 to 53.5% in the Grenfell soil and 31.6 to 50.4% in the Camden soil. Within the monoester region, spectral peaks characteristic of *myo*-inositol hexakisphosphate were present in the malate extractions across all three soils (Figure 3; Turner et al. 2003). A signal close to  $\delta = 4.2$  ppm in several soils (e.g., Grenfell soil and to a lesser extent the Camden soil) could similarly be assigned potentially to *scyllo*-inositol hexakisphosphate (Turner and Richardson 2004). Of the

phosphodiester, phospholipids constituted a considerable fraction of the extracted P (up to 12.9%) in the Robertson and Camden soils especially with malate and oxalate extracts. By contrast only trace amounts of phospholipids were evident in extracts from the Grenfell soil. Nucleic acids (potentially as DNA) accounted for a considerable proportion of the extracted P in the oxalate extracts from all three soils (6.3 to 17.7%). An additional unidentified phosphodiester was detected near  $\delta = -2.1$  ppm which accounted for 4.0 to 4.6% of the extracted P in malate and oxalate extracts of the Camden soil, and trace amounts in the malate extracts of the other two soils. Pyrophosphate was detected in all extracts (6.6 to 25.6%) of the Robertson and Camden soils, particularly in the citrate and malate extracts, but only in the malate extract of Grenfell soil (Table 4).

The P speciation in the extraction spectra were supported by NMR analysis of total P extracted in NaOH–EDTA for the Robertson (1013 mg P kg<sup>-1</sup>) and Grenfell (153 mg P kg<sup>-1</sup>) soils (Figure 3; the Camden soil was not analyzed by NaOH–EDTA extraction). These concentrations of the NaOH-extracted P represented 66% and 87% of that extracted by H<sub>2</sub>SO<sub>4</sub> for the Robertson and Grenfell soils, respectively (Table 1), and were considerably greater (by 30 to 126-fold) than the amount of P extracted by the organic anions (Table 4). For both soils, NaOH–EDTA extracts were similarly dominated by orthophosphate (~48% of extracted P) and phosphomonoesters (36.7 to 50.8% for Robertson and Grenfell, respectively). Nucleic acids (as DNA) were present at smaller proportions in both soils (1.2 to 2.2%), while the Robertson soil contained a larger proportion of pyrophosphate (13.6%). Phospholipids were not detected in NaOH–EDTA extracts of either soil, presumably due to their degradation during Na–OH extraction (Turner et al. 2003), which is in direct contrast to their predicted presence (up to 13%) in the organic anions extracts in the Robertson and Grenfell soils (Table 4).

## Discussion

Across six contrasting soils we showed that citrate, oxalate and malate were effective in extracting P and that this reaction was dependent on both soil type and extractant concentration. Extracted P was evident as both MRP (presumably mostly as orthophosphate) and MUP, which would be expected to include a range of organic and condensed forms of P. These extractions were analyzed with NMR and the presence of orthophosphate, pyrophosphate and various monoester and diester forms of P was confirmed. Importantly, we also showed that the addition of the PHY, PME and PDE phosphatases, either separately or in combination, significantly increased the MRP content of most extracts, with up to ~60% of the MUP being enzyme labile (depending on the treatment). This provides strong evidence for the biological potential of a combination of organic anions and phosphatases to mobilize soil P from both inorganic and organic pools.

## Effectiveness of organic anions for extraction of soil P

Both the total amount of P extracted by organic anions and relative contribution of MUP as a proportion of the total P extracted, varied considerably across the soils. The total amount extracted was related to total soil P content, and the proportion within the MUP fraction was generally less in the cropping soils which had lower total organic P contents (e.g., Greenthorpe and Berthong). The effectiveness of each organic anion to extract P thus varied with soil type. For example, 10 mM citrate was more effective than similar concentrations of malate or oxalate at mobilizing P in the Robertson and Wallaroo soils, whereas oxalate and citrate were equally effective in several soils, particularly the cropping soils (i.e., Grenfell, Greenthorpe and Berthong). Malate was generally less effective than oxalate, except on the Robertson and Wallaroo soils which are the most acidic. The Robertson soil (Ferrosol) also has high Fe and Al contents. The finding that the organic anions differ in their capacity to mobilize P was not unexpected since many reports have shown that tricarboxylic acids like citrate are generally more effective than dicarboxylates like oxalate and malate. Furthermore, the pH of the organic anion solutions were adjusted to match the water-based pH of each soil (Table 1).

This means that the pH of the extraction solutions were different and, therefore, the degree of dissociation (or protonation) of the carboxyl groups of the extractants would also vary according the pKa profile of each organic acid. This would be particularly important for citrate with three pKa at pH ~3.1, 4.8 and 6.4. Nonetheless, the interaction between soil type and the capacity of different organic anions to release P suggest that mobilization of P occurred from different soil pools of the inorganic and/or organic P fractions.

Whilst our study investigated each organic anion in isolation, it would be interesting to investigate the effectiveness of combining the organic anions since this would better mimic the conditions in the rhizosphere (Nuruzzaman et al. 2006; Pearse et al. 2006; Wang et al. 2013; Wouterlood et al. 2004). Whether or not specific organic anions, or different compositions of organic anions, provide a 'niche' advantage for different crops (or genotypes) to mobilize different pools of sparingly available P in different soils has been subject of much speculation and remains unclear (Lambers et al. 2002; Pearse et al 2007; Veneklaas et al. 2003). Niche differentiation, with regard to P acquisition, may have significance for ecological function and the evolutionary adaption of plants to different soils and environments (Turner 2008).

## Positive interaction of phosphatases and organic anions

This study clearly demonstrated that the effectiveness of phosphatases to increase P availability from organic pools was enhanced after the soil was first extracted with organic anions. This was evident from the 'phosphatase-mediated' increase in MRP content in most organic anion extracts and by the 'increase in the lability' of the organic P mobilized, as indicated by a greater proportion of the MUP that was amenable to hydrolysis by enzymes (Figure 2). There were, however, strong interactions between the enzyme preparations, soil types and extractants. For example, while phytase (PHY) was generally the most effective phosphatase across all soils and extractants, it was most effective when used in combination with citrate except for the Greenthorpe and Berthong cropping soils. With few exceptions, the effectiveness of the monoesterase (PME) was no different to the combination of PME with the diesterase (PDE). In this regard some 'endogenous' phosphatase activity in the soil extracts cannot be excluded and this was supported by the low levels of activity detected in the water controls. Nonetheless, direct addition of the phosphatase enzymes (either PME, PDE or PHY) increased the concentrations of MRP above the water controls indicating additional P mobilization occurred. Such observations provide further insights into the forms of P mobilized in the soils by the organic anions.

The observation that the PHY preparation was the most effective phosphatase suggests that the organic anions were particularly effective in mobilizing inositol phosphates that were either adsorbed in the soil or possibly associated with high molecular weight organic matter and/or sparingly-soluble precipitates. Indeed, Celi and Barberis (2005) have shown that, similar to orthophosphate, inositol hexakisphosphates are readily adsorbed in soils through interactions with four of the six negatively charged monoester-phosphate moieties. Importantly, Giaveno et al. (2010) demonstrated that inositol hexakisphosphates were not amendable to dephosphorylation by phytases when adsorbed to the solid phase (soil clays and iron-oxide minerals) presumably due to steric hinderance between substrate and enzyme. Precipitated forms of inositol phosphates (e.g., Al- and Fe-phytates) have similarly been shown to be less susceptible to dephosphorylation by a range of phytases (Tang et al. 2006). Tang et al. (2006) also showed that the addition of citrate, and to a lesser extent malate and oxalate, improved the lability of substrates to phytase activity. Interestingly, in these experiments the presence of  $\text{Al}^{3+}$  and  $\text{Fe}^{2+/3+}$  also inhibited the hydrolysis of Ca-phytate but this inhibition could be reduced when citrate was added. Citrate may influence the desorption kinetics of inositol hexakisphosphates from soil minerals just as it does for orthophosphate (Martin et al. 2004). These studies, in combination with our findings here, suggest that the amenability of inositol hexakisphosphates and other forms of organic P to hydrolysis is enhanced by the presence of organic anions.

The complementary activity of organic anions and phosphatase enzymes for mobilizing P has important implications for the bioavailability of P in soil for plant nutrition and microbial processes. For example, it has been widely suggested that the effectiveness by which white lupins are able to mobilize and acquire P is a combination of root structural and functional traits (Lambers et al. 2013). Indeed 'the success' of white lupin to acquire P appears to rely on both the formation of cluster roots and 'their cocktail' of exudates, comprised of organic anions, protons, phosphatases and secondary metabolites to inhibit microbial activity (Neumann et al. 1999; Weiskopf et al. 2006). The phosphatases released by white lupin include isoforms with a broad specificity including those with phytase activity (Tadano et al. 1993; Maruyama et al. 2012). Moreover, when tobacco plants were genetically modified to enhance citrate and phytase exudation together, they showed a greater ability to utilize organic P from soils than lines modified to release citrate or phytase alone (Giles et al. 2017). The evolution of combinatory approaches such as these by plants to mobilize and acquire soil P is therefore expected to improve their resilience to P stress and, potentially, to provide a competitive advantage through resource partitioning as proposed by Turner (2008).

## Direct evidence for extraction of organic phosphate compounds by organic anions

This study used  $^{31}\text{P}$  NMR to confirm and identify the forms of P extracted from soil by organic anions. The compounds released depended on soil type and the organic anion used but included a range of monoesters (including inositol hexakisphosphates), diesters (including phospholipids, DNA and unidentified compounds that are likely diesters) and pyrophosphate. Even though the release of these compounds with organic anions was small compared with a NaOH-EDTA-extraction, it is the first example, to our knowledge, of NMR analysis being applied to demonstrate directly the mobilization of soil organic P by organic anions. The 'quality' of the NMR spectra was considered to be high for the Grenfell and Camden soils (Figure 3) but more variable for the Robertson soil. Nevertheless, compounds across all soils could be assigned to the peaks according to chemical shifts obtained from the NaOH-EDTA extractions (Turner et al. 2003). These peaks should be verified in future experiments with 'spiked' controls. Furthermore, EDTA had to be included in the extraction samples to obtain reliable NMR spectra (data not shown), which was particularly important for the Robertson (Ferralsol) soil where high  $\text{Fe}^{2+/3+}$  and  $\text{Al}^{3+}$  content may have contributed to the poorer spectral quality.

The effectiveness of the organic anions in mobilizing different P compounds varied both within and across the soils and often contrasted with the NaOH-EDTA extractions. For example, phospholipids were present in all the organic anion extracts of the Robertson soil, but they were not evident in the NaOH-EDTA extractions. Moreover, phospholipids were absent in extracts of Grenfell soil but were more variable in the Camden soil. Nucleic acids as DNA was most efficiently extracted by oxalate across all three soils while pyrophosphate was most prevalent in the citrate and malate extracts. Whether these differences are a direct effect of an extraction efficiency from different soil pools or are a consequence of varying degrees of degradation during and post-extraction across the different soils remains to be determined. Indeed it is feasible that the organic anions were effective in extracting a large component of microbial P as indicated by generally high content of DNA and phospholipids across the various extracts. Moreover, monoester forms of organic P were consistently identified (32–53% of total extracted P) from the three soils extracted with the three organic anions. Spectral signals that were indicative of both *myo*- and *scyllo*-inositol hexakisphosphate were particularly evident in the majority of organic anion extracts, especially in the Robertson and Grenfell soils extracted with malate (Figure 3).

Importantly, the variable detection of monoester and diester P substrates, including inositol phosphates, in the organic anion extracts is consistent with the observed increases in MRP and the lability of MUP to dephosphorylation by combinations of PHY, PME and PDE. Further work is needed to better link activity of the different phosphatases with the dephosphorylation of specific organic P compounds. Ideally this would require the use of highly purified enzymes with more defined substrate specificities, as compared to the commercial enzyme preparations that were used in the present study. Assessment of different organic anions extracts both before and after phosphatase treatment would also be

informative for identifying specific substrates that were enzyme-labile. Whilst this could initially be achieved using laboratory incubations, as conducted here, in the longer term these experiments should be directed at rhizosphere samples from plants that show different exudation profiles for organic anions and phosphatases. Based on a range of observations from different experimental systems, we further propose that increasing the release of both organic anion exudation and phosphatases (particularly phytase) from roots may be a viable strategy for increasing the P-use efficiency of plants through greater mobilization of soil P (e.g., Giles, et. 2018; Giles et al. 2016, Ryan et al. 2004, Richardson et al. 2011, George et al. 2005).

Collectively, our findings demonstrate the potential importance of combining organic anion and phosphatase exudation from roots for manipulating P dynamics in the soil and for enhancing plant nutrition. This study provides insights into the soil chemistry of natural and agroecosystems and contributes to ongoing efforts to improve the P-use efficiency of crop plants.

## Declarations

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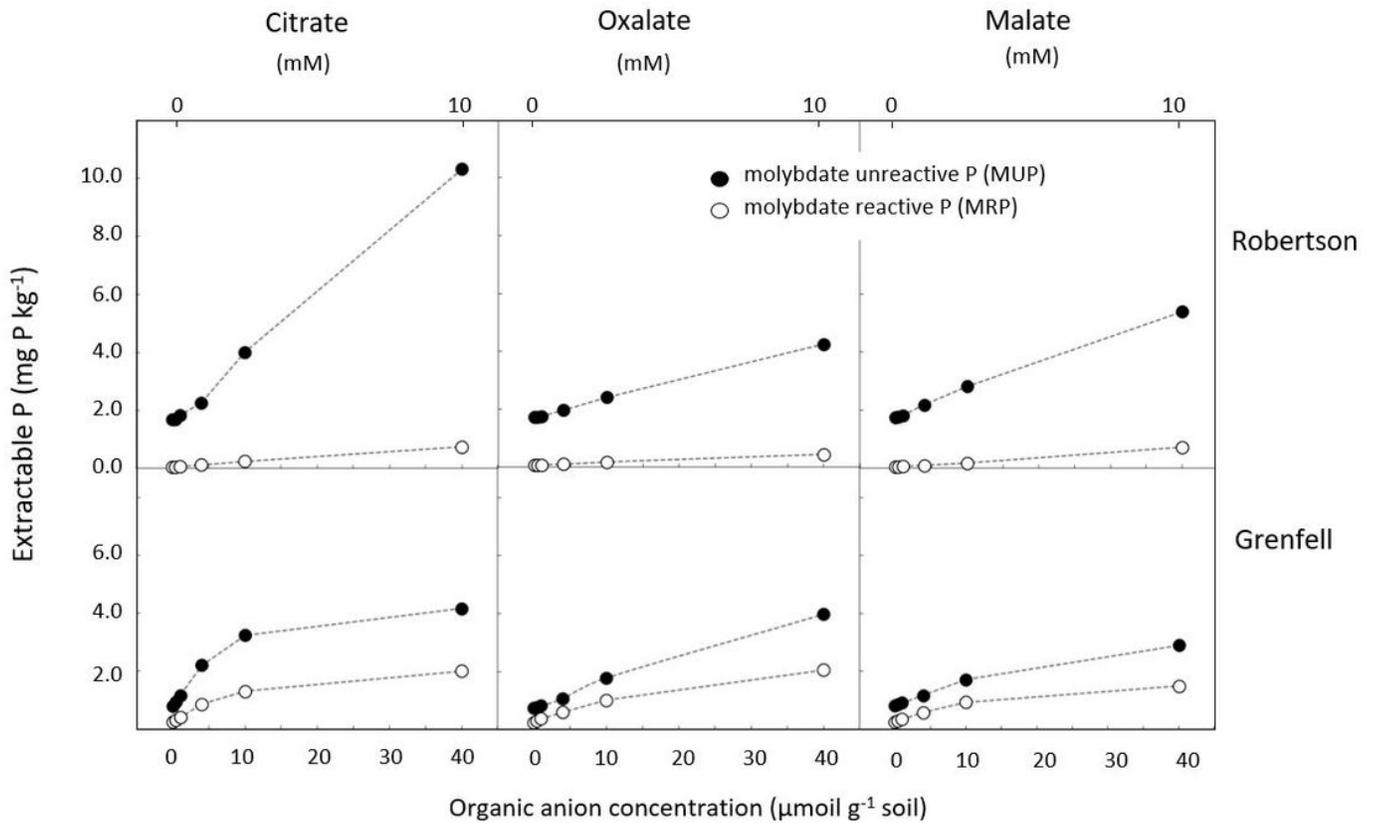
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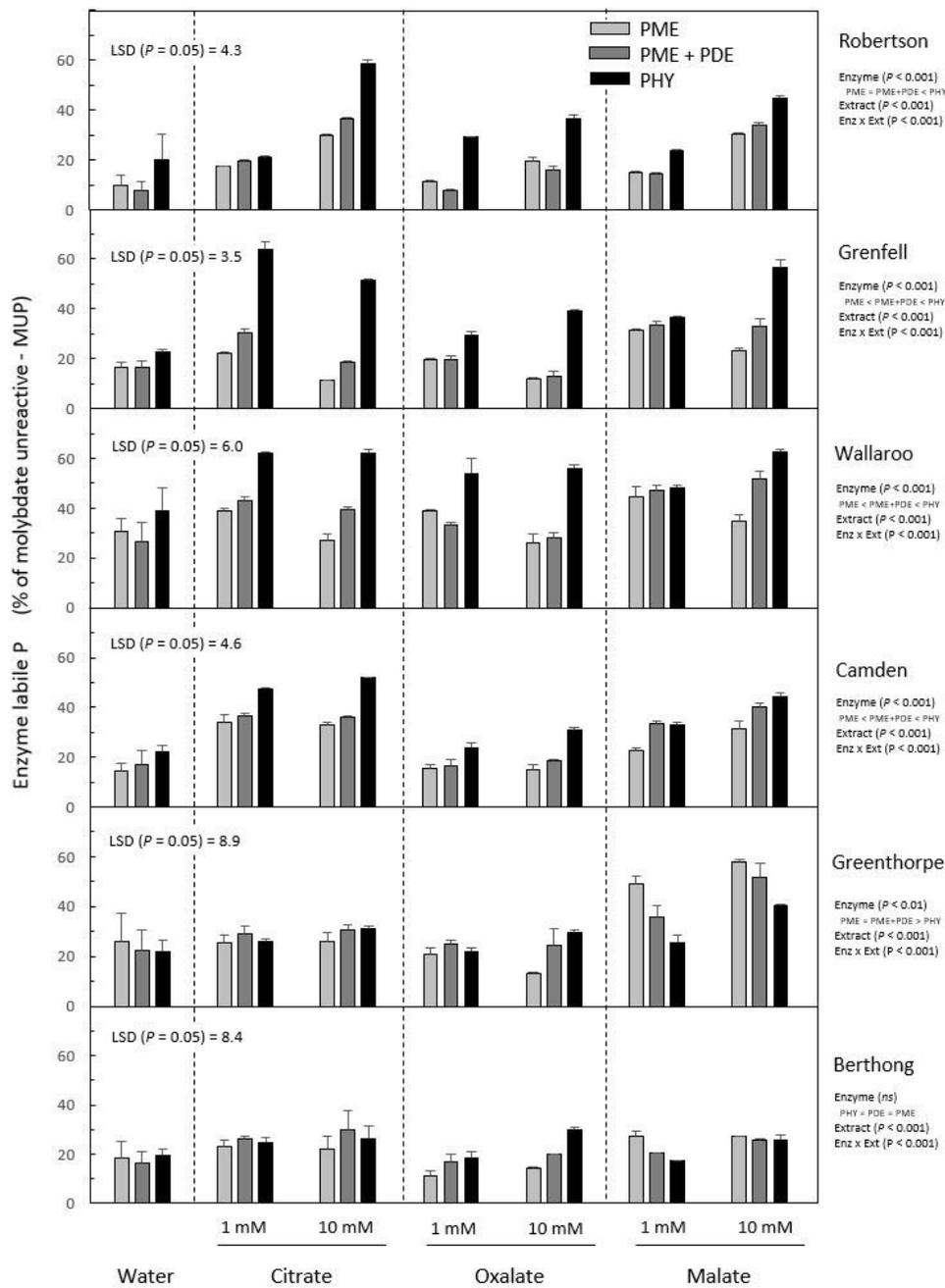
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## Figures



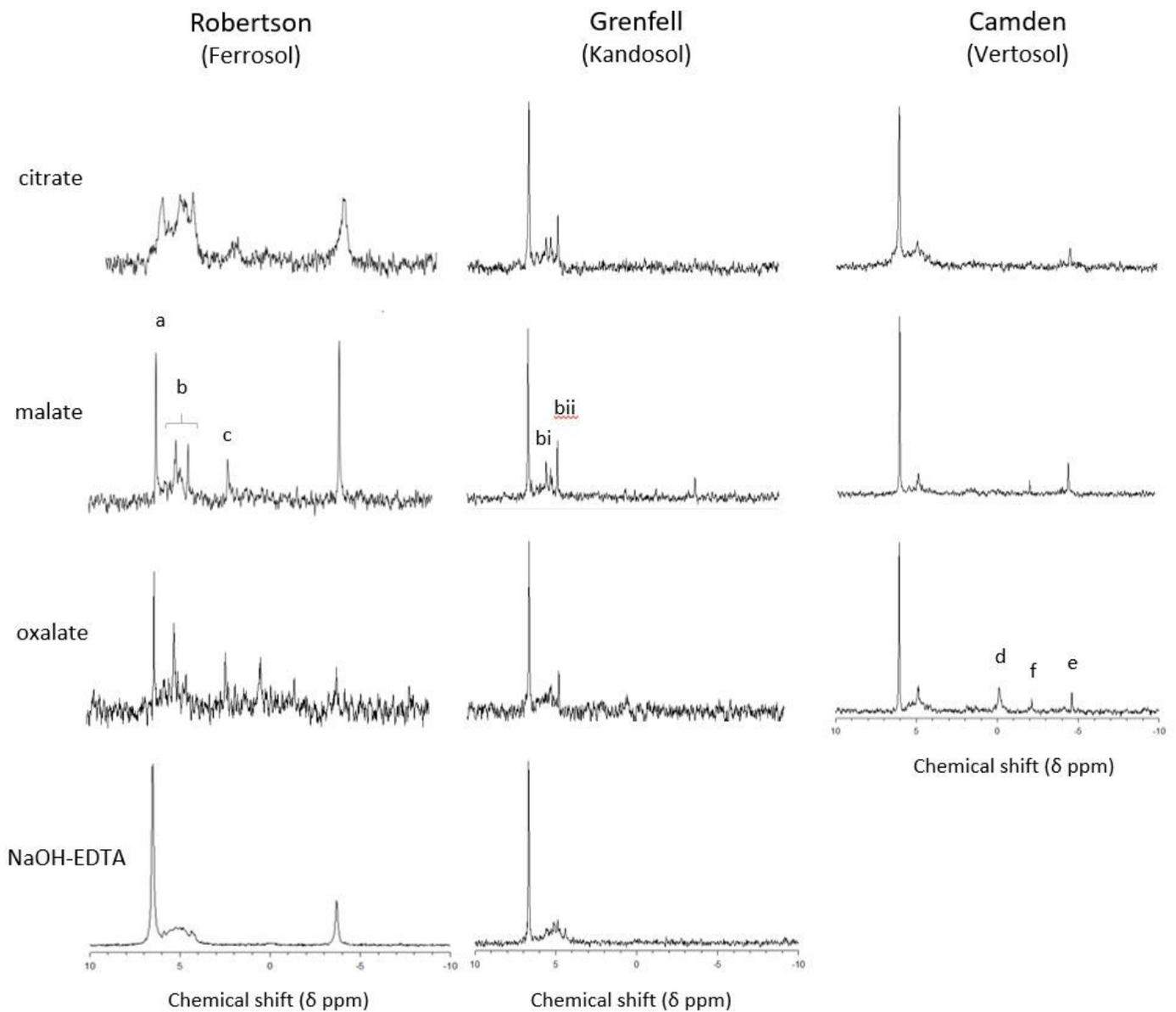
**Figure 1**

Organic anion extraction of phosphorus (P) from the Robertson and Grenfell soils. Shown are the concentrations (mg P kg<sup>-1</sup> soil) of molybdate-reactive P (MRP) and molybdate-unreactive P (MUP) extracted by water (zero value) and by citrate, oxalate, and malate each at 5 concentrations. Each data point is the mean of 3 replicates ( $n = 3$ ) with an average coefficient of variation (standard deviation/mean) of 2.9% (range 0.1 to 6.9%) across all of the means.



**Figure 2**

Enzyme labile phosphorus (P) following extraction of soil with organic anions and incubation with phosphatases. Shown is the percentage (%) of that total molybdate unreactive P (MUP) that was hydrolysed to molybdate reactive (MRP) after incubation of soil extracts with phosphomonoesterase (PME), PME in combination with phosphodiesterase (PME + PDE), or with phytase (PHY). Soils were extracted by water and by citrate, oxalate, and malate each at two concentrations (1 and 10 mM). Values are the mean of 3 replicates ( $n=3$ ) and bars on each column (where shown) show 1 standard deviation. For each soil the significance of Enzyme and Extract (as main effects) and the Enzyme x Extract (interaction) is shown with indication of the least significant difference (LSD) value for the Enzyme x Extract interaction ( $P = 0.05$ ) shown for each soil.



**Figure 3**

Solution  $^{31}\text{P}$  NMR spectra for extracts from the Robertson, Grenfell and Camden soils. The soils were extracted with 10mM solutions of organic anions (citrate, malate, oxalate) and, for the Robertson and Grenfell soils, NaOH-EDTA. Identifiable peaks were assigned to major classes of P according to spectral shifts based on NaOH-EDTA spectra, and are indicated as (a) orthophosphate, (b) phosphomonoesters (bi and bii, potentially as *myo*- and *scyllo*-inositol hexakisphosphate, respectively), (c) phospholipids, (d) DNA, (e) pyrophosphate and (f) as other (a non-identifiable potential diester).