Geoderma 284 (2016) 93–102

Contents lists available at ScienceDirect

Geoderma

journal homepage: www.elsevier.com/locate/geoderma

Assessment of bioavailable organic phosphorus in tropical forest soils by organic acid extraction and phosphatase hydrolysis

Tegan Darch [a,*], Martin S.A. Blackwell [a], David Chadwick [c], Philip M. Haygarth [b], Jane M.B. Hawkins [a], Benjamin L. Turner [d]

[a] Rothamsted Research, North Wyke, Okehampton, Devon EX20 2SB, UK
[b] Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK
[c] School of Environment, Natural Resources and Geography, Environment Centre Wales, Deiniol Road, Bangor University, Bangor LL57 2UW, UK
[d] Smithsonian Tropical Research Institute, Apartado 0843-03092, Balboa, Ancon, Republic of Panama

ARTICLE INFO

Article history:
Received 4 April 2016
Received in revised form 30 June 2016
Accepted 26 August 2016
Available online 7 September 2016

Keywords:
Citric acid
Organic acid
Organic phosphorus
Phosphatase hydrolysis
Tropical
Bioavailable

ABSTRACT

Soil organic phosphorus contributes to the nutrition of tropical trees, but is not accounted for in standard soil phosphorus tests. Plants and microbes can release organic anions to solubilize organic phosphorus from soil surfaces, and synthesize phosphatases to release inorganic phosphate from the solubilized compounds. We developed a procedure to estimate bioavailable organic phosphorus in tropical forest soils by simulating the secretion processes of organic acids and phosphatases. Five lowland tropical forest soils with contrasting properties (pH 4.4–6.1, total P 86–429 mg P kg^{-1}) were extracted with 2 mM citric acid (i.e., 10 μmol g^{-1}, approximating rhizosphere concentrations) adjusted to soil pH in a 4:1 solution to soil ratio for 1 h. Three phosphatase enzymes were then added to the soil extract to determine the forms of hydrolysable organic phosphorus. Total phosphorus extracted by the procedure ranged between 3.22 and 8.06 mg P kg^{-1} (mean 5.55 ± 0.42 mg P kg^{-1}), of which on average three quarters was unreactive phosphorus (i.e., organic phosphorus plus inorganic polyphosphate). Of the enzyme-hydrolysable unreactive phosphorus, 28% was simple phosphomonoesters hydrolyzed by phosphomonoesterase from bovine intestinal mucosa, a further 18% was phosphodiesters hydrolyzed by a combination of nuclease from *Penicillium citrinum* and phosphomonoesterase, and the remaining 51% was hydrolyzed by a broad-spectrum phytase from wheat. We conclude that soil organic phosphorus can be solubilized and hydrolyzed by a combination of organic acids and phosphatase enzymes in lowland tropical forest soils, indicating that this pathway could make a significant contribution to biological phosphorus acquisition in tropical forests. Furthermore, we have developed a method that can be used to assess the bioavailability of this soil organic phosphorus.

1. Introduction

Productivity in lowland tropical forests is often considered to be limited by phosphorus (P) availability, in part because concentrations of plant-available inorganic phosphate determined by conventional agronomic soil tests are extremely low (e.g. Clinebell et al., 1995; Condit et al., 2013). However, organic P is abundant in tropical forest soils (e.g. Turner and Engelbrecht, 2011) and can contribute to the nutrition of tropical trees following hydrolysis to inorganic phosphate by phosphatase enzymes synthesized by plants and microbes (George et al., 2006). Indeed, much of the soil organic P in tropical forests is dynamic over relatively short timescales (Turner et al., 2015; Vincent et al., 2010) and its turnover can account for the majority of the P uptake by tropical forest trees on an annual basis (Chen et al., 2008; Tiessen et al., 1994).

Despite the importance of organic P in the nutrition of tropical forest trees, it is not considered in standard soil tests for "plant-available P", such as Mehlich-III, Bray, or Olsen P. These procedures were developed to predict annual fertilizer requirements by quantifying a pool of soil inorganic P in temperate agricultural soils that correlates with crop growth. Sequential extraction schemes (e.g., Hedley fractionation; Hedley et al., 1982) equate bicarbonate extractable organic P with plant-available organic P (Bowman and Cole, 1978; Cross and Schlesinger, 1995). This pool can be large in tropical forest soils and it has been suggested that failure to account for bicarbonate organic P might explain the high productivity in tropical forests growing on what appear to be soils with low bioavailable P content (Johnson et al., 2003). However, only a fraction of the organic P in bicarbonate

Abbreviations: (IP_6), *myo*-inositol hexakisphosphate; (P), phosphorus; (RP), reactive phosphorus; (TP), total phosphorus; (UP), unreactive phosphorus.

* Corresponding author.
E-mail address: tegan.darch@rothamsted.ac.uk (T. Darch).

http://dx.doi.org/10.1016/j.geoderma.2016.08.018

extracts is amenable to hydrolysis by phosphatase enzymes (Hayes et al., 2000; Turner et al., 2003).

An alternative approach to estimating bioavailable soil organic P is to simulate the mechanisms used by organisms to acquire organic P from the soil. Plants and microbes can solubilize soil organic P by secreting organic acids, including citrate, malate, and oxalate (Begum and Tofazzal, 2005). The rate of organic acid secretion increases under P deficiency in some plants (Gerke, 2015), and many species of non-mycorrhizal plants (e.g. the Proteaceae) that grow on the some of the most infertile soils in the world exude large quantities of organic acids to 'mine' soil P (Lambers et al., 2008). Once organic P is solubilized, it must be hydrolyzed by phosphatase enzymes to release inorganic phosphate for plant uptake (Nash et al., 2014; Richardson et al., 2005). Plants can secrete a series of phosphatase enzymes that target different organic P compounds, including phosphomonoesterase, phosphodiesterase, and phytase (Steidinger et al., 2015; Turner, 2008a). A procedure combining organic acids and phosphatase hydrolysis might therefore provide a more accurate assessment of bioavailable soil P than conventional soil P tests, especially for tropical soils.

Organic acids have previously been used to extract a pool of bioavailable soil P, but extraction conditions vary markedly among studies and no standardized protocol exists. As an example of the variation that exists among protocols, the organic acid concentration, which affects the quantity of P extracted (Strom et al., 2005), has varied in published studies by an order of magnitude, from 1 mM (Lan et al., 1995) to 50 mM (Hayes et al., 2000). Surprisingly, few attempts have been made to use organic acid concentrations typical of those found in the rhizosphere, which are in the order of μM rather than mM (Jones, 1998). Likewise, P extraction is affected by the choice of organic acid (Gerke et al., 2000), extraction pH (Strom et al., 2005), extraction time (Turner, 2008b) and solution to soil ratio (Chapman et al., 1997). Published research has used a variety of conditions, making it difficult to compare among studies, or to determine which extraction conditions best approximate those in the soil. Finally, whilst phosphatase hydrolysis of organic P is a well-established method to assess the potential bioavailability of soil organic P (e.g. Bünemann, 2008; Turner et al., 2003), it has only rarely been applied to organic acid extracts (Hayes et al., 2000) and no standard protocol exists. However, citrate appears to extract a more enzyme-labile pool of organic P compared to other chemical extractants such as sodium bicarbonate, and is therefore more likely to be representative of organic P likely to be utilized by plants (Hayes et al., 2000; Otani and Ae, 1999). A recently developed extraction protocol, designed to account for rhizosphere processes, uses both citric acid and phosphatases to extract soil P as part of a suite of extractions run in parallel (DeLuca et al., 2015), but indications are that organic acids and phosphatases are most effective when used in series (Clarholm et al., 2015).

Here we report the development of a standard protocol for the determination of bioavailable organic P using sequential organic acid extraction and phosphatase hydrolysis. Our aim was to develop a protocol that optimized organic P extraction, but remained biologically meaningful. We examined a number of methodological aspects of the procedures for extraction (organic acid concentration, extractant pH, extraction time, solution to soil ratio) and phosphatase hydrolysis (enzyme concentration, source, and optimal pH). The protocol was then applied to five different soils under lowland tropical forest in Panama, to determine the proportion of potentially bioavailable P as organic P in soils with low concentrations of readily-extractable orthophosphate.

2. Materials and methods

2.1. Soils

We studied eight soils under lowland tropical forest in Panama. Three soils were used for method development and a further five to quantify bioavailable P. The soils were rich in clay and represented three orders (Oxisols, Ultisols and Alfisols) in Soil Taxonomy (Soil Survey Staff, 1999). The soils were selected to have contrasting pH, P and carbon concentrations (Table 1). Samples were taken from the upper 10 cm of soil, air-dried, and sieved (<2 mm). Total P and organic P were determined as reported previously (Turner and Engelbrecht, 2011).

2.2. Selection of organic acids

Plants secrete a number of different organic acids, the composition of which varies among plant species and with plant age (Jones, 1998). Based on previous studies (e.g. Gerke et al., 2000; Wei et al., 2010) we initially tested maleic acid, citric acid, and oxalic acid. In particular, maleic acid and citric acid are among the most quantitatively important organic acids exuded by plants (Roelofs et al., 2001; Veneklaas et al., 2003).

2.3. Method development

2.3.1. Effect of solution to soil ratio

Deionized water, 2 mM citric acid, 2 mM maleic acid, and 2 mM oxalic acid (organic acid solutions were adjusted to pH 4 using dilute NaOH) were shaken for 16 h at 180 oscillations min^{-1} (Model E6010 - Fixed Speed Reciprocal Shaker; Eberbacht, Ann Arbor, MI) with the three soils (Table 1). For each solution–soil combination, 30 mL of solution was shaken with either 6.0 g, 4.0 g, 2.0 g or 1.5 g of soil, to give solution to soil ratios of 5:1, 7.5:1, 15:1 and 20:1 (equivalent to 10, 15, 30 and 40 μmol organic acid g^{-1} soil, respectively). After extraction, samples were centrifuged at 8000 × *g* for 10 min and the solution was analyzed for molybdate-reactive P (RP) and total P (TP) (see Section 2.5). Each treatment was replicated three times.

2.3.2. Extraction time

Soil (6 g) was extracted in 30 mL of 2 mM citric acid or 2 mM oxalic acid (i.e. a 5:1 solution to soil ratio, 10 μg organic acid g^{-1} soil) adjusted to pH 4. Soils were extracted for 0.5, 1, 2, 4, 8 and 16 h. Each treatment was repeated three times. The samples were centrifuged and analyzed for RP and TP as described in Section 2.5.

2.3.3. Extractant pH

The effect of extractant pH on P release was tested only for citric acid. Citric acid monohydrate (2 mM) and sodium citrate dihydrate (2 mM) were mixed in varying proportions to generate solutions of pH 3.3, 4.0, 4.6, 5.0, 5.4, and 6.0. These citrate solutions (30 mL) were added to 6 g of soil, shaken for 2 min, and the pH was measured. The samples were then shaken for a further hour, centrifuged, and the solution was analyzed for RP and TP (Section 2.5). Each treatment was repeated three times.

2.3.4. Phosphatase hydrolysis

A range of enzyme sources and hydrolysis conditions were tested to ensure complete hydrolysis of target compounds, and to confirm that non-target compounds were not hydrolyzed. Enzymes (obtained from Sigma Aldrich, St Louis, Missouri, USA) were diluted in sodium acetate buffer ($NaC_2H_3O_2$, 0.5 M, pH 5) or tris buffer ($C_4H_{11}NO_3$, 0.1 M, pH 8). The buffers contained 2 mM magnesium chloride ($MgCl_2$) and 2 mM zinc sulphate ($ZnSO_4$) as enzyme activators. The enzymes were: acid phosphomonoesterase from potato (EC 3.1.3.2, Sigma P1146), alkaline phosphomonoesterase from bovine intestinal mucosa (EC 3.1.3.1, Sigma P7640), alkaline phosphomonoesterase from *Escherichia coli* (EC 3.1.3.1, Sigma P5931), nuclease from *Penicillium citrinum* (EC 3.1.30.1, Sigma N8630), phosphodiesterase from *Crotalus atrox* (Western Diamondback Rattlesnake) venom (EC 3.1.4.1, Sigma P4506), and phytase from wheat (EC 3.1.3.26, Sigma P1259). The phytase preparation contained phosphate, which was removed by repeated dialysis (12,000 Da membrane) in sodium acetate buffer until the RP