Rhizosphere acid and alkaline phosphatase activity as a marker of P nutrition in nodulated *Cyclopia* and *Aspalathus* species in the Cape fynbos of South Africa

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A R T I C L E   I N F O

A B S T R A C T

Cyclopia and *Aspalathus* species are important economic legumes in the Cape fynbos of South Africa, as they are used for making Honeybush and Rooibos tea, and for trade in the cut wild flower industry. The aim of this study was to assess acid and alkaline phosphatase activity in the rhizosphere of *Cyclopia genistoides*, *Cyclopia subternata*, *Aspalathus caledonensis* and *Aspalathus aspalathoides* as an indicator of P supply and P nutrition in the nutrient-poor soils of the Cape fynbos. Whether at Kokrivier or Kanetberg, the P enzyme activities were much higher in the rhizospheres of the legumes *C. genistoides*, *C. subternata*, *A. caledonensis*, and *A. aspalathoides* compared to those of the non-legumes *Leucadendron strictum*, *Elegia thyrsoidea* and *Mimetes cucullatus*, or bulk soil. As a result, plant-available P concentration in the rhizosphere, as well as shoot P levels closely mirrored acid and alkaline phosphatase activity in the rhizosphere of each plant species. Relative to younger plants, older *Cyclopia* species exhibited, much greater acid and alkaline phosphatase activity in the rhizosphere and this again resulted in much higher plant-available rhizosphere P. *C. subternata* plants developed from cuttings at Kanetberg showed greater rhizosphere acid and alkaline phosphatase activity than seedlings and bulk soil. As a result, the concentration of plant available-P and organic P were much higher in the rhizosphere of cuttings than seedlings, leading to greater shoot P in cuttings than seedlings. Taken together, these data suggest that rhizosphere P enzyme activity can be used as a good indicator of P supply and P nutrition in *Cyclopia* cuttings and seedlings, but less so in *Aspalathus* species in the Cape fynbos. The enhanced P nutrition in plants from cuttings probably accounts for the higher tea yields obtained by farmers when they use cuttings instead of seedlings in their plantations.

1. Introduction

Phosphorus is the second most important mineral nutrient for plant growth after N. Although total phosphate is abundant in many

and/or incorporated into organic matter by microbes (Tran et al., 2010; Vance et al., 2003). Even where P is applied as fertilizer, more than 80% becomes unavailable for plant uptake through adsorption, precipitation, or microbial immobilization (Holford, 1997; Mózner et al., 2012). Thus crop yield on the 30−40% of the world's arable land is limited by P availability (Kochian et al., 2004; Lambers et al.,

2006; Vance et al., 2003). In low P soils, both plants and microbes have developed various strategies for enhancing P supply. These include acidification of the rhizosphere, exudation of organic acids (Dakora and Phillips, 2002), and secretion of extracellular phospha-

teria, fungi and earthworms (Hebrien and Neal, 1990) and function catalytically above pH 7. These enzymes catalyze the cleavage of mineral P from organic phosphate esters, in acidic and alkaline soils that are low in P (Nannipieri et al., 2011), thus making P more available in these soils. Organic P from soil organic matter is therefore the main substrate for P supply by phosphatases in natural and agricultural ecosystems.

Soils of the Cape fynbos are characterized by low organic matter, low pH (pH 3–5), and low nutrient levels (Dakora, 2012), especially plant-available N (0.001–0.002% N, or 1 to 2 mg N g^{-1} soil) and P (0.00004 to 0.00037% P, or 0.4–3.7 μg P g^{-1}; Cramer, 2010). Furthermore, soils of the fynbos biome contain between 58–77% organic P and 2–88% inorganic P, which is Fe-bound and unavailable to plants (Straker, 1996). Despite these nutrient constraints, the fynbos is rich

in many indigenous legume genera and species. For example, the genus *Aspalathus* consists of 281 species that are endemic to the Cape Floristic Region (Boatwright and Cupido, 2011), while *Cyclopia* has 24 species that uniquely occur only in the Cape fynbos (Du Toit et al., 1998). N limitation in legumes of the fynbos is overcome by high levels of N_2 fixation. For example, *Aspalathus linearis* and *Cyclopia* species can contribute over 100 kg N ha^{-1} annually to the fynbos biome (Moufhe and Dakora, 1999; Spriggs and Dakora, 2008). However, little is known about P nutrition in fynbos legumes. Different mechanisms have been proposed for enhanced P supply and mineral uptake by fynbos species. Mycorrhizal infection of fynbos legumes has been suggested to enhance P supply by the symbiosis (Harrison, 1999), just as formation of cluster roots has been regarded as a way of increasing P nutrition in fynbos legumes (Kanu et al., 2013; Lambers and Shane, 2007; Lamont, 1982). We have, in fact, shown that cluster root formation by *A. linearis* in the Cape fynbos does not only improve P nutrition, but also increases the assimilation of other nutrients such as Ca, K, Mg, Na, Al, Fe, Mn, Zn and Cu (data not shown), clearly indicating that cluster root formation is a major mechanism for nutrient acquisition in the nutrient-poor fynbos.

So far, little information exists on the mechanisms of P supply to native legumes of the fynbos. Yet some of them have economic importance. The 24 *Cyclopia* species, for example, are a source of Honeybush tea, a herbal beverage that is emerging as a major income earner that contributes about R12 million to the South African economy (DAFF, Department of Agriculture, Fisheries and Forestry, 2012). Similarly, some of the 281 *Aspalathus* species and most *Leucadendron* species are exported as cut wild flowers (Criley, 2000; Reinten et al., 2011), while *A. linearis* produces Rooibos tea, another herbal beverage that contributes about R500 million to the South African economy (Joubert and de Beer, 2011; http://www.busrep.co.za). *A. linearis* is among the many species of the genus *Aspalathus* that is perceived to be suitable for phytoremediation (Kanu et al., 2013). There is therefore a need to understand the mechanisms underlying P supply to native species in a very low P environment. The aim of this study was to measure rhizosphere acid and alkaline phosphatase activity as an indicator of P supply and availability. This was achieved by measuring acid and alkaline phosphatase activity in bulk and rhizosphere soils and comparing that with P concentrations in both systems. Finally, a measure of increased P availability in the rhizosphere was used to track P levels in plant shoots. For comparison, non-legume plant species were sampled from each site as the legumes and included in this study. The test plant species used in this study are shown in Table 1, together with their collection sites, uses and nutrition-related biological traits. The data showed a relationship between acid and alkaline phosphatase in the rhizosphere and P availability in the rhizosphere, which was ultimately linked to P concentration in shoots.

2. Materials and methods

2.1. Collection and processing of rhizosphere and bulk soils

Rhizosphere soil (defined as soil rich in roots, and/or soil adhering to the roots and influenced by root activity) was collected together with bulk soils from 0 to 30 cm depth at Koksrivier near Gansbaai, and Kanetberg near Riversdale, in the Western Cape Province of South Africa. A minimum of 5 and maximum of 10 soil samples were cored from each farm as rhizosphere or non-rhizosphere bulk soils. The rhizosphere soil was cored at a distance of 1 to 2 mm from the plant roots, while bulk non-rhizosphere soil (free from roots) was collected in between rows of *Cyclopia* and *Aspalathus* plants. The samples were transferred into pre-labelled plastic bags and transported to the laboratory, where they were stored at −4 °C until bioassay of enzyme activity. Subsamples of soil were however air-dried, sieved (2.0 mm), and used for measuring soil P concentration. Rhizosphere soils of non-legumes were similarly collected and processed for comparison with those of legumes.

2.2. Plant harvest and processing

Young shoots of 5 to 10 replicate legume plants (10 shoots per plant) (from which rhizosphere soils were collected) were harvested at crown level, oven-dried (60 °C), weighed, and milled to a fine powder (0.85 mm) for analysis of P. Adjacent non-legume plants were

Table 1
Plant species studied, their sites of collection, families, tribes, uses and nutrition-related traits.

Plant species	Family	Tribe	Plant age	Site	Uses	Nutrition-related traits	References
Cyclopia genistoides and *Cyclopia subternata*	Fabaceae	Podalyrieae	10 & 2 8 & 5	Koksrivier Kanetberg	Honeybush tea, fruit juice mixtures, cosmetic industry, food flavourant, food dye	N_2-fixing, mycorrhizal, cluster root-forming	Spriggs et al. (2003) Spriggs (2004) Power et al (2010) DAFF, Department of Agriculture, Fisheries and Forestry (2012) Van Wyk (2011)
Aspalathus caledonensis	Fabaceae	Crotalarieae	2	Koksrivier	Harvested as fresh and cut dried flowers	N_2-fixing, mycorrhizal cluster root-forming	Allsopp and Stock (1994) Maseko (2013) Maseko and Dakora (2013)
Aspalathus aspalathoides	Fabaceae	Crotalarieae	2	Koksrivier	Harvested as fresh and cut dried flowers	N_2-fixing, mycorrhizal, cluster root-forming	Allsopp and Stock (1994) Maseko (2013) Maseko and Dakora (2013)
Leucadendron strictum	Proteaceae	Senecioneae	8	Kanetberg	Harvested as fresh and cut dried flowers	Non-N_2-fixing, mycorrhizal, cluster root-forming	Criley (2000) Lambers and Shane (2007) Reinten et al (2011) Maseko and Dakora (2013)
Mimetes cucullatus	Proteaceae	Corotocina	2	Koksrivier	Harvested as fresh and cut dried flowers	Non-N_2-fixing	Reinten et al (2011)
Gnidia anomala	Thymelaeceae	Gnidieae	10	Koksrivier	Medicinal, thatching, wood, clothing, dyeing leather	Non-N_2-fixing	Bhandurge et al (2013)
Tetraria bromoides	Cyperaceae	Schoeneae	2	Koksrivier	Grazing, erosion control, water purification	Non-N_2-fixing	Lambers and Shane (2007) http://www.inarchive.com
Elegia thyrsoidea	Restionaceae	Restioneae	5	Kanetberg	Thatching, horticultural industry	Non-N_2-fixing	Kubitzki and Huber (1998)
Erica multiflora	Ericaceae	Ericeae	5	Kanetberg	Grazing, medicinal	Non-N_2-fixing, mycorrhizal	Straker (1996) Rogosic et al (2006) Harnafi et al (2007)

similarly harvested and processed for P analysis for comparison with legumes.

2.3. Determination of pH and plant-available P in rhizosphere and bulk soils

To measure pH, 50 mL 0.01 M $CaCl_2$ was added to 10 g of soil sample which had been air-dried and sieved (2 mm). The mixture was shaken for 30 min, allowed to settle, and the pH measured using a pH meter.

Extractable P in each of the 5 to 10 replicate soils was determined by the citric acid method, using the modification of Du Plessis and Burger (1964). A 20 g of the air-dried soil sample was extracted in 200 mL of 1% (w/v) citric acid, heated to 80 °C shaken for 2 min at 10-min intervals over 1-h period and filtered. A 50 mL aliquot was heated to dryness on a water bath, digested with 5 mL of concentrated HCl and HNO_3, evaporated to dryness on water bath, and 5 mL of concentrated HNO_3 and 20 mL of deionized water added. The mixture was then heated to dissolve the dry residue, and the sample filtered. Measurement of P was done directly by aspiration on a calibrated simultaneous inductively coupled plasma-mass spectrometer (IRIS/AP HR DUO Thermo Electron Corporation, Franklin, Massachusetts, USA).

2.4. Measurement of P in plant shoots

Measurement of P in plant shoots was determined by ashing 1.0 g of finely ground sample (0.85 mm) in a porcelain crucible at 500 °C overnight. The ash was dissolved in 5 mL of 6 M HCl and placed in an oven at 50 °C for 30 min, followed by addition of 35 mL deionised water. The mixture was filtered through Whatman #1 filter paper, and the concentration of P in plant extracts determined using inductively coupled plasma mass spectrometry (Giron, 1973).

2.5. Assay of acid and alkaline phosphatase activity in bulk and rhizosphere soils

Bioassay of phosphatase activity was done as described by Tabatabai (1994). About 1 g of moist soil sample (stored at −4 °C) was weighed in duplicates into polypropylene vials for each of the 5 to 10 replicate soil samples. Acid or alkaline phosphatase activity was determined by adding 4 mL of a pH buffer (pH 6.5 for acid and pH 11 for alkaline phosphatases), and 1 mL of 0.1 M disodium phenylphosphate as a substrate. The mixture in polypropylene vials was incubated at 37 °C for 1 h. At the end of incubation, 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH were added to stop the reaction, the mixture was filtered through Whatman #2 filter paper and the supernant analyzed using a UV–VIS spectrophotometer at 420 nm. Absorbance of filtrates was compared with p-nitrophenol standards. For each assay, a control was included to account for non-enzymatic substrate hydrolysis.

2.6. Statistical analysis

Statistical analyses were carried out using a STATISTICA 2 analytical software program 2007 (StatSoft Inc, 2005). A 1-Way ANOVA was performed to compare the means of phosphatase enzyme activity as well as P concentration in soil and shoots of each species. Where significant differences were found, the Duncan Multiple Range Test was used to separate treatment means at p ≤ 0.05.

3. Results

3.1. Soil pH

Although the rhizosphere pH of each species was generally lower than that of the corresponding bulk soil, these were not statistically significant. The mean values of rhizosphere and bulk soil were respectively 4.32 and 4.48, 4.12 and 4.48, and 4.12 and 4.20 for *Cyclopia genistoides*, *Aspalathus* species and *Cyclopia subternata*. The rhizosphere pH of the test species were also similar in magnitude.

3.2. Soil P, shoot P and Acid/alkaline phosphatase activity in rhizosphere and bulk soils from Koksrivier and Kanetberg

Acid and alkaline phosphatase activity in rhizosphere soil of *C. genistoides* established in 1997 and 2005 at Koksrivier were measured and found to be significantly (p ≤ 0.05) higher than that of bulk soil (Table 2). A comparison of phosphatase activity between years showed that *C. genistoides* planted in 1997 had higher rhizosphere acid and alkaline phosphatase activity than those established in 2005 (Table 2). P concentration was measured in both bulk and rhizosphere soil of *C. genistoides* to determine whether the differences in acid and alkaline phosphatase activity affected P availability. Plant-available inorganic P concentration was much greater in the rhizosphere soil of *C. genistoides* than bulk soil (Table 2). Organic P levels however were greater in non-rhizosphere bulk soil compared to rhizosphere of *C. genistoides* established in 1997 (Table 2). The greater enzyme activity in the rhizosphere of *C. genistoides* from the 1997 plantation resulted in equally greater rhizosphere inorganic P concentration compared to *C. genistoides* in 2005 plantation (Table 2). A comparison of shoot P concentration between *C. genistoides* grown in 1997 and a non-legume, *Gnidia anomala* showed no differences, younger shoots of the legume however accumulated greater P concentration compared to another non-legume, *Tetraria bromoides* (Table 2). A similar assay of enzymes was done for *C. subternata* planted at Kanetberg in 1999 and 2002. The data showed that acid phosphatase activity was again higher in the rhizosphere soils of *C. subternata* compared to bulk soil (Table 3). Rhizosphere soil of *C. subternata* established in 1999 showed significantly greater acid and alkaline phosphatase activity to that grown in 2002 (Table 3). Measurement of inorganic and organic P levels in both bulk and rhizosphere soil revealed significantly greater P levels in rhizosphere soil than bulk soil (Table 3). Determination of P concentration in

Table 2
P nutrition in two fynbos species at Koksrivier. Acid (APase) and alkaline (Alk) phosphatase activity and plant-available inorganic phosphorus (Pi) and organic phosphorus (Po) associated with rhizosphere and non-rhizosphere soil of *Cyclopia genistoides* established in 1997 and 2005 at Koksrivier farm. Values (Mean ± SE) followed by dissimilar letters in a column for year of planting and in a row for enzyme activity for soil or plant P are significantly different at p ≤ 0.05. Lower case letters compare means of phosphorus level in a column and upper case letters compare means in a row. 1997 (non-legume: *Gnidia anomala*) and 2005 (non-legume: *Tetraria bromoides*).

Plant age	Soil APase activity		Soil Alk activity		Pi		Po		Shoot P	
	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Legume	Non-legume
	μg ρ-nitrophenol g^{-1} F wt soil h^{-1}				mg.kg^{-1}		g.kg^{-1}		mg.g^{-1}	
10	826 ± 37.3aA	379 ± 17.9aB	168 ± 9.9aC	75 ± 10.8aD	10 ± 2.0aa	3.5 ± 1.8aB	372 ± 34.4aB	782 ± 25.6aA	0.57 ± 0.0aA	0.625 ± 0.1aA
2	586 ± 35.4bA	263 ± 90.5aB	93 ± 4.9bC	64 ± 28.3aD	8 ± 0.9bA	3.7 ± 1.8aB	499 ± 74.5aA	604 ± 58.6bA	0.88 ± 0.0aA	0.575 ± 0.1aB
F-Statistics	21.86**	1.58ns	46.56***	0.13ns	4.83*	0.13ns	2.41ns	6.47*	0.00ns	0.00ns

Table 3
P nutrition in two fynbos species at Koksrivier. Acid (APase) and alkaline (Alk) phosphatase activity and plant-available inorganic phosphorus (Pi) and organic phosphorus (Po) associated with rhizosphere and non-rhizosphere soil of *Cyclopia subternata* established in 1997 and 2005 at Koksrivier farm. Values (Mean ± SE) followed by dissimilar letters in a column for the year of planting and in a row for enzyme activity for soil or plant P are significantly different at $p \leq 0.05$. Lower case letters compare means of phosphorus level in a column and upper case letters compare means in a row.1999 (non-legume: *Leucadendron strictum*) and 2002 (non-legume: *Elegia thyrsoidea*).

Plant age	Soil APase activity		Soil Alk activity		Pi		Po		Shoot P	
	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Legume	Non-legume
	µg ρ-nitrophenol g^{-1} F wt soil h^{-1}				mg.kg^{-1}		g.kg^{-1}		mg.g^{-1}	
8	839 ± 50.3aA	644 ± 51.9aB	266 ± 15.9aC	242 ± 9.7aC	20.4 ± 1.8aA	14.8 ± 1.4aB	2064 ± 92.8aA	1706 ± 121.0aB	1.1 ± 0.0aA	0.75 ± 0.0aB
5	726 ± 48.8bA	561 ± 34.4aB	193 ± 21.4bC	138 ± 37.1bC	24.0 ± 2.7aA	8.8 ± 2.1bB	2114 ± 164.3aA	1636 ± 152.4aB	1.1 ± 0.0aA	0.62 ± 0.2aB
F-Statistics	8.85***	1.77ns	7.47**	7.39**	1.24ns	5.92*	0.07ns	0.13ns	0.31ns	0.41ns

shoots of *C. subternata* plants showed higher P levels when compared to the two non-legumes, *Leucadendron strictum* and *Elegia thyrsoidea* (Table 3).

3.3. Acid phosphatase activity, soil P and shoot P of C. genistoides compared with Aspalathus caledonensis, A. aspalathoides and the non-legume Mimetes cucullatus at Koksrivier

The marked differences in acid phosphatase between bulk and rhizosphere soil of *C. genistoides* and *C. subternata* led to further field tests that compared the enzyme activity of *C. genistoides* with two *Aspalathus* species and the non-legume *Mimetes cucullatus* at Koksrivier. The data generally showed much higher acid phosphatase activity in the legumes relative to non-legume. *C. genistoides* had the highest enzyme activity, followed by *A. caledonensis*, *A. aspalathoides*, and *Mimetes cucullatus* (Fig. 1A). For each species, rhizosphere plant-available inorganic P concentration closely mirrored rhizosphere enzyme activity, except for *A. caledonensis* which had high enzyme activity but low rhizosphere inorganic P level (Fig. 1B). In a trend almost opposite that of the inorganic P, the non-legume, *Mimetes cucullatus* showed greater rhizosphere organic P (Fig. 1C). To ascertain plant response to the species differences in rhizosphere P availability, shoot P concentration was measured and found to closely mirror rhizosphere inorganic P concentration of *C. genistoides*, *A. aspalathoides* and *M. cucullatus*, but not *A. caledonensis* (Fig. 1D).

3.4. A comparison of acid/alkaline phosphatase activity, soil P, and shoot P in the rhizosphere of Cyclopia cuttings vs. seedlings

Two adjacent *C. subternata* plantations developed from seedlings and cuttings at Kanetberg were assessed for phosphatase enzyme activity in bulk and rhizosphere soil. Rhizosphere soil from both cuttings and seedlings showed greater enzyme activity than bulk soil. However, the acid and alkaline phosphatase activity was higher in cuttings than seedlings (Table 4). Determination of both inorganic and organic P showed both planting material to possess greater P level in rhizosphere than non-rhizosphere bulk soil (Table 4). However, the cuttings showed much greater plant-available inorganic and organic P concentration in rhizosphere soil than seedlings (Table 4). The marked differences in P availability and accumulation in the rhizosphere resulted in statistically greater shoot P levels in cuttings than seedlings, legume shoot P was also statistically greater than those of the non-legumes *Erica multiflora* and *E. thyrsoidea* (Table 4).

4. Discussion

4.1. Can acid/alkaline phosphatase activity in the rhizosphere serve as an indicator of P supply to Cyclopia species?
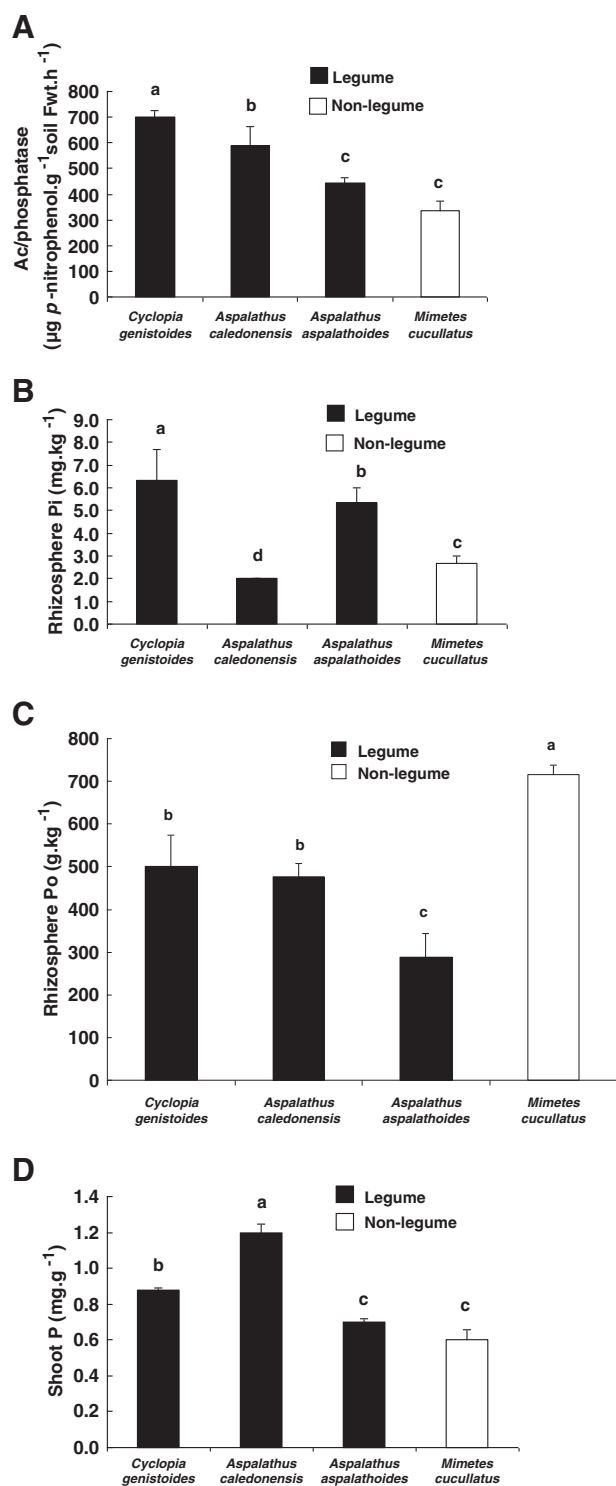
The Cape fynbos is characterized by low concentrations of mineral nutrients in soil, especially P and N. While N requirement in legumes is often met by symbiotic N_2 fixation, P supply is enhanced through a range of mechanisms which include development of mycorrhizal symbiosis, formation of cluster roots, alteration in root architecture, production of root exudates, as well as induction of phosphatase enzyme activity (Maseko and Dakora, 2013). Some nutrition-related biological traits of the test species used in this study are listed in Table 1. The aim of this part of the study was to assess acid and alkaline phosphatase activity as an indicator of P supply in the rhizosphere of *C. genistoides* from a plantation at Koksrivier. The data revealed a significant relationship between acid/alkaline phosphatase activity in the rhizosphere of *C. genistoides* and P concentration in the soil around the roots. Whether planted in 1997 or 2005, both acid and alkaline phosphatase activities in the rhizosphere of *C. genistoides* showed higher activity compared to bulk soils (Table 2). There was also increased P enzyme activity in the rhizosphere of older *C. genistoides* plants compared to their younger counterparts (Table 2). These higher P enzyme activities resulted in markedly greater plant-available inorganic P concentrations in the rhizosphere compared to bulk soil, and in the rhizosphere of older plants compared to younger ones (Table 2). The greater acid/alkaline phosphatase activity also resulted in changes in soil organic P. As the activity of soil phosphatases increased (Table 2), there was a marked and significant decrease in rhizosphere organic P compared to the bulk soil, and a decrease in rhizosphere organic P of older plants compared to their younger counterparts (Table 2). Similar findings have been reported for a number of agricultural species (Asmar et al., 1995; Chen et al., 2002; Radersma and Grierson, 2004; Tarafdar and Jungk, 1987). Our data therefore suggest that the increase in acid/alkaline phosphatase activity in the rhizosphere of *Cyclopia* resulted in greater inorganic P supply from organic sources relative to bulk soil, where there was low P enzyme activity, and therefore low P concentration. The greater acid phosphatase activity in the rhizosphere of older *Cyclopia* plants also resulted in increased Pi supply in the rhizosphere of older compared to younger plants (Table 2). However, the increased plant-available P in the rhizosphere was not reflected in tissue P concentrations, perhaps suggesting that plants adapted to low-nutrient soils such as fynbos biome, inherently take up small quantities of nutrients even with high nutrient supply (Witkowski, 1991).

To confirm that rhizosphere P enzyme activity is a proxy for P supply in the rhizosphere, a similar study was conducted at Kanetberg using *C. subternata* planted in 1999 and 2002. The data again revealed markedly high acid phosphatase enzyme activity in the rhizosphere than bulk soil, and in the rhizosphere of older than younger *C. subternata* plants. As a result, organic and inorganic P levels were lower in magnitude in the rhizosphere of older plants, but not statistically (Table 3). There was also much greater organic and inorganic and P concentrations in the rhizosphere relative to bulk soil (Table 3).

It is important to note that the non-legume *T. bromoides* recorded lower P concentration in shoots at Koksrivier in 2005 compared to nodulated *C. genistoides* (Table 2). Similarly, the non-legumes *L. strictum* and *E. thyrsoidea* also showed much lower P levels in

generally accumulate greater mineral nutrients in their organs than cereals (Broadley et al., 2003; Fageria, 2004; Pedersen et al., 2002). While the mechanism remains unknown, the greater P concentration in *Cyclopia* shoots over the non-legumes *T. bromoides*, *L. strictum* and *E. thyrsoidea* could well be further evidence that N_2-fixing shrub/tree legumes also accumulate more minerals than their non-fixing counterparts. Whatever the case, the greater P assimilation by *C. genistoides*, *C. subternata*, *A. caledonensis* and *A. aspalathoides* over non-legumes at Koksrivier and Kanetberg could suggest that, for these legume species endemic to the low-P fynbos environment, rhizosphere acid/alkaline phosphatase activity is potentially a good measure of P availability.

To further assess if the increase in P supply observed here enhances P nutrition in other fynbos legumes, rhizosphere soils of two *Aspalathus* species growing in the same field at Koksrivier as *C. genistoides* were sampled together with the non-legume *Mimetes cucullatus* and *C. genistoides* as control. The rhizosphere soils were assayed for acid/alkaline phosphatase activity. The data revealed marked differences between and among the four test species, with *C. genistoides* exhibiting significantly higher acid phosphatase activity, followed by *A. caledonensis*, *A. aspalathoides* and *M. cucullatus* (Fig. 1A). Although the rhizosphere soil of *Aspalathus caledonensis* had the second highest P enzyme activity, rhizosphere Pi was lowest for that species (Fig. 1B). However, of the other test plants, the rhizosphere concentration of plant-available Pi was higher in *C. genistoides* and *A. aspalathoides*, and least in the non-legume, *M. cucullatus* (Fig. 1B). Because of the low acid phosphatase activity in the rhizosphere of the non-legume *M. cucullatus*, the level of organic P substrate was much greater compared to *C. genistoides*, *A. caledonensis* and *A. aspalathoides* (Fig. 1C). However, measuring P in shoots revealed significantly greater concentrations in *A. caledonensis* (Fig. 1D) [the species with the lowest rhizosphere Pi], suggesting that the low concentration in the *A. caledonensis* rhizosphere was due to rapid uptake by roots, thus creating a depletion in rhizosphere inorganic P (Fig. 1B). Such variation in P assimilation has been reported for other plant species and genotypes (Betrand et al., 1999; Chen et al., 2002; Hedley et al., 1994; Shi et al., 2004; Zhao et al., 2010; Zoysa et al., 1997, 1999), and could be inherently related to a greater P demand by *A. caledonensis* compared to the other co-occurring fynbos species such as *A. aspalathoides* and *C. genistoides*. As found by Silberbush et al. (1981), the intensity of acid phosphatase enzyme exudation is strongly influenced by the P demand of the plant species.

4.2. Is the expression of acid/alkaline phosphatase activity different in the rhizosphere of cuttings and seedlings of Cyclopia species?

The Honeybush tea farmers in the Cape of South Africa use both seeds and cuttings as planting material in their tea plantations with greater preference for cuttings. From interviews with farmers regarding the choice of Honeybush tea planting material, Gleason (2004) found that farmers have observed a huge variation in tea yield during the second and third year of tea production from seeds. Moreover, the majority of *Cyclopia* plants grown from seeds generally exhibited retarded growth. Furthermore, less than 15% of *C. subternata* plants established from seed were productive by the fifth year of cultivation. By contrast, *C. subternata* plants established from cuttings generally produced twice the dry matter yield of seedlings of the same age.

The aim of this part of the study was to assess whether P nutrition is a factor in the variation of tea yield on farmers' fields. As described before, this was done using bioassays of P enzyme activity in rhizosphere soils, as well as P in shoots of tea plants. The data revealed higher acid and alkaline phosphatase activity in the rhizosphere soil of cuttings than seedlings (Table 4). As a result, there was markedly greater organic and inorganic P concentrations in the rhizosphere soil of cuttings relative to seedlings (Table 4). Possibly due to increased uptake of this available P, shoot P concentrations were also greater in *C. subternata* plants

Fig. 1. Phosphorus nutrition in four fynbos species: A) Acid phosphatase activity associated with rhizosphere soils of *Cyclopia genistoides*, *Aspalathus caledonensis*, *Aspalathus aspalathoides*, and *Mimetes cucullatus* sampled from Koksrivier farm; B) Pi levels in the rhizosphere soils of the four fynbos species, C) Po percentage in rhizosphere of the test species and D) P in shoots of *Cyclopia genistoides*, *Aspalathus caledonensis*, *Aspalathus aspalathoides*, and *Mimetes cucullatus*. Bars followed by dissimilar letters are significantly different at p ≤ 0.05. Vertical lines on bars represent SE (n = 5).

shoots at Kanetberg in 1999 and 2002, respectively, relative to the N_2-fixing *C. subternata* (Table 3). While these high P concentrations in shoots of *Cyclopia* species could be attributed to P enzyme activity in the rhizosphere, it is well documented that N_2-fixing legumes

Table 4

P nutrition in two fynbos species at Koksrivier. Acid (APase) and alkaline (Alk) phosphatase activity and plant-available inorganic phosphorus (Pi) and organic phosphorus (Po) associated with rhizosphere and non-rhizosphere soil of *Cyclopia subternata* planted using cuttings and seeds in 2002 at Kanetberg farm. Values (Mean ± SE) followed by dissimilar letters in a column for year of planting and in a row for enzyme activity for soil or plant P are significantly different at $p \leq 0.05$. Lower case letters compare means of phosphorus level in a column and upper case letters compare means in a row. Cuttings (non-legume: *Elegia thyrsoidea*) and seedlings (non-legume: *Erica multiflora*).

Treatment	Soil APase activity		Soil Alk activity		Pi		Po		Shoot P	
	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	>Legume	Non-legume
	µg ρ-nitrophenol g^{-1} F wt soil h^{-1}				mg kg^{-1}		g kg^{-1}		mg g^{-1}	
Cuttings	726.1 ± 48.8aA	561.2 ± 34.4aB	229.5 ± 19.9aC	102.5 ± 15.3aC	24.0 ± 2.7aA	8.8 ± 2.1aB	2114 ± 164.3aA	1636 ± 152.4aB	1.1 ± 0.0aA	0.63 ± 0.2aB
Seedlings	528.2 ± 46.4bA	318.8 ± 51.6bB	136.6 ± 20.0bC	42.7 ± 1.5bD	13.2 ± 1.1bA	7.8 ± 2.5aB	1642 ± 839.3bA	1154 ± 100.1bB	1.0 ± 0.0bA	0.68 ± 0.1aB
F-Statistics	8.63*	15.30**	10.85**	15.09**	13.99**	0.09^{ns}	6.55*	6.99*	6.60**	0.06^{ns}

established from cuttings than seedlings. As observed at Koksrivier and Kanetberg, *Cyclopia* grown from seedlings also showed greater P levels in shoots compared to the non-legumes *E. thyrsoidea* and *Erica multiflora*, a finding consistent with data for N$_2$-fixing grain legumes and non-fixing cereals (Broadley et al., 2003; Fageria, 2004; Pedersen et al., 2002). At Koksrivier, the shoot P levels of nodulated *A. caledonensis*, *A. aspalathoides* and *C. genistoides* were also greater than those of the non-legume *M. cucullatus* (Fig. 1D), suggesting that even with tree/shrub species, legumes accumulate more nutrient elements than non-legumes.

Using an agricultural legume, Makoi et al (2010) reported significant correlations between plant-available P and acid phosphatase activity in the rhizosphere of cowpea grown in fynbos soils. In this study, we also found a significantly positive correlation between acid phosphatase activity and organic P levels in the rhizosphere of the test legumes (Table 5). This could be interpreted to mean that the more the organic-P substrate in the rhizosphere, the greater the enzyme activity, and hence P availability in the rhizosphere for root uptake. However, with accumulation of inorganic P in the rhizosphere, acid phosphatase activity can get suppressed (Nannipieri et al., 2011; Treseder and Vitousek, 2001).

In conclusion, our results suggest that acid/alkaline phosphatase activity in the rhizosphere of *Cyclopia* and *Aspalathus* species growing in the low-P soils of the Cape fynbos can be used as a predictor of P supply and P assimilation in the rhizosphere of those legumes. We, however, acknowledge that other mechanisms such as cluster root formation and mycorrhizal infection could have contributed to P uptake by the test legumes (Table 1), as cluster root-forming *A. linearis* has been found to accumulate P in addition to other essential nutrient elements in the nutrient-poor Cape fynbos.

Acknowledgements

Table 5

Correlation analysis of acid phosphatase activity against organic P (Po) and inorganic P (Pi) in rhizosphere soils of *C. genistoides*, *C. subternata*, *Aspalathus caledonensis* and *A. aspalathoides*. Mean values are significantly different at *P≤0.05, **P≤0.01, ***P≤0.001.

Species name/Year	Significance	
	Po	Pi
C. genistoides (1997)	0.9995***	−0.8995*
C. genistoides (2005)	0.9141*	−0.8138*
C. subternata (1999)	0.9884***	−0.9399**
C. subternata (2002)	0.9100*	−0.8902*
A. aspalathoides	0.9612*	−0.9940***
Cuttings	0.9100*	−0.8724*

References

Allsopp, N., Stock, W.D., 1994. VA mycorrhizal infection in relation to edaphic characteristics and disturbance regime in three lowland plant communities in the south-western Cape, South Africa. Journal of Ecology 82, 271–279.

Asmar, F., Gahoonia, T.S., Nielsen, N.E., 1995. Barley genotypes differ in activity of soluble extracellular phosphatase and depletion of organic phosphorus in the rhizosphere soil. Plant and Soil 172, 117–122.

Betrand, I., Hinsinger, P., Jaillard, B., Arvieu, J.C., 1999. Dynamics of phosphorus in the rhizosphere of maize and rape grown on synthetic, phosphate calcite and goethite. Plant and Soil 211, 111–119.

Bhandurge, P., N, R., Ganapaty, S., Pattanshetti, S., 2013. The Gnidia genus: a review. Asian Journal of Biomedical & Pharmaceutical Sciences 3, 1–31.

Boatwright, J.S., Cupido, C.N., 2011. Aspalathus crewiana (Crotalarieae, Fabaceae), a new species from the Western Cape Province of South Africa. Nordic Journal of Botany 29, 513–517.

Broadley, M.R., Bowen, H.C., Cotterill, H.L., Hammond, J.P., Meacham, M.C., Mead, A., White, P.J., 2003. Variation in the shoot calcium content of angiosperms. Journal of Experimental Botany 54, 1431–1446.

Chen, C.R., Condron, L.M., Davis, M.R., Sherlock, R.R., 2002. Phosphorus dynamics in the rhizosphere of perennial ryegrass (Lolium perenne L.) and radiata pine (Pinus radiata D. Don.). Soil Biology and Biochemistry 34, 487–499.

Cramer, M.D., 2010. Phosphate as a limiting resource: introduction. Plant and Soil 334, 1–10.

Criley, R.A., 2000. Leucospermum: botany and horticulture. In: Janick, J. (Ed.), Horticultural Reviews. John Wiley, Oxford, pp. 28–88.

DAFF, Department of Agriculture, Fisheries and Forestry, 2012. Report, Honeybush tea, Honeybush tea industry profile. http://www.nda.agric.za (15/09/2012).

Dakora, F.D., 2012. Root-nodule bacteria isolated from native Amphithalea ericifolia and four Aspalathus species from the acidic soils of the South African fynbos are tolerant to very low pH. African Journal of Biotechnology 11, 3766–3772.

Dakora, F.D., Phillips, D.A., 2002. Root exudates as mediators as mineral acquisition in low-nutrient environments. Plant and Soil 245, 35–47.

Du Plessis, S.F., Burger, R.D.T., 1964. A comparison of chemical extraction methods for the evaluation of phosphate availability of top soils. South African Journal of Agricultural Science 8, 11–13.

Du Toit, J., Joubert, E., Britz, T.J., 1998. Honeybush tea — a rediscovered indigenous South African herbal tea. Journal of Sustainable Agriculture 12, 67–84.

Fageria, N.K., 2004. Dry matter yield and shoot nutrient concentration of upland rice, common bean, corn, and soybean grown in rotation on an Oxisol. Communications in Soil Science and Plant Analysis 35, 961–964.

Giron, H.C., 1973. Comparison between dry ashing and wet digestion in the preparation of plant materials for atomic absorption analysis. Atomic Absorption Newsletters 12, 28–29.

Gleason, G., 2004. Cuttings are key to commercial Cape honeybush production. Farmers' Weekly No. 94029, 30 July. 40–41.

Harnafi, H., Bouanani, N.H., Serghini, C.H., Ghalim, N., Amrani, S., 2007. The hypolipidaemic activity of aqueous Erica multiflora flowers extract in Triton WR−1339 induced hyperlipidaemic rats: a comparison with fenofibrate. Journal of Ethnopharmacology 3, 156–160.

Harrison, M.J., 1999. Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Plant Physiology and Plant Molecular Biology 50, 361–389.

Hebrien, S.A., Neal, J.L., 1990. Soil pH and phosphatase activity. Communications in Soil Science and Plant Analysis 21, 439–456.

Hedley, M.J., Kirk, G.J., Santos, M.B., 1994. Phosphorus efficiency and the forms of soil phosphorus utilized by upland rice cultivars. Plant and Soil 158, 53–62.

Holford, I.C.R., 1997. Soil phosphorus: its measurement, and its uptake by plants. Australian Journal of Soil Research 35, 227–239.

http://www.busrep.co.za/business-news/25/08/10 (23 March 2011).
http://www.inarchive.com (12 June 2013).
StatSoft Inc, 2005. STATISTICA (data analysis software System), version 7.1. www.statsoft.com.
Joubert, E., De Beer, D., 2011. Rooibos (Aspalathus linearis) beyond the farm gate: from herbal tea to potential phytopharmaceutical. South African Journal of Botany 77, 869–886.
Kanu, S.A., Dakora, F.D., 2009. Thin-layer chromatographic analysis of lumichrome, riboflavin and indole acetic acid in cell-free culture filtrate of Psoralea nodule bacteria grown at different pH, salinity and temperature regimes. Symbiosis 48, 173–181.
Kanu, S.A., Dakora, F.D., 2012. Effect of N and P nutrition on extracellular secretion of lumichrome, riboflavin and indole acetic acid by N₂-fixing bacteria and endophytes isolated from Psoralea nodules. Symbiosis 57, 15–22.
Kanu, S.A., Matiru, V.N., Dakora, F.D., 2007. Strain and species differences in rhizobial secretion of lumichrome and riboflavin, measured using thin-layer chromatography. Symbiosis 43, 37–43.
Kanu, S.A., Okonkwo, J.O., Dakora, F.D., 2013. Aspalathus linearis (Rooibos tea) as potential phytoremediation agent: a review on tolerance mechanisms for aluminium uptake. Environmental Reviews 21, 85–92.
Kochian, L.V., Hoekenga, O.A., Pineros, M.A., 2004. How do crop plants tolerate acid soils? Mechanism of aluminium tolerance and phosphorus efficiency. Annual Review of Plant Biology 55, 459–493.
Kubitzki, K., Huber, H., 1998. Flowering plants, monocotyledons: Lilianae (except Orchidaceae). In: Kubitzki, K. (Ed.), The Families and Genera of Vascular Plants. , vol. 3. Springer, Berlin, Heidelberg.
Lambers, H., Shane, M.W., 2007. Role of root clusters in phosphorus acquisition and increasing biological diversity in agriculture. In: Spiertz, J.H.J., Struik, P.C., Van Laar, H.H. (Eds.), Scale and Complexity in Plant Systems Research: Gene-Plant-Crop Relations. Springer, Dordrecht, The Netherlands, pp. 237–250.
Lambers, H., Shane, M.W., Cramer, M.D., Pearse, S.J., Veneklaas, E.J., 2006. Root structure functioning for efficient acquisition of phosphorus: matching morphological and physiological traits. Annals of Botany 98, 693–713.
Lambers, H., Raven, J.A., Shaver, G.R., Smith, S.E., 2008. Plant nutrient-acquisition strategies change with soil age. Trends in Ecology & Evolution 23, 95–103.
Lamont, B., 1982. Mechanisms for enhancing nutrient uptake in plants, with particular reference to Mediterranean South Africa and Western Australia. The Botanical Review 48, 597–689.
Makoi, J.H.J.R., Chimphango, S.B.M., Dakora, F.D., 2010. Elevated levels of acid and alkaline phosphatase activity in roots and rhizosphere of cowpea (Vigna unguiculata L. Walp.) genotypes grown in mixed culture and at different densities with sorghum (Sorghum bicolor L.). Crop & Pasture Science 61, 279–286.
Maseko, S.T., 2013. N₂ fixation, P nutrition, C and mineral accumulation, and δ¹³C of nodulated natural stands of legumes in the Cape fynbos, South Africa. D-Tech. Agric. (Crops Sciences) Thesis Tshwane University of Technology.
Maseko, S.T., Dakora, F.D., 2013. Plant enzymes, root exudates, cluster roots and mycorrhizal symbiosis are the drivers of P nutrition in native legumes growing in P deficient soil of the Cape fynbos in South Africa. Journal of Agricultural Science and Technology A 3, 331–340.
Moufhe, M.L., Dakora, F.D., 1999. Nitrogen nutrition in nodulated field plants of the shrub tea legume Aspalathus linearis assessed using ¹⁵N natural abundance. Plant and Soil 209, 181–186.
Mózner, Z., Tabi, A., Csutora, M., 2012. Modifying the yield factor based on more efficient use of fertilizer-the environmental impacts of intensive agricultural practices. Ecological Indicators 16, 58–66.
Nannipieri, P., Giagnoni, L., Landi, L., Renetla, G., 2011. Role of phosphatase enzymes in soil. In: Bünemann, E.K., Oberson, A., Frossard, E. (Eds.), Phosphorus in action, soil biology 26. Springer, Berlin, pp. 215–243.
Pedersen, G.A., Brink, G.E., Fairbrother, T.E., 2002. Nutrient uptake in plant parts of sixteen forages fertilized with poultry litter. Agronomy Journal 94, 895–904.

Power, S.C., Cramer, M.D., Verboom, G.A., Chimphango, S.B.M., 2010. Does phosphate acquisition constrain legume persistence in the fynbos of the Cape Floristic Region? Plant and Soil 334, 33–46.
Radersma, S., Grierson, P.F., 2004. Phosphorus mobilization in agroforestry: Organic anions, phosphatase activity and phosphorus fractions in the rhizosphere. Plant and Soil 259, 209–219.
Reinten, E.Y., Coetzee, J.H., Van Wyk, B.-E., 2011. The potential of South African indigenous plants for the international cut flower trade. South African Journal of Botany 77, 934–946.
Richardson, A.E., Barea, J.M., Mcneill, A.M., Prigent-Combaret, C., 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant and Soil 321, 305–339.
Rogosic, J., Pfister, J.A., Provenza, F.D., Grbesa, D., 2006. Sheep and goat preference for and nutritional value of Mediterranean maquis shrubs. Small Ruminant Research 64, 169–179.
Shi, W.M., Wang, X.C., Yan, W.D., 2004. Distribution patterns of available P and K in rape rhizosphere in relation to genotypic difference. Plant and Soil 261, 11–16.
Silberbush, M., Shomer-Ilan, A., Waisel, Y., 1981. Root surface phosphatase activity in ecotypes of Aegilops peregrine. Physiologia Plantarum 53, 501–504.
Spriggs, A.C., 2004. Symbiotic N₂ fixation in Cyclopia Vent. spp. (honeybush): Towards sustainable cultivation in the Western Cape of South Africa. Doctoral degree Thesis University of Cape Town.
Spriggs, A.C., Dakora, F.D., 2008. Field assessment of symbiotic N₂ fixation in wild and cultivated Cyclopia species in the South African fynbos by ¹⁵N natural abundance. Tree Physiology 29, 239–247.
Spriggs, A.C., Stock, W.D., Dakora, F.D., 2003. Influence of mycorrhizal associations on foliar δ¹⁵N values of legume and non-legume shrubs and trees in the fynbos of South Africa: implications for estimating N₂ fixation using the ¹⁵N natural abundance method. Plant and Soil 255, 495–502.
Straker, C.J., 1996. Ericoid mycorrhiza: ecological and host specificity. Mycorrhiza 6, 215–225.
Tabatabai, M.A., 1994. Soil enzymes. In: Weaver, R.W., Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A., Wollum, A. (Eds.), Methods of soil analysis. Part 2. Microbiological and biochemical properties. Soil Science Society of America, Madison, pp. 775–833.
Tarafdar, J.C., Jungk, A., 1987. Phosphatase activity in the rhizosphere and its relation to the depletion of soil organic phosphorus. Biology and Fertility of Soils 3, 199–204.
Tarafdar, J.C., Yadav, R.S., Meena, S.C., 2001. Comparative efficiency of acid phosphatase originated from plant and fungal sources. Journal of Plant Nutrition and Soil Science 164, 279–282.
Tran, H.T., Hurley, B.A., Plaxton, W.C., 2010. Feeding hungry plants: the role of purple acid phosphatases in phosphate nutrition. Plant Science 179, 14–27.
Treseder, K.K., Vitousek, P.M., 2001. Effects of soil nutrient availability on investment in acquisition of N and P in Hawaiian rain forests. Ecology 82, 946–954.
Van Wyk, B.-E., 2011. The potential of South African plants in the development of new food and beverage products. South African Journal of Botany 77, 857–868.
Vance, C.P., Uhde-Stone, C., Allan, D.L., 2003. Phosphorus acquisition and use: critical adaptations by plants for securing a non renewable resource. The New Phytologist 157, 423–447.
Witkowski, E.T.F., 1991. Growth and competition between seedlings of Protea repens (L.) L. and the alien invasive, Acacia saligna (Labill.) Wendl. in relation to nutrient availability. Functional Ecology 5, 101–110.
Zhao, Q., Zeng, D.H., Fan, Z.P., 2010. Nitrogen and phosphorus transformations in the rhizospheres of three tree species in a nutrient-poor sandy soil. Applied Soil Ecology 46, 341–346.
Zoysa, A.K.N., Loganathan, P., Hedley, M.J., 1997. A technique for studying rhizosphere processes in tree crops: soil phosphorus depletion around camellia (Camellia japonica L.) roots. Plant and Soil 190, 253–265.
Zoysa, A.K.N., Loganathan, P., Hedley, M.J., 1999. Phosphorus utilization efficiency and depletion of phosphate fractions in the rhizosphere of three tea (Camellia sinensis L.) clones. Nutrient Cycling in Agroecosystems 53, 189–201.