

Growth, carboxylate exudates and nutrient dynamics in three herbaceous perennial plant species under low, moderate and high phosphorus supply

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Abstract

Background and aims Australian herbaceous native species have evolved in phosphorus (P) impoverished soils. Our objective was to explore shoot and root adaptations of two of these species with potential to be developed as pasture plants, at low, moderate and high P supply after 4 and 7 weeks of growth.

Methods A glasshouse experiment examined the effect of 5, 20 and 80 mg P kg⁻¹ air-dry soil on growth, rhizosphere carboxylate content, and mineral nutrition of two Australian native perennials, *Kennedia nigricans*

(Fabaceae) and *Ptilotus polystachyus* (Amaranthaceae), and the exotic *Medicago sativa* (Fabaceae).

Key results Leaf P concentrations at P80 were 6, 14 and 52 mg P g⁻¹ leaf dry weight for *M. sativa*, *K. nigricans* and *P. polystachyus*, respectively. As soil P concentration increased, rhizosphere carboxylate content decreased for *M. sativa*, increased and then decreased for *K. nigricans* and was unchanged for *P. polystachyus*. For all species, the contribution of malic acid declined at the second harvest. For all species and P treatments, the amount of rhizosphere carboxylates per unit root length decreased as root length of a plant increased. Plant P content was determined more by P uptake rate per unit root length and time than by root length. Uptake of Mo for all species, and uptake of K, Mg and Mn for *P. polystachyus*, increased with soil P concentration. Uptake of Fe and S was higher when the content of carboxylates in the rhizosphere was higher. **Conclusion** Root physiological adaptations (i.e. rhizosphere carboxylate content and P-uptake rate) are more important than morphological adaptations (i.e. root length and diameter) to enhance the uptake of P and cations.

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Introduction

Phosphorus (P) in soil is relatively immobile and often poorly available to most plants (Vance et al. 2003).

Therefore, morphological, physiological and biochemical mechanisms have evolved in plants to allow them to respond to P deficiency (Vance et al. 2003; Suriyagoda et al. 2010a). Typical morphological responses to low P availability include increased root development, higher root:shoot ratios, finer roots, longer root hairs and formation of mycorrhizas, all of which facilitate exploration of a greater soil volume (Wissuwa 2005; Smith and Read 2008).

In shoots, P deficiency can trigger a reduction of leaf expansion, photosynthetic rate and stomatal conductance (Jacob and Lawlor 1991; Ghannoum and Conroy 2007; Suriyagoda et al. 2010b), and thereby decrease plant growth. In roots, P availability can affect root morphological (e.g., architecture) and physiological (e.g., rhizosphere carboxylate dynamics) responses (Gahoonia and Nielsen 2004; Ho et al. 2005; Hammond et al. 2009; Liang et al. 2010; Suriyagoda et al. 2010a, 2011; Lynch 2011).

Plants can affect P acquisition by altering their root physiology (Neumann and Martinoia 2002; Wissuwa 2005), such as by an enhanced P-uptake rate (*PUR*- mg P cm⁻¹ root day⁻¹), and increased exudation of carboxylates (e.g., malate and citrate) and phosphohydrolases (Richardson et al. 2000; Wouterlood et al. 2005). Carboxylates can enhance the solubility of soil P and this is particularly important when the inherent soil P fertility is poor and/or most of the soil P is strongly sorbed. Carboxylates not only increase the uptake of P, but also contribute to the solubilisation of cations in the soil and, as a consequence, may lead to higher contents of these cations in plants (Page et al. 2006). When considering roots, little attention has been given to study of the relationship between the amount of carboxylates present in the rhizosphere of a plant and root length. In most instances the amount of carboxylates in the rhizosphere is reported per unit root dry weight (DW) (Pearse et al. 2007; Pang et al. 2010; Suriyagoda et al. 2010a; 2011). Even in the few instances where the relationships between carboxylate content and root length were established, only a small fraction of the root, near the root tip, was used (Peñalosa et al. 2002; Wouterlood et al. 2005). Due to the difficulty in assessing rhizosphere soil and roots from bulk soil, and because often only a single harvest was made, changes in rhizosphere carboxylate content (i.e. the balance between the amount exuded and that decomposed) with plant age are not well understood. Moreover, contrasting responses have

been reported (Wouterlood et al. 2004; Marschner et al. 2011 and references therein). In summary, the dynamics of carboxylates in the rhizosphere (i.e. changes in content and composition) as affected by soil P supply, root length and plant growth (i.e. time) have rarely been explored and therefore warrant further investigation.

Australian native plant species have evolved in P-impooverished environments (Handreck 1997) and in some of these species, P uptake is little down-regulated at increased P supply (Shane et al. 2004; Ryan et al. 2009; Pang et al. 2010). Many native species, but by no means all, are therefore sensitive to P toxicity (Handreck 1997; Ryan et al. 2009; Pang et al. 2010). Because of inherently low fertility, large quantities of P fertilisers have been applied to agricultural soils in Australia to improve crop yields; this has resulted in substantial increases in soil P in many regions (Bolland et al. 1997). An understanding of the morphological and physiological traits that affect P acquisition of native plant species under high P availability is now important to facilitate the effective revegetation of native Australian agricultural landscapes with native species (Standish et al. 2007) and the development of native plants as pasture and crop species (Pang et al. 2010; Bell et al. 2011; Suriyagoda et al. 2011). However, knowledge to assist in the selection of species with enhanced P acquisition under low-P conditions is still required for low-input agroecosystems (Cornish 2009), and may be utilised to improve the productivity and sustainability of high-input agroecosystems (Lambers et al. 2011).

Two perennial herbaceous species native to Australia, *Kennedia nigricans* Lindl. (Fabaceae) and *Ptilotus polystachyus* (Gaudich.) F. Muell. (Amaranthaceae) were used in the current experiment. *K. nigricans* is considered to have potential for use as a grain crop due to its large seed size and favourable seed chemical composition (Rivett et al. 1983; Bell et al. 2011). *Kennedia nigricans* also has high amounts of rhizosphere carboxylates (i.e. >100 μmol g⁻¹ root DW) (Ryan et al. 2012). *P. polystachyus* exhibits a remarkable P physiology, being very tolerant of low-P conditions, for reasons that are not yet known, but can accumulate very high leaf [P] (40 mg g⁻¹) at high P supply, without any P-toxicity symptoms (Ryan et al. 2009). In *P. polystachyus*, high concentrations of Mg and K (greater than 23 and 136 mM, respectively) were found in the vacuoles of leaf cells which also contained high [P] (Ryan et al. 2009). Indeed, for a wide range of

plant species, uptake of various nutrients is correlated (Norvell and Welch 1993; Conn and Gilliam 2010), possibly due to their solubilisation in soil through release of carboxylates into the rhizosphere by the plant (Page et al. 2006). Thus it appears that *K. nigricans* and *P. polystachyus* are both well adapted to grow in low-P soils and probably possess a range of adaptations to low soil [P] and, for *P. polystachyus*, to high soil [P].

The objective of the present study was to investigate the growth of *K. nigricans* and *P. polystachyus* at low, moderate and high P supply, with a focus on root morphological (i.e. root length, diameter) and root physiological (i.e. nutrient-uptake rate, rhizosphere carboxylates) characteristics after 4 and 7 weeks of growth. *Medicago sativa* L. (lucerne), a widely grown perennial pasture legume, was also included. We investigated six hypotheses: (i) an increase in soil [P] and thereby tissue [P] would decrease the amount of carboxylates in the rhizosphere; (ii) the composition of carboxylates would not change across P treatments and plant age/harvests; (iii) the total amount of carboxylates in the rhizosphere would increase as root length increased; (iv) higher tissue [P] at increased soil P supply would be due to both an increased root length and an increased *PUR* ($\text{mg P cm}^{-1} \text{ root day}^{-1}$); (v) plants would accumulate high concentrations of mineral elements (i.e. both cations and anions) at high soil [P]; and, (vi) the accumulation of high amounts of mineral elements per plant would also be correlated with the amount of carboxylates in the rhizosphere.

Materials and methods

Growth conditions

The experiment was a fully crossed factorial design of two factors: plant species (*K. nigricans*, *M. sativa*, *P. polystachyus*) and P (P5, 5 mg P kg⁻¹ air-dry soil; P20, 20 mg P kg⁻¹ air-dry soil; P80, 80 mg P kg⁻¹ air-dry soil). In previous experiments, growth of most of the Australian native perennial pasture species was maximum at a soil P supply of ~20 mg P kg⁻¹, and growth was reduced at a P supply over 50 mg P kg⁻¹ (Pang et al. 2010; Suriyagoda et al. 2010a). Therefore, we expected P5 and P80 to be at the sub- and supra-optimal range of P supply. There were two harvests, at 4 and 7 weeks after seedling emergence, with four replicates at each harvest. *K. nigricans* accession NS19026, *M.*

sativa cv. SARDI-10 and *P. polystachyus* accession MGP001 were used in the experiment. A sandy loam soil was collected from the top 10-cm layer of a native, virgin forest near Perth (32°2'S, 115°8'E). Soil was air dried and sieved through a 3-mm diameter mesh to remove debris such as plant roots and other organic matter. Initial soil nutrient status as determined by CSBP FutureFarm analytical laboratories, Bibra Lake, Australia, is given in Table 1. As the bicarbonate-extractable soil [P] (Colwell-P) in its native state was 5 mg P kg⁻¹ air-dry soil, the three P treatments received 0, 15 and 75 mg P kg⁻¹ air-dry soil as KH₂PO₄, in order to obtain P5, P20 and P80, respectively. Additional potassium was supplied in the P5 and P20 treatments as KCl to balance the level of potassium. NH₄NO₃ (40 mg kg⁻¹ dry soil) was also added to all pots. To prepare the soil for each P treatment, soil was thoroughly mixed using a mechanical mixer until homogeneous. Mixing was done for P5 soil first and then for the P20 and P80 treatments. Pots of 10-cm diameter and 17-cm length were filled with 1.8 kg of soil to a bulk density of 1.4 g cm⁻³. De-ionised water was added to each pot to reach the field capacity and left for 3 days. The experiment was carried out in a glasshouse at the University of Western Australia, Perth (31°59'S, 115°53'E) as a randomised complete block design. Three seedlings, which were raised on a sterilised sand bed, were planted in each pot and thinned to one plant at 3–4 days. From week 4, 50 mL of 2 mM NH₄NO₃ was added twice a week to ensure adequate nitrogen supply. Each pot was weighed and watered to reach the field capacity daily. The glasshouse was unheated under natural light and had an average day and night time temperatures of 23 and 13 °C,

Table 1 Initial properties of the soil used in the experiment, n=4

	Mean±std.err
NH ₄ ⁺ -N (mg kg ⁻¹)	2.0±0.1
NO ₃ ⁻ -N (mg kg ⁻¹)	2.3±0.3
Bicarbonate-extractable K (mg kg ⁻¹)	29.0±8.5
Bicarbonate-extractable P (Colwell-P) (mg kg ⁻¹)	5.3±1.9
S (mg kg ⁻¹)	1.5±0.06
Organic carbon (g kg ⁻¹)	11±1.3
Conductivity (dS m ⁻¹)	0.02±0.002
pH (CaCl ₂)	5.0±0.03
pH (H ₂ O)	5.9±0.03

respectively during the experiment, which was conducted from August to September 2010.

Plant analyses

Harvests were conducted 4 and 7 weeks after establishment. At each harvest, the shoot was removed from the base (i.e. collar area) and used for leaf area measurements with a LiCor LI-3000 Portable Area Meter, which was equipped with a LI-3050A Transparent Belt Conveyor Accessory (LiCor, Lincoln, NE, USA). Soil was taken out of the pot and roots were shaken lightly to remove the excess soil; remaining soil was defined as the rhizosphere soil (Veneklaas et al. 2003). Roots were transferred to a 200-mL vial and washed in a measured amount of 0.2 mM CaCl₂ solution ranging from 20 to 100 mL depending on the root volume. The root system was gently dunked in the solution until as much rhizosphere soil as possible was removed. Care was taken to minimise root damage. A 1-mL subsample of the rhizosphere extract was then filtered using a 0.2- μ m syringe filter into a 1-mL HPLC vial. The vial was acidified with one drop of concentrated phosphoric acid, placed in dry ice, and transferred to a -20 °C freezer until HPLC analysis. Detailed methodology for the carboxylate analysis is given in Suriyagoda et al. (2010a). The root system was then washed more thoroughly to remove any residual soil. These roots were used to measure the root length, diameter and the distribution of the root length according to diameter classes (i.e. <0.5, 0.5–1, 1–2, 2–3, 3–4, and >4 mm) using the commercial software package WinRHIZO 4.1 (Regent Instruments Inc., Quebec, Canada, 2000). Calculated root length and the soil volume in a pot were used to calculate the root density (i.e. half distance between roots) as given in Bhadoria et al. (2004).

Root, stem and leaf dry weights (DW) were determined after drying at 60 °C for a week. Root mass ratio (*RMR*) was calculated as the ratio between root dry weight and total plant dry weight. Roots, stems and leaves of each plant were ground separately in a stainless steel ball mill. For analysis of leaf nutrient concentrations, approximately 100 mg of leaf was taken and digested in nitric/perchloric acid and analysed using inductively coupled plasma (ICP) atomic absorption with a Perkin Elmer Optima 5300 DV optical emission spectrometer (OES; Shelton, CT, USA). For stem and root [P] determination, an approximately 100-mg

subsample was taken and digested in nitric/perchloric acid, and analysed using the molybdate and malachite green method (Motomizu et al. 1983). Leaf, stem and root [P] and DWs were used to calculate the amount of P taken up by each plant at both harvests. The Δ P uptake and Δ root length for each treatment combination were calculated by taking the difference of mean P content and mean root length, respectively between the second (7 wk) and first (4 week) harvest. The mean *PUR* (mg P cm⁻¹ root day⁻¹) for each treatment combination was calculated by taking the difference of total amount of P present in plant tissues (i.e. leaves, stems and roots) between the second and first harvest and dividing that value by the difference of average root length at the two harvests.

Soil analyses

Ammonium-N and nitrate-N concentrations were determined as described by Searle (1984), “plant-available” phosphorus (P) and potassium (K) were measured using the Colwell test (Colwell 1965; Rayment and Higginson 1992), available sulfur (S) according to Blair et al. (1991), organic carbon according to Walkley and Black (1934), with the remainder of soil analyses using protocols outlined in Rayment and Higginson (1992). Cation-exchange capacity (CEC) was calculated from exchangeable soil cations (Black 1968).

Statistical analyses

Data were subjected to 2-way analyses of variance in SAS/STAT software Version 9.1 (SAS Institute Inc., Cary, NC, USA) to examine the impact of species, P treatments and their interaction on response variables at each harvest separately. No transformations were needed to meet ANOVA assumptions. Comparisons between means were made using Tukey’s Honest Significant Difference procedure. Means are presented with standard errors of the mean (std.err) and significance is expressed at $p < 0.05$. Shoot and root DW, root length, root diameter and tissue nutrient concentrations are presented for the second harvest only. When estimating net *PUR*, Δ P uptake and Δ root length from measured variables, means of the measured variables within each treatment combination at each harvest were used. When establishing the relationship between root length and rhizosphere

carboxylate content, heteroscedasticity was present. Therefore, log-transformed root carboxylate data were used to correct this heteroscedasticity.

Results

For both shoot and root DW there was an interaction between P treatment and species (Table 2). Shoot and root DW of *K. nigricans* and *M. sativa* increased from P5 to P20, while *P. polystachyus* was less responsive (Fig. 1). At P80, shoot DW of *K. nigricans* and *P. polystachyus* was reduced compared with that at P20. Root DW of *P. polystachyus* was reduced at P80 compared with that at P20. At the first harvest, the impact of P treatment on growth of all species was similar to that at the second harvest (data not shown). Leaf area followed a similar trend to shoot DW (data not shown). Root mass ratio (i.e. root to plant DW ratio) of *K. nigricans* (0.17) and *P. polystachyus* (0.17) was much lower than that of *M. sativa* (0.40) and was unaffected by P treatment (data not shown).

The effect of P treatment on root length varied with species, with root length of all species increasing from

Table 2 Significance of different sources of variability after 7 weeks of growth (second harvest)

Variable	Source of variability			R^2
	P	S	P×S	
Shoot DW	***	*	*	0.59
Root DW	***	***	***	0.87
Leaf area	**	*	*	0.46
Root mass ratio	***	ns	ns	0.90
Root length	***	***	*	0.67
Root area	**	***	*	0.66
Root diameter	ns	***	*	0.55
Specific root length (SRL)	ns	**	***	0.80
% root length distribution	ns	***	ns	0.52
Carboxylate content (at first harvest)	**	**	**	0.63
Carboxylate content (at second harvest)	**	***	**	0.59
Leaf [P]	***	***	***	0.98
Total P (plant ⁻¹)	**	***	***	0.96

Significant effects are indicated for phosphorus (P) and species (S) treatments, and their interaction (ns., no significant difference; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

R^2 for the fitted full model is given

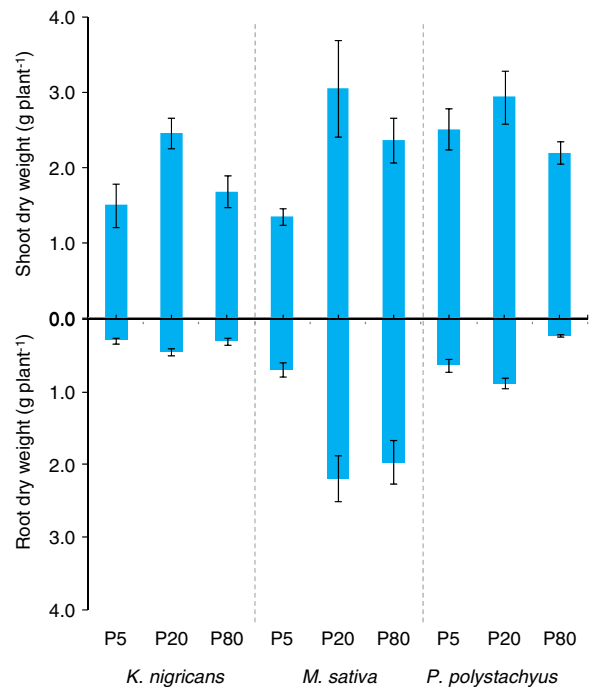


Fig. 1 Shoot and root dry weight of *Kennedia nigricans*, *Medicago sativa* and *Ptilotus polystachyus* grown at 5, 20 or 80 mg P kg⁻¹ air-dry soil after 7 weeks of growth (harvest 2)

P5 to P20 and that of *P. polystachyus* then declining greatly to P80 (Fig. 2a, Table 2). *K. nigricans* had a smaller root system (i.e. total root length and root DW were less) than that of *M. sativa* and *P. polystachyus* in most instances (Figs 1 and 2a). Root surface area showed a similar trend to root length (data not shown).

For root diameter, there was an interaction between species and P treatment (Fig. 2b, Table 2). For *M. sativa* only, root diameter increased from P5 to P80 (Fig. 2b). Due to this change, specific root length (SRL; root length per unit root DW) of *M. sativa* changed among P treatments from 121±11 mg⁻¹ at P5 to 58±6 mg⁻¹ and 59±9 mg⁻¹ at P20 and P80, respectively. For *K. nigricans* and *P. polystachyus*, SRL remained at 115±12 mg⁻¹ and 157±11 mg⁻¹, respectively, across all treatments.

Root length distribution of a plant based on root diameter classes differed only with species (Fig. 2c, Table 2), with the distribution for *K. nigricans* and *P. polystachyus* being very similar and including a higher proportion of fine roots than for *M. sativa*. For instance, the proportion of root length with a diameter of <1 mm was 21 % for *K. nigricans*, 27 % for *P. polystachyus* and 10 % for *M. sativa*.

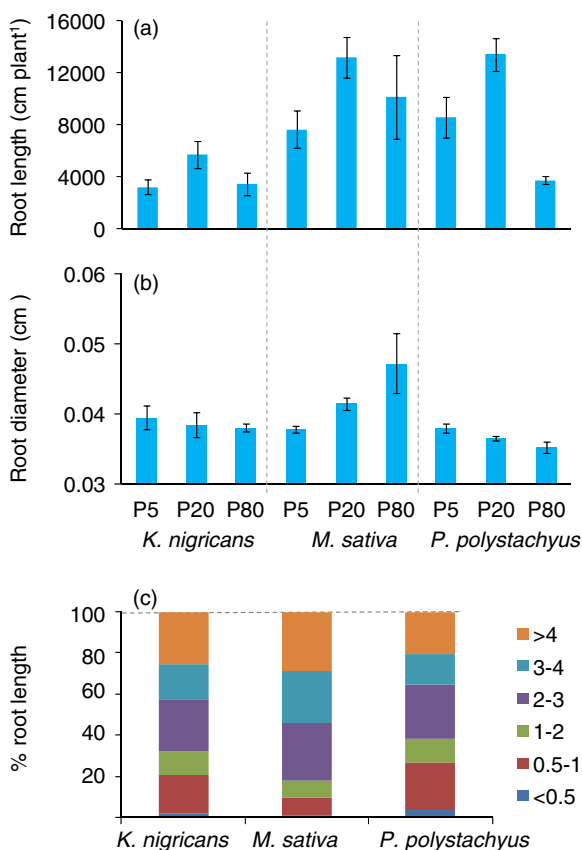


Fig. 2 **a** Root length and **b** root diameter of *Kennedia nigricans*, *Medicago sativa* and *Ptilotus polystachyus* grown at 5, 20 or 80 mg P kg⁻¹ air-dry soil, and **c** root length distribution based on diameter classes (in mm) for *Kennedia nigricans*, *Medicago sativa* and *Ptilotus polystachyus* after 7 weeks of growth (harvest 2). Note: P treatment had no effect on root length distribution

Rhizosphere carboxylate content measured at 4 and 7 weeks after seedling emergence showed a species and P treatment interaction (Fig. 3, Table 2). At both harvests, carboxylate content ($\mu\text{mol g}^{-1}$ root DW) of *K. nigricans* increased from P5 to P20 and then decreased at P80. For *M. sativa*, rhizosphere carboxylate content decreased as soil [P] increased, while rhizosphere carboxylate content of *P. polystachyus* was unchanged with P treatment. For *M. sativa* and *P. polystachyus* only, there was a reduction in rhizosphere carboxylates at the second harvest compared with that at the first harvest across P treatments ($F_{1, 55}=18.7$, $P<0.001$). Carboxylates in the rhizosphere mainly comprised citric, malic, malonic and oxalic acid, with the contribution from other organic acids (i.e. acetic, cis-aconitic, fumaric, lactic, succinic and trans-aconitic)

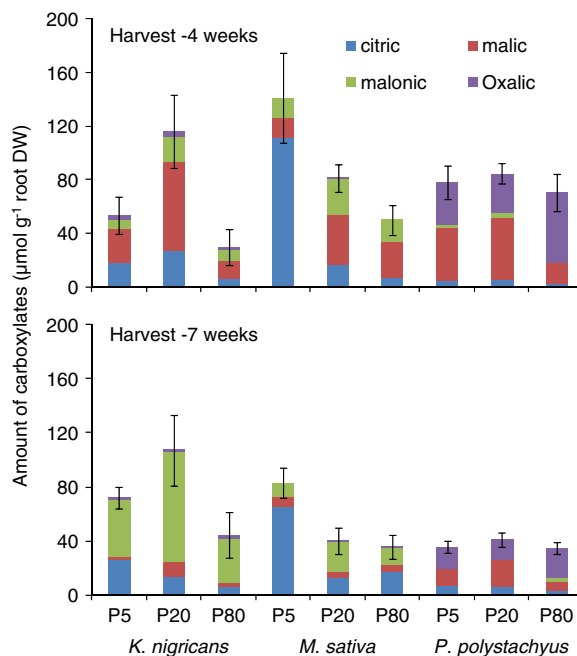


Fig. 3 Rhizosphere carboxylates (citric- blue, malic- red, malonic- green, oxalic- purple) at 4 and 7 weeks after seedling emergence for *Kennedia nigricans*, *Medicago sativa* and *Ptilotus polystachyus* grown at 5, 20 or 80 mg P kg⁻¹ air-dry soil

less than 8 % (Fig. 3). Composition differed among harvests and species. Contribution of malic acid was reduced from the first to the second harvest for all species. At the second harvest, malonic, citric and oxalic acids were the main rhizosphere carboxylates for *K. nigricans*, *M. sativa* and *P. polystachyus*, respectively (Fig. 3).

Heteroscedasticity observed in root carboxylate content with increase in root length could be corrected for by using the log-transformed root carboxylate data (Fig. 4a). Root carboxylate content (or log-transformed root carboxylate content) in the rhizosphere of a plant increased at a decreasing rate when root length increased, irrespective of species and P treatment (Fig. 4a). Moreover, when higher root lengths (see Fig. 2a) caused roots to be in closer proximity, rhizosphere carboxylates increased (Fig. 4b).

For leaf [P] there was an interaction between species and P treatment (Fig. 5a, Table 2). For all species, leaf [P] increased with increasing soil [P]. However, the increase from P20 to P80 was much greater for *P. polystachyus* than for the other two species. The total amount of P taken up by each plant showed a similar response to P treatment as leaf tissue [P]

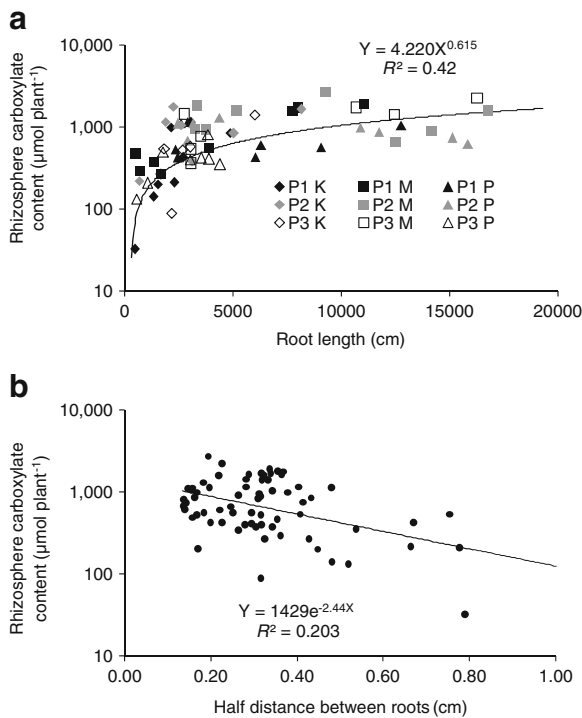


Fig. 4 **a** Relationship between log-transformed rhizosphere carboxylate content and root length of *Kennedia nigricans*, *Medicago sativa* and *Ptilotus polystachyus* grown at 5, 20 or 80 mg P kg⁻¹ air-dry soil, and **b** log-transformed rhizosphere carboxylate content and half the distance between roots across P treatments and species. Note: only for **b** P treatment and species had no effect

(Table 2, data not shown). Therefore, *P. polystachyus* achieved a leaf [P] and total P content that was 6.7 and 3.5 times higher, respectively, than those of *M. sativa*, and 3.1 and 3.4 times higher, respectively, than those of *K. nigricans*.

Net P-uptake rate (*PUR*- mg P cm⁻¹ root day⁻¹) increased with P supply for all species (Fig. 5b). However, *M. sativa* had the lowest *PUR* at all levels of P supply, while *P. polystachyus* had the highest. The relationship between ΔP taken up (i.e. amount of P taken up by a plant between two harvests) and *PUR* was stronger ($r=0.98$) than the relationship between ΔP taken up and Δ root length (i.e. difference in root length between two harvests) ($r=-0.50$) (Fig. 6).

Leaf tissue Fe and S contents per plant were correlated with rhizosphere carboxylate content (Fig. 7). However, there was no relationship with leaf tissue concentrations of K, Mg, Al, Co, Na, Ca, Mn, Cu, Zn or Mo.

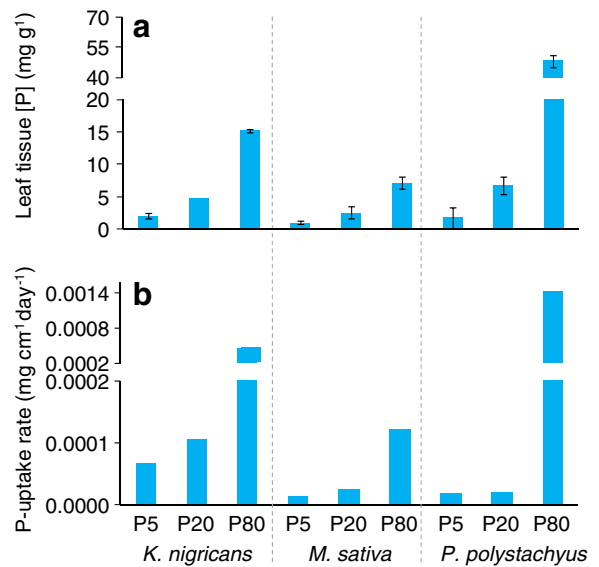


Fig. 5 **a** Leaf tissue P concentration after 7 weeks of growth (harvest 2), and **b** net P-uptake rate (*PUR*) for *Kennedia nigricans*, *Medicago sativa* and *Ptilotus polystachyus* grown at 5, 20 or 80 mg P kg⁻¹ air-dry soil

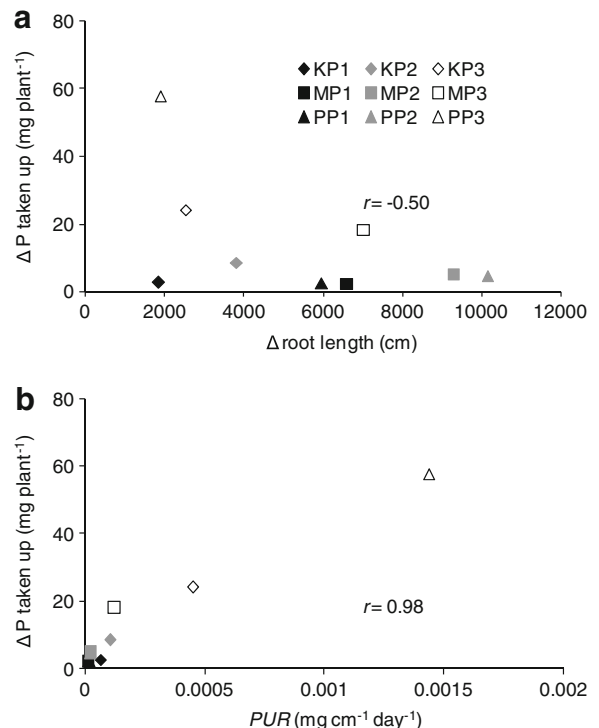


Fig. 6 **a** Relationship between ΔP taken up and Δ root length between two harvests and **b** ΔP taken up and *PUR* of *Kennedia nigricans*, *Medicago sativa* and *Ptilotus polystachyus* grown at 5, 20 or 80 mg P kg⁻¹ air-dry soil

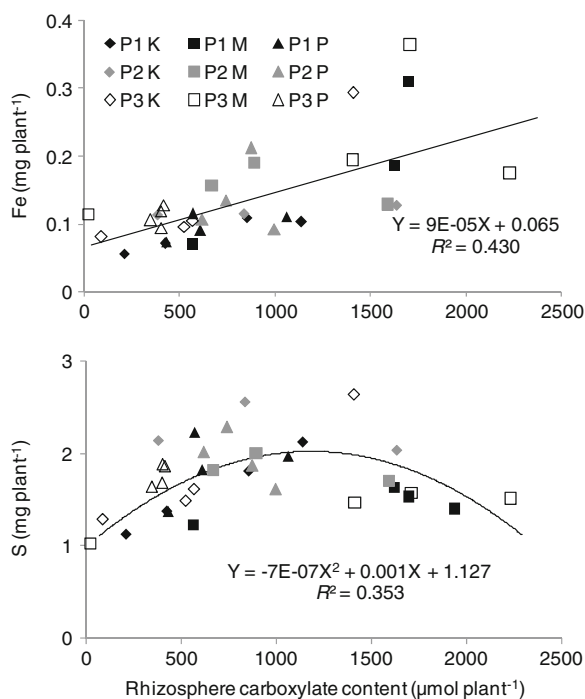


Fig. 7 Relationship between leaf tissue Fe and S content per plant, and rhizosphere carboxylate content of *Kennedia nigricans*, *Medicago sativa* and *Ptilotus polystachyus* grown at 5, 20 or 80 mg P kg⁻¹ air-dry soil after 7 weeks of growth (harvest 2)

Leaf tissue concentrations of most elements were affected by species and P treatment (Tables 3 and 4, Fig. 8). For *P. polystachyus* at P80, [K], [Mg], [Mo] and [Mn] were higher, and, irrespective of the P treatment, [Na] and [Zn] were higher than those of *K. nigricans* and *M. sativa* (Fig. 8). Therefore, the uptake of both cations (i.e. K, Mg, Mn and Na) and anions (i.e. Mo) of *P. polystachyus* was highly correlated with P uptake, irrespective of P treatment ($r=0.82, 0.51, 0.33, -0.32$, and 0.72 for K, Mg, Mn, Na and Mo, respectively, $p<0.05$). For *K. nigricans*, only K and Mo uptake were correlated with P uptake ($r=0.65$ and 0.62 , respectively, $p<0.05$) and for *M. sativa* only Mo uptake was correlated with P uptake ($r=0.36$, $p<0.02$). Moreover, in a narrow range of leaf [P] (i.e. within each soil P treatment) there were numerous correlations between leaf [P] and other mineral elements (e.g., Mg, Na and Zn) for all the species (Fig. 8).

Discussion

Only *K. nigricans* and *M. sativa* increased growth from P5 to P20, and only *K. nigricans* and *P. polystachyus*

Table 3 Significance of different sources of variability for leaf nutrient concentrations after 7 weeks of growth (second harvest)

Nutrient	Source of variability			R^2
	P	S	P×S	
Na	ns	***	ns	0.91
Ca	ns	***	ns	0.52
Mn	*	***	*	0.66
Fe	ns	*	ns	0.46
Cu	***	***	***	0.86
Zn	ns	***	ns	0.86
Mo	**	ns	ns	0.45
Mg	*	***	**	0.83
Al	*	*	*	0.56
S	**	***	**	0.77
K	***	***	***	0.92
Co	*	ns	ns	0.29

Significant effects are indicated for phosphorus (P) and species (S) treatments, and their interaction (ns., no significant difference; * $p<0.05$; ** $p<0.01$; *** $p<0.001$)

R^2 for the fitted full model is given

decreased growth from P20 to P80. Therefore, for *K. nigricans* P5 and P80 were in the sub- and supra-optimal range of soil [P], respectively. For *M. sativa*, P5 was a sub-optimal soil [P], but plants grew equally well at P20 and P80. For *P. polystachyus* P5 and P20 can be considered as optimal, while P80 was supra-optimal. Thus the growth of both Australian native species was negatively affected by the higher soil [P], while *M. sativa* grew well even at the higher soil [P] of P80. The contrasting response of *K. nigricans* and *M. sativa* to increasing P supply is consistent with that reported for *K. prostrata*, *K. prorepens* and *M. sativa* by Pang et al. (2010). P toxicity at high P supply is relatively common for Australian native plant species that have evolved on P-impoorished soils (Shane et al. 2004; Pang et al. 2010).

Soil [P] and rhizosphere carboxylate dynamics

Hypothesis (i) that an increase in soil [P] and thereby tissue [P] will decrease the amount of carboxylates in the rhizosphere, was only partly supported. Only for *K. nigricans* (only from P20 to P80) and *M. sativa* did the amount of carboxylates in the rhizosphere decrease when soil [P] increased. Recently, Pang et al. (2010) and Suriyagoda et al. (2010a, 2011) reported that carboxylate composition did not change with soil [P]

Table 4 Effect of P treatments on concentrations of mineral elements in the leaf tissue of *Kennedia nigricans*, *Medicago sativa* and *Ptilotus polystachyus* after 7 weeks of growth (second harvest), n=4 (see Table 3 for statistics)

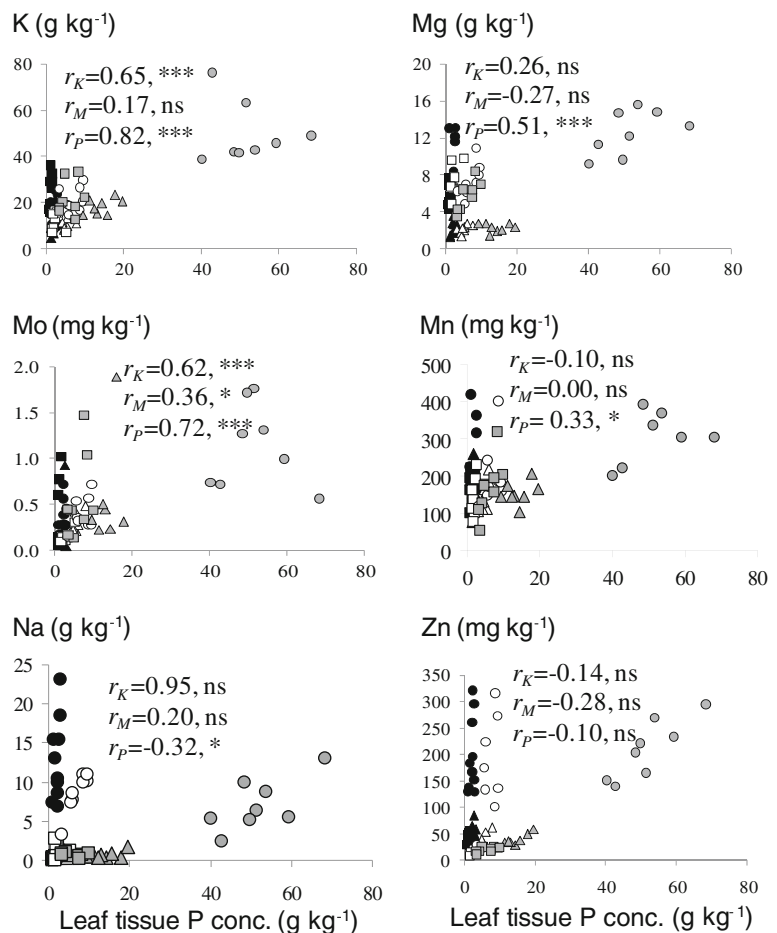
Species	P treatment	Mg (mg g ⁻¹)	Na (mg g ⁻¹)	P	S	K	Ca	Mn (mg kg ⁻¹)	Fe (mg kg ⁻¹)	Cu	Al	Co	Zn	Mo
<i>K. nigricans</i>	P5	2.5	0.6	2.4	1.31	10.6	14.8	161	65	19.7	17.9	0.057	52	0.2
	P20	2.5	0.5	5.2	1.05	12.8	13.0	148	64	8.5	18.7	0.075	37	0.3
	P80	2.5	0.7	14.2	1.55	19.7	13.8	153	86	4.2	19.2	0.125	38	1.0
<i>M. sativa</i>	P5	6.1	0.2	1.3	2.50	26.9	20.6	174	219	2.8	23.6	0.171	43	0.1
	P20	7.6	1.4	2.4	1.07	15.1	20.3	147	107	2.4	17.0	0.075	15	0.3
	P80	5.6	0.7	6.2	1.25	26.0	17.4	166	129	2.6	12.0	0.125	20	0.5
<i>P. polystachyus</i>	P5	10.7	12.9	2.2	0.97	21.1	8.5	233	53	9.1	24.5	0.067	197	0.4
	P20	8.3	8.6	7.2	0.87	24.7	8.6	201	54	8.3	28.7	0.100	174	0.4
	P80	12.9	7.0	51.8	1.25	58.5	15.2	330	66	12.7	39.7	0.175	210	1.14
LSD($\alpha=0.05$) ^a		0.7	0.9	3.3	0.14	2.0	1.8	24	28	1.6	7.8	0.022	15	0.19

^a LSD for comparison between treatment combinations is given

for *Kennedia* spp. and *M. sativa*. However, using a range of plant species, Lambers et al. (2002) and

Pearse et al. (2007) showed that the composition of carboxylates did change with the source of P. Moreover,

Fig. 8 Relationship between leaf tissue concentrations of K, Mg, Mo, Mn, Na and Zn and P for *Kennedia nigricans* (diamonds), *Medicago sativa* (squares) and *Ptilotus polystachyus* (circles) grown at 5, 20 or 80 mg P kg⁻¹ air-dry soil (black, white and grey symbols, respectively). Correlation coefficients (r) for *K. nigricans*, *M. sativa* and *P. polystachyus* are given as r_K , r_M and r_P , respectively, where, ns no significant difference; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$



Veneklaas et al. (2003) found that composition of *Cicer arietinum* L. carboxylates changed when grown in different soils with varying P availability. When considering the composition of carboxylates along the root length of *Lupinus albus* L., malate was predominantly exuded from apices of both seedling taproots and proteoid roots, whereas citrate exudation was restricted to proteoid root clusters (Peñaloza et al. 2002). Most of these experiments have tested the amount of carboxylates in the rhizosphere only once during the growth period (i.e. at final harvest) and contrasting results are reported. Moreover, changes in rhizosphere carboxylate composition with time/plant age have been paid little attention. Therefore, we tested hypothesis (ii) that composition of carboxylates would not change across P treatments and plant age/harvests. When considering soil [P] only for *K. nigricans*, carboxylate composition did not change with soil [P] while for all species, especially *K. nigricans*, carboxylate composition changed with harvest. Overall, the present findings support previous findings (Lambers et al. 2002; Veneklaas et al. 2003; Pearse et al. 2007) that the amount and composition of carboxylates in the rhizosphere is variable and depend on the plant species, soil [P] and growth stage of the plant. However, the impact of changes in amount and composition of rhizosphere carboxylates on P uptake is not yet clear and needs further attention as not all carboxylates may have a similar effect on plant P uptake. For instance, Ryan et al. (2012) found that plant P content in a range of *Kennedia* species was strongly correlated with citric acid, but not malic or malonic acids.

Root length and rhizosphere carboxylate content

Roots exude carboxylates into the rhizosphere to enhance uptake of P for plant growth (Lambers et al. 2002). Therefore, our third hypothesis was that the total amount of carboxylates in the rhizosphere of a plant would increase as the root length increased. However, the hypothesis was only partially supported as the rate of increase of rhizosphere carboxylate content decreased and varied (i.e. heteroscedasticity) as the root length increased (Fig. 4a). This might be due to several reasons. First, mature and senescing roots may have had reduced carboxylate exudation compared with active root tips (Marschner et al. 1997; Marschner et al. 2011 and references there in) as we examined plants that grew

for 7 weeks and had root lengths up to over 150 m. A decrease in the rhizosphere malate content along the root length was found in *L. albus* while the response of citrate changed depending on the [P] in soil (Peñaloza et al. 2002). However, Wouterlood et al. (2004) found that carboxylate content of *C. arietinum* increased with increasing distance from the root apex only up to 2.5 cm from the root tip. Decomposition of carboxylates also can reduce the amount of carboxylates present in the rhizosphere (Jones 1998; Van Hees et al. 2002; Marschner et al. 2011). Second, plants might decrease carboxylate exudation from root regions where soil P has already been depleted. Third, the reduced rate of increase of rhizosphere carboxylate content as the root length increases can also be affected by root morphology, soil characteristics and rhizosphere microbial properties (Suriyagoda et al. 2010a; Marschner et al. 2011 and references there in). As far as we are aware this is the first attempt to establish relationships between total amount of rhizosphere carboxylate content of a plant and root length and this concept warrants further testing.

Root growth and morphology

Hypothesis (iv) that increased tissue [P] at higher soil [P] would be due to both an increased *PUR* (mg P cm⁻¹ root day⁻¹) and an increased root length was only partly supported. Even though there was an increase in root length from P5 to P20 for all the species, supporting the hypothesis, root length of *K. nigricans* and *P. polystachyus* was reduced at P80 compared with that at P20 which did not support the hypothesis. Reduced root length of *K. nigricans* and *P. polystachyus* at P80 was correlated with reduced shoot and root growth (Figs 1 and 2). Reduced root length at P80 might be an adaptive response to avoid P toxicity at a higher P supply (Shane et al. 2004). Increased root growth and relative allocation to root growth (higher RMR) is beneficial for P acquisition, since inorganic P is relatively immobile in soil, but may slow overall plant growth because of the increased respiratory burden of root tissue (Lynch and Ho 2005, and references there in). At low- to optimal soil P supply, root length increase of *Oryza sativa* L. was better correlated with the amount of P taken up than with *PUR* (Wissuwa 2005). However, in the present experiment, as hypothesised, the *PUR* increased from P5 to P80 for all species (Fig. 5).

Therefore, the amount of P taken up between the two harvests (ΔP) was more strongly correlated with *PUR* ($r=0.98$) than with root length ($r=-0.5$) (Fig. 6). Moreover, when comparing among species at P80, the *PUR* of the two Australian native legumes, *K. nigricans* and *P. polystachyus* was 2–6 times higher than that of *M. sativa*. The higher *PUR* of *K. nigricans* and *P. polystachyus* may reflect a higher proportion of fine roots than for *M. sativa* (Fig. 2), and/or reduced root length as a morphological adaptation coupled with an inability to down-regulate P uptake proportionately. However, Shane and Lambers (2006) reported that the net-P uptake of *Grevillea crithmifolia* R. Br. was reduced at higher P supply which was considered an adaptation to decrease the rate of P influx at increased P supply.

Nutrient accumulation

Accumulation of one nutrient often has consequences for the accumulation of others, with changes seemingly driven largely by a need to maintain charge balance, although it may also serve as a detoxification mechanism (Zaharieva and Römheld 1991; Ryan et al. 2003; Conn and Gilliam 2010). *P. polystachyus* has previously been found to accumulate high [P], [K] and [Mg] in its leaf cell vacuoles (Ryan et al. 2009). Therefore, we tested hypothesis (v) plants would accumulate high concentrations of ions (i.e. both cations and anions) at high soil P supply. As hypothesised, *P. polystachyus* accumulated high concentrations of K, Mg and Mo at P80 (Fig. 8). Furthermore, irrespective of the P treatment, *P. polystachyus* accumulated very high concentrations of Na, Mn and Zn. Therefore, correlations of ion uptake (i.e. K, Mg, Mo, Mn and Na) of *P. polystachyus* with P uptake, irrespective of the P treatment, were significant (i.e. $r=0.82$, 0.51, 0.72, 0.33 and -0.32 for K, Mg, Mo, Mn and Na, respectively) (Fig. 7, Table 4). For all the species, numerous strong positive correlations were present between a narrow range of leaf [P] (i.e. within each P treatment) and mineral elements. However, to test hypothesis (v) we considered correlations across all P treatments (i.e. wide range of leaf [P]) (Fig. 8) in order to study broad, general trends. Brennan et al. (2000) found that at the seedling stage of *P. polystachyus*, the critical concentration for shoot growth to avoid deficiency is about 15 mg g⁻¹ DW for K, and 9 mg g⁻¹ DW for P. Adequate

concentrations in shoots were 17–27 mg g⁻¹ DW for K and 10–16 mg g⁻¹ DW for P. In the present experiment, at P20, similar results were found, while at P80 concentrations of K and P were higher than those reported by Brennan et al. (2000). Therefore, the P80 treatment was in the supra-optimal range of P for *P. polystachyus*. However, *P. polystachyus* did not show any visual P toxicity symptoms, apart from the reduced DW at P80.

Hypothesis (vi) that the accumulation of high amounts of mineral elements would be correlated with the amount of carboxylates in the rhizosphere was only supported for Fe and S (Fig. 8). Argyriadis et al. (1976) found similar results for S. Even though rhizosphere carboxylate dynamics and plant nutritional status were measured in many previous studies, their correlation was not tested (Shen et al. 2004; Suriyagoda et al. 2011). This merits further detailed study.

Concluding remarks

The response of rhizosphere carboxylate content and composition varied with soil P supply, plant species and plant age. For all species and P treatments, the amount of rhizosphere carboxylates per unit root length decreased as root length of a plant increased. Higher rhizosphere carboxylates corresponded with enhanced uptake of Fe and S from soil. The amount of P taken up by plants was determined more by the *PUR* (i.e. physiological adaptation) than by the root length (i.e. morphological adaptation). When P (i.e. an anion) was taken up in greater quantities, cation uptake was also increased.

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