Annals of Botany **89**: 435–442, 2002
doi:10.1093/aob/mcf066, available online at www.aob.oupjournals.org

The Effect of Nitrogen Nutrition on Cluster Root Formation and Proton Extrusion by *Lupinus albus*

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Received: 25 June 2001 Returned for revision: 1 November 2001 Accepted: 6 January 2002

Nitrogen nutrition can influence cluster root formation in many wild species, but the effect of N form on cluster root formation and root exudation by white lupin is not known. In a solution culture study, we examined the effect of N nutrition (ammonium, nitrate, both or N_2 fixation) on cluster root formation and H^+ extrusion by white lupin plants under deficient and adequate P supply. The number of cluster roots increased greatly when plants were supplied with 1 μM P compared with 50 μM P, the increase being 7·8-fold for plants treated with $(NH_4)_2SO_4$, 3-fold for plants treated with KNO_3 and NH_4NO_3, and 2·4-fold for N_2-fixing plants. Under P deficiency, NH_4^+-N supply resulted in production of a greater number and biomass of cluster roots than other N sources. Dry weight of cluster roots was 30 % higher than that of non-cluster roots in P-deficient plants treated with $(NH_4)_2SO_4$ and NH_4NO_3. In plants treated with sufficient P (50 μM), the weight of non-cluster roots was approx. 90 % greater than that of cluster roots. Both total (μmol per plant h^{-1}) and specific (μmol g^{-1} root d. wt h^{-1}) H^+ extrusions were greatest from roots of plants supplied with $(NH_4)_2SO_4$, followed by those supplied with NH_4NO_3 and N_2 fixation, whereas plants receiving KNO_3 had negative net H^+ extrusion between the third and fifth week of growth (indicating uptake of protons or release of OH^- ions). The rate of proton extrusion by NH_4^+-N-fed plants was similar under P-deficient and P-sufficient conditions. In contrast, proton exudation by N_2-fixing plants and KNO_3-treated plants was ten-fold greater under P deficiency than under P sufficiency. In comparison with P deficiency, plants treated with 50 μM P had a significantly higher concentration of P in roots, shoots and youngest expanded leaves (YEL). Compared with the N_2 fixation and KNO_3 treatments, total N concentration was highest in roots, shoots and YEL of plants supplied with $(NH_4)_2SO_4$ and NH_4NO_3, regardless of P supply. Under P deficiency, K concentrations in roots decreased at all N supplies, especially in plants treated with $(NH_4)_2SO_4$ and NH_4NO_3, which coincided with the greatest H^+ extrusion at these P and N supplies. In conclusion, NH_4-N nutrition stimulated cluster root formation and H^+ extrusion by roots of P-deficient white lupin.

Key words: Cluster roots, H^+ extrusion, mineral composition, N_2 fixation, N nutrition, P deficiency, white lupin.

INTRODUCTION

Formation of cluster roots is one adaptation to nutrient acquisition (Gerke *et al*., 1994; Marschner, 1995; Keerthisinghe *et al*., 1998; Neumann *et al*., 1999, 2000; Neumann and Rõmheld, 1999). The release of large amounts of citrate from cluster roots of P-deficient white lupin is an efficient strategy for chemical mobilization of sparingly soluble P sources in the rhizosphere (Neumann *et al*., 1999). It has been shown that phosphorus deficiency increases proton extrusion by roots, results in an excess cation concentration in roots and shoots, and enhances the exudation of citrate and malate by white lupin plants (Sas *et al*., 2001). Localized rhizosphere acidification and organic acid anion extrusion not only mobilize phosphorus, but also iron, manganese and zinc in the rhizosphere and increase their rates of uptake and their contents in plants (Marschner, 1995). Thus, white lupin may be regarded as a model system for plant adaptations related to chemical mobilization of nutrients in the rhizosphere (Neumann *et al*., 1999, 2000; Neumann and Römheld, 1999).

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Many environmental factors may stimulate cluster root formation. Generally, cluster roots are produced under conditions of low phosphate, while their initiation is generally suppressed at higher levels of phosphate supply (Lamont, 1972*a*, *b*; Gardner *et al*., 1983; Marschner *et al*., 1987). However, it has been demonstrated by Marschner *et al*. (1987), Johnson *et al*. (1996*a*) and Keerthisinghe *et al*. (1998) that cluster roots may form at moderate or even adequate P levels.

In several species, cluster root formation is also influenced by nitrogen nutrition. *Myrica gale* seedlings supplied with urea formed root clusters more quickly than seedlings supplied with nitrate (Crocker and Schwintzer, 1993). In *Gymnostoma papuanum*, formation of root clusters increased with nitrogen supply in the order: NH_4^+-N < N_2 fixation < nitrate-N (Racette *et al*., 1990). At low P supply, cluster root formation was enhanced by low N supply and depressed by high N supply in *Hakea* spp. (Lamont, 1972*a*, *b*). In contrast, cluster root formation in P-deficient *Myrica gale* (Crocker and Schwintzer, 1993) and *Grevillea robusta* (Moore and Kairaitis, 1966) was greater when plants were supplied with adequate N compared with no nitrogen. The form of nitrogen had no effect on cluster root formation

in *Myrica cerifera* (Louis *et al*., 1990). In white lupin, low levels of N enhanced P deficiency-induced formation of cluster roots, whereas high N supply had inhibitory effects (Dinkelaker *et al*., 1995). It is not known, however, how different N forms influence cluster root formation in this species.

The aims of this study were to elucidate the effects of N nutrition on cluster root formation, root morphology and mineral composition of white lupin plants grown under P deficiency or P sufficiency. Special emphasis was placed on characterizing the relationship between P and N supply and root extrusion of H^+.

MATERIALS AND METHODS

Plant cultivation

Seeds of white lupin (*Lupinus albus* L., 'Kiev Mutant') were pre-germinated on a stainless-steel mesh in a 15 l plastic container containing an aerated solution of 1 mM $CaCl_2$ and 5 μM H_3BO_3. Plants in the N_2-fixation treatment were inoculated with *Bradyrhizobium* sp. (*Lupinus*) WU 425 during the germination stage as well as in the first week of the experiments. Plants were grown in nutrient solution in a glasshouse to facilitate daily pH control. The nutrient solution had the following composition (in μM): 600 K_2SO_4, 200 $MgSO_4$, 600 $CaCl_2$, 10 FeEDDHA, 0·2 $CoSO_4$, 0·03 Na_2MoO_4, 5 H_3BO_3, 0·75 $ZnSO_4$, 1 $MnSO_4$ and 0·2 $CuSO_4$.

N and P treatments

White lupin plants, with their cotyledons removed, were treated with 0·5 mM N as either $(NH_4)_2SO_4$, KNO_3 or NH_4NO_3 (all treatments non-inoculated with *Bradyrhizobium* sp.), while N_2-fixing plants were inoculated with *Bradyrhizobium* sp. No nitrogen was added to the solution for the initial period before N_2 fixation took over. All solutions were supplied with either 1 or 50 μM P as KH_2PO_4. The lowest P concentration causing intensive formation of cluster roots is 1 μM P, while 50 μM P is twice the amount (25 μM) that suppresses cluster root formation by white lupin plants (Keerthisinghe *et al*., 1998; Sas *et al*., 2001). Each experimental combination consisted of triplicate 5·5 l pots, each with five plants. The pH of the nutrient solution was buffered at 5·6 by 0·2 mM MES and was adjusted daily using 0·1 M KOH or 0·1 M HCl. The volume of KOH used was recorded to enable calculations of proton extrusion. Nutrient solutions were aerated continuously and renewed every 2 d. Root temperature was maintained at 20 °C using a water bath.

Plant harvests

Starting from the first week of treatments (7-d-old plants), one plant from each pot was harvested weekly. At each harvest, the number of cluster roots was recorded. Cluster roots were defined as those portions of secondary lateral roots bearing bottlebrush-like clusters with a density of ten or more rootlets per cm (Johnson *et al*., 1996*a*). At the final harvest, after 35 d of N and P treatments, total root length, root area, root volume and the number of root tips were determined separately for cluster and non-cluster roots using a Hewlett Packard scanner (HP ScanJet 6100C) controlled by WinRhizo software (Regent Instruments Inc., Quebec, Canada; Arsenault *et al*., 1995).

Determination of dry biomass

Dry biomass was determined for roots, shoots and young expanded leaves (YEL) separately, in accordance with the analytical procedure of Ostrowska *et al*. (1991). Approx. 7 g of air-dried plant material was dried at 105 °C for 6 h, cooled for 0·5 h in a desiccator filled with silica gel and subsequently weighed. The weighing and drying procedure was repeated until the samples achieved constant weight.

Wet mineralization of plant material to determine total nitrogen (Polish standard PN-73C-04576/12)

Plant material for the analysis of mineral composition was dried at 60 °C for 48 h, ground to pass through a 1-mm sieve, mixed thoroughly, mineralized in Kjeldahl flasks using the wet method with H_2SO_4 and catalysts H_2O_2 and $CuSO_4.5\ H_2O$ (Ostrowska *et al*., 1991), cooled and diluted with deionized water to the specified volume. Total nitrogen was determined using the standard Kjeldahl method based on distillation of ammonia with steam from the alkalized solution, and determining the ammonia content in the distillate by titration.

Wet mineralization of plant material to determine macro- and micro-elements

Ground plant material (see above) was mineralized in teflon-coated containers in a microwave oven in a 5 : 1 mixture of HNO_3 and H_2O_2 under controlled temperature and pressure. The macro- and micro-element content in diluted digests was determined by an inductively coupled plasma emission spectrometer (ICP) (Cygañski, 1997).

Statistical analysis

Statistical analysis was carried out using single factor ANOVA. Comparisons of means were performed at $P < 0·05$.

RESULTS

The effect of N and P supply on formation and biomass of cluster roots

The number of cluster roots increased greatly when plants were supplied with 1 μM P in comparison with 50 μM P, the increase being 7·8-, 2·9-, 3·1- and 2·4-fold for $(NH_4)_2SO_4$, KNO_3, NH_4NO_3 and N_2-fixation treatments, respectively (Fig. 1). These differences were statistically significant. The dry root biomass of cluster roots increased significantly when plants were treated with 1 μM P in comparison with 50 μM P, the increase being 11·5-, 3·7-, 6·7- and 3·7-fold for $(NH_4)_2SO_4$, KNO_3, NH_4NO_3 and N_2-fixation treatments,

respectively (Fig. 2). In contrast, under P sufficiency (50 μM P), the dry weight of non-cluster roots was 1·2–2·9-times greater than under P deficiency (depending on the N source). At 1 μM P, NH_4-N nutrition increased the dry biomass of cluster roots 1·4-fold, whereas KNO_3 treatment and N_2 fixation decreased their biomass by approx. two-fold in comparison with P sufficiency. Phosphorus supply considerably influenced root length and the ratio of cluster to non-cluster roots (Fig. 3). Under P deficiency, cluster roots were significantly longer than non-cluster roots, the increase being greater in the case of $(NH_4)_2SO_4$ and NH_4NO_3 nutrition compared with other N sources (Fig. 3). However, for non-cluster roots, the reverse relationship was observed.

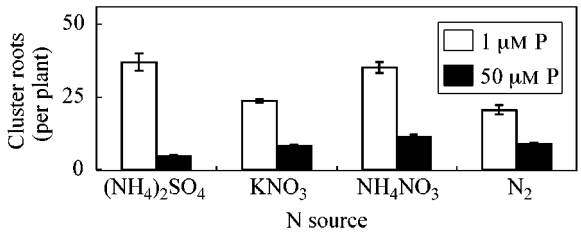

FIG. 1. Number of cluster roots formed per *L. albus* ('Kiev Mutant') plant after 35 d of growth in nutrient solutions containing different N forms and P concentrations. Bars represent ± s.e. (n = 3).

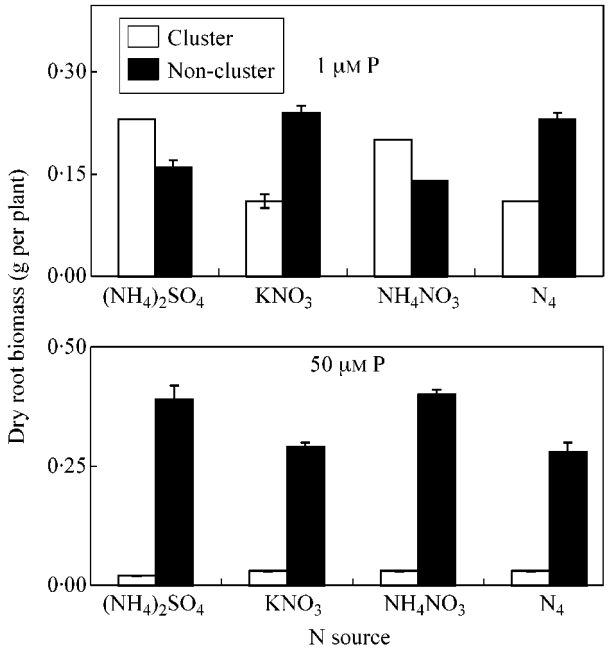

FIG. 2. Dry biomass of cluster and non-cluster roots of *L. albus* ('Kiev Mutant') after 35 d of growth in nutrient solutions containing different N forms and P concentrations. Bars represent ± s.e. (n = 3).

Proton extrusion in relation to N and P treatment

Proton excretion from roots of white lupin was highly dependent on P and N supplies (Figs 4 and 5). Total H^+ extrusion (μmol per plant h^{-1}) was highest when plants were supplied with $(NH_4)_2SO_4$ and decreased under NH_4NO_3, N_2 fixation and KNO_3 supply (Fig. 4). Similarly, the highest specific H^+ exudation (μmol g^{-1} root d. wt h^{-1}) (Fig. 5) was observed when plants were supplied with $(NH_4)_2SO_4$ or NH_4NO_3. In contrast, KNO_3 nutrition caused a release of OH^- ions.

The effect of P and N supply on mineral composition of white lupin

Mineral element concentrations in plants were greatly influenced by P and N nutrition. Treatment of plants with 50 μM P increased the concentration of P in roots and shoots (Fig. 6). Compared with KNO_3 or N_2 fixation treatments,

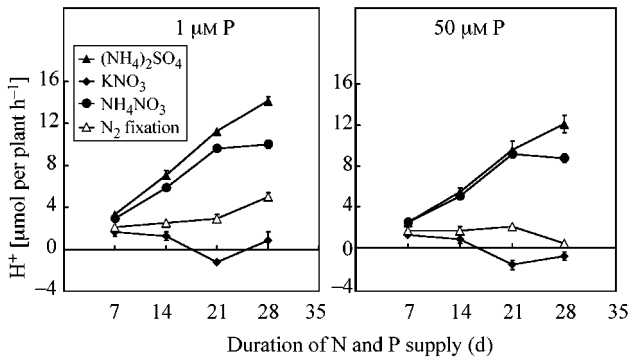

FIG. 4. Total H^+ extrusion by *L. albus* plants grown in nutrient solutions under different N forms and at 1 and 50 μM P for 7–35 d. Bars represent ± s.e. (n = 3).

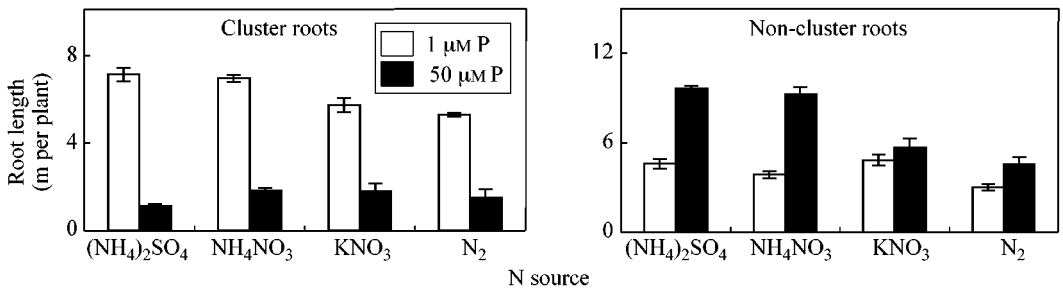

FIG. 3. Total length of cluster and non-cluster roots of *L. albus* plants after 35 d of growth in nutrient solutions containing different N forms and P concentrations. Bars represent ± s.e. (n = 3).

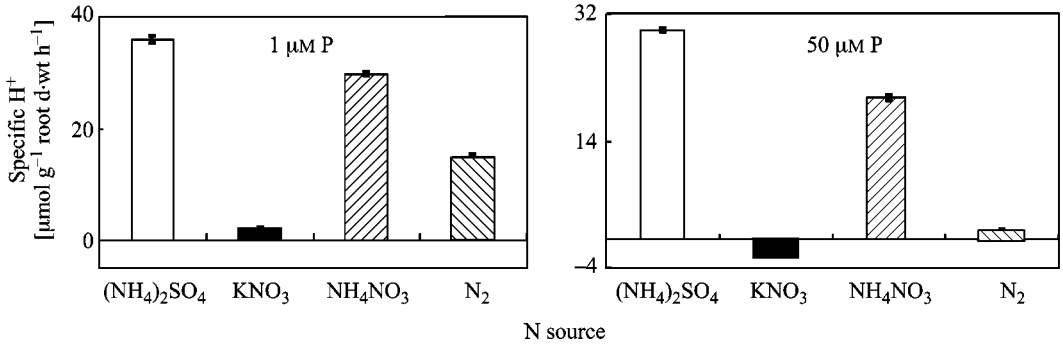

FIG. 5. Specific H^+ extrusion by *L. albus* plants after 35 d of growth in nutrient solutions containing different N forms and either 1or 50 μM P. Bars represent ± s.e. (n = 3).

total nitrogen concentrations in roots and shoots were highest in the case of $(NH_4)_2SO_4$ and NH_4NO_3 nutrition (Fig. 6). Root potassium concentration increased significantly when plants were supplied with 50 μM P, the increase being 1·6-, 2·0-, 1·3- and 1·2-fold for $(NH_4)_2SO_4$, NH_4NO_3, KNO_3 and N_2-fixation treatments, respectively (Fig. 6).

Magnesium and calcium concentrations in roots increased under P deficiency, whereas the reverse was observed in shoots. Regardless of P and N treatment, Fe and Zn concentrations were significantly higher in roots than in shoots, but the reverse was true for Mn (Fig. 7). Compared with phosphorus sufficiency, P deficiency increased the concentration of Mn in roots and shoots at all N supplies. Irrespective of N nutrition, insufficient P supply decreased the Zn concentration in roots and increased it in shoots in comparison with P sufficiency. Generally, P deficiency increased Fe, Mn and Zn in shoots, regardless of N treatment (Fig. 7). Irrespective of N supply, treatment of plants with 1 μM P significantly decreased the P concentration in young expanded leaves, as compared with P sufficiency (Fig. 8). Phosphorus deficiency and $(NH_4)_2SO_4$ nutrition significantly increased concentrations of K, Mg, Ca, Fe and Mn in YEL, whereas this tendency was not clear for other N treatments.

DISCUSSION

The effect of N and P supply on formation and biomass of cluster roots

The large increase in cluster root number under 1 μM P (Fig. 1) confirmed earlier findings that phosphorus deficiency induces cluster root formation in white lupin (e.g. Gardner *et al*., 1983; Marschner *et al*., 1987; Johnson *et al*., 1994, 1996*a*, *b*; Keerthisinghe *et al*., 1998; Neumann *et al*., 1999, 2000; Neumann and Römheld, 1999; Sas *et al*., 2001). However, we showed for the first time that under P deficiency the greatest number of cluster roots (37 per plant) was obtained when plants were supplied with $(NH_4)_2SO_4$, followed by NH_4NO_3 (35 per plant), KNO_3 (24 per plant) and N_2 fixation (20 per plant). It is suggested, therefore, that NH_4^+-N is a powerful stimulant of cluster root formation in P-deficient white lupin. Similarly, species in the Proteaceae utilize NH_4^+ as the predominant N source and also form extensive cluster roots (Stock and Lewis, 1984). Conversely, Diem and Arahou (1996) found that *Casuarina glauca* only produces cluster roots when grown in nitrate, with no cluster roots being produced in ammonium. Riley and Barber (1971) suggested that the N source might lead to a feedback on cluster root formation in soil via P. If the N source was ammonium, the excess protons released into the rhizosphere could free up P, thus preventing cluster root formation because the threshold level needed for cluster root production would not be crossed due to adequate P being available via the assimilatory consequences of the N source. However, Reddell *et al*. (1986) found that cluster root production in Casuarinaceae is unaffected by the available forms of N used.

Under 1 μM P, the dry biomass of cluster roots was significantly greater than that of non-cluster roots only in plants treated with $(NH_4)_2SO_4$ and NH_4NO_3, whereas for other P sources the reverse was observed (Fig. 2). Under P deficiency, cluster roots were significantly longer than non-cluster roots. However, for non-cluster roots, the reverse relationship was observed (Fig. 3). The number of root tips, root area and root volume followed similar relationships as shown for root length (data not shown). Similarly, in a previous study, Sas *et al*. (2001) showed that P deficiency (1 μM) significantly increased the total root length and the number of root tips of white lupin as compared with higher levels of P (5 and 25 μM).

Proton extrusion in relation to N and P treatment

The form of nitrogen supplied has a strong impact on the uptake pattern of nutrients, cellular pH regulation and the rhizosphere pH (Raven 1985; Marschner, 1995). Rhizosphere acidification can be caused by excess uptake of cations over anions, and alkalization occurs when anion uptake exceeds cation uptake. Ammonium uptake is generally associated with acidification, while nitrate nutrition induces an increase in rhizosphere pH.

Little is known about the mechanism of citrate exudation from cluster roots. Since P deficiency in white lupin (Dinkelaker *et al*., 1989; Neumann *et al*., 1999) and citrate

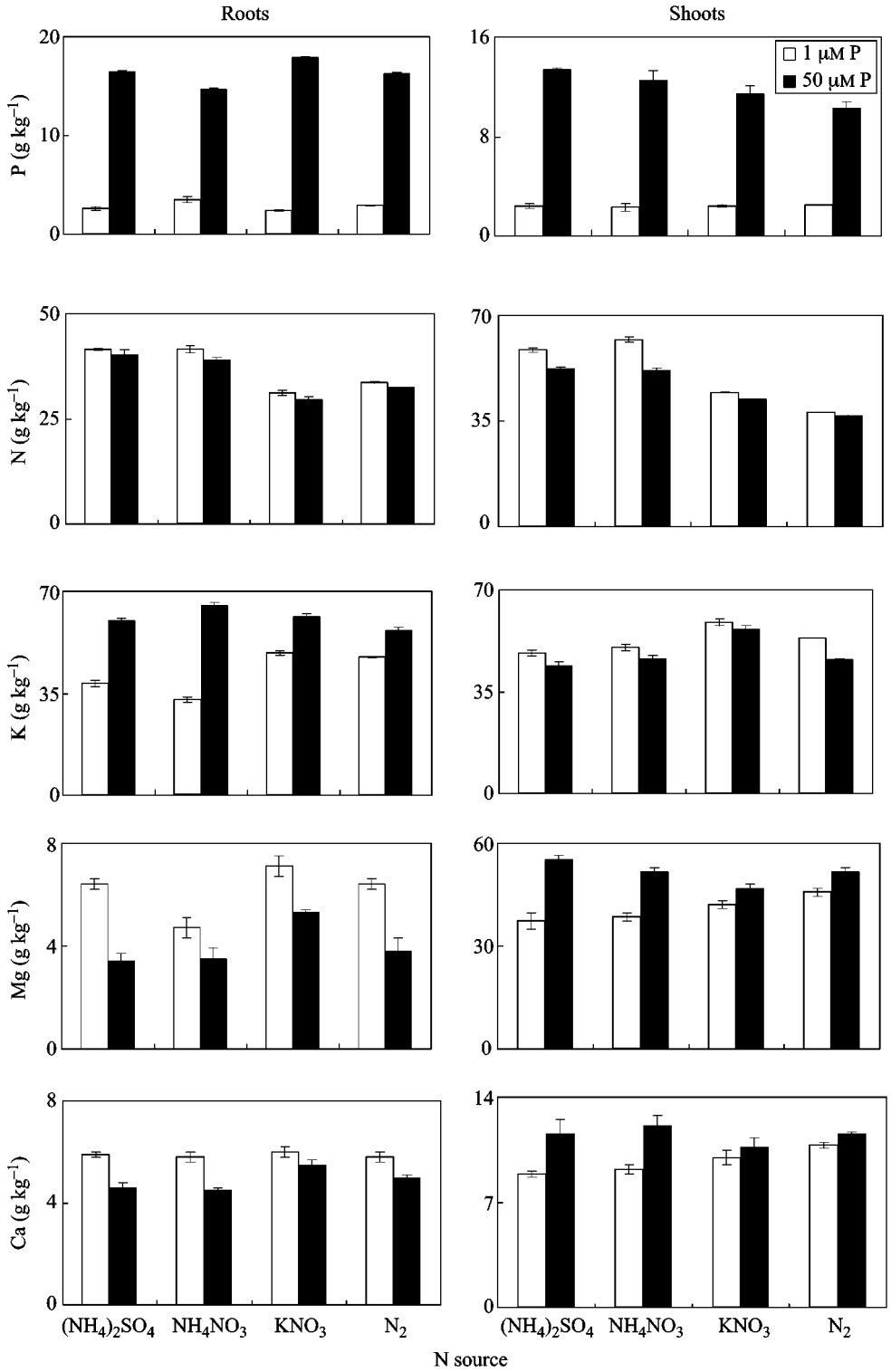

FIG. 6. Root and shoot concentrations of P, N, K, Mg and Ca in white lupin after 35 d of growth in nutrient solutions containing different N forms and P concentrations. Bars represent ± s.e. (n = 3).

excretion from cluster roots coincide with rhizosphere acidification (Marschner *et al*., 1987; Dinkelaker *et al*., 1989), it has been suggested that citrate may be excreted via an anion channel, with a concomitant release of protons to maintain charge balance (Dinkelaker *et al*., 1989; Johnson *et al*., 1996*b*; Neumann *et al*., 1999; Sas *et al*., 2001). However, the relationship between N nutrition and proton release in white lupin has not yet been elucidated.

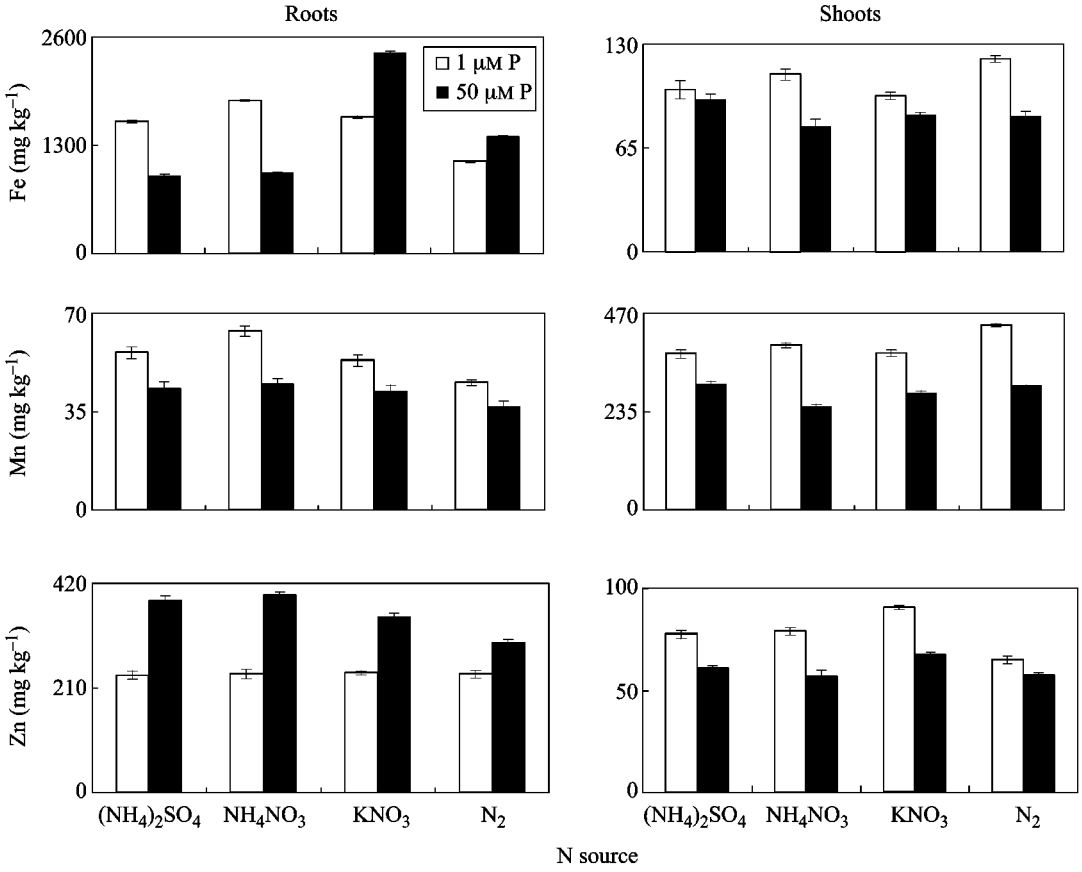

FIG. 7. Root and shoot concentrations of Fe, Mn and Zn in white lupin after 35 d of growth in nutrient solutions containing different N forms and P concentrations. Bars represent ± s.e. (n = 3).

In our study proton excretion from roots of white lupin was highly dependent on P and N supplies (Figs 4 and 5). Both total and specific H^+ extrusion increased under P deficiency and $(NH_4)_2SO_4$ or NH_4NO_3 nutrition. In contrast, KNO_3 nutrition caused a release of OH^- ions between days 21 and 35. Interestingly, after 35 d in the NH_4^+ treatment, the amounts of proton released were similar in P-deficient and P-adequate plants, whereas in N_2-fixing plants and those treated with KNO_3 phosphorus deficiency increased proton release ten-fold (Fig. 4). Similarly, in the previous study, P deficiency increased proton extrusion, with the greatest H^+ extrusion by N_2-fixing white lupin plants being associated with both organic acid anion exudation and excess uptake of cations over anions (Sas *et al*., 2001).

The effect of P and N supply on mineral composition of white lupin

In comparison with P deficiency, 35-d-old plants treated with 50 μM P had a 6·3-, 4·2-, 7·5- or 5·6-fold higher concentration of P in roots and a 5·6-, 5·4-, 4·8- and 4·1-fold higher concentration in shoots when supplied with $(NH_4)_2SO_4$, NH_4NO_3, KNO_3 and N_2-fixation, respectively (Fig. 6). Sas *et al*. (2001) reported that increasing P supply led to an increased concentration of P and also of K, S, Zn, Fe and Cu in whole roots. At 1 μM P, phosphorus concentrations in roots and shoots were similar (between 2·4 and 3·5 g kg^{-1} in roots and 2·3 and 2·5 g kg^{-1} in shoots), regardless of the N form supplied to the plants. The highest N concentrations in roots and shoots under P deficiency and ammonium nutrition (in both $(NH_4)_2SO_4$ and NH_4NO_3 treatments) coincided with the greatest number and largest biomass of cluster roots (Figs 1 and 2) and most H^+ extrusion (Figs 4 and 5). Conversely, Lamont (1973) reported that increasing N status in *Hakea* shoots led to a decrease in cluster root formation.

In comparison with the 1 μM P treatment, the root potassium concentration increased significantly when plants were supplied with 50 μM P (Fig. 6). However, in shoots, the reverse was observed. Sas *et al*. (2001) reported that K concentration in roots was significantly lower at 1 μM P than at 25 μM P, although the K concentration in shoots was not affected by P supply. In the present study, under P deficiency, treatment of plants with $(NH_4)_2SO_4$ and NH_4NO_3 decreased K in roots and shoots, which coincided with the greatest H^+ extrusion at these N supplies. Since cluster roots of white lupin are the main site for release of H^+ and organic anions under P deficiency (Keerthisinghe *et al*., 1998; Neumann *et al*., 1999), it is speculated that the release of organic anions is accompanied by K^+ efflux (co-transport). This deserves further investigation. Further work could include a comparison of K levels in cluster and non-

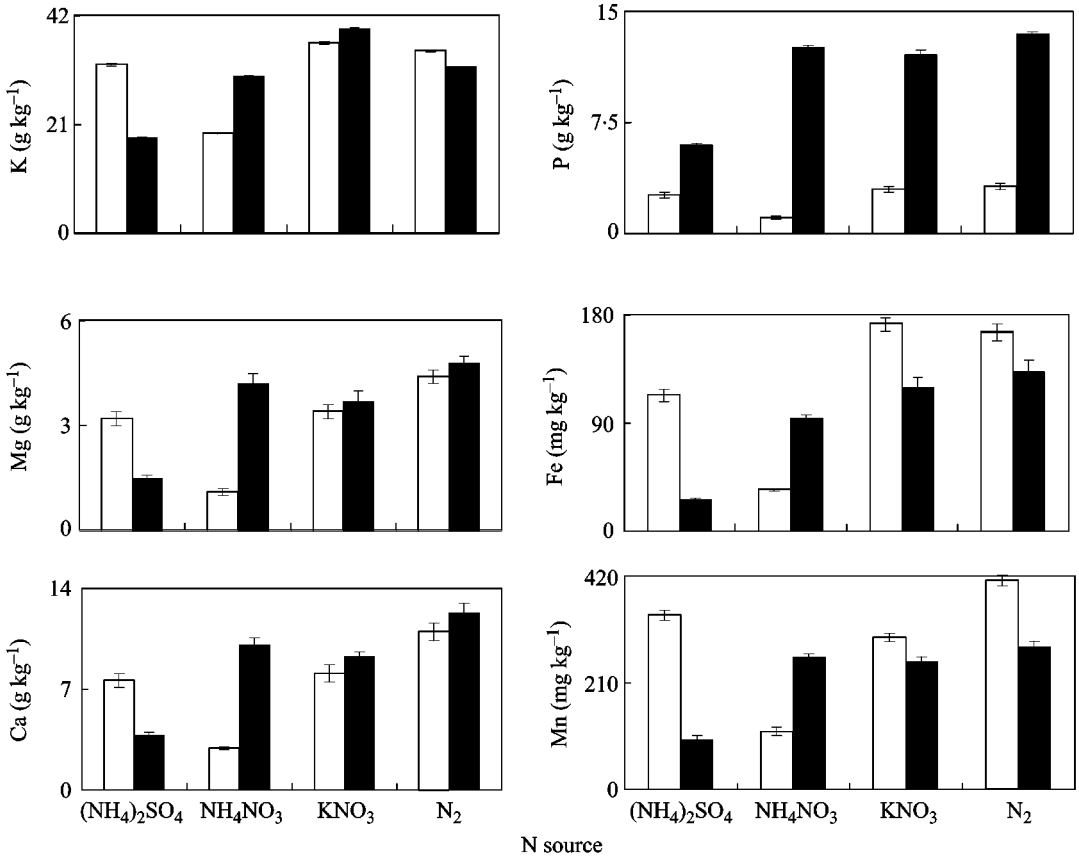

FIG. 8. Concentrations of P, K, Mg, Ca, Fe and Mn in young expanded leaves of white lupin after 35 d of growth in nutrient solutions containing different N forms and P concentrations. Bars represent ± s.e. (n = 3).

cluster roots under low P. Phosphorus deficiency increased Fe, Mn and Zn in shoots, regardless of N treatment (Fig. 7). We found that phosphorus deficiency and $(NH_4)_2SO_4$ nutrition significantly increased K, Mg, Ca, Fe and Mn concentrations in young expanded leaves (Fig. 8). Lu and Zhang (1995) also reported that P deficiency led to an increase in Fe, Cu and Zn absorption and transport in lupin plants.

CONCLUSIONS

Phosphorus deficiency increased cluster root formation and the ratio of cluster roots to non-cluster roots. NH_4^+-N stimulated production of a greater number, length and biomass of cluster roots compared with other N sources. Under P deficiency, K concentration in roots decreased at all N supplies, but especially in plants treated with $(NH_4)_2SO_4$ and NH_4NO_3, which coincided with the greatest H^+ extrusion in these P and N treatments. Total and specific H^+ extrusion occurred in the order: $(NH_4)_2SO_4 > NH_4NO_3 > N_2$ fixation > KNO_3 nutrition. The mechanisms of cluster root initiation and formation, and root exudation in relation to N nutrition deserve further investigation.

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