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Nitrogen and carbon costs of soybean and lupin root systems during phosphate starvation

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Abstract
Phosphate (P) starvation is one of the most limiting nutrients to N_2 fixation in legumes. Soybeans and lupins present different climatic origins, nodule morphologies and metabolic complexities, which may have various adaptive responses to short-term P starvation. Lupins and soybeans were cultivated hydroponically for 3 weeks. Short-term P starvation was induced for 14 days by switching the P-supply to 2 μM P. During P starvation, the lupins showed a lower decline in nodular P concentrations and maintained their biological N_2 fixation (BNF), in contrast to the soybeans. The lupins also maintained their photosynthetic rates and the nodular construction and growth respiration costs under P starvation, whilst soybeans showed a decrease in photosynthetic rates and an increase in nodular construction and growth respiration costs under P starvation. There was a also a shift towards more organic acid synthesis, relative to amino acid synthesis in lupin nodules than soybean nodules under P starvation. The lupins had higher amino acid concentrations in their nodules, whilst the soybean nodules maintained their ureide levels at the expense of a decline in amino acids. These results indicate that lupins may to be better adapted to maintaining BNF during short-term P starvation than the soybeans.

Keywords: Nitrogen metabolism, carbon costs, phosphate, soybean, lupin

1. Introduction

P limitation is one of the most notable environmental constraints for legumes (Jakobsen, 1985; Israel, 1987; Høgh-Jensen et al., 2002). The high sensitivity of the N_2 fixation process to environmental conditions, may be attributed to the C costs (Mengel, 1994). Legumes relying on N_2 fixation require more P than when N is acquired from soil mineral N (Sa and Israel, 1991; Ribet and Drevon, 1995; Al Niemi et al., 1997, 1998; Tang et al., 2001). The high requirement of P may be linked to its role in nodule carbon and energy metabolism, with at least the plant cell fraction being energy limited under low P supply (Sa and Israel, 1991).

The effect of P starvation on N_2 fixation and nodule O_2 permeability has been demonstrated for both amide and ureide exporting nodule types (Ribet and Drevon, 1995; Drevon and Hartwig, 1997; Schulze and Drevon, 2001). Most temperate legumes (e.g. lupin, pea, clover) translocate fixed N as amides, notably asparagine and glutamine (Streeter, 1991), whilst tropical legumes (e.g. soybean, cowpea, common bean) export ureides, most commonly allantoin and allantoic acid. Lupins and soybeans also differ in other means, such as being of temperate and tropical origin and having morphologically distinct nodules. However, as noted by Streeter (1991), the two different sets of metabolic capabilities of amino acid and ureide exporting legumes, present a metabolic complexity that is perhaps unsurpassed by some other more typical plant systems. Although the ureide exporters require several more enzymes for ureide biosynthesis, compared to only a few

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Table 1. Biomass and nutrients of lupins (*Lupinus angustifolius*) and soybeans (*Glycine max*). All plants were grown 3 weeks in hydroponic culture with N-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 ppm CO_2 in the root zone. A low P condition was induced for 14 days in the P starved plants by switching the P-supply to 2 µM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n = 4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Biomass	Lupin		Soybean	
	Control	-P	Control	-P
Plant growth				
Plant DW (g.plant^{-1})	0.90 c	0.77 b	0.75 b	0.62 a
Shoot DW (g.plant^{-1})	0.65 b	0.58 b	0.30 a	0.25 a
Root DW (g.plant^{-1})	0.18 b	0.12 a	0.40 d	0.33 c
Root:Shoot	0.28 a	0.26 a	1.60 b	1.21 b
Nodule growth				
Nodule DW (g.plant^{-1})	0.07 b	0.06 ab	0.04 a	0.04 a
Nodule number	171 b	169 b	12 a	15 a
Normalized nodule weight (g.g^{-1} shoot)	0.10 a	0.10 a	0.16 b	0.13 ab
Nodule C cost				
Nodule construction cost (mmol C.g^{-1} dw)	281.28 c	295.87 c	218.51 a	264.83 b
Nodule growth respiration (mol CO_2.g^{-1})	0.28 a	0.27 a	0.22 a	0.37 b
N concentrations (mmol N.g^{-1} dw)				
Root N:Shoot N	0.35 a	0.40 a	0.72 c	0.60 b

for asparagine synthesis, the ATP and reductant expenditure per N assimilated is similar to that of amide exporting legumes (Smith and Atkins, 2002).

Due to the energy costs of N_2 fixation, overall C expenditure is high under non-stress conditions and varies considerably among legume species and varieties (Vance, 1998; Schulze, 2004). Since lupins and soybeans are from temperate and tropical origins, and present a difference in N-metabolism and nodule morphology, it is likely that they may show different physiological responses to P starvation. The aim of this study was to draw comparative analyses between the nitrogen and carbon costs imposed on the two legume systems during short-term P starvation.

2. Material and Methods

Plant material and growth conditions

Seeds of *Lupinus angustifolius* (cv. Tanjil) and *Glycine max* (var. PAN 626) were germinated in vermiculite. At planting, seeds were inoculated with a rhizobial inoculum specific for each species obtained from a commercial seed company. Seeds were coated in a saturated sucrose solution and 2 g of inoculum / 150 seeds were added and mixed. The seeds were spread out, away from direct sunlight to allow the inoculum to dry, before the seeds were planted.

Seedlings were transferred seven to 10 d after germination to 20 l hydroponic tanks that was purged with ambient air. The base nutrient solution consisted of 4 mM $CaCl_2$, 1.5 mM $MgSO_4$, 2 mM K_2SO_4, 2 mM NaH_2PO_4/ Na_2HPO_4, 139 µM H_3BO_3, 21 µM $MnSO_4$, 2 µM $ZnSO_4$, 3 $CuSO_4$ µM, 0.2 Na_2MoO_4 µM, 89 µM FeEDTA and no N (pH 5.8) (Hewitt, 1966). Solutions were changed once a week. Seedlings were fixed in the lid of the hydroponic tank with foam rubber at their bases and inserted through holes in the lids of the tanks. Since the aim of the experiments were to investigate the effects of abrupt P deprivation, plants were initially grown on a non-limiting P supply (2 mM P) for approximately three weeks. Short-term P starvation was induced for 14 days by switching the P-supply to 2 µM P, after which plants were harvested. Although this represents a short period of P starvation after 3 weeks of normal growth, the control group of plants, was maintained at sufficient P supply, so that the short-term departure from these sufficient conditions could be physiologically recorded. The plants were grown in an east-facing glasshouse in Cape Town, South Africa. The range of midday irradiances were between 500 and 670 µmol $m^{-2}.s^{-1}$ and the average day/night temperatures and humidities were 21/16°C and 34/73%, respectively.

Photosynthesis

The youngest fully expanded leaves for each plant were used for the photosynthetic determinations. Light-response curves were used to determine the irradiance (1100 µmol.$m^{-2}.s^{-1}$) at which to conduct the photosynthetic rates. Readings were taken at midday, using a portable infrared gas analyzer (LCA-Pro, ADC, Herts SG12 9TA, England).

P, N and C determination

Inorganic orthophosphate (P*i*) and pyrophosphate (PP*i*) measurements were made on fresh nodules. Approximately 0.5 g of fresh nodule mass was ground to a fine powder inn liquid nitrogen and was extracted in 500 µl 10% trichloroacetic acid (TCA) at 4°C as described by Rychter and Mikulska (1990). This was diluted 3 times with cold 5% TCA and centrifuge for 10 min at 2 500 g at 4°C. The supernatant was removed and centrifuged a further 10 min at 13,000 g at 4°C. The resultant supernatant was kept on ice (or stored -20°C) until ready for use. PP*i* and P*i* concentrations were determined indirectly via pyrophosphatase activity, which was assayed by incubating 10 µl of extract with 190 µl of 50 mM Tris-HCl (pH 8.0) containing 2.4 U/assay inorganic pyrophosphatase, 20 mM $MgCl_2$ and 1.3 mM Na-PP_i for 15 min (Mustroph et al., 2005).

For total C, N and P concentrations, dried and milled samples were analysed by a commercial laboratory (BemLab, De Beers Rd, Somerset West, South Africa), using inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyser with Spectrascan standards (Norway).

Construction cost and growth respiration

Tissue construction costs (mmol $C.g^{-1}$ dw) and growth respiration (mol $CO_2.d^{-1}$) were calculated according to Mortimer et al. (2005, 2008), as modified from the equations used by Peng et al. (1993). Construction costs represent the C required for tissue growth, whilst growth respiration is the daily respiration associated with new growth (Peng et al., 1993).

Nitrogen fixation

The $\delta^{15}N$ analyses were carried out at the Archeometry Department, University of Cape Town, using a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. The seed corrected $\delta^{15}N$ values (Boddey et al., 1995) were used to determine the percentage N derived from the atmosphere (NDFA). % NDFA was calculated according to Shearer and Kohl (1986):

$$\%NDFA = 100 * ((\delta^{15}N_{reference\ plant} - \delta^{15}N_{legume}) / (\delta^{15}N_{reference\ plant} - B))$$

where *B* is the $\delta^{15}N$ natural abundance of the N derived from biological N fixation of the above-ground tissue of *Lens vulgaris*, grown in a N-free culture, according to Shearer and Kohl (1986). The *B*-value of *Lens vulgaris* was determined as -0.76‰.

Amino acid and ureide concentrations

Amino acid concentrations were determined according to the ninhydrin method by Rosen et al. (1957) using leucine as a standard. Ureides were measured as allantoin concentration according to Trijbels and Vogel (1966).

Enzyme activities

The extraction of the organic acid synthesizing enzymes, phospoenol pyruvate carboxylase (PEPC) and NADH-malate dehydrogenase (MDH) was performed according to Ocaña et al. (1996). The procedure was modified so that 0.5 g of tissue was extracted in 2 ml of extraction buffer consisting of 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM dithiothreitol (DTT), 20% (v/v) ethylene glycol, plus 2% (m/v) insoluble polyvinylpoly pyrrolidone (PVPP) and one Complete Protease Inhibitor Cocktail tablet (Roche) per 50 ml of buffer. The extraction of N assimilating enzymes, aspartate amino transferase (AAT), glutamine synthetase (GS) and glutamate synthase (GOGAT) were prepared according to the method of Olivera et al. (2004). All extractions were carried out at 4°C and the crude extract was used in colourometric assays for PEPC, MDH, GS, GOAT and AAT according to Le Roux et al. (2008).

Statistical analysis

The percentage data were arcsine transformed (Zar, 1999). Significant differences between treatments the means were separated using a *post hoc* Student Newman Kuehls (SuperAnova, Abacus Concepts, USA), multiple range test ($P \leq 0.05$). Different letters indicate significant differences between treatments.

3. Results

Nodular P concentration and utilization efficiency

Nodular P*i* (Fig. 1b) and PP*i* (Fig. 1c) concentrations remained unchanged during P deficiency, but the total P (Fig. 1a) concentrations declined and this decline was more pronounced for soybeans (60%) than for lupins (35%).

The specific total P (Fig. 2a) and P*i* (Fig. 2b) utilisation rates for soybean nodules, increased significantly under P starvation, compared to the soybean controls and the P starved lupins. Indeed in spite of the decline in root growth under P starvation, the growths of nodule and shoot remained unaffected in both legumes (Table 1).

Nodule respiration and photosynthesis

During P starvation, the nodule construction costs and

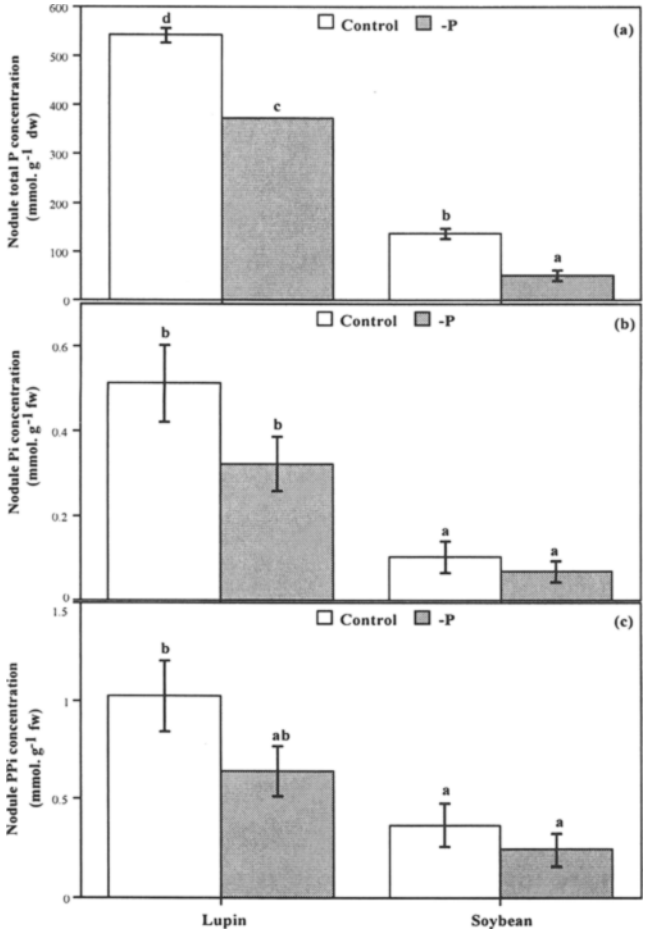

Figure 1. (a) The nodular total phosphate (P), (b) orthophosphate (P*i*), and (c) pyrophosphate (PP*i*) concentrations of lupin (*Lupinus angustifolius*) and soybean (*Glycine max*). All plants were grown 3 weeks in hydroponic culture with N-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P-supply to 2 μM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n=4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

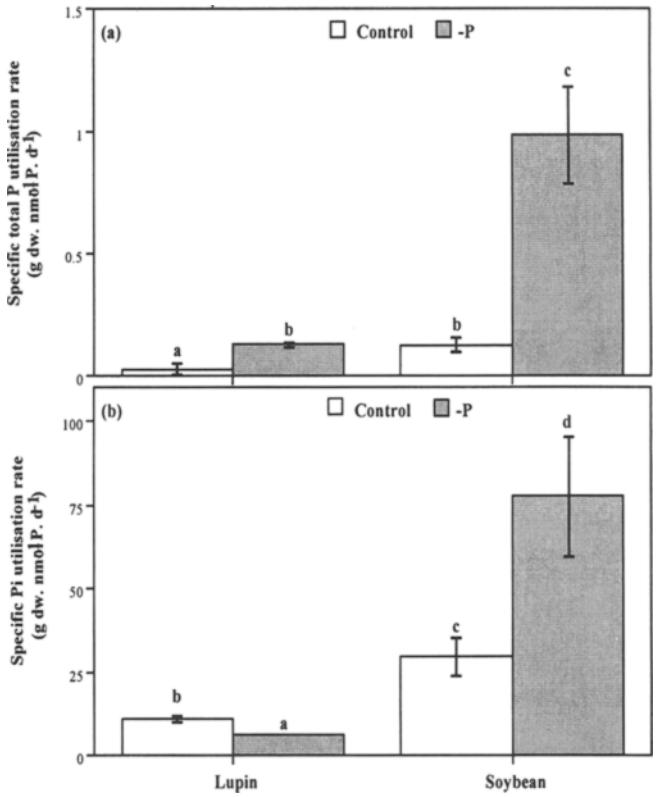

Figure 2. (a) The specific total phosphate (P) utilisation rate and (b) specific orthophosphate (P*i*) utilisation rate of lupin (*Lupinus angustifolius*) and soybean (*Glycine max*). All plants were grown 3 weeks in hydroponic culture with N-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P-supply to 2 μM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n=4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

growth respiration of lupins remained unchanged, in contrast with those of soybeans which increased with P deficiency (Table 1).

Lupins generally had higher photosynthetic rates that soybeans (Fig. 3). Although the photosynthetic rates in lupins remained unchanged during P deficiency, soybeans showed a decrease during P starvation (Fig. 3).

Nitrogen metabolism

During P starvation, lupins maintained the % nitrogen derived from atmosphere (Ndfa), compared to soybeans which showed a decline in the % Ndfa (Fig. 4).

Consequently, the tissue N levels remained unchanged for lupins, whilst soybeans had a decline in root and nodule N concentrations (Figs. 5a–c). Furthermore, the ratio of N between roots and shoots was unaffected by P starvation in lupins, but in soybeans the root N: shoot N ratio declined (Table 1).

The activities of the enzymes associated with organic acid synthesis, PEPC (Fig. 6a) and MDH (Fig. 6b) increased for both lupin and soybean nodules under P starvation. In addition, there were generally also increases in the amino acid synthesizing enzyme, GS (Fig. 6c), GOGAT (Fig. 6d) and AAT (Fig. 6e), with P starvation in both lupin and soybean nodules. In spite of these increases in both organic acid and amino acid synthesizing enzymes, the ratio of the major organic acid synthesizing enzyme, MDH to the amino acid synthesizing enzymes, was enhanced in lupins, but not in soybeans (Figs. 7a–c).

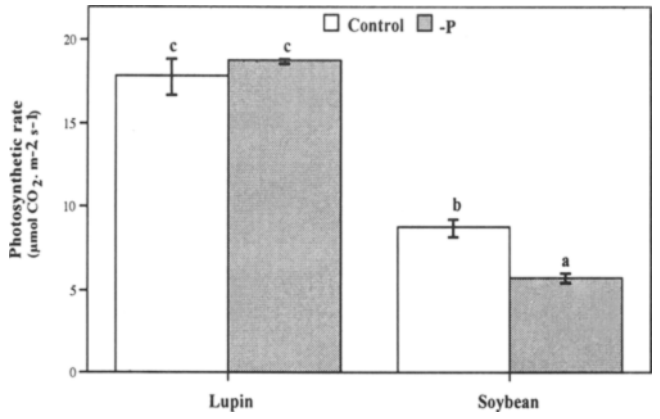

Figure 3. The photosynthetic rates of lupin (*Lupinus angustifolius*) and soybean (*Glycine max*). All plants were grown 3 weeks in hydroponic culture with N-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P-supply to 2 μM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n=4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

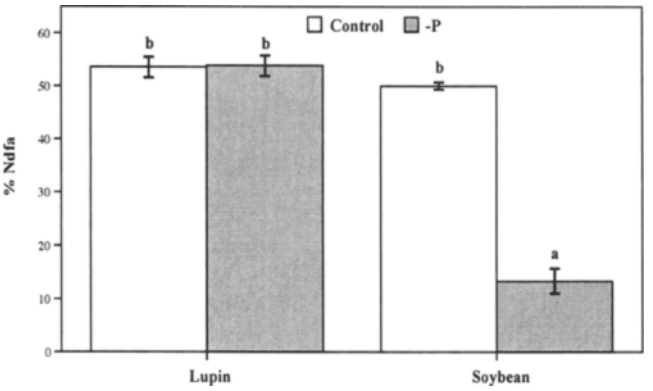

Figure 4. The percentage (%) of nitrogen derived from atmosphere (% Ndfa) of lupin (*Lupinus angustifolius*) and soybean (*Glycine max*). All plants were grown 3 weeks in hydroponic culture with N-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P-supply to 2 μM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n=4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

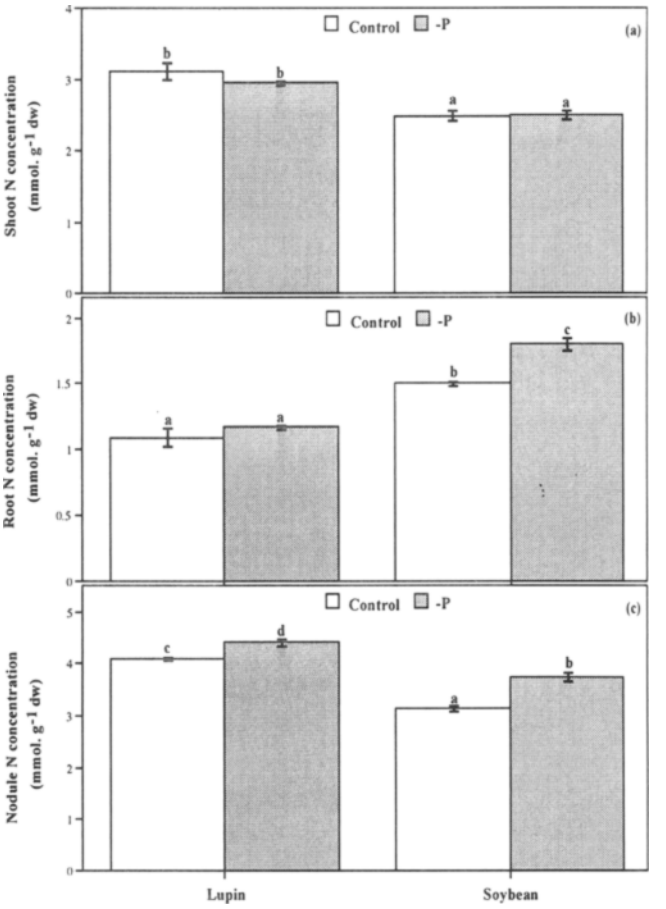

Figure 5. (a) Shoot N, (b) Root N, and (c) Nodule N concentrations of lupin (*Lupinus angustifolius*) and soybean (*Glycine max*). All plants were grown 3 weeks in hydroponic culture with N-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P-supply to 2 μM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n=4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

These increased activities of amino acid synthesizing enzymes during nodular P deficiency, were associated with the enhanced concentrations of nodular amino acid levels in lupins (Fig. 8a), but not in soybeans (Fig. 8b). The maintenance of the nodule ureide concentrations in soybeans under P starvation, appeared to have been at the expense of amino acid levels soybean nodules (Fig. 8c). This accounted for the increase in the ratio of ureides: amino acids concentrations in soybean nodules during P deficiency (Fig. 8d).

4. Discussion

During short-term P starvation, the lupins were physiologically better adapted for maintaining biological N_2-fixation (BNF) than the soybeans.

The maintenance of metabolic PP*i* (Fig. 1c) and P*i* (Fig. 1b) concentrations in nodules of both legumes under P starvation, suggest that nodules function optimally at these

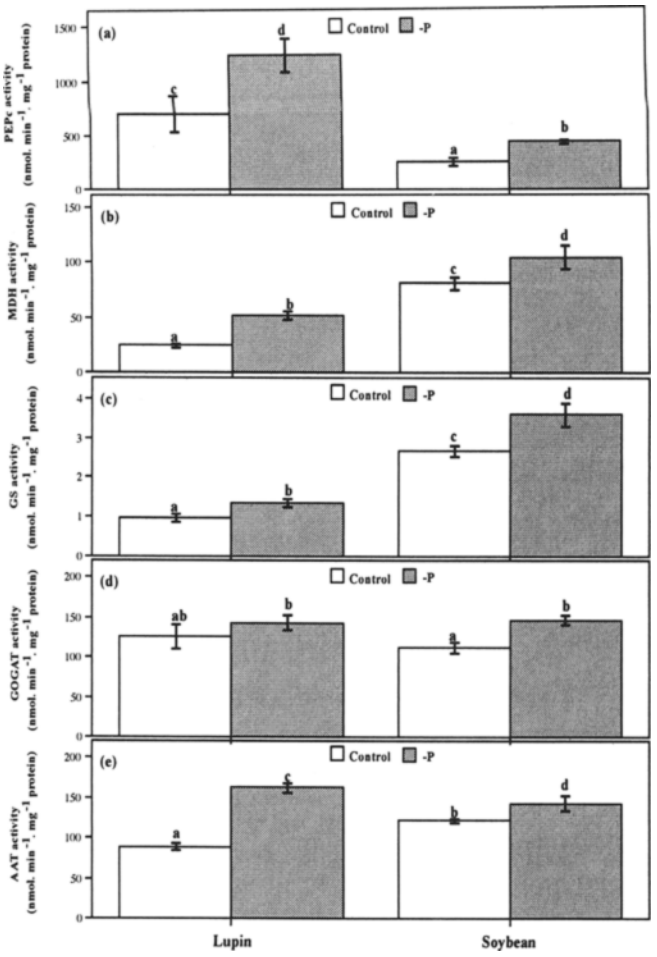

Figure 6. *In vitro* specific activities of (a) PEPc, (b) MDH, (c) GS, (d) GOGAT, and (e) AAT of lupin (*Lupinus angustifolius*) and soybean (*Glycine max*). All plants were grown 3 weeks in hydroponic culture with N-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P-supply to 2 μM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n=4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

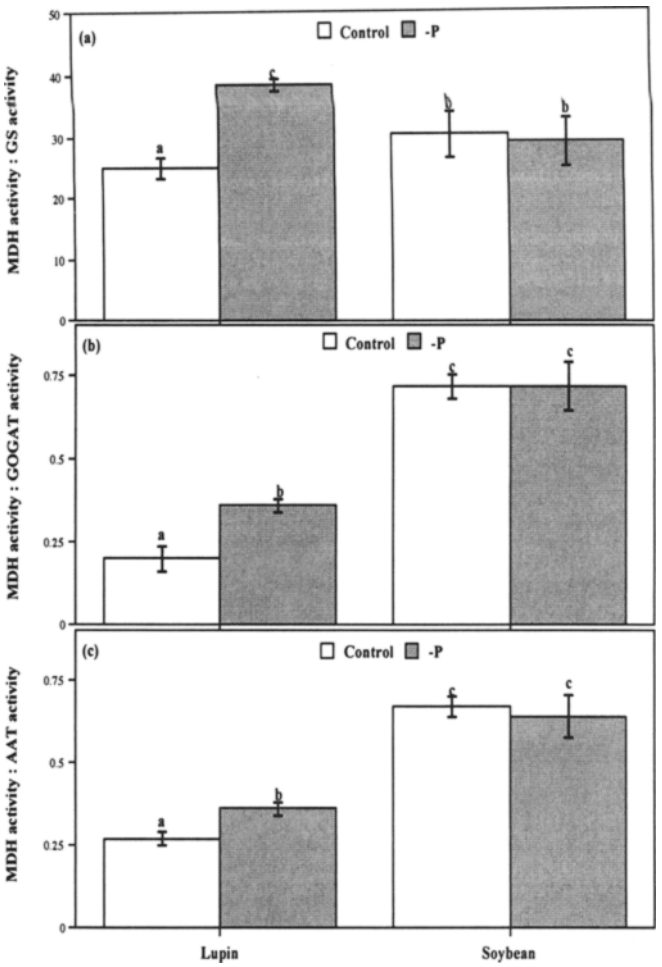

Figure 7. The relative ratios of *in vitro* specific activities of (a) MDH to GS, (b) MDH to GOGAT, and (c) MDH to AAT of lupin (*Lupinus angustifolius*) and soybean (*Glycine max*). All plants were grown 3 weeks in hydroponic culture with N-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P-supply to 2 μM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n=4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

P levels (Tang et al., 2001; Høgh-Jensen et al., 2002; Colebatch et al., 2004; Le Roux et al., 2006). Lupins showed a lower decline (35%) in nodular total P concentration (Fig. 1a) compared to the soybeans (64%) and this may be related to lupin nodules being more efficient at P recycling and having a greater sink strength during P starvation. In this regard, nodules are known to have a strong sink capacity for P incorporation during P starvation (Israel, 1993; Høgh-Jensen et al., 2002).

Although soybeans had fewer nodules per plant than lupins (Table 1), the soybean nodules had a greater efficiency to utilise P for growth under limiting P conditions (Figs. 2a,b). This increase in P utilisation efficiency of soybean nodules may be compensating for the relatively, more pronounced decline in the total P concentration of soybean nodules, compared to lupin nodules (Fig. 1a). The enhanced nodule efficiency for P utilization is considered to be a pivotal coping strategy during P starvation (Vadez et al., 1999). The alteration in biomass allocation is another strategy for coping with P starvation (Høgh-Jensen et al., 2002; Le Roux et al., 2006; Le Roux et al., 2008).

The maintenance of nodule and shoot growth for both lupins and soybeans appeared to have been at the expense of root growth (Table 1). Such adaptations in legumes have been reported previously, where growth of nodules were

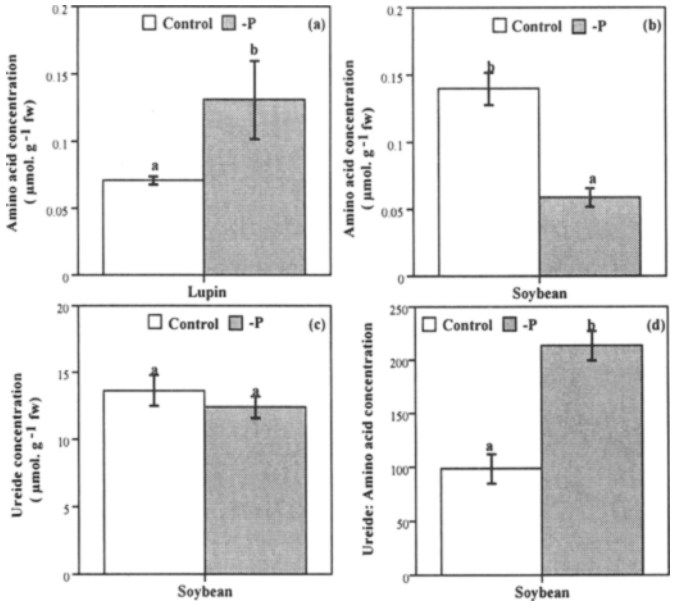

Figure 8. The nodular amino acid concentrations of (a) *Lupinus angustifolius*, (b) *Glycine max*, (c) nodular ureide concentrations of *Glycine max*, and (d) nodular ureide:amino acid ratio of *Glycine max*. All plants were grown 3 weeks in hydroponic culture with N-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 ppm CO_2. A low P condition was induced for 14 days in the P starved plants by switching the P-supply to 2 µM P (-P). The P sufficient plants remained at 2 mM P (Control) supply. Values are presented as means (n=4). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

less affected than the growth of other plant organs such as roots, after abrupt P withdrawal in white clover plants (Høgh-Jensen et al., 2002).

The % N derived from atmosphere (% Ndfa) in lupins and soybeans may have been directly affected by a limitation in P supply during P starvation, or indirectly by means of other physiological responses. During P starvation, the unchanged % Ndfa in lupins compared to the decline in % Ndfa in soybean (Fig. 4), may be related to lupins having a lower decline in total nodular P, compared to soybean. However, the P starved lupins were also able to maintain nodule growth C costs, leaf photosynthetic rates and to increase the activities of enzymes related to the synthesis of organic acids, relative to amino acids.

In this regard, the C-costs of nodule growth (Table 1) in lupins did not compete with C costs of BNF (Fig. 4), as it seems to have been the case with soybeans. This has been found in common bean where C costs associated with growth was able to compete for C with BNF (Mortimer et al., 2008). Furthermore, the unchanged shoot and root N (Figs. 5a,b) concentrations and the constant root N: shoot N concentrations of lupins under P starvation (Table 1), may have underpinned the sustained photosynthetic rates (Fig. 3). By maintaining shoot N allocation relative to the roots N under P starvation, it is likely that the sustained photosynthetic rates in lupins were able to provide C to the nodules for BNF.

Although both lupins and soybean had an increase in nodular organic acid and amino acid synthesizing enzymes under P starvation (Fig. 6), only lupin nodules showed a shift of increasing the activities of the major organic acid synthesizing enzyme MDH, relative to amino acid synthesizing enzymes (Fig. 7) in lupins during P starvation. This shift in lupin nodules during P starvation, may have provided more organic acids in the form of malate for the maintenance of bacterial metabolism and the synthesis of certain amino acids. This concurs with previous work (Le Roux et al., 2008) where a shift towards organic acid metabolism specifically malate, occurred in lupin nodules under P starvation. This may explain the increase in amino acid synthesis of lupin nodules, compared to soybean nodules (Figs. 8a,b), as also found by Le Roux et al. (2008).

In spite of the decline in %Ndfa in soybeans, their nodules accumulated more ureides relative to amino acids under P starvation. This was primarily achieved due to a decline in amino acid synthesis, rather than an increase in ureide production (Figs. 8b–d). The amino acid (lupins) and ureide (soybeans) exporting legumes present an unusual metabolic complexity among plant systems (Streeter, 1991). In addition, it has also been suggested that different regulatory principles might have to be adopted for amino acid exporting and ureide exporting legumes (Schulze, 2004), and some of these may be of importance to P starvation adaptation. At this stage it is not possible to conclude whether one strategy for N assimilation is more cost effective than the other, because this needs to be substantiated by more comprehensive comparative analyses (Smith and Atkins, 2002). Furthermore, lupins and soybeans also differ in other means, such as being of temperate and tropical origin and having determinate and indeterminate types of nodules.

In conclusion, the ability of lupins to maintain BNF under short-term P starvation compared to soybean, may be related to a variety of differences between the species. The data from the current study show that lupins have more physiological alteration than soybean during P starvation, which include the changes in nitrogen and carbon metabolism and showing a relatively lower decline in total P concentration of nodules. Although the findings are based on two weeks of P starvation, they represent the adaptations to short-term P stress.

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