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Nitrogen Fixation by White Lupin under Phosphorus Deficiency

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• *Background and Aims* White lupin is highly adapted to growth in a low-P environment. The objective of the present study was to evaluate whether white lupin grown under P-stress has adaptations in nodulation and N_2 fixation that facilitate continued functioning.
• *Methods* Nodulated plants were grown in silica sand supplied with N-free nutrient solution containing 0 to 0·5 mM P. At 21 and 37 d after inoculation (DAI) growth, nodulation, P and N concentration, N_2 fixation ($^{15}N_2$ uptake and H_2 evolution), root/nodule net CO_2 evolution and CO_2 fixation ($^{14}CO_2$ uptake) were measured. Furthermore, at 21 DAI *in-vitro* activities and transcript abundance of key enzymes of the C and N metabolism in nodules were determined. Moreover, nodulation in cluster root zones was evaluated.
• *Key Results* Treatment without P led to a lower P concentration in shoots, roots, and nodules. In both treatments, with or without P, the P concentration in nodules was greater than that in the other organs. At 21 DAI nitrogen fixation rates did not differ between treatments and the plants displayed no symptoms of P or N deficiency on their shoots. Although nodule number at 21 DAI increased in response to P-deficiency, total nodule mass remained constant. Increased nodule number in P-deficient plants was associated with cluster root formation. A higher root/nodule CO_2 fixation in the treatment without P led to a lower net CO_2 release per unit fixed N, although the total CO_2 released per unit fixed N was higher in the treatment without P. The higher CO_2 fixation was correlated with increased transcript abundance and enzyme activities of phosphoenolpyruvate carboxylase and malate dehydrogenase in nodules. Between 21 and 37 DAI, shoots of plants grown without P developed symptoms of N- and P-deficiency. By 37 DAI the P concentration had decreased in all organs of the plants treated with no P. At 37 DAI, nitrogen fixation in the treatment without P had almost ceased.
• *Conclusions* Enhanced nodulation in cluster root zones and increased potential for organic acid production in root nodules appear to contribute to white lupin's resilience to P-deficiency.

Key words: White lupin, *Lupinus albus*, nitrogen fixation, P deficiency, H_2 evolution, $^{15}N_2$ uptake.

INTRODUCTION

White lupin (*Lupinus albus*) has evolved elegant adaptations for growth under P-deficient soil conditions (Dinkelaker *et al*., 1995; Keerthisinghe *et al*., 1998; Watt and Evans, 1999; Neumann and Martinoia, 2002). Rather than forming a mycorrhizal symbiosis, white lupin has a highly synchronous, co-ordinated expression of genes which results in proliferation of cluster roots, root exudation of organic acids and acid phosphatase, as well as the induction of numerous transporters (Gilbert *et al*., 1999; Neumann and Martinoia, 2002; Vance *et al*., 2003; Uhde-Stone *et al*., 2003*a*). The exudation of organic acids and acid phosphatase solubilize bound forms of P thus increasing the availability of P and micronutrients in cluster root zones (Lamont, 2003). Moreover, the formation of cluster roots results in a striking increase in root surface area, thereby providing enhanced zones for P uptake (Dinkelaker *et al*., 1989, 1995; Gerke *et al*., 1994; Neumann *et al*., 1999; Lamont, 2003).

In agricultural systems, white lupin is readily nodulated by *Bradyrhizobium lupini* and forms effective nodules. Thus, it is surprising that there are few if any studies of white lupin nodulation and N_2 fixation when grown in low P environments. Symbiotic nitrogen fixation has a high P demand because the process consumes large amounts of energy (Schulze *et al*., 1999), and energy-generating metabolism strongly depends upon the availability of P (Israel, 1987; Plaxton, 2004). Several reports have documented that nodules are a strong P sink and nodule P concentration normally exceeds that of roots and shoots (Sa and Israel, 1991; Drevon and Hartwig, 1997). Legumes frequently respond to mild P-stress through an increase in nodule number but nodules generally are smaller. Mechanistically, the increase in nodule number under low P has been linked to increasing nodule surface area and thereby facilitating oxygen diffusion into the

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nodule (Ribet and Drevon, 1995). Oxygen diffusion into and availability in the nodule-infected cell zone is regarded as a primary regulatory factor for effective N_2 fixation (Layzell *et al*., 1990) and, as such, is subject to tight regulation (Minchin, 1997; Denison, 1998). Oxygen diffusion into soybean (Ribet and Drevon, 1995), common bean (Vadez *et al*., 1996) and alfalfa (Schulze and Drevon, 2005) root nodules is facilitated under P-stress conditions. Oxygen diffusion is thought to be facilitated in these species through a change in nodule cortical cell size mediated by osmoregulatory reactions (Drevon *et al*., 1998). By contrast, regulation of oxygen diffusion in white lupin nodules appears to be brought about by occlusion of nodule cortical-cell free space (De Lorenzo *et al*., 1993; Iannetta *et al*., 1993).

It is noteworthy that the plant enzymes phosphoenolpyruvate carboxylase (PEPC; EC 4·1·1·31) and malate dehydrogenase (MDH; EC 1·1·82) play a key role in carbon metabolism of both root nodules and P-deficiency-induced cluster roots. In root nodules, PEPC and MDH are integral enzymes in providing carbon in the form of organic acids for rhizobial bacteroids in infected cells (Rosendahl *et al*., 1990). Moreover, PEPC and MDH in nodules have been implicated in osmoregulation of the nodule variable-oxygen diffusion barrier (Miller *et al*., 1998). In P-deficiency-induced cluster roots, expression of PEPC and MDH is increased and a portion of the carbon skeleton of the organic acids exuded from cluster roots is derived from CO_2 fixed by PEPC (Johnson *et al*., 1994, 1996; Uhde-Stone *et al*., 2003*a*). Whether root nodules on P-deficient white lupin show any change in expression of PEPC and MDH is not known. Neither is it known whether a relationship exists between cluster root development and root nodule formation in P-stressed white lupin. The objectives of this study were (*a*) to determine the nodulation profile of white lupin grown under P-stress conditions; (*b*) to assess if nodule PEPC and MDH were affected by P-stress; and (*c*) to determine how P-stress affects white lupin nodule physiology.

MATERIALS AND METHODS

Growth of plant material

Lupinus albus L. 'Ultra' plants were grown in a growth chamber at 20/15 °C with 16-h/8-h day/night cycles. Photosynthetic active radiation was approx. 210 µmol m^{-2} s^{-1} at plant height. Seeds were surface sterilized by placing them for 20 min in H_2O_2. Subsequently, they were rinsed with sterile water and planted in pots (100 × 100 mm) (four plants per pot singled out to two plants per pot after emergence) containing 1 mm diameter silica sand. Plants were watered daily with 200–500 mL of the appropriate nutrient solution. The base nutrient solution consisted of 2 mM MES [2-(*N*-morpholino) ethane-sulfonic acid] buffer, 1 mM $MgSO_4$, 3 mM K_2SO_4, 0·95 mM $CaCl_2$, 12 µM Fe (as FeEDTA), 4 µM $MnCl_2$, 22 µM H_3BO_3, 0·4 µM $ZnSO_4$, 0·05 µM $NaMoO_4$ and 1·6 µM $CuSO_4$. Two treatments were grown, one in which phosphate as $Ca(H_2PO_4)_2$ was added to the nutrient solution to a concentration of 0·5 mM (+P) and a second without phosphate (−P). The appropriate amount of $CaSO_4$ was added to the −P nutrient solution to ensure equal calcium concentrations. All nutrient solutions were nitrogen free.

Bradyrhizobium lupini (WU 425) was grown in YEM at 28 °C for 1 week. The strain has no uptake hydrogenase (Layzell *et al*., 1979; Murillo *et al*., 1989). Plants were inoculated twice at 4 and 6 d after emergence (DAE) with the application of 1 mL of inoculum at the stem of each plant. First nodules appeared 5 d after inoculation. An uninoculated control remained nodule free.

On a separate set of plants grown as described above, nodules were classified into those in or adjacent (10 mm) to proteoid (cluster) root zones and those on normal roots. In addition to the +P and −P treatments, nodules from plants which had been additionally treated with the synthetic auxin naphthalene acetic acid (NAA) or the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) were counted and classified. The application of NAA and TIBA followed the procedure outlined in Gilbert *et al*. (2000).

$^{15}N_2$ uptake and H_2- and CO_2-evolution

At 19–21 d after inoculation (DAI) (subsequently referred to as 21 DAI) and 35–37 DAI (subsequently referred to as 37 DAI), plants were carefully removed from the silica sand. Due to its large grain diameter, the sand could be removed easily without damaging roots. The plants were transferred to 50 mL (21 DAI) or 150 mL (37 DAI) darkened glass containers. The stem base was carefully placed in a hole (diameter 5 mm) through a rubber stopper. The rubber stopper had been sliced to allow the stem to be placed inside the hole. After sealing the cut as well as the stem against the rubber stopper with non-plant toxic silicon rubber, the root/nodule system was placed inside the container and the container was closed. An inlet and outlet tube to the container was placed through the rubber stopper and 25 mL (21 DAI) or 75 mL (37 DAI) of the appropriate nutrient solution were added. An airflow of two times the container volume min^{-1} was connected to the inlet and outlet for 24 h, sealing the root/nodule container to allow the plants to adapt. The airflow was bubbled through water before entering the root compartment.

For measuring $^{15}N_2$ uptake the container was filled with nutrient solution and the nutrient solution was then replaced by a $^{15}N_2$ (99 atom% $^{15}N_{exc}$)/O_2 (80/20) mixture. The root/nodule system was exposed to the mixture for 2 h, after which the labelled air was removed by water and the plants were immediately harvested. Nodules were detached, counted, weighed, then macerated in a mortar and pestle and, along with shoots and roots, immediately dried to a constant weight at 70 °C.

For measuring root/nodule H_2 and CO_2 evolution the container was connected to an airflow (2 vols min^{-1}) from a gas blender (80 % N_2/20 % O_2). The out-flowing air was analysed for H_2 and CO_2 with a flow-through H_2 analyser and a flow-through CO_2 analyser. Apparent nitrogenase activity (ANA) was measured as H_2 evolution in an N_2/O_2

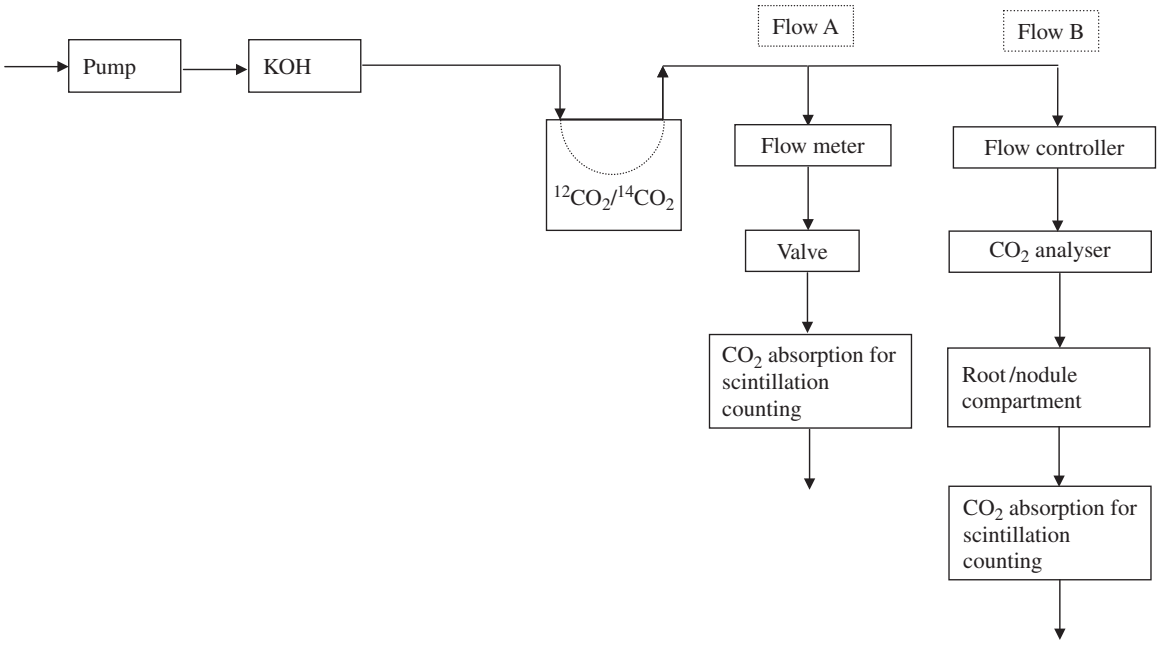

FIG. 1. Flow scheme of an experimental set-up for measuring short-term root/nodule CO_2 fixation. Further explanations are given in the text.

(80/20) mixture while total nitrogenase activity (TNA) was defined as the peak value of H_2 evolution after switching to an Ar/O_2 (80/20) mixture (Blumenthal *et al*., 1997). The maximum value was reached about 10–12 min after replacing N_2 by Ar. CO_2 evolution was measured in an N_2/O_2 (80/20) mixture.

Calculation of nitrogen fixation and electron allocation from ANA, TNA and $^{15}N_2$ uptake

The process of nitrogen fixation can be described by the following equations:

$$N_2 + 8e^- + 10H^+ + 16ATP \rightarrow 2NH_4^+ + H_2 + 16ADP + 16P_i \quad (1)$$

H^+ is reduced concomitantly with N_2 on which the measurement of ANA is based. If N_2 is replaced by Ar, all electrons will be directed towards H^+ reduction and the resulting H_2 evolution represents TNA. In a N_2/O_2 mixture the electron allocation between N_2 and H^+ can vary. The electron allocation coefficient (EAC) is defined as:

$$EAC_{H2} = 1 - ANA/TNA \quad (2)$$

If the process follows eqns (1) and (2), the EAC would be 0·75 ($1-2e^-/8e^-$) and in this case 1 mol N_2 would be reduced (fixed) with every mol H_2 evolving (see eqns 1 and 2).

To calculate the amount of N_2 reduced from an ANA and TNA value, the number of mol H_2 evolving along with 1 mol N_2 was calculated as follows:

$$x/(6 + x) = 1 - EAC_{H2} \quad (3)$$

with x = mol e^- flowing onto H^+ along with the reduction of 1 mol N_2.

Accordingly, the mol H_2 evolving (y) along with the reduction of 1 mol N_2 would be:

$$y = 1/2x \quad (4)$$

The ANA value in mol H_2 divided by y consequently results in mol N_2 reduced (fixed).

The amount of N fixed was calculated from the $^{15}N_2$ uptake as:

$$\text{N fixed} = \frac{\text{total N in plant} \times \text{atom \%}^{15}N_{exc} \text{ in total plant N}}{\text{atom \% } ^{15}N_{exc} \text{ in } N_2 \text{ of the } N_2/O_2 \text{ mixture}} \quad (5)$$

To calculate the EAC_{15N} from $^{15}N_2$ uptake and ANA the following equation was used:

$$EAC_{15N} = 1 - \frac{(ANA \times 2)}{(\text{mol } N_2 \text{ fixed[based on } ^{15}N_2 \text{ uptake]} \times 6) + (ANA \times 2)} \quad (6)$$

Root/nodule CO_2 fixation

Root/nodule CO_2 fixation was measured at 21 DAI on a separate set of plants grown under similar conditions. The experimental set-up is outlined in Fig. 1. A constant, CO_2-free airflow was directed through a CO_2-permeable silicon rubber tubing located in an airtight mixing chamber. The chamber was filled with a $^{12}CO_2/^{14}CO_2$ mixture. Depending upon the diameter and length of the tubing and the airflow rate, a constant rate of $^{12}CO_2/^{14}CO_2$ mixing was achieved. The airflow emerging from the mixing chamber was split into two streams. The stream designated Flow A was passed directly through a scintillation fluid that absorbs CO_2 and counted. The stream designated Flow B was passed sequentially through a CO_2 analyser, the compartment containing the nodulated root, and then

through scintillation fluid for CO_2 capture and counting. The flow control and valve system allowed the airflow to be adjusted so that it contained 400 ppm CO_2 (CO_2 analyser) and the airflow rate through the root/nodule container was 1 × the container volume min^{-1}.

The specific activity of the CO_2 (Bq mol^{-1} CO_2) in the airflow could be calculated from the CO_2 content (CO_2 analyser), the radioactivity collected in scintillation fluid, and the flow rate of Flow A. The root/nodule system was exposed to the $^{12}CO_2/^{14}CO_2$-containing air for 30 min. The plants were immediately frozen in liquid nitrogen, lyophilized, and the ^{14}C determined after oxidization and subsequent liquid scintillation counting. The amount of fixed CO_2 (a) was calculated as:

$$a = \frac{\text{total radioactivity in the plant (Bq)}}{\text{specific activity of the } CO_2 \text{ in the airflow } \left(\text{Bq mol}^{-1}\text{CO}_2\right)} \quad (7)$$

Enzyme activities

For enzyme assays, nodules were harvested at 21 DAI and kept on ice (10–30 min) until extracted. Approximately 100 mg fresh weight nodules were extracted as previously described (Egli *et al*., 1989). Total protein in the extract was then determined using Bradford's reagents. PEPC (EC 4·1·1·31), MDH (EC 1·1·1·37), glutamate synthase (GOGAT) (1·4·1·13) and aspartate aminotransferase (AAT) (EC 2·6·1·1) activity was determined by measuring the disappearance of NADH at A_{340}, using protocols previously described (Egli *et al*., 1989).

RNA gel blot

Nodule RNA extraction, gel separation, blotting and analysis of PEPC and MDH transcript abundance was performed following the procedures outlined in detail in Uhde-Stone *et al*. (2003*b*). Equal loading of RNA in each lane was assessed by staining with ethidium bromide.

N and ^{15}N analyses

The dried plant material was weighed and ground to a fine powder. C, N and ^{15}N in the ground material was measured with a combination of a C/N analyser (vario EL Elementaranalysensysteme Hanau) and a ^{15}N emission spectrometer (NOI 7, Fischer Analysen Instrumente, Leipzig).

Statistics

Unless otherwise noted, data were subjected to analyses of variance and the mean values were compared with the *t*-test, if applicable.

RESULTS

Plant growth, N and P content and nodulation

As can be seen in Table 1, at 21 DAI plants grown under P-deficient conditions did not differ in dry matter (DM) accumulation, % N, total N or root : shoot ratio compared with plants grown under P-sufficient conditions. There was, however, a striking difference in P concentration and total plant P with plants grown under P-deficiency conditions having significantly less P than those grown under P-sufficient conditions. Notably, nodules had a higher P concentration than either shoots or roots at 21 DAI. Plants grown under P-deficient conditions at 21 DAI had significantly more nodules than plants grown under P-sufficient conditions, but the nodules on P-deficient plants were smaller than those on P-sufficient plants (Table 2). Nodules in the −P treatment grew predominantly on lateral roots in or in the vicinity of proteoid root clusters (Figs. 2 and 3), while in the +P treat ment most nodules were found on the primary root (Fig. 4).

By comparison, at 37 DAI plants grown under P-deficient conditions were strikingly reduced in shoot and nodule DM accumulation (Table 3). However, root DM accumulation did not differ resulting in a greater root : shoot ratio for −P plants. The N concentration and total N of −P plants was significantly less than that of +P plants. It was notable again that nodules had a greater N and P concentration than either leaves or roots. Similarly to N, at 37 DAI, P concentration and total P were reduced in −P plants as compared to +P plants. Nodule number and weight were also reduced on −P plants. Total plant P in the −P treatment at both 21 and 37 DAI corresponded to the amount of P that could on average be found in one seed (1·89 ± 0·31 mg P g DM^{-1}, n = 10).

NITROGEN FIXATION

Nitrogen fixation per unit nodule dry matter as measured by $^{15}N_2$ uptake did not differ between both treatments at 21 DAI. However, at 37 DAI $^{15}N_2$ uptake was lower in the +P treatment as compared with +P at 21 DAI, and drastically reduced in the −P treatment (Table 4). This pattern was confirmed by nodule H_2 evolution, even though the total amount of N_2 fixed calculated on the basis of H_2 evolution is lower compared to that based on $^{15}N_2$ uptake (Table 4). This holds true with the exception of −P at 37 DAI where nitrogen fixation had almost ceased. The lower data for nitrogen fixation calculated from H_2 evolution results from a lower EAC measured on the basis of ANA and TNA when compared with the EAC calculated from ANA and $^{15}N_2$ uptake.

At 21 DAI the −P plants had a significantly higher EAC_{H2} compared with the +P plants, while based on $^{15}N_2$ uptake, there was no significant difference in EAC_{15N}. Nitrogen fixation per nodule, which was significantly lower in the −P treatment at 21 DAI, was compensated for on a whole-plant basis by the higher nodule number. In terms of P efficiency, the −P treatment fixed the same amount of nitrogen with much less P, both on a plant total-P as well as a nodule-P basis (Table 4).

ROOT/NODULE CO_2 EVOLUTION AND FIXATION AND ENZYME ACTIVITIES

As shown in Table 5, total nodule/root CO_2 evolution at 21 DAI was fairly comparable in −P and +P plants despite the fact that net nodule/root CO_2 evolution was significantly

TABLE 1. *Dry matter (DM) accumulation and N- and P-assimilation of white lupin plants grown in silica sand with +P or −P nutrient solution at 21 DAI*

	DM (mg)		% N		Total N (mg)		P-concentration (mg P g^{-1} DM)		Total P (mg)	
	+P	−P	+P	−P	+P	−P	+P	−P	+P	−P
Shoot	446	444	3·91	3·98	17·4	17·6	8·31	3·05*	3·68	1·36*
Root	193	206	1·94	1·92	3·7	3·9	8·72	2·58*	1·68	0·54*
Nodules	13·5	12·7	5·95	5·34	0·86	0·69	12·3	6·53*	0·16	0·08*
Total plant	652	663	–	–	21·9	22·3	–	–	5·51	1·98*

Data are means of six replicates.
* Significantly different from the +P treatment, *t*-test ($P \leq 0{\cdot}05$).

TABLE 2. *Nodule number and dry matter of white lupin plants grown in silica sand with +P or −P nutrient solution*

	21 DAI		37 DAI	
	+P	−P	+P	−P
Nodule number	36	55*	97	80*
Nodule dry matter (mg)	13·5	12·7	80·8	47·3*
Nodule dry matter/nodule number (mg)	0·37	0·23*	0·83	0·59*

Data are means of six replicates.
* Significantly different from the +P treatment, *t*-test ($P \leq 0{\cdot}05$).

less in −P plants as compared with +P plants. The much greater CO_2 fixation rate of −P nodules/roots offset the lower net CO_2 evolution of −P plants. Thus, total CO_2 evolution per N fixed was fairly equal in +P and −P plants. By 37 DAI net CO_2 evolution remained high in +P roots/nodules but decreased in −P roots/nodules. Net CO_2 evolution per N fixed at 37 DAI in −P plants was 10-fold that in +P plants, reflecting the continuing respiration of −P roots/nodules but little accompanying N_2 fixation.

Measurements of MDH and PEPC enzyme activity and gene transcript at 21 DAI reflect the root/nodule *in-vivo* CO_2 fixation. Both enzyme activities and transcripts for PEPC and MDH (Figs. 5 and 6) and AAT enzyme activity (Fig. 5) increased more in −P nodules as compared with +P nodules. By comparison, *in vitro* enzyme activity for NADH-GOGAT did not increase (Fig. 5). Probing of the same blot with radiolabel showed the increase in PEPC and MDH transcript was not an artefact of RNA loading.

DISCUSSION

The understanding of P-stress on nodulation and N_2-fixation has been extended by showing (*a*) white lupin nodulation and continuing N_2-fixation is extremely tolerant of insufficient P for a comparatively extended time in its growth cycle (21–27 DAE); (*b*) individual nodule dry mass accumulation is more sensitive to P-deficiency than either nodule number or total nodule mass per plant; (*c*) the P-concentration threshold for nodule function is much higher than that for leaves or roots; and (*d*) P stress induces nodule enzyme activities of PEPC and MDH, two key enzymes involved in carbon cycling and energy substrates for N_2 fixation.

Remarkably, nodulated white lupin shoots did not show any symptoms of nutrient stress when grown in the presence of N- and P-free medium for a period as long as 27 DAE. Although seeds provided some P, the −P treatment led to markedly low P concentrations by 21 DAI but N concentration and total N remained unaffected. Because −P plants were grown under symbiotic conditions, nodulated by *Bradyrhizobium* and fixing N_2, these results clearly show that white lupin is well adapted for growth under P-deficiency stress conditions. In fact, at 21 DAI, N_2-fixation measured as both $^{15}N_2$ uptake and H_2 evolution clearly demonstrate that total N_2 fixation in +P and −P plants is equivalent. This occurs even though N_2 fixed per nodule is less in −P plants because nodule number at 21 DAI is much greater in −P plants. Thus, total nodule mass is equivalent in −P and +P plants. At 37 DAI, this picture changes. As P deficiency becomes more pronounced and P deficiency symptoms are more evident N_2 fixation had almost ceased, as confirmed by $^{15}N_2$ uptake and H_2 evolution. It is quite apparent that a certain internal threshold of P concentration is required for lupin plants to continue optimal N_2 fixation and physiological function. This threshold lies between 3·05 and 1·12 mg P g DM^{-1} for shoots and 6·53−3·33 mg P g DM^{-1} for nodules. The existence of such thresholds is supported by observations relating legume dry matter accumulation to P content (Bell *et al*., 1990). Moreover, the shoot P threshold reported for clover, *Medicago* and soybean is approx. 2·7 mg P g DM^{-1} (Israel, 1987; Tang *et al*., 2001; Hoch-Jensen *et al*., 2002). By contrast, a shoot P concentration of 2·5 mg P g DM^{-1} resulted in a 50 % dry matter reduction in common bean (Olivera *et al*., 2004).

Similar to legume nodules in other studies (Jakobson, 1985; Israel, 1987; Hoch-Jensen *et al*., 2002), lupin nodules contained a greater P concentration than other tissues. Whether the difference is due to selective partitioning of P to nodules or P uptake by nodules is not clear. A particularly high demand of the bacterial part of the nodule might also play a role. It is very apparent that the nodule P concentration of 6·5 mg P g DM^{-1} found at 21 DAI in plants grown without P supported N_2 fixation rates equal to that of plants receiving P fertilization and having a nodule P concentration of 12·3 mg P g DM^{-1}. However, by 37

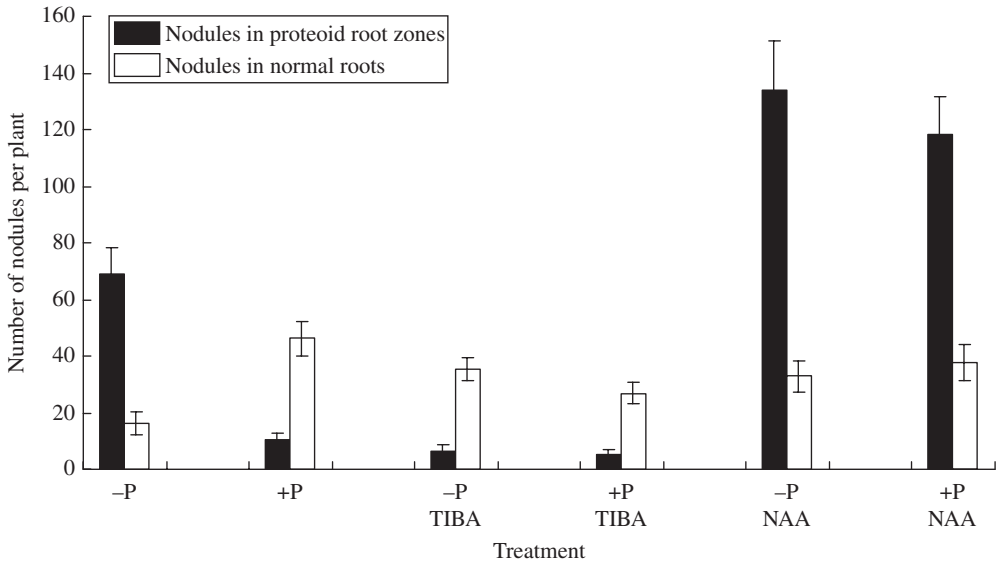

FIG. 2. Nodulation in cluster root zones of P-deficient white lupin treated with either TIBA or NAA. Black columns show nodules in proteoid (cluster) root zones. White columns show nodules in normal root zones. Data are means of nine replicates. Error bars indicate the s.e.m.

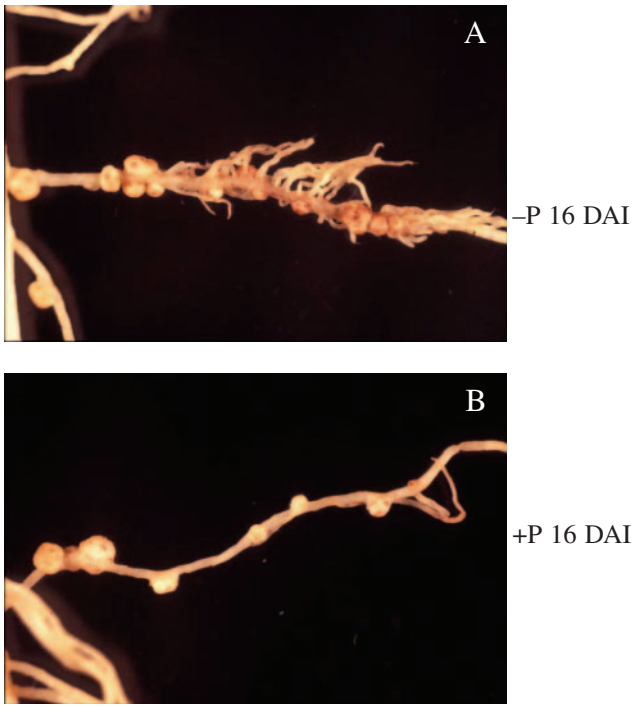

FIG. 3. Nodules of white lupin plants in proteoid (cluster) root zones and on normal root segments at 16 d after inoculation (DAI).

DAI the P concentration of nodules from plants receiving no P had decreased to 3·3 mg P g DM^{-1} accompanied by a severe impact on nodule N_2 fixation. In *Medicago truncatula* (Tang *et al*., 2001) nodule functioning was fairly efficient when nodule P concentration was 4–5 mg P g DM^{-1}. Similar nodule P concentrations support adequate N_2 fixation in pea and soybean.

Phosphorus stress had a greater impact on shoot growth as compared with that of root growth. In fact, root dry matter accumulation was comparable in P-stressed and P-sufficient plants. Shoot dry matter accumulation increased 3-fold in P-stressed plants between 21 and 37 DAI as compared with the 4·2-fold increase in P-sufficient plants. Thus, roots seem to have a more pliable response to P deficiency than shoots.

The present observations with white lupins appear to support the view that phosphorus deficiency affects nitrogen fixation through secondary effects rather than direct involvement of P in nitrogenase functioning (Jakobson, 1985; Israel, 1987; Ribet and Drevon, 1995). However, this is at most an indirect indication and the question can only be adequately addressed when P deficiency is experimentally achieved through continuous low supply, which would more readily reflect the conditions in a soil with low P availability. Nevertheless, up to 21–27 DAI the plants showed clear adaptative features, comprising both morphological and physiological phenomena.

Consistent with previous reports for other legumes, it was found that mild P stress in white lupin leads to the formation of more and smaller nodules (Ribet and Drevon, 1995), i.e. individual nodule mass is initially more affected by emerging P-deficiency than nodule number or nodule dry matter per plant. Additionally, in white lupin, these nodules seem to be located in the vicinity of cluster (proteoid) root zones where P uptake is presumably highest, especially at low soil P. In separate experiments, nodule number and cluster root formation were evaluated in response to P-stress and auxin treatment (Fig. 2). Consistent with earlier experiments (Tables 1 and 2) nodule number increased in response to P-deficiency and the greatest number of nodules was associated with cluster root

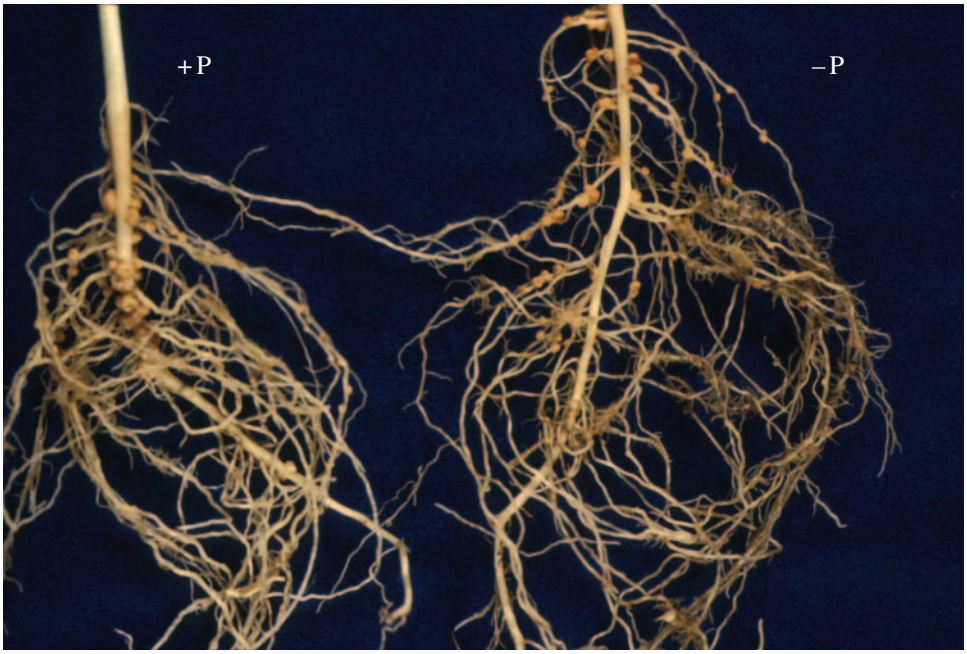

FIG. 4. Nodulated root of white lupin at 21 d after inoculation grown with +P or −P nutrient solution.

TABLE 3. *Dry matter (DM) accumulation and N- and P-assimilation of white lupin plants grown in silica sand with +P or −P nutrient solution at 37 DAI*

	DM (mg)		% N		Total N (mg)		P-concentration (mg P g^{-1} DM)		Total P (mg)	
	+P	−P	+P	−P	+P	−P	+P	−P	+P	−P
Shoot	1884	1325*	3·22	2·16*	60·7	28·6*	8·3	1·2*	15·6	1·6*
Root	378	376	1·53	0·56*	5·8	2·1*	10	1·1*	3·8	0·4*
Nodules	80·8	47·3*	5·69	4·15*	4·6	1·9*	13·2	3·3*	1·1	0·2*
Total plant	2342	1748*	–	–	71·1	32·7*	–	–	20·4	2·1*

Data are means of six replicates.
* Significantly different from the +P treatment, *t*-test ($P \leq 0{\cdot}05$).

TABLE 4. *N_2 fixation and H_2 evolution of white lupin plants grown in silica sand with +P or −P nutrient solution*

	21 DAI		37 DAI	
	+P	−P	+P	−P
N fixed based on $^{15}N_2$ uptake [nmol N (mg DM nodule)$^{-1}$ h^{-1}]	75·7	73·6	50·2	2·34*
N fixed based on H_2 evolution [nmol N (mg DM nodule)$^{-1}$ h^{-1}]	58·6	65·2	40·52	3·89*
H_2 evolution [nmol H_2 (mg DM nodule)$^{-1}$ h^{-1}]				
ANA	61	55	44	15*
TNA	149	153	107	21*
EAC	0·59	0·64*	0·58	0·28*
EAC based on $^{15}N_2$ uptake and H_2 evolution (ANA)	0·65	0·67	0·63	0·19*
N fixed ($^{15}N_2$ uptake) nodule^{-1} [nmol N nodule^{-1} h^{-1}]	50·29	34·58*	83·84	2·76*
N fixed ($^{15}N_2$ uptake) total P^{-1} [nmol N (mg P in plant)$^{-1}$ h^{-1}]	235·9	321·1*	400·4	105·34*
N fixed ($^{15}N_2$ uptake) nodule P^{-1} [µmol N (total P in nodules)$^{-1}$ h^{-1}]	12·26	23·23*	7·58	1·85*

Data are means of six replicates.
ANA, Apparent nitrogenase activity; TNA, total nitrogenase activity; EAC, electron allocation coefficient.
* Significantly different from the +P treatment, *t*-test ($P \leq 0{\cdot}05$).

TABLE 5. *CO_2 evolution and CO_2 fixation of nodules and roots of white lupin plants grown in silica sand with +P or −P nutrient solution*

	21 DAI		37 DAI	
	+P	−P	+P	−P
Net CO_2 evolution				
[nmol CO_2 (mg DM nodule + root)$^{-1}$ h^{-1}]	187	127*	157	75*
[μmol CO_2 plant^{-1} h^{-1}]	38·5	27·2*	71·9	31·7*
CO_2 fixation				
[nmol CO_2 (mg DM nodule + root)$^{-1}$ h^{-1}]	45	87*	n.d.	n.d.
Total CO_2 evolution				
[nmol CO_2 (mg DM nodule + root)$^{-1}$ h^{-1}]	232	214*	n.d.	n.d.
[μmol CO_2 plant^{-1} h^{-1}]	47·8	46·8	n.d.	n.d.
Net CO_2 evolution/N fixed ($^{15}N_2$ uptake)				
[μmol CO_2 (mol N fixed)$^{-1}$ plant^{-1} h^{-1}]	37·21	29·73*	17·7	173·2*
Total CO_2 evolution/N fixed ($^{15}N_2$ uptake)				
[μmol CO_2 (μmol N fixed)$^{-1}$ plant^{-1} h^{-1}]	46·8	50·1	n.d.	n.d.

Data are means of six replicates.
* Significantly different from the +P treatment, *t*-test ($P \leq 0{\cdot}05$).

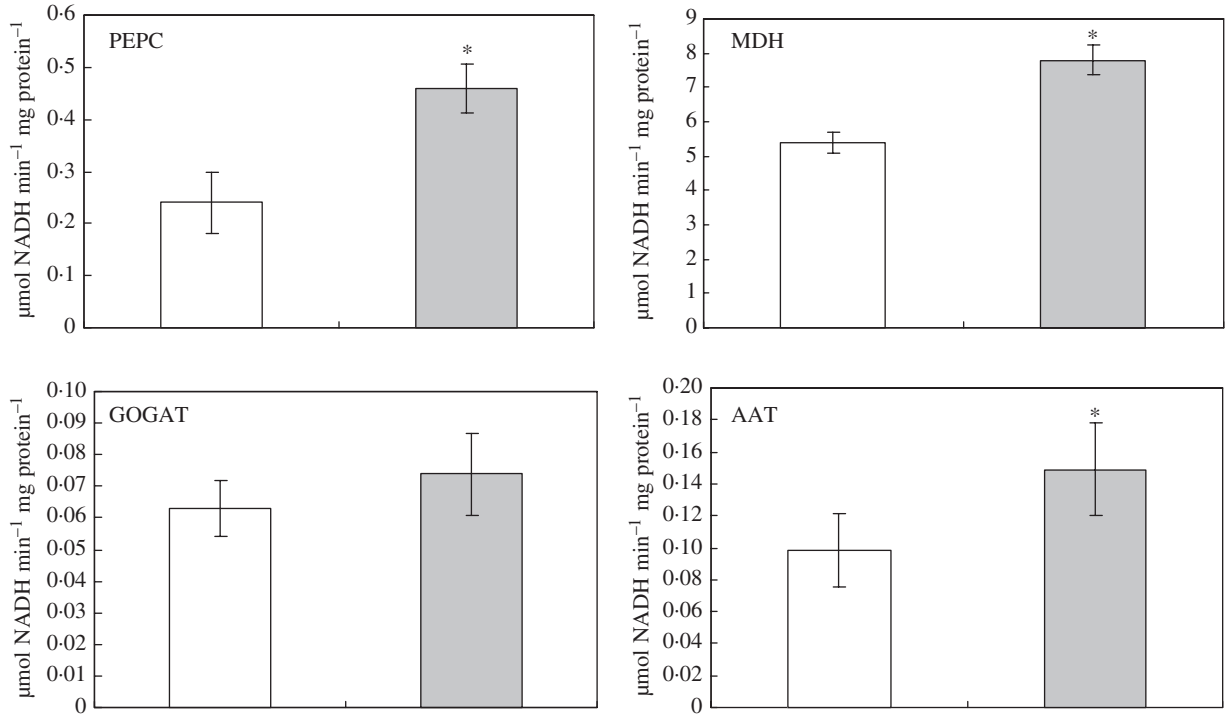

FIG. 5. *In-vitro* activity of key enzymes of the C- and N-metabolism in nodules of white lupin grown with +P or −P nutrient solution at 21 d after inoculation. Mean values of six replicates. * Significantly different to the +P treatment, *t*-test ($P \leq 0{\cdot}05$).

zones. When nodule number was calculated on the basis of cluster root weight, P-deficient plants had 3-fold more nodules per gram of cluster root than P-sufficient plants (54 nodules g^{-1} cluster root *vs*. 17 nodules g^{-1} cluster root). In addition, an auxin analogue NAA stimulated cluster root formation and nodule development in cluster root zones. In comparison, TIBA an auxin transport inhibitor, inhibited cluster root formation and nodule development in cluster root zones. The effects of NAA and TIBA on cluster roots were independent of P, similar to the findings of Gilbert *et al*. (2000). These results show that cluster root zones appear to be more susceptible to nodulation than normal roots. Whether an increased susceptibility to nodulation in cluster root zones is due to exudation of nodulation signals or the prolific emergence of root and root hairs combined with enhanced organic acid synthesis is worthy of study. The altered location of nodules under P stress indicates that tissue P concentration might be involved in regulation of nodule initiation (Almeida *et al*., 2000), although the underlying mechanisms are unclear. Recent

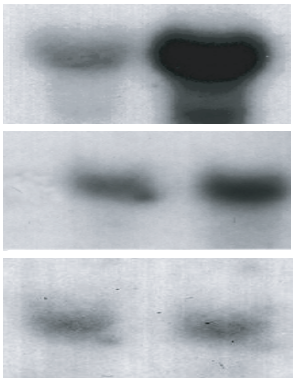

FIG. 6. Gel blot analysis of RNA isolated from nodules of +P- and −P-treated white lupin. LaPEPC1 cDNA was used as a probe in the left panel while in the right panel LaMDH1 was used as a probe. Plants grown in presence of sufficient P are designated by (+) and those grown under P stress are designated by (–).

studies (Liu *et al*., 2005) have shown that the expression of P stress-induced genes are activated in nodules of plants grown with adequate P, suggesting that nodules may frequently be P-limited even under adequate fertilizer.

P-deficiency stress is accompanied by increased respiration per fixed N_2 in nodules (Schulze and Drevon, 2005) which may result in a carbon shortage in nodules of −P plants. This could become more severe as photosynthesis is impaired due to reduced phosphorylation processes in shoots (Foyer and Spencer, 1986). With respect to nitrogen fixation white lupin plants reacted to any potential carbon shortage in the −P treatment in two ways. First, the EAC was slightly increased, leading to a more (carbon) efficient nitrogen fixation in the first half of the growth cycle. Secondly, PEPC and MDH expression and activity were strongly increased, with the result of a nearly doubled root/nodule CO_2 fixation. Although the measurement of CO_2 fixation, based on applied $^{14}CO_2$, will most probably underestimate the actual fixation, in the present case that would even increase the difference between the treatments. Nodule CO_2 fixation has been shown to be closely connected to nitrogen fixation activity (Schulze *et al*., 1998) and nodule organic acid production is central for functioning nitrogen fixation (Vance, 1998). In addition, CO_2 fixation by cluster roots contributes a significant amount of carbon for organic acid synthesis (Johnson *et al*., 1994, 1996). Due to the strong CO_2 uptake in the −P treatment, a lower net CO_2 release was observed, although the CO_2 release per unit fixed N was higher (Table 5). Thus, in fact, a shift towards the PEPC-MDH pathway could result in greater carbon efficiency of N assimilation when the recovered carbon is used for asparagine formation and N-transport. AAT activity was increased, possibly leading to a higher portion of C derived from CO_2-fixation in the N-exporting compounds and maybe a increased portion of asparagine versus glutamine as the N export compound resulting in a lower C : N ratio of the N-transporting compounds. Furthermore, if additional malate was produced, it could be used to feed the bacteroid respiration (Schulze *et al*., 2002). These combined effects may contribute to more efficient carbon fixation and assimilation of nitrogen in the −P treatment.

Finally, a further notable aspect of the present results is that in the system described here the TNA value based on H_2 evolution after a switch to Ar/O_2 seems to underestimate the actual total nitrogenase activity by about 20 % when compared with $^{15}N_2$ uptake. The reasons are not immediately apparent, yet could be various. The mechanism that brings about the argon-induced decline in nitrogenase activity (Witty *et al*., 1984) might have an immediate effect before the onset of the time-dependent decline, which is probably due to oxygen diffusion restriction (Schulze, 2004). Moreover, a certain unavoidable delay of every open-flow gas-exchange measurement system might somewhat moderate the H_2 evolution peak after switching to an Ar/O_2 mixture.

In conclusion, it has been shown that white lupin can maintain sufficient nitrogen fixation rates solely on the basis of seed P for as long as 3 weeks after nodulation. Adaptation is achieved by a shift to a more carbon efficient N assimilation and increased nodulation in cluster root zones. These characteristics add to the high adaptability of this species to a low P environment.

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