N-FEEDBACK REGULATION OF N_2 FIXATION IN *MEDICAGO TRUNCATULA* UNDER P-DEFICIENCY

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Summary. The objective of the present study was to determine a possible interconnection between a P-deficiency induced decline in nitrogen fixation of *Medicago truncatula* Gaertn. (barrel medic) cv. Jemalong and the accumulation of nitrogen or amino acids in different organs of the plants as a possible agent of a N-feedback mechanism. Nodulated plants were grown in N-free nutrient solution at increasing levels of P-concentration in the solution (0.2-5 µM P). Increased P-supply improved plant growth and nodule functioning. When nitrogenase activity (H_2-evolution) was measured at the 1 and 5 µM P-level, the activity was more than doubled at the 5 µM P supply. Coinciding with the improved nitrogenase activity, nodule number and plant P- and N-content improved with higher P-supply. With increasing P-supply the N-concentration in shoots and roots declined, while the C/N ratio decreased. In nodules the total amount of free amino acids was higher at low P and this was almost exclusively a result of higher asparagine concentrations. Thus, asparagine might be a substance that exerts N-feedback effects in nodules and it might be increasingly retranslocated to nodules via the phloem when the nitrogen content in the shoot is high as in our experiments under conditions of low P supply. Overall, the results are consistent with an effect of P-deficiency on nitrogen fixation that is mediated through an N-feedback mechanism.

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INTRODUCTION

Phosphorus (P) is one of the most important elements significantly affecting plant growth and metabolism. It is, along with N, a major yield-limiting nutrient in many regions of the world. The crop production on more than 30 % of the world arable land is limited by P availability, with the acid, weathered soils of the tropics and subtropics particularly prone to P deficiency (Tesfaye et al., 2007). Phosphorus may be a critical constraint of legumes under low-nutrient environments because there is a substantial need for P in the N_2 fixation process (Schulze et al., 2006; Tsvetkova and Georgiev, 2007). The high requirement for P in legumes is consistent with the involvement of P in the high rates of energy transfer that must take place in the nodule. Under P shortage conditions, legumes may lose the distinct advantage of an unlimited source of symbiotic N (Hartwig et al., 1996; Lüscher et al., 1996, 1998).

A low and limiting P supply eventually reduces plant growth and thus reduces N demand and N_2 fixation, but evidence concerning the regulating mechanisms is conflicting. It is unclear whether P has a direct or indirect effect on the N_2 fixation process (Hellsten and Huss-Danell, 2000; Vance et al., 2000). Some studies suggest that regulation takes place in the photosynthetic apparatus, thereby affecting the production and supply of non-structural carbohydrates to the nodules (Robson et al., 1981; Jakobsen, 1985; Sa and Israel, 1991; Gordon et al., 1997); others indicate that the regulation of N_2 fixation has a direct effect on nitrogenase activity in the nodules (Ribet and Drevon, 1995; Drevon and Hartwig, 1997; Almeida et al., 2000). At present there are three competing theories as how SNF is regulated under various environmental stresses (Schulze, 2004). In this report, we will address the involvement of the N-feedback theory as a master regulating mechanism for the rate of N_2 fixation under P-deficiency.

The concept of feedback regulation of N_2 fixation was developed in the last decade of the previous century as a general mechanism governing regulation of N_2 fixation by environmental factors (Parsons et al., 1993; Serraj et al., 1999; Schulze, 2004). This concept is a very common feature in biochemical pathways (Hartwig and Nösberger, 1994). Product feedback regulation is a widespread and effective principle particularly in biochemical

reaction chains. The importance of such a mechanism is strengthened if N_2 fixation in excess of the N demand not only leads to a waste of carbon, but to ammonium intoxication, because plants are hardly capable of storing excess nitrogen (Hartwig, 1998). It is accepted that P in conjunction with the plant demand for N controls the nodule growth and modulates the symbiotic processes of the legume and *Rhizobium* (Wall et al*.,* 2000; Hellsten and Huss-Danell, 2000; Le Roux et al., 2008). The hypothesis suggests that when the shoot receives more N than it can use in growth, some of this excess N 'spills over' into the phloem where it is translocated to the nodulated roots and interacts with nodule physiology so as to reduce N_2 fixation (Schulze, 2003, Fischinger et al., 2006). This mechanism has been thought to explain the reduction in nodulation and nodule growth in white clover (Almeida et al., 2000; Høgh-Jensen et al., 2002) and pigeonpea (Fujita et al., 2004), *Lotu*s (Keller et al., 2002), *Acacia* (Nguyen et al., 2006), alfalfa (Schulze and Drevon, 2005) under P-deficiency.

The objective of the present work was therefore to show whether there is a relation between P starvation and a possible N-feedback regulatory effect on nodules in the model legume *Medicago truncatula*. Nodulated *Medicago truncatula* plants were grown at different P levels and N_2 fixation and its relation to the accumulation of N in shoots and roots, and amino acids in nodules were studied.

MATERIALS AND METHODS

Plant growth conditions

Seeds of *Medicago truncatula* Gaertn: (barrel medic) cv. Jemalong A17 were chemically scarified in H_2SO_4 95 % for 6 min, surface sterilized with sodium hypochlorite 5 % for 3 min, cold-treated at 4 °C in water overnight, and then sown in quartz sand (Ø = 0.1 – 0.5 mm, 'Quarzsandwerke Weferlingen', Germany) and irrigated manually with normal water. Four plantlets were transferred (day 0) to 3 L plastic pots containing a vigorously aerated basal nutrient solution containing the following composition (µM): K_2SO_4, 700; $MgSO_4$, 500; $CaCl_2$, 800; H_3BO_3, 1.3; Na_2MoO_4, 0.03; $ZnSO_4$, 0.3; $MnCl_2$, 0.7; $CoCl_2$, 0.07; $CuCl_2$, 0.3 and FeNaEDTA (ferric

monosodium salt of ethylenediamine tetraacetic acid), 3.3. The pH was buffered with 0.2 mM MES [2-(*N*-morpholino) ethane-sulfonic acid]. A dense water suspension of *Sinorhizobium meliloti* 102F51 was added to the solution at a rate of approx 10^8 cells mL^{-1} two times and solutions were changed every third day. Inoculum was prepared by growing the bacteria in YEM at 28 °C for 4 d. The first nodules became visible about 10-14 days after the first inoculation. Urea (0.2 mM N) was added for the first 14 d after transplanting (DAT). Thereafter, the plants did not receive any external source of N. Solution pH was adjusted daily with KOH to 6.5. Plants were grown in a growth chamber with day/night temperatures of approx. 23/18 °C and a 16 h photoperiod and a relative humidity of about 70 %. During the light periods, photosynthetic active radiation was about 360 µmol $m^{-2}s^{-1}$ at plant height. The pots were arranged in a completely randomized block design with three replicates for each treatment. Phosphorus was applied at the following levels (µM P): 0.2, 0.5, 0.8, 1.0, 3.0 and 5.0. KH_2PO_4 was the source of P in the experiment. Plants were harvested at 52 DAT and were fractioned into shoots, roots and nodules. The nodule samples were frozen immediately in liquid nitrogen and stored at – 20 °C until analysis.

H_2 evolution measurements

A separate set of plants was grown with the according nutrient solution at two P levels (1 and 5 µM P) and otherwise comparable conditions. On the 76th day of growth the attached plant root part was transferred to a 150 ml glass bottle, which was darkened by aluminium foil. The lower end of the shoots was fixed to a rubber stopper with a whole. The nutrient solution was bubbled with 2 volumes 60 min^{-1} air. Prior to measurement, the root/nodule compartment was airtightly sealed with non plant toxic modelling clay. Hydrogen evolution from the nodules was measured in the outgoing air as described in Schulze et al. (1998).

Free amino acid analyses

Frozen nodule material was ground to a fine powder and homogenized with a mortar and pestle in liquid N. Around 0.5 mg of the material was

extracted with 3 ml 50 % (v/v) ethanol for 20 min at 50 °C water bath. The solution sample was subsequently centrifuged at 4 °C for 20 min at 8000 rpm (6810 g). The supernatant was immediately used for HPLC analyses after filtration (0.45 mm). Free amino acids concentration was detected with a fluorescence detector (Waters, Milford, MA, USA) after precolumn derivatization by ortho-phthaldialdehyde (Chen et al., 1979).

Nitrogen and phosphorus determinations

Plants were harvested and dried at 65 °C for 48 h. Dried material was ground to a fine powder and its N and P contents were measured. Nitrogen was determined in a C/N analyzer (Vario EL, Elementar GmbH, Hanau). For P, milled and dried plant material was digested in a microwave oven for 3 h with a mixture of concentrated HNO_3 and H_2O_2 (30 %) (4/2 v/v). Phosphate concentration was measured calorimetrically following the molybdenum-vanadate method (Scheffer and Pajenkamp, 1952).

Statistics

The data were subjected to one-way analysis of variance using ANOVA procedure of the Sigmastat analytical software. Mean separation procedures were carried out using the Tukey test.

RESULTS

Plant growth nitrogen fixation

Phosphorus deficiency reduced significantly plant dry weight because of its adverse effect on shoot and root growth (Fig. 1). By 52 DAT, plant growth increased faster with increasing solution P level. The dry weight of the shoots increased proportionally as the solution P level increased from 0.2 to 5.0 µM while for roots the dry weight reached its maximum at 3.0 µM and then decreased with increasing further the P level. A comparison of the apparent nitrogenase activity at 1 and 5 µM P supply revealed a significantly higher activity at 5 µM P supply after 76 days of growth at

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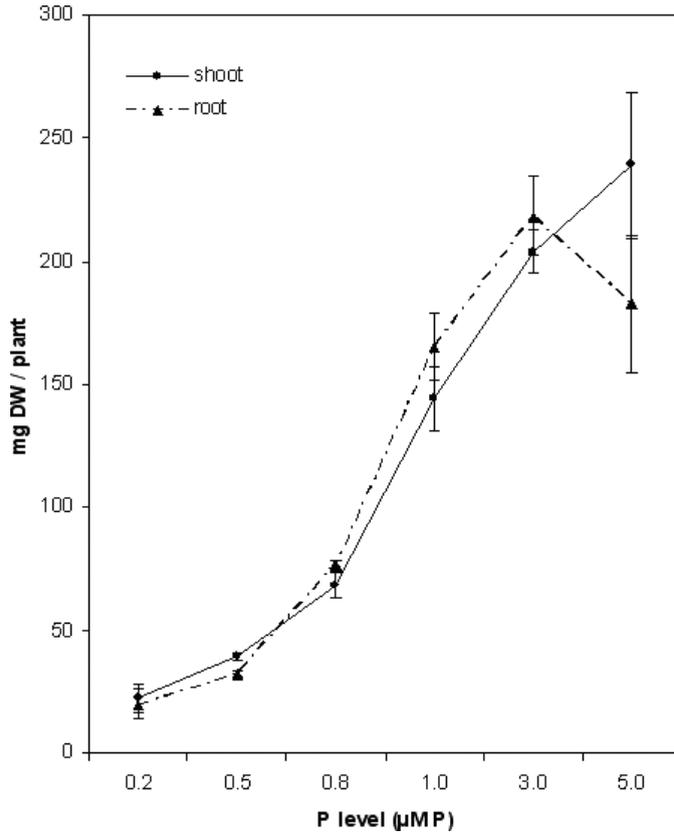
Fig. 1. Dry weights of shoot (■) and roots (▲) of Medicago truncatula grown at 0.2 -5.0 µM P for 52 d. The effect of P supply is significant at P≤0.001. Bars represent ± SE of three replicates.

the two P levels (Table 1). Nitrogenase activity per plant was more than doubled in the sufficiently P-supplied treatment.

Nodulation

Nodules were clearly visible on the roots between 8 and 11 DAT. Very low P concentrations (0.2, 0.5 and 0.8 µM P) in the nutrient solution prevented nodulation persistence from the beginning of the plant growth. Various

Table 1. Dry matter (DM), P-concentration and apparent nitrogenase activity of *Medicago truncatula* plants grown at 1 or 5 µM P in a nutrient solution. Measurements were done at 74-76 days of growth. Data are per plant as means of 6 replicates. * indicates a statistically significant difference at $P \leq 0.05$, (Tukey-test).

	Growth in nutrient solution at 1 µM concentration of P	Growth nutrient solution at 5 µM concentration of P
DM [mg]		
Shoot	210	336*
Root	170	327*
Nodules	2.1	4.3*
P-concentration [mgP mgDM^{-1}]		
Shoot	0.14	1.23*
Root	n.d.	n.d.
Nodules	0.28	0.43*
Apparent nitrogenase activity [µmol H_2-evolution plant^{-1} and h^{-1}]	1.7	4.2*

nodule initials were noticed on the root surface of the plants grown under such P levels. One µM P was found to be sufficient to establish nodulation and the number of nodules tended to increase with increasing P supply (Table 2). Nodule fresh weight was markedly increased with increasing P supply and reached the maximum at 5 µM P (Table 2). Compared to 1 µM P, the supply of 5 µM P increased the nodule fresh biomass 6.4-fold on the 52nd DAT.

Nitrogen and phosphorus concentration

As expected, P content in plant tissues increased as P concentration in the nutrient solution increased (Fig. 2). P content was much higher in roots than in shoots at all P levels except for 5.0 µM P where shoot P exceeded the content in the roots. Generally N content in shoots and roots increased with increasing P supply in the nutrient solution (Fig. 3). Plants grown at

Table 2. Nodule number and fresh weight by N_2-fixing plants of *Medicago truncatula* grown at 0.2 – 5.0 µM P for 52 DAT.
Different letters indicate a statically significant difference among the different treatments ($P \leq 0.001$). Data are the means ± S.E. of three replicates. (n.d. = not determined).

P-level *(µM P)*	Nodule Number *(Number/plant)*	Nodule FW *(mg/plant)*
0.2	n.d.	n.d.
0.5	n.d.	n.d.
0.8	n.d.	n.d.
1.0	5.7 ± 2.5 a	6.2 ± 2.3 a
3.0	22.7 ± 4 b	19.4 ± 1.4 b
5.0	60 ± 3.6 c	39.6 ± 3.7 c

the highest P level were given 6.7 times as high N accumulation compared with plants grown at the lowest P level (Fig. 3). In contrast, N concentration in both shoots and roots showed a slight decrease with rising P level (Fig. 4). Increasing the P supply in the nutrient solution decreased the N/P ratio significantly and the reduction was much pronounced in the shoots than the roots (Fig. 5). C/N ratios were more reduced when P supply was decreased in the nutrient solution (Fig. 6). C/N ratios showed a reversible trend to the pattern of N accumulation under different P levels. Therefore, we could speculate that the photosynthetic capacity was not much affected.

Nodule free amino acids

Decreasing P supply in the nutrient solution was followed by a proportional increase in the total free amino acid content of the nodules (Fig. 7). Plants grown at 1 µM P showed about 2-fold increase in the concentration of the total free amino acids over 5 µM P. Despite such higher increment in P concentration, no significant difference in the AA
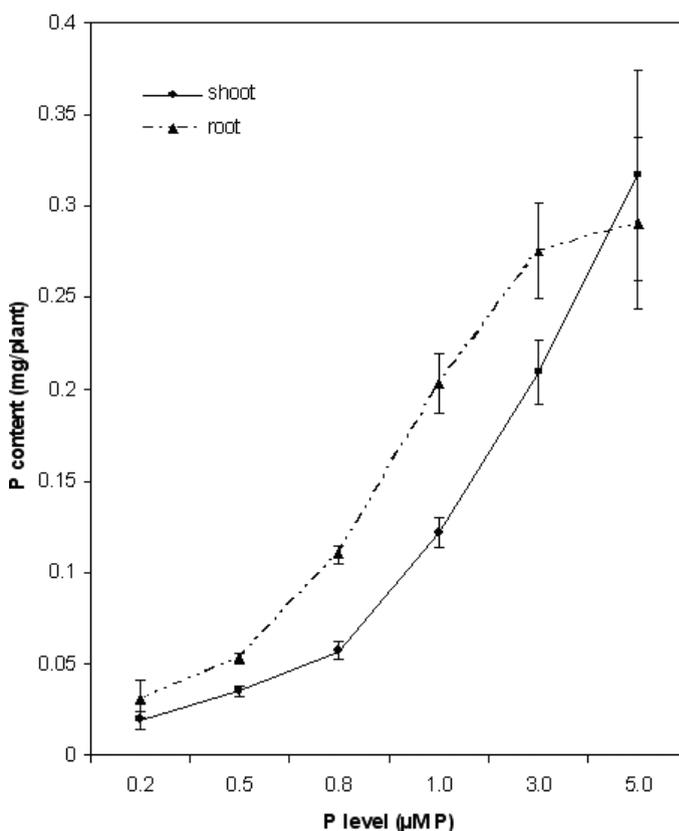
Fig. 2. Phosphorus accumulations of shoot (■) and roots (▲) of *Medicago truncatula* grown at 0.2 -5.0 µM P for 52 d. The effect of P supply is significant at P≤0.001. Bars represent ± SE of three replicates.

concentration was revealed. When investigating the profile of the free amino acids in the nodules, asparagine (Asn) showed the most dramatic increase in concentration in nodules under severe P deficiency treatment (Fig 8). The concentration of this free amino acid was two-times higher at 1 µM P than 5 µM P.

DISCUSSION

In the present study, nodule formation and development were evaluated

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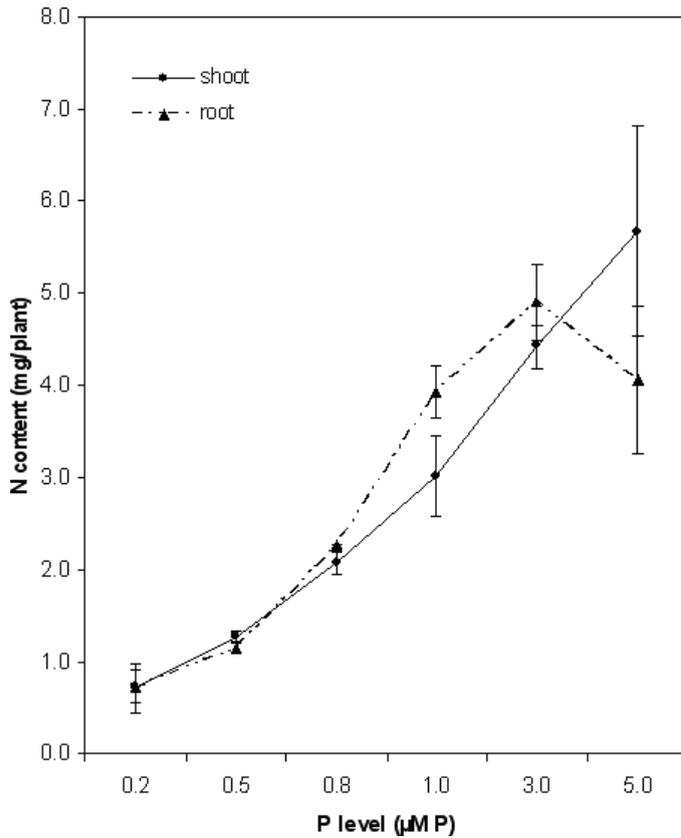
Fig. 3. Nitrogen accumulations of shoot (■) and roots (▲) of *Medicago truncatula* grown at 0.2 -5.0 μM P for 52 d. The effect of P supply is significant at P≤0.001. Bars represent ± SE of three replicates.

using the number of nodules and nodule fresh biomass. The results demonstrated that nodule formation in *Medicago truncatula* had higher P than the host plant growth. Supply of 1 μM P appeared to be sufficient for optimal nodulation. This finding is in agreement with a previous report of Tang et al. (2001) for *Medicago truncatula*. Alternatively, the seed P might be sufficient to support the rhizobial infection on the roots. Thomson et al. (1992) supported the fact that seed P might play a critical role in early nodule formations in soybean. Since P is essential for nodule development,
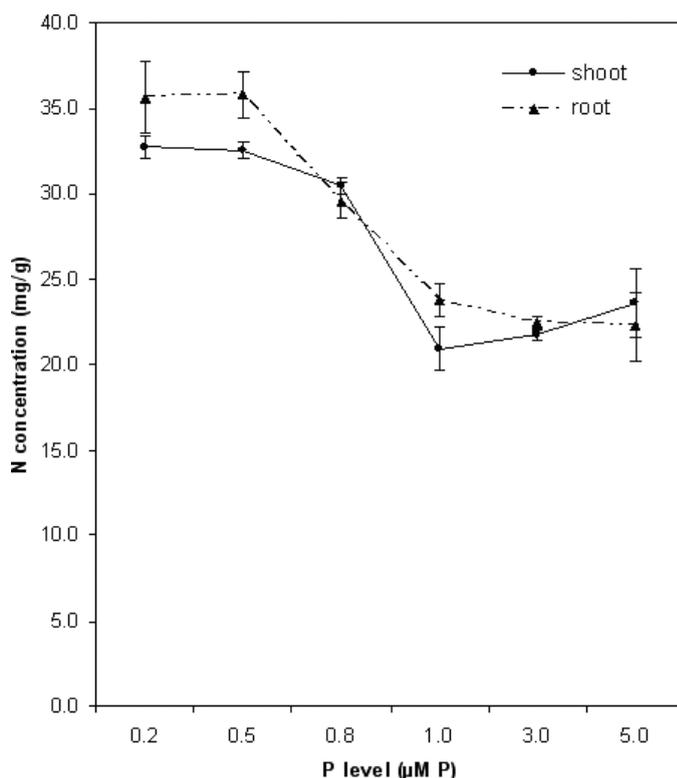
Fig. 4. Nitrogen concentrations of shoot (■) and roots (▲) of *Medicago truncatula* grown at 0.2 -5.0 µM P for 52 d. The effect of P supply is significant at P≤0.001. Bars represent ± SE of three replicates.

more P is required for the complete development of nodule initials formed at an early stage. Under P deficiency, only a portion of these initials would develop which is not the case under severe P shortage. For the mechanism of nodulation inhibition, it has been found that root infection is under host plant control (systematic) control and is mediated through the balance between stimulatory and inhibitory compounds that affect the induction of *nod* genes (Djordjevic et al., 1987). The level of the inhibitory compound secretion is regulated by a signal from the phloem (Djordjevic et al., 1987). This study also showed that nodule number and biomass increased with increasing P supply, suggesting that nodulation had a high requirement for
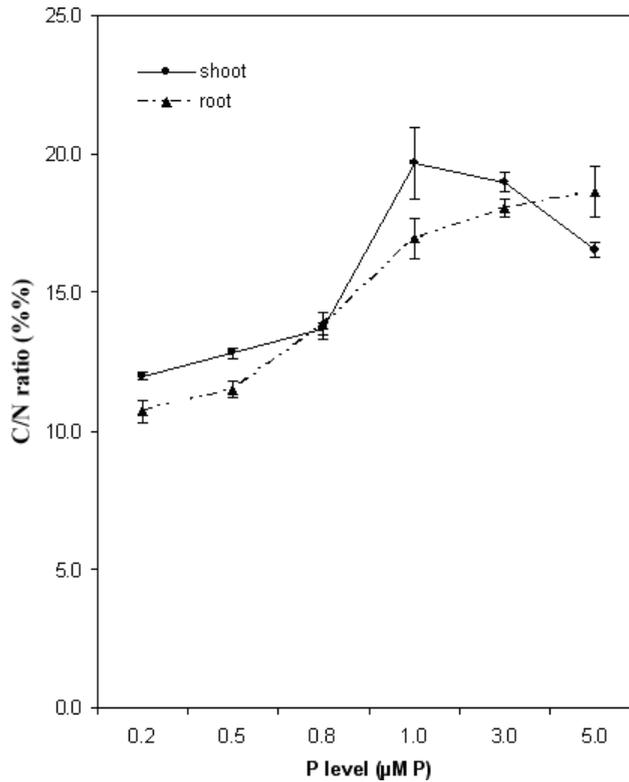
Fig. 5. C/N ratios of shoot (■) and roots (▲) of *Medicago truncatula* grown at 0.2 -5.0 μM P for 52 d. The effect of P supply is significant at P≤0.001. Bars represent ± SE of three replicates.

external P (Vance, 2001).

Increasing P application was followed by more dry matter accumulation in the plant. However, the different behavior shown by shoot and root growth indicated that shoot growth had a higher P requirement (Fig. 1). Studies with several legumes showed consistently a positive response to P application and plant dry matter was found to increase in response to phosphate in the growth media (Pereira and Bliss, 1987). The lower shoot biomass induced by P deficiency has been suggested to be mainly due to the reduction in leaf expansion and reduced leaf initiation in different legumes (Radin and Eidenbock, 1984; Chaudhary and Fujita, 1998; Liao and Yan,
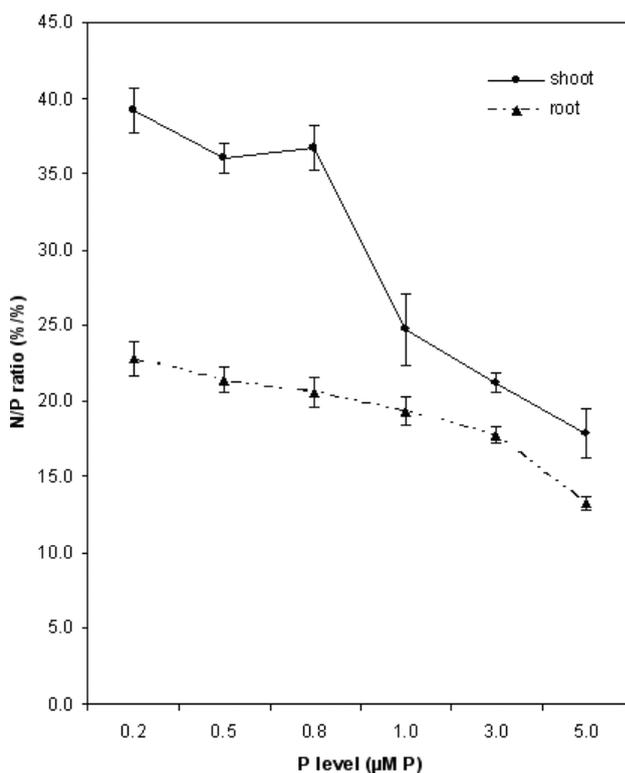
Fig. 6. N/P ratios of shoot (■) and roots (▲) of *Medicago truncatula* grown at 0.2 -5.0 μM P for 52 d. The effect of P supply is significant at P≤0.001. Bars represent ± SE of three replicates.

1999; Olivera et al., 2004) in addition to specific leaf area (Nguyen et al., 2006). The reduction in plant growth leads to further reduction of N sink strength (N-demand) under P deficiency (Almeida et al., 2000; Høgh-Jensen et al., 2002). This was also noticed under other environmental stresses such as leaf darkening (Schulze, 2003) and defoliation (Hartwig and Trommler, 2001). Accordingly, we could expect fewer requirements for further fixed-N for the host plant. The hypothesis that N$_2$ fixation of *Medicago truncatula* under P shortage is regulated by an N-feedback mechanism is supported by several results from the present study. The total N concentration in the plant and in the leaves and the N/P ratio were highest under severe P deficiency.

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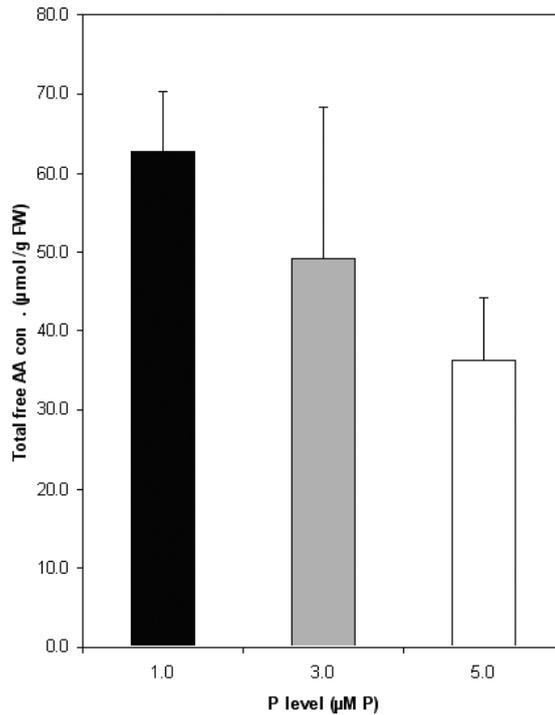
Fig. 7. Nodule total free amino acid composition of *Medicago truncatula* grown at 0.2 -5.0 µM P for 52 d. Bars represent ± SE of three replicates.

This probably indicates a reduction in the plant N demand. However, low P had no effect on nodule activity while it significantly reduced nodule biomass. According to Marschner (1995), the average optimal N/P ratio in plant tissue is around 7, with optimal N and P concentrations of 2–5 and 0.3–0.5 % of DM, respectively. The increased N/P ratio under P shortage level is consistent with the results of Schulze and Drevon (2005) for alfalfa and Almeida et al. (2000) and Høgh-Jensen et al. (2002) for white clover. The amount of N fixed normally depends on the N/P ratio that is sensed by the plant through a mechanism which is not yet fully understood. Additionally, reducing P supply led to increased shoot and root % N while decreasing the C/N ratio. Almeida et al. (2000) proved for white clover plants grown under similar conditions that such an increase in N concentration was not a result of a lower plant size (Farrar and Williams, 1991). Nurnberger et al. (1990)
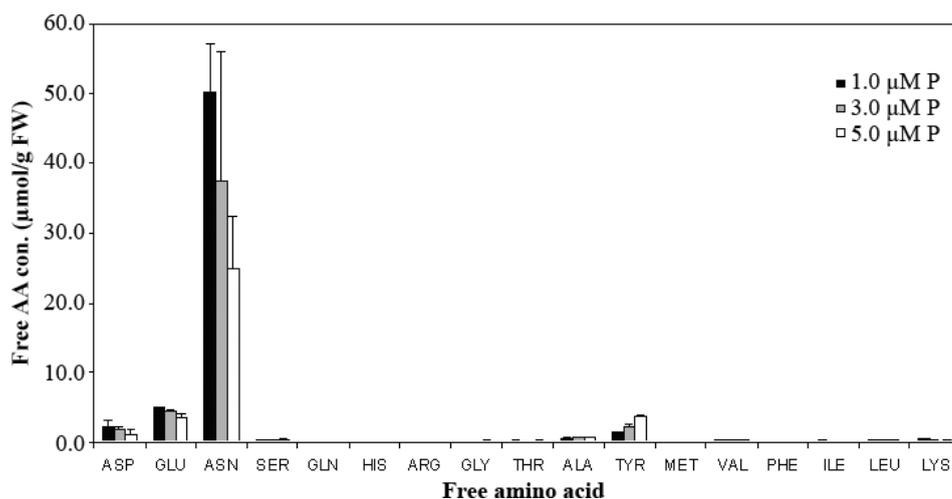
Fig. 8. Nodule free amino acid composition of *Medicago truncatula* grown at 0.2 -5.0 µM P for 52 d. Bars represent ± SE of three replicates.

reported that in P-stressed tissue, RNA levels were reduced resulting in impaired protein synthesis and free amino acid accumulation. In agreement with the feedback concept, higher concentrations of free amino acids and particularly of Asn were found in nodules and phloem sap (S. Sulieman and J. Schulze, unpublished data) under low P supply. These results are in line with previous findings of Almeida et al. (2000) and Høgh-Jensen et al. (2002). It was generally assumed that a control of nodule activity based on the plant nitrogen status would involve nitrogen compounds being transported to the nodules via the phloem (e.g. Parsons et al., 1993).

In several studies, Asn was indicated as a feedback regulatory agent for symbiotic nitrogen fixation (SNF). For example Vadez et al. (2000) found Asn accumulation in nodules and shoots of soybean plants after imposing various stresses (e.g., water deficit). Asparagine was also indicated as a feedback agent when applied exogenously to broad beans (Oti-Boateng and Silsbury, 1993; Oti-Boateng et al., 1994). Stewart and Larher (1980) reported an accumulation of Asn in leguminous and non leguminous plants deficient in various essential minerals (P, K, S, Mg). In soybean, a 35-fold increase in shoot Asn concentration was reported after 15 mM

NO$_3^-$ application (Bacanamwo and Harper, 1997) while Hartwig and Trommler (2001) noticed a two-fold increase in Asn concentration in the lupins phloem sap after defoliation. Moreover, Fougère et al. (1991) argued that Asn might have an osmoregulatory function in nodule tissues after observing higher concentration of this amide under salt stress. Asparagine in cowpea nodules is an example of an amide which is delivered in the phloem, and not produced within the nodule (Atkins et al., 1988; Atkins et al., 1990). Recently, Keller et al. (2002) found in *Lotus japonicus* three different clones encoding asparagine synthetase that were down-regulated significantly under P deficiency and they suggested a feedback regulatory mechanism of Asn on the expression of asparagine synthetase.

In conclusion, the presented results indicate that P is of predominant importance for intensive nitrogen fixation. Under low P supply, nitrogen fixation is strongly impaired, both in respect to nodule functioning and initiation. The down-regulatory effect of low P might be mediated by an N-feedback mechanism. The presented data are in full compliance with such a concept. Given the high share of Asn on total amino acids in nodules and the fact that this share indeed increases at low P supply, it appears evident that Asn plays a crucial role in the N-feedback mechanism.

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