P-deficiency increases the $O_2$ uptake per $N_2$ reduced in alfalfa

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Abstract

Nodulated alfalfa (Medicago sativa L. cv. Saranac) plants were grown in hydroponics at $P$-sufficient and $P$-deficient supply levels. After 5 weeks of growth, dry matter accumulation, nodulation, total $N$ and $P$ accumulation, as well as $^{15}N_2$ uptake, were measured. Moreover, the response of nodule $O_2$-uptake to raising external $pO_2$ was determined in an open-flow measurement system and nodule permeability was calculated. Plants in the $P$-deficient supply treatment had a lower $P$ concentration in all organs. In both treatments the highest $P$ concentration was found in nodules. In the $P$-deficient treatment plants formed less dry matter, had a lower shoot/root ratio, less nodulation, decreased total $N$ accumulation, and lower $^{15}N_2$ uptake per dry matter nodule. Nodules in the $P$-deficient treatment were, on average, smaller and had a higher $O_2$ uptake per $N_2$ reduced, coinciding with increased nodule permeability and conductance. Thus increased oxygen uptake appears to be a mechanism to adjust nodule metabolism to $P$ deficiency in indeterminate $N_2$-fixing nodules such as in alfalfa, as has previously been shown for determinate nodule forms.

Key words: Alfalfa, nitrogen fixation, nodule conductance, oxygen diffusion, phosphorus, respiration, rhizobia, symbiosis.

Introduction

$P$-availability can limit legume productivity in the fields by a negative impact on nitrogen fixation. Römer and Lehné (2004) showed that in a German loess (CAL-$P$=5 mg kg$^{-1}$ soil) after long-term organic farming with no $P$ fertilization, $P$ was the principal limiting factor for broad-bean (Vicia faba L.) growth through strongly reduced $N_2$ fixation giving reduced nitrogen availability for a subsequent crop. In tropical soils, $P$ availability is one major restriction to legume crop productivity (Andrew and Robins, 1969). Growing $N_2$-fixing root-nodules are strong phosphorus ($P$) sinks in legumes. $P$ concentrations in nodules can reach up to 3-fold those of other plant parts (Sa and Israel, 1991). Thus the fast and positive growth reaction of legumes to $P$ supply becomes understandable (Hoch-Jensen et al., 2002).

The metabolic functions of $P$ in nodules are probably multifold and mostly related to intensive carbon and energy turnover. Nodule $O_2$ permeability is thought to be involved in the regulation of nitrogen fixation (Hunt and Layzell, 1993). Restricted $O_2$ supply to the infected zone functions as a widespread stress response of nodules (Denison, 1998). By contrast, $P$ deficiency has been shown to increase nodule $O_2$ conductance and uptake in soybean (Ribet and Drevon, 1995a) and common bean (Vadez et al., 1996).

The objective of the present study was to clarify whether an increased $O_2$ conductance of nodules as a response to deficient $P$ supply also occurs in the pasture legume alfalfa.

Materials and methods

Biological material and growth conditions

Seeds of Medicago sativa L. cv. Saranac were surface-sterilized with 70% ethanol for 10 min, washed in sterile water, and germinated on water-saturated vermiculite. At 7 d after emergence (DAE) plants were transferred to glass cylinders (h=600 mm, inner diameter=20 mm) with an air-tight sealed cover (Fig. 1). Plants were held at their stem bases on the open side of the cylinder with sterilized cotton wool leaving roots in the nutrient solution. Plants

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Photosynthetic flux density was 665 mol m⁻² s⁻¹. Deficiency symptoms occurred.

The nutrient solution was lowered to 1/3 of its original volume 24 h prior to any measurement to allow the plants to acclimate. The air volume in the cylinder was 115 ml and an airstream (20 kPa O₂) of 15 ml min⁻¹ was pumped through the root/nodule compartment. The airflow was kept constant by a flow controller (MKS instruments).

At 34 and 35 DAE the root compartments were connected to an open-flow gas exchange measurement system to measure root/nodule O₂ uptake. Data for O₂ (Oxynos 100, Rosamount) in the outflowing air were taken and O₂ uptake could be calculated from the difference in the O₂ content in the in- and out-flowing air and the flow rate (Fig. 1). The flow rate for the open flow measurement of O₂-uptake was selected to have at least a difference of 0.1 kPa between the in- and out-flowing air, which could conveniently be measured. Following the measurement at 20 kPa, the O₂ pressure in the airstream was altered to 15 kPa (85/15, N₂/O₂, v/v). The airflow for the root/nodule was taken from a larger flow in which O₂ pressure was regulated by changing the N₂ and O₂ mixture of the flow (Fig. 1). In this way a constant flow through the root/nodule compartment could be maintained. Five to eight minutes after changing the O₂ content of the inflowing air, a new constant O₂ content of the outflowing air was reached. Thus measurements of O₂ in the outflowing air were, in all cases, taken at 15 min after switching to a new mixture. Preliminary experiments had shown that the O₂ content in the outflowing air remained stable for at least 1 h at all O₂ levels once the equilibrium was reached. Accordingly, measurements were made after changing to 25, 30, 40, and 50 kPa O₂, in that sequence. Subsequently, the root compartment was disconnected, filled with fresh nutrient solution, and connected to an airstream of 1 vol. min⁻¹.

15N₂ uptake measurement

Forty-eight hours after the O₂-uptake measurements, the root/nodule compartments were disconnected from the airstream and totally filled with fresh nutrient solution for the 15N₂ uptake measurements of all replicates. Subsequently, 75% of the nutrient solution was replaced by a mixture of 15N₂ (98 at%exc.)/O₂ (80/20, v/v) so that all nodules were above the nutrient solution and the root/nodule compartment was sealed for 1 h. The 15N₂ uptake was terminated by replacing the 15N₂ containing atmosphere with water. This was followed by immediate harvest and drying of the plants.

Biomass parameter and statistical analysis

At harvest, plants were separated into nodules, roots, and shoots. Roots and shoots were immediately dried at 70 °C to a constant weight, while nodules were previously counted and classified according to their length (0.5–2 mm, 2–3 mm, above 3 mm). Nodule surface area (S) was calculated assuming that all alfalfa nodules are cylinders as S = Σ [πh (n Rdn)] where n, d, and h are the nodule number, mean diameter, and mean length, respectively, in each class. Based on sample measurements, nodule diameter was assumed to be 1 mm for nodules shorter than 2 mm and 1.5 mm for nodules longer than 2 mm.

After drying, all plant material was weighed and ground to a fine powder. N and 15N was determined with a combination of an elemental analyser (Vario EL, Firma Elemental Analysen GmbH, Hanau) and an emission spectrometer (NOI 7, Fischer Analysetechnik, Leipzig). P was determined according to Murphy and Riley (1962) after ashing the plant dry material.

All data were subjected to analysis of variance and mean values of the two P treatments were compared by the t-test. Regression analysis was performed for the dependence of root/nodule O₂ uptake as a function of pO₂. In all cases the Sigmastat analytical software was used.

Fig. 1. Experimental set-up for measuring O₂ uptake in an open-flow system.

were inoculated with 1 ml Sinorhizobium meliloti (102F51) inoculum at transfer, and were re-inoculated during the first 10 d at each solution change. Inoculum was prepared by growing the bacteria in YEM at 28 °C for 3 d at an approximate cell density of 10⁷ ml⁻¹. The first nodules became visible 5–7 d after inoculation. Inoculation resulted in intensive nodulation and effective N₂ fixation while an uninoculated control remained nodule free.

The cylinder contained 180 ml of an N and P-free basic nutrient solution consisting of 1 mM MgSO₄, 0.7 mM K₂SO₄, 1.65 mM CaCl₂, 16 µM Fe (as Fe-EDTA), 4 µM MnCl₂, 22 µM H₂BO₃, 0.4 µM ZnSO₄, 0.05 µM NaMoO₄, and 1.6 µM CuSO₄ buffered with 2 mM MES [2-(N-morpholino) ethane-sulphonic acid]. Urea to a concentration of 0.5 mM N was added to the nutrient solution during the initial 10 d of growth to avoid N-deficiency during nodule development. The solution was intensly aerated by an airflow of normal air of about 1 vol. min⁻¹ during the experiment.

Two P treatments were applied as follows: sufficient P received KH₂PO₄ to the nutrient solution in the cylinder to a final concentration of 0.5 mM N was added to the nutrient solution -morpholino) ethane-sulphonic acid]. Urea to a concentration of 0.5 mM N was added to the nutrient solution during the initial 10 d of growth to avoid N-deficiency during nodule development. The solution was intensly aerated by an airflow of normal air of about 1 vol. min⁻¹ during the experiment.

Two P treatments were applied as follows: sufficient P received KH₂PO₄ to the nutrient solution in the cylinder to a final concentration of 20 µM P while the P concentration in deficient P was 5 µM. An appropriate amount of potassium as K₂SO₄ was added to the low-P solution to ensure equal potassium supply. In all cases the pH was adjusted to 6.0. The nutrient solution was renewed 2 h after transfer, and were reinoculated during the first 10 d at each solution change.

Roots and shoots were immediately dried at 80 °C and a constant O₂ content of the inflowing air, a new constant O₂ content of the outflowing air was reached. Thus measurements of O₂ in the outflowing air were, in all cases, taken at 15 min after switching to a new mixture. Preliminary experiments had shown that the O₂ content in the outflowing air remained stable for at least 1 h at all O₂ levels once the equilibrium was reached. Accordingly, measurements were made after changing to 25, 30, 40, and 50 kPa O₂, in that sequence. Subsequently, the root compartment was disconnected, filled with fresh nutrient solution, and connected to an airstream of 1 vol. min⁻¹.

15N₂ uptake measurement

Forty-eight hours after the O₂-uptake measurements, the root/nodule compartments were disconnected from the airstream and totally filled with fresh nutrient solution for the 15N₂ uptake measurements of all replicates. Subsequently, 75% of the nutrient solution was replaced by a mixture of 15N₂ (98 at%exc.)/O₂ (80/20, v/v) so that all nodules were above the nutrient solution, and the root/nodule compartment was sealed for 1 h. The 15N₂ uptake was terminated by replacing the 15N₂ containing atmosphere with water. This was followed by immediate harvest and drying of the plants.

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Fig. 1. Experimental set-up for measuring O₂ uptake in an open-flow system.
Results

Plant growth and nodulation

The level of P-deficiency applied in this work induced a significant growth reduction (Table 1). Total growth and the shoot/root ratio were significantly lower under P deficiency. Moreover, the slope of a linear regression of total dry matter accumulation as a function of total plant P was increased in the P-deficient treatment (Fig. 2).

Nodule dry matter was higher in the P-sufficient treatment (164%) (Table 1). This difference was only due to the larger number of nodules in the class of greatest length (Fig. 3), whereas nodule number per plant did not differ between treatments (Table 1). Thus, overall surface and dry matter of nodules per plant were higher as a result of larger individual nodule size (Fig. 3) and dry matter (Table 1) under P sufficiency.

By contrast, neither the shoot per nodule nor the total plant dry matter per nodule ratios differed between both P treatments (Table 1). The linear regression of total dry matter formation as a function of nodule dry matter revealed no difference between treatments (Fig. 4).

P distribution and total P-uptake

Whatever the P treatment, P was preferentially transported into nodules since, even under P deficiency, P concentration (mg g⁻¹) was higher in nodules than in roots or shoots. Under P deficiency, nodules had only about half the P concentration of those in the P-sufficient treatment (Fig. 5).

Under P sufficiency, nodule P concentration was also twice as high as that in roots, and in shoots to a lesser extent, and P concentration was much higher in all plant organs (Fig. 5). Thus, total P-uptake was increased by 374% when compared to the P-deficient treatment.

Table 1. Dry matter and nodulation of alfalfa grown at sufficient and deficient P supply in nutrient solution culture over a period of 35 DAE

<table>
<thead>
<tr>
<th>Dry matter and nodule characteristics</th>
<th>Sufficient P</th>
<th>Deficient P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot (mg plant⁻¹)</td>
<td>960±72</td>
<td>570±83*</td>
</tr>
<tr>
<td>Root (mg plant⁻¹)</td>
<td>353±26</td>
<td>289±32*</td>
</tr>
<tr>
<td>Shoot/root (mg mg⁻¹)</td>
<td>2.45±0.19</td>
<td>1.82±0.23*</td>
</tr>
<tr>
<td>Nodule (mg plant⁻¹)</td>
<td>39.9±3.2</td>
<td>24.3±3.0*</td>
</tr>
<tr>
<td>Nodule number plant⁻¹</td>
<td>320±58</td>
<td>298±31</td>
</tr>
<tr>
<td>Individual nodule DM (µg)</td>
<td>127±14</td>
<td>82±4*</td>
</tr>
<tr>
<td>Nodule surface (mm² plant⁻¹)</td>
<td>4293±676</td>
<td>2087±251*</td>
</tr>
<tr>
<td>Nodule surface/DM (mm² g⁻¹ DM)</td>
<td>107±13</td>
<td>86±7</td>
</tr>
<tr>
<td>Shoot/nodule (mg mg⁻¹)</td>
<td>24±1.3</td>
<td>23.6±3.5</td>
</tr>
<tr>
<td>Total dry matter/nodule (mg mg⁻¹)</td>
<td>33.9±1.3</td>
<td>36.5±4.2</td>
</tr>
</tbody>
</table>

P treatments (Table 1). The linear regression of total dry matter formation as a function of nodule dry matter revealed no difference between treatments (Fig. 4).

Fig. 2. Dependence of dry matter formation on plant total P content of alfalfa plants grown at sufficient and deficient P supply. Data are means of six replicates.

Fig. 3. Distribution of nodules among length classes from alfalfa plants grown at sufficient and deficient P supply. Data are means of six replicates. Error bars represent standard deviation. Nodule number in the individual length classes did in all cases differ between the treatments (t-test, P <0.05).

Fig. 4. Dependence of dry matter formation on nodule dry matter per plant of alfalfa plants grown at sufficient and deficient P supply. Data are means of six replicates.
**N assimilation and $^{15}$N$_2$-uptake**

Total N uptake calculated from the N concentration and plant dry matter was increased in the P-sufficient treatment by 225% when compared to the P-deficient treatment (Table 2). Since N-concentration in the P-deficient treatment was lower in all plant organs, the difference in N accumulation was even stronger than that in dry matter. Total N uptake per individual nodule was increased in the P-sufficient treatment whereas total N uptake per total plant P was decreased (Table 2).

In order to evaluate the nodule nitrogenase activity at one stage of the growth curve, $^{15}$N$_2$-uptake was measured per individual intact plant. The corresponding values in the P-sufficient treatment were increased by 274% or 167%, as expressed per plant or nodule dry matter, respectively. However, nitrogen fixation per nodule P was significantly increased in the deficient P treatment (Table 2).

**Table 2.** *Alfalfa N-assimilation and $^{15}$N$_2$ uptake at sufficient and deficient P supply in nutrient solution culture over a period of 35 DAE*

Data are means of six replicates ± standard deviation. An asterisk indicates a significant difference when comparing both P treatments ($t$-test, $P < 0.05$).

<table>
<thead>
<tr>
<th>N-assimilation and $^{15}$N$_2$ uptake</th>
<th>Sufficient P</th>
<th>Deficient P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N concentration in dry matter (mg g$^{-1}$)</td>
<td>Shoot: 3.8±0.2</td>
<td>2.7±0.3*</td>
</tr>
<tr>
<td></td>
<td>Root: 1.8±0.6</td>
<td>1.2±0.3*</td>
</tr>
<tr>
<td></td>
<td>Nodules: 7.4±0.4</td>
<td>4.7±0.6*</td>
</tr>
<tr>
<td>$^{15}$N$_2$/nodule DM (mg mg$^{-1}$)</td>
<td>Shoot: 1.14±0.04</td>
<td>0.82±0.11*</td>
</tr>
<tr>
<td></td>
<td>Root: 10.1±1.1</td>
<td>16.9±4*</td>
</tr>
<tr>
<td></td>
<td>Nodules: 5.7±5.4</td>
<td>16.7±2.2*</td>
</tr>
<tr>
<td>Plant total $^{15}$N$_2$ uptake (µg h$^{-1}$)</td>
<td>45.7±5.4</td>
<td>16.7±2.2*</td>
</tr>
<tr>
<td>$^{15}$N$_2$ uptake/nodule DM (µg mg$^{-1}$ h$^{-1}$)</td>
<td>1.15±0.13</td>
<td>0.69±0.07*</td>
</tr>
<tr>
<td>$^{15}$N$_2$ uptake/nodule P (µg mg$^{-1}$ h$^{-1}$)</td>
<td>209±23</td>
<td>281±34*</td>
</tr>
</tbody>
</table>

**Table 3.** *$O_2$-uptake of alfalfa grown at sufficient and deficient P supply in nutrient solution culture over a period of 35 DAE*

Data are means of six replicates ± standard deviation. An asterisk indicates a significant difference when comparing both P treatments ($t$-test, $P < 0.05$).

<table>
<thead>
<tr>
<th>$O_2$ uptake (µmol O$_2$ plant$^{-1}$ h$^{-1}$)</th>
<th>Sufficient P</th>
<th>Deficient P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>39.3±2.3</td>
<td>34.8±3.3*</td>
</tr>
<tr>
<td>Maintenance and growth</td>
<td>13.2±1.7</td>
<td>15.4±2.1</td>
</tr>
<tr>
<td>$N_2$ fixation</td>
<td>26.1±2.3</td>
<td>19.4±1.3*</td>
</tr>
<tr>
<td>Nitrogenase-linked $O_2$-uptake per nodule dry matter (µmol O$_2$ g$^{-1}$ DM nodule h$^{-1}$)</td>
<td>656±45</td>
<td>805±94*</td>
</tr>
<tr>
<td>Nitrogenase-linked $O_2$-uptake per $^{15}$N$_2$ fixed (µmol O$_2$ µg$^{-1}$ $^{15}$N fixed h$^{-1}$)</td>
<td>0.58±0.09</td>
<td>1.18±0.18*</td>
</tr>
</tbody>
</table>

**$O_2$ uptake linked to nitrogen fixation**

Since nitrogenase activity depends upon nodule respiration, the nodulated-root $O_2$ uptake per plant was measured on intact plants, the day before the above nitrogen fixation measurement. The corresponding values were significantly higher by 13% in the P-sufficient treatment at ambient oxygen pressure when compared to the P-deficient treatment (Table 3).

In order to assess the effect of P supply on nodule permeability, and the subsequent nitrogenase-linked respiration, the response of nodulated-root $O_2$ uptake to variation of rhizospheric $pO_2$ was measured following the principles described in detail in Jebara and Drevon (2001). The $O_2$ uptake to $pO_2$ showed a typical saturation curve indicating that nitrogenase activity was oxygen limited at ambient oxygen pressure (Fig. 6). In both treatments the increase in $O_2$ uptake appeared to be linear between 20 kPa and 30 kPa $O_2$. Thus, nodule permeability was calculated as the slope of the linear response of $O_2$ uptake as a function of $pO_2$ in the

**Fig. 5.** $P$ concentration of shoots, roots, and nodules of alfalfa plants grown at sufficient and deficient $P$ supply. Data are means of six replicates. Error bars represent standard deviation.

**Fig. 6.** Oxygen uptake at different kPa $O_2$ of roots and nodules of alfalfa plants grown at sufficient and deficient $P$ supply. Data are means of six replicates. Error bars represent standard deviation. Dotted lines show regression of root/nodule oxygen uptake as a function of oxygen pressure between 20 kPa and 30 kPa around the roots.
20–30 kPa O₂ interval. From data in Fig. 6, P deficiency decreased the overall permeability of the nodule population from a mean value of 2133±325 mm² h⁻¹ compared with 3339±332 for P sufficiency.

The nodule conductance could be calculated by dividing the above permeability values per individual plant by the nodule area calculated from nodule number per plant in Table 1. P deficiency significantly increased the nodule conductance with a mean value of 17.3±1.9 µm s⁻¹ compared with 13.2±1.5 µm s⁻¹ under P sufficiency. Consequently, nitrogenase-linked respiration per unit nodule in the P-deficient treatment was 123% of that in the P-sufficient treatment (Table 3). Thus nodules consumed about twice as much O₂ per unit fixed N₂ in the P-deficient treatment when compared with the P-sufficient treatment.

Discussion

Increased nodule O₂ permeability (Table 3) confirms, with undeterminate nodules of alfalfa, earlier similar reports on determinate nodules of soybean (Ribet and Drevon, 1995a) and common bean (Vadez et al., 1996). In this work, the methodical approach was developed further by using ¹⁵N₂ uptake to measure nitrogenase activity, thus avoiding problems of the flow-through acetylene reduction or H₂ evolution assays (Minchin et al., 1983; King and Layzell, 1991; Ribet and Drevon, 1995b), and by using a differential, open-flow O₂ uptake measurement for the nodulated-root compartments. The nodules developed under submerged conditions, but did not show visible differences to those grown in soil or sand. The measurement was subsequently done in a gaseous environment after lowering the nutrient solution. It cannot be totally excluded that this somehow influenced nodule permeability, although the plants were allowed to adapt for 24 h.

The physiological importance of increased O₂ permeability as a response to P-deficient supply is not yet understood. In P-deficient nodules the adenylate charge, at least of the plant fraction, appears to be decreased (Sa and Israel, 1991). Thus the high O₂ uptake might contribute to maintaining a sufficient adenylate charge for high N₂ fixation rates. In addition, alternative oxidases are increasingly expressed in P-starved tissues (Rychter et al., 1992). A similar increase in nodule tissues where alternative oxidases are expressed (Millar et al., 1997), could contribute to changes in respiratory costs of nitrogen fixation (Schulze et al., 2000; Adgo and Schulze, 2002). Higher O₂ consumption would create an O₂ sink which would then, in turn, induce the observed increase in nodule O₂ permeability. Increases in nodule permeability were previously associated with nodule cortex-cell expansion (Drevon et al., 1998). A triggering mechanism for such changes involving a low P status in those cells has not yet been elucidated. Inorganic P accumulation in the nodule cortex during nodule growth was shown with ³¹P-NMR studies (Rolin et al., 1989) and might be involved in osmoregulatory changes in cell size. However, in the case of these experiments the proportion of P in nodules to plant total P did not differ between both treatments, although P concentration in nodules was much higher compared with roots or shoots in both P treatments. Moreover, during recovery from P deficiency, nodules are the primary P sink (Israel, 1993). If the legume adaptation to deficient P comprises a preferential allocation of P to nodules, a relatively higher flow of P into nodules might occur at more severe P restrictions than in this study’s experiment. In this work, the most apparent morphological adaptation of the plants grown at deficient P was a smaller individual nodule size, although nodule number per plant did not differ (Table 1).

The nodulated-root compartment had to be as small as possible to allow a differential O₂ measurement against a large O₂ background pressure. The chosen size of 180 ml for this compartment required the nutrient solution containing 20 µM P to be changed twice a day for a sufficient P supply, since concentrations above 25 µM P would induce P toxicity (Bell et al., 1990; Tang et al., 2001). At 5 µM P for the P-deficient supply, N₂ fixation had been particularly affected compared with other growth processes (Table 2), although nitrogenase activity per unit nodule dry matter is maintained for much longer (Almeida et al., 2000; Hoch-Jensen et al., 2002). The increased N/P ratio under deficient P supply (Table 2), consistent with the results of Almeida et al. (2000) and Hoch-Jensen et al. (2002), may indicate a N-feedback effect. In P-stressed tissue, RNA levels are reduced (Numberger et al., 1990), resulting in impaired protein synthesis and free amino acid accumulation (Johnson et al., 1996), in particular asparagine in nodules (Almeida et al., 2000). However, total N concentration in the P-deficient nodules was much lower, which might have been the result of less infected tissue in this treatment. The way by which a putative amino acid accumulation would exert its feedback effect is largely unknown (Schulze, 2003), although Neo and Layzell (1997) demonstrated that oxygen diffusion into the nodule is involved, but the data from this study indicate that a P-deficient supply increases rather than decreases nodule O₂ permeability.

In conclusion, alfalfa adapt by forming smaller nodules with higher O₂ permeability and O₂ consumption per unit fixed nitrogen as well as O₂ consumption per nodule P. The primary metabolic effect, if any, of P status on nitrogenase functioning remains obscure, and it is unclear in which way it would translate into nodule permeability. Addressing these questions appears to be worthwhile since there is considerable variability in the tolerance of legumes to P deficiency in which the adjustability of nodule O₂ permeability is involved (Vadez et al., 1996; Vadez and Drevon, 2001).
Acknowledgements

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