

Evidence that P deficiency induces N feedback regulation of symbiotic N₂ fixation in white clover (*Trifolium repens* L.)

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Abstract

Trifolium repens L. was grown to test the following hypotheses: when P is deficient (i) N₂ fixation decreases as a result of the plant's adaptation to the low N demand, regulated by an N feedback mechanism, and (ii) the decrease in the photosynthetic capacity of the leaves does not limit N₂ fixation. Severe P deficiency prevented nodulation or stopped nodule growth when the P deficiency occurred after the plants had formed nodules. At low P, the proportion of whole-plant-N derived from symbiotic N₂ fixation decreased, whereas specific N₂ fixation increased and compensated partially for poor nodulation. Leaf photosynthesis was reduced under P deficiency due to low $V_{c,max}$ and J_{max} . Poor growth or poor performance of the nodules was not due to C limitation, because (i) the improved photosynthetic performance at elevated p_{CO_2} had no effect on the growth and functioning of the nodules, (ii) starch accumulated in the leaves, particularly under elevated p_{CO_2} , and (iii) the concentration of WSC in the nodules was highest under P deficiency. Under severe P deficiency, the concentrations of whole-plant-N and leaf-N were the highest, indicating that the assimilation of N exceeded the amount of N required by the plant for growth. This was clearly demonstrated by a strong increase in asparagine concentrations in the roots and nodules under low P supply. This indicates that nodulation and the proportion of N derived from symbiotic N₂ fixation are down-regulated by an N feedback mechanism.

Key words: Elevated CO₂, nodule, photosynthesis, carbohydrate, asparagine.

Introduction

In field experiments on fertile soil, grassland legumes respond much more strongly to elevated atmospheric partial pressure of carbon dioxide (p_{CO_2}) than grasses (Hebeisen *et al.*, 1997; Lüscher *et al.*, 1998). The specific advantage of the response of legumes to p_{CO_2} is the result of symbiotic fixation of di-nitrogen (N₂) (Zanetti *et al.*, 1996, 1997; Lüscher *et al.*, 2000), while the response of grasses to elevated p_{CO_2} was limited by N (Fischer *et al.*, 1997; Zanetti *et al.*, 1997; Rogers *et al.*, 1998; Daepf *et al.*, 2000). However, under unfavourable environmental conditions (e.g. low P or K), legumes may lose the distinct advantage of an unlimited source of symbiotic N (Hartwig *et al.*, 1996; Lüscher *et al.*, 1996, 1998). Indeed, when P is very deficient, legumes no longer respond to elevated p_{CO_2} by increasing biomass (Stöcklin *et al.*, 1998; Almeida *et al.*, 1999), and fixation of symbiotic N₂ is greatly reduced (Cadisch *et al.*, 1989, 1993). As a result, two crucial questions arise: (1) When P is deficient, do legumes such as white clover (*Trifolium repens* L.) no longer respond to elevated p_{CO_2} because symbiotic N₂ fixation is severely hindered? (2) How is the effect of low P availability on N₂ fixation regulated?

It is well known that the rate of N₂ fixation in plants deficient in P is reduced. This is usually explained by an effect of low P supply on the growth of the host plant,

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Abbreviations: DAP, days after planting; DM, dry mass; J_{max} , apparent potential rate of electron transport at saturated light; %N_{sym}, relative contribution of symbiotically fixed N to total plant N; p_{CO_2} , partial pressure of atmospheric CO₂; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; *RubP*, ribulose 1,5-bisphosphate; $V_{c,max}$, apparent maximum rate of carboxylation with non-limiting ribulose-1,5-bisphosphate; WSC, water-soluble carbohydrate.

on the growth and functioning of the nodule, or on the growth of both the plant and the nodule (Robson *et al.*, 1981; Jakobsen, 1985; Israel, 1987, 1993; Sa and Israel, 1991). However, some of these experiments were conducted under field conditions, or the plants were grown in soil in pots. Therefore, the effect ascribed to the addition of mineral P on N₂ fixation may actually be due to the interactive effects of P and N in the soil; i.e. P fertilization would stimulate the growth of soil organisms or non-leguminous plants which, in turn, would compete for soil mineral N and thus, indirectly, lead to increased symbiotic N₂ fixation in legumes (Hartwig, 1998). Moreover, in all these experiments, N₂ fixation was assessed indirectly by estimating the short-term nitrogenase activity (acetylene reducing assays), which is a non-integral method.

Early studies suggested that low P reduces shoot growth and, as a consequence, that the lower rate of photosynthesis limits the supply of C to the nodules (Jakobsen, 1985), thereby decreasing N₂ reduction (Israel, 1987, 1993; Sa and Israel, 1991, 1998). The N₂ reduction process requires about 2.9 mg C mg⁻¹ fixed N; this demand can account for up to 32% of the current photosynthetic rate (Minchin and Pate, 1973; Warembourg and Roumet, 1989). Studies on the effect of P deficiency report that the supply of photosynthates from the shoots to the nodules does play a key role in N₂ fixation. However, the effects of P supply on important parameters such as photosynthesis, non-structural carbohydrate, N₂ fixation, and the N status of the plant were not considered together in any of those studies. Reports on the effect of other stress factors, however, suggest that the supply of non-structural carbohydrate to the nodules does not primarily control N₂ fixation (Hartwig *et al.*, 1990, 1994; Weisbach *et al.*, 1996; Gordon *et al.*, 1997; Curioni *et al.*, 1999), whereas N demand does control N₂ fixation (Heim *et al.*, 1993; Parsons *et al.*, 1993; Hartwig *et al.*, 1994; Neo and Layzell, 1997; Hartwig, 1998).

P deficiency decreases the rate of plant growth and, thus, the plant's N requirement. Therefore, several reports concluded that P-deficient plants, even when fully dependent on N₂ fixation, are not limited by N (Robson *et al.*, 1981; Jakobsen, 1985; Israel, 1987). This indicates a balancing mechanism between N source and N sink in the legume. If this were to apply to P deficiency, then (1) the decrease in N₂ fixation would be the result of the adaptation to a lower N requirement, as mediated by an N feedback mechanism, and (2) photosynthesis would be decreased by a separate mechanism that balances the reduced C demand due to P-limited plant growth, but would not directly control nodule growth and functioning. Thus, (3) the biomass of P-deficient white clover plants would no longer respond to elevated p_{CO_2} , not because N supply limits growth as a result of C-limited N₂ fixation, but primarily because P directly limits growth. This chain

of hypotheses was tested by growing white clover in a controlled environment. A wide range of P supply levels were combined with two atmospheric CO₂ partial pressures (p_{CO_2}) to achieve a large variation in the C source and in the requirement of N for growth in order to assess their involvement in controlling N₂ fixation. The asparagine concentration in the roots was measured as an indicator of the concentration of free asparagine in the vascular tissue in order to evaluate a possible N feedback regulation of symbiotic N₂ fixation (Hartwig, 1998). To avoid interactions between P supply and soil nutrients, quartz sand was used as the substrate together with a nutrient solution with a low concentration of ¹⁵N-enriched N.

Materials and methods

In the main experiment with nodulated plants the following key factors were measured: photosynthesis, C and N partitioning, N derived from symbiosis, and plant and nodule growth. In the nodulation experiment, the interactive effect of sub-optimal P concentrations and p_{CO_2} on the establishment of nodules was investigated.

Plant material and growth conditions

The main experiment with nodulated plants has been described in full previously (Almeida *et al.*, 1999). Briefly, uniform plants from cuttings of white clover (*Trifolium repens* L. cv. Milkanova) were grown in growth rooms (PGV-36, Conviron, Winnipeg, Canada) for 25 d (25 DAP, days after planting) in boxes (40 × 17.5 × 12.5 cm depth), filled with quartz sand (0.7–1.2 mm), irrigated with 0.075 mM KH₂PO₄ and 1.5 mM N in a nutrient solution, at an atmospheric p_{CO_2} of 35 Pa, day/night temperatures of 18/13 °C, relative humidity of 75%, light/dark periods of 16/8 h, and an irradiance of 550 μmol m⁻² s⁻¹ PAR. At 25 DAP, the nutrient solution was replaced by four solutions with different concentrations of P (0.0027, 0.075, 0.67, and 2 mM KH₂PO₄) and combined with two atmospheric p_{CO_2} (35 Pa = ambient, 70 Pa = elevated), and the other conditions were maintained for an additional 30 d (55 DAP). In our experience, the P concentration in a standard nutrient solution of about 0.5 mM is optimal under similar growth conditions at ambient p_{CO_2} . The plants were inoculated with *Rhizobium leguminosarum* biovar *trifolii* (strain RBL 5020, Leiden, NL) at 0, 7 and 14 DAP. From 25 to 55 DAP, the 1.5 mM N in the nutrient solution was labelled with 0.9% U-¹⁵N NH₄NO₃ (Isotec, Mimsburg, OH and Matheson, Secaucus, NJ, USA). The experiment was conducted in a randomized complete block design with four replicates.

Nodulation experiment: white clover cuttings were grown and inoculated under the same conditions as in the main experiment. However, from the beginning (0 DAP), two levels of suboptimal P (0.0027 and 0.075 mM KH₂PO₄) were combined with two levels of atmospheric p_{CO_2} (35 and 70 Pa) for 44 d. The experiment was conducted in a randomized complete block design with four replicates.

Plant sampling

Plants were harvested at 25 and 55 DAP in the main experiment with nodulated plants and at 0, 22 and 44 DAP in the nodulation experiment; they were separated into root and shoot fractions at each harvest (Almeida *et al.*, 1999). The samples of the first plant harvested from each pot were frozen

immediately on dry ice and stored at -20°C until lyophilization. After lyophilization, nodules were separated from roots and cleaned under a dissection microscope. The samples were then used for analysing non-structural carbohydrates and for determining total P and N content. The samples of the second plant harvested from the same pots were oven-dried at 65°C for 48 h, weighed, and pooled for analysis of whole plant contents of N, ^{15}N , and P.

Chemical analyses

Water-soluble carbohydrate (WSC), starch, and total P contents were determined as described previously (Almeida *et al.*, 1999). Total N and ^{15}N were determined from 1 mg ground plant material using a continuous-flow mass spectrometer (Europa Scientific, Cambridge, UK). The concentrations of N in leaves, roots, and nodules were calculated on the basis of the dry mass corrected for starch content. Asparagine concentrations in freeze-dried, ground material were determined using a standard enzymatic assay (Boehringer, Mannheim, Germany). The relative contribution of symbiotically fixed N to total plant N ($\%N_{\text{sym}}$) was determined using the ^{15}N -isotope-dilution method (Sangakkara *et al.*, 1996a) from 25 to 55 DAP.

Leaf gas exchange

Rates of photosynthetic CO_2 assimilation (A) on the youngest fully unfolded leaf were determined on each plant in the morning at 25 and 55 DAP with an infrared gas analyser (Ciras-1, PP-Systems) equipped with a Parkinson leaf cuvette (type Broad Leaf, PP-Systems) and an incandescent light unit operating at near saturating PAR ($870\mu\text{mol m}^{-2}\text{ s}^{-1}$ PAR). The p_{CO_2} concentration in the leaf cuvette was set to different values (5, 10, 15, 25, 35, 70, 90, 110, and 150 Pa) in order to obtain different internal concentrations (C_i) in the leaf.

The apparent maximum rate of carboxylation with non limiting ribulose-1,5-biphosphate ($V_{c,\text{max}}$) and the apparent potential rate of electron transport at light saturation (J_{max}) were calculated for each leaf from the fit of the A/C_i values to the biochemical model of Farquhar and von Caemmerer (Farquhar and von Caemmerer, 1982) as described by Kirschbaum and Farquhar (Kirschbaum and Farquhar, 1984). Stomatal limitation of photosynthesis (l) was calculated using A recorded at the growing p_{CO_2} regime (C_a) and the photosynthetic rate (A_0) estimated from the A/C_i response at $C_i=C_a$ according to $l=100\times(A_0-A)/A_0$ (Long and Drake, 1992).

Statistical data analysis

Statistical analyses were carried out using the ANOVA procedure of the SAS statistical analysis package (SAS Institute, Cary, NC, USA). If needed, data were transformed to correct for non-normal distribution and non-homogeneity of variance. The 95% confidence level was used in all tests.

Results

Nodule mass and plant growth

In the main experiment, low P concentration in the nutrient solution strongly reduced the growth of white clover plants (Fig. 1A). The effect of elevated p_{CO_2} on total plant mass depended on the P concentration of the nutrient solution; at low P no increase in plant mass occurred, whereas at the highest P concentration plant mass increased by 30%. When plants had developed

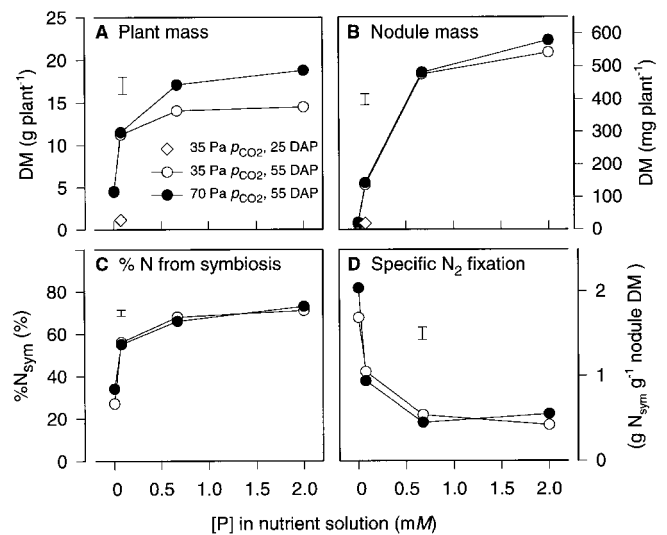


Fig. 1. Total plant mass (A), nodule mass per plant (B), percentage N derived from symbiosis (C), and specific N₂ fixation rate (D) of white clover plants grown for 30 d (25 DAP to 55 DAP, main experiment) at four concentrations of P in the nutrient solution (0.0027, 0.075, 0.67, and 2 mM) and at two atmospheric p_{CO_2} . $n=4$; bar=standard error of mean.

nodules, by 25 DAP at 0.075 mM P (Fig. 1B), lowering the P concentration to 0.0027 mM stopped further nodule growth for the remaining 30 d of the experiment (no increase in nodule mass between 25 and 55 DAP). At 0.075 mM P, nodule growth continued from 25 to 55 DAP and increased further with increasing P concentration. Atmospheric p_{CO_2} had no effect on nodule mass (Fig. 1B). As a result of the stronger effect of the P concentration of the nutrient solution on nodule mass (Fig. 1B) than on total plant mass (Fig. 1A), the mass ratio (nodule/total plant) strongly increased with increasing P concentration, independent of p_{CO_2} (data not shown).

In the nodulation experiment, a very low concentration of P in the nutrient solution (0.0027 mM P) from the beginning of growth prevented nodulation at both elevated and ambient p_{CO_2} , while nodulation occurred at 0.075 mM P (Table 1). With 0.075 mM P in the nutrient solution, plant mass increased considerably compared to 0.0027 mM P in the nutrient solution.

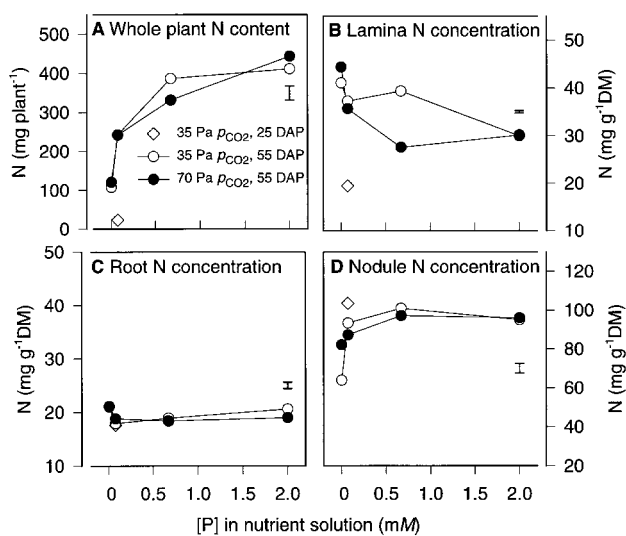
$\%N_{\text{sym}}$ and N partitioning

The $\%N_{\text{sym}}$ integrated from 25 to 55 DAP increased with increasing P in the nutrient solution, but was not affected by p_{CO_2} (Fig. 1C). Specific N₂ fixation (N from symbiosis/nodule mass) decreased with increasing P concentration but was not affected by p_{CO_2} (Fig. 1D).

Total N per plant at 55 DAP increased with increasing P in the nutrient solution (Fig. 2A). Reducing the P concentration to 0.0027 mM for 30 d impaired N assimilation to a much lesser extent (N content at 25 DAP versus 55 DAP) than it did plant growth (Fig. 1A). As a result,

Table 1. Nodule and plant mass of white clover plants grown for 44 d (0–44 DAP, nodulation experiment) at two concentrations of P in the nutrient solution and at two atmospheric p_{CO_2} Inoculation with *Rhizobium leguminosarum* bv. *trifolii* took place at 0, 7 and 14 DAP. $n=4$.

P (mM)	p_{CO_2} (Pa)	Nodules (mg DM)	Plant (g DM)
0.0027	35	0 a	0.3 a
	70	0 a	0.3 a
0.075	35	50.9 b	3.7 b
	70	34.6 b	5.1 c
ANOVA	p_{CO_2}	P ns	$P < 0.05$
	P	$P < 0.001$	$P < 0.001$
	$p_{\text{CO}_2} \times \text{P}$	P ns	$P < 0.05$

Values are re-transformed means; when referenced with the same letter, no significant differences at $P < 0.05$.**Fig. 2.** N content per plant (A) and N concentrations of leaf laminae (B), roots (C), and nodules (D) based on DM corrected for the content of starch of white clover plants grown for 30 d (25 DAP to 55 DAP, main experiment) at four concentrations of P in the nutrient solution (0.0027, 0.075, 0.67, and 2 mM) and at two atmospheric p_{CO_2} . $n=4$; bar=standard error of mean.

the concentration of N in the whole plant (not shown) and in starch-free leaf dry mass at 55 DAP was highest at 0.0027 mM P and decreased with increasing P concentration (Fig. 2B). At 0.67 mM P, the concentration of leaf N was lower under elevated p_{CO_2} than under ambient p_{CO_2} . The concentration of N in the roots was affected neither by P concentration nor by p_{CO_2} (Fig. 2C). The concentration of N in the nodules was lower at a concentration of 0.0027 mM P in the nutrient solution than at the other P concentrations, especially at ambient p_{CO_2} (Fig. 2D).

Photosynthesis and carbohydrate partitioning

Increasing the concentration of P in the nutrient solution enhanced leaf photosynthesis by increasing $V_{\text{c,max}}$ and

J_{max} (Table 2). As compared to ambient p_{CO_2} , white clover leaves at elevated p_{CO_2} had a lower $V_{\text{c,max}}$ only at 0.67 mM P. At elevated p_{CO_2} , the rate of photosynthesis increased (Almeida *et al.*, 1999) due to reduced stomatal limitation of photosynthesis (Table 2). Under ambient p_{CO_2} , stomatal limitation of photosynthesis (l) was not significantly affected by the P concentration, but limitation tended to increase at 2 mM P. Under elevated p_{CO_2} , however, stomatal limitation of photosynthesis decreased with increasing P concentration.

Doubling the p_{CO_2} increased the concentration of starch in the leaves at all P concentrations in the nutrient solution (Table 3). By contrast, the concentrations of starch and WSC in the roots were not affected by p_{CO_2} or by P concentration (Fig. 3A, C). The concentration of starch in the nodules was lower and the concentration of WSC higher at the lowest P concentration than at higher P concentrations (Fig. 3B, D).

Asparagine concentration in roots and nodules

Compared to an ample P concentration in the nutrient solution, plants grown under severe P deficiency showed a 7-fold increase in the concentration of asparagine in the roots; this response was not affected by atmospheric p_{CO_2} (Table 4). There was a higher asparagine concentration in the nodules than in the roots. Plants at severe P deficiency showed a 2.5-fold increase in the concentration of asparagine in the nodules (determined only at elevated p_{CO_2} due to insufficient sample material, Table 4).

Discussion

Growth of white clover and its response to elevated p_{CO_2} was not limited by N assimilation under P deficiency

The total N concentration in the plant (not shown) and in the leaves (Fig. 2B) and the N/P mass ratio (Fig. 4) were highest under severe P deficiency. This clearly suggests that plant growth and growth response to elevated p_{CO_2} were not limited by N assimilation under P deficiency. The same conclusion for plant growth at ambient p_{CO_2} has been reached by other authors (Robson *et al.*, 1981; Jakobsen, 1985; Israel, 1987). In this experiment, the higher concentration of N in the lowest P treatment was not a developmental effect linked to the smaller plants (Farrar and Williams, 1991). This is evident from the fact that, at the lowest P supply, the concentration of N at 55 DAP was higher than that of the younger and smaller plants harvested at 25 DAP. Under severe P deficiency, N assimilation from the mineral and symbiotic sources obviously exceeded the amount of N required for plant growth during the experimental period in both p_{CO_2} treatments; it was the capacity of the plant to use the total assimilated N that was limited. This is strongly supported by the N/P ratio in the plants (Fig. 4), indicat-

Table 2. Apparent maximum rate of carboxylation with non-limiting RubP ($V_{c,max}$), apparent potential rate of electron transport at saturated light (J_{max}), and stomatal limitation of leaf photosynthesis (l) of white clover plants grown for 30 d (25–55 DAP, main experiment) at four P concentrations in the nutrient solution and at two atmospheric p_{CO_2} ; $n=4$

DAP	P (mM)	p_{CO_2} (Pa)	$V_{c,max}$ ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	J_{max} ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	l (%)
25 ^a	0.075	35	93.4 ± 7.1	248 ± 29.5	31.9 ± 5.1
55 ^b	0.0027	35	36.6 a	95.9 a	31.9 ab
		70	39.2 a	120 ab	26.7 ab
	0.075	35	64.1 b	153 bc	37.9 a
		70	53.4 ab	149 b	19.6 bc
	0.67	35	118 c	221 d	34.0 ab
		70	82.5 bd	192 cd	14.3 c
	2	35	130 c	218 d	48.0 a
		70	105 cd	214 d	12.4 c
ANOVA	p_{CO_2}		$P < 0.001$	P ns	$P < 0.001$
	P		$P < 0.001$	$P < 0.001$	P ns
	$p_{CO_2} \times P$		$P < 0.05$	$P < 0.05$	$P < 0.01$

^aResults from 25 DAP are means ± standard error of mean.

^bResults from 55 DAP are re-transformed means; when referenced with the same letter, no significant differences at $P < 0.05$. ANOVA calculated for 55 DAP.

Table 3. Concentration of starch in the leaves of white clover plants grown for 30 d (25–55 DAP, main experiment) at four P concentrations in the nutrient solution and at two atmospheric p_{CO_2} ; $n=4$

DAP	p_{CO_2} (Pa)	P (mM)			
		0.0027	0.075	0.67	2
		(mg glucose equivalent g ⁻¹ DM)			
25 ^a	35	278 ± 18.7			
55 ^b	35	191a	147 a	312 b	241 c
	70	368 bd	299 bc	425 d	372 bd
ANOVA:		p_{CO_2} , $P < 0.001$; P, $P < 0.001$; $p_{CO_2} \times P$, $P < 0.01$			

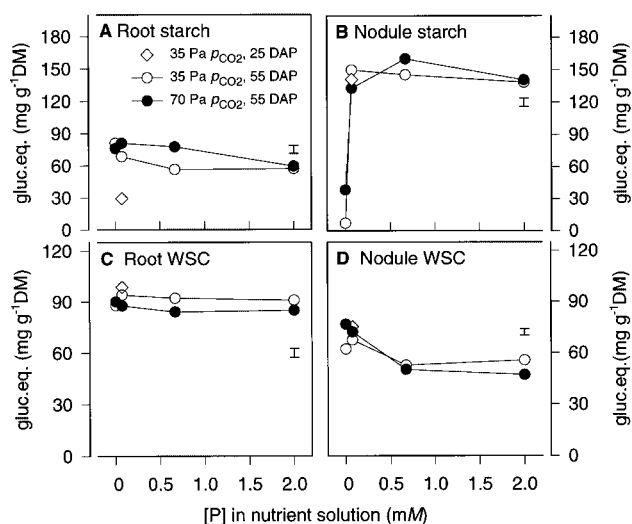
^aResult from 25 DAP is mean ± standard error of mean.

^bResults from 55 DAP are re-transformed means; when referenced with the same letter, no significant differences at $P < 0.05$.

ing that the plants in the 0.075 mM P treatment were slightly limited, whereas those in the 0.0027 mM P treatment were severely limited by P nutrition (Dunlop and Hart, 1987). Accordingly, P concentration in the leaves increased strongly with increasing P supply (Almeida *et al.*, 1999). Therefore, the fact that P limited plant growth and the plant's response to elevated p_{CO_2} (Fig. 1A; Almeida *et al.*, 1999) was not due to a limiting effect of low P on the assimilation of N.

Supply of photoassimilates did not limit growth and functioning of nodules

The decrease in the rates of leaf photosynthesis with decreasing P supply (Almeida *et al.*, 1999) was due to a decrease in the Rubisco activity (decline of $V_{c,max}$) and a simultaneous reduction in the regeneration of RubP (decline of J_{max}) (Brooks, 1986; Rao, 1997). The lower $V_{c,max}$ may have been due to a decrease in the activation state of Rubisco rather than to a decrease in the total concentration of Rubisco, since the concentration of N

**Fig. 3.** Concentration of starch in the roots (A) and nodules (B) and concentration of water-soluble carbohydrate (WSC) in the roots (C) and nodules (D) of white clover plants grown for 30 d (25 DAP to 55 DAP, main experiment) at four concentrations of P in the nutrient solution (0.0027, 0.075, 0.67, and 2 mM) and at two atmospheric p_{CO_2} . $n=4$; bar = standard error of mean.

in the leaves increased with decreasing P supply. Indeed, P-deficient tobacco does not show lower levels of protein or lower concentrations of Rubisco (Paul and Stitt, 1993). The mechanisms that reduce $V_{c,max}$ are unclear, because low P limits growth directly and the expression of photosynthetic genes usually responds to N nutrition (Paul and Stitt, 1993).

The lower $V_{c,max}$ under elevated p_{CO_2} at medium P supply suggests an adaptive response of the photosynthetic apparatus to elevated p_{CO_2} . The balance between the C source and the C sink was probably maintained even when N was allocated for other processes (Stitt,

Table 4. Concentration of asparagine in the roots and nodules of white clover plants grown for 30 d (25–55 DAP, main experiment) at four P concentrations in the nutrient solution and at two atmospheric p_{CO_2} ; $n=4$

	p_{CO_2} (Pa)	P (mM)			
		0.0027 ($\mu\text{mol asparagine g}^{-1} \text{DM}$)	0.075	0.67	2
Roots	35	588	106	107	74
	70	650	76	57	108
	ANOVA:	SE: 30.5 p_{CO_2} , P ns; P, $P < 0.0001$; $p_{CO_2} \times P$, P ns			
Nodules	35	nd	500	509	479
	70	1115	391	457	526
	ANOVA	nd ^a	SE: 106		

^and: Not determined (due to insufficient sample material).

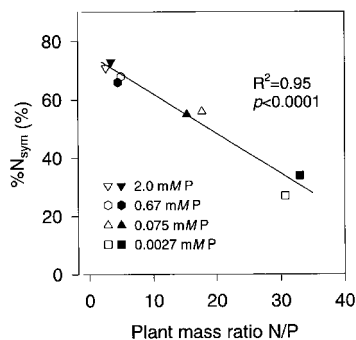


Fig. 4. Relationship between percentage N derived from symbiosis ($\%N_{\text{sym}}$) and total plant mass ratio N/P of white clover plants grown for 30 d (25 DAP to 55 DAP, main experiment) at four concentrations of P in the nutrient solution and at two atmospheric p_{CO_2} . (35 Pa = open symbols; 70 Pa = filled symbols).

1991; Long and Drake, 1992), as suggested by the concomitant decrease in the starch-corrected concentration of N in the leaves under elevated p_{CO_2} . It is likely that the reduction in $V_{c,\text{max}}$, observed at the lower P concentration in the nutrient solution or under elevated p_{CO_2} , was due to a C sink limitation (Rao, 1997), as suggested by the high starch contents in the leaves and the lower growth rate of the plants. This interpretation is further supported by the fact that photosynthesis in these leaves was stimulated by CO_2 only in the morning (Almeida *et al.*, 1999). This increased photosynthetic performance at elevated p_{CO_2} , however, had no effect on the growth and functioning of the nodules.

Assuming that the root buffers the supply of water soluble carbohydrate (WSC) to nodules, the higher concentration of WSC in the roots than in the nodules suggests that a large source of WSC is available for the nodules in all treatments. Furthermore, when the concentration of P was low in the nutrient solution, the concentration of WSC in the nodules was highest compared with the other P treatments (Fig. 3D), indicating that P-deficient nodules were the weakest sink for WSC. With

increasing P supply, the concentration of WSC in the nodule decreased, indicating a stronger sink which may have resulted in the increase in the concentration of starch to the initial value (Fig. 3, at 25 DAP). Hence, poor growth or performance of the nodules in our experiment did not appear to be due to a limited supply of C.

Low P inhibits nodulation and N_2 fixation to a greater extent than plant growth

Nodulation of clover roots did not occur when roots were inoculated with rhizobia under severe P deficiency (Table 1). As far as is known, this is the first time that such an absolute response of nodulation to P deficiency has been reported. In nodulated plants subjected to severe P deficiency, the further growth of already established nodules apparently stopped (Fig. 1B). The extent of this effect compared to that reported in other studies might be due to the very low concentration of P that it was found possible to maintain since pure silica sand was used. In experiments with soil, an uncontrolled supply of P from the soil probably affects test results. Similarly, other environmental stresses appear to prevent nodulation: K deficiency (Sangakkara *et al.*, 1996a, b), high temperatures (Purwantari *et al.*, 1995; Sangakkara *et al.*, 1996b), and high salt concentration (Banet *et al.*, 1996).

Under P deficiency, nodulation and nodule growth were inhibited to a greater extent than plant growth; this confirms the results of studies of other plant species (Drevon and Hartwig, 1997). Thus, when the external P supply is low, nodules might not receive sufficient P to sustain growth. However, under severe P deficiency, the concentration of P in the nodule is generally much higher than in the host plant (Jakobsen, 1985; Israel, 1987, 1993; Sa and Israel, 1991; Drevon and Hartwig, 1997), and nodules appear to take up P directly from the nutrient solution (Al-Niemi *et al.*, 1998). This may explain why a relatively high concentration of P is maintained in the nodule. Thus, it is unlikely that the concentration of P in the nodules directly limits nodule growth and functioning. In fact, specific N_2 fixation increased strongly under P deficiency (Fig. 1D), indicating that the nodule continued to function even when P supply was very low.

Even though the nodules did not grow under severe P deficiency, approximately 30% of the total N assimilated by these plants was due to symbiotic N_2 fixation (Fig. 1C). Specific N_2 fixation increased (Fig. 1D), consistent with the increased apparent O_2 permeability of the nodule under P deficiency (Ribet and Drevon, 1995a, b; Drevon and Hartwig, 1997). Similar increases in specific N_2 fixation under conditions that hindered the development of nodules to a greater extent than plant growth are found in white clover under high mineral N supply (Zanetti *et al.*, 1998) and in faba bean and common bean under K deficiency (Sangakkara *et al.*, 1996a).

Nodulation and N₂ fixation are regulated by an N feedback mechanism induced by low P

The proportion of N derived from symbiosis (%N_{sym}) decreased as the N/P mass ratio in the plant increased due to the reduced P concentration in the nutrient solution (Fig. 4), suggesting that the plant's N demand had decreased. Indeed, as the concentration of P in the nutrient solution decreased, the plant mass decreased by a factor of four (Fig. 1A), leading to a very low apparent demand for N by the plant at P deficiency. However, when the requirement for N was low due to cutting (Seresinhe *et al.*, 1994), potassium deficiency (Sangakkara *et al.*, 1996a), high temperature (Sangakkara *et al.*, 1996b), or low pCO₂ (Zanetti *et al.*, 1998; Fig. 1C), then a simultaneous reduction in the assimilation of N from symbiosis and mineral sources was observed, i.e. %N_{sym} remained stable. The decrease in %N_{sym} under P deficiency (Figs 1C, 4; Cadisch *et al.*, 1993) indicates that, in this case, N from mineral sources was preferred; the mechanistic understanding of the regulation of N uptake from the different sources has yet to be established.

There are two possible explanations for this behaviour. As plants grow faster under sufficient P, the gap between the mineral N uptake from the growth substrate and the plant's N demand increases, resulting in an increase in %N_{sym} because of a depletion of mineral N in the growth substrate. Since, however, plants were supplied with nutrient solution twice a day, the more plausible explanation is that a poor P supply leads more directly to a specific reduction of symbiotic N₂ fixation. In this case, the high plant N concentration (Fig. 2B, C) and the fast senescence of leaves (Almeida *et al.*, 1999) would result in high concentrations of amino acids in the vascular tissues. Such a dramatic increase was observed in the asparagine concentration in roots and nodules (Table 4) at low P supply. Rabe and Lovatt and Rufty *et al.* also found a strong increase in the concentration of amino acids in P-deficient plants (Rabe and Lovatt, 1986; Rufty *et al.*, 1993). Fixation of N₂ is believed to be regulated by an N feedback mechanism (Hartwig, 1998). Thus both nodulation (Parsons *et al.*, 1993) and nodule performance (Heim *et al.*, 1993; Hartwig *et al.*, 1994; Neo and Layzell, 1997) are considered to be controlled by a signal of organic N from the vascular tissue.

It is reported here for the first time that there is an increase in the asparagine concentration and a concomitant reduction in %N_{sym} under P deficiency. Although it is assumed that the assimilation of mineral N is also regulated by an N feedback mechanism (Rufty *et al.*, 1990, 1993; Imsande and Touraine, 1994; Marschner *et al.*, 1996; Jeschke *et al.*, 1996, 1997), this process may have a stronger effect on symbiotic N₂ fixation compared to its effect on nitrate assimilation. It is proposed that, at low P supply, an N feedback mechanism, related to

increased organic N compounds in the vascular tissues, is induced, which down-regulates or even prevents nodulation and nodule growth, thus leading to a decrease in the %N_{sym} even if the specific N₂ fixation rate of the nodules is increased.

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