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The Influence of Phosphorus Deficiency on Growth and Nitrogen Fixation of White Clover Plants

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The effects of P deficiency on growth, N_2-fixation and photosynthesis in white clover (*Trifolium repens* L.) plants were investigated using three contrasting relative addition rates of P, or following abrupt withdrawal of the P supply. Responses to a constant below-optimum P supply rate consisted of a decline in N_2-fixation per unit root weight and a small reduction in the efficiency with which electrons were allocated to the reduction of N_2 in nodules. Abrupt removal of P arrested nodule growth and caused a substantial decline in nitrogenase activity per unit root weight, but not per unit nodule mass. Similarly, the rate of photosynthesis per unit leaf area was unaffected by abrupt P removal, whereas CO_2 acquisition for the plant as a whole decreased due to a decline in total leaf area, leaf area per unit leaf weight and utilization of incoming radiation. These changes followed the decline in tissue P concentrations. The ratio between CO_2-fixation and N_2-fixation was maintained under short-term P deprivation but increased under long-term low P supply, indicating a regulatory inhibition of nodule activity following morphological and growth adjustments. It is concluded that N_2-fixation did not limit the growth of clover plants experiencing P deficiency. A low P status induced changes in the relative growth of roots, nodules and shoots rather than changes in N and/or C uptake rates per unit mass or area of these organs.

Key words: Fodder legumes, grassland, phosphorus, phosphorus deficiency, photosynthetic efficiency, N_2-fixation, nitrogenase activity, relative addition rate, specific shoot area, *Trifolium repens* L., white clover.

INTRODUCTION

All cultivated legumes possess the ability to reduce atmospheric dinitrogen (N_2) symbiotically and to make it available to plants. Generally, restrictions on plant growth are caused by scarcity of other resources, such as phosphorus (P), which is strongly bound in soil, resulting in a low rate of diffusion towards the root surface (Tinker and Nye, 2000). The phytoavailability of P in grassland is characterized by both spatial and temporal fluctuations, following variations in soil organic matter turnover and in the diffusion of low-molecular weight inorganic and organic P compounds (Veresoglou and Fitter, 1984; Jackson and Caldwell, 1993). P deficiency occurs in semi-natural and low-input managed grasslands and may affect the ability of fodder legumes, particularly white clover, to obtain an input of N that is sufficient to ensure a productive system.

Under environmental stress, most N_2-fixing legumes are capable of maintaining a high metabolic activity in their root nodules (Walsh, 1995). However, nitrogenase activity is particularly sensitive to abiotic stresses such as salinity (Serraj *et al*., 1994) and drought (Durand *et al*., 1987).

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The relationship between low P supply and N_2-fixation is not clear. 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. 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; Jacobsen, 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).

Environmental stress causes species-specific responses of N_2-fixation activity. The responses will reflect the duration and severity of the stress, the plant growth history, and effects on nodulation and nodule anatomy (Walsh, 1995). In the short term there may be considerable decoupling of plant nutrient uptake and plant growth as it may take some time before a specific nutrient becomes limiting. In such dynamic cases, theories such as the resource-ratio hypothesis (Hunt and Nicholls, 1986) and the multiple-limitation hypothesis (Chapin *et al*., 1987) do not necessarily provide adequate explanations as they have been developed to predict plant responses under steady-state conditions. The theories may, however, be valuable tools for the prediction of longer-term adaptations where plants are expected to allocate resources

preferentially to those functions that most strongly limit growth (Bloom *et al*., 1985).

The aim of the present studies was to investigate the responses of N_2-fixation, growth and morphology of white clover plants to P deficiency. To obtain an integrated view of the effects of P deficiency, experiments that simulated long-term steady-state situations were conducted using different relative addition rates of P (Ingestad, 1982; Ingestad and Ågren, 1988). Allowing plants to adapt to specific P conditions, however, gives no indication of when and where primary metabolic regulation takes place. Therefore, in subsequent experiments, the P supply was withdrawn abruptly and plants were fully deprived of external P for 3 weeks to study the short-term dynamic responses.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of white clover (*Trifolium repens* L. 'Milkanova') were germinated in vermiculite. After 1 week, plants were inoculated with a *Rhizobium leguminosarum* biovar *trifolii* strain (WPBS5 of IGER, Aberystwyth, Wales) that is known to lack uptake hydrogenase activity, thus enabling the measurement of H_2 evolution for nitrogenase activity. Seed germination and plant growth were carried out in a controlled environment chamber at 75 % relative humidity, with a 16/8 h day/night length, a 20/15 °C day/night temperature and a light intensity (photosynthetically active radiation) of approx. 300 μmol photons m^{-2} s^{-1} at plant level (Powerstar HQI-T 400 W/D, Osram, Germany).

While growing in the vermiculite medium, plants were watered twice a week with an N-free nutrient solution containing (mmol m^{-3}): 400 $CaCl_2$; 200 $MgSO_4$; 400 K_2SO_4; 100 NaH_2PO_4; 50 H_3BO_3; 50 $FeC_6H_5O_7$; 20 $MnSO_4$; 2 $ZnSO_4$; 1 Na_2MoO_4; 0·5 $CuSO_4$; 0·5 $NiSO_4$; and 0·5 $CoCl_3$. Four weeks after planting, samples of six trifoliate plants were transplanted to 4-l containers that were purged by ambient air. The plant samples were fixed in the lid by an inert plastic material (Terostat®; Henkel Surface Technologies, Philadelphia, PA, USA), positioning most of the nodules above the nutrient solution. The solution was renewed twice a week until the experimental period started and was then changed three times per week to maintain the nutrient content. After renewal, the biological buffer MES [2-(*N*-Morpholino)-ethanesulfonic acid; 10 ml of a 750 mmol m^{-3} solution at pH 6·0] was added to control the pH in the nutrient solution.

Experimental protocols

Steady-state experiments were conducted after white clover plants had adapted to three different growth rates controlled by relative addition rates (RAR), four containers per RAR, following the equation (Ingestad, 1982; Ingestad and Ågren, 1988):

$$\text{net daily P addition} = P(t+1) - P(t) = P(t) \times (e^{RAR} - 1) \quad (1)$$

where $P(t)$ is the P content per unit plant dry weight at time, t.

Treatments started when plants were 7 weeks old (after sowing) and continued for 3 weeks. The RAR treatments of 0·03, 0·06 and 0·10 g P g^{-1} P d^{-1} were obtained by supplying the required amounts of P each morning [eqn (1)]. Before adding P, the nutrient solution was sampled and kept frozen (–20 °C) until analysis. After the plants had adapted to these treatments for 21 d, they were subjected to gas exchange measurements (completed within 2 d).

After transfer from the 100 mmol m^{-3} P concentration in the pre-experimental nutrient solution, plants adjusted to the new lower P supply of the RAR treatments by effluxing P during the first days after transfer. Assessed on the basis of the amount of P added and the amount remaining just before the next addition on the following day, it was evident that roots from plants in RAR 0·03, 0·06 and 0·10 treatments showed a net efflux of P during the first 12, 6 and 4 d, respectively. Thereafter, plants at RAR 0·03 and 0·06 had depleted the P content of the nutrient solution entirely by the end of the 24 h period between P additions, whereas plants subjected to the 0·10 RAR treatment were not able to absorb all of the P supplied on a daily basis.

The short-term experiment, which aimed to investigate the effects of abrupt P deprivation, was conducted after growing plants with a non-limiting P supply (100 mmol m^{-3}) for 10 weeks after sowing, after which the P supply was withdrawn from half of the plants.

For both groups of plants, controls and P-deprived, measurements started at the point of P withdrawal. Subsequently, measurements was carried out every 3–4 d until the final sampling after 17 d. Four replicate containers, each with six clover plants, were sampled at random on each measurement day.

Shoot measurements

Shoot CO_2 and water vapour exchanges were measured using a differential CO_2/H_2O infrared gas analyser (Ciras-1; PP Systems, Hitchin, UK), recording the difference between the inlet and outlet concentration of the two gases. Each single container was placed in a 25-l perspex cuvette, subjected to a flow rate of 30 l air min^{-1}.

Chlorophyll fluorescence (FMS-1; Hansatech Instruments Ltd, Pentney, UK) was determined on three replicate leaves per pot over a period of 6 min following 30 min dark-adaptation. The youngest fully expanded leaves were selected and a light intensity equivalent to the ambient light of 300 μmol photons (PAR) m^{-2} s^{-1} was used.

Phloem sap was sampled using the exudation in EDTA technique (Urquhart and Joy, 1981). Sampling starting at 0900 h by excising three leaves per container, immersing their petioles immediately into 5 ml 20 mol m^{-3} EDTA (pH 7·0), and placing them in a dark container in a water-saturated atmosphere (Caputo and Barneix, 1997). After 6 h, leaves were blotted dry and weighed. The solution containing the phloem exudates was stored at –80 °C until analysed for amino acid composition using HPLC (Waters AccQ-Tag

TABLE 1. *Nitrogen and phosphorus accumulation (mg per plant) and content (%) in dry matter of white clover plants adapted to grow under different long-term steady-state P treatments controlled by the relative daily addition rate of P*

Treatment (g P g^{-1} P d^{-1})	Root N (mg)	Shoot N (mg)	Root P (mg)	Shoot P (mg)
0·03	9·99 (0·61)[a]	18·98 (0·58)[a]	0·75 (0·06)[a]	0·85 (0·04)[a]
0·06	12·90 (0·17)[a]	25·54 (1·62)[ab]	1·12 (0·08)[b]	1·49 (0·10)[b]
0·10	12·27 (1·44)[a]	31·27 (4·12)[b]	1·31 (0·10)[b]	3·07 (0·29)[c]
	(%)	(%)	(%)	(%)
0·03	3·24 (0·11)[a]	2·59 (0·10)[a]	0·24 (0·00)[a]	0·12 (0·00)[a]
0·06	3·96 (0·06)[b]	3·30 (0·05)[b]	0·34 (0·02)[b]	0·19 (0·01)[b]
0·10	4·25 (0·12)[b]	3·60 (0·19)[b]	0·46 (0·02)[c]	0·36 (0·03)[c]

Values shown in parentheses are means of four observations ± s.e. Different superscripts within a column indicate differences on a 5 % level.

3·9 × 150 mm column; Waters 474, Milford, MA, USA).

Root measurements

The lids in which the plants were fixed were transferred to the containers for gas exchange measurements and left to adjust for 1 h. The tightly fitting lid and the plastic material around the stem bases enclosed the root compartment effectively. All root gas exchange measurements were conducted using an open-flow system in conjunction with a flow-through H_2 analyser (Layzell *et al*., 1984) with a flow-rate of 1000 ml min^{-1} and a root volume of 1200 ml of which 200 ml contained a P-free nutrient solution. CO_2 emission from nodulated roots was measured using a differential CO_2/H_2O infrared gas analyser (Ciras-1). Nitrogenase (EC 1.7.99.2) activity was determined by measuring H_2 evolution in 79 : 21 (v/v) mixtures of $N_2 : O_2$ and $Ar : O_2$ obtained by mixing high purity (99·999 %) gases using mass-flow controllers. To obtain the correct rate of N_2-fixation, the values for nitrogenase activity were adjusted using the electron allocation coefficient (EAC) defined as 1-(ANA/TNA), where ANA and TNA denote the apparent and total nitrogenase activity, measured as H_2 evolution in the $N_2 : O_2$ and $Ar : O_2$ mixtures, respectively. ANA and the initial root respiration were measured for 5 min in the $N_2 : O_2$ gas mixture and then in the $Ar : O_2$ mixture. TNA was determined as the peak H_2 evolution. Following the peak, the argon-induced decline was determined as the decline after 30 min. The EAC values obtained were between 0·55 and 0·69, which corresponds with the near-optimum values reported by Hunt and Layzell (1993).

The H_2 concentration in the dried gas stream was determined using a calibrated H_2 sensor (Qubit Systems Inc., Kingston, Canada). The sensor was reconditioned each morning (Layzell *et al*., 1989) by injecting 2 ml pure H_2 into the gas stream.

Tissue analysis

Following gas exchange measurements, roots were rinsed in de-ionized water, excised and blotted dry. Fresh weight (f. wt) of roots and shoots was determined. Nodules were removed from the root material and weighed. The shoot leaf area was measured (one-side) using an LI-3100 Area Meter (LICOR Inc., Lincoln, NE, USA). The plant material was frozen (–20 °C), freeze-dried to constant weight and weighed again before being ground (mesh size of 0·2 mm).

Nitrogen and phosphorus determination

Nitrogen in the ground plant material was analysed using an ANCA-SL Elemental Analyser coupled to a 20-20 Tracermass Mass Spectrometer (Europa Scientific Ltd, Crewe, UK) using the Dumas combustion method. The remaining plant material was dry-ashed at 550 °C for 3 h, solubilized in 3 M HCl, dried and solubilized again in 1 M HNO_3 before filtering. The P concentration in the plant digests and in samples taken from the nutrient solution in the long-term steady-state experiment was determined by UV-VIS spectrophotometry using the molybdo-phosphoric blue method of Murphy and Riley (1962).

Statistical analysis

Data were analysed by regression analysis using the SAS ANOVA procedure (SAS Institute Inc., 1993). Comparison of the means for the individual treatments was done using a Waller–Duncan *t*-test.

RESULTS

Steady-state responses to different P supply rates

Total dry matter production was reduced (P = 0·07) from 1·17 g per plant at RAR 0·10 to 1·10 and 1·05 g per plant at RAR 0·06 and 0·03, respectively. The shoot : root ratio decreased from 2·94 to 2·38 when RAR was reduced from 0·10 to 0·03 (data not shown). Plants that had adapted to the lowest P supply rate also contained the smallest amount of N and P in the shoots and roots (Table 1). As the dry matter yield was only slightly decreased as a result of reduced P supply, this difference was mainly due to a lower N (P < 0·05) and P (P < 0·005) concentration in the plant dry matter at

TABLE 2. *N_2-fixation and net photosynthesis in white clover plants grown under different long-term steady-state P treatments controlled by the relative daily addition rate of P*

Relative addition rate (g P g^{-1} P d^{-1})	N_2-fixation* (µmol N_2 g^{-1} root f. wt h^{-1})	Net photosynthesis (µmol CO_2 m^{-2} shoot surface area s^{-1})	Net photosynthesis (µmol CO_2 s^{-1} on total shoot basis)	Ratio net photosynthesis : N_2-fixation (mmol CO_2 $µmol^{-1}$ N_2)	Specific leaf area (cm g^{-1} DM)
0·03	3·02 (0·21)[a]	10·6 (2·2)[a]	0·47 (0·09)[a]	302 (86)[a]	103 (6)[a]
0·06	3·50 (0·08)[a]	9·4 (0·7)[a]	0·58 (0·05)[a]	158 (14)[ab]	134 (6)[b]
0·10	4·68 (0·53)[b]	8·1 (0·5)[a]	0·59 (0·06)[a]	94 (14)[b]	142 (4)[b]

Values are means of four observations (± s.e.). Different superscripts within a column indicate differences on a 5 % level.
* Nodules were mistakenly not separated from the roots, thus N_2-fixation could not be calculated on a nodule basis.

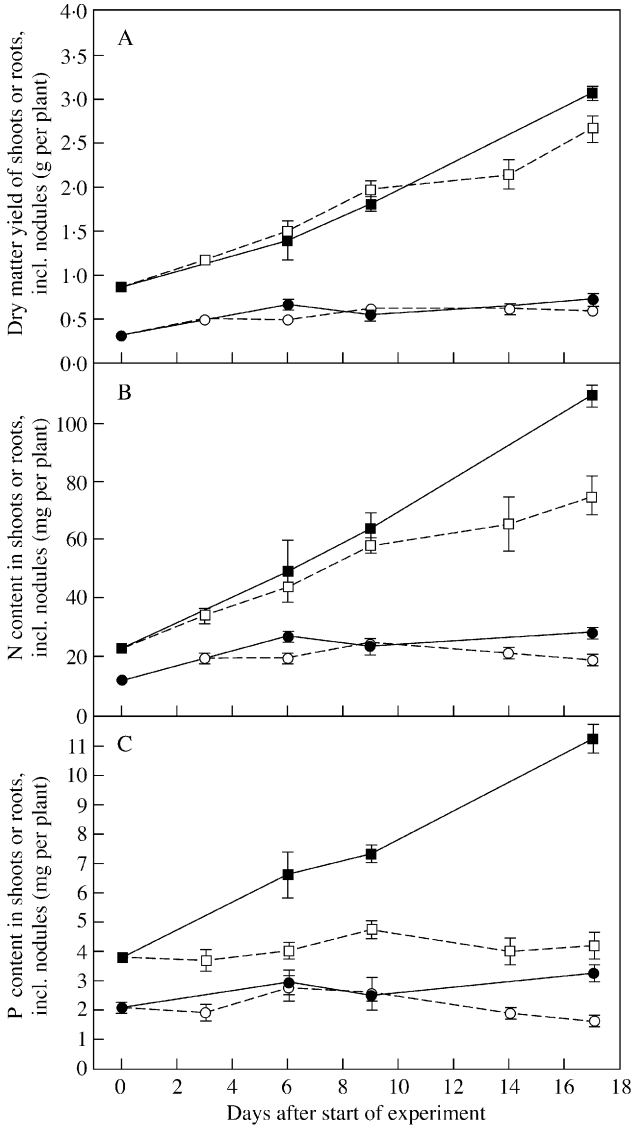

FIG. 1. Dry matter yield (A), and content of nitrogen (B) and phosphorus (C) in shoots (squares) and roots (circles) of white clover plants grown with a continuous supply of phosphorus (closed symbols) or abruptly deprived of phosphorus (open symbols) following withdrawal of the external P supply. Values are means of four observations. Bars represent ± s.e.

the lowest RAR. There was a decline in N_2-fixation from 4·68 to 3·02 µmol N_2 g^{-1} root f. wt h^{-1} when the P supply was reduced from 0·10 to 0·03 g P g^{-1} P d^{-1} (Table 2). This was associated with an electron allocation efficiency (EAC) of 55 % (s.e. = 0·3 %) at RAR 0·03 compared with 62 % (s.e. = 2·0 %) in the other two cases. The argon-induced decline in nitrogenase activity was 3 % at RAR 0·03, and approx. 8 % in the other two treatments.

Net photosynthesis per unit shoot surface area declined with decreasing RAR on a total shoot basis (Table 2). Mean rates of root respiration were 628, 774 and 798 µmol CO_2 g^{-1} root f. wt h^{-1} at RAR 0·03, 0·06 and 0·10. This decrease (P = 0·09) may indicate a lower growth rate following reduced P supply.

The ratio of total net CO_2-fixation to N_2-fixation was much higher at a lower P supply than at a higher P supply (Table 2), suggesting a regulatory inhibition of N_2-fixation following reduced N demand. Furthermore, shoot morphology changed with P supply in the sense that the specific leaf area (SLA) was reduced (P < 0·05) to 103 cm^2 g^{-1} at RAR 0·03 compared with 142 cm^2 g^{-1} at the highest RAR (Table 2).

Short-term dynamic responses to P withdrawal

Growth. Total withdrawal of the P supply for the last 3 weeks of the experiment did not (P > 0·05) affect the total dry matter yield of single white clover plants (Fig. 1A). The shoot : root ratio was not (P > 0·05) affected by P withdrawal within the experimental period, with mean values increasing from 2·76 at the onset of P deprivation to 4·52 on day 17. The relative growth rates of shoots and roots were similar for P-starved and control plants at 0·163 d^{-1} for shoots and 0·185 d^{-1} for roots.

Withdrawal of P did, however, result in lower N (P < 0·008) and P (P < 0·05) concentrations in the dry matter compared with concentrations in plants with full access to P in the nutrient solution (Fig. 1B and C). Thus, while control plants continued to accumulate P throughout the experimental period, accumulation of P in the shoots of P-deprived plants ceased and that in the roots even declined slightly. Nodule N constituted 33 and 6·3 % of total root-N and total plant-N, respectively; corresponding values for nodule P were 24 and 5·4 %.

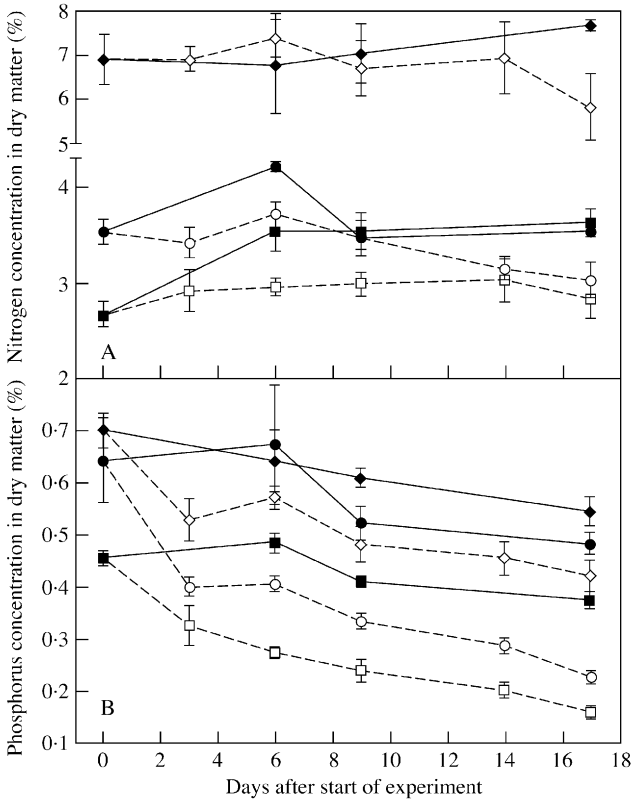

FIG. 2. Concentration of nitrogen (A) and phosphorus (B) in shoots (squares), roots (circles) and nodules (diamonds) of white clover plants grown with a continuous supply of phosphorus (closed symbols) or abruptly deprived of phosphorus (open symbols) following withdrawal of the external P supply. Values are means of four observations. Bars represent ± s.e.

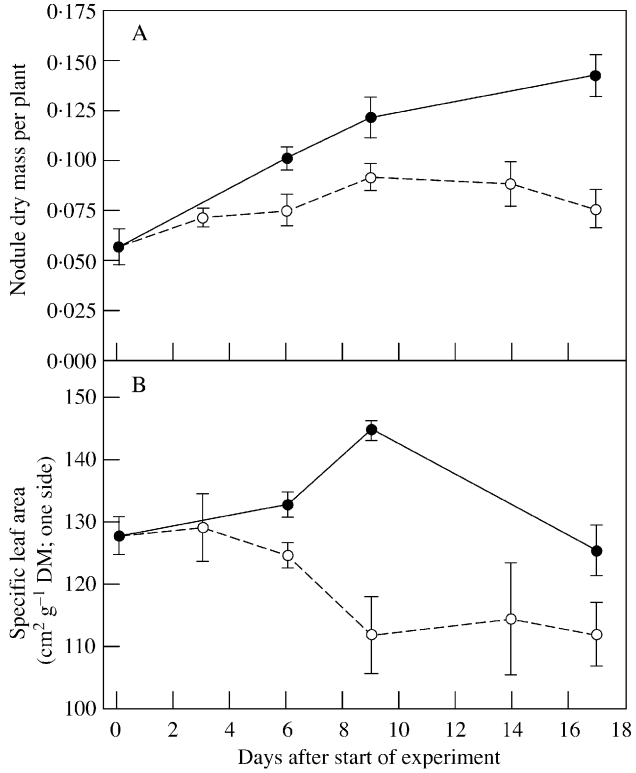

FIG. 3. Nodule dry mass (A) and specific leaf area (B) of white clover plants grown with a continuous supply of phosphorus (closed symbols) or abruptly deprived of phosphorus (open symbols) following withdrawal of the external P supply. Values are means of four observations. Bars represent ± s.e.

Nutrient concentrations in the dry matter. The N concentration in the dry matter of roots, including nodules, declined by approx. 20 % (Fig. 2A) following P withdrawal. The P concentration in the dry matter was initially high, at 0·46, 0·65 and 0·70 % in shoots, roots and nodules, respectively. Following P withdrawal, tissue P concentration decreased to a greater extent than tissue N concentration, and the decline was greater in roots (65 %) and shoots (53 %) than in nodules (40 %; Fig. 2B). The P concentration in the P-sufficient control plants declined by 21 % with plant age. The concentration of P in nodules was consistently higher than that in roots and shoots, and roots had a higher P concentration in the dry matter than shoots.

Nodule dry mass and specific leaf area. Nodule dry mass continued to increase in control plants, whereas values for P-deprived plants reached their highest levels around 9 d after P withdrawal (Fig. 3A). At 6 d after P deprivation, the SLA was lower (P = 0·06) in P-deprived plants than in control plants, and this difference persisted (P < 0·05) (Fig. 3B).

N_2-fixation and photosynthesis. Nitrogenase activity declined by approx. 17 % under continuous P supply and by approx. 40 % under P deprivation when expressed as N_2-fixation per unit root f. wt (Fig. 4A). However, when expressed on a per unit nodule mass basis, P-deprived plants tended to maintain a higher specific nitrogenase activity than control plants (Fig. 4B). This difference was already significant (P < 0·05) 9 d after P withdrawal. The EAC and argon-induced decline of H_2 efflux from the nodules were not affected by P supply (data not shown), maintaining mean values of 69 and 12 %, respectively.

In the case of photosynthesis, the efficiency with which incoming radiation was utilized (ΦPSII) was lower in P-deprived plants than in controls by the end of the experimental period (Fig. 5). Non-photochemical quenching (i.e. radiating heat; qNP) was higher (P < 0·05) in P-deprived plants (Fig. 5), whereas photochemical quenching remained unaffected (data not shown).

Specific CO_2 exchange tended to decrease with plant age in both treatments throughout the 18 d experimental period, but net CO_2-fixation per shoot of P-starved plants was lower than that of controls (P < 0·01), whereas the specific CO_2 exchange per unit shoot area was not affected by P supply (Fig. 6). Specific root respiration also decreased with time after withdrawal of P, from a mean of 670 μmol CO_2 g^{-1} root f. wt s^{-1} at the start of the experiment to 582 μmol CO_2 g^{-1} root f. wt s^{-1} in controls and 365 μmol CO_2 g^{-1} root f. wt s^{-1} in P-deprived plants, respectively, after 17 d (data not shown)

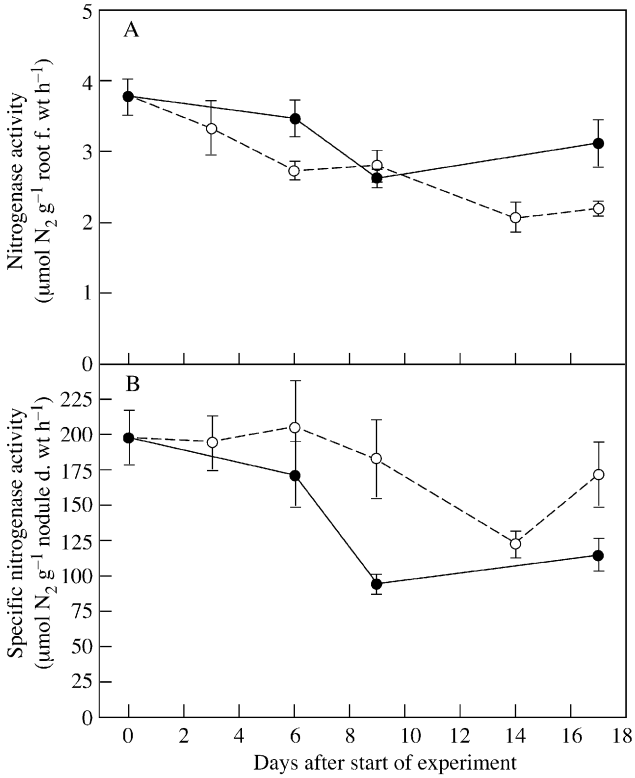

FIG. 4. Nitrogenase activity per unit root fresh weight (A) and nitrogenase activity per unit nodule dry weight (B) in white clover plants grown with a continuous supply of phosphorus (closed circles) or abruptly deprived of phosphorus (open circles) following withdrawal of the external P supply. Values are means of four observations. Bars represent ± s.e.

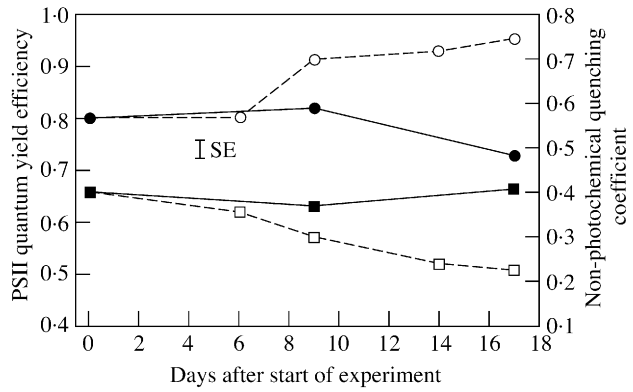

FIG. 5. PSII quantum yield efficiency (squares) and non-photochemical quenching coefficient (circles) in leaves of white clover plants grown with a continuous supply of phosphorus (closed symbols) or abruptly deprived of phosphorus (open symbols) following withdrawal of the external P supply. Mean s.e. is indicated; $n = 12$.

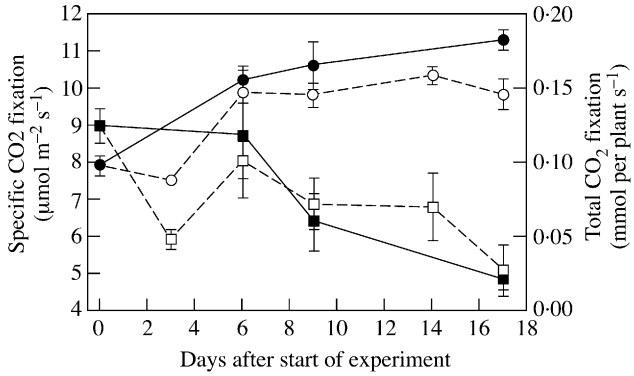

FIG. 6. Photosynthetic net CO_2 fixation per unit leaf area (squares) and per total plant (circles) in white clover plants grown with a continuous supply of phosphorus (closed symbols) or abruptly deprived of phosphorus (open symbols) following withdrawal of the external P supply. Bars represent ± s.e.; $n = 4$.

The ratio of total net CO_2-fixation in shoots to total N_2-fixation in roots was independent of P supply, suggesting a finely tuned interregulation of N_2-fixation and photosynthetic CO_2-fixation (Fig. 7).

Phloem exudate composition. Under steady-state conditions the concentration of asparagine in the EDTA-solution was higher (approx. 22 pM) at RAR 0·03 and 0·06 than at RAR 0·10 (approx. 8 pM; $P < 0·05$). No differences in phloem amino acid concentration were observed under short-term P-deprivation.

DISCUSSION

Steady-state P supply experiment

In plants with different growth rates, determined by the relative daily P additions, root growth was favoured compared with shoot growth at low P supply. In addition, N_2-fixation per plant and per unit root mass decreased with reduction in the rate of P supply, as did SLA (Table 2). However, net photosynthesis per unit leaf area (Table 2) was not significantly affected by P supply although plants experiencing a withdrawal of their P supply showed a slight reduction in the concentration of N in the shoots, from 3·63 to 2·83 % (Fig. 2A).

As both root respiration and N_2-fixation per unit root mass were lower at low P supply, the assimilates partitioned to the root system were used mainly to increase the size of the root system which, under field conditions, would be a key factor in the uptake of P from the soil solution. The higher ratio of net CO_2 to N_2-fixation, following adjustment to lower P supply, together with the reduction in the N_2-fixation per unit root mass (Table 2), suggests that P deficiency induced a feedback reduction of symbiotic N_2-fixation (Oti-Boateng and Silsbury, 1993; Parsons *et al.*, 1993; Hartwig *et al.*, 1994). As the concentration of asparagine in the phloem extracts was higher at the lower RARs (0·03 and 0·06) than at the highest RAR ($P < 0·05$), this inhibition may have been caused by a reduced demand for N by the clover plants under long-term steady-state conditions, as found by Neo and Layzell (1997). A similar conclusion was reached by Almeida *et al.* (2000), who measured the asparagine concentration in P-deficient and control white clover plants grown with inorganic N supply.

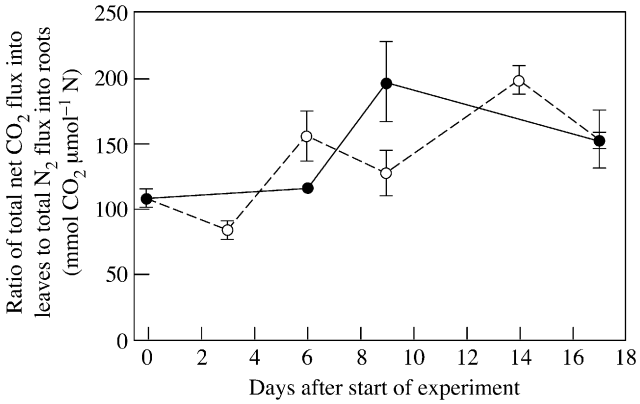

FIG. 7. Ratio of total net CO_2 fixation and total N_2-fixation per white clover plant grown with a continuous supply of phosphorus (closed symbols) or abruptly deprived of phosphorus (open symbols) following withdrawal of the external P supply. Bars represent ± s.e.; $n = 4$.

The inhibition of N_2-fixation was associated with a small reduction in the efficiency with which electrons were allocated to the reduction of N_2 in nodules. The argon-induced decline, in particular under the lowest P supply, indicates that such a reduction in efficiency did take place; this is in agreement with results of previous studies (Drevon and Hartwig, 1997; Vadez *et al*., 1997; Tang *et al*., 2001). In contrast to the present study, low P supply to clover plants supplied with inorganic N caused an increase in the N concentration of shoot dry matter (Fig. 2A; Almeida *et al*., 2000).

Short-term P deprivation experiment

As clover plants in this experiment were older and, therefore, larger when subjected to P deficiency than plants in the steady-state experiments, responses to P starvation were expected to occur after some delay. However, despite having a relatively high P status (Fig. 2), plants responded rapidly to the withdrawal of P. Changes in photosynthetic activity were observed 6 d after P withdrawal and they coincided with adjustments of SLA and nodule mass (Figs 3 and 6). The increase in nodule mass stopped after approx. 9 d of P deprivation, whereas nodule growth was maintained in control plants, whose final nodule mass was approximately double that of P-starved plants (Fig. 3A). This result is in agreement with findings of previous studies (e.g. Gordon *et al*., 1990; Almeida *et al*., 2000; Tang *et al*., 2001).

The amount of P in nodules at the onset of P-deprivation constituted a significant proportion of whole plant (5·4 %) and root (33 %) P content. Owing to the decline in nodule mass of P-deprived plants, by day 17 nodules contributed less (22 %; $P = 0{\cdot}003$) to total root-P in the deprived plants than in control plants (38 %). Nevertheless, the proportional decline in P concentration was greater in the dry matter of shoots and roots (Fig. 2B) than in nodules, confirming results of studies on other species (Jacobsen, 1985; Sa and Israel, 1991; Drevon and Hartwig, 1997; Tang *et al*., 2001). The measured trends in P concentration thus indicate that nodules do constitute a preferential sink for P in white clover (Fig. 2B) or that nodules can absorb P directly from the nutrient solution (Al-Niemi *et al*., 1998).

Phosphorus concentrations in the dry matter of white clover plants used in the present experiments (Fig. 2B) were within the range shown by Sa and Israel (1991) to cause decreases in specific nitrogenase activity in soybean. However, in the present work P-deprived white clover plants increased the level of N_2-fixation per unit nodule mass compared with that of controls (Fig. 4B). This result corresponds to conclusions by Almeida *et al*. (2000) concerning the exposure to a low P content of clover plants grown in the presence of inorganic N.

The hypothesis that nodule growth undergoes regulatory inhibition by an N feedback mechanism is not directly supported by results obtained from the analysis of amino acid contents of the phloem (Caputo and Barneix, 1997). Total amino acid and asparagine contents in the EDTA-promoted phloem exudates in P-deprived plants did not differ from those of the controls; this finding is in contrast to that of Neo and Layzell (1997). However, the EDTA-promoted exudation technique may not be sufficiently sensitive to reveal changes in the phloem composition that could include a regulatory response in relation to nodule activity under adaptation to P deficiency (Parsons *et al*., 1993; Bacanamwo and Harper, 1996).

The continued N_2-fixation in white clover under P starvation was also reflected by an unchanged EAC and argon-induced decline in nitrogenase activity. This indicates that there is no direct effect of P on the diffusion barrier of the nodule (Sheehy *et al*., 1983) within the duration of the experiment, since an increase in the resistance to gas diffusion in this barrier would have increased the internal concentration of H_2 and, subsequently, reduced the EAC below the theoretical optimum of 75 %. The mean values of EAC obtained in the present work (69 %) correspond to near-optimal values (Hunt and Layzell, 1993). A fast reduction following a significant change in plant N status (e.g. Neo and Layzell, 1997) could not be expected. The present experiments demonstrate that clover plants can reduce the capacity of their N_2-fixation apparatus, i.e. nodules, within a few days (Fig. 3B) whilst maintaining the specific N_2-fixation activity (Fig. 4B).

Leaf photosynthesis per unit leaf area (Fig. 6) and nitrogenase activity per unit root mass (Fig. 4) displayed parallel declines with time in both control and P-deprived plants. This was presumably a consequence of an increasing degree of self-shading, leading to a growth curve that was less than exponential. It is likely that this self-shading effect restricted both specific nitrogenase activity and the magnitude of the argon-induced decline in control and P-deprived plants (Faurie and Soussana, 1993).

Contrary to the findings of Almeida *et al*. (2000), a reduction in the rate of photosynthesis per unit leaf area with decreasing P supply was not observed. The lack of an effect of P on the specific shoot CO_2 exchange is in accordance with the observation that photochemical quenching remained unaffected. An increase in non-photochemical quenching following P deficiency (Fig. 5) has also been found in sunflower and maize (Jacob, 1995). The efficiency with which incoming radiation was utilized decreased with

increasing P starvation (Fig. 5; Lauer *et al*., 1989). This suggests a slower adjustment of PSI and PSII to the relatively low light intensity, which follows a slower oxidation of the traps upon reduction from a strong light pulse (Schreiber *et al*., 1995).

Comparison of the short-term and long-term responses of white clover to P supply

Comparisons of the results obtained from two complementary experimental approaches in the present study show that the response of white clover to a lack of P supply depends on the duration of the stress. The effects displayed by clover plants subjected to short-term P deprivation were similar to, but less marked than, those observed in clover plants adjusting to steady-state low P supplies.

As total dry matter yield was not reduced significantly within the duration of the experiments, the white clover plants were able to cope with P deprivation. The adaptive response of white clover plants to continuously low P-supply consisted, first, of changes in plant morphology (phenotypic plasticity) resulting in a proportional reduction in shoot mass, which is in accordance with the resource-ratio hypothesis (Bloom *et al*., 1985; Hunt and Nicholls, 1986). In addition, the morphological adjustments included a reduction in SLA. These changes in morphology imply that the leaf area per plant was smaller under P deprivation leading to a decline in whole plant photosynthesis since the photosynthetic rate per unit leaf area was not affected significantly. Similar morphological changes in the root system caused major reductions in nodule mass, leading to a decline in the nitrogenase activity per unit root mass, but, as indicated by the response to short-term P-deprivation, not per unit nodule mass. Therefore, the clover plants responded to P deprivation by increasing partitioning to the roots (for P acquisition), and by reducing leaf area (C acquisition) and nodule mass (N acquisition). Such regulatory behaviour is in accordance with the phenotypic plasticity (Grime, 1979) that is typical of many grassland genotypes (Fransen *et al*., 1999). In both experiments, phenotypic adaptation appeared to be triggered at a threshold of approximately 0·27 % P in shoots (Table 1; Figs 2B and 3).

As a consequence of tight regulation (Bloom *et al*., 1985), the ratio of total CO_2-fixation to total N_2-fixation was maintained in the short-term deprivation experiment (Fig. 7). However, under a severe steady-state P deficiency, this ratio increased (Table 2), suggesting an inhibition of N_2-fixation caused by an N supply feedback mechanism, as indicated by an increased asparagine concentration in the phloem. Almeida *et al*. (2000) reached the same conclusion, and in both investigations nodule growth was hindered, whereas nitrogenase activity per unit nodule mass remained unaffected. It is concluded that the rate of N_2-fixation does not limit white clover growth under P-deficient conditions.

The observed responses to P deprivation are in contrast to well known reversible short-term inhibition effects on N_2-fixation caused by external nitrate supply (Minchin *et al*., 1986). In the present experiments, the short-term changes in P supply affected nodule growth only 6 d after P withdrawal, whereas nitrate is known to act on the gas diffusion barrier of nodules within hours. This implies that restoring N_2-fixation would take longer under P deprivation than under excess nitrate. Clearly, legumes need to resume N_2-fixation quickly whenever N availability is reduced, as this is the basis of their competitive advantage in environments where N supply is low or fluctuating. A rapid re-establishment of N_2-fixation after a period of P starvation might be less critical for white clover, especially under low-input conditions where co-existing grasses grow slowly.

In summary, this work has shown that a low P status initiates whole plant adaptive responses in white clover. Changes in the relative growth of shoots, roots and nodules, rather than changes in the resource uptake rates per unit mass or per unit area of these plant parts, appear to be involved. Thus, the duration of P-deprivation and plant age can influence experimental results significantly. Although the exact mechanism is not yet fully understood, an N-feedback mechanism is apparently involved in the regulation of nodule growth in P-deficient white clover plants. Since the P-deprived plants were apparently not N-deficient, except possibly at the lowest steady-state P supply rate, the observed reductions in shoot : root ratio and in SLA were directly induced by a low P content in the plant tissue, rather than by a low N status. Finally, the observed phenotypic changes arose below a threshold of approximately 0·27 % P content in the dry matter of white clover shoots.

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