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Organic acid accumulation may inhibit N_2 fixation in phosphorus-stressed lupin nodules

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Summary

• Nodulated lupins (*Lupinus angustifolius* cv. Wonga) were hydroponically grown under conditions of low phosphate (LP) or adequate phosphate (HP) to assess the effect of phospho*enol*pyruvate carboxylase (PEPC)-derived organic acids on nitrogen assimilation in LP nodules.

• LP conditions are linked to altered organic acid metabolism, by the engagement of PEP metabolism via PEPC. In LP nodules, the enhanced organic acid synthesis may reduce the available organic carbon for nitrogen assimilation. The diversion of carbon between the organic acid- and amino acid pools was assessed through key nodular enzymes and $^{14}CO_2$ metabolism.

• Under LP conditions, increased rates of organic acid synthesis via PEPC and malate dehydrogenase (MDH), coincided with reduced nitrogen assimilation via aspartate aminotransferase (AAT), aspartate synthetase (AS) and glutamine synthetase (GS)/glutamate synthase (GOGAT) activities. There was a preferential metabolism of nodular $^{14}CO_2$ into organic acids and particularly into malate. High malate levels were associated with reduced N_2 fixation and synthesis of amino acids.

• These results indicate that phosphorus deficiency can enhance malate synthesis in nodules, but that excessive malate accumulation may inhibit N_2 fixation and nitrogen assimilation.

Key words: amino acids, legume, N_2 fixation, nodules, organic acids, phosphorus (P) deficiency.

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Introduction

Nodulated legumes are self-sufficient at acquiring nitrogen (N), but are particularly sensitive to a wide range of other environmental limitations. In studies on mineral requirements of the legume–Rhizobia symbiosis, phosphorus (P) has received considerable attention as a result of the greater responsiveness of traits associated with biological N_2 fixation compared with host-plant growth (Israel, 1987; Olivera *et al.*, 2004). In spite of this, the relationship between low P supply and N_2 fixation remains obscure, primarily because of an indifferent response to P deficiency by some legume species (Vadez *et al.*, 1996). Furthermore, the responses to suboptimal P supply differ between the individual partners involved in the symbiosis, with nodules being less affected by fluxes in P supply than either roots or shoots (Tang *et al.*, 2001; Høgh-Jensen *et al.*, 2002; Olivera *et al.*, 2004; Le Roux *et al.*, 2006).

Although numerous studies have reported the effects of P nutrition on host plant growth and on parameters that quantify the N_2-fixing capacity of legumes, very few have considered P in nodule metabolism (Jakobsen, 1985; Israel, 1987; Sa & Israel, 1991; Al-Niemi *et al.*, 1997, 1998; Tang *et al.*, 2001; Le Roux *et al.*, 2006). Low P supply eventually reduces plant growth, with subsequent reduction in N demand and N_2 fixation. Other responses to limiting P in the growth medium in nonlegumes range from an increase in root carbohydrate content (Rychter & Randall, 1994), to reduced rates of total respiration and root ATP concentrations (Wanke *et al.*, 1998; Rychter *et al.*,

1992). Furthermore, for nonlegumes a typical response to P deprivation at the physiological level is carbon (C) channeling via alternative routes (Duff *et al.*, 1989, Theodorou & Plaxton, 1993). These are engaged as an adaptive measure to facilitate mitochondrial respiration by inorganic phosphate (P_i)-deficient plant cells (Plaxton, 1996; Sieger *et al.*, 2005). For C metabolism, a major point of divergence in glycolysis is at phospho*enol*pyruvate (PEP). This divergence involves the engagement of nonadenylate-requiring steps via the sequential action of phospho*enol*pyruvate carboxylase (PEPC) and malate dehydrogenase (MDH), which serve to circumvent the conventional adenylate-requiring pyruvate kinase (PK) (EC 2.7.1.40) route. Similar data for symbiotic legumes are scant. Evidence suggests that P deficiency impacts negatively on the energy metabolism of root nodules for optimal nodule functioning (Sa & Israel, 1991), which may be detrimental to any one of the aforementioned assimilative routes. A potential drawback at this PEP branch-point would be the constant competition for C skeletons between the organic acid pool (for tri-carboxylic acid (TCA) cycle intermediates and energy) and the amino acid pool (for N assimilation) (Olivera *et al.*, 2004; Le Roux *et al.*, 2006).

A natural extension of microaerobic conditions is the altered metabolism of C to malate, instead of to pyruvate, as under nonrestricted conditions (Vance & Heichel, 1991). The major C substrates for bacteroid respiration and N_2 fixation are dicarboxylic acids, primarily in the form of malate, with effective nodules accumulating high levels of this metabolite (Kouchi & Yoneyama, 1986; McRae *et al.*, 1989; Rosendahl *et al.*, 1990). Furthermore, malate is rapidly labeled when nodules are exposed to $^{14}CO_2$, which is indicative of the importance of this particular metabolite in nodules (Streeter, 1987; Rosendahl *et al.*, 1990). Moreover, metabolomic and transcriptomic analyses indicate that elevated levels of malate in root nodules are mainly the result of very low oxygen and P concentrations (Colebatch *et al.*, 2004).

Given the role of organic acids in nodule metabolism, it is unclear how P deficiency affects C partitioning between the organic acid pools and amino acid pools in nodules. The aim of the present study was to assess the effect of PEPC-derived organic acids on N-assimilation nodules under prolonged P_i deprivation. This was assessed through the diversion of C between the organic acid pools and amino acid pools through key nodular enzymes and $^{14}CO_2$ metabolism.

Materials and Methods

Plant growth conditions

All seeds were germinated in vermiculite, which was commercially irradiated by a cobalt C-60 source of gamma radiation at a dose of 18 kGray. Pots measuring 10 cm in diameter were washed in Ekon-D (Merck Chemicals, Wadeville, South Africa), rinsed in distilled water, then dried. The pots were then filled with vermiculite. For all experiments, seeds of *Lupinus angustifolius* L. (cv. Wonga) were inoculated with a rhizobial inoculum containing *Bradyrhizobium* sp. (*Lupinus*) bacteria. Seeds of lupins were coated in a saturated sucrose solution and then 2 g of inoculum/150 seeds was added and mixed. The seeds were spread out, away from direct sunlight, to allow the inoculum to dry until manageable. Once dry, the seeds were planted in pots containing vermiculite.

Seeds were germinated in an east-facing glasshouse at the University of Stellenbosch (Stellenbosch, South Africa). The range of midday irradiances was between 540 and 600 μmol m^{-2} s^{-1}, and the average day and night temperatures and humidities were 23 and 15°C and 35 and 75%, respectively. Pots were watered daily with distilled water until seeds germinated. Seedlings were transferred, 10 d after germination, to 20-l hydroponic tanks under the same glasshouse conditions. The tanks contained a modified Long Ashton nutrient solution modified to contain no N, 2 mM PO_4^{3-} and 0.05 mM 2-(N-Morpholino)ethanesulfonic acid (MES) (pH 5.8). Solutions were changed every 3–4 d. The hypocotyls of seedlings were wrapped with foam rubber at their bases and inserted through holes in the lids of the tanks. Each tank was supplied with an air supply line, which bubbled air containing 360 parts per million (ppm) CO_2. Once nodules were established and of adequate size, usually in the third or fourth week of hydroponic growth, plants were divided into two treatments. Half of the tanks were maintained at an adequate P concentration (2 mM PO_4^{3-}; HP treatment), whereas the other half were switched to a low-P nutrient regime (2 μM PO_4^{3-}; LP treatment). Phosphorus starvation was induced for 14 d, after which plants were harvested.

Inorganic phosphate determination

Inorganic phosphate levels were determined using a modified Fiske–Subbarow method (Rychter & Mikulska, 1990). Approximately 0.5 g of root or nodule was homogenized in 500 μl of 10% trichloroacetic acid (TCA) at 4°C. This was diluted three times with cold 5% TCA and centrifuged for 10 min at 2500 *g* and 4°C. The supernatant was removed and centrifuged for a further 10 min at 13 000 *g* and 4°C. The resultant supernatant was kept on ice (or stored at −20°C) until ready for use.

Protein extraction and determination

The extraction for PEPC, PK, MDH and malic enzyme (ME) was performed according to Ocaña *et al.* (1996), modified so that 0.5 g of tissue was extracted in 2 ml of extraction buffer consisting of 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM dithiothreitol (DTT), 20% (v/v) ethylene glycol, plus 2% (mass per volume (m/v)) insoluble polyvinylpolypyrrolidone (PVPP) and one Complete Protease Inhibitor Cocktail tablet (Roche) per 50 ml of buffer. Crude extracts of N-assimilating

enzymes, glutamine synthetase (GS)/glutamate synthase (GOGAT) and aspartate aminotransferase (AAT), were prepared according to the method of Olivera *et al.* (2004). Aspartate synthetase (AS) was extracted according to Egli *et al.* (1989). The protein concentration was determined by the procedure of Bradford (1976), using a protein assay reagent (Bio-Rad) and bovine serum albumin (BSA) as a standard. Amino acid concentrations were determined according to the ninhydrin method of Rosen *et al.* (1957), using leucine as a standard. In the same extract, NH_4^+ was measured using a phenol-hypochloride method (Solorzano, 1969).

Enzyme assays

Phosphoenolpyruvate carboxylase (PEPC) PEPC activity was determined spectrophotometrically by coupling the carboxylation reaction with exogenous NADH-malate dehydrogenase and measuring NADH oxidation at 340 nm and 30°C. The standard assay mixture contained 100 mM Tris (pH 8.5), 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 4 mM PEP, 0.20 mM NADH and 5 U of MDH (Ocaña *et al.*, 1996). The blanks consisted of a reaction medium without PEP.

Pyruvate kinase (PK) PK activity was assayed in a buffer containing 75 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 1 mM ADP, 3 mM PEP, 0.18 mM NADH and lactate dehydrogenase (3 U) (Smith, 1985). The blanks consisted of one reaction in which ADP was omitted and then another from which PEP was absent.

NADH-Malate dehydrogenase (MDH) MDH was assayed as described by Appels & Haaker (1988). The reaction mixture contained 25 mM KH_2PO_4, 0.2 mM NADH and 0.4 mM oxaloacetic acid (OAA). The pH was adjusted to 7.5 with 1 mM HCl (Appels & Haaker, 1988). The blanks consisted of a reaction medium without OAA.

Malic enzyme (ME) This assay monitored the increase in absorption at 340 nm as a result of the formation of NADPH or NADH. The assay mixture contained 80 mM Tris-HCl (pH 7.5), 2 mM $MnCl_2$, 1 mM malate and 0.4 mM NADP or NAD^+ (Appels & Haaker, 1988). The blanks consisted of a reaction medium without malate.

Aspartate amino transferase (AAT) AAT activity was determined in a reaction medium containing 4 mM $MgCl_2$, 10 mM aspartic acid, 0.2 mM NADH and 1 mM 2-oxoglutarate in 50 mM Tris-HCl buffer (pH 8.0) (Olivera *et al.*, 2004).

Glutamine synthetase (GS) GS activity was measured in a final volume of 250 µl of imidazole (pH 7.2) 20 mM $MgCl_2$, 25 mM hydroxylamine, 100 mM glutamate and 10 mM ATP, in accordance with Olivera *et al.* (2004). Absorbance was determined at 540 nm.

Glutamate synthase (GOGAT) GOGAT activities were measured spectrophotometrically, monitoring absorbance, as a result of NADH oxidation, at 340 nm. NADH-GOGAT was assayed in 100 mM KH_2PO_4 (pH 7.6), containing 0.1% (v/v) 2-mercaptoethanol. NADH-GOGAT activity was measured using 100 mM NADH, 2.5 mM a-ketoglutarate, 10 mM L-glutamine and 1 mM aminooxyacetate (to inhibit aminotransferase activities) (Olivera *et al.*, 2004). GOGAT activities were corrected for endogenous NAD^+ (NADH) reduction (oxidation) in the absence of added substrate. All measurements of NADH-GOGAT activity were made within 3–4 h of extraction.

Nodule host plant enzyme activities were determined as initial rates of reaction at 25–30°C. All the reactions were initiated by adding 30 µl of crude extract to reaction mixture in a total volume of 250 µl. Initial reaction rates have been shown to be proportional to the concentration of enzyme used under the conditions used.

Whole-plant ^{14}C incorporation

Roots of intact plants were supplied with 42 nmol $NaHCO_3$ containing 0.093 MBq $Na^{14}HCO_3^-$, and pulsed with air for 30 s thereafter, every 15 min for 1 h. In addition to the 15-min intervals of air pulses, the solutions in the cuvettes were also swirled by hand every 5 min. After 1 h the plants were harvested. Roots were rinsed twice in separate containers of distilled water and blotted dry. Plants were then separated into root, nodule and shoot components, which were immediately weighed and quenched in liquid N before storage at −80°C.

^{14}C fractionation

Components were homogenized with 80% (v/v) ethanol and separated into soluble and insoluble components. The soluble component was subsequently separated into water-soluble and chloroform-soluble components. The water-soluble component was further fractionated into amino acid, organic acid and carbohydrate fractions, as described by Atkins & Canvin (1971).

Carbon cost determinations

Nodule construction costs (mmol C g^{-1} dry weight (DW)) were calculated according to Mortimer *et al.* (2005). $Cw = [C + (kN/14) \times (180/24)](1/0.89)(6000/180)$, where Cw is the construction costs of the tissue (mmol C g^{-1} DW), C is the carbon concentration (mmol C g^{-1} DW), k is the reduction state of the N substrate (NH_4^+, derived from N_2 reduction, was taken as the source of N for amino acid synthesis and therefore k is −3) and N is the organic nitrogen content of the tissue (g g^{-1} DW). The constant (1/0.89) represents the fraction of the construction cost that provides reductant which is not incorporated into biomass (Williams *et al.*, 1987; Peng *et al.*, 1993) and (6000/180) converts units of g glucose g^{-1} DW to mmol C g^{-1} DW. Growth respiration (mmol CO_2 g^{-1} DW),

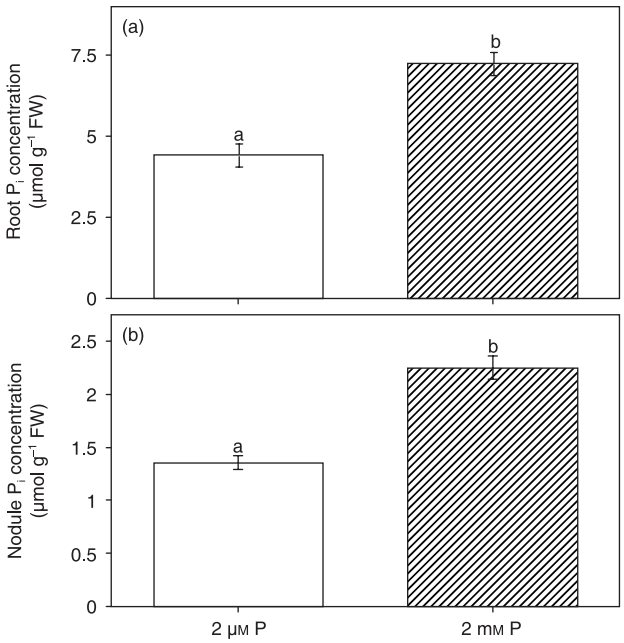

Fig. 1 (a) Root inorganic phosphate (P_i) concentrations and (b) nodule P_i concentrations of phosphorus (P)-starved and P-sufficient lupin plants. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 wk in hydroponic culture in a nitrogen-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 parts per million (ppm) CO_2 at the roots zone. A low-P condition was induced for 24 d in the P-starved plants by switching the P supply to 2 mM P (LP). The P-sufficient plants remained at 2 mM P (HP) supply. Values are presented as means ($n = 4$). Different letters indicate significant differences between each treatment ($P \leq 0.05$). FW, fresh weight.

representing the C respired for the biosynthesis of new tissue, was calculated according to Peng *et al.* (1993).

Measurement of nodule respiration

Oxygen uptake by nodulated root segments was measured at 25°C for 2–5 min using a Clark oxygen electrode. Although excision can affect the nodule metabolism, we attempted to reduce the impact of the detachment. This was achieved by using nodulated root segments and limiting the time of the assay to under 5 min after detachment. According to Vessey (1994), these precautions can reduce the potential artifacts associated with nodule excision. Detached nodulated root segments were rinsed in distilled water and placed in a cuvette containing 3 ml of aerated nutrient medium (sufficient or deficient). Upon completion of the measurements, nodules were oven dried at 80°C for 48 h. The nodules were weighed and respiration expressed on a DW basis.

Nitrogenase activity

Nitrogenase activity of nodules (attached to a piece of root) was measured according to Parsons & Baker (1996). Ethylene and acetylene concentrations were measured using a Varian gas chromatograph (Varian, Inc., Palo Alto, CA, USA) with a 1200 × 1.6 mm Porapak T (80–100) mesh column (Varian). Gas samples were injected directly into the gas chromatograph and analysis was completed within 5 min. Although excision can affect the nodule metabolism, we attempted to reduce the impact of the detachment. This was achieved by using nodulated root segments and limiting the time of the assay to under 5 min after detachment. According to Vessey (1994), these precautions can reduce the potential artifacts associated with nodule excision.

Table 1 Nodular growth, biomass and respiratory costs, and nitrogen nutrition of phosphorus (P)-starved and P-sufficient lupin nodules

	2 μM P	2 mM P
Nodule biomass parameters		
Nodule dry weight (g per plant)	0.047 a	0.071 b
Construction costs (mmol C g^{-1} DW)	3413 a	3614 a
Growth respiration costs (mmol CO_2 g^{-1} d^{-1})	6059 a	7535 b
Nodule nitrogen nutrition		
N_2 fixation via acetylene reduction activity (nmol g^{-1} FW)	60.70 a	78.23 b
N_2 fixation via acetylene reduction activity (nmol g^{-1} protein)	15.45 a	67.15 b
Nitrogen concentration (mmol N g^{-1} DW)	41.42 a	56.11 b

Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 wk in hydroponic culture in a nitrogen-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 parts per million (ppm) CO_2 at the roots zone. A low-P condition was induced for 24 d in the P-starved plants by switching the P supply to 2 μM P. The P-sufficient plants remained at 2 mM P supply. Values are presented as means ($n = 4$). Different letters indicate significant differences between each treatment ($P \leq 0.05$). DW, dry weight; FW, fresh weight.

Statistical analysis

Averages for the data are means of separate replicates ($n = 4$). All data were analysed by single ANOVA. Percentage data was arcsine transformed before analysis and ratios were square root transformed before analysis. All data was then subjected to a post-hoc LSD test to determine significance.

Results

Growth and respiration following P starvation

The concentration of P_i decreased markedly after 25 d of P starvation in both roots and nodules (Fig. 1a,b). The P_i decrease for both compartments was approx. 40%. Nodule biomass accumulation and growth respiration costs decreased with growth under LP, although the construction costs of tissues remained almost unchanged (Table 1).

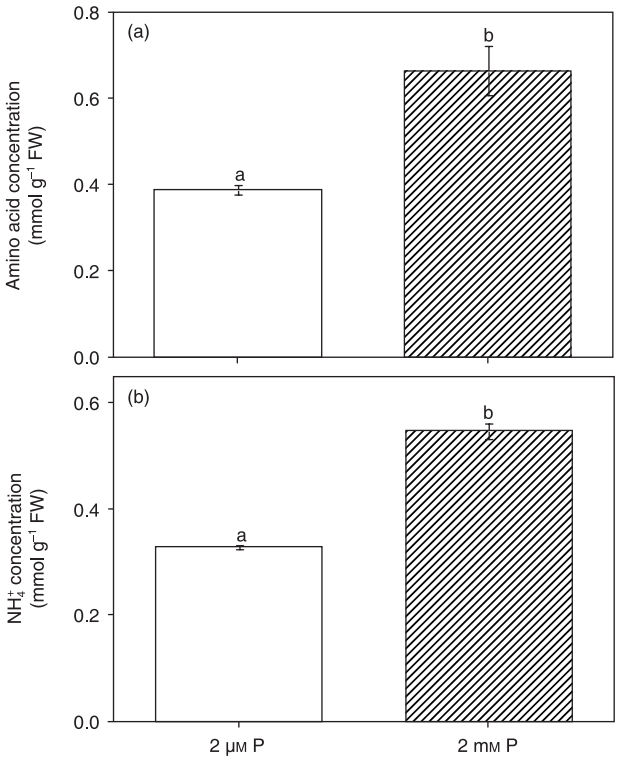

Fig. 2 (a) Amino acid concentrations and (b) NH_4^+ concentrations of phosphorus (P)-starved and P-sufficient lupin nodules. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 wk in hydroponic culture in a nitrogen-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 parts per million (ppm) CO_2 at the roots zone. A low-P condition was induced for 24 d in the P-starved plants by switching the P supply to 2 mM P (LP). The P-sufficient plants remained at 2 mM P (HP) supply. Values are presented as means ($n = 4$). Different letters indicate significant differences between each treatment ($P \leq 0.05$). FW, fresh weight.

Table 2 Specific activities of selected enzymes involved in amino acid and organic acid synthesis of phosphorus (P)-starved and P-sufficient lupin nodules

Nodular enzyme activities	2 μM P	2 mM P
Amino acid synthesizing enzymes (μmol min^{-1} mg^{-1} protein)		
Aspartate aminotransferase (AAT)	32.51 a	28.39 a
Glutamine synthetase (GS)	1.26 a	1.19 a
Glutamate synthase (GOGAT)	0.64 a	0.40 a
Organic acid synthesizing enzymes (μmol min^{-1} mg^{-1} protein)		
Phospho*enol*pyruvate carboxylase (PEPC)	0.254 b	0.169 a
Malate dehydrogenase (MDH)	0.15 b	0.06 a
Pyruvate kinase (PK)	0.12 a	0.28 b
Malate dehydrogenase : amino acid synthesizing enzymes		
MDH : AAT	0.004 b	0.002 a
MDH : GS	0.115 b	0.053 a
MDH : GOGAT	0.240 b	0.156 a

Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 wk in hydroponic culture in a nitrogen-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 parts per million (ppm) CO_2 at the roots zone. A low-P condition was induced for 24 d in the P-starved plants by switching the P supply to 2 μM P. The P-sufficient plants remained at 2 mM P supply. Values are presented as means ($n = 4$). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

Nitrogen metabolism

Nodules receiving LP showed a decline in nitrogenase activity via the acetylene reduction assay (ARA), both on a fresh mass basis and when expressed per mg of protein (Table 1). There was a marked reduction in the concentration of amino acids and NH_4^+ in the nodules of plants that were P deprived (Fig. 2a,b).

In all P treatments, GS activity was approx. two- to threefold higher than GOGAT enzyme activity. However, both enzyme activities were unaffected by P treatment (Table 2). Similarly, AAT activity remained unchanged under P-deficient conditions (Table 2).

Carbon metabolism

The activities of enzymes of the alternative route of PEP metabolism (i.e. PEPC and MDH) were both increased markedly under P_i deprivation. PEPC registered an approximate increase of twofold in activity as compared with PEPC of nodules at sufficient P (Table 2). The activity of MDH was also increased approx. threefold under P deficiency (Table 2). By comparison, the activity of PK, which constitutes the conventional route of C incorporation, decreased twofold under P-deficient conditions. Under LP conditions this PK activity constituted approx. 50% that of the PEPC activity, whereas under HP conditions, PK activity was almost twofold higher than PEPC activity. There was an increase in the ratio of MDH activities compared with all the enzyme activities (GS, GOGAT, AAT) of the amino acid-synthesizing pathway (Table 2).

Nodular ^{14}C fractionation and respiration

There was a higher incorporation of $^{14}CO_2$ in P_i-deficient nodules and their constituent fractions (Table 3). During P_i deficiency the ratio of incorporation of ^{14}C shifted towards nonnitrogenous organic compounds (Table 3).

Most of the incorporated ^{14}C was recovered in organic acids, and was higher under LP supply than under HP supply (Fig. 3a). The percentage of organic acids incorporated into nodules under LP conditions was approx. twofold higher than that for amino acids under the same conditions (Fig. 3a–c).

Table 3 Incorporation and assimilation of dissolved $^{14}CO_2$ in detached nodules from phosphorus (P)-starved and P-sufficient *Lupinus angustifolius* (cv. Wonga) plants after 5 min of $^{14}CO_2$ labelling

Dissolved inorganic carbon (DI ^{14}C) concentration (nmol ^{14}C g^{-1} FW)	2 μM P	2 mM P
Soluble fractions	5.86 b	2.82 a
Insoluble fractions	10.57 b	3.40 a
Lipid fractions	0.29 b	0.12 a
Total incorporation	14.11 b	9.32 a
^{14}C incorporation C : N ratio	4.70 b	2.23 a

Nodulated *L. angustifolius* (cv. Wonga) plants were grown for 5 wk in hydroponic culture in a nitrogen-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 360 parts per million (ppm) CO_2 at the roots zone. A low-P condition was induced for 24 d in the P-starved plants by switching the P supply to 2 μM P. The P-sufficient plants remained at 2 mM P supply. Values are presented as means ($n = 4$). Different letters indicate significant differences between each treatment ($P \leq 0.05$).

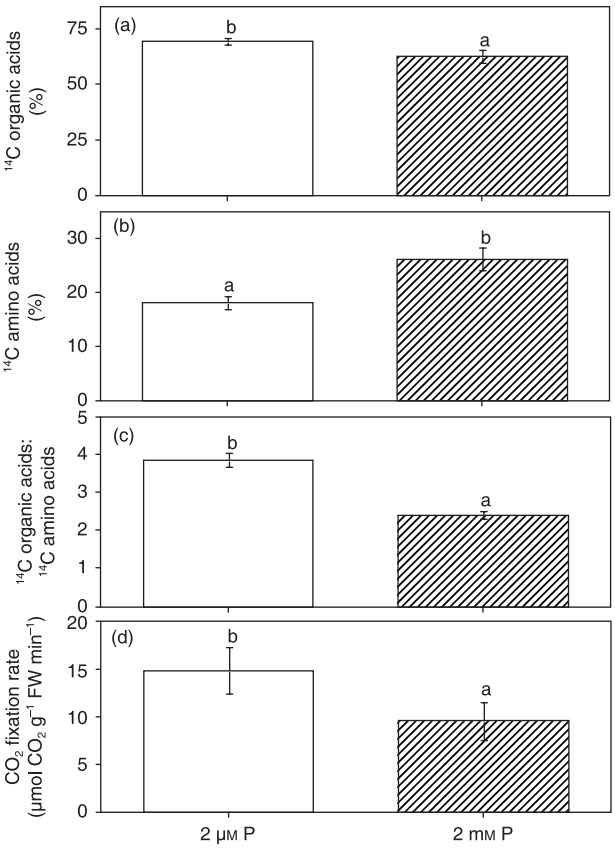

Fig. 3 Dark $^{14}CO_2$ fixation into (a) organic acid fraction, (b) amino acid fractions, (c) the ratio of organic acids to amino acids and (d) the $^{14}CO_2$ fixation rate of detached nodules from phosphorus (P)-starved and P-sufficient lupins after 5 min of $^{14}CO_2$ labelling. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 wk in hydroponic culture in a nitrogen-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 parts per million (ppm) CO_2 at the roots zone. A low-P condition was induced for 24 d in the P-starved plants by switching the P supply to 2 mM P (LP). The P-sufficient plants remained at 2 mM P (HP) supply. Values are presented as means ($n = 4$). Different letters indicate significant differences between each treatment ($P \leq 0.05$). FW, fresh weight.

This increase in organic acid concentration was inversely related to the amino acid concentration in nodules (Fig. 3a–c). The higher incorporation into organic acids coincided with a higher fixation rate of $^{14}CO_2$ by LP nodules compared with HP nodules (Fig. 3d). The long-term P-starvation period also caused an increase in the O_2 consumption rate of nodules (Fig. 4). There was a pronounced increase in total malate levels (derived from PEP and the TCA cycle) in nodules subjected to LP conditions as opposed to nodules of P sufficient controls (Fig. 5a). However under LP conditions, most of the malate appeared to be derived from PEP, as determined by $^{14}HCO_3^-$ incorporation via PEPC (Fig. 5b).

Discussion

During the long-term P deficiency of nodules investigated in the present study, the reduced N_2 fixation and N assimilation were found to be associated with an increase in the allocation of PEP-derived C into organic acids, relative to amino acids.

Nodules are known to be strong sinks for P, particularly under conditions of P deficiency (Al-Niemi *et al.*, 1997, 1998; Tang *et al.*, 2001, Høgh-Jensen *et al.*, 2002). It has been reported that nodules often respond relatively more slowly than roots to P deficiency (Israel, 1993; Le Roux *et al.*, 2006). This was evidenced in the present study by the delayed reduction of P_i in root nodules 25 d after P starvation, compared with a previous study where 14 d of P withdrawal had no apparent effect on the P_i concentration of nodules (Le Roux *et al.*, 2006). In this study, prolonged P deficiency decreased nodule DW, which concurs with previous reports of the effects of low P supply on legumes (Drevon & Hartwig, 1997, Olivera *et al.*, 2004). Although the growth respiration rate, an indication of the respired C associated with the biosynthesis of new tissue (Peng *et al.*, 1993), declined with the decreased nodular growth, the construction costs of these nodules were unaffected. Because construction costs represent the amount of simple carbohydrate required to build new tissue (Peng *et al.*, 1993), this implies that new nodular growth was limited not by C allocation, but by P supply.

The low P-induced decreases in nodular growth have been attributed to the decline of N_2 fixation (Ribet & Drevon, 1995), but the present study implicates changes in C allocation between amino acid and organic acid pools. The decline in P_i and adenylates during P stress can increase the engagement of alternative routes to circumvent the adenylate- and P_i-requiring pathways of conventional glycolysis and mitochondrial respiration (Rychter *et al.*, 1992; Theodorou & Plaxton, 1993).

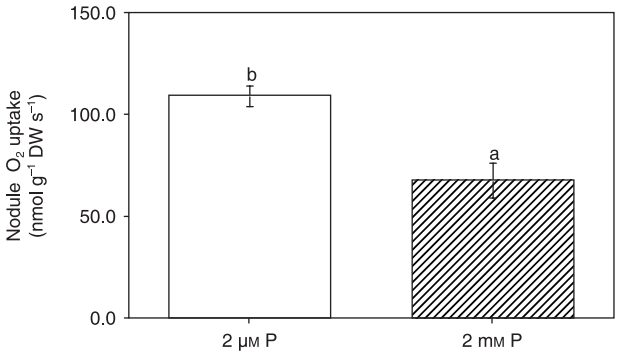

Fig. 4 O_2 uptake rate of phosphorus (P)-starved and P-sufficient lupin nodules. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 wk in hydroponic culture in a nitrogen-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 parts per million (ppm) CO_2 at the roots zone. A low-P condition was induced for 24 d in the P-starved plants by switching the P supply to 2 mM P (LP). The P-sufficient plants remained at 2 mM P (HP) supply. Values are presented as means ($n = 4$). Different letters indicate a significant difference between each treatment ($P \leq 0.05$). DW, dry weight.

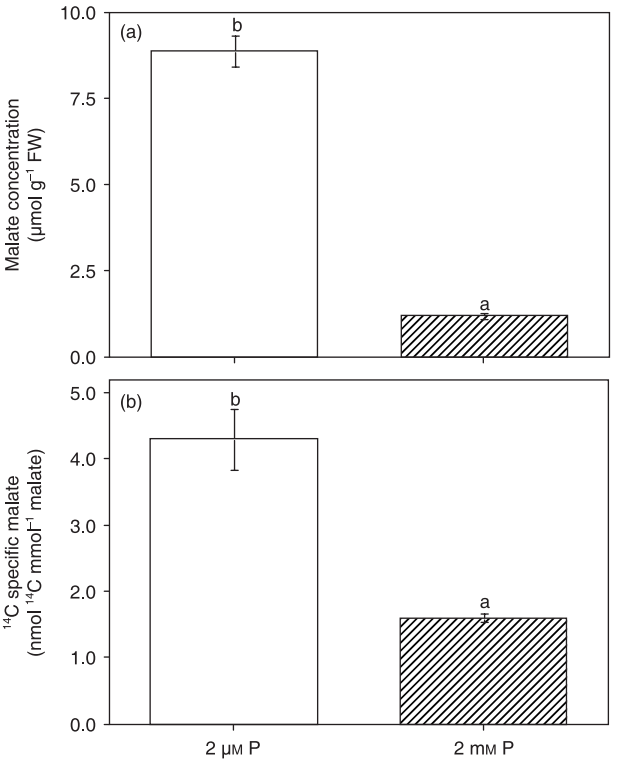

Fig. 5 (a) Total malate concentration and (b) ^{14}C-enriched malate of phosphorus (P)-starved and P-sufficient lupin nodules. Nodulated *Lupinus angustifolius* (cv. Wonga) plants were grown for 5 wk in hydroponic culture in a nitrogen-free Long Ashton nutrient solution containing 2 mM P. The solution was aerated with ambient air containing 380 parts per million (ppm) CO_2 at the roots zone. A low-P condition was induced for 24 d in the P-starved plants by switching the P supply to 2 mM P (LP). The P-sufficient plants remained at 2 mM P (HP) supply. Values are presented as means ($n = 4$). Different letters indicate significant differences between each treatment ($P \leq 0.05$). FW, fresh weight.

In nodules, the subsequent increased engagement of an alternative glycolytic route at a low P_i concentration, most notably PEP metabolism via PEPC, is thought to contribute to the elevated malate levels typical of nodules (Colebatch *et al.*, 2004, Le Roux *et al.* 2006). Previous work, on shorter-term P deficiency in nodules, found no engagement of this PEPC bypass, when cellular P_i levels were maintained for 14 d of P starvation (Le Roux *et al.*, 2006). However, the P_i decline in nodules under 25 d of P starvation, observed in the present study, was correlated with an increase in PEP metabolism via PEPC.

The increase in oxygen consumption with decreasing nutritional P supply concurs with the respiratory responses of previous work on P-deficient plants (Bingham & Farrar, 1989; Usuda & Shimogawara, 1993). This was mainly ascribed to O_2 consumption being mediated via the nonphosphorylating alternative pathway (Rychter *et al.*, 1992). In addition, P deficiency may upset the favorable energy status of the plant cell fraction where N assimilation into amino acids and ureides occur (Sa & Israel, 1991; Israel, 1993).

In this regard, the present study found that nodular enzymes associated with N metabolism (GS/GOGAT, AAT and AS) had decreased activities under LP conditions, which resulted in lower N assimilation. Despite the current study's focus on amino acid-exporting legumes, a similar down-regulation of comparable N-assimilation enzymes has previously been reported for the ureide-exporting *Phaseolus vulgaris* under LP conditions (Olivera *et al.*, 2004). Although Almeida *et al.* (2000) found that nitrogenase activity undergoes regulatory inhibition by an N-feedback mechanism under LP conditions, the decrease in total amino acid and NH_4^+ concentrations found in the present study were not associated with this predicted relief of the inhibition of N fixation under LP. Furthermore, it was found that the accumulation of specific amino acids (e.g. asparagine), rather than changes in total amino acids, could elicit a reduction in specific nitrogenase activity (Almeida *et al.*, 2000).

The nodule metabolite levels and enzyme activities strongly suggest that LP nodules have an increased C flux towards organic acids. Because nodules have a high requirement for dark CO_2 fixation (Rosendahl *et al.*, 1990), the enhanced uptake and assimilation of dark CO_2 under P deficiency, and the subsequent preferential incorporation into organic acids, concurs with the engagement of the P-deficient bypass in plant cells (Theodoroux & Plaxton, 1993). The PEP-derived organic acids, particularly malate, are widely accepted to be the primary source of C for bacteroids (Rosendahl *et al.*, 1990; Kaur & Singh, 1999). In the present study, this shift in C metabolism towards organic acid synthesis was at the expense of C assimilation into amino acids. Therefore, it is proposed that altered organic acid metabolism may account for

the reported decline in N_2 fixation in this study. In particular, the increase in malate synthesis and dark CO_2 incorporation into malate can further substantiate this assertion. This increased malate concentration in nodules of plants grown under LP conditions is matched by the increase in MDH activities of these nodules. Elevated malate concentrations under LP conditions may have been inhibitory to N_2 fixation.

In vitro studies have demonstrated that even modest increases (0.5–2 mM) in malate supply to isolated bacteroids could heavily impede N_2 fixation (Bergersen & Turner, 1990, 1993). This effect on N_2 fixation was only reversed once substrate levels were lowered in isolated bacteroids (Bergersen & Turner, 1990, 1993). Although there has been a study on whole nodules to resolve the malate concentrations between the cytolsolic and bacteroid components, these findings were not implicated in N_2 fixation (Rosendahl *et al*., 1990).

In conclusion, the engagement of the PEPC bypass route in P_i-limited nodules may ensure the continuation of respiration, but the preferential shift of PEPC-derived C to organic acid synthesis may have resulted in malate accumulation to inhibit symbiotic N_2 fixation. Furthermore, it is clear that more detailed information is required on the malate distribution in various nodular tissues in order to assess the relationship between malate levels and N_2 fixation.

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References

Almeida JPF, Hartwig UA, Frehner M, Nösberger J, Lüscher A. 2000. Evidence that P deficiency induces N feedback regulation of symbiotic N_2 fixation in white clover (*Trifolium repens* L.). *Journal of Experimental Botany* **51**: 1289–1297.

Al-Niemi TS, Kahn ML, McDermott TR. 1997. Phosphorus metabolism in the bean-*Rhizobium tropici* symbiosis. *Plant Physiology* **113**: 1233–1242.

Al-Niemi TS, Kahn ML, McDermott TR. 1998. Phosphorus uptake by bean nodules. *Plant and Soil* **198**: 71–78.

Appels MA, Haaker H. 1988. Identification of cytoplasmic nodule-associated forms of malate dehydrogenase involved in the symbiosis between *Rhizobium leguminosarum* and *Pisum sativum*. *European Journal of Biochemistry* **171**: 515–522.

Atkins CA, Canvin DT. 1971. Photosynthesis and CO_2 evolution by leaf discs: gas exchange, extraction, and ion-exchange fractionation of ^{14}C–labelled photosynthetic products. *Canadian Journal of Botany* **49**: 1225–1234.

Bergersen FJ, Turner GL. 1990. Bacteroids from soybean root nodules: respiration and N_2-fixation in flow chamber reactions with oxyleghaemoglobin. *Proceedings of the Royal Society of London B* **228**: 296–320.

Bergersen FJ, Turner GL. 1993. Effects of concentration of substrates supplied to N_2-fixing soybean bacteroids in flow chamber experiments. *Proceedings of the Royal Society of London B* **251**: 95–102.

Bingham IJ, Farrar JF. 1989. Activity and capacity of respiratory pathways in barley roots deprived of inorganic nutrients. *Plant Physiology and Biochemistry* **27**: 847–854.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248–254.

Colebatch G, Desbrosses G, Ott T, Krusell L, Montanari O, Kloska S, Kopka J, Udvardi M. 2004. Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. *Plant Journal* **39**: 487–512.

Drevon J-J, Hartwig UA. 1997. Phophorus deficiency increases the argon-induced decline of nodule nitrogenase activity in soybean and alfalfa. *Planta* **201**: 463–469.

Duff SMG, Moorhead GBG, Lefebvre DD, Plaxton WC. 1989. Purification and characterisation of a phospho*enolpyruvate* phosphatase from *Brassica nigra* suspension cells. *Plant Physiology* **90**: 1275–1278.

Egli MA, Griffith SM, Miller SS, Anderson MP, Vance CP. 1989. Nitrogen assimilating enzyme activities and enzyme protein during development and senescence of effective and plantgene-controlled ineffective alfalfa nodules. *Plant Physiology* **91**: 898–904.

Høgh-Jensen H, Schjoerring JK, Soussana J-F. 2002. The influence of phosphorus deficiency on growth and nitrogen fixation of white clover plants. *Annals of Botany* **90**: 745–753.

Israel DW. 1987. Investigation of the role of phosphorus in symbiotic dinitrogen fixation. *Plant Physiology* **84**: 835–840.

Israel DW. 1993. Symbiotic dinitrogen fixation and host-plant growth during development of and recovery from phosphorus deficiency. *Physiology Plantarum* **88**: 294–300.

Jakobsen I. 1985. The role of phosphorus in nitrogen fixation by young pea plants (*Pisum sativum*). *Physiologia Plantarum* **64**: 190–196.

Kaur K, Singh R. 1999. Sugar metabolism and partitioning in cytosol and bacteroid fractions of chickpea nodules. *Plant Physiology and Biochemistry* **37**: 685–692.

Kouchi H, Yoneyama T. 1986. Metabolism of [^{13}C]-labeled photosynthate in plant cytosol and bacteroids of root nodules of *Glycene max*. *Physiologia Plantarum* **68**: 238–244.

Le Roux MR, Ward CL, Botha FC and Valentine AJ. 2006. The route of pyruvate synthesis under P_i starvation in legume root systems. *New Phytologist* **169**: 399–408.

McRae DG, Miller RW, Berndt WB, Joy K. 1989. Transport of C_4-dicarboxylates and amino acids by *Rhizobium meliloti* bacteroids. *Molecular Plant Microbe Interactions* **2**: 273–278.

Mortimer PE, Archer E, Valentine AJ. 2005. Mychorrhizal C costs and nutritional benefits in developing grapevines. *Mychorrhiza* **15**: 159–165.

Ocaña A, del Pilar Cordovilla M, Ligero F, Lluch C. 1996. Phospho*enolpyruvate* carboxylase in root nodules of *Vicia faba*: partial purification and properties. *Physiologia Plantarum* **97**: 724–730.

Olivera M, Tejera N, Iribarne C, Ocaña A, Lluch C. 2004. Growth, nitrogen fixation and ammonium assimilation in common bean (*Phaseolus vulgaris*): effect of phophorus. *Physiologia Plantarum* **121**: 498–505.

Parsons R, Baker A. 1996. Cycling of amino compounds in symbiotic lupin. *Journal of Experimental Botany* **47**: 421–429.

Peng S, Eissenstat DM, Graham JH, Williams K, Hodge NC. 1993. Growth depression in mychorrhizal citrus at high phosphorus supply. I. Analysis of carbon costs. *Plant Physiology* **101**: 1063–1088.

Plaxton WC. 1996. The organization and regulation of plant glycolysis. *Annual Review Plant Physiology and Plant Molecular Biology* **47**: 185–214.

Ribet J, Drevon J-J. 1995. Increase in permeability to oxygen and in oxygen uptake of soybean nodules under limiting phosphorus nutrition. *Physiologia Plantarum* **94**: 298–304.

Rosen H. 1957. A modified ninhydrin colorimetric analysis for amino acids. *Archives of Biochemistry and Biophysics* **67**: 10–15.

Rosendahl L, Vance CP, Pedersen WB. 1990. Products of dark CO_2 fixation

in pea root nodules support bacteroid metabolism. *Plant Physiology* **93**:12–19.

Rychter AM, Chauveau M, Bomsel JL, Lance C. 1992. The effect of phosphate deficiency on mitochondrial activity and adenylate levels in bean roots. *Plant Physiology* **84**: 80–86.

Rychter AM, Mikulska M. 1990. The relationship between phosphate status and cyanide-resistant respiration in bean roots. *Physiologia Plantarum* **79**: 663–667.

Rychter AM, Randall DD. 1994. The effect of phosphate deficiency on carbohydrate metabolism in bean roots. *Physiologia Plantarum* **91**: 383–388.

Sa T-M, Israel DW. 1991. Energy status and functioning of phosphorus–deficient soybean nodules. *Plant Physiology* **97**: 928–935.

Sieger SM, Kristensen BK, Robson CA, Amirsadeghi S, Eng EWY, Abdel-Mesih A, Moller IM, Vanlerberghe GC. 2005. The role of alternative oxidase in modulating carbon use efficiency and growth during macronutrient stress in tobacco cells. *Journal of Experimental Botany* **56**: 1499–1515.

Smith AM. 1985. Capacity for fermentation in roots and *Rhizobium* nodules of *Pisum sativum* L. *Planta* **166**: 264–270.

Solorzano L. 1969. Determination of ammonium in natural waters by the phenol-hypochlorite method. *Limnology and Oceanography* **14**: 799–801.

Streeter JG. 1987. Carbohydrate, organic acid and amino acid composition of bacteroids and cytosol from soybean nodules. *Advances in Boatanical Research* **18**: 129–187.

Tang C, Hinsinger P, Drevon, JJ, Jaillard B. 2001. Phosphorus deficiency impairs early nodule functioning and enhances proton release in roots of *Medicago truncatula* L. *Annals of Botany* **88**: 131–138.

Theodorou ME, Plaxton, WC. 1993. Metabolic adaptation of plant respiration to nutritional phosphate deprivation. *Plant Physiology* **101**: 339–334.

Usuda H, Shimogawara K. 1993. Phosphate deficiency in maize. IV. Changes in amounts of sucrose phosphate synthase during the course of phosphate deprivation. *Plant and Cell Physiology* **34**: 767–770.

Vadez V, Beck DP, Lasso JH, Drevon J-J. 1996. Utilization of the acetylene reduction assay to screen for tolerance of symbiotic N_2 fixation to limiting P nutrition in common bean. *Physiologia Plantarum* **99**: 227–232.

Vance CP, Heichel GH. 1991. Carbon in N_2 fixation: limitation or exquisite adaptation. *Annual Review Plant Physiology and Plant Molecular Biology* **42**: 373–392.

Vessey JK. 1994. Measurement of nitrogenase activity in legume root nodules: in defense of the acetylene reduction assay. *Plant and Soil* **158**: 151–162.

Wanke M, Ciereszko I, Podbiekowska M, Rychter AM. 1998. Response to phosphate deficiency in bean (*Phaseolus vulgaris* L.). Respiratory metabolism, sugar localization and changes in ultrastructure of bean root cells. *Annals of Botany* **82**: 809–818.

Williams K, Percival F, Merino J, Mooney HA. 1987. Estimation of tissue construction cost from heat of combustion and organic nitrogen content. *Plant Cell and Environment* **10**: 725–734.

