Phosphorus Stress-Induced Proteoid Roots Show Altered Metabolism in *Lupinus albus*\(^1\)

Jane F. Johnson, Deborah L. Allan*, and Carroll P. Vance

Department of Soil Science, (J.F.J., D.L.A.), and United States Department of Agriculture-Agricultural Research Service, Department of Agronomy and Plant Genetics (C.P.V.), University of Minnesota, St. Paul, Minnesota 55108–6028

Proteoid roots develop in *Lupinus albus* L. in response to nutrient stress, especially P. Proteoid roots excrete citrate and thus increase the availability of P, Fe, and Mn in the rhizosphere. In an effort to understand citrate synthesis and organic acid metabolism in proteoid roots of lupin, we have evaluated in vitro enzyme activities and in vivo dark CO\(_2\) fixation were higher in proteoid roots of plants grown with or without P. Organic acid concentrations, phosphoenolpyruvate carboxylase (PEPC) in proteoid and normal roots of citrate synthase (CS), malate dehydrogenase (MDH), and phosphoenolpyruvate carboxylase (PEPC) in proteoid and normal roots of plants grown with or without P. Organic acid concentrations, respiration rates, and dark \(^{14}\)CO\(_2\) labeling patterns were also determined. The in vitro specific activities of CS, MDH, and PEPC and in vivo dark \(^{14}\)CO\(_2\) fixation were higher in proteoid roots compared to normal roots, particularly under P stress. Western blot analysis showed that PEPC enzyme protein was more highly expressed in \(-P\) proteoid roots compared to other tissues. The majority of the fixed \(^{14}\)C was found in organic acids, predominantly malate and citrate. A larger fraction of citrate was labeled in P-stressed proteoid roots compared to other root tissue. Respiration rates of proteoid roots were 31% less than those of normal roots. The data provide evidence for increased synthesis of citrate in proteoid roots compared to normal roots, particularly under P stress. A portion of the carbon for citrate synthesis is derived from nonautotrophic CO\(_2\) fixation via PEPC in proteoid roots.

Induction of proteoid root or cluster root morphology has been reported in response to nutrient stress (P, Fe, and N) in numerous species, including white lupin (*Lupinus albus* L.) (Lamont, 1972; Gardner et al., 1981; Marschner et al., 1986; White and Robson, 1989; Louis et al., 1990; Moraghan, 1991; Rosenfield et al., 1991). Gardner et al. (1983) noted that roots of white lupin excrete large quantities of citrate. Marschner et al. (1987) and Dinkelaker et al. (1989) later reported citrate released as root exudate by lupin in response to P stress enhancement of citrate exudation by alfalfa (*Medicago sativa* L.) is only 0.3% of the total dry matter production (Lipton et al., 1987). In white lupin, the high rates of citrate exudation suggest a modification of carbon metabolism in proteoid roots.

Hoffland et al. (1990) reported an increase in leaf PEPC (EC 4.1.1.31) activity in P-stressed rape plants, which coincided with an accumulation of citrate in the shoot. They also reported that application of \(^{14}\)CO\(_2\) to the shoots resulted in a 9-fold increase in the specific radioactivity of organic acids exuded by \(-P\) roots compared to \(+P\) (0.25 mM P) roots. However, they did not identify whether this carbon was derived from CO\(_2\) fixation by PEPC or photosynthate. Increased leaf PEPC activity has also been reported for black mustard (*Brassica nigra*) suspension cells (Duff et al., 1989) and a green alga (*Selenastrum minutum*) (Theodorou et al., 1991) in response to insufficient Pi. Theodorou et al. (1991) suggested that increased PEPC activity in green algae caused by Pi limitation may be a response to increased demands for pyruvate and/or Pi production rather than strictly for anaerobic replenishment of TCA cycle intermediates. The PEPC reaction forms OAA and releases Pi; it also provides a mechanism to bypass pyruvate kinase, which requires Pi and ADP.

Malate rather than pyruvate may be the terminal product of glycolysis and the primary substrate of the TCA cycle in plants, thus bypassing the control point at pyruvate kinase (Lance and Rustin, 1984). Malate in roots is derived from two separate pathways (Fig. 1). The first involves traditional

---

\(^1\) This is Minnesota Agricultural Experiment Station Publication No. 20.167.

* Corresponding author; fax 1–612–625–2208.

---

Abbreviations: CS, citrate synthase; DAE, days after emergence; LSC, liquid scintillation counter; MDH, malate dehydrogenase; OAA, oxaloacetic acid; +P, with 1.0 mM phosphorus; \(-P\), phosphorus withheld; PEPC, phosphoenolpyruvate carboxylase.
glycolysis with conversion of PEP to pyruvate and subsequent entry into the TCA cycle. The second involves carboxylation of PEP to OAA by PEPC and conversion of OAA to malate by MDH (EC 1.1.1.37). To maintain the flow of carbon through glycolysis, malate must either be used (TCA cycle, citrate excretion, amino acid synthesis, etc.) or stored in the vacuole. Accumulation of PEP, malate, and/or citrate in the plant cytosol would lead to inhibition of glycolysis (Douce and Neuberger, 1989; Gietl, 1992). Conditions in which organic acids are being released from roots (i.e. proteoid roots releasing citrate) require that carbon be replaced either by photosynthesis in the shoots or nonautotrophic CO\textsubscript{2} fixation via PEPC in roots. Citrate that has been synthesized can be converted to isocitrate in the mitochondria as part of the TCA cycle, moved to the cytosol, and ultimately be used in amino acid synthesis or excreted into the rhizosphere.

We hypothesized that specific alterations of organic acid metabolism are necessary to supply the carbon excreted from proteoid roots as citrate. The objectives of this study were to determine (a) whether proteoid root formation was specific to P stress, (b) whether the development of proteoid roots in response to nutrient stress was accompanied by changes in organic acid metabolism (i.e. changes in enzyme activity), and (c) whether nonautotrophic CO\textsubscript{2} fixation contributed to organic acid metabolism in proteoid roots.

**MATERIALS AND METHODS**

**Plant Material**

*Lupinus albus* L. var Ultra plants were grown in a Conviron (model E15; Controlled Environments, Pembina, ND) growth chamber at 20/15°C and 16-/8-h day/night cycles, 300 μmol of photons m\textsuperscript{-2} s\textsuperscript{-1}, and approximately 50% RH. Three seeds were surface sterilized and planted in pots containing 3 kg of 1.0-mm-diameter and 5 kg of 0.625-mm-diameter acid-washed silica sand for a total volume of 3.5 L. The pots were thinned to one plant per pot after emergence, and plants were grown for an additional 20 to 25 d. Pots were watered every 2 to 3 d with 300 mL of the appropriate nutrient solution. The control nutrient solution consisted of 3.0 mM KNO\textsubscript{3}, 2.5 mM Ca(NO\textsubscript{3})\textsubscript{2}, 0.5 mM Ca(H\textsubscript{2}PO\textsubscript{4})\textsubscript{2}, 1.0 mM MgSO\textsubscript{4}, 12.0 μM Fe (as FeEDTA), 4.0 μM MnCl\textsubscript{2}, 22.0 μM H\textsubscript{3}BO\textsubscript{3}, 0.4 μM ZnSO\textsubscript{4}, 0.05 μM NaMoO\textsubscript{4}, and 1.6 μM CuSO\textsubscript{4}. The -P solution did not receive the 0.5 mM Ca(H\textsubscript{2}PO\textsubscript{4})\textsubscript{2}. In the macronutrient stress experiment, KCl and CaSO\textsubscript{4} were substituted to maintain K and Ca concentrations in the -N solution. In the -K solution, the amount of Ca(NO\textsubscript{3})\textsubscript{2} was increased to maintain the N concentration. In the micronutrient stress experiment, Fe, Mn, or Zn were withheld from the nutrient solution. The concentration of the low-strength nutrient solution was one-twentieth that of the control for all nutrients except P, which was maintained at the same concentration as the control. In the enzyme assay, labeling, and respiration experiments, half the plants received the control (+P) solution, and the remaining half received the -P solution. The pH was adjusted to 5.5 with NaOH or HCl for all solutions.

**Proteoid Root Development in Response to Nutrient Stress**

Experiments were conducted to determine whether selected nutrient stresses would induce proteoid roots. Each experiment had four replications arranged in a randomized complete block design. The first experiment tested the effect of withholding N, P, or K or the use of a low-strength solution, and the second tested the effect of withholding P, Fe, Mn, or Zn, on proteoid root development. Plants were harvested at 23 and 22 DAE, respectively. Plants were separated into shoot, normal roots, and proteoid roots, dried at 68°C for at least 24 h, and weighed after all adhering sand was removed.

**Preparation of Root Extracts for Enzyme Assays**

Enzyme assay experiments were repeated three times to confirm the results. Plant material was harvested 17, 19, and 23 DAE when symptoms of P stress were visible. About 1.5 g fresh weight each of proteoid and normal root tissue and shoot tissue (at the 23-DAE harvest only) were collected from each plant and kept on ice. Approximately 1.0 g fresh weight of tissue was then placed into a prechilled mortar and pestle,
frozen with liquid N, and pulverized to a fine powder. The tissue was homogenized to a well-blended slurry with a small amount of acid-washed sand in cold Tris-HCl (0.25 M, pH 8.0, with 0.5 mM Suc and 0.01 mM DTT) (Bogin and Wallace, 1969). The homogenate was filtered through four to five layers of cheesecloth, brought to a volume of 4 mL, and centrifuged for 10 min at 10,000g. The supernatant was saved, and the pellet was discarded. The supernatant was used to assay for PEPC, CS (EC 4.1.3.7), MDH, and total protein.

In Vitro Enzyme Assays

CS (Srere, 1967; Kurz and LaRue, 1977) was assayed spectrophotometrically by monitoring the reduction of acetyl-CoA to CoA with 5,5’-dithio-bis-2-nitrobenzoic acid at 340 nm for 2 min. MDH (Sulebele and Silverstein, 1969) and PEPC (Vance et al., 1983) were assayed spectrophotometrically by monitoring the disappearance of NADH at 340 nm in a direct and coupled assay, respectively. The protein was precipitated with acetone by thoroughly mixing 200 μL of root extract with 800 μL of acetone. The solution was stored at −20°C for at least 2 h before centrifuging (20 min, 14,000g) and evaporating the acetone. The protein was resuspended in 0.5 M NaOH; heat and physical agitation were used as necessary to solubilize the protein. Total protein was then determined using a BCA protein assay reagent kit (Pierce Biochemical, Rockford, IL).

SDS-PAGE and Western Blot Immunnoanalysis for PEPC

Plant material was grown as described above and harvested 23 to 25 DAE. The normal and proteoid root tissue from +P and −P treatments was pooled from three plants. The PEPC activity and total protein were determined for each tissue type as described above. Soluble protein (50 μg of protein for each tissue) from cell-free extracts was separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. Rabbit polyclonal antibodies to alfalfa nodule PEPC were used to detect lupin root PEPC protein on western blots (Miller et al., 1987). Protein extracted from alfalfa nodules was used as a positive control for antibody detection. Miller et al. (1987) demonstrated that this antibody cross-reacts with the 100-kD lupin nodule PEPC protein and the 100-kD alfalfa root PEPC protein.

14CO2 Labeling and Extraction Procedure

The protocol for the in vivo CO2 fixation assay for excised roots was modified from that used by Christeller et al. (1977) and Maxwell et al. (1984). Excised root tissue (350 mg fresh weight) was labeled with 7.4 × 10−6 Bq of aqueous NaH14CO3 (2.1 × 105 Bq mmol−1; ICN Radiochemicals, Irvine, CA) for 20 min at 23°C. The samples were homogenized in 50% (v/v) ethanol, followed by extraction in a 45°C water bath for 20 min and centrifugation at 10,000g for 20 min. Phenols and alkaloids were removed with a chloroform separation (2:1, supernatant:chloroform ratio). An aliquot of the water-soluble layer was treated with HCl, and the acid-stable radioactivity was determined by LSC (model LS8000; Beckman, Fullerton, CA).

Separation of Labeled Root Extracts

After chloroform partitioning, a 0.5-mL aliquot of the water-soluble fraction was separated into neutral, acidic, and basic fractions using ion-exchange chromatography (Dowex 50-X8 H+ 200–400 mesh and Dowex 1-X8 200 mesh, Sigma) as described by Atkins and Canvin (1971) and Vance et al. (1983). Sugars elute into the neutral fraction, carboxylic acids elute in the acid fraction, and amino acids elute in the basic fraction (Stumpf and Burris, 1979). The total radioactivity of each fraction was determined by LSC. At least 70% of the acid-stable radioactivity was recovered in the ion-exchange fractions.

Analysis of Organic Acids

The organic acid fraction (25 mL) was evaporated to dryness using a Savant Speedvac auto evaporator (Savant Instruments, Inc., Farmington, NY), equipped with both a chemical and charcoal trap. The residue was resuspended in 0.5 mL of 0.008 N H2SO4 and refrigerated. The individual organic acids were separated by HPLC (model SP8800 pump and SP4290 integrator; Spectra-Physics, San Jose, CA) equipped with an Aminex HPX-87H, 300 × 7.8-mm column (Bio-Rad, Richmond, CA) and an organic acid guard column (Bio-Rad). The eluant was 0.008 N H2SO4 with a flow rate of 0.6 mL min−1 at ambient temperature (about 23°C). The acids were detected at 210 nm on a Spectroflow 757 UV/VIS detector (ABI Analytical Kratos Division, Ramsey, NJ). Standard solutions were tested to determine retention time and retention factors for oxalic, citric, α-ketoglutaric, malic, succinic, and fumaric acids. Formic acid was detected, but because of the contamination during formic acid elution from the ion-exchange procedure, it was not included for calculation of the results. The detector was interfaced to a Gilson fraction collector (model FC203; Gilson, Middleton, WI) such that individual peaks could be resolved and collected. The total radioactivity of each fraction was determined by LSC. About 70% of the total radioactivity determined in the acidic fraction after the Dowex ion-exchange procedure was recovered in the collected HPLC fractions.

Measurement of Respiration Rate (O2 Consumption)

O2 consumption was measured for 8 min at 20°C using an O2 electrode in a Gilson K-IC oxygraph (Gilson, Middleton, WI). Respiration rate was measured for proteoid and normal root (50–100 mg fresh weight) tissue from both P treatments, and then dry weight was determined. The experiment was repeated using plants harvested 26 and 23 DAE. Calculation of respiration rate was based on the assumption that the water solubility of O2 at 20°C is 1.46 mol m−3. The linear decrease in O2 over 5 to 7 min was determined from a strip chart recorder. The rate of decrease was divided by the dry weight to obtain the specific respiration rate.

Statistical Analysis

Treatments in the nutrient stress experiments were arranged in a randomized complete block design with three replications. The differences among treatments were deter-
mined by analysis of variance and LSD of the means. Enzyme assay, respiration, and root-labeling experiments were arranged in a randomized complete block design, in which run of the experiment was the block effect. Each block had three replications, and roots were a repeated measure. There were only two root treatments, resulting in one degree of freedom for this level of variation. Under such circumstances, the H-F conditions (Huynht and Feldt, 1970) for multivariate analysis hold automatically; therefore, univariate and multivariate significance probabilities are equal (Littell, 1989). Thus, it is possible to analyze the data set as split-plot with P treatment as the main plot and root tissue as the subplot unit. The LSD values for the interaction terms within or across P treatment used the weighted t values and sd values as described by Gomez and Gomez (1984).

RESULTS

Nutrient Stress Effects on Development of Proteoid Roots

The operational definition of a proteoid root was a secondary root with densely clustered rootlets (Fig. 2), following the morphological description of previous authors (Purnell, 1960; Lamont, 1972; Gardner et al., 1981). Proteoid roots were present in all treatments, ranging from 3 to 56% (w/w) (Table I). The amount of total root mass made up of proteoid roots for control plants averaged 7.4% (w/w) for both experiments. The —K treatment had only 3% (w/w) proteoid roots, significantly less than the other nutrient treatments in the macronutrient experiment. In both the —Mn and —P treatments, the incidence of proteoid roots was higher than the control. The —P-treated plants had the highest incidence of proteoid roots with an average of 54% (w/w) in the macronutrient and micronutrient experiments. Typically, root segments with proteoid morphology from plants receiving P were less than 2.5 cm in length, and those from the —P treatments were greater than 5 cm in length. In the —P plants, the sand surrounding the proteoid roots was especially difficult to remove.

Enzyme Activity

The specific activities of CS, PEPC, and MDH were significantly higher in proteoid roots compared to normal roots (Table II), but there was no difference due to P treatment when averaged over the two tissue types (data not shown). The higher CS activity of proteoid roots (P = 0.056) could be accounted for by the 40 to 60% higher CS activity in the —P proteoid roots compared to the other three tissues (Fig. 3). For PEPC and MDH, there was no interaction between P treatment and tissue type. Protein concentrations did not differ with P treatment or tissue type. The overall protein concentration was 1.32 mg of protein g⁻¹ fresh weight tissue (data not shown). When enzyme activities were expressed in units of fresh weight instead of protein, the same trends among treatments were observed for all three enzymes.

PEPC Immunoblot Analysis

Immunoblot analysis showed that proteoid roots contain an intensely staining 104-kD band that reacts with highly specific PEPC antibodies (Fig. 4, lanes 2 and 4). Lanes containing comparable amounts of normal root protein (Fig. 4, lanes 1 and 3) showed much less intense bands. These bands correspond to the 104-kD PEPC subunits in alfalfa and lupine root nodules (Miller et al., 1987). The PEPC activity of extracts used for immunoblot analysis was similar to that in Table II, with PEPC activity of proteoid roots being significantly greater than normal roots. Because the amount of soluble protein in proteoid and normal roots is the same, the increased intensity of the PEPC band in proteoid roots indicates that PEPC enzyme protein is increased in proteoid roots compared to normal roots.

¹⁴CO₂ Labeling

As shown by incorporation of ¹⁴CO₂ into acid-stable products, in vivo nonautotrophic CO₂ fixation was significantly higher in proteoid roots compared to normal roots, but there was no significant difference between P treatments (Table III). The increased amount of label incorporated into the proteoid root tissue was completely accounted for by the acidic fraction. Although label incorporated into the basic fraction did not differ in proteoid versus normal root tissue, the percentage in the basic fraction was reduced in the —P treatment. In the —P proteoid roots, significantly less label was incorporated into the basic fraction compared to the other three treatments, whereas the +P proteoid roots incorporated significantly more label into the basic fraction.

The distribution of the radioactivity among the ion-exchange fractions (Table III) indicated that about 74% of the radioactivity was in the acidic fraction, with the remainder in the basic fraction and none in the neutral fraction. Similar to the pattern for amount of label incorporated, there was no effect of P treatment, but the type of root tissue was significant. The proteoid roots had a higher percentage of label in the acidic fraction but significantly less in the basic fraction compared to the normal roots. In the +P treatment, more label was fixed in the proteoid roots, but there was no change in the distribution into acidic and basic fractions. In the —P treatment, the normal roots had less label incorporated into
Table I. Effects of selected nutrient stresses on the occurrence of proteoid roots (percentage of root mass) and shoot and root weight

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Proteoid Root</th>
<th>Shoot Mass</th>
<th>Root Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient stress macronutrients</td>
<td>Control</td>
<td>9.4b*</td>
<td>2.38a</td>
<td>1.15a</td>
</tr>
<tr>
<td></td>
<td>-N</td>
<td>7.1bc</td>
<td>0.86b</td>
<td>0.51bc</td>
</tr>
<tr>
<td></td>
<td>-P</td>
<td>51.9a</td>
<td>1.17b</td>
<td>0.25c</td>
</tr>
<tr>
<td></td>
<td>-K</td>
<td>2.9c</td>
<td>1.26b</td>
<td>0.78b</td>
</tr>
<tr>
<td></td>
<td>1/20</td>
<td>8.6b</td>
<td>0.81b</td>
<td>0.34c</td>
</tr>
<tr>
<td>Nutrient stress micronutrients</td>
<td>Control</td>
<td>5.6c</td>
<td>2.21a</td>
<td>1.04a</td>
</tr>
<tr>
<td></td>
<td>-P</td>
<td>55.7a</td>
<td>0.77c</td>
<td>0.35b</td>
</tr>
<tr>
<td></td>
<td>-Fe</td>
<td>11.4bc</td>
<td>2.11ab</td>
<td>0.94a</td>
</tr>
<tr>
<td></td>
<td>-Mn</td>
<td>17.1b</td>
<td>2.13ab</td>
<td>0.87a</td>
</tr>
<tr>
<td></td>
<td>-Zn</td>
<td>7.0e</td>
<td>2.13ab</td>
<td>1.02a</td>
</tr>
</tbody>
</table>

* Values in a column followed by different letters were significantly different as calculated by LSD for P = 0.05.

The acidic fraction and more into the basic fraction than the +P roots, whereas the -P proteoid roots had more in the acidic and less in the basic fraction than the +P roots (Table III).

HPLC Analysis of Organic Acids

Citric, malic, formic, and fumaric acids were consistently detected in both normal and proteoid roots. Three unidentified fractions were routinely detected with retention times of about 11.1, 11.9, and 12.8 min and were designated unknowns 1, 2, and 3, respectively. Oxalic and α-ketoglutaric acids were detected in samples when a doubled amount of the typical sample concentration was loaded.

The total concentration of detected organic acids was significantly less in the -P treatment compared to the +P treatment because of the significant reduction in malate concentration (Table IV). The two types of root tissue did not differ in total amounts of organic acids, but they did differ in composition. The amount of citrate in proteoid roots was 60% greater than in normal roots, whereas the amount of malate and fumarate was 21 and 47% less, respectively. Fumarate was less than 1% of the total for both tissues.

Treatment interactions indicated that -P proteoid roots had lower total concentration of organic acids than the -P normal roots, whereas +P tissues did not differ. Citrate concentrations did not differ between tissues for the -P treatment but were highest in the +P proteoid roots and lowest in the +P normal roots. Citrate made up a larger percentage of the total organic acids in both -P and proteoid tissues, with the highest value (50%) in -P proteoid roots. Citrate/malate ratios were also more than twice as high in the -P and proteoid tissues, with the highest ratio greater than 1:1 in the -P proteoid roots.

Citrate and malate represented more than 90% of the total radioactivity recovered in the HPLC-separated organic acids for every treatment. Unknown 1 accounted for less than 2%, and fumarate accounted for less than 3% of the detectable radioactivity recovered (Fig. 5). The percentage of radioactivity recovered in citrate was significantly higher, whereas the percentage recovered in malate was significantly lower in the -P treatment compared to the +P treatment and in the proteoid roots compared to the normal roots (data not shown). These differences were mostly due to the -P proteoid roots, in which the percentage of radioactivity in citrate was higher than the other three tissues, and the percentage

Table II. Specific activity on a mg of protein basis for CS (μmol of acetyl-CoA min⁻¹ mg⁻¹ of protein), determined spectrophotometrically at A₄₁₂, MDH, and PEPC (μmol of NADH min⁻¹ mg⁻¹ of protein), determined spectrophotometrically at A₃₄₀

<table>
<thead>
<tr>
<th>Tissue measure averaged across both P treatments.</th>
<th>Normal</th>
<th>Proteoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>0.102</td>
<td>0.128</td>
</tr>
<tr>
<td>MDH</td>
<td>38.3</td>
<td>48.0</td>
</tr>
<tr>
<td>PEPC</td>
<td>0.30</td>
<td>0.48</td>
</tr>
</tbody>
</table>

* Significant at the 0.1 probability level.  ** Significant at the 0.05 and 0.01 probability levels, respectively.

Figure 3. Specific activity on a mg of protein basis for CS (μmol of acetyl-CoA min⁻¹ mg⁻¹ of protein) determined spectrophotometrically at A₄₁₂ for normal and proteoid roots of L. albus plants grown with +P or -P. Each bar represents the treatment mean (n = 9). Bars labeled with the same letter are not different as determined by analysis of variance and LSD (P ≤ 0.05).
of radioactivity in malate was lower (Fig. 5). The percentage of radioactivity in fumarate was significantly lower in the proteoid compared to normal roots of the —P treatment, but there was no difference between root tissues for fumarate in the +P treatment.

Specific radioactivity of individual organic acids is an indicator of the amount of carbon derived from direct $^{14}$CO$_2$ fixation. The specific activity of citrate, malate, and fumarate was higher in the proteoid roots compared to normal roots (Table V), but there was no difference due to P treatment when averaged over the two tissue types (data not shown). Although there were no differences in citrate-specific radioactivity between normal and proteoid roots of the +P-treated plants, the specific radioactivity of citrate from proteoid roots of the —P plants was 5-fold greater than that of the —P normal roots (Fig. 6). The interaction between P treatment and root tissue was not significant for the specific radioactivity of malate or fumarate. Fumarate had the highest specific radioactivity but made up less than 3% of the total radioactivity in the sample (Fig. 5) and less than 1% of the organic acids recovered (Table IV).

### Table III. Total radioactivity (kBq g$^{-1}$ fresh weight) and the distribution of radioactivity for the acidic (carboxylic acids), basic (amino acids), and combined fractions after separation by ion-exchange chromatography for plants grown with +P or —P

Excised roots were exposed to aqueous NaH$^{14}$CO$_3$ for 20 min.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No.</th>
<th>Total</th>
<th>Acidic</th>
<th>Basic</th>
<th>Acidic</th>
<th>Basic</th>
</tr>
</thead>
<tbody>
<tr>
<td>+P</td>
<td>30</td>
<td>30.3</td>
<td>22.9</td>
<td>7.4</td>
<td>74</td>
<td>25</td>
</tr>
<tr>
<td>—P</td>
<td>30</td>
<td>23.9</td>
<td>19.1</td>
<td>4.8</td>
<td>74</td>
<td>25</td>
</tr>
<tr>
<td>Root</td>
<td></td>
<td></td>
<td>NS*</td>
<td>NS*</td>
<td>NS</td>
<td>NS*</td>
</tr>
<tr>
<td>Normal</td>
<td>30</td>
<td>20.4</td>
<td>14.2</td>
<td>6.2</td>
<td>67</td>
<td>31</td>
</tr>
<tr>
<td>Proteoid</td>
<td>30</td>
<td>33.8</td>
<td>27.8</td>
<td>6.0</td>
<td>81</td>
<td>19</td>
</tr>
<tr>
<td>Root</td>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>+P normal</td>
<td>15</td>
<td>24.4</td>
<td>17.7</td>
<td>6.7</td>
<td>72</td>
<td>26</td>
</tr>
<tr>
<td>+P proteoid</td>
<td>15</td>
<td>36.2</td>
<td>28.1</td>
<td>8.0</td>
<td>76</td>
<td>24</td>
</tr>
<tr>
<td>—P normal</td>
<td>15</td>
<td>16.4</td>
<td>10.7</td>
<td>5.7</td>
<td>62</td>
<td>36</td>
</tr>
<tr>
<td>—P proteoid</td>
<td>15</td>
<td>31.4</td>
<td>27.5</td>
<td>3.9</td>
<td>86</td>
<td>14</td>
</tr>
</tbody>
</table>

**Notes:**
- *NS* or *Not significant or significant, respectively, at the 0.05 probability level.
- NA, LSD value not applicable as the interaction term was not significant as calculated with analysis of variance.

**DISCUSSION**

Consistent with previous reports, we found that nutrient stress, particularly P, enhances the development of proteoid root tissue in white lupin (Gardner et al., 1981, 1982b; Marschner et al., 1986; Dinkelaker et al., 1989; Moraghan, 1991). Carbon metabolism and the partitioning of carbon into organic and amino acid fractions are modified by P treatment and root type. When the plants are P stressed, there appears to be a shift toward increased organic acid synthesis in the proteoid roots, with PEPC providing a portion of the required carbon. The shift toward organic acid synthesis in the P-stressed proteoid roots is accompanied by an increase in the activities of PEPC, MDH, and CS as well as an increase in nonautotrophic CO$_2$ fixation and reduced O$_2$ consumption. Increased specific radioactivity of citrate, malate, and fumarate in proteoid roots labeled via root $^{14}$CO$_2$ support this interpretation. Citrate in particular constitutes a higher percentage of the organic acids and is more highly labeled in the —P proteoid roots compared to other tissues. These modifications in carbon metabolism in proteoid roots from P-stressed plants are consistent with reports showing localized excretion of citrate into the proteoid rhizosphere of...
Table IV. Concentrations of citrate, malate, and fumarate detected by HPLC in root tissue from plants grown with +P or -P

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No.</th>
<th>Total Citrate</th>
<th>Malate</th>
<th>Fumarate</th>
<th>Citrate</th>
<th>Citrate/Malate</th>
</tr>
</thead>
<tbody>
<tr>
<td>+P</td>
<td>30</td>
<td>24.5</td>
<td>6.53</td>
<td>18.4</td>
<td>0.14</td>
<td>26.1</td>
</tr>
<tr>
<td>-P</td>
<td>30</td>
<td>17.0</td>
<td>7.27</td>
<td>9.6</td>
<td>0.12</td>
<td>42.9</td>
</tr>
</tbody>
</table>

Root
Normal 30 21.1 5.34 15.6 0.17 25.4 0.41
Proteoid 30 21.8 8.58 12.4 0.09 44.6 0.95

Root
+P normal 15 23.4 3.3 19.9 0.17 14 0.18
+P proteoid 15 25.8 10.1 17.0 0.10 39 0.67
-P normal 15 18.9 7.4 11.4 0.16 36 0.64
-P proteoid 15 15.2 7.2 7.9 0.08 50 1.20

LSD = 0.05 3.1 1.2 NA NA NA NA

* NS, **, *** Not significant or significant at the 0.05 and 0.01 probability levels, respectively.

Table V. The specific radioactivity (kBq µmol⁻¹ of carboxylic acid) of the three major acids detected by HPLC from the normal or proteoid roots averaged across P treatments

<table>
<thead>
<tr>
<th>Root</th>
<th>No.</th>
<th>Citrate</th>
<th>Malate</th>
<th>Fumarate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18</td>
<td>1.4</td>
<td>1.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Proteoid</td>
<td>18</td>
<td>3.2</td>
<td>3.7</td>
<td>6.1</td>
</tr>
</tbody>
</table>

** Significant at the 0.05 and 0.01 probability levels, respectively.
produced. This could be verified by assaying the activity of respiration rates measured in proteoid roots provide indirect specific activity of P-stressed proteoid roots was 0.54 Imol-1 min-1 of protein, nearly twice that of P-stressed shoots. The PEPC-specific activity of P-stressed proteoid roots was 0.54 μmol min-1 mg-1 of protein, nearly twice that of P-stressed shoots. Coupled with the rapid labeling of the citrate and malate pools (Fig. 5) and the high specific radioactivity of citrate in P-stressed proteoid roots (Fig. 6), these data provide good evidence that altered root metabolism plays a key role in the exudation of citrate from proteoid roots under P stress. Lower respiration rates measured in proteoid roots provide indirect evidence that TCA cycle activity may also be altered, such that less NADH and flavin adenine dinucleotide H2 are produced. This could be verified by assaying the activity of TCA enzymes subsequent to CS, such as aconitate hydratase and isocitrate dehydrogenase.

Label incorporated into citrate can either continue through the TCA cycle, be excreted, or be converted to α-ketoglutarate and transaminated to amino acids (Fig. 1). In proteoid roots, the 14C-labeling pattern of the ion-exchange fractions showed that more 14C was incorporated into the acidic fraction (carboxylic acids) compared to the basic fraction (amino acids, Table III). The most striking difference was in the −P proteoid roots, where only 14% of the label was found in the basic fraction. One of the roles of PEPC in higher plants is to replenish OAA that is removed from the TCA cycle during amino acid synthesis (Latzko and Kelly, 1983). We propose that the increased PEPC activity in −P proteoid roots is replenishing carbon from the TCA cycle for the synthesis of citrate rather than amino acids. This is also supported by the high specific radioactivity of citrate in the −P proteoid roots.

Carbon fixed in roots via PEPC may reduce demands for photosynthetically fixed carbon. In general, CO2 uptake for assimilation in root systems is considered negligible (Farmer and Adams, 1991). However, fixation of carbon in roots may be of great importance for carbon balance in roots (Vuorinen et al., 1992) and may result in increased biomass production (Vapaavuori and Pelkonen, 1985). Significant amounts of CO2 fixed by tomato (Lyceopersicon esculentum L.) roots was transported to the stem and leaves (Bialzyk and Lechowski, 1992). Carbon fixed by nodules has been estimated to provide 25 to 30% of the carbon required for N assimilation (Vance et al., 1983; Maxwell et al., 1984). The activity of PEPC in the P-stressed proteoid roots was about 10-fold greater than activities reported for L. albus roots and about 4-fold less than the activity of nodule PEPC (Christeller et al., 1971). In P-stressed lupin, the increased PEPC may buffer against losses of photosynthetic carbon during citrate excretion from the roots.

The ratio of citrate/malate was higher in the −P treatment compared to +P and in proteoid roots compared to normal roots, but the interaction was not significant (Table IV). Similar changes in the ratio of citrate to malate have been reported in alfalfa (Lipton et al., 1987) and rape (Hoffland et al., 1990) in response to P stress, in white lupin (Jeschke, 1986) and in alfalfa (Fougere et al., 1991) in response to salinity stress, and in response to Fe stress (Landsberg, 1981). We hypothesize that cellular citrate concentration is maintained during citrate excretion in P-stressed plants via higher activities of PEPC, MDH, and CS with malate converted to citrate rather than being stored in the vacuole. Although the total citrate concentration of −P proteoid roots is less than +P proteoid roots, cellular citrate concentration probably underestimates the citrate produced, especially in the P-stressed proteoid roots, which excrete citrate (Marschner et al., 1987). The cellular concentration we measured is about 250-fold less than the rhizosphere concentration of P-stressed proteoid roots reported by Marschner et al. (1987) and Dinkelaker et al. (1989). A comparison of radioactive label in citrate versus malate (Table V) supports our hypothesis that malate is converted to citrate in the proteoid roots of P-stressed lupin, where the ratio is about 1:1. By comparison, ratios for the other tissues range from 1:3 to 1:5.

Our data show that proteoid roots develop in response to P stress and to a lesser extent to Mn and Fe stress. Such proteoid roots have altered metabolism with increased PEPC, MDH, and CS activity but reduced O2 consumption. With P stress, there is evidence for a change in the allocation of carbon between organic and amino acid fractions in the proteoid roots. The higher amount of label incorporated into the acidic fraction of P-stressed proteoid roots compared to normal roots as well as the increased PEPC activity are evidence that nonaotrophic CO2 fixation contributes to organic acid metabolism in roots. Our experimental results are consistent with the hypothesis that proteoid roots develop in response to P stress, and such roots have altered metabolism that will facilitate citrate production and excretion.

**ACKNOWLEDGMENTS**

The authors would like to thank Dr. Albert Markhart III for use of his O2 electrode and constructive comments, Dr. Ed Nater for use of his Gilson fraction collector, Sue Miller for her technical assistance and valuable advice, and Larry Oldham and Dr. Gary Oehlert for statistical advice.
Received August 25, 1993; accepted October 13, 1993.

LITERATURE CITED


White PF, Robson AD (1989) Rhizosphere acidification and Fe2+ reduction in lupin and peas: iron deficiency in lupin is not due to a poor ability to reduce Fe3+. Plant Soil 119: 163–175