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Physiological Aspects of Cluster Root Function and Development in Phosphorus-deficient White Lupin (*Lupinus albus* L.)

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Cluster root formation in white lupin (*Lupinus albus* L.) is induced mainly by phosphorus (P) starvation, and seems to be regulated by the endogenous P status of the plant. Increased formation of cluster roots, when indole acetic acid is supplied to the growth medium of P sufficient plants, and inhibitory effects of kinetin application suggest the involvement of endogenous phytohormones (auxins and cytokinins), which may act in an antagonistic manner in the P-starvation response. Phosphorus deficiency-induced adaptations of white lupin, involved in P acquisition and mobilization of sparingly available P sources, are predominantly confined to the cluster roots, and moreover to distinct stages during their development. Increased accumulation and exudation of citrate and a concomitant release of protons were found to be mainly restricted to mature root clusters after prolonged culture (3–4 weeks) under P-deficient conditions. Inhibition of citrate exudation by exogenous application of anion channel antagonists such as ethacrynic- and anthracene-9-carboxylic acids may indicate involvement of an anion channel. Phosphorus deficiency-induced accumulation and subsequent exudation of citric acid seems to be a consequence of both enhanced biosynthesis and reduced turnover of citric acid in the cluster root tissue, indicated by enhanced expression of sucrose synthase, fructokinase, phosphoglucomutase, phosphoenol-pyruvate carboxylase, but reduced activity of aconitase and slower root respiration. The release of acid phosphatase and of phenolic compounds (isoflavonoids) as well as the induction of a putative high-affinity P uptake system was more highly expressed in juvenile, mature and even senescent cluster regions than in apical zones of non-proteoid roots. An AFLP-cDNA library for cluster root-specific gene expression was constructed to assist in the identification of further genes involved in cluster root development.

Key words: Acid phosphatase, auxin, citric acid, cluster roots, cytokinin, *Lupinus albus* L., P acquisition, P uptake, root exudates.

INTRODUCTION

White lupin (*Lupinus albus* L.) is highly efficient with respect to P uptake and the utilization of sparingly available sources of soil phosphorus. This efficiency cannot be attributed to a high rate of root growth or to mycorrhizal associations. However, white lupin has developed effective mechanisms for the chemical mobilization of sparingly available P sources in the rhizosphere, involving the formation of cluster roots. During the last decade, our work on white lupin and other cluster-rooted species in the genus *Hakea* has focused on the chemical effects of cluster roots on mineral nutrient acquisition in the rhizosphere, and on the related changes in root physiology which occur during cluster root formation. The present work summarizes the results of our research activities on adaptations to P deficiency in white lupin.

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MOBILIZATION OF MINERAL NUTRIENTS IN THE RHIZOSPHERE

When exposed to P starvation, white lupin and other cluster-rooted plant species excrete large amounts of citric and malic acids from closely-spaced lateral rootlets arranged in clusters along first order lateral roots, so-called proteoid roots. These root clusters exhibit limited growth, reaching an average length of 5 mm, and are densely covered with root hairs (Dinkelaker *et al.*, 1989, 1995, 1997). Accumulation of 50–90 μmol of citric acid per g soil has been reported in the rhizosphere soil of cluster roots in white lupin (Dinkelaker *et al.*, 1989; Gerke *et al.*, 1994; Li *et al.*, 1997). This concentration level was sufficient to mediate a significant desorption of phosphorus from sparingly soluble Ca-, Al- and Fe-P and from P-adsorbing Fe/Al humic acid complexes, mainly by mechanisms of ligand exchange, and dissolution of P sorption sites in the soil matrix (Gardner *et al.*, 1983; Gerke *et al.*, 1994). Citrate excretion from cluster roots of white lupin was also associated with a marked acidification of the rhizosphere to pH 4–5 (Fig. 1), which was detectable even in a

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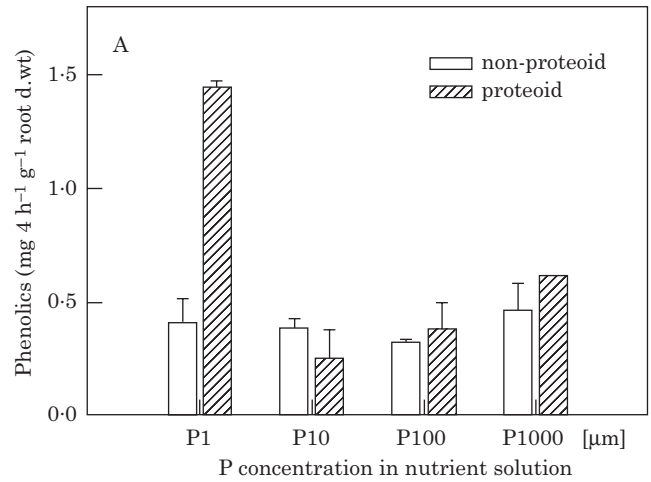
FIG. 1. Root system of white lupin grown in hydroponic culture for 28 d without P supply. Spatial variation of pH along the root surface of cluster roots in different developmental stages detected by application of agar sheets containing bromocresol purple as a pH indicator: pH 6·5–7·0 = purple, pH 4–5 = yellow.

TABLE 1. *Soil pH and concentrations of citrate, phosphate and micronutrients in bulk and proteoid rhizosphere soil of white lupin grown in a P-deficient calcareous soil*

	Bulk soil	Rhizosphere soil (cluster roots)
pH (H_2O)	7·5	4·8
Citrate (μmol g^{-1} soil)	1·1 ± 0·2	47·7 ± 7·2
Phosphorus (μmol kg^{-1} soil)		
H_2O-extractable	178 ± 28	61 ± 7
CAL	904 ± 80	581 ± 76
Olsen	581 ± 93	484 ± 68
DTPA-extractable (μmol kg^{-1} soil)		
Fe	34 ± 6	251 ± 43
Mn	44 ± 8	222 ± 23
Zn	2·8 ± 0·4	16·8 ± 2·4

Means ± s.d. of at least three independent replicates. Source: Dinkelaker *et al*, 1989.



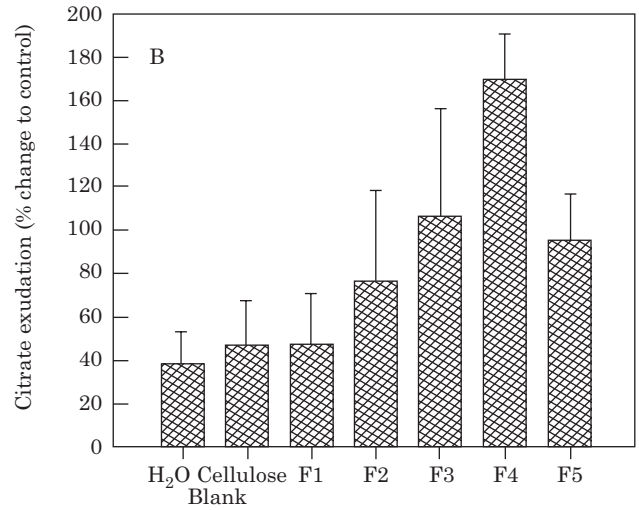


FIG. 2. A, Exudation of phenolic compounds in different root zones of hydroponically-grown white lupin in response to variations of P supply in the nutrient solution. Determination of total phenolics with Folin-Ciocalteus phenol reagent. Bars represent means and s.d. of three to four independent experiments. B, Effect of partially purified phenolics isolated from root exudates of white lupin on citrate excretion in cluster roots. Exudate phenolics were isolated from root washings after a collection period of 2 h by solid phase extraction with Sep-Pak RP-18 cartridges (Millipore, Milford, USA), and subsequently eluted with methanol. The concentrated methanolic solution was separated by preparative thin-layer chromatography (TLC) on Avicel cellulose plates using 30% (v/v) acetic acid as a solvent. Flavonoids were detected by spraying one part of the TLC plates with indicator reagents according to Linskens (1959), and colour reactions were examined under UV/Vis light. Separated bands were scraped off from the untreated parts of the TLC plates and extracted with 80% (v/v) methanol. Methanol was removed under vacuum and the aqueous phenolic solutions were applied to filter papers used for 1·5 h of exudate collection from single root clusters (Neumann *et al*, 1999). To account for the high variability of citrate exudation from different clusters, exudation rates were recorded relative to a preceeding 1·5 h control incubation of the same clusters using filter papers without phenolics. Means and s.d. of four independent replicates are presented.

well-buffered calcareous soil with pH 7·5, and may dissolve acid-soluble Ca-P (Table 1; Dinkelaker *et al*., 1989).

High stability constants for the formation of metal complexes with citrate (Jones, 1998) account for the intense mobilization of various metal ions reported in the rhizosphere of cluster roots (Table 1; Gardner *et al*., 1983; Dinkelaker *et al*., 1989, 1997; Gerke *et al*., 1994). A marked reduction of iron and manganese is detectable at the rhizoplane of cluster rootlets by application of agar gels or filter papers containing metal-specific redox indicators (Marschner *et al*., 1987; Dinkelaker *et al*., 1993*a*,*b*, 1995). This may be attributed to an increased activity of membrane-bound reductases, stimulated by low pH levels in the rhizosphere of cluster roots, but also to the excretion of large amounts of reducing compounds such as malate, citrate and phenolics (Fig. 2A), identified mainly as isoflavonoids (Neumann, unpubl. res.; Wojtaszek *et al*., 1993). Iron solubilization, mediated by reduction of FeIII bound in Fe-P, might thereby also increase the availability of phosphorus. Antibiotic properties of isoflavonoids in root exudates (Rao, 1990) may not only counteract infection by root pathogens but also prevent the microbial degradation of organic exudate compounds involved in mobilization of mineral nutrients (Dinkelaker *et al*., 1995)

or even directly affect the exudation process. Accordingly, root excretion of citrate in mature root clusters was rapidly stimulated within 1·5 h by exogenous application of certain fractions of partially purified root exudate phenolics (Fig. 2B). Effects of flavonoids and other phenolics on membrane transport of inorganic and organic compounds are well documented, and depending on concentration and type of phenolics, stimulatory as well as inhibitory effects have been observed (for review see Rao, 1990). Recently, Pinton *et al.* (1997) reported a stimulation of proton extrusion associated with increased activity of root plasmalemma H^+-ATPase by exogenous application of humic acids to intact oat seedling roots. It remains to be established whether the increased exudation of root phenolics (Fig. 2A) or citrate-mediated mobilization of humic acids (Gerke *et al.*, 1994) in the rhizosphere of cluster roots impacts on changes of root exudation or ion uptake during cluster root development.

Phosphorus mobilization from the organic soil P fraction in the proteoid rhizosphere seems to be related to the release of large amounts of a P deficiency-inducible isoform of acid phosphatase (APase) (Li *et al.*, 1997; Gilbert *et al.*, 1999; Neumann *et al.*, 1999), predominantly restricted to cluster root zones of P-deficient white lupin (Fig. 3A). Accordingly, AFLP analysis of gene expression in cluster roots revealed enhanced expression of a clone with 78% homology to a purple acid phosphatase precursor in mature root clusters (see Table 7), which may represent the secretory precursor form of the isoenzyme released into the rhizosphere.

In many soils, however, the availability of organic P is limited mainly by the low solubility of recalcitrant organic P forms such as Ca/Mg-, and Fe/Al-phytates, which can comprise a major proportion of the soil-organic P (Adams and Pate, 1992), and by the limited mobility of the root-borne phosphohydrolases (APase, phytase), mainly associated with the root cell wall and with mucilage in apical root zones (Dinkelaker *et al.*, 1997). The concomitant release of carboxylates and phosphohydrolases in the proteoid rhizosphere may help to mobilize sparingly soluble organic P esters and thereby increase their availability for enzymatic hydrolysis by phosphohydrolases adsorbed on the root surface. Accordingly, in a P-deficient sandy soil, more Pi was liberated by simultaneous application of acid phosphatase combined with organic acids in a concentration range determined for the rhizosphere soil of cluster roots (Dinkelaker *et al.*, 1997; Li *et al.*, 1997), than by separate application of organic acids or acid phosphatase alone (Fig. 3B).

Citrate-mediated mobilization of P-adsorbing Fe/Al humic acid complexes in the rhizosphere of cluster roots has been reported by Gerke *et al.* (1994). Phosphorus complexed with Fe and Al in this kind of organic P fraction is not susceptible to enzymatic hydrolysis by root-secretory or microbial phosphohydrolases due to the absence of P esters. It is not clear to what extent P-adsorbing humic acid complexes are plant available.

Another function of enhanced root secretion of phosphohydrolases under P-deficient conditions may be the rapid retrieval of P from organic P forms permanently released into the rhizosphere from damaged or sloughed-off root cells and microorganisms (Lefebvre *et al.*, 1990).

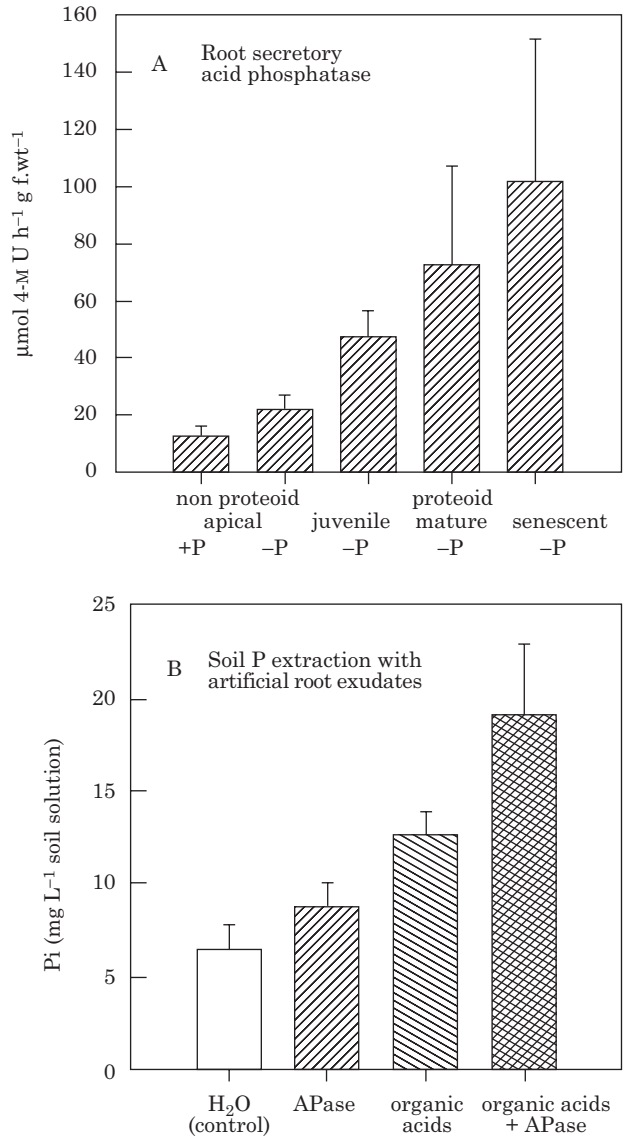


FIG. 3. A, Activity of root-secretory acid phosphatase in different root zones of P-deficient white lupin. Means and s.d. of three to five replicates are presented (Source: Neumann *et al*, 1999). B, Water extractable Pi in a P-deficient sandy soil from Niger (West Africa) after separate or simultaneous application (24 h) of acid phosphatase and of organic acids according to concentration levels detected in the rhizosphere soil of cluster roots. Organic acids: Malic 7·5 mM; Citric 2 mM; Fumaric 1 mM, t-Aconitic 0·6 mM Acid phosphatase: Wheat germ APase (0·7 U g^{-1} soil). Means and s.d. of four independent replicates. (Source: Neumann and Römheld, 2000).

INDUCTION OF CLUSTER ROOTS

Phosphorus deficiency is a major factor for induction of cluster roots in *Lupinus albus* but these structures may be stimulated to a lesser extent by low Fe- Mn- and Zn-supply. Low levels of N enhance P deficiency-induced formation of cluster roots, whereas high N supply has inhibitory effects (Dinkelaker *et al.*, 1995). A limited number of root clusters

are formed even at moderate or adequate P levels (Marschner *et al.*, 1987; Johnson *et al.*, 1994; Keertisinghe *et al.*, 1998). Increased formation of cluster roots is the earliest visible symptom of P deficiency in seedlings of white lupin grown in a hydroponic culture system without P supply, appearing 12–14 d after seed imbibition (see Fig. 4B). Suppression of cluster root formation by foliar P application (Marschner *et al.*, 1987) suggests that induction is determined by internal P concentration rather than by P levels of the substrate.

There is increasing evidence that, similar to lateral root initiation in other plant species, cluster root formation might be under hormonal control. Exogenous application of indole-3-acetic acid (IAA) to the nutrient solution of hydroponically-grown white lupin stimulated cluster root formation even in the presence of high P levels (Fig. 4A). In contrast, induction of cluster roots in P-deficient plants was suppressed by application of TIBA (2,3,5-triiodobenzoic acid) and NPA (N-(1-naphtyl)phtalamic acid), known to be potent inhibitors of the polar shoot-to-root transfer of auxins in higher plants (Gilbert *et al.*, 1998).

Similarly, a drastic inhibition of cluster root formation and of lateral rootlet elongation was observed when kinetin was applied to the growth medium of P-deficient white lupin at concentration levels $\geqslant 0{\cdot}01$ mg l^{-1} (Fig. 4B), suggesting a possible role of cytokinins as well. A similar auxin-cytokinin antagonism has been previously reported for lateral root formation in other plant species (Wightman *et al.*, 1980; Hinchee and Rost, 1986). Elevated concentrations of the zeatin/zeatin riboside cytokinin fraction were detected in the root tissue of 4 week old P-deficient white lupin compared with P-sufficient control plants (Fig. 5). Based on these findings it may be speculated that auxin-stimulated emergence of juvenile cluster rootlets in P-deficient plants results in increased production of cytokinins due to the large number of root tips. Enhanced accumulation of cytokinins in the juvenile clusters may in turn contribute to inhibition of rootlet elongation during cluster root development.

Nutrient deficiency is also frequently associated with increased ethylene production (Lynch and Brown, 1997), and ethylene has been implicated in alterations of root morphology under P-deficient conditions (Borch *et al.*, 1999). However, an involvement of ethylene in cluster root induction remains to be established. In view of the highly differentiated morphology of cluster roots, hormonal interactions during cluster root development are likely to be quite complex.

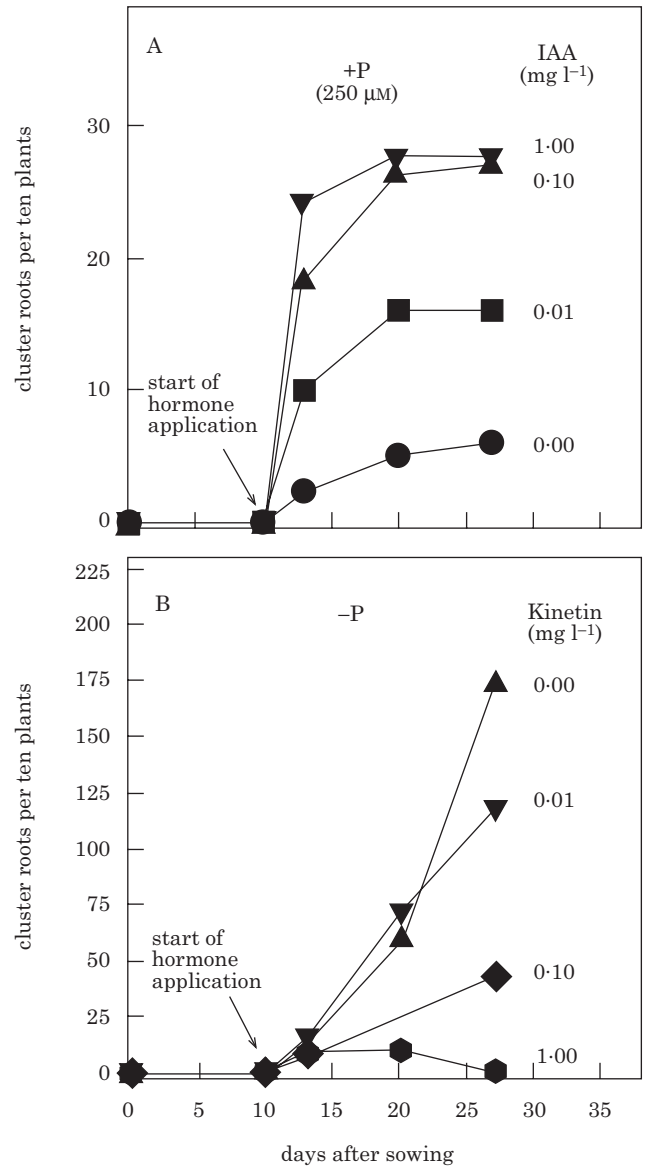


FIG. 4. A, Effects of exogenous application of indole-3-acetic acid (IAA) on cluster root formation in white lupin grown in a hydroponic culture system under P-sufficient conditions. IAA and nutrient solution supplied in 2 d intervals. B, Effects of exogenous application of kinetin on cluster root formation in white lupin grown in a hydroponic culture system under P-deficient conditions. Kinetin and nutrient solution supplied in 2 d intervals. Means of two experiments with each three replicates of ten plants.

MECHANISM OF CARBOXYLATE EXUDATION

In contrast to cluster root induction, which is an early response to P starvation (12–14 d after seed imbibition, Fig. 4B), increased carboxylate accumulation in the root tissue and enhanced excretion of citrate and protons from root clusters is a symptom associated with later stages of P deficiency (21–28 d after seed imbibition, Fig. 6). These findings suggest that organic acid excretion is related to P deficiency-induced metabolic changes during cluster root development.

In white lupin grown in hydroponic culture without P supply over a period of 5–6 weeks, a distinct spatial distribution pattern with different stages of cluster root development could be distinguished along single first-order lateral roots (Neumann *et al.*, 1999). Emergence of the youngest white-coloured juvenile cluster rootlets started approximately 2–3 cm from the lateral root tip. In the light-brown coloured, mature clusters, located 4–7 cm from the root tip, lateral rootlets had reached their final length of

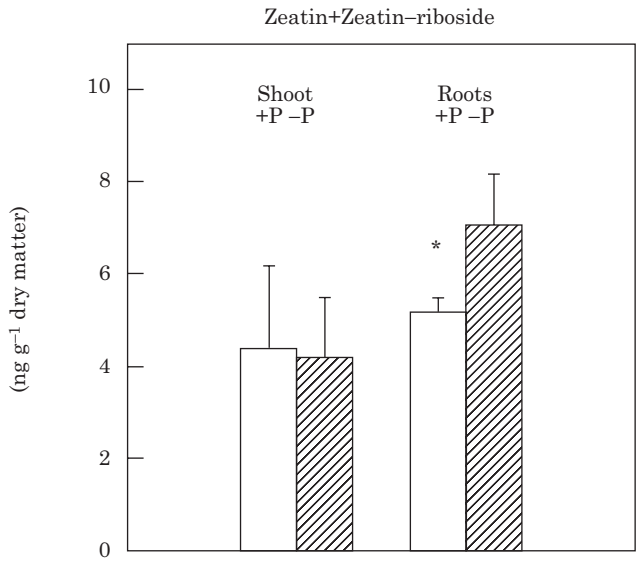


FIG. 5. Tissue concentrations of zeatin and zeatin riboside in shoot and roots of white lupin grown in a hydroponic culture system for 28 d with or without P supply. Cytokinins were determined by radioimmunoassay according to Bohner and Bangerth (1988). Means and s.d. of three independent replicates are presented. Significant differences are marked by an asterisk.

approx. 5 mm, and the colour turned to grey brown in senescent clusters close to the lateral root base.

Citric acid accumulated at high levels mainly in mature and senescent cluster roots, whereas malic acid was dominant in root tissue and in root exudates of non-proteoid roots and juvenile root clusters (Fig. 7). A peak of citrate exudation (up to 1 μmol h^{-1} g^{-1} f.wt) was induced when citrate accumulation in mature root clusters reached a threshold level of approx. 25–30 μmol g^{-1} f.wt, and was associated with a simultaneous acidification of the rhizosphere (Figs 1 and 7).

In senescent clusters, however, almost no citrate exudation was detectable despite high internal citric acid concentrations in the root tissue, indicating that a high rate of citrate release in mature root clusters is not simply leakage as a result of P deficiency-induced impaired plasma membrane integrity (Ratnayake *et al.*, 1978). Citric acid applied exogenously during a 1·5 h period of localized root exudate collection (using the filter paper technique of Neumann *et al.*, 1999) in senescent clusters, was 90% recoverable, suggesting that there was little effect of increased microbial degradation due to a higher microbial colonization rate at the surface of older root tissues (Foster, 1986). These findings suggest that the transient release of

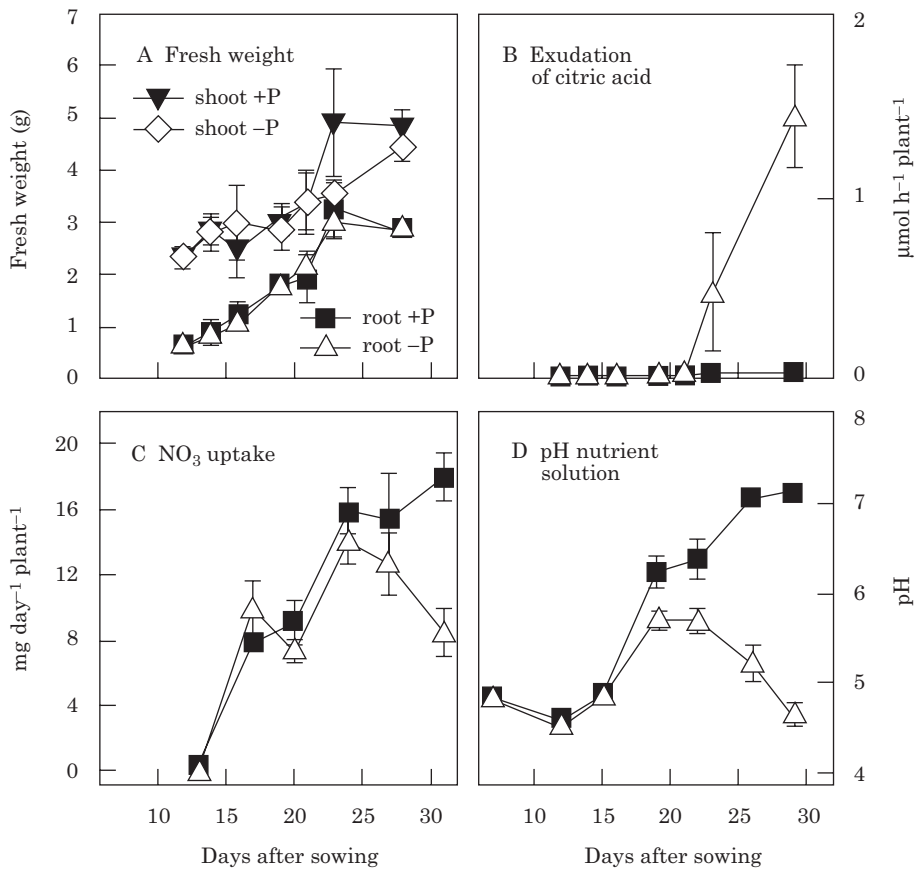


FIG. 6. Root and shoot biomass (A), root exudation of citrate (B), nitrate uptake (C) and pH (D) of the growth medium during seedling development of white lupin grown in hydroponic culture under P-sufficient or P-deficient conditions supplied with NO_3^- as nitrogen source. Means ± s.d. of three independent replicates (Source: Neumann *et al*, 1999).

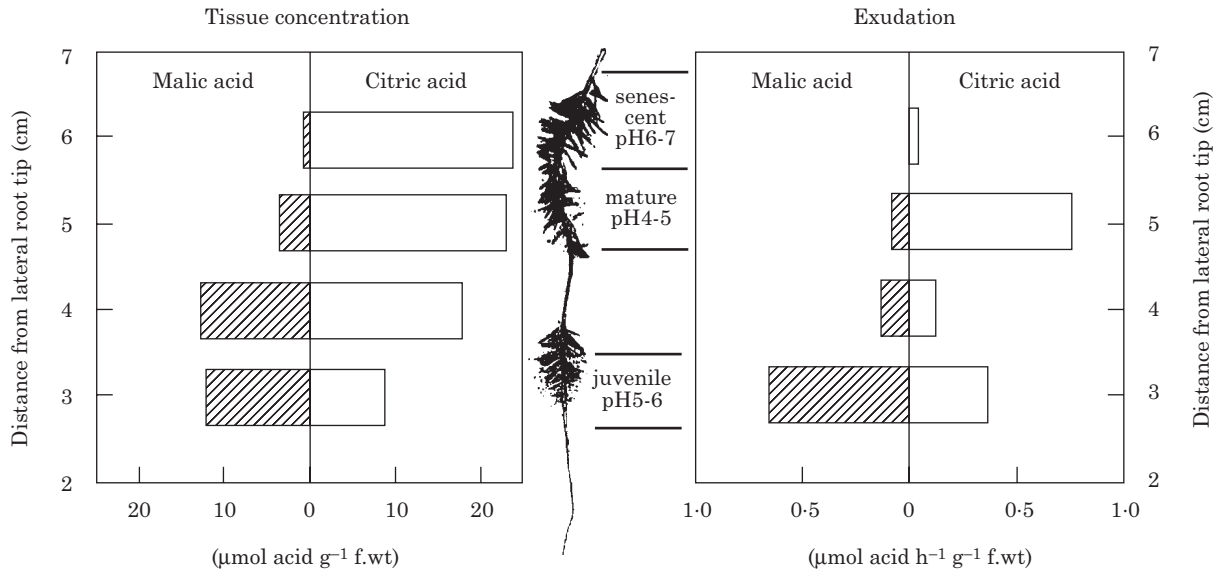


FIG. 7. Spatial variation of tissue concentrations and root exudation of major carboxylates (malate, citrate) and rhizoplane pH in different developmental stages of cluster roots in white lupin grown in hydroponic culture for 35 d without P supply. Means of three replicates (each including four root segments) are presented (adapted from Neumann *et al*, 1999).

high amounts of citrate in mature root clusters of white lupin is mediated by a specific transport mechanism located at the plasma membrane. At the cytosolic pH of approx. 7·1–7·4 (Marschner, 1995), carboxylic acids usually exist as anions with low plasma membrane permeability. When the root systems of P-deficient white lupin were exposed for 1·5 h to the anion channel inhibitors ethacrynic acid and anthracene-9-carboxylic acid, the release of citrate declined by 50% (Table 2). This strongly suggests that citrate excretion from cluster roots is mediated by anion channels, with a concomitant release of protons in order to maintain charge balance, and accounting for rhizosphere acidification (Neumann *et al*., 1999). Similarly, inhibitory effects of anion-channel antagonists on root excretion of carboxylates have been reported for the Al-stimulated exudation of malate in root apices of wheat (Ryan *et al*., 1995), and the diurnal release of mugineic acid phytosiderophores in sub-apical root zones of Fe-deficient barley and maize (Sakaguchi *et al*., 1999; Neumann and Römheld, 2000). Future characterization of this transport mechanism will require studies of membrane physiology using patch clamp techniques and other tools.

TABLE 2. *Effect of anion-channel inhibitors on root exudation of citrate in P-deficient white lupin grown in a hydroponic culture system*

Treatment	H_2O	Ethacrynic acid (50 μM)	Anthracene-9-carboxylic acid (50 μM)
Citrate exudation (% of control)	77·8[a] ±15·1	41·6[b] ±15·3	32·1[b] ±19·7

Release of citrate was monitored during 1·5 h immersion of the whole root system into aerated inhibitor solutions or distilled H_2O and compared to the exudation rates during a preceeding control incubation (1·5 h) of the same plants in distilled H_2O. Due to large variations of citrate exudation between single plants (depending on activity status and number of root clusters per plant), changes in exudation rates were measured with the same plants before and after inhibitor application. Means ± s.d. of three independent replicates. Significant differences are indicated by different superscripts (Source: Neumann *et al*, 1999).

P DEFICIENCY-INDUCED CHANGES IN CARBOXYLATE METABOLISM

Differential $^{14}CO_2$ pulse-chase labelling experiments with shoots and roots of white lupin revealed biosynthesis of carboxylates in the root tissue and particularly in cluster roots under P-deficient conditions (Johnson *et al*., 1996*b*). This is in good agreement with enhanced expression and *in vitro* activities of enzymes such as sucrose synthase, phosphoglucomutase, fructokinase (Table 3), and PEP carboxylase (Table 4), involved in the catabolism of carbohydrates and in the biosynthesis of organic acids particularly in juvenile and mature root clusters (Johnson *et al*., 1996*a*; Keerthisinghe *et al*., 1998; Neumann *et al*., 1999; Watt and Evans, 1999). Compared with conventional glycolysis in P-sufficient plants, these enzymes may operate as an alternative pathway of carbohydrate catabolism under conditions of P starvation, which facilitates a more efficient P utilization by P recycling, reduced P consumption and utilization of alternative P pools such as PPi (Theodoru and Plaxton, 1993; Plaxton, 1998). The ability to express those P-independent metabolic bypass reactions under P-deficient conditions determines a high sink strength of cluster roots for carbohydrates supplied by the shoot, which is a prerequisite for the biosynthesis of carboxylates in the root tissue.

Additionally, non-photosynthetic CO_2 fixation via PEPC can contribute a substantial proportion of carbon (>30%) to carboxylate production in cluster roots (Johnson *et al*.,

TABLE 3. *Representative distribution of specific* in vitro *activities of glycolytic enzymes in different root zones of P-sufficient and P-deficient white lupin grown in hydroponic culture*

	Sucrose synthase	Phosphoglucomutase	Fructokinase
Root type/treatment	(nmol substrate turnover h^{-1} mg^{-1} protein)		
+ P non-proteoid	103	47551	52
− P non-proteoid	139	140355	158
− P proteoid juvenile	307	295339	337
− P proteoid mature	396	230196	159
− P proteoid senescent	20	28602	33

Similar distribution patterns were obtained in three independent experiments. For enzyme extraction, roots were ground in liquid nitrogen and homogenized in 0·1 M Hepes (pH 7·5), 5 mM $MgCl_2$, 2·5 mM DTT, 3 mM Na-DEDTC, 1 mM EDTA, 1 mM benzamidine, 1 mM PMSF, 3% PVPP K30, in the ratio of 3 ml per g of fresh tissue. After two centrifugation steps (12000 *g*, 15 min and 10 min, 4°C) the final supernatant was used to detect enzymatic activities. All enzymes were assayed spectro-photometrically by monitoring the changes of NADH concentrations at A_{340} nm.

TABLE 4. *Specific* in vitro *activities of phosphoenolpyruvate carboxylase (PEPC), and aconitase in relation to tissue concentrations of citrate and* cis*-aconitate in roots of P-sufficient and P-deficient white lupin 23 d after sowing*

	PEPC	Aconitase	Citrate	*cis*-Aconitate
Root tissue	(nmol product min^{-1} mg^{-1} protein)		(μmol g^{-1} f.wt)	
+ P non-proteoid	$116^{a} \pm 15$	$19^{a} \pm 3$	$2{\cdot}5^{a} \pm 0{\cdot}5$	$0{\cdot}012^{a} \pm 0{\cdot}005$
− P non-proteoid	$212^{b} \pm 35$	$14^{b} \pm 1$	$8{\cdot}8^{b} \pm 2{\cdot}0$	$0{\cdot}016^{a} \pm 0{\cdot}004$
− P proteoid	$341^{c} \pm 71$	$14^{a,b} \pm 2$	$22{\cdot}1^{c} \pm 4{\cdot}3$	$0{\cdot}036^{b} \pm 0{\cdot}010$

Means ± s.d. of three independent replicates. In each column significant differences (one way ANOVA) are indicated by different superscripts. (Source: Neumann *et al*, 1999).

TABLE 5. *Relationship between P status (intracellular soluble Pi and RNA), respiration (O_2 consumption), fermentation (specific activity of alcohol dehydrogenase), and citrate accumulation in different root zones of P-sufficient and P-deficient white lupin*

	+P non-proteoid apical (2 cm)	−P non-proteoid apical (2 cm)	−P proteoid juvenile	−P proteoid mature	−P proteoid senescent
Soluble Pi (μmol g^{-1} f.wt)	n.d.	$1{\cdot}29^{a}$	$1{\cdot}86^{b}$	$0{\cdot}95^{ac}$	$0{\cdot}51^{c}$
RNA (mg g^{-1} f.wt)	$0{\cdot}25^{a}$	$0{\cdot}36^{a}$	$0{\cdot}57^{a}$	$0{\cdot}06^{b}$	$0{\cdot}02^{b}$
Oxygen consumption (μmol min^{-1} g^{-1} f.wt)	$0{\cdot}90^{a}$	$0{\cdot}88^{ab}$	$1{\cdot}22^{a}$	$0{\cdot}53^{b}$	$0{\cdot}47^{b}$
Alcohol dehydrogenase (μmol NAD^{+} h^{-1} mg^{-1} protein)	25^{a}	18^{a}	18^{a}	81^{b}	61^{b}
Citrate (μmol g $f.wt^{-1}$)	n.d.	$10{\cdot}22^{a}$	$13{\cdot}70^{b}$	$32{\cdot}16^{c}$	$28{\cdot}23^{c}$

n.d. = not determined.

Means of three independent replicates are presented. In each row, different superscripts indicate significant differences by one-way ANOVA (adapted from Neumann *et al*, 1999).

1996*a*,*b*). Thus, PEPC-mediated CO_2 fixation was interpreted as an anapleurotic carbon supply to compensate for carbon losses associated with root exudation of carboxylates, which can comprise up to 25% of the photosynthetic CO_2 net fixation (Dinkelaker *et al*., 1989; Johnson *et al*., 1996*a*,*b*).

Increased citrate accumulation in mature and senescent root clusters was associated not only with enhanced activity of PEPC but also with a reduced aconitase activity (Table 4, Neumann *et al*., 1999). Aconitase is involved in the turnover of citrate within the TCA cycle and probably also in the cytosol (Brouquisse *et al*., 1987). Thus, citrate accumulation is probably a consequence of both increased biosynthesis and reduced metabolism of citrate under P-deficient conditions. Induction of PEPC, reduced activity of aconitase, and increased accumulation of citric acid in the root tissue in response to P starvation has been similarly reported for other plant species such as tomato and chickpea (Neumann and Römheld, 1999).

Citrate accumulation during proteoid root development in white lupin was also associated with reduced respiration (Johnson *et al*., 1994; Neumann *et al*., 1999) and a concomitant decrease of soluble intracellular Pi (Table 5, Neumann *et al*., 1999). Phosphorus limitation of the respiratory chain may induce feed-back inhibition of citrate turnover in the TCA cycle in order to prevent excessive production of reducing equivalents (Lance and Rustin, 1984). Accordingly, in roots of *Phaseolus vulgaris* L.,

P deprivation decreased the capacity of the respiratory cytochrome pathway, but increased the ratio of NADH/NAD and cyanide-resistant respiration (Juszczuk and Rychter, 1997).

A P deficiency-induced inhibition of nitrate uptake in white lupin, both at the whole plant level (Fig. 6C) and based on root biomass (different rates of NO_3^- uptake per plant but identical root fresh weight in +P and −P treatments, Fig. 6A,C), may further contribute to increased citrate accumulation in the root tissue by down-regulation of citrate conversion to 2-oxoglutarate, which is an important acceptor for amino N as a product of NO_3^- reduction (Lancien *et al*., 1999). Inhibition of NO_3^- uptake and assimilation has been previously reported as a common P-deficiency response in many other plant species (Le Bot *et al*., 1990; Rufty *et al*., 1990; Pilbeam *et al*., 1993; Gniazdowska *et al*., 1999), and was frequently associated with enhanced root accumulation of citric acid (Neumann and Römheld, 1999).

From these findings, it may be concluded that increased accumulation of citrate in the mature root clusters of white lupin is a P deficiency-induced metabolic disorder, and the release of high amounts of citrate and protons into the rhizosphere might serve as a detoxification mechanism to prevent cytoplasmic acidosis and over-accumulation of citrate exceeding the vacuolar storage capacity. A similar mechanism has been discussed for the detoxification of lactic acid, which accumulates in root tips of maize under hypoxic conditions (Xia and Roberts, 1994) with comparable intracellular carboxylate concentrations (20–30 μM) and similar exudation rates (2000–5700 nmol h^{-1} g^{-1} root f.wt) as observed in cluster roots of P-deficient white lupin (Xia and Roberts, 1994; Jones, 1998; Neumann *et al*., 1999). However, accumulation of the tri-carboxylic citric acid in the cluster roots of white lupin may cause much stronger cytosolic acidification than similar concentrations of the mono-carboxylic lactic acid in the apical root zones of maize under hypoxia. Moreover, due to the chelating properties of citrate anions, cytosolic over-accumulation may interfere with the cytosolic Ca- and Mg-homeostasis in the root tissue. In both hypoxic maize root tips and mature root clusters of P-deficient white lupin, enhanced activity of alcohol dehydrogenase (AlDH) was detectable (Table 5). This enzyme is induced by energy limitation and low cytosolic pH (Pfister-Sieber and Brändle, 1994). In hypoxic plant tissues, AlDH induction is associated with enhanced production of ethanol, which is membrane permeable and easily released into the environment. This response is interpreted as an alternative pathway of carbohydrate fermentation to avoid excessive lactic acid accumulation (Pfister-Sieber and Brändle, 1994). Similarly, in P-deficient white lupin, AlDH induction may: (1) reflect severe energy shortage due to P limitation of root respiration; (2) indicate acidification of the cytosol by excessive accumulation of citric acid; or (3) counteract overproduction of NADH. Catabolism of sucrose, mediated by sucrose synthase instead of invertase, is another obvious similarity between metabolic alterations induced by limited oxygen supply (Guglielminetti *et al*., 1997) and P deficiency in cluster roots of white lupin.

Metabolic changes related to P deficiency are particularly expressed in mature and senescent cluster roots where concentrations of soluble Pi and RNA are extremely low. In contrast, Pi and RNA levels in juvenile clusters are comparatively high (Table 5). This may be attributed to re-mobilization and re-translocation of Pi from the older cluster regions, which exhibit no more growth activity (Watt and Evans, 1999), to the emerging juvenile clusters which include a high proportion of meristematic tissue with a high P demand. As a consequence, P limitation becomes more severe in mature and senescent clusters, associated with enhanced expression of P deficiency-induced metabolic alterations such as induction of PEPC and P-independent glycolytic bypass reactions, inhibition of NO_3^- uptake and respiration, and finally increased accumulation of citric acid in the root tissue.

P UPTAKE MECHANISMS

$^{33}P_i$ uptake per unit root fresh weight in P-deficient white lupin was approx. 2-fold higher in juvenile and mature root clusters compared to the apices of non-proteoid roots (Fig. 8, Neumann *et al*., 1999). Similar results were obtained for $^{33}P_i$ uptake based on root surface area of juvenile root clusters compared with non-proteoid root apices (Table 6). This implies that mature cluster roots are the sites of both P mobilization and of effective P uptake as well. Kinetic studies revealed a Km of 30·7 μM for P uptake of non-proteoid root apices in P-sufficient plants *vs*. K_m values of 8·5–8·6 μM for non-proteoid and juvenile root clusters in white lupin exposed to P starvation (Table 6, Neumann *et al*., 1999), suggesting the induction of a high-affinity P uptake system under P-deficient conditions (Schachtman *et al*., 1998). A higher V_{max} (6·5 nmol h^{-1} cm^{-2} root

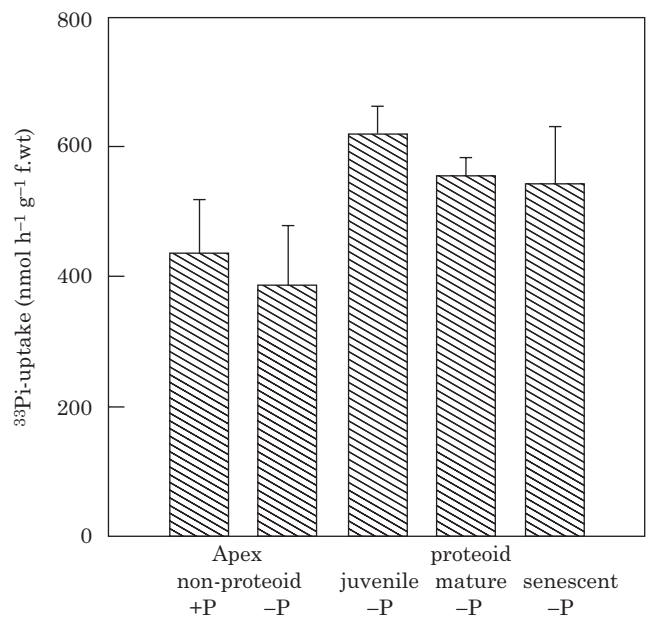


FIG. 8. Uptake of ^{33}Pi in different root zones of P-sufficient and P-deficient white lupin after a culture period of 4 weeks in a hydroponic culture system. Means and s.d. of three to five replicates. (Source: Neumann *et al*, 1999).

TABLE 6. *Uptake characteristics of ^{33}Pi in different root zones of P-sufficient and P-deficient white lupin based on root surface area*

Root zone/treatment	Surface area ($cm^2\ g^{-1}$ root f.wt)	^{33}Pi uptake (nmol $h^{-1}\ cm^{-2}$ root surface)	Km (μM)	Vmax (nmol $h^{-1}\ cm^{-2}$ root surface)
Non-proteoid Apical (2 cm) +P	137·5	3·2 ± 0·6	30·7	4·8
Non-proteoid Apical (2 cm) −P	137·5	2·8 ± 0·7	8·6	2·0
Proteoid juvenile −P	75·1	8·3 ± 0·3	8·5	6·5

Only 2 cm-apical root zones and juvenile root clusters are compared since reliable surface area determination of mature and senescent clusters was not possible due to strong proliferation of root hairs in these root zones. Calculations are based on average surface area determinations of ten apical root segments and ten juvenile clusters respectively. Pi uptake data represent means ± s.d. of three to five independent replicates.

TABLE 7. *Homology analysis and putative functions of selected AFLP cDNA clones predominantly expressed in different stages of cluster root development*

Clones identified	Putative function
Proteoid juvenile	(Pi-independent glycolytic bypass reactions)
Sucrose synthase (EC 2.4.1.13) (86% for 666 bp)	Increased carbohydrate catabolism
Fructokinase (EC 2.7.1.4) (77% for 361 bp)	Increased carbohydrate catabolism
Phosphoglucomutase (EC 5.4.2.2) (56% for 277 bp)	Increased carbohydrate catabolism
Proteoid mature	
Purple acid phosphatase precursor (EC 3.1.3.2) (78% for 135 bp)	Precursor for root-secretory APase
Chitinase (77% for 88 bp)	Anti-fungal activity
Proteoid senescent	
Chorismate synthase precursor (80% for 296 bp)	Increased activity of shikimate pathway, synthesis of phenolics, P recycling

surface) for P uptake in juvenile root clusters compared with apical root zones of non-proteoid roots (2·0 nmol $h^{-1}\ cm^{-2}$ root surface) may indicate a higher density of high-affinity P transporters in the plasma membrane of cluster roots (Table 6).

CONCLUSIONS

In P-deficient white lupin, adaptations towards enhanced spatial acquisition of available P (e.g. stimulation of root growth, enhanced formation of fine roots and root hairs, mycorrhizal associations) are poorly expressed (Marschner *et al.*, 1987). In contrast, white lupin exhibits all the adaptations which have been shown to play a role in root-induced chemical mobilization of sparingly available P sources in soils. These include rhizosphere acidification, release of chelating root exudates, root-excretion of phosphohydrolases and induction of high-affinity P uptake systems. This system offers the opportunity to study both chemical modifications in the rhizosphere as well as physiological changes induced by limited P supply, in comparison with other plant species. Moreover, white lupin may be a good model system to investigate the molecular biology of plant adaptations to P starvation, which could help to define new targets for plant breeding or genetic engineering. The analysis of root zone-specific gene expression in different stages of cluster root development using the AFLP approach revealed more than 80 clones with homologies to genes encoding for protein kinases and phosphatases, transcription factors, membrane transport proteins, pathogen-related proteins, cell wall-modifying enzymes, and enzymes of carbohydrate and phenolic metabolism. Table 7 represents a selection of those clones, most closely related to P deficiency-induced metabolic changes associated with the production and release of P mobilizing root exudates. A detailed understanding of these processes will be required to develop strategies for genetic manipulation to result in improved acquisition efficiency for P by crop plants. These investigations also have a bearing on the acquisition of iron and other micronutrients, aluminium tolerance, phytomining and phytoremediation strategies.

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