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Original article

Exudation of low molecular weight organic acids by *Lupinus albus* L., *Lupinus angustifolius* L. and *Lupinus luteus* L. as affected by phosphorus supply

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Abstract – The objective of this investigation was to study the influence of *P* fertilizer application on the quality and quantity of organic acid exuded by six cultivars of three lupin species: *Lupinus albus* L. (cultivars Minori and Nelly), *Lupinus angustifolius* L. (cultivars Borweta and Bordako) and *Lupinus luteus* L. (cultivars Borsaja and Borselfa). We also investigated the influence of the exudate collection medium (deionized water, 0.05 mM $CaCl_2$ solution) on the composition and the intensity of exuded organic acids in *L. albus* (cultivar Minori). Plants were grown in quartz sand with (+ *P*) and without (– *P*) fertilization for 21 days. The plants were removed carefully from the sand, the roots washed, and then allowed to release exudates into deionized water or 0.05 mM $CaCl_2$ solution twice for two hours or twice for four hours. Root length was measured. Root morphology was also reported. The results show that *L. albus* formed dense root clusters known as proteoid roots. The proteoid root formation was greatest under *P* deficiency conditions. A similar root system was observed in yellow lupin but this was less dense than in white lupin. Blue lupin did not form root clusters. Regardless of *P* nutrition, eight organic acids (citric, 2-oxoglutaric, malic, succinic, lactic, formic, acetic and fumaric acids) were identified in the collection solution of all six cultivars. The collection medium did not affect the amount and composition of released carboxylates within four hours. Exudates collected in $CaCl_2$ solution over an 8-hour period contained citric, malic and succinic acids as the most important organic acids. *L. angustifolius* showed the greatest average carboxylate efflux. Cultivated under *P* deficiency conditions, five cultivars increased their citrate exudation rate (on average, 67% for *L. albus*, 37% for *L. angustifolius* and 72% for *L. luteus*).

exudation / *Lupinus albus* L. / organic acid / phosphorus nutrition / root

Résumé – Effet de l'apport de phosphore sur l'exudation d'acides organiques de faible poids moléculaire par *Lupinus albus* L., *Lupinus angustifolius* L. et *Lupinus luteus* L. L'objectif de cette recherche était d'étudier l'influence d'une application de fertilisant phosphaté sur la qualité et la quantité d'acides organiques exudés par six variétés de trois espèces de lupins : *Lupinus albus* L. (variétés Minori et Nelly), *Lupinus angustifolius* L. (variétés Borweta et Bordako) et *Lupinus luteus* L. (variétés Borsaja et Borselfa). Nous avons également étudié l'influence du milieu de collecte d'exudat (eau déionisée, solution à 0,05 mM $CaCl_2$) sur la composition et l'intensité des acides origaniques exudés par *L. albus* (variété Minori). Les plantes ont été cultivées dans un sable quartzeux avec (+ *P*) et sans (– *P*) fertilisation pendant 21 jours. Les plantes étaient extraites du sable soigneusement, les racines lavées, puis conditionnées pour libérer les exudats dans l'eau déionisée ou la solution à 0,05 mM $CaCl_2$ deux fois pendant deux heures ou deux fois pendant quatre heures. La longueur des racines a été mesurée. La morphologie des racines a aussi été relevée. Les résultats montrèrent que *L. albus* formait des amas denses de racines dénommés racines protéoides. La formation de racines protéoides était plus importante dans des conditions de carence de *P*. Un système de racines similaire fut observé chez le lupin jaune mais il était moins dense que chez le lupin blanc. Le lupin bleu ne formait pas d'amas de racines. Indépendamment de la nutrition phosphatée, huit acides organiques (les acides citrique, 2-oxoglutarique, malique, succinique, lactique, formique, acétique et fumarique) furent identifiés dans la solution de collecte de six variétés. Le milieu de collecte n'a pas affecté la quantité et la composition des hydrates de carbone libérés pendant les quatre heures. Les exudats collectés dans la solution de $CaCl_2$ sur une durée de 8 heures contenaient principalement des acides citrique, malique et succinique. *L. angustifolius* a montré la moyenne la plus élevée d'exudation d'hydrates de carbone. Cultivées dans des conditions de carence en *P*, cinq variétés ont augmenté leur taux d'exudation de citrate (en moyenne 67 % pour *L. albus*, 37 % pour *L. angustifolius* and 72 % pour *L. luteus*).

exudation / *Lupinus* / acide organique / nutrition phosphatée / racine

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1. INTRODUCTION

Mechanistic models are used to improve understanding of nutrient acquisition by plants [2, 37]. At high *P* supply in the soil, measurement of *P* uptake by plants agrees relatively well with calculations using mechanistic models, but at low *P* supply, the models fail: plants show a higher *P* uptake than the model predicts [18, 35]. It may indicate that at low *P* supply in the soil, plants have evolved different strategies to cope with restricted *P* supplies that help them to extract more phosphorus from the soil, leading to enhanced soil phosphorus acquisition [34]. These strategies include changes to cellular metabolism and root development; initiation of mycorrhizal associations; acidification of the rhizosphere, which can promote the release of nutrients from soil minerals; and the exudation of root extracellular acid phosphatase activity and organic anions [1, 10, 16, 31]. Organic anions can also stimulate microbial activity in the rhizosphere [36], which is likely to influence the availability of nutrients. These strategies are not taken into account in the mechanistic models. Several authors have shown that protons and organic anions are released into the rhizosphere soil, thereby enhancing the solubility of phosphate [4, 5, 7, 9–11, 17, 21]. This beneficial effect of organic acids in the rhizosphere is assumed to be caused by the complexing ability of carboxylate anions through three chemical mechanisms: (a) direct ligand exchange in which the *P* bound to Fe or Al oxyhydroxides is replaced by the organic anions; (b) complexation of Al/Fe species which cause *P* sorption, and (c) an increase in negative charges at the soil solid phase through the adsorption of organic acids [9]. Phosphorus mobilizing substances (for example, organic acids) exuded by plant roots affect parameters that are important in mechanistic models of nutrient uptake (e.g., diffusion coefficient, buffer capacity and exchange capacity for nutrient ions), and must therefore be quantified and understood in order to improve these models.

Claassen et al. [3] established assessments of phosphorus mobilization by some organic acids and the quantitative effects of these anions on *P* uptake. The result of these calculations, however, depends on data of root exudation rates. As yet, only few comparative inter-species studies have been carried out. Some important data have been obtained about the formation of proteoid roots and the exudation of organic acids by white lupin roots under *P* deficiency [8, 16, 24], but other lupin species have not been investigated.

Considering that the methods employed for collection and analysis of root exudates play an important role in the qualitative and quantitative interpretations of measured exudate data, the present study includes a test of the influence of the collection medium on measured exudation rates. For roots placed in distilled water, the lack of Ca^{2+} can severely and irreversibly damage cell membranes, which in some cases can lead to the bursting of root cells and loss of cell contents into the external medium [12]. Therefore the objectives of this investigation were: (1) to investigate first the influence of two different collection media (deionized water and 0.05 mM $CaCl_2$ solution) on the composition and intensity of organic acid exudation by *Lupinus albus* L. (cultivar Minori), and then (2) to study the rate and composition of excreted organic anions by six cultivars of 3 lupin species: white lupin (*Lupinus albus* L.: cultivars Minori and Nelly), blue lupin (*Lupinus angustifolius* L.: cultivars Borweta and Bordako) and yellow lupin (*Lupinus luteus* L.: cultivars Borsaja and Borselfa) after cultivation in quartz sand at two levels of *P* supply.

2. MATERIALS AND METHODS

2.1. Plant cultivation

Seeds of white lupin (*L. albus*: cultivars Minori and Nelly), blue lupin (*L. angustifolius*: cultivars Borweta and Bordako) and yellow lupin (*L. luteus*: cultivars Borsaja and Borselfa) were pre-germinated in quartz sand, which was moistened with deionized water. After 6 days, seedlings of each cultivar were transplanted into plastic containers filled with 4 kg quartz sand in the growth chamber and cultivated for 21 days (day/night rhythm 14/10 h, temperature 24/18 °C, relative air humidity 70%, photo-synthetically active radiation during the day 240 $\mu E \cdot m^{-2} \cdot s^{-1}$). There were four plants per pot. The quartz sand was fertilized with 600 mg N as $Ca(NO_3)_2 \cdot 4H_2O$, 500 mg K as K_2SO_4, 500 mg as KCl, 120 mg Mg as $MgSO_4 \cdot 7H_2O$, Fe as sequestrene and micronutrients according to Hoagland [34]. Half of the pots received 48 mg P as NaH_2PO_4 (+ *P* treatment) and in the other half *P* was omitted (– *P* treatment). Each treatment had four replicates. During plant cultivation, distilled water was added to maintain a moisture content of 70% of the maximum water holding capacity. To test the influence of the exudate collection medium, one cultivar of white lupin (*L. albus*, cultivar Minori) was cultivated in the same controlled conditions with full macro- and micro-nutrient application and eight replications.

2.2. Collection of exudates and determination of plant parameters

Figure 1 shows the procedure of the exudate collection. The whole root systems of intact plants were washed carefully to remove the quartz sand and transferred to Erlenmeyer flasks (4 plants in 250 ml). The flasks were filled with deionized water or 0.05 mM $CaCl_2$ to study the influence of the medium on the exudation intensity. Later on, for the effect of *P* nutrition on the exudation, only 0.05 mM $CaCl_2$ was used as collection solution. The pH of the collection solution was 5.5. The flasks were darkened with aluminium foil and the collection experiment was carried out in the same controlled climate conditions as where plants were grown. The solutions were permanently aerated. The roots were stored for one hour in solution (water or $CaCl_2$ solution) to remove exudates from possibly injured cells caused by washing the roots free of sand. Solutions obtained during the first hour were not considered and not analyzed. After the first hour, solutions were renewed and the plants were allowed to exude into the collection solution for over two hours (twice) for the study of the influence of the collection medium. For the comparative study of the three lupin species and the investigation of *P* supply on the root exudation, plants were allowed to exude for four hours (twice: 2nd–5th, and 6th–9th h) after washing the sand from the roots. Here also, the collection solutions were renewed after the first hour. At the end of the exudate collection time,

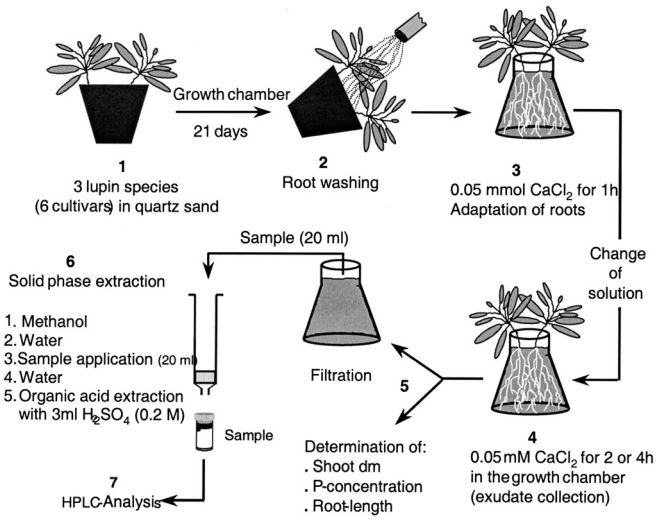

Figure 1. Schematic illustration of the procedure of root exudate collection as well as their preparation for HPLC analysis.

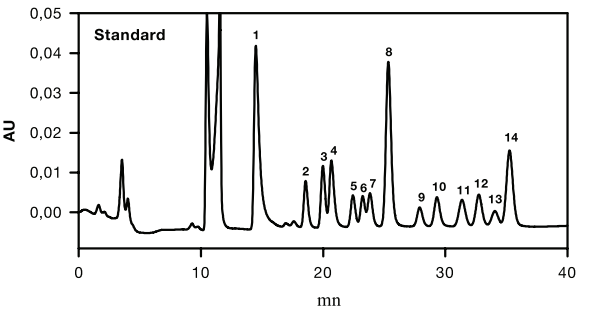

Figure 2. Chromatogram for the separation of 14 different organic acid standard solutions: *1 = oxalic acid, 2 = citric acid, 3 = tartaric acid, 4 = 2-oxoglutaric acid, 5 = malic acid, 6 = pyruvic acid, 7 = malonic acid, 8 = trans-aconitic acid, 9 = succinic acid, 10 = lactic acid, 11 = formic acid, 12= glutaric acid, 13 = acetic acid, 14 = fumaric acid.*

shoots and roots were harvested. Shoots were dried and digested in Teflon bombs with HNO_3 at 180 °C. Phosphate was determined using the vanadate-molybdate method and analyzed according to Scheffer and Pajenkamp [33]. Root lengths were determined with the scanner using the "Wurzel.exe" program.

2.3. Recovery of root exudates and their quantification

Aliquots of the collection solutions were filtered through filter paper (602 h ½, Schleicher & Schuell). 20 ml of filtered exudate solutions of each treatment were concentrated by using solid phase extraction (SPE) for selective sample preparation (Chromabond columns from Macherey-Nagel; Duren, Germany). Organic anion residues were dissolved in 0.2M H_2SO_4 filtered through a 0.45 µm membrane and analyzed by high performance liquid chromatography (HPLC). The analysis of organic anions followed procedures described by Gerke [9] and Keller [17]. A separation method was developed, so that 14 mono-, di- and tricarboxylates could be identified and quantified.

The HPLC system (Waters; Toronto, Canada) consisted of a low-pressure gradient pump 600E, an automatic sample injector WISP 712, a photodiode array detector PDA 996 and an EDP-supported evaluation unit (Waters Millennium 2010-Software). Separation was conducted on a 300 × 7.8 mm reverse phase column (Merck Polyspher OAKC; Darmstadt, Germany). Sample solutions (40 µl) were injected into the column with a flow rate of 0.3 $ml \cdot min^{-1}$ at 52 °C and UV detection at 210 nm. 0.015 M H_2SO_4 solution was used for isocratic elution. Identification of organic acids was performed by comparing retention times and absorption spectra with those of known standards of 14 different organic acids. Figure 2 shows the chromatogram for the separation of the organic acid standards.

3. RESULTS

3.1. Shoot dry matter, *P* concentration in shoots, and root morphology

Phosphate fertilizer application significantly increased shoot dry matter of cultivars Nelly, Bordako and Borselfa only, by 13%, 24% and 28%, respectively (Tab. I). For the other cultivars, *P* supply did not affect shoot biomass. Shoot dry matter was 47% and 140% higher for white lupin compared with blue

Table I. Influence of phosphorus application on shoot dry matter (dm) and root length of the plants (six cultivars of three lupin species) 21 days after sowing.

Plant species	Cultivars	Shoot dry matter [$g \cdot plant^{-1}$]		Root length [$m \cdot plant^{-1}$]		Root length/shoot dry matter-ratio [$m \cdot g^{-1}$]	
		– *P*	+ *P*	– *P*	+ *P*	– *P*	+ *P*
Lupinus albus	Minori	0.41 a	0.45 a	9.1 a	10.1 a	22	22
	Nelly	0.48 b	0.54 a	9.5 a	10.8 a	20	20
Lupinus angustifolius	Borweta	0.18 a	0.22 a	3.4 a	3.7 a	19	17
	Bordako	0.25 b	0.31 a	3.1 b	5.8 a	13	19
Lupinus luteus	Borsaja	0.27 a	0.29 a	7.8 a	7.2 a	29	25
	Borselfa	0.25 b	0.32 a	6.9 a	5.1 b	28	16

Each value is the mean of 4 replicates. Values for each cultivar followed by the same letter are not significantly different at $P = 0.05$, Newmann Keuls test.

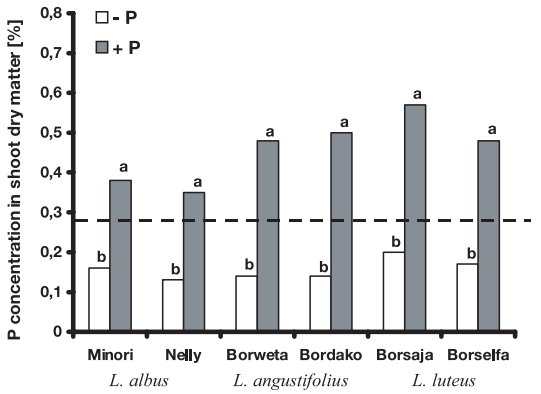

Figure 3. Influence of phosphorus application on *P* concentration in shoot dry matter by three six cultivars of three lupin species, 21 days after sowing (--- = *0.28% P: critical P concentration in shoot dry matter* (*according to Reuter and Robinson 1997*)).

and yellow lupin, respectively. Figure 3 shows the *P* contents of the shoots. According to Reuter and Robinson [28], *P* concentrations in the shoots were below the critical *P* content (0.28%) in the treatment without *P* fertilizer, whereas fertilized plants were *P*-sufficient. *P* supply caused a significant increase in root length in Bordako *(L. angustifolius)* and a decrease in Borselfa (*L. luteus*) (Tab. I). On average, *L. albus* showed higher root length per plant in comparison with the others: 9.9 m root·(plant)$^{-1}$ for *L. albus,* 4.0 m root·(plant)$^{-1}$ for *L. angustifolius* and 6.7 m root·(plant)$^{-1}$ for *L. luteus.* This greater root length of *L. albus* plants can be seen in illustration 4, showing a higher proportion of proteoid roots (root clusters). It seems that total root length was increased due to an increase in the number of first lateral roots from the tap root, and mainly due to a high rootlet density of the proteoid root clusters. A similar root system was observed for yellow lupin but less dense cluster segments were observed than for white lupin. Blue lupin did not form root clusters (Fig. 4).

3.2. Influence of collection solution on exudation

The two collection media produced highly similar results for the exudation rate and composition during both two-hour collection periods (Tab. II). Regardless of the collection medium, citrate, 2-oxoglutarate, malate, succinate, lactate, formate, acetate and fumarate were identified. There were no significant differences between the two collection solutions (deionized water compared with 0.05 mM $CaCl_2$) in exuded organic anion concentrations. The hypothesis that the root exudation could be higher in the deionized water medium was not confirmed, at least during our collection time. In short, the exudation rate of roots was relatively stable over four hours and the effect of the collection medium was small.

3.3. Influence of *P* nutrition on the composition and exudation rate of organic acids

The composition of organic acids excreted by all three lupin species was highly similar (Tabs. III and IV). The following carboxylates were identified in the collection solution: citrate, malate, formate, acetate, succinate, 2-oxoglutarate, lactate and fumarate. Phosphorus nutrition did not influence the spectrum of released organic acids. From the quantitative point of view, blue lupin showed on average the highest exudation rates (amount of excreted organic acids per root length and hour) for all identified organic anions (Tabs. III and IV). Citrate, malate and succinate were found as the predominant compounds in root exudates of all three lupin species. Fumarate and 2-oxoglutarate were detected in small amounts.

Phosphorus deficiency in the plants led to a significant increase in the exudation rate of citrate for all cultivars with the exception of Borweta (blue lupin). *P*-deficient white lupin plants showed a 113% (cv. Minori) and 73% (cv. Nelly) greater citrate exudation rate than plants with adequate *P* supply (Tab. III). The two cultivars of yellow lupin increased their citrate exudation rate by 73% and 47%, respectively. With regard to blue lupin, *P*-deficient plants of the cultivar Bordako increased their citrate exudation rate by 86%, while for the same treatment the cultivar Borweta decreased the citrate

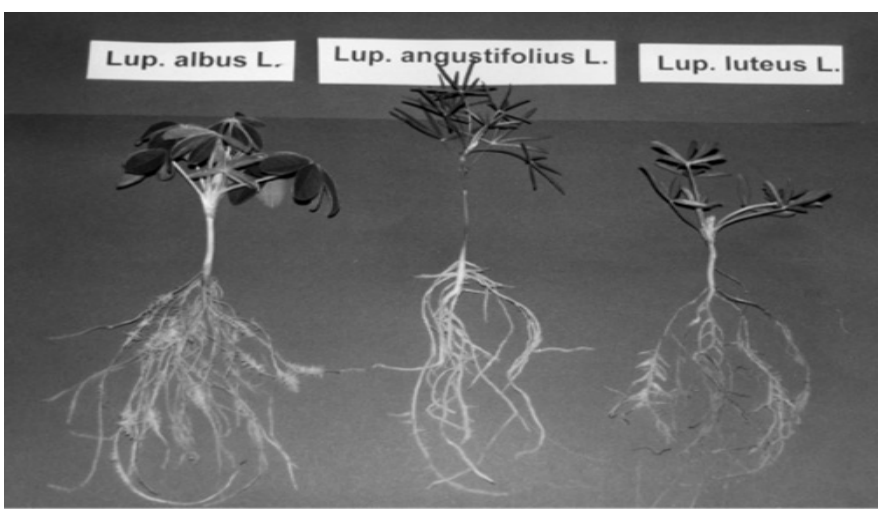

Figure 4. Root morphology by three lupin species, 21 days after sowing: the plants were cultivated in quartz sand (see chapter 2.1).

Table II. Effect of exudate collection medium on the composition and concentration of released organic acids by the cultivar Minori (*Lupinus albus*), 21 days after sowing (4 plants in 250 ml).

	First collection period (2 h)		Second collection period (next 2 h)	
	water	0.05 mM $CaCl_2$	deionized water	0.05 mM $CaCl_2$
	mM			
Citric acid	0.122 a	0.113 a	0.093 a	0.079 b
2-oxoglutaric acid	0.015 a	0.016 a	0.013 a	0.015 a
Malic acid	0.478 a	0.466 a	0.460 b	0.586 a
Succinic acid	0.042 a	0.039 a	0.042 a	0.051 a
Lactic acid	0.048 a	0.053 a	0.058 a	0.053 a
Formic acid	0.080 a	0.091 a	0.080 a	0.085 a
Acetic acid	0.127 a	0.100 a	0.072 b	0.093 a
Fumaric acid	0.005 a	0.009 a	0.007 a	0.008 a

Means of each organic acid within the same time of collection followed by the same letter are not significantly different at $P = 0.05$, Newmann Keuls test.

Table III. Influence of *P* nutrition on the organic acid exudation rate per cm root length during a first 4-hour exudation collection period (2nd to 5th h) by six cultivars of three lupin species (21 days after sowing).

		L. albus		*L. angustifolius*		*L. luteus*	
		Minori	Nelly	Borweta	Bordako	Borsaja	Borselfa
		nM $(cm \cdot root)^{-1} \cdot h^{-1}$					
Citric acid	– *P*	0.32 a	0.45 a	4.27 a	6.80 a	0.73 a	1.19 a
	+ *P*	0.15 b	0.26 b	5.23 a	3.64 b	0.42 b	0.81 b
2-oxoglutaric acid	– *P*	0.07 a	0.09 a	0.19 a	0.27 a	0.10 a	0.11 a
	+ *P*	0.07 a	0.08 a	0.22 a	0.14 b	0.13 a	0.16 a
Malic acid	– *P*	0.43 b	1.18 b	1.75 b	7.19 a	0.88 b	1.46 a
	+ *P*	0.87 a	1.65 a	7.02 a	5.23 b	1.22 a	0.83 b
Succinic acid	– *P*	0.27 a	0.43 a	0.77 a	1.23 a	0.46 a	0.53 a
	+ *P*	0.29 a	0.44 a	0.88 a	0.65 b	0.43 a	0.61 a
Lactic acid	– *P*	0.27 a	0.26 a	0.71 a	0.73 a	0.34 a	0.38 a
	+ *P*	0.23 a	0.28 a	0.57 a	0.40 b	0.44 a	0.53 a
Formic acid	– *P*	0.67 a	0.67 a	1.66 a	2.31 a	0.91 a	-
	+ *P*	0.59 a	0.63 a	1.70 a	1.11 b	0.97 a	0.99
Acetic acid	– *P*	0.61 a	0.64 a	1.08 b	1.83 a	0.61 a	1.04 b
	+ *P*	0.68 a	0.97 a	1.53 a	1.14 b	0.93 a	1.57 a
Fumaric acid	– *P*	0.01 a	0.04 a	0.06 b	0.23 a	0.03 a	0.07 a
	+ *P*	0.02 a	0.09 a	0.51 a	0.37 a	0.04 a	0.09 a

Different letters show for the same carboxylate in each cultivar significant differences between + *P* and – *P* treatments, $P = 0.05$, Newmann Keuls test.

exudation rate by 18%. Under *P* deficiency, the cultivars Bordako (blue lupin) and Borselfa (yellow lupin) significantly increased their malate exudation rate by 72% and 57%, respectively. In contrast, we observed in the other cultivars a significant increase in the malate exudation rate due to *P* fertilization.

Table IV shows the exudation rate during the second 4-hour period (5th–9th h) of the exudate collection. The composition of exuded organic anions remained the same: citrate and malate were the dominating compounds, followed by formate, acetate, succinate, 2-oxoglutarate, lactate and fumarate. Exudation rates decreased during the second 4-h period, compared with the first 4-h period for all cultivars (Tabs. III and IV). On average, the citrate exudation rate of white, blue and yellow lupins dropped to 67%, 37% and 72%, respectively. Nevertheless, *P*-deficient plants continued to show higher citrate excretion rates than non-deficient plants.

4. DISCUSSION

Regardless of the level of *P* nutrition, eight organic acid anions were identified for all six cultivars: citrate, 2-oxoglutarate, malate, succinate, lactate, formate, acetate and fumarate.

Table IV. Influence of *P* nutrition on the organic acid exudation rate per cm root length during a second 4-hour exudation collection period (6th to 9th h) of six cultivars of three lupin species (21 days after sowing).

		L. albus		*L. angustifolius*		*L. luteus*	
		Minori	Nelly	Borweta	Bordako	Borsaja	Borselfa
		nM(cm·root)$^{-1}$·h^{-1}					
Citric acid	– *P*	0.22 a	0.27 a	1.61 a	2.44 a	0.75 a	0.64 a
	+ *P*	0.14 b	0.16 b	1.62 a	1.71 b	0.57 b	0.31 b
2-oxoglutaric acid	– *P*	0.07 a	0.08 a	0.23 a	0.23 a	0.09 a	0.11 a
	+ *P*	0.07 a	0.07 a	0.18 b	0.11 b	0.12 a	0.14 a
Malic acid	– *P*	0.21 b	0.41 a	0.69 b	1.67 a	0.66 b	0.56 a
	+ *P*	0.43 a	0.49 a	1.41 a	1.34 a	1.11 a	0.47 a
Succinic acid	– *P*	0.26 a	0.44 a	0.78 a	1.04 a	0.47 a	0.52 a
	+ *P*	0.30 a	0.46 a	0.58 a	0.54 b	0.41 a	0.48 a
Lactic acid	– *P*	0.26 a	0.28 a	0.76 a	0.85 a	0.33 a	0.30 a
	+ *P*	0.25 a	0.29 a	0.59 a	0.38 b	0.37 a	0.48 a
Formic acid	– *P*	0.60 a	0.59 a	1.43 a	1.64 a	0.67 a	-
	+ *P*	0.56 a	0.64 a	1.30 a	0.77 b	0.61 a	1.04
Acetic acid	– *P*	0.56 a	0.85 a	1.38 b	2.07 a	0.84 a	0.94 b
	+ *P*	0.82 a	0.99 a	1,91 a	1.54 b	0.95 a	1.64 a
Fumaric acid	– *P*	-	0.02 a	0.01 b	0.07 a	0.13 a	0.03 a
	+ *P*	-	0.03 a	0.10 a	0.10 a	0.06 b	0.02 a

Different letters show for the same carboxylate in each cultivar significant differences between + *P* and – *P* treatments, $P = 0.05$, Newmann Keuls test.

Citrate, malate and succinate were the major organic acids excreted from the roots. Kahm et al. [15] found similar results for white lupin. The spectrum of identified organic acids in this study exceeds the composition indicated by Neumann et al. [24] for *Lupinus albus* L. They observed only citrate and malate as the most significant organic acids. The higher amount of excreted citrate and malate was associated with their greater concentrations in the roots [25]. Hoffland et al. [11] obtained similar results with *P*-deficient oilseed rape (release of malic and citric acid). Of interest was the finding of Gerke [9], who identified oxalacetate in other legume plants (*Trifolium pratense, Trifolium repens, Medicago sativa* and *Lotus corniculatus*) and oxalate in *Lotus corniculatus*. Contrary to the results of Gerke [9], oxalacetate was not found in *Medicago sativa* in the experiments of Lipton et al. [20]. For ryegrass (*Lolium multiflorum* Lam.), Römer et al. [29] found only citrate, malate succinate and acetate. Gerke [9] attributed the finding or not of certain organic acids to dissimilarities in the analytical methods used for collecting and identifying the excreted organic acids.

The collection media (deionized water or 0.05 mM $CaCl_2$ solution) did not affect the composition or amount of released organic acids during the two first hours (2nd–3rd h) of collection (Tab. II). During the fourth and fifth h of collection, the citrate concentration in the deionized water was slightly higher; acetate and malate concentrations were lower than in 0.05 mM $CaCl_2$ solution. The exudation rates for the other organic anions were similar. We therefore do not believe that a lack of Ca^{2+} during our collection periods destabilized cell membranes, leading to the instability and bursting of root cells and loss of cell contents into the external medium. Similar results were found by comparing deionized water and 0.5 mM $CaSO_4$ for short-term incubations of two hours [25]. Although our results did not indicate a deleterious effect of aerated deionized water within four hours of exposure, we decided to use 0.05 mM $CaCl_2$ solution in the eight-hour experiment, because maintenance of stability may not be guaranteed without Ca^{2+} for this longer period of time. A long collection time may increase the impact of microbial degradation on the recovery of root exudates. In our study the collection period was relatively short in comparison with other authors who collected exudates for over 12 h [23, 27]. We suppose that during the short time period, effects of microbial degradation on exuded organic acids which may occur on the root surface during the collection time might be minimized. Neumann and Römheld [26] found 90% recovery of citric acid released from roots of white lupin into aerated distilled water under non-sterile conditions after four hours' incubation time. Furthermore, the recovery rates were 86% for malate, 91% for fumarate and 87% for aconitate after 24 h of incubation time at 22 °C [23]. We therefore suggest that microbial degradation did not contribute to the observed differences in exudation rates when comparing collection periods, species or treatments. Despite this suggestion, we assume that in our collection medium microbial degradation could occur, and further investigations under sterile conditions will be needed to confirm our results.

Citrate and malate have been shown to be highly efficient for nutrient acquisition by plants and heavy metal chelation/complexation (detoxification) [6, 9, 10, 17]. Particularly with heavy metals, citrate and malate form highly stable complexes [22]. For these reasons, most attention has been focused on the exudation rate of both organic anions. In this study, Tables III and IV show that citrate, malate and succinate were the most significant released carboxylic anions for all six investigated cultivars. White lupin showed exudation rates of 0.15–0.45 nM·(cm root)$^{-1}$·h^{-1} citrate and 0.43–1.65 nM·(cm root)$^{-1}$·h^{-1} malate. In particular, the citrate exudation rate was lower than the results from Neumann: 6.7 nM·(cm root)$^{-1}$·h^{-1}

for separated proteoid roots and 0.1–0.2 $nM \cdot (cm\ root)^{-1} \cdot h^{-1}$ for non-proteoid roots [24]. Here, it is to be considered that exudation rates were calculated on the total root system and not on the sections of the root clusters or root tips. Moreover, the proportion of cluster roots of the total root system in the present study was not quantified, so it is not possible to give an estimation of the exudation rate from proteoid roots only. Consequently, the exudation rate might be greatly underestimated for root tip regions and cluster root segments.

Blue and yellow lupin excreted higher amounts of organic acids per root length and per hour than white lupin. The blue lupin showed the highest exudation rate of all the eight identified carboxylic anions and its citrate and malate exudation rates were around ten times higher than those of white lupin. As yet, we do not know the underlying biochemical reason for these large differences.

In different plant species, organic acid exudation is directly related to biosynthesis/accumulation and to transport out into the medium [14, 19]. We suppose that the greatly different exudation rates observed between the three lupin species were due to the differences in their physiological mechanism of biosynthesis/accumulation of organic acids in roots and/or to the transport of the synthesized organic acids of roots towards the collection medium.

Phosphorus nutrition did not influence the composition of released organic acids from roots into the collecting solution. Phosphorus deficiency in the plants led to a significantly increased exudation rate of citrate by the cultivars with the exception of Borweta (blue lupin). The cultivars Bordako (blue lupin) and Borselfa (yellow lupin) significantly increased their malate exudation rate. Many authors have observed the same response in different plant species (*Lupinus albus, Medicago sativa, Trifolium pratense, Zea mays, Beta vulgaris* and *Spinacia oleracea)*, which increased their citrate exudation under *P* deficiency [1, 9, 13, 16, 17, 22, 24, 29, 30]. Neumann and Römheld [28] investigated the mechanisms responsible for this response in white lupin. They found an increase in citrate synthesis, supported by higher activity of phosphoenolpyruvate carboxylase (PEPC), and a decreased rate of conversion of citric acid into aconitate through inhibited aconitase activity. The release of citrate might not only function as a strategy for *P* mobilization in the soil or for reducing the concentration of toxic cations in the rhizosphere but also as a detoxification mechanism to prevent excessive accumulation of citric acid and cytoplasmic acidosis, which could result from the excessive accumulation of citric acid in root cells [28]. We propose that similar mechanisms exist in the other investigated lupin species.

5. CONCLUSION

The present study shows that not only white lupin, but also blue and yellow lupin, were able to release organic anions into the rhizosphere. The most significant released carboxylic anions were citrate, malate and succinate for all the six investigated cultivars of the three lupin species. Cultivated under *P* deficiency conditions, five cultivars increased their citrate exudation rate. This investigation quantified exudation rates in solution as a medium. Extrapolation of the exudation rates to soil conditions should be treated with some degree of caution. Special procedures have to be developed to collect exudates directly under soil conditions, although these measurements will represent the quasi steady-state levels between root excretion, microbial excretion and degradation, soil sorption and leaching. Nevertheless, for the modeling of nutrient uptake, it would be of interest to study the diffusion of released organic acids away from the root, their decomposition by soil microbes, their temporal change in soil, and their reaction with the soil in solubilizing nutrients or chelating/complexing heavy metals.

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