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REGULAR ARTICLE

Bioavailability of zinc and phosphorus in calcareous soils as affected by citrate exudation

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Abstract
Aims Zinc (Zn) and phosphorus (P) deficiency often occurs at the same time and limits crop production in many soils. It has been suggested that citrate root exudation is a response of plants to both deficiencies. We used white lupin (*Lupinus albus* L.) as a model plant to clarify if citrate exuded by roots could increase the bioavailability of Zn and P in calcareous soils.
Methods White lupin was grown in nutrient solution and in two calcareous soils in a rhizobox. Rhizosphere soil solution was sampled to determine citrate, metals and P. Based on the measured citrate concentrations, a soil extraction experiment with citrate as extractant was done.
Results Absence of Zn triggered neither cluster root formation nor citrate exudation of white lupin grown in nutrient solution, whereas low P supply did. The maximum citrate concentration (~1.5 m*M*) found in the cluster rhizosphere soil solution of one soil mobilized P, but not Zn. In the other soil the highest citrate concentration (~0.5 m*M*) mobilized both elements.
Conclusions White lupin does not respond to low Zn bioavailability by increasing citrate exudation. Such a response was observed at low P supply only. Whether Zn and P can be mobilized by citrate is soil-dependent and the possible controlling mechanisms are discussed.

Keywords Rhizosphere · Bioavailability · White lupin · Citrate · Zinc · Calcareous soils

Abbreviations

DAG	Days after germination
CE	Capillary electrophoresis
ICP-MS	Inductively coupled plasma mass spectroscopy
SOC	Soil organic carbon
CEC	Cation exchange capacity
CBD	Citrate-bicarbonate-dithionite
OM	Organic matter

Introduction

Zinc (Zn) deficiency is limiting crop production in ±30 % of the world's soils (Sillanpää and Vlek 1985). Most of these soils are calcareous. In these areas, Zn deficiency is often caused not by low total soil Zn contents but by low bioavailability of Zn (Alloway 2009). Zinc deficiency often co-occurs with P deficiency, because the bioavailability of both elements decreases with increasing pH (Zhu et al. 2001). At neutral pH (pH<8.4) Zn solubility is mainly

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controlled by adsorption reactions (Catlett et al. 2002) and P can precipitate as Ca-phosphate (Weng et al. 2011). Quijano-Guerta et al. (2002) studied the tolerance of rice (*Oryza sativa* L.) germplasm to Zn deficiency. They showed that genotypes tolerant to Zn deficiency also have at least a moderate tolerance to P deficiency. Hoffland et al. (2006) related these characteristics of multiple tolerance to citrate exudation.

Mobilization of Zn and P by citrate is based on different mechanisms. The free Zn ion (Zn^{2+}) is considered as the main bioavailable species (Kalis et al. 2007). Zinc mobilization is based on acidification due to the exudation of protons (Hinsinger et al. 2003) and on formation of soluble complexes of exuded citrate with Zn^{2+} in solution, which reduces the activity of Zn^{2+} in the soil solution. As a consequence Zn^{2+} desorbs from soil surfaces or dissolves from labile solid phases to replenish Zn^{2+} in solution (Lindsay and Norvell 1978). Phosphate mobilization from soils by citrate and proton exudation is attributed to (i) the enhanced dissolution of P minerals caused by acidification and complexation of cations such as calcium (in alkaline soils), aluminum and iron (in acid soils) from phosphate minerals by citrate, and (ii) the competitive adsorption of phosphate and citrate on metal (hydr)oxides (Geelhoed et al. 1998).

Although it is well documented that citrate (and protons) is able to mobilize P in soil (Hoffland 1992; Geelhoed et al. 1999; Kirk et al. 1999; Hinsinger 2001; Oburger et al. 2011), there are contrasting reports on root exudation of citrate or other low molecular weight organic acids under Zn deficiency and/or their potential to mobilize Zn in soil. On the one hand Rose et al. (2011) proposed that enhanced exudation of malate, but not citrate, is a response of Zn-efficient rice genotypes to Zn deficiency. Widodo et al. (2010) stated that the high tolerance of rice cultivars to Zn deficiency is most likely a result of malate exudation. Neither of these studies, however, proved Zn mobilization from soil by citrate. Degryse et al. (2008) showed that carboxylates exuded from roots of spinach (*Spinacia oleracea* L.) and tomato (*Solanum lycopersicum* L.) were able to mobilize Cu and Zn from a calcareous soil. On the other hand Gao et al. (2009) showed that malate concentrations observed in the rhizosphere of rice had a negligible effect on the concentration of Zn in soil solution of a low Zn soil.

We aimed to test the effectiveness of citrate exudation as response to low Zn and P in calcareous soils. White lupin (*Lupinus albus* L.) is a useful model plant to study mobilization, because it is known from non-calcareous soils that white lupin shows a strong and effective response to low P supply by the formation of cluster roots (bottlebrush-like clusters of rootlets (Purnell 1960)) and exudation of citrate and protons (Dinkelaker et al. 1995; Weisskopf et al. 2006). Citrate concentrations can reach m*M* levels in the rhizosphere solution of cluster roots (Dessureault-Rompré et al. 2008). Gardner et al. (1982) showed that white lupin grown on an alkaline soil also responds to iron stress by the development of a higher proportion of cluster roots and an increase in proton and reducing and chelating compound production. However, to our best knowledge, such a response was not yet shown for white lupin under Zn deficiency conditions.

There are different approaches to determine a possible multiple stress response to low Zn and P bioavailability in the rhizosphere. One is the use of rhizoboxes in combination with micro-suction cups (Göttlein et al. 1996) which were already used with different plants and soils (Wenzel et al. 2001; Dessureault-Rompré et al. 2008; Gao et al. 2009). In the present study we determined *in situ* Zn and P mobilization by citrate in the rhizosphere of calcareous soils and linked potential Zn and P mobilization to plant uptake and the results of a nutrient solution and a soil extraction experiment.

Material and methods

Nutrient solution experiment

With a nutrient solution experiment we studied the formation of cluster roots and the exudation of citrate by white lupin in absence of Zn and P. White lupin (*Lupinus albus* L. *var.* Feodora) seeds were germinated in quartz sand and were transplanted after 5 days to 50 L containers filled with a continuously aerated nutrient solution. The composition of the nutrient solution was based on Jaitz et al. (2011). The pH of the nutrient solution was adjusted to 7.3 (±0.2). In addition to the control treatment containing all nutrients, there were two other treatments: either without P or without Zn. After 21 days of growth, each plant was put individually in a Petri dish with 30 mL 1 m*M* $CaCl_2$ solution to determine the citrate exudation rate (Neumann and Römheld 2007). Two hours later, a

sample of 10 mL was taken from the solution. After filtration (0.45 μm), 50 μL of $CHCl_3$ was added to avoid microbial degradation and the samples were analyzed for citrate and nitrate using capillary electrophoresis (CE) (see below). The citrate exudation rate was determined for four individual plants per treatment (n=4). After collecting root exudates, roots were scanned on a root scanner followed by drying the roots at 70 °C for root dry weight determination.

Soils

Soil samples were collected in Hofuf (Saudi Arabia) and Anatolia (Turkey) from the top layer (0–20 cm). The soils are named after the location of collection. The sampling sites were used for crop cultivation and both soils were classified as Aridisols. In crops grown on Hofuf soil Fe chlorosis (Schenkeveld et al. 2008) and in crops grown on Anatolia soil Zn chlorosis (Cakmak et al. 1996) was manifested, respectively. The pre-treatment of the soil samples consisted of drying (40 °C), sieving (2 mm) and homogenizing. The soil characteristics are shown in Table 1. The pH and *aqua regia* extractable Zn fractions of the two soils are comparable. The DTPA extractable Zn (Lindsay and Norvell 1978) in the loamy Anatolia soil was five times lower than in the sandy Hofuf soil (Table 1). For quality assurance, reference samples from clay and sandy soils were analyzed (respectively ISE 989 and ISE 949, WEPAL, www.wepal.nl).

Rhizobox experiment

With a rhizobox experiment we investigated how the rhizosphere is affected by the response of white lupin to Zn and P deficiency in the two soils (Anatolia and Hofuf). The experiment was conducted in a climate chamber at Wageningen University (photoperiod of 15 h (6:00–21:00 h), light intensity 525 $\mu M\ m^{-2}\ s^{-1}$, day/night temperature 25/18 °C, rel. humidity 70 %). Rhizoboxes, so-called "Hohenheim" boxes (Luster et al. 2009) (33 cm long, 20 cm wide, and 1.5 cm deep) were filled with moistened soil to a dry bulk density of 1.1 g cm^{-3}. Treatments included a combination of two Zn and two P levels (i.e. −P/−Zn, +P/−Zn, −P/+Zn and +P/+Zn). Except for the −P and −Zn treatments the soil was fertilized with 100 mg N kg^{-1} (as $Ca(NO_3)_2$ $4H_2O$), 80 mg P kg^{-1} (as KH_2PO_4), 100 mg K kg^{-1} (as KCl), 10 mg Zn kg^{-1} (as $ZnCl_2$) and 1.5 mg Fe kg^{-1} (as Fe-HBED,(Chaney 1988)), respectively. In the −P and −Zn treatment, P or Zn, respectively was omitted. Each treatment was duplicated. The soil was adjusted to 60 % of its water holding capacity every day.

Seeds of white lupin (*Lupinus albus* L. *var.* Feodora) were soaked in 10 % H_2O_2 for 10 min. After a germination period of 4 days on moist (double-distilled water) paper tissue, the seedlings were transplanted to the rhizoboxes (two plants per rhizobox), which was defined as the first Day After Germination (DAG). The rhizoboxes were standing in racks at an angle of 30° to force the roots to grow along the Plexiglas plate side, which enabled us to observe and localize the roots. The Plexiglas was covered most of the time with a plastic sheet to exclude any light effect on root growth. The other side of the rhizobox consisted of a non-transparent PVC sheet with a 5×5 mm grid of holes (1.8 mm diameter) for insertion of micro-suction cups, which were used for *in situ* soil solution sampling. The design, pre-cleaning- and sampling procedure with the micro suction cups was done according to Shen and Hoffland (2007).

Soil solution was sampled in the rhizosphere of the cluster roots and in the bulk soil. Around the cluster roots 6–8 micro-suction cups were inserted per rhizobox at a distance of ±1 mm from the rootlet. We considered soil which was more than 5 cm away from the roots as bulk soil. Between the 1st and the 11th day after the emergence of the cluster root (17–27 days after germination (DAG)) the soil solution was sampled every second day at 14:00 h (Dessureault-Rompré et al. 2007) for 2.5 h, which yielded a volume of ±0.2 mL per micro-suction cup. To obtain enough soil solution for analysis, the samples collected by 6–8 micro-suction cups per cluster root were mixed. In the Hofuf soil only one replicate was analysed 7 days (+P/−Zn treatment) and 9 days (−P/+Zn and −P/−Zn treatment) after cluster emergence, because the sample volume was too small to allow for replicates. The pH was determined immediately after sampling. The solution was subsequently divided into subsamples for the analysis of citrate and nitrate with Capillary Electrophoresis (see below) and of Zn, Fe and P with Inductively Coupled Plasma Mass Spectroscopy (ICP-MS, Elan 6000, Perkin Elmer). The detection limit for P and Zn with ICP MS was 1 μg L^{-1} and 0.3 μg $L^{-1,}$ respectively. Phosphorus analysis with ICP-MS includes the inorganic and the organic P fractions. Roots and shoots were harvested 27 DAG.

Table 1 Soil characteristics

Soil	pH[a]	Clay	$CaCO_3$[b]	SOC[c]	CEC[d]	CBD[e]	0.005 *M* DTPA[f]		*Aqua regia*[g]
		g kg^{-1}	g kg^{-1}	g kg^{-1}	$cmol^{+}$ kg^{-1}	Fe g kg^{-1}	Zn mg kg^{-1}	Fe	Zn
Hofuf [#]	7.7	40	62	7.1	3.5	0.6	5.03	6.7	28
Anatolia	7.9	220	93	10	32.1	5.1	0.09	11.2	33

[#] Data of Hofuf soil are from Schenkeveld et al. (2008)

[a] 0.01 *M* $CaCl_2$, ISO/DIS 10390; [b] ISO 10693, [c] Soil organic carbon, Walinga et al. (1992) ;[d] Cation exchange capacity, ISO/DIS 11260; [e] Citrate Bicarbonate Dithionite, Holmgren (1967); [f] Lindsay and Norvell (1978); [g] ISO 11466

Roots were washed thoroughly with distilled water to remove all soil particles and the number of cluster roots per plant was counted followed by washing the roots for 1 min in a 0.01 *M* EDTA solution to remove the root surface adsorbed metals (Kalis et al. 2007). Afterwards the roots and shoots were dried at 70 °C for 3 days to determine their dry weight and milled and digested according to the procedure described by Novozamsky et al. (1996). The Zn and P concentrations in digests were measured with ICP-MS. Plant uptake was calculated by summing the products of root and shoot dry weight and their respective tissue content of P and Zn, minus the product of seed dry weight and seed tissue content of P and Zn.

The rhizobox experiment was done twice, yielding highly similar results. Results of one of the two experiments are shown.

Soil extraction experiment

The soil extraction experiment with the Anatolia and Hofuf soils aimed to determine the Zn and P mobilizing capacity of citrate. Air-dried soil (3 g) was added to a 50 mL centrifugation tube and 30 mL extraction solution was added (Houba et al. 2000). The extraction solution consisted of citric acid with a concentration range from 0 to 7 m*M* in 0.01 *M* $CaCl_2$. Hundred μL of $CHCl_3$ was added to the extraction solution to avoid microbial degradation The tubes were shaken horizontally for 24 h at 20 °C. The pH in the suspensions was measured immediately after shaking, then the suspensions were centrifuged at 3000×g for 10 min, and the supernatants were filtered (0.45 μm) before determining the concentrations of citrate (Capillary Electrophoresis, see below), Zn and P (ICP-MS). The experiment was done in duplicate.

Citrate and nitrate analysis

Citrate and nitrate were analyzed with capillary electrophoresis (CE) in adaptation to the method of Westergaard et al. (1998). The instrument (Waters Corp., Milford, MA, USA) was equipped with a UV detector (indirect) and a 254 nm filter. All separations were performed in a fused-silica capillary (accu-Sep 75 μm×60 cm capillary, Waters Corp., Milford, MA, USA). Between two samples the capillary was rinsed with deionized water for 0.5 min, with 0.1 *M* NaOH for 1 min and with deionized water for 0.5 min, respectively, after which it was preconditioned with the background electrolyte for 3 min. The background electrolyte was prepared with 1,2,4-benzenetri-carboxylic acid [TriMellitic Acid, (TMA)]. The electrolyte consisted of 3 m*M* TMA and 0.02 % (*v/v*) DiEthyleneTriAmine (DETA) with the pH adjusted to 5.8 with NaOH. Cation interference (metal-organic anion complexes) was prevented by adding 50 μL of a 25 m*M* tetra-sodium-ethyleneditetraacetic (Na_4EDTA, pH >10) solution to each sample of 450 μL. The CE was run at 20 °C and the high voltage was set to 25 kV. The detection limit was 10 μ*M*.

Statistical analysis

Statistical analysis of data was performed with SPSS analytical software (SPSS Inc., Chicago, IL, USA; version 17). A two-way ANOVA was done to analyze main effects of the P and Zn treatments on shoot dry weight, on Zn and P plant uptake and on Zn, Fe and P concentrations in the soil solution. The variation among data was homogeneous (Levene's test). Bivariate correlation was used to determine the Pearson correlation coefficient (*r*) between the P or Zn contents

in the shoot tissue and the number of cluster roots, respectively, with a two tailed test for significance ($P<0.05$).

Results

Nutrient solution experiment

Cluster root formation was observed neither on plants grown in the nutrient solution without Zn nor on those grown in the complete nutrient solution. In the solution without added P considerable cluster root formation was observed (Fig. 1). Citrate exudation by root systems grown in -Zn and in complete nutrient solution was below the detection limit (10 μ*M*), which means that the exudation rate was below ~0.8 μmol g^{-1} h^{-1}. In the −P treatment, the exudation rate was 3.9 μmol g^{-1} h^{-1}. The plants grown on the −Zn treatment showed Zn deficiency symptoms (chlorotic leaves with brown necrotic spots). Neither the plants grown in the nutrient solution without P nor those grown in the complete nutrient solution showed visible deficiency symptoms apart from growth reduction.

Rhizobox experiment

Plants of all treatments looked similar. No stress symptoms could be observed. Shoot dry weight was increased significantly ($P=0.002$) by P fertilization in the Anatolia soil, but not by Zn fertilization. Shoot dry weight of the plants grown in the Hofuf soil showed neither a response to P nor to Zn fertilization ($P>0.05$) (Fig. 2a). Plant uptake of P and Zn increased significantly due to P ($P\leq0.0001$) and Zn ($P\leq0.0001$) fertilization on the Anatolia soil (Fig. 2b,c). Plants grown on the Hofuf soil responded to P ($P=0.002$), but not to Zn fertilization. Zinc uptake was significantly higher in the −P/−Zn treatment than in the +P/−Zn treatment from this soil (Fig. 2b,c).

Cluster root abundance in both soils was around 2.5 times higher in the −P compared to the +P treatments. The total number of cluster roots per plant in the −P treatment was about 20 and 8 in the Anatolia and the Hofuf soil, respectively. Phosphorous content in the shoot tissue of the Anatolia ($r=-0.88$, $P=0.04$, $n=8$) and Hofuf soil ($r=-0.78$, $P=0.02$, $n=8$) correlated negatively with the number of cluster roots per plant. The Zn content in the shoot tissue did not correlate with the number of cluster roots ($P>0.05$).

Citrate concentrations in the soil solution of the cluster rhizosphere peaked 3 days after emergence of the cluster roots (Fig. 3). Maximum citrate concentrations in the Anatolia and Hofuf soils were about 1.5 m*M* and 0.5 m*M*, respectively. At this point in time citrate concentrations in the -P treatments were significantly higher than in the +P treatments. Zn

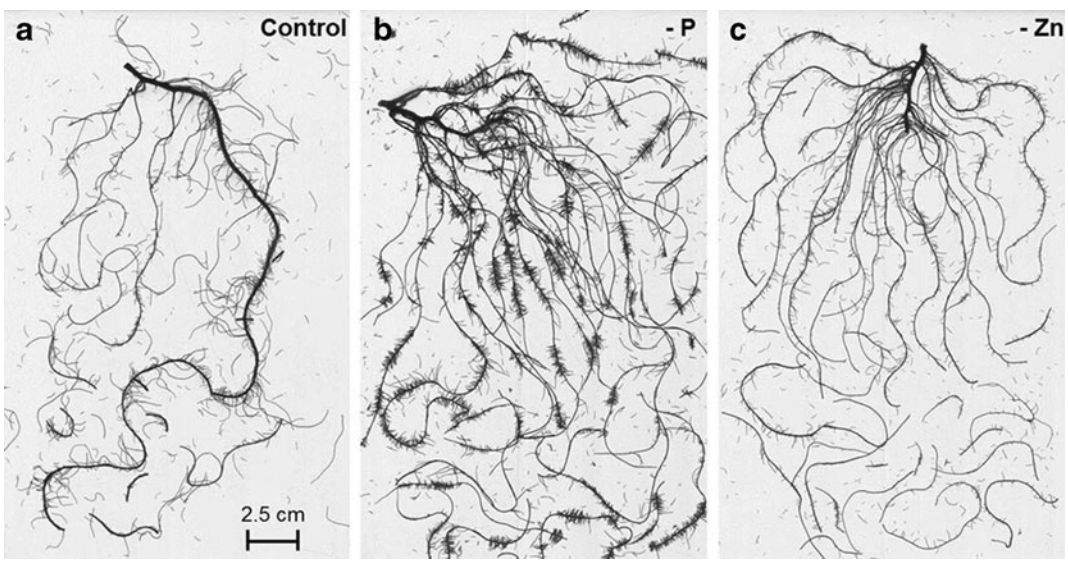

Fig. 1 Representative root system scans of white lupin plants grown in nutrient solution with all nutrients (**a**), without P (**b**), or without Zn (**c**). The bottle-brush-like clusters of rootlets in (b) are cluster roots

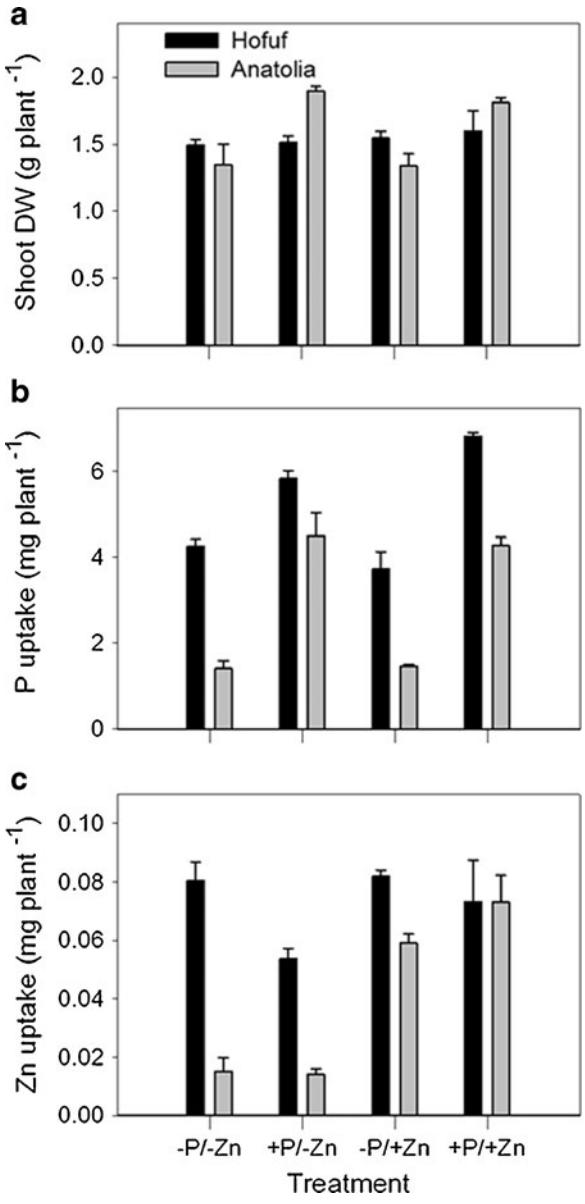

Fig. 2 Shoot dry weight (**a**), P plant uptake (**b**), and Zn plant uptake (**c**) of white lupin plants (27 days after germination) grown on two calcareous soils with (+) and without (−) fertilization with Zn or P. Error bars are standard errors (n=2)

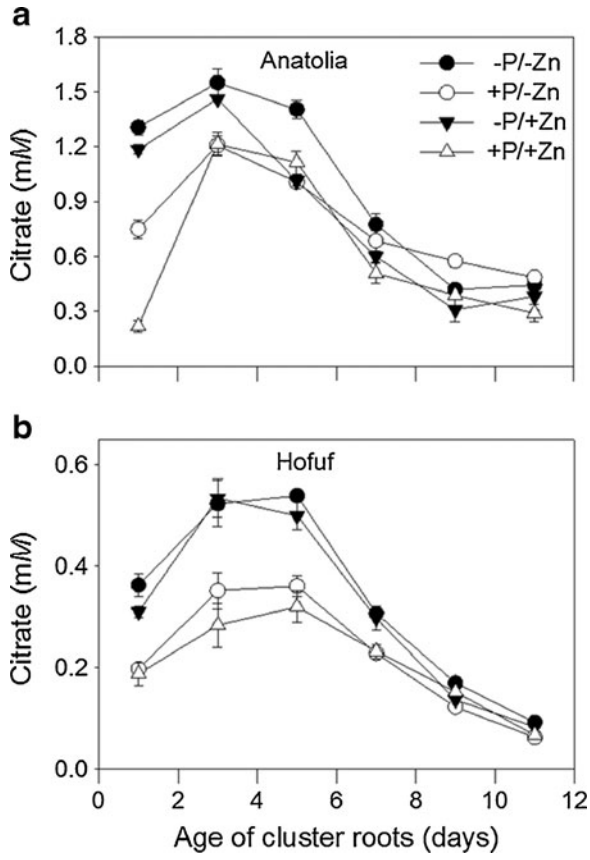

Fig. 3 Citrate concentrations in the cluster rhizosphere soil solution of the Anatolia (**a**) and Hofuf (**b**) soil depending on the age of the cluster roots. Error bars represent standard errors (n=2)

fertilization did not affect the citrate concentrations in the cluster rhizopshere (Fig. 3). At the same time Zn and P application had increased the respective Zn and P concentrations in the cluster rhizosphere solution of both soils compared with the bulk soil (−P/−Zn) (Table 2). However, except for the Zn concentrations in the Anatolia cluster rhizosphere soil solution, the Zn and Fe concentrations in the cluster rhizosphere of the −P treatments (−P/−Zn; −P/+Zn) were significantly higher than in the +P (+P/−Zn; +P/+Zn) treatments and the bulk soil (−P/−Zn), respectively (Table 2). The P concentrations in the cluster rhizosphere of the −P treatments (−P/−Zn; −P/+Zn) were also significantly higher than in the −P/−Zn bulk soil.

The bulk soil pH was almost constant over time at ~7.8 and ~7.6 in the Anatolia and Hofuf soil, respectively. The pH values in the soil solution of the cluster rhizosphere were increasing ~3 days after the emergence of the cluster roots (Fig. 4). Nitrate concentrations in the soil solution of the cluster root rhizosphere were higher in the −P treatments compared to the +P treatments (data not shown). In all treatments, a sharp decline of the NO_3 concentrations could be observed around 3 days after the emergence of the cluster roots (data not shown).

Table 2 Metal micronutrients and phosphorus (μg L-1) in the soil solution of the cluster rhizosphere and the bulk soil 3 days after cluster root emergence (19 days after germination). Values in brackets are standard errors (n=2)

Element	Rhizosphere				Bulk
	−P/−Zn	+P/−Zn	−P/+Zn	+P/+Zn	−P/−Zn
			Anatolia soil		
Zn	3.7 (0.1)	3.7 (0.4)	7.6 (0.3)	7.1 (0.2)	4.4 (0.2)
Fe	124.0 (9.0)	71.2 (7.0)	121.5 (13.5)	108.5 (12.5)	101.7 (12.0)
P	29.6 (5.3)	423.5 (34.5)	36.9 (3.4)	438.5 (17.5)	17.8 (3.5)
			Hofuf soil		
Zn	19.9 (0.6)	9.9 (0.3)	21.8 (0.6)	13.4 (0.5)	7.8 (0.8)
Fe	131.0 (5.2)	98.9 (4.7)	136.9 (11.3)	107.6 (16.5)	119.0 (5.6)
P	143.2 (27.4)	563.3 (9.6)	192.5 (9.1)	582.2 (20.3)	68.9 (3.6)

Extraction experiment

After shaking soil samples in a citrate solution, adsorbed citrate (difference between the added amount of citrate and the remaining citrate in solution) appeared to be linearly related to citrate concentration in the extract. The highest added concentration of citric acid (7 mM) resulted in 1.3-1.7 mM citrate in solution after reaching equilibrium (24 h) (Fig. 5a). The pH values in the extractants of the two soils were decreasing with an increasing citrate concentration in solution. In the

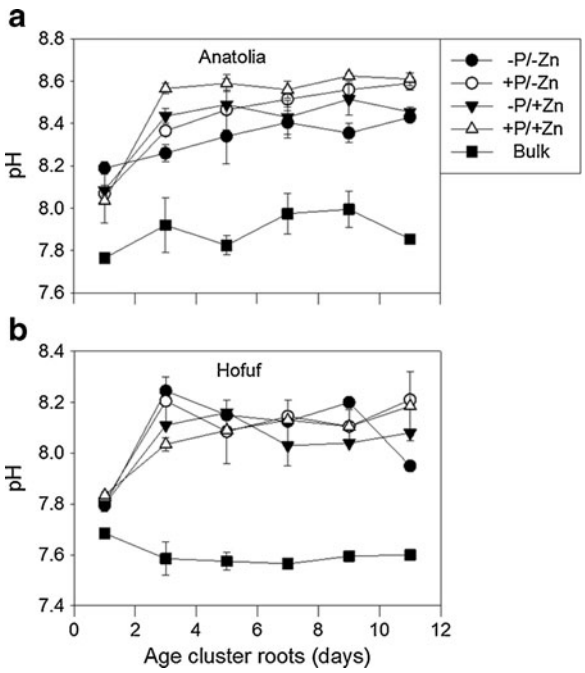

Fig. 4 pH in the soil solution of cluster rhizosphere or bulk soil of the Anatolia (**a**) and Hofuf (**b**) soil depending on the age of the cluster roots. Error bars represent standard errors (n=2)

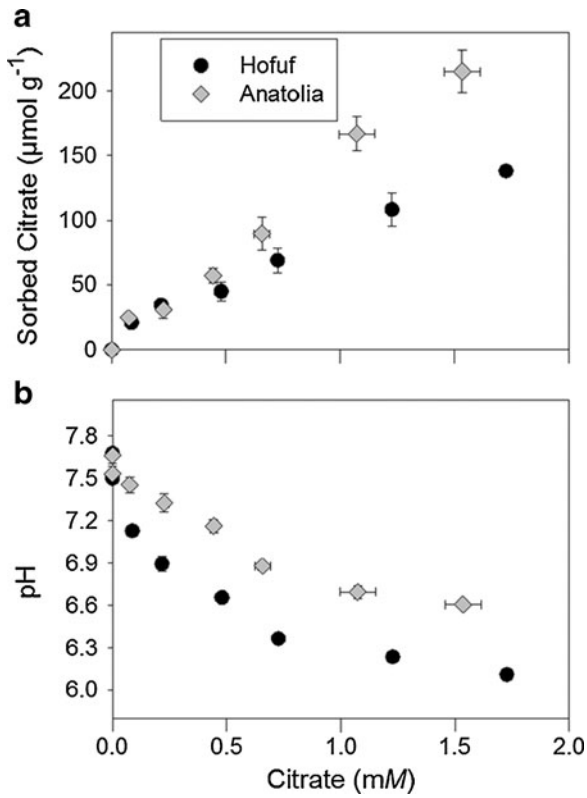

Fig. 5 Adsorption isotherms of citrate (**a**) on the Anatolia and Hofuf soil and the pH (**b**) after 24 h shaking (20 °C). pH changes following shaking the soils in solutions of citric acid in 10 mM CaCl2 for 24 h at 1:10 soil:solution ratio. Citrate concentrations in solution (x axis) were measured after centrifuging the resulting suspensions, and the amounts of citrate sorbed were inferred from the changes in solution concentrations. Error bars represent standard errors (n=2)

Anatolia soil, the pH decreased approximately 1 unit at the highest citrate concentrations in solution, whereas in Hofuf soil, the pH decreased approximately 1.3 units (Fig. 5b). Extractable P increased linearly with the citrate concentration added. Extracted P from the Hofuf soil was about 40 times higher compared to the Anatolia soil (Fig. 6a). Extractable Zn increased with the citrate concentration added in the Hofuf soil. No Zn was extracted at all from the Anatolia soil regardless of the original citrate concentration in the extractant (Fig. 6b).

Discussion

We showed that white lupin did not respond to Zn deficiency with cluster root formation and citrate exudation. Even plants grown in nutrient solution in the absence of any Zn neither formed cluster roots (Fig. 1) nor exuded any detectable citrate. Zn deficiency caused no increase in citrate concentration in the soil solution around cluster roots, even though Zn shoot tissue content was at a critical level (Reuter et al. 1997). Neumann et al. (2000) studied the physiological aspects of cluster root function and development of white lupine and suggested that cluster root induction may be stimulated by low Zn-supply. Our experiments did not confirm this suggestion. Cluster root formation and citrate exudation were increased as a response to low P shoot tissue concentrations (Fig. 3) as shown before (Dinkelaker et al. 1995; Neumann et al. 2000). The citrate concentration in the soil solution peaked 3 days after the cluster root emergence, followed by a rapid decline which was also observed by Peñaloza et al. (2002) and Dessureault-Rompré et al. (2006).

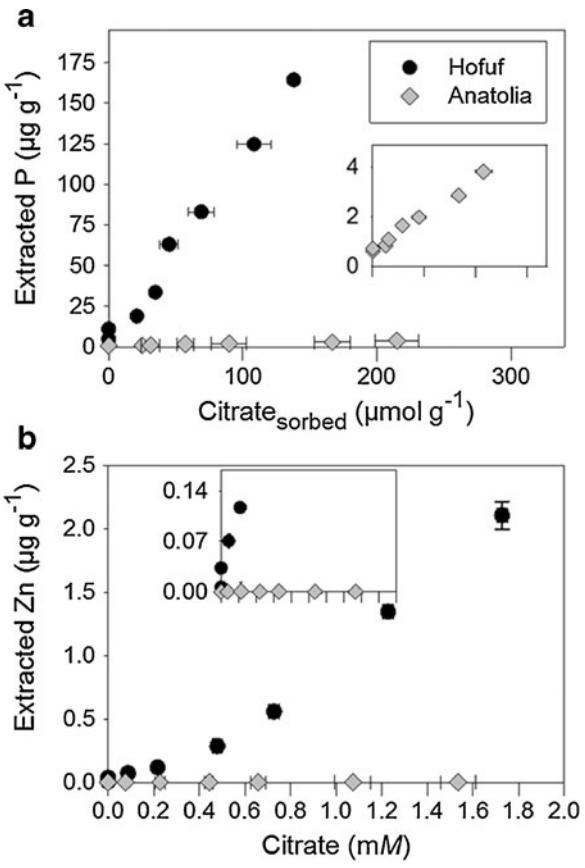

Fig. 6 Relationships between the extractable P in the extractant and sorbed citrate (**a**) and between the extractable Zn in solution and the citrate concentration in the extractant (**b**) after 24 h shaking at 20 °C of the Anatolia and Hofuf soil. The x-axis ticks and values of the inserted small graphs are the same as of the main graphs, respectively. Error bars represent standard errors (n=2)

To our knowledge we for the first time determined citrate, metal and P concentrations in the rhizosphere of calcareous soils. The maximum citrate concentrations we detected can be considered as high compared with other studies in a non-calcareous soil (Dessureault-Rompré et al. 2007). The highest citrate concentration (~1.5 mM), which was detected in the cluster rhizosphere soil solution of the Anatolia soil (−P treatments) (Fig. 6), did mobilize P, but not Zn (Table 2). In the Hofuf soil the highest citrate concentration (~0.5 mM) (−P treatment) (Fig. 6) mobilized both P and Zn (Table 2). The conclusion that P (and Zn) were mobilized is based on a comparison of P and Zn concentrations in the soil solution of the cluster rhizosphere of the −P treatments with the +P treatments and with the −P/−Zn bulk soil (Table 2). The P concentrations in the cluster rhizosphere solution in the −P treatments were, due to mobilization, 5–7 times higher in the Hofuf soil than in the Anatolia soil (Table 2) and resulted in adequate shoot P tissue contents in all treatments (Reuter et al. 1997). Zinc mobilization by citrate in the −P treatments of the Hofuf soil (Table 2) was the reason that the Zn fertilization had no significant main effect due to the adequate Zn shoot tissue contents in the −P/−Zn treatment. This shows that the exuded citrate in the Hofuf soil was able to increase Zn mobilization and Zn plant uptake.

Zinc was not mobilized by citrate in the Anatolia soil neither in the rhizobox (Table 2) nor in the extraction experiment (Fig. 6b), even though the citrate

concentrations in the cluster rhizosphere solution were ~3 times higher than in the Hofuf soil. This difference in Zn mobilization can be explained by Fe-Zn competition. Both soils had similar *aqua regia* extractable Zn concentrations. The DTPA-extractable Zn was about a fifth of that in the Anatolia soil, whereas the DTPA-extractable Fe was twice that high as in the Hofuf soil (Table 1). Considering the stability constants (log K, based on MINTEQA2 (Allison et al. 1991)) of citrate with Fe(III) (13.1) and Zn (6.21), citrate probably mainly formed complexes with Fe. This is in line with the changes in Fe and Zn concentrations in the soil solution of the cluster root rhizosphere when the citrate concentrations reached their maximum (Table 2). Hence, in the Anatolia soil, less Zn was complexed with citrate in solution and, by inference, less Zn was desorbed or dissolved from the soil solid to replenish Zn taken up by the roots. The Zn extraction efficiencies of citrate (% release as mol Zn per mol citrate in solution) in Hofuf and Anatolia soils were 0.19 % and <0.00 %, respectively. Therefore, metal competition in the soil solution should be considered when evaluating the mobilization capacity of citrate (or another ligand) for Zn (Kinniburgh et al. 1999).

Phosphorus, in contrast, was most probably mobilized due to competitive adsorption of phosphate and citrate on metal (hydr)oxides (Geelhoed et al. 1998). In the extraction experiment, 40 times more P was extracted from the Hofuf soil (Fig. 6a), which had about 8.5 times less Citrate Bicarbonate Dithionite (CBD) extractable Fe (Table 1) than the Anatolia soil. Since the CBD-extractable Fe is considered to represent the total iron (hydr)oxide content (Hiemstra et al. 2010), there was a stronger competition between P and citrate in the Hofuf soil. In the Anatolia soil, more citrate was bound in total (Fig. 5a), but the competition was less strong.

Other authors reported that, due to P deficiency, an increased exudation of citrate leads to a decrease of the soil pH (Neumann and Römheld 1999; Sas et al. 2001) and a correspondingly higher mobility of P and Zn. However, in our rhizobox experiment, citrate exudation caused no pH decrease since the high NO_3^- uptake of the plant and the corresponding excess of negative charges was counterbalanced by releasing equivalent amounts of OH^- or HCO_3^- into the rhizosphere (Imas et al. 1997). This implies that the citrate release from the root was small compared with the intake of nutrient ions and that organic anion release not necessarily acidifies the rhizosphere. Therefore, a pH decrease was excluded to explain the mobilization of P and Zn. In the extraction experiment without plant-soil interactions, the pH value decreased (Fig. 5). This explains why the relative amount of mobilized P and Zn in the extraction experiment was higher than in the rhizobox experiment. The stronger pH decrease in the Hofuf soil was explained with the lower $CaCO_3$ content compared with the Anatolia soil.

We conclude that white lupin does not respond to low Zn supply with increased citrate exudation by cluster root formation, as was shown at low P supply, which is not supportive of a multiple stress response. However, citrate exudation can, dependent on the type of soil, mobilize both Zn and P. To improve the understanding of the mechanisms which control Zn mobilization by root exudates in (calcareous) low Zn soils requires metal speciation techniques and mechanistic multicomponent modeling such as those used in studies with polluted soils (Koopmans et al. 2008).

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