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The effect of lime on the rhizosphere processes and elemental uptake of white lupin

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ABSTRACT

Acid soils cover 30–40% of the world's arable land and represent one of the major constraints to agricultural production. Lime is routinely added to soil to improve fertility and to reduce the solubility of elements such as aluminum (Al) and cadmium (Cd). White lupin is cultivated globally, however, this is done mainly on acidic soils because of its calcifuge characteristics resulting from its limited ability to compartmentalize calcium (Ca). In abiotic stress conditions, lupins exude organic acids and flavonoids from cluster roots. This can increase the availability of essential soil nutrients to the plant but also exacerbate the uptake of contaminants. We aimed to determine the effect of liming on the rhizosphere processes of white lupin plants in two high-fertility soils, which were treated with seven levels of lime. Nutrient availability and plant uptake was assessed with a pot experiment. Three lime levels have been chosen for a further rhizotron study. Diffusive gradient in thin layers (DGT) gels were applied on selected root zones and then analyzed by laser ablation inductively-coupled plasma mass spectrometry (LA ICP-MS).

The results showed that lime affected the solubility of extractable elements and the plant uptake. In soils treated with different levels of lime, the uptake of nutrients was sufficient to avoid nutrient deficiency. However, analysis of the DGT gels only showed mobilization around the cluster root of the plant grown in the untreated soil. The results indicate that white lupin can be grown at pH as high as 7.50 with 10 wt% lime without suffering from nutrient deficiencies.

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1. Introduction

Soil pH is one of the most important chemical parameters influencing element sorption and dissolution processes in soil (Comerford, 2005) and thus the bioavailable fraction to plants. Many authors reported that plant element uptake is highly correlated with pH (Dakora and Phillips, 2002; Hinsinger et al., 2003; Marschner, 1991). The bioavailability of trace element cations such as copper (Cu), zinc (Zn), nickel (Ni), cadmium (Cd) and lead (Pb) and their concentration in plants is significantly reduced at pH > 7.0. Acidic pH can enhance the bioavailability of these elements, as well as essential plant micronutrients, such as iron (Fe), manganese (Mn) and boron (B). However, these benefits can be offset by increased bioavailability of potentially phytotoxic elements such as aluminum (Al^{3+}). Acid soils, covering 30–40% of the world's arable land, represent one of the major constraints in agricultural production due to plant growth inhibition and yield reduction (Marschner, 1991). Several factors could further increase soil acidification, such as large inputs of inorganic fertilizers, high rainfall, acid deposition and greenhouse gases. In addition to toxic concentrations of Al^{3+} and protons (H^+), acid soils can provoke deficiencies in anionic plant nutrients such as molybdate (MoO_4^{2-}) and phosphate (PO_4^{3-}). The ongoing application of PO_4^{3-} containing fertilizers to overcome phosphorus (P) deficiency in acid soils, can lead to the accumulation of Cd (Williams and David, 1976), that exists naturally as an impurity in phosphate rocks, from which phosphate fertilizers are obtained. The entry of toxic metals, such as Cd, from soils into the food

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chain through plant uptake is of primary concern in agricultural production systems because of the potential threats to food quality, crop growth, and environmental health (McLaughlin et al., 2000).

Liming ($CaCO_3$) is routinely used as long-term agricultural practice to improve soil quality by increasing nutrient bioavailability, as well as improving soil structure and increasing rates of infiltration. In addition, liming has been demonstrated to be effective in reducing the solubility of cationic trace elements in soils by increasing the negative charge on oxides, clays, and organic matter (Kirkham, 2006) and/or leading to the pH driven precipitation of mineral phases (Fe, Mn oxides, Ca-phosphates). However, excessive carbonate concentrations may lead to toxic effects besides reducing the plant-available fraction of essential macro- and micronutrients, such as P, Fe, Mn and Zn.

White lupin (*Lupinus albus* L.) is adapted to well drained, light to medium textured soil, can tolerate moderately alkaline soils (up to pH 8.0), provided that the free lime or Ca content of the soil is low (the accepted maximum soil level of $CaCO_3$ is 30–50 g kg^{-1}) (Jansen, 2006), since lupin species are unable to regulate Ca uptake (De Silva et al., 1994). Such typical calcifuge behavior may be related to an insufficient capacity for compartmentation and/or physiological inactivation of Ca (Hawkesford et al., 2011). In addition, an immobilization of P in the tissues of calcifuge plants may occur, since the excessive uptake of Ca may cause precipitation of Ca phosphate in plant tissues (Zohlen and Tyler, 2004). The concentration of carbonate (Brand et al., 2000) and especially the so-called free lime concentration as previously shown in *L. angustifolius* L. (Jessop et al., 1990) is the limiting factor for the plant growth. White lupin is known to cope with abiotic stresses by releasing organic compounds (organic acids and flavonoids) into the rhizosphere (Neumann et al., 1999). However, whether these substances can have beneficial effects increasing the availability of nutrients or might even counteract the liming effect by mobilizing toxic elements needs to be elucidated.

We aimed to determine the effect of lime concentration on the rhizosphere processes of white lupin in two high-fertility soils. Specifically, we sought to measure the bioavailability of nutrients as well as trace elements in selected root zones of the plants, where the release of root exudates is more pronounced. The final objective was to define a so-called "ideal [Ca]/pH zone" where lupin is still able to mobilize nutrients without suffering from Ca toxicity and trace elements such as Cd.

2. Materials and methods

2.1. Soils

We selected two high-fertility soils with contrasting chemical–physical characteristics (Table 1), referred to as "Pukekohe" and "Levin" soils. Both soils were slightly acidic (Pukekohe pH 5.45; Levin pH 6.46), rich in macro- and micronutrients meeting plant requirements for an optimal growth. Seven different lime ($CaCO_3$, Thermo Fischer Scientific NZ Ltd.) treatments (T1–T7) were applied to both soils, as follows T1: 0 wt%, T2: 0.31 wt%, T3: 0.61 wt%, T4: 1.25 wt%, T5: 2.50 wt%, T6: 5.00 wt%, T7: 10.00 wt%. The $CaCO_3$ amended soils were well mixed in buckets and then transferred into pots. For each treatment five replicates consisting of 250 g of soil were used. After filling, pots were moved to a greenhouse, watered to field capacity and left there for a week in order to allow the lime to react with soil.

2.2. Pot experiment

White lupin seeds (*L. albus* L.), were soaked for 24 h in ASTM Type I ultrapure water and then transferred to the pots containing the different soil treatments. Subsequently, plants were grown in a greenhouse for 6 weeks, watered every two days with tap water and weeds were carefully removed every week. After 6 weeks, plants were harvested and the above ground biomass was assessed. The above-ground biomass of the plants was thereby cut 3 cm above the soil level and it was carefully washed with deionised water. Plants were dried at 60 °C for one week until constant weight was reached and then ground for subsequent elemental analyses. Rhizosphere samples were obtained from each pot by gently uprooting the plants and by removing the soil adhering to the roots by shaking and brushing. These samples were oven dried, ground and sieved using a 2 mm sieve. All sample processing was carried out ensuring that there was minimal metal contamination.

Table 1
Chemical–physical characteristics of Pukekohe and Levin soils; LOD = limit of detection.

	Pukekohe	Levin
Parameter		
pH (H_2O)[a]	5.95 ± 0.04	6.46 ± 0.06
CEC (me/100g)[b]	22.00	15
Base saturation [%][c]	70.00	88
C [%][d]	2.10	1
N [%][e]	0.23	0.13
Olsen P [me/100g][f]	290.00	229
N available [kg/ha][g]	50.00	53
Total P [mg/kg][h]	3414 ± 26	2247 ± 20
Total S [mg/kg][h]	491 ± 6	296.46 ± 1.32
Total Ca [mg/kg][h]	4147 ± 117	7008 ± 99
Total Mg [mg/kg][h]	2400 ± 95	2873 ± 43
Total K [mg/kg][h]	1951 ± 59	2242 ± 54
Total B [mg/kg][h]	<LOD	8.89 ± 0.15
Total Cd [mg/kg][h]	1.45 ± 0.03	0.47 ± 0.01
Total Cu [mg/kg][h]	65 ± 0.46	20 ± 0.20
Total Mo [mg/kg][h]	<LOD	<LOD
Total Mn [mg/kg][h]	1266 ± 12	387 ± 6
Total Zn [mg/kg][h]	173.21 ± 1.06	66.52 ± 0.72
Total Fe [mg/kg][h]	44606 ± 96	22729 ± 1527
Total Pb [mg/kg][h]	61.02 ± 0.76	13.08 ± 0.18
Cd $Ca(NO_3)_2$-extractable [mg/kg]	0.015 ± 0.002	0.008 ± 0.002
Cu $Ca(NO_3)_2$-extractable [mg/kg]	0.13 ± 0.015	0.123 ± 0.006
Mn $Ca(NO_3)_2$-extractable [mg/kg]	39.75 ± 4.17	8.58 ± 0.25
Zn $Ca(NO_3)_2$-extractable [mg/kg]	0.37 ± 0.06	0.177 ± 0.017
Fe $Ca(NO_3)_2$-extractable [mg/kg]	0.654 ± 0.195	0.511 ± 0.036

[a] 1:2 (v/v) Soil:water slurry followed by potentiometric determination of pH.
[b] Summation of extractable cations (K, Ca, Mg, Na) and extractable acidity.
[c] Calculated from extractable cations and cation exchange capacity.
[d] Determined by NIR, calibration based on total carbon by Dumas combustion.
[e] Determined by NIR, calibration based on Total Nitrogen by Dumas combustion.
[f] Olsen extraction followed by molybdenum blue colorimetry.
[g] Anaerobic incubation followed by extraction using 2 M KCl followed by Berthelot colorimetry.
[h] Pseudo total elemental concentration in the soil, determined by microwave digestion of 0.5 g sieved (2 mm) soil sample with 5 mL conc. HNO_3 and 1 mL H_2O_2.

2.3. Soil and plant analysis

Following microwave-assisted digestion (CEM MARS Xpress, CEM Corporation, NC, USA) of samples (0.3 g each) in concentrated 65% HNO_3 and 30% H_2O_2, the total elemental concentrations in plants were determined by inductively coupled plasma–optical emission spectroscopy (ICP-OES) (Varian 720-ES; Varian, Mulgrave, Australia). Wageningen Evaluating Programs for Analytical Labs (WEPAL) plant was used as reference material. The recoveries for Cd, Fe and Zn were 94%, 94.5% and 91.7% respectively. Soil pH was determined by adding 25 mL of deionised water to 10 g of oven dried soil. Samples were stirred and left to equilibrate overnight. Subsequently the pH was measured in the supernatant.

The plant available element fraction was obtained by extracting soils with 0.05 M $Ca(NO_3)_2$ in a ratio of 1:6 (w/v) for 2 h on an

end-over-end shaker. Samples were then centrifuged at 3000 rpm for 10 min (Hettich Universal 30 RF) after which the supernatant from each extract was filtered with Whatman paper No. 52 and stored at 6 °C until further analysis of elemental concentrations by ICP-OES. Blank extracts representing 5% of the total number of extracts were prepared using the same batch of reagents with the same apparatus, and analysed at the same time and in the same way as soil extracts.

2.4. Rhizotron experiments

Rhizotrons were constructed from clear cast acrylic polymer according to a modified design to that described by Göttlein et al. (1999). The internal dimensions of each rhizotron were 15 × 30 × 2.5 cm (w × h × d) and featured a removable front plate and a fixed back plate fitted with ten ports (in a 2 × 5 configuration at 5 cm intervals) to ensure uniform watering of the soil inside the container. Rhizotron experiments were carried out only with Pukekohe soil due to its crumb structure that was more amenable to plant growth, water infiltration and aeration compared to the Levin soil. The results of the pot trial were used to select two lime treatments (T4 and T7) and a control (T1). The experimental soils were packed in layers into the rhizotrons to ensure a constant bulk density of 1.08 g cm^{-3} across the depth of the rhizotron. White lupin seedlings were transplanted four days after germination on moist tissue paper. The soils were moistened to 30% Maximum Water Holding Capacity (MWHC) before planting the lupin seedlings, after which the rhizotrons were evenly irrigated through the watering ports on the back of the rhizotrons. Moisture content was kept constant for the duration of the growth cycle. The rhizotrons were carefully covered with black plastic film below the level of the soil to limit photo-chemical reduction phenomena in the rhizosphere and biofilm formation on the front plate and were kept in a controlled environment growth chamber with a day/night cycle of 16/8 h and a day/night temperature of 24/16 °C, 70% relative humidity.

2.5. High-resolution diffusive gradients in thin-films measurements (HR-DGT)

The diffusive gradients in thin-films (DGT) technique was used to measure the two-dimensional distribution of metal flux in the lupin rhizosphere in the three rhizotrons. The principles of this method are described in detail by Santner et al. (2012), Kreuzeder et al. (2013), and Williams et al. (2014). The chelating resin used in the DGT gel here is a suspended particulate reagent–iminodiacetate (SPR-IDA; CETAC Technologies Inc., USA) which has been previously identified as a suitable resin for high resolution two dimensional (2D) visualisation of metal supply owing to its capacity to bind trace metals and small resin bead size (Warnken et al., 2004). The SPR-IDA resin was supplied pre-cleaned as 10 mL of a 10% (w/v) suspension. 1.5 mL of the SPR-IDA resin suspension was mixed with 1.5 mL of a solution containing acrylamide (40%, Fisher Scientific, USA) and DGT cross-linker (DGT Research, Ltd., UK) in a ratio of 4:1. To this 3 mL mixture, 21 μL of ammonium persulfate (Merck, Germany) and 6 μL of *N,N,N′,N′*-tetramethylethylenediamine (TEMED, ~99%, Sigma–Aldrich, USA) were added. The gel mixture was then immediately pipetted between two glass plates, where a 0.25 mm spacer was used to ensure that the solution polymerized into a gel sheet with a uniform thickness. The glass plate assembly was then placed in an oven at 45 °C for 1 h, after which the glass plates were separated and the resin gel was placed into 0.5 L of ultrapure water (filtered by Millipore, 18.2 MΩ cm) and allowed to fully hydrate for a minimum of 24 h. The hydrating solution was changed four times in the subsequent 72 h after which the resin gel was stored in 0.5 L of 0.05 M $NaNO_3$ solution. Before deployment, the resin gel sheet was cut down to suitably sized sections using PTFE-coated razor blades and then mounted between a 10 μm-thick polycarbonate filter membrane (Nucleopore, Whatman, 0.4 μm pore size) on one side and a sheet of cellulose acetate (OfficeMax New Zealand Ltd.) on the other to exclude solid particles from the soil and to help manipulate the gels. To minimize contamination, all preparation and processing of gels was carried out using ultraclean trace metal techniques ensuring that all equipment were acid-washed and rinsed in ASTM Type 1 ultrapure water (18.2 MΩ cm).

DGT sampling was performed after 6 weeks of plant growth, when the plants had fully developed cluster roots. For applying the DGT gels, the rhizotrons were laid horizontally with the removable front plate facing upwards. The soil in the rhizotrons was progressively saturated with water over 24 h by allowing water to gently infiltrate. Three hours after the beginning of the light cycle, the front plate was carefully removed and a small amount of water was added until an even thin film of water was visible at the soil surface. The DGT-filter assembly was taped on four sides onto template sheet of transparent cellulose acetate leaving a well-defined window of 10 μm polycarbonate filter to allow diffusion of solute into the DGT. The DGT was then carefully applied on to exposed rhizosphere soil ensuring good and consistent contact between the soil and the DGT gel assembly. The front plate was then placed on top of the gel assembly to provide gentle and even pressure. The DGT gels were deployed for 24 h. After the deployment, the DGT gel assembly was gently peeled off the rhizosphere interface and any soil particles attached to the assembly were removed using a combination of clean laboratory tissues and a steady stream of ultrapure water. The exposed areas of the deployed DGT gels were then cut away from the assembly using PTFE-coated razor blades, separated from the filter paper and stored horizontally in acid washed zip-lock polyethylene bags at 6 °C for no longer than one week. The gels were then dried using the protocol described by Stockdale et al. (2008) and mounted flat onto glass slides using double-sided tape.

2.6. Laser ablation ICP-MS

In this study, the same DGT calibration standards were used for LA-ICP-MS as in Hoefer et al. (2015). Standard preparation and analysis is described briefly here, for details we refer the reader to Hoefer et al. (2015). Resin gel discs (2.5 cm diameter) were cut out of the SPR-IDA resin gel sheets and assembled with a 0.08 cm diffusion layers and a 0.45 μm pore size membrane filters (Supor 450, Pall Corporation, New York USA) within standard DGT housings (DGT Research Ltd., UK). These DGT units were immersed in triplicate into well-stirred solutions containing Mn, Cu, Zn, Cd and Pb at concentrations of 0.01–17.1 mg L^{-1} for 4, 8 and 20 h. The immersion solutions were prepared by dissolving *p.a.* grade nitrate and chloride salts of these elements in 3 L of ultrapure water containing 1 mmol L^{-1} $NaNO_3$. The pH of the immersion solutions was ~5.6. After the probe specific deployment times, the samplers were retrieved, disassembled and the central resin gel area was cut out using a 20-mm diameter polypropylene stencil. The cut-away resin was cut into two even halves and each half was weighed to confirm the volume of the gel being eluted. One half of each gel replicate was eluted for 24 h in 2 mL 1 mol L^{-1} HNO_3 (*p.a.* grade, Sigma Aldrich, Vienna, Austria) and then analyzed the target analytes using ICP-MS. The eluent concentration was used to calculate the mass of metal bound by per unit area of resin gel based on the metal-specific elution efficiencies reported in Warnken et al. (2004). The other half of each replicate was dried onto a 0.45 μm membrane filter for LA-ICP-MS analysis.

The mass of trace metals bound by the standard and the sample DGT gels was analyzed using laser ablation inductively-coupled

plasma mass spectrometry (LA-ICP-MS) using a UP 193-FX (ESI, NWR Division, CA, US) laser ablation system coupled to a NexION 350D ICP-MS (Perkin-Elmer, MA, USA). Prior to analysis, the performance of the ICP-MS was checked by carrying out a standard performance test in solution mode confirming optimum sensitivity for ^{115}In and minimal formation of oxides and doubly charged ions in the plasma.

The LA-ICP-MS analysis was carried out in line scan mode with a laser beam diameter of 150 μm, scanning speed of 200 μm s^{-1}, laser pulse frequency of 20 Hz and a laser energy output of 40% (resulting in an approximate fluence of 4.35 J cm^{-2}). The ICP-MS was set to detect the following analytes: ^{13}C, ^{58}Fe, ^{55}Mn, ^{63}Cu, ^{66}Zn, ^{111}Cd and ^{208}Pb. The dwell times of the analytes were adjusted to provide sufficient precision for a representative signal for each analyte. This resulted in an ICP-MS analysis time of 0.383 s per reading, giving an along-the-line resolution of 76.6 μm. ^{13}C occurs naturally in the gel matrix and was used as the internal standard for the analysis (Gao and Lehto, 2012; Lehto et al., 2012).

The standards were analyzed in line scan mode by ablating a single 1 mm line (total of 132 readings) on each of the seven standards. For every reading, the metal (M) signal was normalized to the internal standard (IS) signal. The mean normalized signals (M/IS), combined with the analyte mass determined in the eluted standard disc halves, were used to quantify the metal loading on the sample gels.

Following the calibration, specific areas of each sample gel were analyzed using the same instrument settings as for the standards used in the calibration. The line scan was run using a 500 μm interval between the centers of adjacent lines (inter-line spacing of 350 μm). All signals were corrected with corresponding gas blank values. The mean normalized M/IS signals allow the analysis of relative differences in supply of metal to the DGT across the area of analysis (Williams et al., 2014). Where calibrated, the M/IS signals could be related to metal fluxes. Due to the difficulty in preparing Fe standards, we were unable to calibrate the signal to obtain a mass; however the M/IS relationship is directly proportional to the mass bound by the DGT resin (Williams et al., 2014). Data processing was done in MS Excel and the visualization was performed using an open source imaging software (ImageJ, National Institute of Health, Maryland, US. Available to download at http://rsbweb.nih.gov/ij).

2.7. Statistical analysis

The results are presented as means of at least three replicates ± standard deviation (SD). Statistical analysis was performed using Statgraphics (Statpoint technologies, INC., Warrenton, VA, USA). Data were analysed by analysis of variance (ANOVA), and means were compared using the Student–Newman–Keuls (SNK) test at $P < 0.05$ to determine the significance of differences found.

Multivariate analyses were carried out by using STAT Graphic Centurion XV, version 15.1.02. The validity of the Principal Component Analysis (PCA) models were assessed by the cross-validation approach described by Bro et al. (2008).

3. Results

3.1. Pot experiment—$Ca(NO_3)_2$-extractions

Soil pH was measured at harvest after 6 weeks of plant growth (Table 2). Lime treatments significantly increased soil pH, from 5.98 in T1 to 7.50 in T7 in Pukekohe soil and from 6.62 to 7.18 in Levin soil. Unexpectedly, shoot biomass was not affected by lime treatments (Table 2). Plants grown on Pukekohe soil had almost 30% higher dry shoot biomass than plants grown on Levin soil, most likely due to the different texture of the two soils. Here we consider the $Ca(NO_3)_2$-extractable trace element fraction to be representative of the plant-available fraction (Black et al., 2011). Table 3 shows the $Ca(NO_3)_2$-extractable trace element fraction of Pukekohe and Levin rhizospheric soils treated with different lime concentrations. Considering the Levin soil, significant differences were found only for Mn, Ni and Mg concentration, which decreased with increasing lime concentrations. The $Ca(NO_3)_2$-extractable concentration of elements was more affected by lime treatments in Pukekohe soil. For instance, P, K and Cu, increased by 16, 20 and 30%, respectively, comparing the control with the highest lime concentration. In contrast, Mn, Zn and Ni were significantly reduced by 98, 70 and 90%, respectively. Iron represented the only exception since its extractable concentration was not affected by the lime treatment in either soil.

Table 2
pH of the Pukekohe and Levin soils treated with increasing lime concentrations (T1–T7) measured at harvest and shoot dry biomass (DW) of lupin plants expressed in g; mean ± SD ($n = 3$).

	Pukekohe		Levin	
Treatment	pH***	Shoot DW^{ns}	pH***	Shoot DW^{ns}
T1	5.98 ± 0.05^{d}	0.88 ± 0.07	6.62 ± 0.06^{c}	0.56 ± 0.06
T2	6.37 ± 0.12^{c}	0.71 ± 0.17	6.96 ± 0.01^{b}	0.67 ± 0.20
T3	6.93 ± 0.17^{b}	0.73 ± 0.14	7.01 ± 0.04^{ab}	0.58 ± 0.05
T4	7.15 ± 0.03^{b}	0.78 ± 0.09	7.07 ± 0.04^{ab}	0.35 ± 0.26
T5	7.42 ± 0.01^{a}	0.82 ± 0.08	7.07 ± 0.07^{ab}	0.59 ± 0.32
T6	7.47 ± 0.03^{a}	0.73 ± 0.06	7.15 ± 0.04^{ab}	0.46 ± 0.18
T7	7.50 ± 0.04^{a}	0.80 ± 0.05	7.18 ± 0.14^{a}	0.59 ± 0.06

ns = not significant.
*** $p < 0.001$.

The contrasting elemental behaviors induced by the lime treatments were also delineated in the pattern recognition techniques, such as PCA (Fig. 1). The micronutrients Mn, Zn, Fe and Cu together with Ni are the main drivers in separating the different lime treatments along the first component (58% of total variance), whereas Na, Mg and P accounted for the different clusters according to the soil type, *i.e.* Pukekohe and Levin. Within the Pukekohe soil (Fig. 1A), T1 and T2 could be distinguished from the treatments T3–T7. The concentrations of Mn, Zn and Ni decreased by 50%, 60%, 65%, respectively, between the unamended soil (T1) and 0.3 wt% lime application (T2) (Table 3). In the Levin soil, only Mn, Ni and Mg showed significant differences (Table 3) and did not allow a separation between the different lime treatments (Fig. 1B).

3.2. Pot experiment–Plant elemental composition

The measured elemental concentrations in lupin shoots indicate that the lime treatments strongly affected the plant uptake of micronutrients in both soils (Fig. 2A and B), but to a different extent depending on the soil type. There were significant differences in all elements with increasing lime treatments in the Pukekohe soil, while only Fe, Mn, Zn and Ca were significantly changed by lime addition in the Levin soil. Lime addition reduced the plant element uptake by almost 50–60 % in both soils with the only exception of Mn and Mo in the Pukekohe soil grown lupin plants, which were reduced by 75% and increased by 80%, respectively.

The elemental composition of white lupin shoots grown in Pukekohe and Levin soils were further analyzed by PCA (Fig. 3A and B). As shown in Fig. 3A, the different treatments are separated along the first principal component which explains 50% of total the variance. The samples of shoots grown in Pukekohe soil can be clearly clustered in seven groups which correspond at the different treatments. In particular clusters of T1, T4 and T7 are well separated from the other samples. This separation reflects the significant differences of the concentration of the elements in

Table 3
$Ca(NO_3)_2$-extractable element fraction in rhizosphere soils treated with different lime concentrations (T1: 0 wt%, T2: 0.3 wt%, T3: 0.6 wt%, T4: 1.3 wt%, T5: 2.5 wt%, T6: 5 wt%, T7: 10 wt%) expressed as mg kg^{-1} DW; mean ± SD ($n = 3$).

Pukekohe	Fe^{ns}	P***	Cu*	Mn***	Zn***	Ni***	Mg***	K*
T1	0.16 ± 0.03	3.11 ± 0.04^{b}	0.15 ± 0.03^{ab}	21.75 ± 1.63^{a}	0.28 ± 0.02^{a}	0.088 ± 0.010^{a}	155.97 ± 1.35^{a}	160.63 ± 5.85^{b}
T2	0.12 ± 0.03	2.77 ± 0.02^{c}	0.13 ± 0.01^{b}	10.19 ± 1.88^{b}	0.11 ± 0.03^{b}	0.034 ± 0.010^{b}	153.57 ± 1.33^{a}	185.35 ± 23.88^{ab}
T3	0.16 ± 0.05	2.97 ± 0.14^{bc}	0.11 ± 0.01^{b}	2.40 ± 0.85^{c}	0.03 ± 0.01^{c}	0.009 ± 0.003^{c}	143.34 ± 5.31^{b}	210.53 ± 20.76^{a}
T4	0.15 ± 0.05	3.18 ± 0.19^{b}	0.16 ± 0.04^{ab}	1.52 ± 0.35^{c}	0.03 ± 0.01^{c}	0.008 ± 0.003^{c}	137.97 ± 1.47^{b}	219.25 ± 31.96^{a}
T5	0.17 ± 0.03	3.56 ± 0.24^{a}	0.16 ± 0.03^{ab}	0.61 ± 0.03^{c}	0.02 ± 0.01^{c}	0.005 ± 0.003^{c}	127.16 ± 0.42^{c}	217.09 ± 6.58^{a}
T6	0.22 ± 0.15	3.60 ± 0.14^{a}	0.15 ± 0.02^{ab}	0.54 ± 0.05^{c}	0.03 ± 0.02^{c}	0.004 ± 0.006^{c}	121.27 ± 3.69^{c}	207.92 ± 10.77^{a}
T7	0.14 ± 0.05	3.70 ± 0.15^{a}	0.21 ± 0.04^{a}	0.61 ± 0.11^{c}	0.08 ± 0.05^{bc}	0.010 ± 0.002^{c}	115.13 ± 5.69^{d}	182.55 ± 12.95^{ab}
Levin	**Fe^{ns}**	**P^{ns}**	**Cu^{ns}**	**Mn****	**Zn^{ns}**	**Ni***	**Mg*****	**K^{ns}**
T1	0.17 ± 0.03	5.71 ± 0.35	0.18 ± 0.04	6.42 ± 1.38^{a}	0.03 ± 0.01	0.001 ± 0.005^{ab}	123.29 ± 1.32^{a}	97.85 ± 19.96
T2	0.21 ± 0.07	5.37 ± 0.12	0.19 ± 0.04	2.99 ± 0.89^{b}	0.02 ± 0.01	0.002 ± 0.000^{b}	109.55 ± 2.28^{b}	91.42 ± 14.63
T3	0.35 ± 0.15	5.40 ± 0.21	0.25 ± 0.08	2.75 ± 1.77^{b}	0.02 ± 0.01	0.008 ± 0.001^{ab}	109.26 ± 1.16^{b}	95.86 ± 6.53
T4	0.23 ± 0.03	5.49 ± 0.03	0.15 ± 0.01	4.34 ± 1.54^{b}	0.01 ± 0.00	0.000 ± 0.004^{ab}	105.99 ± 0.79^{b}	98.13 ± 4.23
T5	0.22 ± 0.10	5.51 ± 0.11	0.25 ± 0.07	2.17 ± 0.55^{b}	0.02 ± 0.01	0.004 ± 0.007^{ab}	102.23 ± 0.37^{c}	85.65 ± 8.59
T6	0.17 ± 0.03	5.28 ± 0.05	0.21 ± 0.07	1.54 ± 0.73^{b}	0.05 ± 0.05	0.012 ± 0.008^{a}	98.70 ± 0.29^{d}	82.71 ± 8.34
T7	0.16 ± 0.07	5.45 ± 0.16	0.24 ± 0.14	2.42 ± 0.31^{b}	0.01 ± 0.01	0.004 ± 0.002^{ab}	93.06 ± 3.60^{e}	71.21 ± 18.42

ns = not significant.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

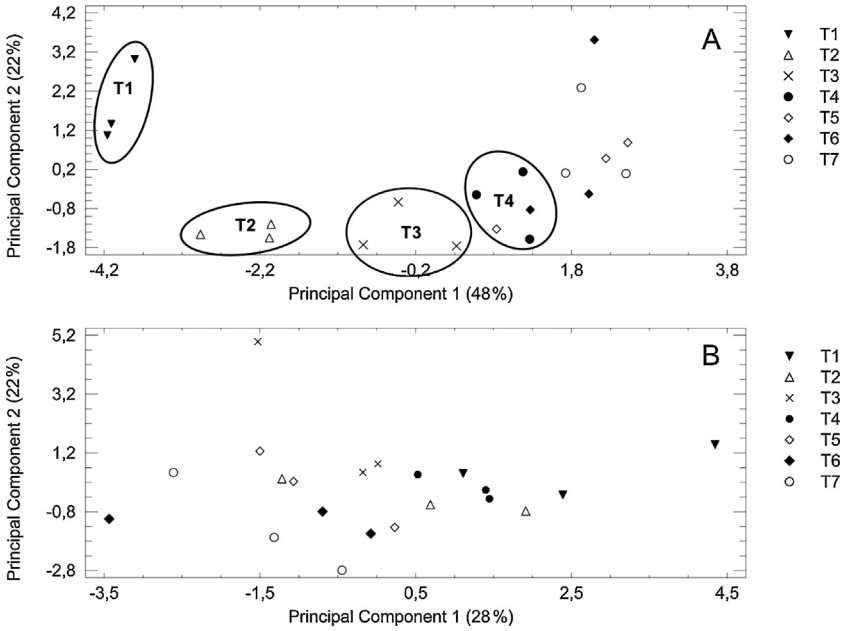

Fig. 1. Score plots of the first two principal components obtained from the dataset of the $Ca(NO_3)_2$-extractable element fraction of the two rhizosphere soils Pukekohe (A) and Levin (B) obtained at the 7 treatments (T1: 0 wt%, T2: 0.3 wt%, T3: 0.6 wt%, T4: 1.3 wt%, T5: 2.5 wt%, T6: 5 wt%, T7: 10 wt%). Clustering of the distinguishable treatments are highlighted by circles.

white lupin shoots. Regarding shoots grown in Levin soil, the PCA (Fig. 3B), grouped the samples of the different treatments in separated clusters. However, the different clusters are not as well separated when compared to plants in the Pukekohe soil. While there was a clear separation between the control and the highest lime treatment, samples from the other treatments are not well distinguished, indicating that the increasing lime levels are not significantly affecting all the components or the plants are compensating for lime addition by mobilizing elements in the rhizosphere.

3.3. HR-DGT and LA ICP-MS analysis

Due to the chemical physical characteristics and to the significant differences of the $Ca(NO_3)_2$-extractable fraction of rhizosphere soil and plant element concentration (Table 2, Table 3, Fig. 1, Fig. 3) caused by lime addition, the Pukekohe soil (T1, T4 and T7) was chosen for the rhizotrons experiment to investigate element bioavailability in response to different lime concentration on some selected root zones of white lupin. However, the high-resolution LA-ICP-MS analysis of the DGT gels (forthwith referred to as "HR-DGT analysis") from the three treatments showed element mobilization only in the rhizosphere of the plant grown in the un-amended (T1) treatment (Fig. 4), where a 21.67 × 23.50 mm area was analyzed at the location of a lupin cluster root. In particular, three distinct areas of elevated Fe supply indicated as HS1–HS3 in Fig. 4 of element mobilization could be observed. These 'hotspots' were defined by measurement points showing Fe mobilization that was greater than two standard deviations of the measurements across the entire area of ablation (Fig. 4). They are

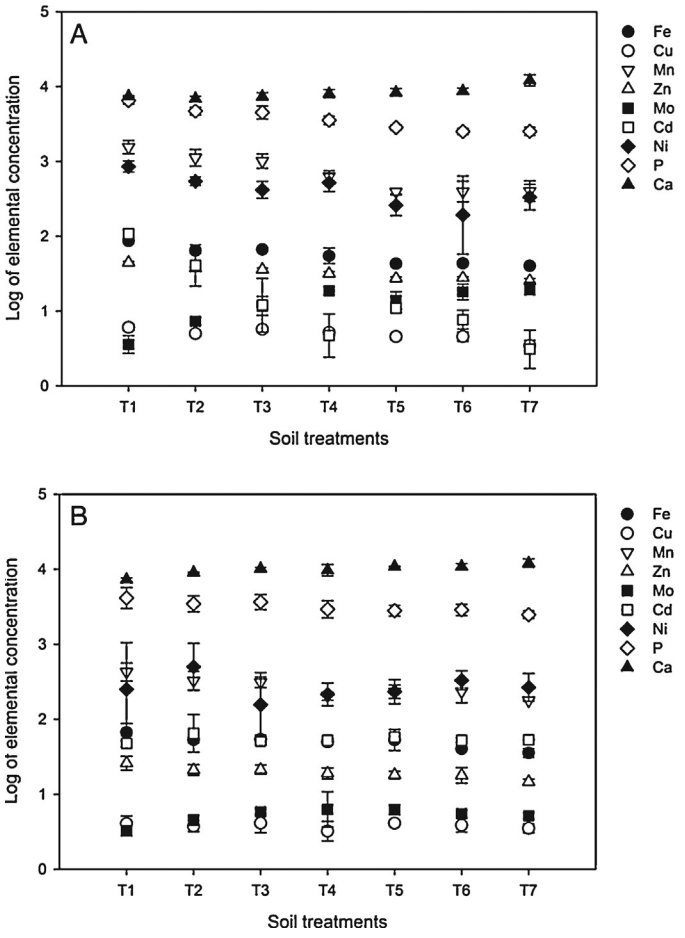

Fig. 2. Elemental concentration expressed as logarithmic scale of shoots of white lupin plants grown in Pukekohe (A) and Levin (B) soils treated with different lime concentrations (T1: 0 wt%, T2: 0.3 wt%, T3: 0.6 wt%, T4: 1.3 wt%, T5: 2.5 wt%, T6: 5 wt %, T7: 10 wt%).

defined by 137, 59 and 62 unique measurements respectively (Table 4) and correspond to the location of the observed cluster root (Fig. 4).

Table 4 shows the distribution of Fe, Cu, Zn and Pb in the entire ablation area and in the three hotspots. The total area of these three hotspots is 1.95% of the entire area of analysis. The relative average fluxes of Fe from the three hotspots was 3.9 (HS1), 3.5 (HS2) and 3.2 (HS3) times higher than the average Fe flux across the entire area of analysis. The areas of high Fe flux to the DGT can also be seen to broadly correspond to localized areas of high Cu, Zn and Pb fluxes. Cu and Zn were also detected in $Ca(NO_3)_2$-extractable fraction of rhizosphere soil whereas Pb was below the limit of detection (LOD). Considered together, the average fluxes of Cu (5.71 pg cm^{-2} s^{-1}), Zn (1.08 pg cm^{-2} s^{-1}) and Pb (4.70 pg cm^{-2} s^{-1}) measured by the HR-DGT within these three areas are 1.29, 1.74 and 3.40 times higher than the respective average fluxes of these metals across the entire area of measurement. Table 4 shows the Mn fluxes in a selected hotspot defined as before by the measurement points showing Mn fluxes greater than those in the bulk soil. The total area of this hotspot is 26 mm^2 in size and consists of 685 unique measurements (5.17% of the total area of analysis). The average flux across these points is 3.40 pg cm^{-2} s^{-1} while the average flux across the entire area of analysis is 2.04 pg cm^{-2} s^{-1} (1.66 times higher than the respective average flux across the entire area of measurement). For all five metals measured using this technique, the highest observed flux across the entire ablation area was found in HS1.

The masses of all of the cationic elements bound by DGT gels deployed on the other treatments were below the detection limits (not shown).

4. Discussion

4.1. Pot experiment—$Ca(NO_3)_2$-extractions

The maximum addition of lime (10% w/w) increased the pH by *ca.*1.52 and 0.56 pH units in the Pukekohe and Levin soil respectively (Table 2), but this increase did not have any significant

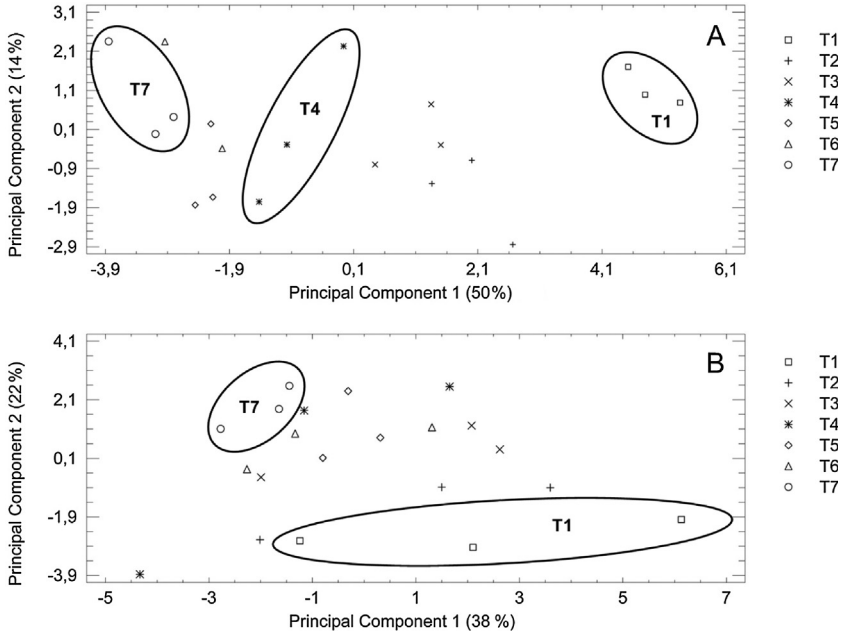

Fig. 3. Score plots of the first two principal components obtained from the dataset of the elemental composition of white lupin shoots of plants grown in Pukekohe (A) and Levin (B) soils at different lime treatments (T1: 0 wt%, T2: 0.3 wt%, T3: 0.6 wt%, T4: 1.3 wt%, T5: 2.5 wt%, T6: 5 wt%, T7: 10 wt%). Clustering of the distinguishable treatments are highlighted by circles.

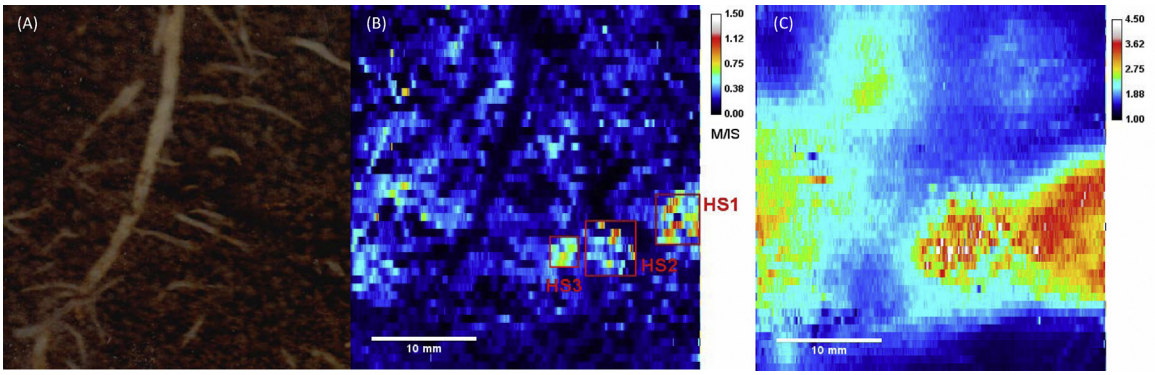

Fig. 4. Selected white lupin cluster root for DGT deployment in T1 treatment (A) and a visual representation of Fe(B) and Mn(C) mobilization around obtained by HR-DGT; each pixel represents a discrete measurement point acquired using this method, where relative Fe flux to the DGT is represented by M/IS data; Mn flux is shown as pg cm^{-2} s^{-1}.

Table 4
Distribution of Fe, Cu, Zn, Pb and Mn taken up by the DGT gel in T1 treatment of Pukekohe soil (M/IS, M: metal of interest, IS: internal standard); mean ± SD.

			Iron		Copper		Zinc		Lead	
Area of interest	No. discrete measurements	Total area	Average M/IS across area of interest	Maximum	Average Flux across area of interest	Maximum flux	Average Flux across area of interest	Maximum flux	Average Flux across area of interest	Maximum flux
Units		(mm^2)	M/IS	M/IS	(pg cm^{-2} s^{-1})	(pg cm^{-2} s^{-1})	(pg cm^{-2} s^{-1})	(pg cm^{-2} s^{-1})	(pg cm^{-2} s^{-1})	(pg cm^{-2} s^{-1})
Entire ablation area	13442	509	0.18 ± 0.13	2.05	4.42 ± 2.09	12.88	0.62 ± 0.56	3.61	1.38 ± 1.52	102.31
HS1	137	5.25	0.71 ± 0.23	2.05	5.51 ± 1.05	12.88	1.15 ± 0.42	3.61	3.31 ± 8.69	102.31
HS2	59	2.26	0.64 ± 0.20	1.13	4.16 ± 1.81	8.80	0.95 ± 0.29	1.93	4.32 ± 2.85	12.35
HS3	62	2.37	0.57 ± 0.09	0.82	7.45 ± 0.91	9.47	1.14 ± 0.23	1.66	6.48 ± 2.30	11.64

Manganese

Area of interest	No. discrete measurements	Total area	Average Flux across area of interest	Maximum flux
Units		(mm^2)	(pg cm^{-2} s^{-1})	(pg cm^{-2} s^{-1})
Entire ablation area	13442	509	2.04 ± 0.51	6.32
HS1	685	26	3.40 ± 0.36	6.32

effect on the lupin shoot biomass (Table 2). This can be explained by comparing the plant-available fractions of the major macro- and micronutrient before and after lupin harvest (Table 1 and Table 3, respectively). Considering treatment T1, *i.e.* both soils without lime addition, the concentrations detected in the $Ca(NO_3)_2$-extractable rhizosphere soil solution at harvest were significantly decreased. For instance, in the Pukekohe soil, Fe was reduced by approximately 70%, Mn by 50%, Zn by 10% and Cu by 20%. Despite this decrease, the plant-available concentration of these nutrients was still in the optimal range for plant growth (Kabata-Pendias, 2010). Iron for instance is one of the nutrients with the lowest solubility in soil usually resulting in soil solution concentrations of about 10^{-7} – 10^{-10} M over a pH range from 5.0 to 8.5 (Kraemer et al., 2006), a concentration too small for optimal plant growth. In the present study, despite plant uptake, the $Ca(NO_3)_2$-extractable rhizosphere soil solution concentration of Fe of white lupin was in the range of 10^{-6} M, meeting both microbe (10^{-5} – 10^{-7} M) and plant (10^{-4} – 10^{-9} M) requirements (Lemanceau et al., 2009). White lupin are highly efficient plants in mobilizing nutrients thanks to the excretions of organic acids, especially citric and malic acid (Neumann et al., 2000), from closely-spaced lateral rootlets arranged in clusters, the so-called "proteoid" roots. Previous studies showed that white lupin releases up to 50 ± 90 mmol of citric acid per g soil in the rhizosphere of cluster roots (Dinkelaker et al., 1989; Gerke et al., 1994), which is sufficient to desorb P from sparingly soluble Ca-, Al- and/or Fe-P and from Fe/Al humic acid complexes by ligand exchange reactions and dissolution of P sorption sites (Gardner et al., 1983; Gerke et al., 1994). Peaks of labile P have also been detected in the depletion zones around *Brassica napus* roots and have been attributed either to organic acids or protons (Santner et al., 2012). In fact, in addition to the organic acid exudation, white lupin releases also protons acidifying its rhizosphere. This has been detected even in a well-buffered calcareous soils in pH ranges similar to the ones of the present study (around 7.50 at the maximum lime addition) dissolving acid-soluble Ca-P (Dinkelaker et al., 1989). Our results indicate that the plant-available P fraction increased significantly between the control and the 10 wt% lime application (Table 3), which is in agreement with previous studies (Dinkelaker et al., 1989; Gardner et al., 1982) that have demonstrated efficiency of white lupin in taking up P and utilizing soil P sources even in calcareous soils. The availability of essential micronutrients as Mn, Zn and Ni decreased with increasing lime content (Table 3). Plant uptake and thus element depletion in the rhizosphere further contributed to this decreasing available fraction. Moreover, it is also to consider that, since rhizosphere was sampled from a pot experiment, the concentrations detected could be lower due to a sort of dilution effect of the bulk soil surrounding the rhizosphere. Regarding Mn, in well-aerated calcareous soils, its solubility decreases due to the adsorption of Mn on $CaCO_3$, its oxidation on MnO_2 surfaces and, probably, to precipitation of Mn calcite (Jauregui and Reisenauer, 1982).

4.2. Pot experiment–Plant elemental composition

Regarding elemental concentration in lupin shoots, lime treatments strongly affected the uptake of micronutrients from both soils (Fig. 2). In particular, Fe, and Zn concentration decreased

significantly in both soils at increasing lime concentration, while Cu, Ni, Mn and Cd decreased only in the Pukekohe soil. These micronutrients were also the main elements contributing to the distinct clustering (Fig. 3A) along the first component which accounted for 50.2% of the total variance. A decrease of Mn concentration as well as Zn and Ni could be a direct consequence of a decrease in the availability of these elements in the rhizosphere (Table 3) even though the plants did not show any deficiency symptoms and biomass was not significantly reduced (Table 1). Zinc ranged from 45 mg kg^{-1} in T1 to 25 mg kg^{-1} in T7 in white lupin shoots, (Fig. 2), concentrations far above those reported for Zn deficient plants (10–20 mg kg^{-1} DW, Boehle and Lindsay, 1969). Even though the Mn concentration decreased with increasing lime addition, the concentrations in lupin shoots were high in all the treatments (ranging from 1400 mg kg^{-1} DW in T1 to 400 mg kg^{-1} DW in T7). This suggests that 0.05 M $Ca(NO_3)_2$-extractable fraction of Mn did not accurately represent the Mn available to the white lupin plant in these treatments. White lupin is known to accumulate Mn in leaves (Zornoza et al., 2010). Reuter & Robinson (1997) and Martínez-Alcalá et al. (2009) have shown Mn concentrations as high as 1300 mg kg^{-1} DW and 4960 mg kg^{-1} DW in leaves and shoots respectively. This high rate of Mn uptake is believed to be due to an increased secretion of organic acids into the plant's rhizosphere, which promotes the metal's solubilization and thus facilitates its uptake by the plant (Dinkelaker et al., 1995). Manganese accumulation in lupin shoots is highest at low and zero lime levels indicating that at the higher liming rates the solubilizing effect of the organic acids might be neutralized by the high calcium carbonate of the soil, thus lowering the uptake.

Although Cd was below the detection limit in the $Ca(NO_3)_2$ extraction, the Cd concentration in the lupin shoots were significantly affected by lime treatment, with a decrease of 97% from T1 to T7 in the plants grown on the lime-treated Pukekohe soils. This is consistent with other studies that reported the effectiveness of liming to minimize the Cd uptake by plants (Oliver et al., 1996).

Organic acids play also a fundamental role in the reduction-based Fe uptake of white lupin as a *strategy I* plant, (Römheld & Marschner, 1983). However, while there was no change in the concentration of the available Fe in the rhizosphere of the soils with the different treatments (Table 3), Fe concentration in shoots decreased significantly ($P < 0.001$). However, the concentration of Fe in green plant tissues normally occurs at 50–100 mg kg^{-1} DW (Mengel and Kirkby, 2001). Only lime treatments >2.5% (w/w) lead in both soils to deficient conditions (<45 mg kg^{-1}).

The discrepancy between the bioavailable Fe concentration in the rhizosphere soil solution and the Fe concentration detected in lupin shoot analysis might be due to a methodological drawback. Even though calcium nitrate is commonly used to determine the bioavailable fraction of elements, it might not be suitable for nutrients such as Fe. Many authors (De Santiago and Delgado, 2006; Gough et al., 1979; Menzies et al., 2007; Walsh and Beaton, 1973) asserted that caution should be used in formulating methods for the determination of the plant-available levels, yet an official and generally accepted method is still missing.

4.3. HR-DGT and LA ICP-MS analysis

The diffusive gradients in a thin film (DGT) technique is a valuable alternative to assess the bioavailable fraction since it represents a well-established in situ, time-integrated, passive sampling method that is designed to accumulate labile cationic and anionic species (Zhang and Davison, 1995). Mimicking a plant root, DGT takes up elements from the soil solution and induces resupply from the solid phase (Lehto et al., 2006) and thus provides valuable data on spatial differences in metal bioavailability when used to provide 2D measurements. In the present study DGT gels were deployed on root tips and cluster roots of white lupins grown in rhizotrons.

The HR-DGT showed metal mobilization only at the location of a cluster root of the plant grown in the un-amended (T1) treatment (Fig. 4). In the lime treatments, all analytes were below the detection limits of LA-ICP-MS analysis. A similar trend was observed in the data obtained with the $Ca(NO_3)_2$ extraction of rhizosphere soil (Table 3) considering Mn, Cu and Zn. Iron on the other hand, showed a different trend. Its concentration did not seem to be affected by lime addition observing the calcium nitrate extract, but could not be detected by HR-DGT analysis of the white lupin rhizospheres in T4 and T7. As previously mentioned, calcium nitrate extraction might not be the most appropriate method to determine Fe availability. This method relies on cation exchange, whereas normally Fe tends to form insoluble species that this extraction method is unlikely to measure; hence no significant changes could be observed in extractable Fe despite the big decrease in plant uptake.

The plant appears to have a highly localized means of mobilizing Fe (despite it is not appearing to be very extractable) as shown by the HR-DGT data, but only without lime. The extent of the exudation-induced rhizosphere effect depends in fact strongly on the soil buffering capacity. For instance, it has been shown that the extent of the acidification by chickpea decreases from several (15% $CaCO_3$) to almost zero mm (60% $CaCO_3$) with increasing concentrations of $CaCO_3$ in the soil (Neumann and Römheld, 2011). Yet, the extent of this effect is most likely related to the type of carbonate, *i.e.* active or total. Usually high lime concentrations are needed to neutralize the root activity, whereas in the present study low concentrations as 1.25% were sufficient to completely suppress the available fraction of Fe below the level where it could be observed by the DGT. However, we can hypothesize that the fine powdery laboratory grade lime used in this experiment can be considered almost 100% active and was able to react immediately with the soil, in contrast with common agricultural lime, which takes more time to react with the soil because constituted by particles with a larger size.

The DGT gel was deployed early in the plant's light-dark cycle to ensure that effects of one cycle of exudation by the roots of the white lupin on the rhizosphere soil were recorded. Previous studies showed that the release of citrate in white lupin follows a diurnal rhythm with a peak of exudation after 5 h of the onset of light (Tomasi et al., 2009). The observed co-mobilization of Cu, Zn and Pb with Fe at the location of the cluster root indicates that similar mechanisms are acting on these metals. Some authors reported that white lupin is able to increase the availability of Cu and Zn (Braum and Helmke, 1995; Dessureault-Rompré et al., 2006; Duffner et al., 2012; Martínez-Alcalá et al., 2010) through the action of root exudates in the rhizosphere. Braum and Helmke (1995) also showed that soybean intercropped with *L. albus* had greater uptake rates of Cu, Fe, and Zn when compared to soybean grown alone. Martínez-Alcalá et al. (2009) found an overall decrease in the EDTA-extractable fraction of Pb, Zn and Fe in the rhizosphere soil of *L. albus* L. They attributed this decrease to the precipitation of Fe into iron oxyhydroxides which also immobilized the Pb and Zn; however, it should be noted that the soils used for their study had a considerably higher pH (7.86) than those considered here, which would be expected to promote the precipitation of Fe into insoluble minerals and the co-precipitation of other trace elements. Furthermore, high-resolution analyses of plant-induced rhizosphere changes are more likely to provide a better representation of small-scale trace element mobilization at the plant-root interface than methods where bulk samples of rhizosphere soils are considered (Hinsinger et al., 2005).

A clear hotspot at the location of the selected cluster roots can be observed also for Mn (Fig. 4c). In fact the average flux of Mn across the area defined as the hotspot is ca. 75% higher compared to the entire ablation area confirming a higher mobilization of the element in this highly localized part of the white lupin rhizosphere. This observation indicates that highly localized exudation processes occurring in the rhizosphere of white lupin may be important factor in this plant's ability to mobilize this element (Dinkelaker et al., 1995; Martínez-Alcalá et al., 2009; Page et al., 2006).

While the ability of white lupin roots to mobilize trace elements nutrients can be beneficial for the plant, the root exudates may also mobilize toxic metals, such as Pb. Yet, Pb could only be detected by HR-DGT and was below the LOD both in lupin shoots and in the Ca $(NO_3)_2$-extractable rhizosphere soil solution most likely due to a dilution during the extraction. Previous studies have found that while the roots of white lupin can take up Pb, it is highly immobile within the plant (Kabata-Pendias, 2010; Martínez-Alcalá et al., 2009) and therefore plant uptake of this metal is unlikely to present a significant risk to human and animal health. However, the mobilization of other metals, that are relatively easily translocated from roots to shoots, such as Cd, Co, Mo and Se, could theoretically pose a threat when present in sufficient concentrations in soils; however, this is unlikely to be an issue in the soils considered here.

5. Conclusions

We found that lime treatment affected both the solubility of Ca $(NO_3)_2$-extractable elements and their plant uptake even though HR-DGT was not able to detect any mobilization when $CaCO_3$ was added to the soil at a rate $\geq$ 1.3 wt%. Yet, plants grown in the pot trials using lime treated soils showed that there was sufficient trace element uptake to avoid nutrient deficiency. While we could not detect nutrient mobilization at the lupin root structures HR-DGT in these cases, we cannot rule out the possibility that root exudates are still present but their ability to mobilize nutrients to a level where they can be detected by HR-DGT is buffered by the lime. Furthermore, lupin shoot elemental concentrations reflect the nutrient uptake through the whole root system whereas HR-DGT was applied only on selected cluster roots. Different areas of the rhizosphere or at the best the whole root system should be investigated by HR-DGT. Longer DGT deployment on root zones might provide further information to overcome the limitations of the method detection limit, especially under circumstances where the availability of nutrients by DGT is reduced by high levels of lime.

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