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Chemical and biological properties in the rhizosphere of *Lupinus albus* alter soil heavy metal fractionation

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ABSTRACT

To understand better the suitability of white lupin (*Lupinus albus* L.) for phytoremediation of heavy metal-contaminated soils, the effect of its roots on chemical and biological properties of the rhizosphere affecting soil metal fractionation was studied. Plants were cultivated in two similar soils, with high levels of Zn, Cd, Cu and Pb but differing pH values (4.2 and 6.8). In the rhizosphere of both soils, its roots induced increases in water-soluble carbon, which influenced the fractionation of heavy metals and ultimately their uptake by plant roots. In the rhizosphere of the acid soil, the concentrations of 0.1 M $CaCl_2$-extractable Mn, Zn and Cu were lower than in the bulk soil, possibly due to their increased retention on Fe (III) hydroxides/oxyhydroxides, while in the neutral soil only the Zn concentration was lower. Higher concentrations of heavy metals were found in plants growing on the acid soil, reflecting their greater availability in this soil. The restricted transfer of heavy metals to the shoot confirms the potential role of this species in the initial phytoimmobilisation of heavy metals, particularly in neutral-alkaline soils.

1. Introduction

After the spillage of mine tailings and acidic, heavy metal-rich water in Aznalcóllar (Seville, Spain) in 1998, a restoration programme was implemented to remediate the affected zone (4286 ha). Although the sludge, together with the top few centimetres of the soil, was mechanically removed, residual pollution remained afterwards in the soil (Bernal et al., 2007; Clemente et al., 2005), due to the high levels of some trace metals (Cd, 0.12–22.0 $\mu g\,g^{-1}$; Cu, 12.5–958 $\mu g\,g^{-1}$; Cr, 26.8–92.4 $\mu g\,g^{-1}$; Mn, 290–940 $\mu g\,g^{-1}$; Ni, 8.04–39.0 $\mu g\,g^{-1}$; Pb, 25.3–4969 $\mu g\,g^{-1}$; Zn, 56.8–5283 $\mu g\,g^{-1}$) and As (9.4–1684 $\mu g\,g^{-1}$) (López-Pamo et al., 1999). The soil background levels of the area were 3.03% Fe and ($\mu g\,g^{-1}$): As 31, Cu 36, Cr 74, Ni 22, Pb 47 and Zn 109 (López-Pamo et al., 1999).

Since the erosion of the zone and the wind transport of particles could increase the risk of pollution in the surrounding areas, action was taken to recover the affected soils that still needs further intervention. Remediation of these pluri-contaminated soils by phytoextraction is not feasible, since the interaction of the different metals regarding plant uptake leads to low metal extraction efficiency (Clemente et al., 2005). Phytostabilisation seems to be the most suitable phytoremediation technology for those soils (Clemente et al., 2006).

White lupin (*Lupinus albus* L.; Fabaceae) is an annual, N_2-fixing species tolerant of drought, heavy metals (Martínez-Alcalá et al., 2009; Pastor et al., 2003; Vázquez et al., 2006), high salinity, excess nitrate and sometimes excess calcium and soil acidity (Kerley, 2000; Vázquez et al., 2006). Under nutrient deficiency, white lupin plants can develop "proteoid roots" on which clusters of rootlets exude chelating agents (organic anions and enzymes such as phosphatase and probably phytase) and hydrogen ions, in order to improve the acquisition of P, Fe, Mn and Zn (Dinkelaker et al., 1989; Ryan et al., 2001; Vance et al., 2003). These factors, together with its ease of cultivation, make this species an excellent candidate for the initial phytoremediation of soils (Martínez-Alcalá et al., 2009; Pastor et al., 2003; Vázquez et al., 2006).

The different chemical and biological conditions of the soil–root interface (rhizosphere) with respect to the bulk soil can affect metal speciation in soil and therefore bioavailability (Bernal et al., 1994; Dessureault-Rompré et al., 2008; Martínez-Alcalá et al., 2009; Tao et al., 2003). Soil pH is a key factor for metal speciation in the soil. Soil pH can decrease in the rhizosphere of white lupin due to several factors: root exudation of organic anions with a concomitant release of H^+; respiration in neutral and alkaline soils, because the build-up of CO_2 can lead to the formation of carbonic acid in the rhizosphere; cation–anion exchange balance by roots; redox-coupled processes; environmental and nutritional constrains, because most plant species respond to P and Fe deficiency or heavy metal (especially Al) toxicity by enhanced H^+

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release (Hinsinger et al., 2003; Ryan et al., 2001). However, in metal-contaminated soils, it has been found that the rhizosphere pH can increase with respect to the bulk soil (Vázquez et al., 2006) or remain unchanged (Martínez-Alcalá et al., 2009), depending on the soil buffer capacity.

Several authors have reported that plant roots effect pH changes in the rhizosphere in order to buffer the effects of hostile surroundings: Loosemore et al. (2004) reported that in spite of the moderate level of Zn pollution and the bulk soil pH values, tobacco plants (*Nicotiana tabacum* L.) took up substantial amounts of Zn as a result of a root-induced pH decrease. However, Bravin et al. (2009) found that durum wheat (*Triticum turgidum durum* L.) induced a rhizosphere alkalinisation so strong that Cu bioavailability was insensitive to the large range of bulk soil pH (4.8–7.5) to which plants were exposed initially. Chaignon et al. (2009), studying Cu availability in Cu-polluted soil where rape plants (*Bassica napus* L.) were grown, showed that this species has the capacity to adapt to adverse soil conditions by inducing significant rhizosphere pH changes: alkalinisation at pH <4.8 and acidification at pH >4.8.

Under nutrient deficiency, white lupin develops proteoid roots that exude organic anions with a concomitant H^+ release, playing an important role in the mobilisation and uptake of nutrients (P, Fe) (Dinkelaker et al., 1989; Ryan et al., 2001; Vance et al., 2003). However, very different levels of exudation of organic anions have been found, depending on the species, environmental constraints, plant age and, above all, nutritional status (Jones, 1998). The release of organic anions must produce a cation–anion imbalance that should be equilibrated by an equivalent influx of OH^- or efflux of H^+ (Dinkelaker et al., 1989). But if such organic anions are protonated, when the external pH is lower than the cytosolic pH (Jones and Darrah, 1994), or are decomposed (Yan et al., 1996), they might produce rhizosphere alkalinisation (Hinsinger et al., 2003). However, Dessureault-Rompré et al. (2008), studying the metal solubility and speciation in the rhizosphere of white lupin, reported than pH did not change even when citrate exudation was maximal.

Nitrogen plays also an important role in the cation–anion balance. It can be taken up as a cation (ammonium) that will counter-balance the excess of positive charges by releasing equivalent amounts of H^+ in the rhizosphere (decreasing rhizosphere pH); as an anion (nitrate) that will counter-balance the excess of negative charges by releasing equivalent amounts of OH^- or HCO_3^- into the rhizosphere (increasing rhizosphere pH); or even as an uncharged species (gaseous N_2) in N_2-fixing species such as white lupin.

Roots could also produce redox changes in the rhizosphere that are associated with the Fe, Mn, N and S dynamics and are intimately coupled with pH changes. Often, electrons are supplied by the oxidation of organic matter and microbial or root respiration. Besides, reduction processes could occur at the root surface, accompanied by rhizosphere acidification (Hinsinger et al., 2003).

In previous work carried out on a metal-contaminated calcareous soil, the more-oxidant conditions in the rhizosphere, with respect to the bulk soil, and organic compounds exuded by roots of white lupin were the main factors responsible for decreasing Zn and Mn solubility in the rhizosphere (Martínez-Alcalá et al., 2009). The aim of this work was to study the concomitant changes in the chemical characteristics and the fractionation of heavy metals in the rhizosphere of white lupin in two non-calcareous, pluri-contaminated soils from the Aznalcóllar area, one of them being acidic and the other of neutral pH. This will contribute to our assessment of the effectiveness of this species for the phytostabilisation of metal-contaminated soils.

2. Materials and methods

2.1. Soil characteristics

Two soils were selected from the same area affected by a pyritic mine sludge, near Sanlúcar la Mayor (longitude W 06°13′00″, latitude N 37°26′21″), 10 km from the Aznalcóllar mine (Seville, Spain). The soils were collected from the top 20 cm, air-dried for 5–6 days and sieved to <2 mm prior to analysis (Section 2.3). The soils were non-calcareous loams with 19.7% clay, 34.3% silt and 46% sand, classified as Typic Xerofluvent (American Soil Taxonomy); the main minerals were quartz (72%), feldespars (albite, 13.7%), phyllosilicates (illite, 4.5%), gypsum (4.1%), jarosite (2.4%), calcite (1.6%) and siderite (1.6%). Their organic matter content was 1.9% and total-N was 1.35 g kg^{-1}. One of them is acid (pH 4.2) while the other is neutral (pH 6.8), having similar total metal concentrations (Table 1). Both soils had total Zn and Cd concentrations above the EU limits for agricultural soils (Council of the European Communities, 1986); Cu and Pb also exceeded these limits in the acid soil.

2.2. Experimental design

For a proper separation of the rhizosphere from the bulk soil, the rhizopots were those used by Martínez-Alcalá et al. (2009), based on the design of Tao et al. (2003). They consist of three compartments. In the first stage, the upper compartment held the soil (170 g) where plant roots grew, and the lower part was filled with the bulk soil (100–120 g), where roots were unable to penetrate as the two compartments were separated by a nylon monofilament gauze with a 45-μm pore size (Sefar Inc., Switzerland). In the second stage, a 2 mm-thick layer of soil was placed between the compartments in contact with the roots; this was considered the rhizosphere (Tao et al., 2003).

Seeds of white lupin (cv. Marta) were surface-sterilised with 10% HClO for 30 min, washed three times with distilled water and then germinated in a plastic container, on filter paper moistened with 0.5 mM $CaSO_4$, in an incubator at 28 °C for 3 days. Two germinated seeds were planted in each rhizopot and were maintained in a growth chamber with a light/dark regime of 14/10 h, temperature of 23/18 °C (day/night) and relative humidity of 50/70%. Plants were watered with deionised water from the base of the rhizopots, using a dripping tray. The soils were not fertilised.

For each soil, three replicated samples were run, made from a total of 60 rhizopots distributed in three trays: 20 rhizopots were pooled to give one replicate. After 57 days of growth in the rhizopots, when a complete development of the root system filled the bottom of the upper part, the 2 mm layer of soil was added, and 14 days later (71 days of growth), the rhizosphere was sampled. The first 30 mm of the bottom part of the rhizopots were removed and the rest of the soil was considered the bulk soil. Plants were harvested, separating roots from the shoot. Shoots were washed with distilled water and roots were first washed with tap water, then with distilled water under sonication (7.5 min) to remove soil particles and finally with 0.1 mM $SrCl_2$ for 30 s, to remove adsorbed metals on the root surface. The used $SrCl_2$ solution was analysed, Ca and Mg were the main elements removed (data not shown), with equivalent average values of Cu, Pb and Zn of 2.4, 0.5 and 7.8 μg g^{-1} of root dry weight, respectively. Fresh and dry (70 °C) weights of roots and shoots were determined before being ground for analysis. Only roots growing at the bottom of the upper part of the rhizopot, in contact with the rhizosphere, were used for chemical analysis, since they were easy to clean as

Table 1
Soil characteristics.

Parameters	Acid soil	Neutral soil
pH	4.20	6.78
EC (dS m^{-1})	2.71	2.22
Buffer capacity (me kg^{-1} pH^{-1})	49.6	63.8
NO_3^--N (μg g^{-1})	15.4	4.9
NH_4^+-N (μg g^{-1})	16.6	10.5
Available-P (μg g^{-1})	34.9	38.3
Exchangeable-Al (μg g^{-1})	120	<0.5
Pseudo-total Fe (g kg^{-1})	44.4	40.2
Pseudo-total Mn (μg g^{-1})	798	906
Pseudo-total Cu (μg g^{-1})	135	122
Pseudo-total Zn (μg g^{-1})	364	400
Pseudo-total Ni (μg g^{-1})	19.0	19.9
Pseudo-total Cd (μg g^{-1})	3.80	4.20
Pseudo-total Pb (μg g^{-1})	291	210
Pseudo-total As (μg g^{-1})	104	103
Available-As (μg g^{-1})	5.16	7.45

EC: Electrical conductivity.

very few soil particles adhered to them. These roots, which had a considerable biomass, can be considered representative of the whole root system. In all cases, 20 sub-samples of soil or plant tissue per tray (2 plants per pot) were combined to give $n=3$.

2.3. Analytical methods

Plant and soil pseudo-total heavy metals (Cu, Fe, Mn, Pb, Zn) were determined by flame atomic absorption spectrometry (AAS) in a UNICAM 969 atomic absorption spectrometer (Thermo Elemental, Cambridge, UK), after nitric–perchloric acid (2:1) digestion. Soil reference material was used to test the analytical procedure (SRM 2711 Montana Soil), the analytical recovery was 91% Cd; 88% Mn; 87% Fe; 86% Cu; 95% Zn; 96% Pb.

Sequential extraction of soil metals had the following steps (McGrath and Cegarra, 1992): 0.1 M $CaCl_2$ (1:10 w/v), metals in soil solution and in exchangeable forms; 0.5 M NaOH (1:10, w/v) followed by aqua regia digestion of the extract, metals associated with organic matter (OM); 0.05 M Na_2H_2EDTA (1:10, w/v), metals which can be extracted only with a strong chelator; acid digestion with aqua regia, residual metals (Table 2). The results were in good agreement with the pseudo-total concentration (Table 1 and Fig. 1). All metal concentrations were adjusted to values for oven-dried (12 h at 105 °C) soil. Total-As was measured by ICP-MS after digestion with aqua regia. A sequential extraction procedure for As could not be performed due to the limited amount of rhizosphere available. Then, available As in soil was determined by ICP-MS after a 0.5 M $NaHCO_3$ extraction (1:10, w/v) (Johnston and Barnard, 1979). Exchangeable Al was extracted with 1 M KCl (1:10, w/v) (Barnhisel and Bertsch, 1982) and determined by ICP-MS. Soil microbial biomass-C (B_C) (fumigation-extraction procedure, Vance et al., 1987) and water-soluble organic carbon (C_W), after a 1:10 water extraction (w/v) for 2 h, were determined in a TOC/Skalar analyser. Biomass-N (B_{NIN}), calculated from the difference between ninhydrin-reactive N in fumigated and non-fumigated soil extracts, was also determined (Joergensen and Brookes, 1990). Soil available-P was determined colorimetrically after a 0.5 M $NaHCO_3$ extraction (1:10, w/v). Total P in plants was determined colorimetrically as molybdovanadate phosphoric acid, after nitric–perchloric acid digestion (210 °C). Ammonium was analysed by the salicylate method after 2 M KCl extraction (1:5 w/v) using dichloroisocyanurate sodium as chlorine source (Kempers and Zweers, 1986). Nitrate was measured in 1:5 (w/v) soil:water extracts by nitrate-ion selective electrode (EPA, 2007). Inorganic anions (Cl^-, NO_3^-, $H_2PO_4^-$, SO_4^{2-}) were determined in the plants by ion chromatography after water extraction (1:200, w/v). The pH and redox potential (Eh) were determined in saturated soil paste: Eh was measured with a combined platinum electrode with an Ag/AgCl reference (Crison, 52-65), and the validity of the method was tested with a redox solution of 220 mV (Crison, 94-00). Soil particle size was assessed by sieving and sedimentation, using the hydrometer method (Gee and Bauder, 1986). The mineralogical composition was determined by XRD. Chemical analyses were performed at least in duplicate.

To assess pH changes in the different root zones after 71 days of growth, agar film with a pH indicator was placed on the intact plant roots. Agar (0.75%) was prepared in a nutrient solution containing only macronutrients, but without a N-source (0.2 mM KH_2PO_4, 0.4 mM $CaCl_2$, 0.4 mM K_2SO_4, 0.1 mM $MgSO_4$), and bromocresol purple indicator (0.006%) and was adjusted with NaOH to pH 6.0 (red) and 7.0 (purple) for testing pH in the rhizosphere of the acid and neutral soils, respectively.

No study was performed involving humans or experimental animals, in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

Table 2
Sequential extraction of heavy metals in the soils (μg g^{-1}).

	Extractants	Acid soil	Neutral soil
Fe	$CaCl_2$	< 0.5	< 0.5
	NaOH	139 ± 6.5	97 ± 9.5
	EDTA	2484 ± 6.5	1436 ± 17
	Residual fraction	41784 ± 458	38731 ± 1374
Cu	$CaCl_2$	4.75 ± 0.02	0.15 ± 0.01
	NaOH	27.9 ± 0.12	21.6 ± 0.23
	EDTA	26.5 ± 0.15	30.4 ± 0.40
	Residual fraction	75 ± 1.5	70 ± 0.51
Zn	$CaCl_2$	138 ± 0.56	1.91 ± 0.06
	NaOH	< 0.5	12.7 ± 0.06
	EDTA	33.5 ± 0.05	91.4 ± 2.3
	Residual fraction	192 ± 2.0	294 ± 5
Pb	$CaCl_2$	0.15 ± 0.05	0.05 ± 0.05
	NaOH	1.24 ± 0.07	0.86 ± 0.05
	EDTA	40.9 ± 0.43	24.4 ± 0.04
	Residual fraction	249 ± 22	185 ± 15
Mn	$CaCl_2$	230 ± 9.1	24.5 ± 0.6
	NaOH	< 0.5	1.1 ± 0.3
	EDTA	74 ± 6.2	255 ± 13
	Residual fraction	494 ± 15	626 ± 15

2.4. Statistical analysis

All data ($n=3$) were analysed by one-way ANOVA using SPSS version 14.0 software. Differences between means were determined using Tukey's test at a probability level of $P<0.05$. Although Principal Component Analysis would be an interesting tool to define the factors affecting metal fractionation, the data of the present experiment (4 "treatments": 2 rhizosphere and 2 bulk soils) for each parameter were not enough to obtain reliable results.

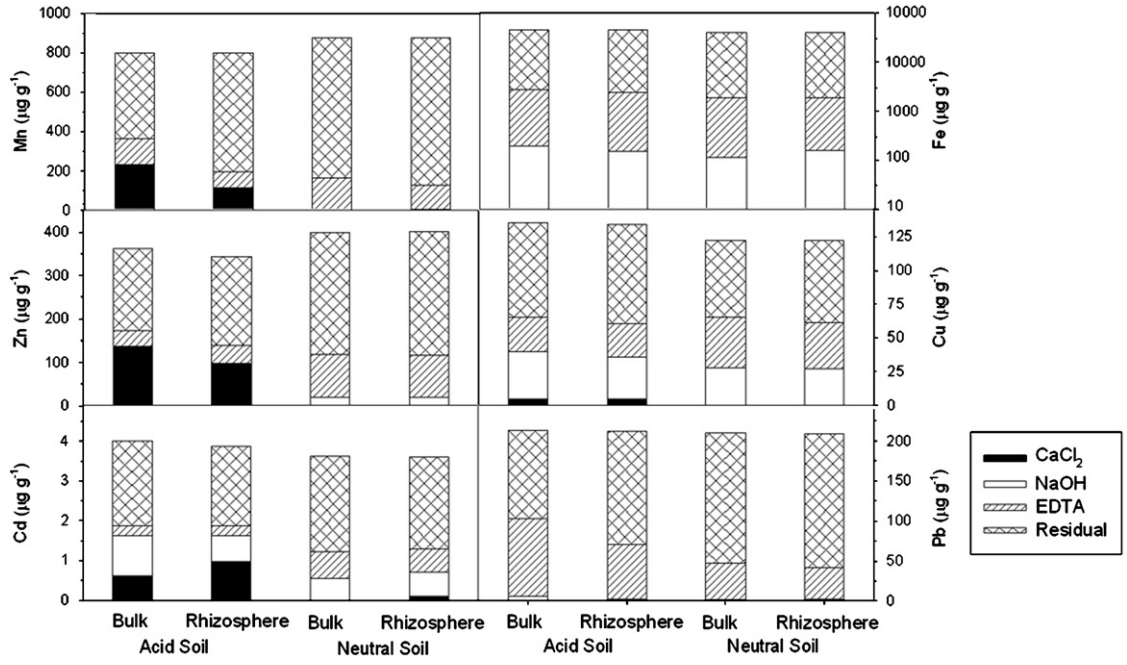

Fig. 1. Heavy metal fractionation by sequential extraction of Mn, Fe, Zn, Cu, Cd and Pb (μg g^{-1}), in the bulk soil and rhizosphere of white lupin grown in a neutral and an acid soil for 74 days ($n=3$).

3. Results

3.1. Chemical and biological changes in the rhizosphere

Values of pH in the rhizosphere of white lupin were decreased significantly ($P<0.05$) with respect to the bulk soil in the acid soil (Table 3). The redox potential (Table 3) increased in the rhizosphere with respect to the bulk soil in both soils, but mainly in the acid soil, indicating more oxidant conditions in the rhizosphere. This lower redox potential in the bulk soil with respect to the rhizosphere could be due also to the design of the irrigation system, where the water goes from the bottom to the upper part of the rhizopot.

The C_W increased in the rhizosphere with respect to the bulk soil in both soils (Table 3). The greatest change in the microbial biomass occurred in the acid soil; here the microbial biomass-C and N in the rhizosphere were increased with respect to the bulk soil. However, in the neutral soil the values of microbial B_C were greater in the bulk soil than in the rhizosphere, but the differences found for the B_{NIN}, measured as ninhydrin-reactive N, were not statistically significant.

3.2. Heavy metal fractionation in the rhizosphere

With respect to the metal fractionation (Fig. 1), the highest concentrations of 0.1 M $CaCl_2$-extractable Mn, Zn and Cu were found in the acid bulk soil (230 ± 9, 138 ± 1 and 4.8 ± 0.1 $\mu g\,g^{-1}$, respectively) and the values decreased significantly in the rhizosphere (115 ± 8, 97 ± 9 and 4.5 ± 0.1 $\mu g\,g^{-1}$ for Mn, Zn and Cu, respectively). The neutral soil had very low concentrations of $CaCl_2$-extractable Mn, Zn and Cu, with significant differences between the bulk soil and rhizosphere only for Zn (0.94 ± 0.08 and 1.36 ± 0.07 $\mu g\,g^{-1}$ in the bulk soil and the rhizosphere, respectively). The Pb concentration in this fraction was very low in both soils (<0.2 $\mu g\,g^{-1}$).

The main metals associated with the organic matter (NaOH-extractable) were Fe and Cu, with significantly-higher concentrations in the acid bulk soil (190 ± 3 and 35.4 ± 0.6 $\mu g\,g^{-1}$, respectively) than in the rhizosphere (152 ± 4 and 31.6 ± 0.3 $\mu g\,g^{-1}$) without significant differences in the neutral soil. Regarding the NaOH-extractable Zn, the neutral soil had similar concentrations in the bulk soil and in the rhizosphere, with a mean value of 18.8 $\mu g\,g^{-1}$, whereas in the acid soil very low values were found for Zn in both the rhizosphere and bulk soil (<0.5 $\mu g\,g^{-1}$). In both soils, the EDTA-extractable concentrations of all metals were decreased in the rhizosphere with respect to the bulk soil, with statistically-significant differences in the acid soil for Fe and Pb, and in the neutral soil for Pb and Mn. For the residual fraction, only significantly-higher concentrations of Mn, Zn, Cu and Pb were found in the rhizosphere with respect to the bulk acidic soil.

Table 3
Properties of the rhizosphere and bulk soils, in the acid and neutral soils ($n=3$): pH, redox potential (Eh), water soluble organic-C (C_W), biomass-C (B_C) and biomass ninhidrin reactive-N (B_{NIN}).

	pH	Eh (mV)	C_W ($\mu g\,C\,g^{-1}$)	B_C ($\mu g\,C\,g^{-1}$)	B_{NIN} ($\mu g\,N\,g^{-1}$)
Acid soil					
Bulk	4.2 ± 0.0	232 ± 9	5.6 ± 0.5	56.5 ± 4.4	2.3 ± 0.9
Rhizosphere	3.9 ± 0.0	299 ± 1	30.5 ± 7.3	96.4 ± 5.9	9.1 ± 0.2
ANOVA	***	*	**	**	**
Neutral soil					
Bulk	7.1 ± 0.0	275 ± 3	19.5 ± 0.4	166 ± 6	11.4 ± 1.1
Rhizosphere	7.0 ± 0.0	294 ± 3	32.9 ± 1.4	130 ± 4	9.4 ± 0.2
ANOVA	N.S.	**	**	**	N.S.

Measurements were made on soil sampled after growth of white lupin for 71 days.
***, **, *: significant at $P<0.001$, 0.01, 0.05, respectively. N.S.: not significant.

3.3. Plant growth and nutritional status

Plant growth, evaluated as dry mass, was greater in the neutral soil than in the acid soil, with values of 0.61 ± 0.01 g and 0.38 ± 0.04 g DM per pot for shoots and 0.47 ± 0.04 and 0.11 ± 0.01 g for roots, for the neutral and the acid soil, respectively, indicating the greater suitability of the neutral soil for white lupin. In both soils cluster roots were observed, while root nodules were observed only in the neutral soil.

There were higher shoot and root concentrations of N and P in the acid soil but greater Mg and Na concentrations in the plants for the neutral soil (Table 4). Higher root concentrations of K occurred in the acid soil. The root NO_3^- concentration in the neutral soil (28.5 $\mu mol\,g^{-1}$) was lower than in the acid soil (39.6 $\mu mol\,g^{-1}$), while the opposite happened in the aerial parts (7.26 and 15.3 $\mu mol\,g^{-1}$ on the acid and neutral soils, respectively).

3.4. Plant heavy metal concentrations

Heavy metal accumulation by *L. albus* (Fig. 2) largely reflected the solubility of the metals in the soil, with lower values in the shoot than in the roots (except for Mn), the usual behaviour of an excluder plant. The Fe concentration (Fig. 2a) was more elevated in the roots, in both soils, with a higher value in the acid soil (3503 ± 169 $\mu g\,g^{-1}$) than in the neutral soil (709 ± 64 $\mu g\,g^{-1}$), while the shoot concentration was 120 ± 12 $\mu g\,g^{-1}$ for the acid soil and 107 ± 7 $\mu g\,g^{-1}$ for the neutral soil. Considering the roots, the highest Zn concentration (Fig. 2b) was found in the acid soil (4117 ± 440 $\mu g\,g^{-1}$), while roots in the neutral soil had 103 ± 1 $\mu g\,g^{-1}$. The shoot Zn concentration was also higher (1014 ± 65 $\mu g\,g^{-1}$) for the acid soil than for the neutral soil (54.3 ± 1.2 $\mu g\,g^{-1}$). The highest Cu and Cd concentrations (Figs. 2b and c) were also for the roots of the plants grown in the acid soil (254 ± 2 $\mu g\,g^{-1}$ for Cu and 30.1 ± 1.6 $\mu g\,g^{-1}$ for Cd), which also gave higher shoot concentrations than the neutral soil. The roots largely retained the Pb (Fig. 2c), its highest concentration (24.9 ± 6.6 $\mu g\,g^{-1}$) being in the plants from the acid soil. Very high, possibly-phytotoxic concentrations of Al were found in roots and shoots of plants grown in the acid soil (Fig. 2a). The concentrations of As in plants were low (Fig. 2c) but with significant differences: the concentrations in shoots and roots of the plants grown in the acid soil were 1.21 ± 0.14 and 11.6 ± 1.5 $\mu g\,g^{-1}$, respectively, while in the neutral soil they were 0.59 ± 0.04 and 5.84 ± 0.48 $\mu g\,g^{-1}$, respectively.

Table 4
Nutrient concentrations ($mg\,g^{-1}$ dry weight) in the shoot and roots of white lupin grown for 71 days in the acid and neutral soils ($n=3$).

	N	P	K	Ca	Mg	Na
Shoot						
Acid soil	47.8 ± 1.2	2.1 ± 0.0	18.6 ± 0.7	3.8 ± 0.1	1.8 ± 0.2	1.2 ± 0.2
Neutral Soil	18.9 ± 0.1	1.9 ± 0.1	23.6 ± 0.9	3.5 ± 0.1	3.4 ± 0.1	4.7 ± 0.2
ANOVA	***	*	*	NS	***	***
Roots						
Acid soil	$36.3\pm1.6.$	4.0 ± 0.2	24.7 ± 2.1	7.6 ± 0.2	0.8 ± 0.0	$4.1\pm0.4.$
Neutral soil	18.9 ± 0.1	0.8 ± 0.1	2.73 ± 0.2	7.0 ± 0.1	4.6 ± 0.2	4.6 ± 0.3
ANOVA	***	***	***	N.S.	***	N.S.

***, **, *: significant at $P<0.001$, 0.01, 0.05, respectively. N.S.: not significant.

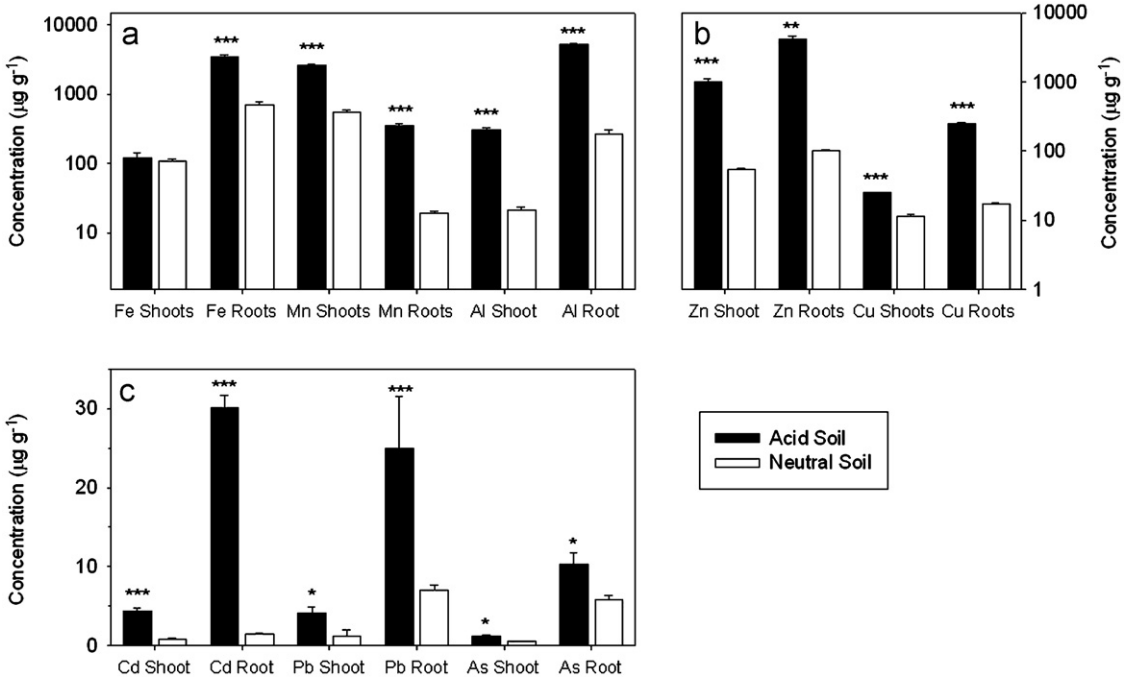

Fig. 2. Heavy metal concentrations ($\mu g\ g^{-1}$) in the shoot and roots of white lupin after growth for 71 days in the acid and neutral soils: (a) Fe, Mn and Al, (b) Zn and Cu, (c) Cd, Pb and As (n=3). In the neutral soil, the shoot concentrations of Cd and As were below the detection limit (0.5 $\mu g\ g^{-1}$).

Manganese was the only heavy metal that accumulated more in the aerial parts of the plant (Fig. 2a), with higher concentrations in the plants grown in the acid soil, $2648 \pm 17\ \mu g\ g^{-1}$ in the shoot and $352 \pm 18\ \mu g\ g^{-1}$ in roots, than in the shoot ($552 \pm 23\ \mu g\ g^{-1}$) and roots ($19.2 \pm 1.1\ \mu g\ g^{-1}$) of plants in the neutral soil.

Taking into account the dry weight of the plants and the concentrations of inorganic cations and anions in the shoots and roots, the cation/anion balance was calculated. For this, cationic macronutrients (Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}), heavy metals (Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+} and Al^{3+}) and anions (NO_3^-, $H_2PO_4^-$, Cl^-, SO_4^{2-}) were considered. The plants grown in the acid soil did not show an excess of inorganic cations (124 ± 8.6 me/100 g) over anions (128 ± 0.5 me/100 g). The concentrations of cations in the plants from the neutral soil were lower than those of anions: 119 ± 2.4 me/100g and 286 ± 7.4 me/100g respectively.

4. Discussion

4.1. Chemical changes in the rhizosphere affecting heavy metal fractionation.

Soil–plant interactions in the rhizosphere cause changes in the chemical soil properties which determine the metal fractionation and therefore bioavailability. Soil pH is the main factor controlling metal solubility and pH changes in the rhizosphere can be due to cation–anion balance, organic anion release, root exudation and respiration and redox-coupled processes (Hinsinger et al., 2003). In the neutral soil, no significant differences were found for pH between the rhizosphere and the bulk soil, while in the acid soil the rhizosphere pH showed a decrease.

The cation–anion balance of plants in the neutral soil indicates that the cation concentration was substantially lower than the inorganic anion concentration so the roots in this soil must have released OH^- or HCO_3^- to compensate the charges (Dinkelaker et al., 1989), while in the acid soil the amount of cations and anions were similar. According to Hinsinger et al. (2003), the nitrogen source also plays an important role in rhizosphere acidification/alkalinisation and thus can influence heavy metal solubility. The soils were not fertilised (Table 1). The presence of root nodules in the neutral soil indicates that N_2-fixation may have occurred. So, the increase of pH in the rhizosphere caused by NO_3^- uptake may have been compensated by the decrease that the N_2-fixation process can cause (Mengel and Kirby, 2001). In plants grown in the acid soil, nodules were not found, and the amount of NO_3^- in plants was higher than in the neutral soil, so NO_3^- might have been the main N-source in this soil. Thus, the N-source utilised by the plants does not seem to be the main factor responsible for the observed differences between the soils regarding effects on rhizosphere pH.

In the present experiment, a local acidification was observed when agar film with the pH indicator bromocresol purple was placed on the intact plant roots (Fig. 3). The decrease of soil pH in the neutral soil was only detected around cluster roots (yellow colour in Fig. 3), while other parts of the roots showed a slight increase in pH (>7, purple marks in Fig. 3), so when rhizosphere soil was collected as a whole, no change in pH was detected in the neutral soil. Organic anions were not measured, but it is assumed that white lupin exudates organic compounds and these may lead to chemical changes in the rhizosphere that affect heavy metal fractionation. Exudation of citric acid by cluster roots has been demonstrated to occur in order to mobilise phosphate under P deficiency (Jones and Darrah, 1994). The available P was similar in both soils of the current work, and should not have been limiting to plant growth (Mengel and Kirby, 2001). However, Li et al. (2008) concluded that critical levels of shoot P concentration that governed cluster-root formation and citrate exudation varied from 2 to 3 mg g^{-1} DW, and that the proportion of cluster roots was negatively and exponentially correlated with shoot P concentration. This indicates that citrate exudation by cluster roots was likely in both soils of the current work, but mainly in the acid soil due to the lower shoot P concentration, and proton

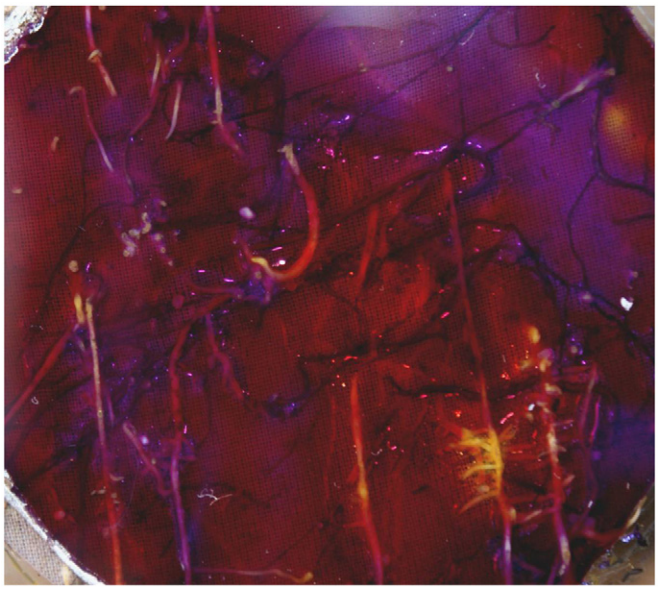

Fig. 3. Distribution of rhizosphere soil pH in the different root zones of white lupin growing in the neutral soil, using agar film with the pH indicator bromocresol purple (0.006%) placed on the intact plant roots. The yellow colour shows soil pH < 5, only detected around cluster roots, while purple marks indicate pH > 7 around other parts of the roots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

exudation seems to be concomitant with citrate exudation (Dinkelaker et al., 1989).

The buffer capacity of the soil is an important parameter controlling pH. In the present experiment, an acid–basic titration curve was obtained from this soil (Hartikainen, 1986). The buffer capacity in the acid range (pH 5.5–3.0) was lower (49.6 me kg^{-1} per pH unit) than near neutrality at pH 6.5–7.5 (63.8 me kg^{-1} per pH unit), corresponding to the neutral soil (Table 1). Thus, the acid soil could be subject to greater rhizosphere pH changes than the neutral soil.

The Eh is considered also a key factor controlling metal mobility in the rhizosphere (Kabata-Pendias, 2001). In the rhizosphere of both soils the Eh was higher than in the bulk soil, the differences being greater in the acid soil. This could have been due to the fact that watering occurred at the bottom of the rhizopot. Usually, heavy metals are at their most mobile at the lower range of pH and at more highly-oxidised states. Only metals which can exist in the soil at different oxidation stages can be affected by the soil redox conditions. In most redox mineral transformations, both Eh and pH play important roles in metal solubility and their effects cannot be separated. The pH–Eh (pe) diagrams of the soil system (Chuang et al., 1996 and Lindsay, 1979) show that the critical pe for reduction reactions depends on the pH, its value decreasing as pH increases. According to this, in the acid soil most Mn should be as Mn (II), and some Fe (III) can be reduced to Fe (II). In the neutral soil Fe should be in the oxidised state while Mn (II) would be dominant over Mn (IV). Copper will be fully-oxidised in both soils. In the neutral soil, the higher Eh values in the rhizosphere, although significant, should not alter the oxidation state of Mn or Fe (pe values 4.65 and 4.97 in bulk soil and rhizosphere, respectively). But the more-highly-oxidised conditions in the rhizosphere of the acid soil (pe 3.92 and 5.05 in the bulk soil and rhizosphere, respectively) could maintain Fe as Fe (III) to a greater extent in the rhizosphere, helping to retain other heavy metals (such as Zn, Cu and Pb) on Fe (III) oxide/hydroxides, thus decreasing their solubility in comparison with the bulk soil. Another element affected by the redox conditions is As. The Eh values of the neutral soil indicate that As was mainly in the oxidised form As (V) in both rhizosphere and bulk soil. In the acid soil, the dominant form should be As (III), which is more toxic and more soluble than As (V).

Our results indicate stronger effects of root exudates in the acid soil (increased C_W, B_C and B_{NIN}) than in the neutral soil (increases in C_W), and thus a greater increase in the microbial biomass in the rhizosphere of the acid soil, this could be quite positive for the remediation of this soil. The release of various forms of organic-C in the rhizosphere (mucilage, free exudates, sloughed-off cells) makes up an important component in the immobilisation of heavy metals. Controversy exists about the effects of root exudates on heavy metal solubility. Dessureault-Rompré et al. (2008) revealed that citrate exudation is concentrated in the cluster roots, that Al, Cu and Zn in the soil solution increased sharply during the exudative burst of citrate and decreased again rapidly after exudation had ceased, and also that mobilisation of soil organic matter by cluster root exudates could solubilise (in a short-lived manner) Cu and Pb as organo-metal complexes (which would be 0.1 M $CaCl_2$-extractable). Degryse et al. (2008) found that root exudates of dicotyledonous plants are able to mobilise Cu and Zn, and plants appear to respond to Zn deficiency by producing root exudates with higher metal affinity. In the neutral soil of present experiment, release of organic compounds from white lupin roots led a mobilisation of Cu and Zn for plant uptake, as low concentrations were found in soluble and exchangeable forms (0.1 M $CaCl_2$-extractable) in the soil. But in the acid soil, soluble levels of Cu and especially Zn and Mn were high and in excess of the concentrations required for plant nutrition. Consideration of the weight of soil in each compartment, the 0.1 M $CaCl_2$-extractable concentrations and the plant uptake showed that the fall in the concentrations of Cu, Mn and Zn in this rhizosphere fraction could not have been due only to plant uptake; this indicates that they were immobilised in the rhizosphere, favouring their precipitation. Cattani et al. (2006) found contrasting effects of soluble organic C with respect to Cu in different soils, depending on the efficiency of uptake processes under deficiency or excess, as excess Cu can be complexed by soluble organic C rendering it hardly-available. Bravin et al., (2009) reported that the free Cu^{2+} concentration decreased with increasing DOC concentration, suggesting that roots could exude Cu-chelating organic compounds that enhance both desorption of Cu from the soil solid phase and complexation of Cu^{2+} in the soil solution.

In the acid soil, the NaOH-extractable concentrations of Cu, Mn and Zn (OM-linked) did not increase in the rhizosphere, indicating that these elements were retained in the hardly-available forms of the residual fraction. The reaction of organic anions with metals in soils depends not only on their complexation ability but also on their sorption/desorption reactions and their microbial degradability. The higher microbial biomass found in the rhizosphere of the acid soil, with respect to the bulk soil, may enhance the degradation of the organic compounds exuded by roots, fixation mechanisms in the soil being more relevant for metal immobilisation. According to Kalbitz et al. (2000), polyvalent cations, including heavy metals, can link negatively-charged functional groups of organic molecules and reduce their solubility by flocculation or by binding them to negatively-charged binding sites.

Aluminium toxicity frequently occurs in plants growing on acid soils (pH < 5) and can be ameliorated in the rhizosphere via interaction with organic anions exuded from the roots (Kochian et al., 2002). There are common characteristics between efflux of organic acids (citrate, malate, oxalate) stimulated by Fe deficiency, P deficiency and Al toxicity (Barceló and Poschenrieder, 2002).

4.2. Heavy metal accumulation in white lupin

The concentrations of Zn, Cu, Cd, Al and As were higher in plants from the acid soil than from the neutral soil, reflecting their greater bioavailability in the former. For plants grown in the acid soil, the values for root Cu and Pb and shoot and root Cd can be considered excessive or toxic (Kabata-Pendias, 2001). Regarding Zn, for plants growing in the acid soil, the root and shoot concentrations were almost certainly toxic, according to the results of Pastor et al. (2003) and Castaldi et al. (2005), whereas the values in the plants grown on the neutral soil can be considered as sufficient for growth (40–70 $\mu g\, g^{-1}$; Kerley, 2000; Pastor et al., 2003).

White lupin plants accumulated Zn, Cu, Cd, Pb, Al and As in their roots, in agreement with previous work (Castaldi et al., 2005; Martínez-Alcalá et al., 2009; Zornoza et al., 2002), due to accumulation within root cell and/or at the cell wall/plasma membrane (Kopittke et al., 2007). The washing procedure was designed to remove apoplastic/free-space heavy metals, so it is possible that heavy metals precipitated at the root cell wall/membrane (for example Pb phosphate) remained. In white lupin, it has been shown that As and Cd are predominantly retained in roots and root nodules (Vázquez et al., 2006), Cd being detoxified principally via binding to the cell wall (Vázquez et al., 2009). There did not seem to be Fe deficiency or toxicity in the plants according to concentrations found previously (in shoots 80–150 $\mu g\, g^{-1}$ and 80–350 $\mu g\, g^{-1}$, Kerley, 2000; Shane et al., 2008, respectively). In legume shoot material, the Al concentration varies from 85 to 3470 ppm on a dry weight basis (Kabata-Pendias, 2001). So, the current values indicate toxicity in the roots and shoots.

White lupin is considered to be a Mn accumulator, and can accumulate high concentrations in the leaves (Braum and Helmke 1995; Kerley, 2000) and shoots (up to 6100 $\mu g\, g^{-1}$ dry weight; Kerley, 2000) without exhibiting any symptoms of Mn toxicity. The concentrations of Mn in the plants of the present experiment, although higher in shoots than in roots, did not reach extremely high values (Dinkelaker et al., 1989; Martínez-Alcalá et al., 2009).

4.3. Macronutrient status of the white lupin plants

Rhizobial infection was not observed in the acid soil, probably due to the low pH or heavy metal toxicity (Kabata-Pendias, 2001; Pastor et al., 2003); so, N acquisition would have occurred mainly via uptake of nitrate or ammonium (Mengel and Kirby, 2001), and the tissue concentrations of N can be considered adequate for white lupin (Braum and Helmke, 1995; Kerley, 2000; Pastor et al., 2003; Sas et al., 2002). The lower tissue N levels on the neutral soil, where nodules were observed, may reflect inhibition of nodule functioning by heavy metals (Carpena et al., 2003; Pastor et al., 2003). The shoot concentrations of Ca and Mg were similar to or greater than those that were found previously for white lupin (Kerley, 2000; Sas et al., 2002) and are sufficient for growth. Shoot K levels were in the range of sufficiency for white lupin (Kerley, 2000).

Despite the chemical similarity of As and P, there was no (inverse) relationship between tissue As concentrations and the available P in the soil or tissue P. In plants, Al toxicity is often manifested as shoot P deficiency, due to precipitation of P in the roots (Simon et al., 1994). In the current work, an elevated concentration of P occurred in the roots of the acid soil (much greater Al solubility) but this had no effect on the shoot P. For plants growing on the acid soil, the lower root concentrations of Mg and the lower shoot concentrations of Mg and K could have been due to excess Al in the soil solution (Wheeler, 1995; Kabata-Pendias, 2001).

5. Conclusions

The mechanism by which white lupin tolerates soil heavy metals seems to involve changes in the chemical environment of the rhizosphere, particularly increased water-soluble organic-C, and a reduction in the solubility and therefore bioavailability of the metals, probably via effects on soil Eh. Although its poorer growth and greater shoot accumulation of heavy metals in the acid soil indicate that this species is more appropriate for neutral-alkaline soils, in which the plants accumulate the contaminants in their roots and limit transport to the shoot, previous work has shown that white lupin performs better than other species on the acid contaminated soils of Aznalcóllar. These factors, together with the nitrogen-fixing capacity of white lupin and the benefit related to increased microbial activity in the acid soil, make this species an excellent candidate for phytoremediation (phytoimmobilisation), enhancing the initial revegetation of contaminated, nutrient-poor soils.

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References

Barceló, J., Poschenrieder, C., 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. Environ. Exp. Bot. 48, 75–92.

Barnhisel, R., Bertsch, P.M., 1982. Aluminium. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties. (2a edition). American Society of Agronomy, Madison, Wisconsin, pp. 275–300.

Bernal, M.P., Clemente, R., Walker, D.J., 2007. In: Gore, R.W. (Ed.), The role of organic Amendments in the Bioremediation of Heavy Metal-polluted Soils. Environmental Research at the Leading Edge, New York, pp. 1–57.

Bernal, M.P., McGrath, S.P., Miller, A.J., Baker, A.J.M., 1994. Comparison of the chemical changes in the rhizosphere of the nickel hyperaccumulator *Alyssum murale* with the non-accumulator *Raphanus sativus*. Plant Soil 164, 251–259.

Braum, S.M., Helmke, P.A., 1995. White lupin utilizes soil phosphorus that is unavailable to soybean. Plant Soil 176, 95–100.

Bravin, M.N., Marti, A.L., Clairotte, M., Hinsinger, P., 2009. Rhizosphere alkalisation—a major driver of copper bioavailability over a broad pH range in an acidic, copper-contaminated soil. Plant Soil 318, 257–268.

Carpena, R.O., Vázquez, S., Esteban, E., Fernández-Pascual, M., De Felipe, M.R., Zornoza, P., 2003. Cadmium-stress in white lupin: effects on nodule structure and functioning. Plant Physiol. Biochem. 41, 911–919.

Castaldi, P., Santona, L., Melis, P., 2005. Heavy metal immobilization by chemical amendments in a polluted soil and influence on white lupin growth. Chemosphere 60, 365–371.

Cattani, I., Fragoulis, G., Boccelli, R., Capri, E., 2006. Copper bioavailability in the rhizosphere of maize (*Zea mays* L.) grown in two Italian soils. Chemosphere 64, 1972–1979.

Chaignon, V., Quesnoit, M., Hinsinger, P., 2009. Copper availability and bioavailability are controlled by rhizosphere pH in rape grown in acidic copper-contaminated soil. Environ. Pollut. 157, 3363–3369.

Chuang, M.C., Shu, G.Y., Lin, J.C., 1996. Solubility of heavy metals in a contaminated soil: effects of redox potential pH. Water Air Soil Pollut. 90, 543–556.

Clemente, R., Almela, C., Bernal, M.P., 2006. A remediation strategy based on active phytoremediation followed by natural attenuation in a soil contaminated by pyrite waste. Environ. Pollut. 143, 397–406.

Clemente, R., Walker, D.J., Bernal, M.P., 2005. Uptake of heavy metals and As by *Brassica juncea* grown in a contaminated soil in Aznalcóllar (Spain): the effect of soil amendments. Environ. Pollut. 38, 46–58.

Council of the European Communities, 1986. Council directive of 12 of June on the protection of the environment, and in particular of the soil when sewage

sludge is used in agriculture. Official Journal of the European Communities, No. L 181/6-12, 4 July 1986.
Degryse, F., Verma, V.K., Smolders, E., 2008. Mobilization of Cu and Zn by root exudates of dicotyledonous plants in resin-buffered solutions and soils. Plant Soil 306, 69–84.
Dessureault-Rompré, J., Nowack, B., Schulin, R., Tercier-Waeber, M.-L., Luster, J., 2008. Metal solubility and speciation in the rhizosphere of *Lupinus albus* cluster roots. Environ. Sci. Technol. 42, 7146–7151.
Dinkelaker, B., Römheld, V., Marschner, H., 1989. Citric acid excretion and precipitation of calcium in the rhizosphere of white lupin (*Lupinus albus L.*). Plant Cell Environ. 12, 285–292.
EPA, 2007. Method 9210A. Potentiometric determination of nitrate in aqueous samples with an ion-selective electrode. Available at: 〈http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/9210a.pdf〉 (accessed 8.11.09).
Gee, G.W., Bauder, J.W., 1986. Particle-size analysis. In: 2nd ed. In: Klute, A. (Ed.), Methods of Soil Analysis. Part 1, 9. ASA and SSSA, Madison, WI, pp. 383–411 Agron. Monogr..
Hartikainen, H., 1986. Acid and base titration behaviour of Finnish mineral soils. Z. Pflanzenernaehr. Bodenkd. 149, 522–532.
Hinsinger, P., Plassard, C., Tang, C., Jaillard, B., 2003. Origins of root-mediated pH changes in the rhizosphere and their responses to environmental constraints: a review. Plant Soil 248, 43–59.
Joergensen, R.G., Brookes, P.C., 1990. Ninhydrin-reactive nitrogen measurements of microbial biomass in 0.5 M K_2SO_4 soil extracts. Soil Biol. Biochem. 22, 1023–1027.
Johnston, S.E., Barnard, W.M., 1979. Comparative effectiveness of fourteen solutions for extracting arsenic from four western New York soils. Soil Sci. Soc. Am. Proc. 43, 304–308.
Jones, D.L., 1998. Organic acids in the rhizosphere—a critical review. Plant Soil 205, 25–44.
Jones, D.L., Darrah, P.R., 1994. Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. Plant Soil 166, 247–257.
Kabata-Pendias, A., 2001. Trace elements in soils and plants. 3rd ed. Boca Raton, Florida.
Kalbitz, K., Solinger, S., Park, J.H., Milchalzik, B., Matzener, E., 2000. Controls on the dynamics dissolved organic matter in soils: a review. Soil Sci. 165, 277–304.
Kempers, A.J., Zweers, A., 1986. Ammonium determination in soil extracts by the salicylate method. Commun. Soil Sci. Plant Anal. 17, 715–723.
Kerley, S.J., 2000. Changes in root morphology of white lupin (*Lupinus albus* L.) and its adaptation to soils with heterogeneous alkaline/acid profiles. Plant Soil 218, 197–205.
Kochian, L.V., Pence, N.S., Letham, D.L.D., Pineros, M.A., Magalhaes, J.V., Hoekenga, O.A., Garvin, D.F., 2002. Mechanisms of metal resistance in plants: aluminium and heavy metals. Plant Soil 247, 109–119.
Kopittke, P.M., Asher, C.J., Blamey, F.P.C., Menzies, N.W., 2007. Toxic effects of Pb^{2+} on the growth of cowpea (*Vigna unguiculata* L.). Environ. Pollut. 150, 280–287.
Li, H., Shen, J., Zhang, F., Tang, C., Lambers, H., 2008. Is there a critical level of shoot phosphorus concentration of cluster-root formation in *Lupinus albus*? Funct. Plant Biol. 35, 328–336.
Lindsay, W.L., 1979. In: Chemical Equilibria in Soils. John Wiley & Sons, New York.
Loosemore, N., Straczeck, A., Hinsinger, P., Jaillard, B., 2004. Zinc mobilisation from a contaminated soil by three genotypes of tobacco as affected by soil and rhizosphere pH. Plant Soil 260, 19–32.
López-Pamo, E., Barettino, D., Antón-Pacheco, C., Ortiz, G., Arránz, J.C., Gumiel, J.C., Martínez-Pledel, B., Montouto, O., 1999. The extent of the Aznalcollar pyritic sludge spill and its effects on soils. Sci. Total Environ. 242, 57–88.
Martínez-Alcalá, I., Clemente, R., Bernal, M.P., 2009. Metal availability and chemical properties in the rhizosphere of *Lupinus albus* L. growing in a high-metal calcareous soil. Water Air Soil Pollut. 201, 283–293.
McGrath, S.P., Cegarra, J., 1992. Chemical extractability of heavy metals during and after long-term applications of sewage sludge to soil. J. Soil Sci. 43, 313–321.
Mengel, K., Kirby, E.A., 2001. In: Principles of Plant Nutrition 5th ed. Kluwer, Dordrecht.
Pastor, J., Hernández, A.J., Prieto, N., Fernández-Pascual, M., 2003. Accumulating behaviour of *Lupinus albus* L. growing in a normal and a decalcified calcic luvisol polluted with Zn. J. Plant Physiol. 160, 1457–1465.
Ryan, P.R., Delhaize, E., Jones, D.L., 2001. Function and mechanism of organic anion exudation from plant roots. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 527–560.
Sas, L., Zed, R., Tang, C., 2002. The effect of nitrogen nutrition on cluster root formation and proton extrusion by *Lupinus albus*. Ann. Bot—London 89, 435–442.
Shane, M.W., Lambers, H., Cawthray, G.R., 2008. Impact of phosphorous mineral source (Al–P or Fe–P) and pH on cluster-root formation and carboxylate exudation in *Lupinus albus* L. Plant Soil 304, 169–178.
Simon, L., Smaley, T.J., Benton Jones Jr., J., Lasseigne, F.T., 1994. Aluminium toxicity in tomato. Part I. Growth and mineral nutrition. J. Plant Nutr. 17, 293–306.
Tao, S., Chen, Y.J., Xu, F.L., Cao, J., Li, B.G., 2003. Changes of copper speciation in maize rhizosphere soil. Environ. Pollut. 122, 447–454.
Vance, C.O., Uhde-Stone, C., Allan, D.L., 2003. Phosphorus acquisition and use: critical adaptations by plants for securing a non-renewable resource. New Phytol. 157, 423–447.
Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biol. Biochem. 19, 703–707.
Vázquez, S., Agha, R., Granado, A., Sarro, M.J., Esteban, E., Peñalosa, J.M., Carpena, R.O., 2006. Use of white lupin plant for phytostabilization of Cd and as polluted acid soil. Water Air Soil Pollut. 177, 349–365.
Vázquez, S., Goldsbrough, P., Carpena, R.O., 2009. Comparative analysis of the contribution of phytochelatins to cadmium and arsenic tolerance in soybean and white lupin. Plant Physiol. Biochem. 47, 63–67.
Wheeler, D.M., 1995. Relative aluminium tolerance of ten species of graminae. J. Plant Nutr. 18, 2305–2312.
Yan, F., Schubert, S., Mengel, K., 1996. Soil pH changes during legume growth and application of plant material. Biol. Fertil. Soils 23, 236–242.
Zornoza, P., Vázquez, S., Esteban, E., Fernández-Pascual, M., Carpena, R.O., 2002. Cadmium-stress in nodulated white lupin: strategies to avoid toxicity. Plant Physiol. Biochem. 40, 1003–1009.