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Research article

Responses of *Noccaea caerulescens* and *Lupinus albus* in trace elements-contaminated soils

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ABSTRACT

Plants exposed to trace elements can suffer from oxidative stress, which is characterised by the accumulation of reactive oxygen species, alteration in the cellular antioxidant defence system and ultimately lipid peroxidation. We assessed the most-appropriate stress indexes to describe the response of two plant species, with different strategies for coping with trace elements (TEs), to particular contaminants.

Noccaea caerulescens, a hyperaccumulator, and *Lupinus albus*, an excluder, were grown in three soils of differing pH: an acidic soil, a neutral soil (both contaminated mainly by Cu, Zn and As) and a control soil. Then, plant stress indicators were measured.

As expected, *N. caerulescens* accumulated higher levels of Zn and Cd in shoots than *L. albus*, this effect being stronger in the acid soil, reflecting greater TE solubility in this soil. However, the shoot concentrations of Mn were higher in *L. albus* than in *N. caerulescens*, while the As concentration was similar in the two species. In *L. albus*, the phenolic content and lipid peroxidation were related with the Cu concentration, whereas the Zn and Cd concentrations in *N. caerulescens* were more closely related to glutathione content and lipid peroxidation. Interestingly, phytochelatins were only found in *L. albus* grown in polluted soils. Hence, the two species differed with respect to the TEs which provoked stress and the biochemical indicators of the stress, there being a close relationship between the accumulation of TEs and their associated stress indicators in the different plant organs.

1. Introduction

Plants have several mechanisms of tolerance that enable them to survive in soils polluted with trace elements (TEs). Some minimise their entry into the roots, while others are capable of reducing the cellular damage once the element is inside the plant. Depending on the prevalent strategy used, plants are classified as (hyper)accumulators, excluders or indicators [1]. Hyperaccumulators of Cd or Zn are capable of accumulating $\geq$100 μg g^{-1} Cd or $\geq$10,000 μg g^{-1} Zn without showing toxicity symptoms. Hyperaccumulator species like *Noccaea caerulescens* (J. & C. Presl) F.K. Mey. have enhanced root uptake and root-to-shoot transport and elevated tolerance of TEs (Zn and Cd) due to internal detoxification processes, such as sub-cellular compartmentation or complexation with cellular ligands [2]. Metal excluder plants restrict the movement of metals from their roots and prevent their translocation to the aerial parts over a broad range of metal concentration in soil. This could be due to the alteration of membrane permeability, changes in metal-binding capacity of cell walls or exudation of chelating substances in the roots, as was proposed for species like *Lupinus albus* L. [3].

Despite the exclusion barriers of the cells, TEs may accumulate in the cytoplasm, causing phytotoxic effects. The TEs are also known to stimulate generation of the reactive oxygen species (ROS) that elicit oxidative stress. In particular, it has been demonstrated that some TEs like Cd, Cr, Zn, Ni, Mn, Cu and Fe are able to induce the formation of ROS in plants [4]. These ROS are highly toxic and can oxidise biological macromolecules such as lipids, proteins and nucleic acids, causing lipid peroxidation, membrane damage and inactivation of enzymes. The antioxidant system response to TEs greatly depends on the plant species and age and the growth conditions [4]. Malondialdehyde (MDA) is a major cytotoxic product of lipid peroxidation and acts as an indicator of free radical production [5].

Abbreviations: Cys, cysteine; GSH, glutathione; MDA, malondialdehyde; PCs, phytochelatins; ROS, reactive oxygen species; TE, trace element.

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Previous studies showed that the presence of MDA in plants like *L. albus* and *N. caerulescens* was related to the accumulation of TEs, and could be considered a reliable biomarker of toxicity [6–9].

Phytochelatins (PCs) and other thiols constitute a family of metabolites regarded as ligands of several metal cations and other metalloid anions such as arsenite, and play an important role in TEs detoxification [10]. Phytochelatins are synthesised from the ubiquitous tripeptide glutathione (GSH), which plays also an important role as an antioxidant metabolite as part of the ascorbate-GSH cycle [11]. Trace elements bind to GSH and/or PCs and these complexes are transported to the vacuole, a mechanism that involves an up-regulation of sulphur metabolism to cope with the demand for S-rich ligands [12]. Therefore, concentrations of PCs and other thiols could be used as an index to reflect the accumulation of TEs.

Phenolic metabolites also have the capability of chelating TEs, mainly Cu and Fe, although they can also form stable complexes with other TEs like Ni, Co, Mn or Al [13]. Among these compounds, flavonoids are known to form chelates with Fe and Cu ions; their concentrations rise under abiotic stresses, as in the case of anthocyanins [14].

One of the characteristic visual symptoms of TEs toxicity is leaf chlorosis, caused by a diminution in chlorophyll concentration [15]. According to Dhir et al. [16], the decline in chlorophyll levels observed under TEs exposure could be due to a reduction of Fe content, lower activity of chlorophyll biosynthesis-related enzymes or replacement of the central Mg^{2+} in the chlorophyll molecule by metal cations [5].

Most of the studies regarding stress indexes of plants with respect to trace elements have been carried out in nutrient solution. Scarce information is available on stress indicators when plants are growing in polluted soils. The aim of this work was to study several stress indexes, as a response to multi-contaminated soils, of two plant species with different strategies for coping with toxic elements: the hyperaccumulator – *N. caerulescens* and the excluder – *L. albus*. In this way, the most-appropriate stress indexes to describe the response of each species to particular contaminants will be determined. The TEs accumulation and several stress indicators were measured: biomass production, lipid peroxidation, chlorophyll and phenolic content and total thiols concentrations (cysteine, GSH and PCs). A correlation analysis was performed to identify the indexes that were related most closely to plant TEs accumulation.

2. Results

2.1. Plant growth

The fresh weight of *L. albus* was reduced with respect to control soil when cultivated in polluted neutral and acid soils, with an average reduction of 31 and 28%, respectively (Fig. 1). Conversely, for *N. caerulescens*, the lowest biomass production occurred in the control soil, probably due to the high TE tolerance of this species.

2.2. Plant trace element concentrations

The TE concentrations in *L. albus* were typical of TEs excluder behaviour (Fig. 2): Zn, Cu, Cd, Pb and As were retained in roots, with low translocation to the aerial parts. Manganese was the exception, as it was accumulated mainly in the aerial parts. The TEs accumulation was greater in the roots of plants grown in the acidic soil, reflecting their higher solubility at low soil pH (Table 1). Only the Fe concentration in roots of *L. albus* was the highest in the control soil. The As concentration was the highest in roots grown in neutral soil. In *N. caerulescens*, the concentrations of TEs were higher in organs of plants grown in the more-polluted (acid) soil, with the exception of Cd. The Zn and Cd concentrations were higher in the aerial parts, showing the accumulator behaviour of this species for these elements (Fig. 3).

2.3. Stress parameters

Lipid peroxidation (MDA) was greatest in the roots of *L. albus* grown in neutral and acid contaminated soils (Fig. 4), whereas in the stems the concentration of MDA was highest for the control soil. In *N. caerulescens*, the highest MDA concentration was found in the aerial parts of plants grown in polluted soils ($P < 0.001$), where Zn and Cd accumulated. The chlorophyll concentration in *L. albus* grown in the control soil was statistically lower than in plants from the polluted soils (Fig. 5). In *N. caerulescens*, although no visual symptoms were observed, a decrease in chlorophyll levels was found in the polluted soils with respect to the control soil.

The concentration of phenolic compounds in roots of white lupin grown in the polluted soils was higher than in control soil, while the opposite happened in leaves (Table 2). In *N. caerulescens*, the

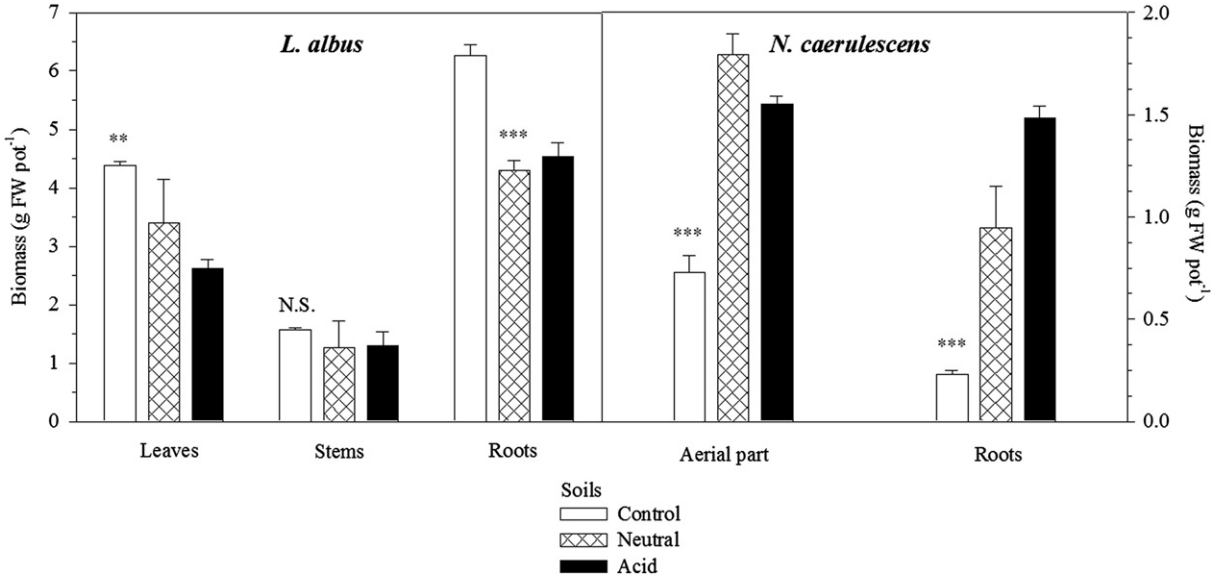

Fig. 1. Plant weight of *L. albus* and *N. caerulescens* grown in the different soils (g FW pot^{-1} ± se). ***, **: significant at $P < 0.001$, 0.01, respectively. N.S.: not significant.

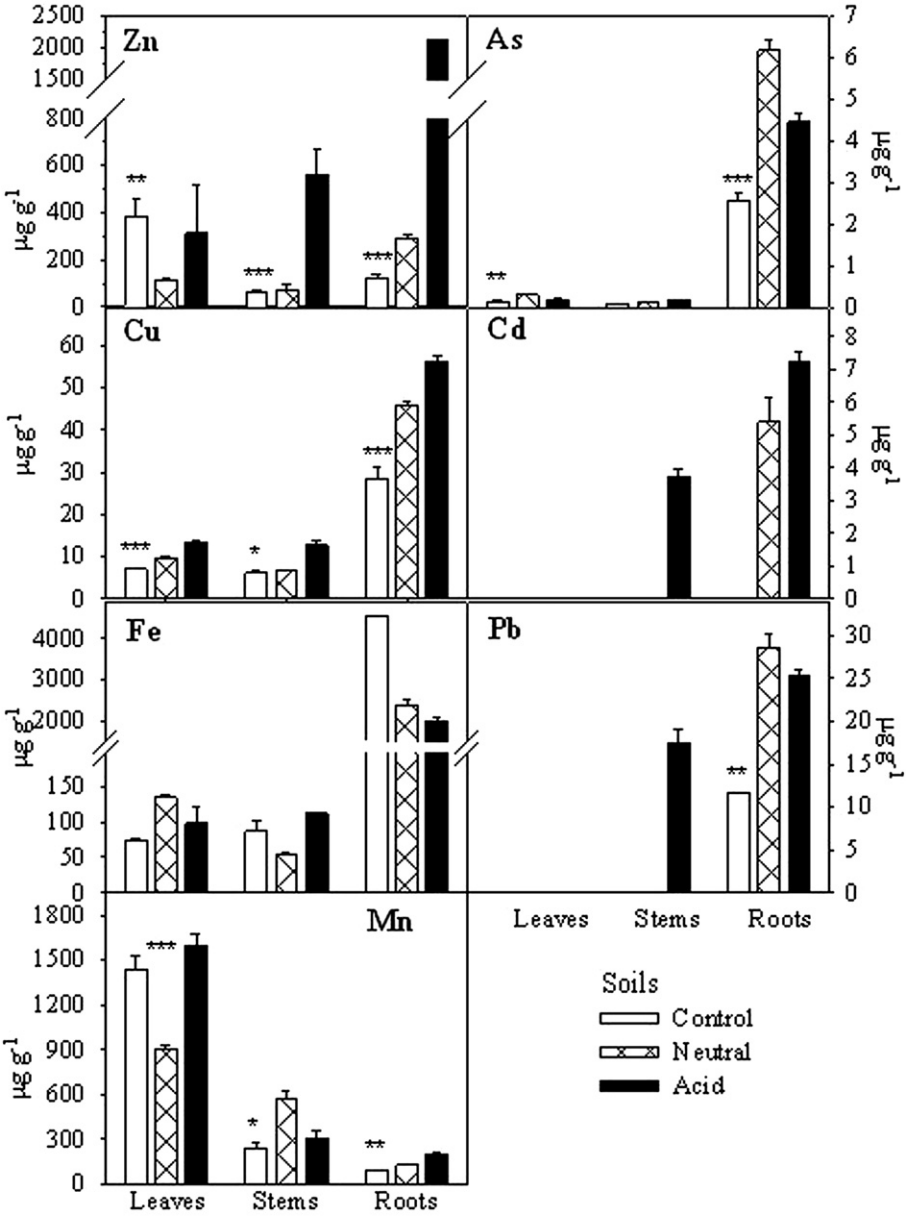

Fig. 2. Trace element concentrations in *L. albus* grown in the different soils (μg g^{-1} DW ± se). Data below the detection limit (0.5 μg g^{-1}) are not shown. ***, **, *: significant at $P < 0.001$, 0.01, 0.05, respectively. N.S.: not significant.

Table 1
Characteristics and trace elements concentrations of the soils.

Parameter	Neutral soil	Acid soil	Control soil
pH	6.8	5.5	7.4
$CaCO_3$ (%)	<0.5	<0.5	2.2
OM (%)	1.8	2.1	1.3
Fe (g kg^{-1})	42.1 (<0.5)[a]	40.5 (<0.5)	34.9 (<0.5)
Mn (μg g^{-1})	834 (0.10)	1024 (32.6)	519 (<0.5)
Zn (μg g^{-1})	268 (0.70)	549 (33.8)	134 (<0.5)
Pb (μg g^{-1})	141 (<0.12)	311 (<0.12)	22.3 (<0.12)
Cu (μg g^{-1})	103 (<0.5)	196 (<0.5)	49.6 (<0.5)
As (μg g^{-1})	69.9 (0.00)	228 (0.02)	14.9 (0.03)
Cd (μg g^{-1})	0.9 (<0.03)	2.0 (0.80)	1.0 (<0.03)

[a] Values in parentheses indicate 0.1 M $CaCl_2$-extractable concentrations (related closely to plant uptake of trace elements).

concentration in the aerial parts of plants grown in the control soil was lower ($P < 0.01$) than for the polluted soils, while the opposite happened for roots. Flavonoid concentrations (data not shown) were similar in the two species: approximately 14.7 μmol g^{-1} DW, with the exception of *N. caerulescens* roots in the control soil, which had the highest concentration (54.0 μmol g^{-1} DW).

2.4. Thiol concentrations

The concentration of total thiols in *L. albus* (Table 3) was 4.6–6.0-fold higher in leaves than in other organs ($P < 0.001$), according to the soil, with the highest values in plants grown in the more-polluted (acid) soil. The *N. caerulescens* shoot total thiol concentration (Table 3) was also higher than in the roots of plants grown in the contaminated soils.

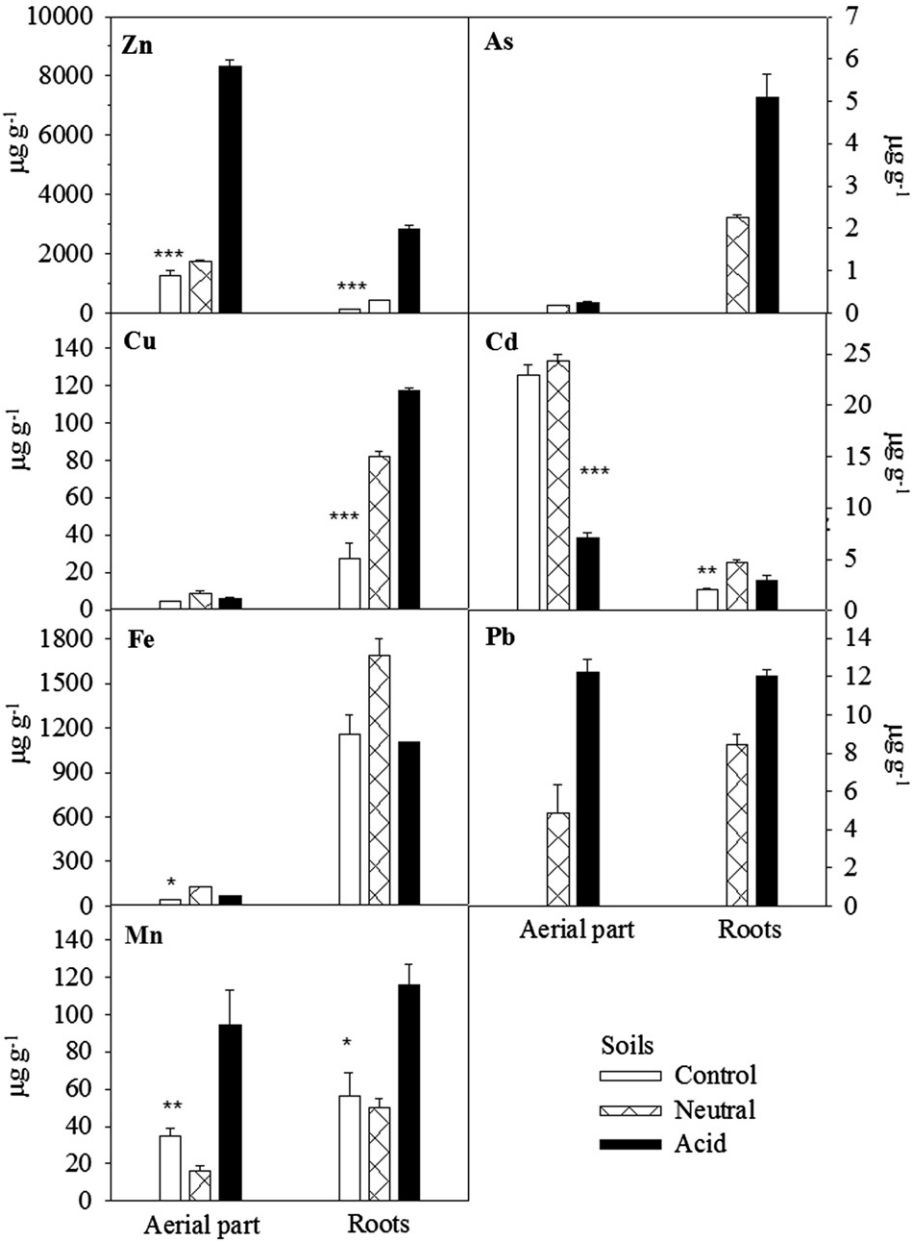

Fig. 3. Trace element concentrations in *N. caerulescens* grown in the different soils (μg g^{-1} DW ± se). Data below the detection limit (0.5 μg g^{-1}) are not shown. ***, **, *: significant at $P < 0.001$, 0.01, 0.05, respectively. N.S.: not significant.

Since changes in the total thiols content were observed, the different classes of thiols were analysed by HPLC. Cysteine (data not shown) was detected only in roots of *L. albus* (19 nmol g^{-1} FW) and in the shoots of *N. caerulescens* (18 nmol g^{-1} FW) grown in the most-contaminated soil, coinciding with the higher TEs concentrations in these organs. For all three soils, the GSH concentration (Table 3) was higher in roots and stems than in leaves of *L. albus*, while the stem and leaf concentrations were highest in plants grown in the acid contaminated soil. The GSH concentrations in *N. caerulescens* were statistically higher for roots of plants grown in the neutral, contaminated soil than for those from control soil (Table 3).

Phytochelatins were found in the roots of white lupin grown in both contaminated soils (PC2, 34 and 42 nmol g^{-1} FW, and PC3, 32 and 38 nmol g^{-1} FW, in the neutral and acid soil, respectively). With regards to the aerial parts of the plant, PC2 and PC3 (99 and 34 nmol g^{-1} FW, respectively) were only detected in stems of plants cultivated in the acid soil; we could not detect PCs in the leaves of white lupin.

2.5. Contribution of trace elements to the levels of stress indicators

The principal component analysis (PCA) of the results obtained for *L. albus* (Table 4) gave three principal components: the first relates positively elements such as Cu, As and Zn with the antioxidant production (total phenolics and MDA), which indicates the physiological response of *L. albus* to TEs (heavy metals and As) stress. The second principal component shows a positive relationship between flavonoids, Fe, weight and anthocyanins. This factor indicates the welfare of the plant, since flavonoids are implicated in protecting the plant against stress and Fe is a micronutrient. The third principal component related negatively phenols and Mn, but positively GSH.

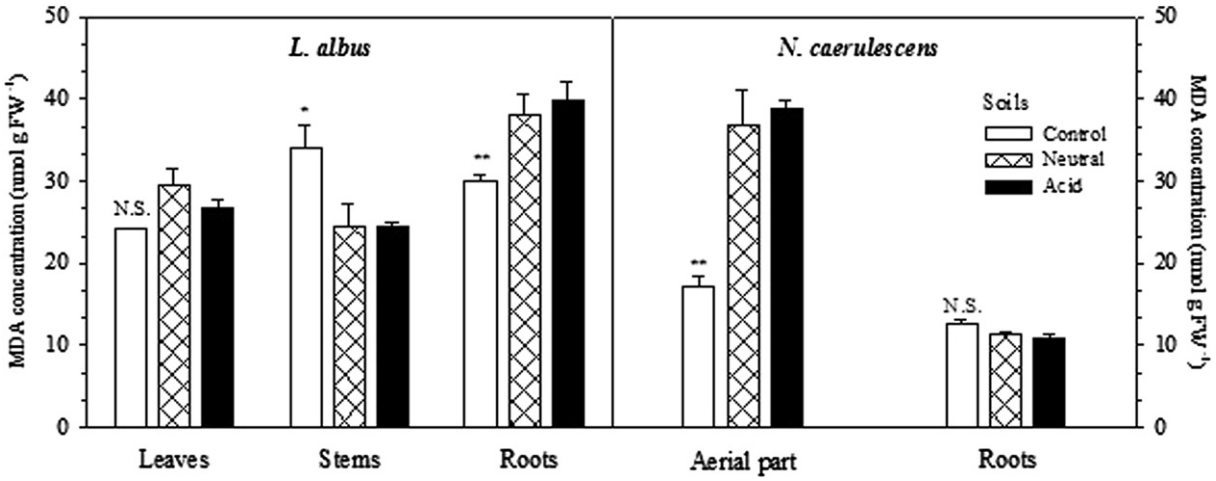

Fig. 4. MDA concentrations in *L. albus* and *N. caerulescens* grown in the different soils (nmol g^{-1} FW ± se). **, *: significant at $P < 0.01$, 0.05, respectively. N.S.: not significant.

The results obtained for *N. caerulescens* (Table 5) gave four principal components. In the first, GSH, MDA and fresh weight are related positively with Zn and Cd, indicating that these TEs could be responsible for higher antioxidant production (GSH). In the second principal component, a positive relationship of Pb, As, Mn, Cu and weight and a negative one for phenolics and flavonoids were observed. The third principal component was related positively with metals such as Mn and Zn. The fourth principal component was anthocyanins, indicating plant stress.

3. Discussion

3.1. Plant growth

As in the present experiment, a severe diminution in plant biomass was also found in *L. albus* grown hydroponically and treated with 18 μM Cd [7] or up to 18 μM As [17]. Pongrac et al. [15] observed an increase of root biomass with the increase of Cd in the substrate for *N. caerulescens*. Whiting et al. [18] found relatively-greater root growth in Cd-enriched soil zones, but only for a Cd-accumulating population of *N. caerulescens*, as well as greater root proliferation of *N. caerulescens* in soil zones with high Zn concentrations. The population used in the current study does not exhibit marked accumulation of Cd, indicating that it was the elevated soil levels of Zn, rather than Cd, which stimulated root growth in the contaminated soils of the present work.

In addition, Brown et al. [19] observed a high variation in biomass production in *N. caerulescens* when grown in polluted soils, the lowest leaf production being in plants cultivated in acidic soils (pH values between 5.06 and 5.84), probably due to increased solubility of several TEs, whereas in our study root and shoot growth were similar in the acid and neutral contaminated soils.

3.2. Plant trace element concentrations

Previous experiments have shown that *L. albus* accumulated heavy metals and As mainly in roots, with the exception of Mn that accumulated mostly in the aerial parts [20]. Relatively-low concentrations of Cd and As were found in the shoots of *L. albus* in the present study, in agreement with data reported by Vázquez et al. [3] for lupin plants also grown in Aznalcóllar soils. In addition, the leaf, stem and root Mn concentrations of plants grown in acid soil were similar to those of white lupin grown cultivated in nutrient solution with 33 μM Mn [21]. The higher Fe concentration in roots of *L. albus* grown in the control soil could be due to inhibition of Fe uptake by other heavy metals in the two contaminated soils [22].

In *N. caerulescens*, the concentrations of Zn and Cd were higher in the aerial part than in roots, but the threshold concentrations of

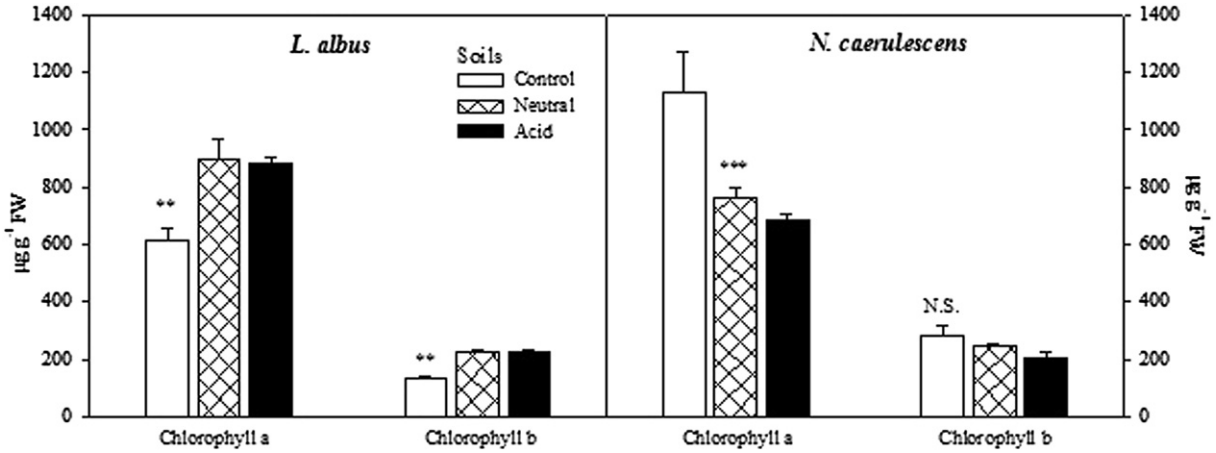

Fig. 5. Chlorophyll concentrations in *L. albus* and *N. caerulescens* grown in the different soils (μg g^{-1} FW ± se). **: significant at $P < 0.01$. N.S.: not significant.

Table 2
Total phenolic concentrations in *L. albus* and *N. caerulescens* grown in the different soils (mg gallic acid g^{-1} DW ± se).

	Control soil	Neutral soil	Acid soil	ANOVA
L. albus				
Leaves	28.6 ± 0.66^{aA}	23.8 ± 0.91^{bB}	24.3 ± 0.56^{bA}	**
Stems	13.8 ± 0.58^{abC}	11.5 ± 0.42^{bC}	15.9 ± 1.30^{aB}	*
Roots	22.9 ± 0.95^{bB}	30.6 ± 1.81^{aA}	27.5 ± 0.92^{abA}	**
ANOVA	***	***	***	
N. caerulescens				
Aerial parts	10.1 ± 0.37^{b}	18.6 ± 0.62^{a}	17.1 ± 0.50^{a}	***
Roots	42.8 ± 3.72^{a}	15.7 ± 0.90^{b}	14.9 ± 0.50^{b}	***
ANOVA	**	*	*	

Different lower-case letters show significant differences among soils according to the Tukey test at $P < 0.05$.
Different upper-case letters show significant differences among organs according to the Tukey test at $P < 0.05$.
***, **, *: significant to $P < 0.001$, 0.01, 0.05, respectively.

Table 4
Matrix of principal components rotated[a] for the results of *L. albus*.

	Components		
	1	2	3
Cu	0.908		
As	0.832		
MDA	0.801		
Zn	0.797		
Phenolics	0.701		−0.556
Flavonoids		0.942	
Fe		0.894	
Fresh weight		0.806	
Anthocyanins		0.729	
GSH			0.858
Mn			−0.832
% of variance	50.7	20.4	15.3

Extraction method: principal component analyses.
Rotation method: Varimax normalisation with Kaiser.
[a] Rotation converged in 5 iterations.

Zn and Cd for hyperaccumulation were not reached (100 μg g^{-1} Cd and 10,000 μg g^{-1} Zn [1]). High rates of Zn and Cd translocation to the leaves of *N. caerulescens* arise from the high levels of HMA4, a plasmalemma P-type ATPase transporter that loads these metals into the xylem from root parenchymatic cells [23]. It is possible that the multi-elemental contamination of the studied soils could have limited the absorption of Cd and Zn by the roots: Walker and Bernal [24] found that the Zn concentration in *N. caerulescens* shoots declined by 87% when 1.0 mg l^{-1} Cu was included in the nutrient solution.

3.3. Stress parameters

Under stress imposed by high solubility of TEs in the polluted soils, MDA concentrations in *L. albus* decreased in stems but increased in roots, where most TEs accumulated. The MDA levels in this experiment were higher than those found by Carpena et al. [7] in nodules and roots (7.05–16.1 nmol g^{-1} FW, respectively) when *L. albus* was grown hydroponically with 18 μM Cd. Carrasco-Gil et al. [25] found that alfalfa plants grown without nitrogen fertilisation suffered a stronger oxidative stress when cultivated in Hg-polluted soils, implying that nutrient-deficient plants might have greater oxidative stress than plants cultivated in a nutrient-rich, hydroponic system. The absence of fertilisation in the present experiment could have enhanced the stress and therefore the MDA concentrations of *L. albus*.

According to the PCA, the presence of Cd will produce a higher plant MDA concentration, as observed by other authors for *N. caerulescens* grown in culture solution with Cd [6,9]. Wang et al. [9] found MDA concentrations of 5–11 nmol g^{-1} FW for plants grown in hydroponics with 400 μM Cd (a concentration much higher than those in the soil solution of even the most-highly-contaminated soils), lower than in the plants of the present experiment, while Boominathan and Doran [6] found similar concentrations in roots treated with 178 μM Cd. Other authors [5] also observed increased MDA concentrations in leaves of the Zn and Cd accumulator *Brassica napus* grown in a medium with Zn and Cd, with positive correlations with the leaf heavy metal concentrations. This suggests that where Zn and Cd accumulate, they induce lipid peroxidation and MDA accumulation: in roots of *L. albus* and in aerial parts of *N. caerulescens*. Metalloids such as As may increase lipid peroxidation and oxidative stress and can decrease chlorophyll levels in leaves [26]. Esteban et al. [8] also found that the chlorophyll concentration was increased when *L. albus* plants were grown in the presence of Hg. Similarly, pea plants grown in an arsenate-polluted soil exhibited an increased chlorophyll concentration [27]. These results could be partially explained by a concentration effect, as the leaves of plants accumulating trace elements were smaller and of lower biomass than those grown in the control soil (Fig. 1). *N. caerulescens* chlorosis has been observed upon exposure to Cd [28] and Zn [15].

L. albus exudes phenolic compounds into the rhizosphere and increases their concentration in the roots as a response to increased Cu bioavailability in the rhizosphere [29]. A similar situation may have existed for the TEs in the soils studied here. Esteban et al. [8] found that the concentration of phenolics in white lupin roots increased, from 2.23 to 4.87 mg g^{-1}, as the Hg dose in the nutrient solution increased. However, the levels were lower than in this experiment. This could be partially due to the younger age of the

Table 3
Total thiols and GSH concentrations in *L. albus* and *N. caerulescens* grown in the different soils (nmol g^{-1} FW ± se).

	Total thiols				GSH			
L. albus	Leaves	Stems	Roots	ANOVA	Leaves	Stems	Roots	ANOVA
Control soil	1128 ± 62.9^{bA}	203 ± 2.96^{bB}	208 ± 6.98^{B}	***	72 ± 8.20^{bB}	150 ± 26.2^{bA}	144 ± 10.6^{A}	*
Neutral soil	1055 ± 14.2^{bA}	192 ± 12.2^{bB}	231 ± 30.9^{B}	***	65 ± 14.2^{bB}	136 ± 29.2^{bAB}	178 ± 57.4^{A}	*
Acid soil	1235 ± 23.3^{aA}	250 ± 6.23^{aB}	206 ± 33.5^{C}	***	127 ± 13.1^{aC}	237 ± 13.4^{aA}	165 ± 16.1^{B}	***
ANOVA	*	**	N.S.		**	**	N.S.	
N. caerulescens	Aerial part		Roots	ANOVA	Aerial part		Roots	ANOVA
Control soil	267 ± 58.5^{b}		324 ± 47.5^{a}	N.S.	434 ± 30.7		132 ± 33.1^{b}	***
Neutral soil	1022 ± 30.2^{a}		155 ± 7.11^{b}	**	375 ± 20.8		293 ± 20.2^{a}	N.S.
Acid soil	1102 ± 44.7^{a}		124 ± 12.1^{b}	***	420 ± 38.8		191 ± 9.81^{b}	**
ANOVA	***		**		N.S.		**	

Different lower-case letters show significant differences among soils according to the Tukey test at $P < 0.05$.
Different upper-case letters show significant differences among organs according to the Tukey test at $P < 0.05$.
***, **, *: Significant at $P < 0.001$, 0.01, 0.05, respectively. N.S.: not significant.

Table 5
Matrix of principal components rotated[a] for the results of *N. caerulescens*.

	Components			
	1	2	3	4
GSH	0.926			
Fe	−0.70			
MDA	0.851			
Cd	0.783			
Phenols	−0.564	−0.544		
Pb		0.957		
As		0.804		
Mn		0.689	0.553	
Cu	−0.661	0.682		
Fresh weight	0.622	0.681		
Flavonoids	−0.619	−0.619		
Zn	0.510		0.703	
Anthocyanins				0.806
% of variance	39.9	32.1	14.4	10.6

Extraction method: principal component analyses.
Rotation method: Varimax normalisation with Kaiser.
[a] Rotation converged in 7 iterations.

plants grown in the nutrient solution (28 days-old) with respect to the plants of the present study (71 days-old) and/or to the different cultivation systems used (nutrient solution *versus* soils). Pongrac et al. [15] found higher anthocyanin concentrations in *N. caerulescens* at higher external Zn concentration; this did not happen in our experiment, where the highest concentration was found in the control soil (data not shown).

The PCA of the results obtained for *L. albus* indicates that phenolics have an important ability to chelate Zn (PCA 1) and Cu, as demonstrated by Martell and Smith [13]. Jung et al. [29] also suggested that phenolic compounds exuded by *L. albus* could chelate Cu in the medium and in root cell walls, restricting Cu toxicity in the plant; so, a higher Cu concentration in the plant could induce a higher internal accumulation of phenolics. The third PCA related negatively phenolics and Mn. This may implicate phenolics in the detoxification of Mn by *L. albus* (implying a higher accumulation capacity), although high Mn concentrations are not toxic for this species and Zornoza et al. [21] observed a protective role of Mn in *L. albus* with respect to Cd toxicity.

The levels of total thiols found in our experiment were much higher than those reported by Pongrac et al. [15], in the aerial parts of *N. caerulescens* (54–60 nmol g^{-1} DW) grown in soils amended with Cd, suggesting a higher degree of stress. Our results are in disagreement with those of other reports [4,8,30] which showed a higher thiol concentration in roots of *L albus* exposed to Hg, Cd and As in a hydroponic system. Interestingly, when alfalfa plants were grown in Hg-polluted soil, the concentration of total thiols in the root was about three-times lower than in the shoot [25]. Therefore, a completely-different behaviour of some TE-related stress parameters might be expected when plants are cultivated in soil or in a hydroponic system.

The Cys concentration of *N. caerulescens* in the present experiment was lower than that found in *N. caerulescens* exposed to Cd [2] and in *Thlaspi goesingense* under Ni stress [31]. Cysteine is the first thiol-containing amino acid that is formed during sulphate assimilation, a metabolic process that is up-regulated under TE stress, in order to permit the synthesis of PCs [12]. Vázquez et al. [30] obtained similar results for the GSH concentration of *L. albus* grown in hydroponics with As or Cd (both 18 μM). Equally, Vázquez et al. [3] did not find differences in GSH when they varied the Cd concentration in the nutrient solution. This indicates the capacity of *L. albus* to maintain its cellular pool of GSH, albeit this important antioxidant metabolite can be used to synthesise PCs. Glutathione is a key factor in TE tolerance, as it contributes to maintenance of the cell viability that is compromised by oxidative stress when TEs accumulate to toxic levels [11]. For all three soils, the GSH concentrations were higher in *N. caerulescens* than in *L. albus*, suggesting that a constitutively-higher level of shoot GSH in the hyperaccumulator plant might contribute to an enhanced tolerance of TEs. Similar responses were reported by Pongrac et al. [15], who found higher GSH concentrations in *N. caerulescens* grown in different substrates supplemented with Zn or Cd. However, some controversy exists, as other researchers observed moderate changes [2], or even a significant decline [6]. These divergences could be explained on the basis of the differences in the TE treatments used and/or the growing conditions of the plants. The first PCA for *N. caerulescens* indicated higher GSH concentrations and plant weight at elevated external Cd concentrations; in accordance with this, Pongrac et al. [15] observed greater biomass production by *N. caerulescens* when exposed to Cd.

Phytochelatins (PCs) can bind metals (Cd, Hg, Zn) and metalloids (As), and are synthesised using GSH as substrate [10]. The importance of PCs in TEs detoxification has been proved in a high number of publications [4,12], but very few studies have been performed in plants grown in multi-polluted soils. Vázquez et al. [30] found that the addition of 18 μM Cd to a hydroponic nutrient solution led to an accumulation of PCs in *L. albus*, PC3 being the most abundant, but when 18 μM As was added PC2 was more abundant than PC3. It must be stressed that the concentrations of PCs in plants grown hydroponically were higher than those of the current study with plants grown in polluted soils, probably related to the lower TE availability in soils. However, the concentrations of PCs were significantly higher in the acid soil, where TE availability was higher.

In *N. caerulescens*, PCs were not found, showing that they may play a secondary role in TEs tolerance in this species, as suggested previously for Cd [28]. Hernández-Allica et al. [2] observed that PCs might not be important for metal tolerance in *N. caerulescens*, but Cys or GSH could be implicated, possibly in maintenance of the cellular redox homeostasis [11], as already discussed. However, in *L. albus*, PCs could be important for TEs detoxification, particularly in roots.

In conclusion, the two species studied (*L. albus* and *N. caerulescens*) had differing behaviour with respect to tolerance of TEs and their relationships with stress indicators:

- There was stronger lipid peroxidation in the organ where trace elements accumulated (in *L. albus* roots and in *N. caerulescens* aerial parts): MDA seems to be the most useful parameter for studying the tolerance of these two species.
- Chlorophylls can be useful as indicators of stress induced by TEs in *L. albus* but not in *N. caerulescens*.
- Total thiols are related to organ Mn concentrations in *L. albus* and to Zn and Cd concentrations in *N. caerulescens*, suggesting that they play an important role in the capacity of these species to accumulate such metals.
- Cys and phenolics seem to be related to accumulation of heavy metals in roots of *L. albus* and in the aerial parts of *N. caerulescens*.
- Phytochelatins may not be related to heavy metal tolerance in *N. caerulescens*, but they do seem to be relevant in *L. albus* for metal/metalloid detoxification.

4. Materials and methods

4.1. Soil characteristics

Three soils were selected from the same area, near Sanlúcar la Mayor (Seville, Spain) (longitude W 06° 13′ 00″, latitude N 37° 26′ 21″), 10 km from the Aznalcóllar mine, the site of a serious pollution

event in 1998. Two of them contain high levels of heavy metals and As; one was acid (pH 5.5) and the other neutral (pH 6.8). The third soil was not affected by the pyritic spillage and was considered as a control, as its TEs concentrations do not exceed the limits stipulated for agricultural soils [32].

The soils were collected from the top 20 cm, air-dried for 5–6 d and sieved to <2 mm prior to analysis (Section 2.3). The soils were non-calcareous loams with 20% clay, 34% silt and 46% sand, classified as typic Xerofluvent (American Soil Taxonomy). The polluted soils have total trace elements concentrations (Table 1) much higher than the control soil. The acid soil had total Zn, Cu and Pb concentrations above the EU limits for agricultural soils [32] and the neutral soil exceeded the Zn level. The As concentrations in both polluted soils are above the guideline limit proposed for agricultural soils [33]. Table 1 shows that the acid soil had higher concentrations of Cd, Mn and Zn extractable with 0.1 M $CaCl_2$; this reflects the greater solubility and potential phytotoxicity of these TEs at low soil pH.

4.2. Plant culture and harvest

Seeds of *L. albus* (cv. Marta) and *N. caerulescens* (J. & C. Presl) F.K. Mey. (formerly *Thlaspi caerulescens* J. & C. Presl) were surface-sterilised with 10% HClO for 30 min, washed three times with distilled water and then germinated for several days before being sown. Two plants of *L. albus* and five of *N. caerulescens* were planted in 7.5-cm-diameter pots, which contained 250 g of soil over 100 g of sand, to aid drainage. The differing numbers of plants per pot were chosen in order to minimise differences in biomass between the species. Six pots were used per soil–species combination and were grouped in pairs to make three replicates. Plants of *L. albus* were grown for 71 d and plants of *N. caerulescens* for 132 d in a growth chamber, as the slower rate of growth of *N. caerulescens* made it necessary to grow them for a longer period of time in order to obtain plants at a similar phenological stage. Pots were maintained with a light/dark regime of 16/8 h, temperature of 25/17 °C (day/night) and relative humidity of 70%, without fertilisation. Plants were watered with deionised water from the base of the pots, using a tray.

Leaves, stems and roots were collected from *L. albus* and shoots and roots from *N. caerulescens*. The samples were washed thoroughly to eliminate adhering soil particles, firstly with tap water and then three times with deionised water. Prior to further processing, all plant material was weighed to determine total fresh weight, and the fresh samples were divided into two: one was frozen in liquid N_2 and stored at −70 °C for chlorophyll, thiols, MDA and PCs determination, and the other was dried at 60 °C for two days and ground to homogeneity for TEs and phenolic compounds determination.

4.3. Trace elements concentrations

Plant and soil pseudo-total heavy metals (Cu, Fe, Mn, Pb, Zn, Cd) and As 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. Trace elements in soil solution and in exchangeable forms were extracted with 0.1 M $CaCl_2$ (1:10 w/v), according to McGrath and Cegarra [34]. The general methods used for soil analysis are indicated in Martínez-Alcalá et al. [20].

4.4. Determination of stress parameters

For the total phenolic concentration in plants, an extract was prepared with 500 mg of dry plant material in 20 ml of acidified methanol (0.1% HCl), autoextracting at room temperature for 24 h. After centrifugation at 200 rpm, the supernatant was adjusted to 25 ml with methanol and then filtered. The total phenolic content was determined using the Folin–Ciocalteu reagent (adapted from Ref. [35]) and expressed in mg of gallic acid g^{-1} DW of plant material. Flavonoids and anthocyanins were measured in the supernatant prepared for the total phenolic analysis, by measuring the absorbance at 300 nm and at 530 and 657 nm, respectively, using a UV–Vis 160A Spectrophotometer (Shimadzu, Tokyo, Japan). The concentrations of flavonoids and anthocyanins (nmol g^{-1} DW) were calculated using an extinction coefficient of 26.9 mM^{-1}.

Chlorophylls, extracted by homogenisation of 0.2 g of fresh leaves with 25 ml of 80% acetone, were calculated according to Wellburn [36], after measuring the absorbance at 645 and 663 nm.

Acid-soluble thiols (total thiols) were determined according to Jocelyn [37], after the homogenisation of 0.1 g of plant material with 0.4 ml of 25 mg ml^{-1} $NaBH_4$, in 0.1 M NaOH, and 0.2 ml of distilled water. Their absorbance at 412 nm was determined spectrophotometrically after the reaction with Ellman reagent. For calibration, a standard curve was elaborated with suitable GSH concentrations.

The thiol profile was analysed by HPLC following the procedure described by Sobrino-Plata et al. [38]. Extracts (100 μl) were injected in a Mediterranea SEA18 column (250 × 4.6 mm; Teknokroma, Sant Cugat del Vallés, Spain), using an Agilent Technologies 1200 series HPLC system (Santa Clara CA, USA). Thiols were detected after post-column derivatisation with Ellman reagent and quantified against a GSH standard curve. A spike of *N*-acetyl cysteine (*N*-AcCys) was added as internal standard prior to homogenisation. The identification of thiols was achieved by comparing the retention times of peaks from each sample with those of commercially-available standards: Cys and GSH were purchased from Sigma–Aldrich (St. Louis, MO, USA) and PC2, PC3 and PC4 from AnaSpec (Fremont, CA, USA).

Lipid peroxidation was measured as the MDA concentration in the plant organs, following the procedure described by Heath and Packer [39]. Frozen material (0.1 g) was homogenised in 1 ml of 15% trichloroacetic acid and 0.37% thiobarbituric acid in 0.25 M HCl. After centrifugation at 12,000×*g* for 10 min, at 4 °C, the MDA concentration in the supernatant was calculated from the absorbance measured at 532 nm (background corrected at 600 nm) using the extinction coefficient of 1.56×10^5 M^{-1}.

4.5. Statistical analysis

The data were analysed using SPSS version 17.0 (Statistical Package for Social Science for Windows, SPSS, Inc., Chicago, IL, USA). All values reported are the means of three replicates (±standard error). Statistical analyses were carried out by analysis of variance (ANOVA): significant differences among mean values were determined by Tukey's test at $P < 0.05$. A principal component analysis (PCA) was performed to detect key variables contributing to data variability and to identify the relationship between TE concentrations in plants and stress indicators. This PCA was performed using Varimax normalised rotation, retaining only eigenvalues higher than 1 (Kaiser criterion). The results obtained for each species were expressed as a matrix of rotary components; coefficients with values lower than 0.5 were eliminated.

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