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Sensitivity of Mediterranean woody seedlings to copper, nickel and zinc

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Abstract

The restoration of heavy metal contaminated areas requires information on the response of native plant species to these contaminants. The sensitivity of most Mediterranean woody species to heavy metals has not been established, and little is known about phytotoxic thresholds and environmental risks. We have evaluated the response of four plant species commonly used in ecological restoration, *Pinus halepensis*, *Pistacia lentiscus*, *Juniperus oxycedrus*, and *Rhamnus alaternus*, grown in nutrient solutions containing a range of copper, nickel and zinc concentrations. Seedlings of these species were exposed to 0.048, 1 and 4 μM of Cu; 0, 25 and 50 μM of Ni; and 0.073, 25 and 100 μM of Zn in a hydroponic silica sand culture for 12 weeks. For all four species, the heavy metal concentration increased in plants as the solution concentration increased and was always higher in roots than in shoots. *Pinus halepensis* and *P. lentiscus* showed a higher capacity to accumulate metals in roots than *J. oxycedrus* and *R. alaternus*, while the allocation to shoots was considerably higher in the latter two. Intermediate heavy-metal doses enhanced biomass accumulation, whereas the highest doses resulted in reductions in biomass. Decreases in shoot biomass occurred at internal concentrations ranging from 25 to 128 $\mu g\ g^{-1}$ of Zn, and 1.7 to 4.1 $\mu g\ g^{-1}$ of Cu. Nickel phytoxicity could not be established within the range of doses used. *Rhamnus alaternus* and *J. oxycedrus* showed higher sensitivity to Cu and Zn than *P. halepensis* and, especially, *P. lentiscus*. Contrasted responses to heavy metals must be taken into account when using Mediterranean woody species for the restoration of heavy metal contaminated sites.

Keywords: Phytotoxicity; Heavy metals; Restoration; Mediterranean seedlings

1. Introduction

Small amounts of heavy metals are present in most natural soils, but human activity has increased their presence to exceptionally high levels on many polluted sites (Adriano, 2001). Restoration of heavy metal contaminated areas must take into account both phytotoxicity and the risk of incorporating heavy metals into food chains. The main sources of heavy-metal pollution in natural soils are waste products from mining and ore-processing operations (Hüttermann et al., 2004), diffuse pollution in industrial and urban areas (Azimi et al., 2003) and biosolid land applications (Smith, 1996). Agricultural activities currently absorb half of the biosolids produced in water treatment plants (USEPA, 1999; European Commission, 2000). Biosolid applications for the reclamation of degraded areas and for the production of growth substrates represent novel scenarios of heavy metal incorporation into natural soils.

Studies on the effects of heavy metals on plant performance have traditionally focused on grasses and agricultural species (Berti and Jacobs, 1996; Cobb et al., 2000).

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Comparatively scant information is available on the response of many of the woody species commonly used in ecological restoration. This is particularly true for Mediterranean woody species. Woody species can be very sensitive to moderate concentrations of heavy metals (Balsberg Påhlsson, 1989). Heavy metals can reduce biomass accumulation in tree seedlings (Kukkola et al., 2000), inhibit root growth (Arduini et al., 1995; Hartley et al., 1999), decrease the availability of essential elements (Kabata-Pendias and Pendias, 1992), and modify root morphology and architecture, thus compromising root capacity to explore soils (Arduini et al., 1994; Schmidt, 1997). Heavy metals can also affect seedling performance indirectly by reducing the ability of the plant to access and transport soil resources, particularly water (Barceló and Poschenrieder, 1990), which could compromise the plant's capacity to withstand adverse climatic conditions.

We have carried out an experiment to assess the sensitivity of four Mediterranean woody species, *Pinus halepensis* Mill., *Juniperus oxycedrus* L., *Pistacia lentiscus* L. and *Rhamnus alaternus* L., when grown in nutrient solutions containing a range of Cu, Ni and Zn concentrations, by defining both the internal plant tissue concentration and the external concentration at which seedling decline begins. These four species are classified as potential natural vegetation in arid and semi-arid Mediterranean areas (Cortina et al., 2005), and they are currently being used in restoration activities on unpolluted sites.

2. Materials and methods

2.1. Plant growing conditions

Our experimental design included 4 species, 3 heavy metals, 3 heavy metal application doses, and 15 replicates in a complete factorial design. Seeds of *P. halepensis* Mill., *P. lentiscus* L., *J. oxycedrus* L., and *R. alaternus* L. from local provenances were germinated in separate 200 ml containers filled with washed silica sand. Two weeks after emergence (once cotyledons opened), the containers were watered on alternate days with a nutrient solution containing 170 μM NH_4NO_3, 42 μM KH_2PO_4, 88 μM KNO_3, 3.1 μM $MgSO_4 \cdot 7H_2O$, 0.27 μM Fe-EDTA, 0.19 μM H_3BO_3, 0.14 μM Mn-EDTA, 0.073 μM Zn-EDTA, 0.047 μM Cu-EDTA, 0.0004 μM $(NH_4)_6Mo_7O_{24}$ to saturation, and allowed to drain freely. The pH of the nutrient solution was 5.5 and the electrical conductivity 55 μS cm^{-1}. This pH value was selected to maintain high heavy metal availability levels in the nutrient solution. Most Mediterranean soils show pH values above this level, but the species used here can be found in soils with pH ranging from 6 to 8.5. We added water-soluble sulfate salts of Cu, Ni and Zn to this solution to obtain concentrations of 0.073, 25 and 100 μM of Zn, 0.047, 1 and 4 μM of Cu, and 0, 25 and 50 μM of Ni. Each metal and dose was applied separately to a different set of seedlings.

Similar doses have evidenced strong effects on woody plant performance in hydroponic cultures (Arduini et al., 1995; Miller and Cumming, 2000; Reichman, 2002), and these solutions can be considered to range from normal to contaminated soil, depending on the extraction method used (Kabata-Pendias and Pendias, 1992; Baker and Senft, 1995; Adriano, 2001). They can be found in sludged areas (Knight et al., 1997; Walker et al., 2003), near mining zones (Poschenrieder et al., 2001), or on restoration sites where biosolid application is used, i.e., in reforestation amendments or artificially created quarry soils (Barrera et al., 2001; Ortiz and Alcañiz, 2006). Arduini et al. (1994, 1995) suggested that heavy metal accumulation in the upper forest soil layers, even at relatively low concentrations, could impair the natural regeneration of forest species. Our seedlings were kept in a glasshouse for three months at a 20–25 °C ambient temperature and a 14-h photoperiod.

2.2. Seedling response

At the end of the growing period we harvested all the seedlings, separated shoots from roots, and carefully washed the root system with distilled water. Rooting systems from 10 seedlings per species, metal and dose were randomly selected and digitized by scanning on a flatbed scanner fitted with a transparency adapter. The images were analyzed with specific software (WinRhizo, Regent Instruments, Québec, Canada) to assess root length and average root diameter. Finally, we dried all biomass fractions at 65 °C for 48 h, and determined the dry weight of all the seedlings. Specific root length was calculated as the ratio between total length and root biomass. Biomass fractions from five seedlings per species, heavy metal and dose were later digested in a heating block at 250 °C with a mixture of sulfuric acid (96%) and hydrogen peroxide (30%) (1:1, v:v) (Jones and Case, 1990). Digests were then analyzed for Cu, Ni and Zn by ICP-OES (Perkin Elmer Optima 4300 Inductively Coupled Plasma-Optical Emission Spectrometry). *Olea europaea* leaf standard reference materials (BCR: CRM 062, Commission of the European Communities Bureaus of Reference, Brussels) were digested and analyzed for quality control.

2.3. Heavy-metal translocation to shoots

We calculated the percentage of heavy-metal translocation to shoots as the ratio between the heavy metal content in shoots divided by the total metal content in the whole seedling.

2.4. Phytotoxicity estimation

We evaluated the phytotoxic effects of heavy metal accumulation by estimating both the tissue concentration corresponding to the onset of the decrease in dry matter accumulation (phytotoxic threshold; PT) for each heavy metal, and the tissue concentration resulting in a 10% reduction of the estimated maximum shoot and root biomass accumulation (PT10) (Alloway, 1995; Reichman, 2002). These parameters were estimated by fitting the response curves to peak models using SigmaPlot 7.0 SPSS Inc. The model providing the best fit was used for these calculations. When no model could be adjusted, we assumed the absence of measurable phytotoxic effects under the assessed experimental conditions.

2.5. Data analysis

We evaluated the effect of each heavy metal on plant traits by applying one-way ANOVA for one fixed factor with three levels (doses). Levels within each factor were compared by applying Tukey's test at a 0.05 significance level when ANOVA showed a significant treatment effect. Data were transformed when needed to ensure homoscedasticity. All statistical analyses were carried out with the SPSS v.10.0 statistical package (SPSS Inc., Chicago, USA).

3. Results

3.1. Heavy metal bioaccumulation

Shoot Cu accumulation was below 7 μg g^{-1} in all species (Table 1). The effect of Cu addition on shoot Cu concentration was significant in *J. oxycedrus* and *P. lentiscus*, but not in *P. halepensis* and *R. alaternus*. Nickel and Zn concentration in shoots increased in all species in response to Ni and Zn application, especially at the highest application rates. *Rhamnus alaternus* showed higher heavy metal concentration in shoots than the other species tested, except for Zn in seedlings treated with 25 μM Zn.

Heavy metal concentration in roots was higher than in shoots in all species. Heavy metal concentration in roots increased in response to heavy metal application in the nutrient solution, but the significance of these differences depended on the species and the heavy metal used. *Pinus halepensis* showed the highest heavy metal accumulation in roots, except for the Ni concentration in seedlings with the lowest Ni application rate.

Rhamnus alaternus showed the highest capacity for heavy metal transport to shoots, ranging from 23% to 47% of the total plant accumulation (Table 2). *Pistacia lentiscus* and *P. halepensis* showed the lowest rates of metal transport to shoots, whereas *J. oxycedrus* showed intermediate values. All three heavy metals showed similar behavior patterns but different mobility within the seedlings. Except for *R. alaternus*, the increased uptake and accumulation of Cu and Zn in the root system was coupled with a decreased translocation of Cu and Zn to the shoot, although few differences were found between the two higher doses applied. Copper translocation in *R. alaternus* decreased only with the application of 4 μM Cu, while in the case of Zn, this value did not decrease, even at the highest dose. In all cases, metal transport rates were highest for Zn, followed by Cu and Ni, except for *R. alaternus* which showed similar values for the three heavy metals.

3.2. Biomass accumulation and root morphology

The effect of heavy metal application on biomass accumulation showed a positive trend at intermediate application rates, particularly for Cu and Zn, and a negative trend at the highest doses, mainly for Zn (Fig. 1). Application of 4 μM Cu in the nutrient solution decreased *P. halepensis* shoot and root biomass by 27% and 33% respectively,

Table 1
Copper, nickel and zinc concentration in seedlings of four Mediterranean woody species exposed to different doses of these elements for three months

Dose (μM)		*J. oxycedrus*		*P. halepensis*		*P. lentiscus*		*R. alaternus*	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Cu concentration (μg g^{-1})									
Cu	0.047	1.0 ± 0.2a	7.7 ± 2.0a	3.0 ± 1.0	33 ± 5a	0.9 ± 0.4a	17 ± 2a	2.5 ± 0.2	9 ± 1a
	1	1.7 ± 0.4ab	37.0 ± 8.0b	1.2 ± 0.2	122 ± 16b	2.0 ± 0.4ab	96 ± 7b	4.1 ± 1.2	18 ± 2b
	4	3.9 ± 0.9b	71.0 ± 5.0c	4.0 ± 0.9	216 ± 44b	3.6 ± 0.5b	135 ± 21b	6.8 ± 2.0	51 ± 10c
	F	6.73^{*}	32.6^{**}	2.66	16.8^{**}	8.9^{**}	79.6^{**}	2.20	35.6^{**}
Ni concentration (μg g^{-1})									
Ni	0	0.1 ± 0.0a	0.9 ± 0.2a	<0.05a	0.8 ± 0.5a	<0.05a	0.9 ± 0.4a	0.2 ± 0.2a	0.1 ± 0.1a
	25	3 ± 0b	68 ± 20b	12 ± 1b	350 ± 23b	10 ± 2b	322 ± 70b	18 ± 4b	71 ± 13b
	50	6 ± 1c	137 ± 28b	14 ± 3b	569 ± 97c	11 ± 2b	446 ± 42b	33 ± 4c	130 ± 31b
	F	139^{**}	65.2^{**}	10.4^{**}	24.9^{*}	135^{**}	15.7^{**}	30.2^{**}	158^{**}
Zn concentration (μg g^{-1})									
Zn	0.073	10 ± 4a	28 ± 12a	33 ± 10a	88 ± 18a	5 ± 2a	64 ± 3a	5 ± 3a	55 ± 13a
	25	22 ± 5b	236 ± 35b	81 ± 7a	721 ± 67b	19 ± 4b	330 ± 56b	45 ± 16b	351 ± 174b
	100	272 ± 53c	2405 ± 368c	240 ± 26b	3444 ± 686c	182 ± 20c	2753 ± 330c	531 ± 83c	2016 ± 331c
	F	42.8^{**}	96.5^{**}	31.9^{**}	122^{**}	51.5^{**}	240^{**}	48.9^{**}	24.4^{**}

Means and standard errors of $n = 4–5$ plants and results of one-way ANOVA for each heavy metal, species and biomass fraction are shown. Asterisks denote significant differences at the 0.05 (one) and 0.01 (two) levels. Results of Tukey's test ($P < 0.05$) are indicated by letters within the same column and heavy metal.

Table 2
Percentage of Cu, Ni and Zn translocated to shoots of four Mediterranean woody species exposed to different doses of these elements for three months

Dose (μM)		Translocation to shoots (%)			
		J. oxycedrus	*P. halepensis*	*P. lentiscus*	*R. alaternus*
Cu	0.047	19.0 ± 5.4a	9.4 ± 3.8a	12.6 ± 2.9a	40.2 ± 9.9ab
	1	9.3 ± 1.9ab	0.7 ± 0.2b	3.0 ± 0.6b	46.9 ± 7.8a
	4	8.2 ± 2.3a	1.8 ± 0.6b	4.2 ± 1.2b	23.5 ± 6.2b
	F	4.41*	11.40*	4.50*	4.26*
Ni	0[a]	–	–	–	–
	25	5.2 ± 1.9	3.8 ± 0.4	3.8 ± 0.9	44.1 ± 17.5
	50	6.8 ± 2.2	2.7 ± 1.0	4.4 ± 1.1	30.3 ± 6.3
	F	2.64	2.56	0.17	2.48
Zn	0.073	46.0 ± 14.5a	33.1 ± 6.2a	17.7 ± 8.0	31.2 ± 17.5
	25	17.6 ± 4.4b	11.8 ± 1.4b	13.5 ± 5.4	24.8 ± 12.8
	100	13.5 ± 3.9b	6.2 ± 1.5b	10.0 ± 2.0	40.9 ± 8.3
	F	14.83**	38.50**	0.56	2.60

Means, standard errors of $n = 4$–5 plants and results of one-way ANOVA are shown. Asterisks denote significant differences at the 0.05 (one) and 0.01 (two) levels. Results of Tukey's test ($P < 0.05$) are indicated by letters within the same column and heavy metal.
[a] Concentration in shoots and roots <1 μg g^{-1}.

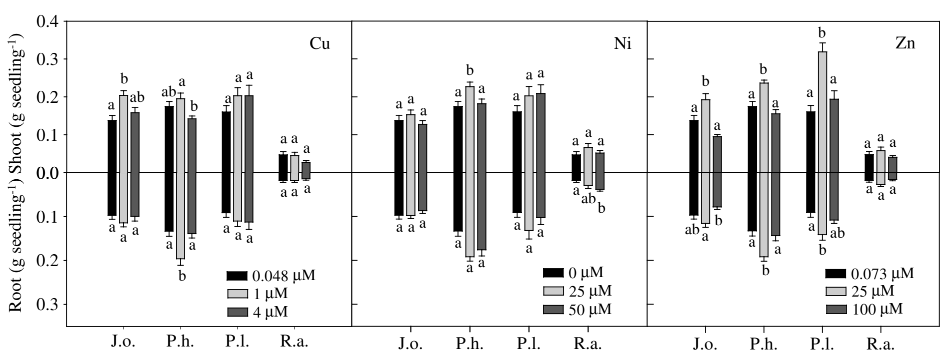

Fig. 1. Biomass accumulation of *Juniperus oxycedrus* (J.o.), *Pinus halepensis* (P.h.), *Pistacia lentiscus* (P.l.), and *Rhamnus alaternus* (R.a.) seedlings exposed to different doses of Cu (left), Ni (center) and Zn (right) for three months in hydroponic culture. Means and standard errors of $n = 14$–15 replicated seedlings are shown. Results of Tukey's tests ($P < 0.05$) for a given heavy metal, biomass fraction and species are indicated by letters.

as compared with the 1 μM Cu dose. In *J. oxycedrus*, the shoot biomass of seedlings exposed to 1 μM Cu was significantly higher than that found in seedlings receiving the lowest dose, and it decreased at 4 μM Cu. *Pistacia lentiscus* and *R. alaternus* were not significantly affected by Cu application. Exposure to 100 μM Zn reduced shoot and root biomass (by ca. 35% and 24%, respectively) in all species, as compared with exposure to 25 μM Zn. *Juniperus oxycedrus* showed the highest reductions in shoot (50%) and root (33%) biomass as the Zn dose increased from 25 to 100 μM. This effect was marginally significant both in *P. lentiscus* roots and in *R. alaternus* roots and shoots. When Ni was applied at 50 μM, it reduced shoot biomass in *P. halepensis* seedlings by 20% and caused a significant increase in *R. alaternus* root biomass.

Root length in *J. oxycedrus* seedlings treated with Zn and in *P. halepensis* seedlings treated with the three heavy metals evaluated in this study, increased at intermediate application rates, and decreased at the highest rates, as compared with the maximum root length achieved (Table 3). Copper application resulted in a gradual increase in root length in *J. oxycedrus*. In contrast, *R. alaternus* seedlings responded to Zn application by decreasing root length at the highest application dose. *Pistacia lentiscus* showed increased root length after application of the three metals, but this effect was only significant when applied at 25 μM Ni. Specific root length (SRL) showed very limited response to heavy metal application. Only *P. halepensis* showed a significant decrease in this variable when exposed to 50 μM Ni.

3.3. Phytotoxicity levels

A significant peak model could be fitted to 13 of the 24 combinations of species, biomass fractions and heavy metals. Log normal and Gaussian distribution functions were used in all cases. The amount of variability in biomass accumulation explained by these functions ranged from 32% to 65%. *Juniperus oxycedrus* and *R. alaternus* were very sensitive to Cu, as they showed reductions in shoot biomass when Cu concentration was above 1.75 and 4.1 μg g^{-1}, respectively (Table 4). Critical Cu concentration

Table 3
Root length (L) and specific root length (SRL) of four Mediterranean woody species exposed to different doses of Cu, Ni and Zn for three months

Dose (μM)		*J. oxycedrus*		*P. halepensis*		*P. lentiscus*		*R. alaternus*	
		L (cm)	SRL (m g^{-1})	L (cm)	SRL (m g^{-1})	L (cm)	SRL (m g^{-1})	L (cm)	SRL (m g^{-1})
Cu	0.048	137 ± 12a	19.0 ± 1.2	251 ± 14a	19.8 ± 0.9	357 ± 53	50 ± 6	115 ± 25	44 ± 5
	1	178 ± 24ab	20.0 ± 1.4	384 ± 21b	19.3 ± 0.4	495 ± 50	51 ± 7	122 ± 25	57 ± 8
	4	208 ± 17b	18.9 ± 1.3	269 ± 23a	21.1 ± 1.0	569 ± 121	56 ± 6	97 ± 24	57 ± 10
	F	3.99^{*}	0.23	10.9^{**}	1.21	2.57	0.22	0.28	0.64
Ni	0	137 ± 12	19.0 ± 1.2	251 ± 14a	19.8 ± 0.9a	357 ± 53a	50 ± 6	115 ± 25	44 ± 5
	25	154 ± 23	16.0 ± 1.0	329 ± 19b	16.8 ± 0.4ab	631 ± 72b	46 ± 3	90 ± 24	42 ± 5
	50	157 ± 13	16.9 ± 0.6	229 ± 22a	15.8 ± 0.9b	564 ± 80ab	52 ± 5	111 ± 24	29 ± 4
	F	0.45	2.74	5.99^{*}	6.81^{**}	4.16^{*}	0.39	0.30	2.71
Zn	0.073	137 ± 12a	19.0 ± 1.2	251 ± 14a	19.8 ± 0.9	357 ± 53	50 ± 6	115 ± 25a	44 ± 5
	25	217 ± 30b	17.5 ± 1.3	410 ± 15b	21.5 ± 1.2	517 ± 61	41 ± 3	114 ± 15a	37 ± 3
	100	142 ± 12a	19.0 ± 1.5	222 ± 25a	17.4 ± 1.5	488 ± 68	44 ± 4	57 ± 6b	42 ± 4
	F	5.91^{*}	0.34	29.4^{**}	2.77	1.93	1.01	5.39^{*}	0.71

Means, standard errors of $n = 8$–10 replicates and results of one-way ANOVA are shown. Asterisks denote significant differences at the 0.05 (one) and 0.01 (two) levels. Results of Tukey's test ($P < 0.05$) for each heavy metal are indicated by letters within the same column.

Table 4
Models describing the relationship between heavy metal concentration in shoots and roots, and biomass accumulation for four Mediterranean woody species

Biomass fraction	Metal	Species	R^2	Model	PT[a]	PT10[a]
Shoot	Cu	*J. oxycedrus*	0.551^{*}	$y = 0.16 + 0.113 * \exp(-0.5 * (\ln(x/1.53)/0.23)^2)$	1.7	2.0
		R. alaternus	0.480^{*}	$y = 0.063 * \exp(-0.5 * (\ln(x/4.06)/0.59)^2)$	4.1	5.3
	Zn	*J. oxycedrus*	0.563^{**}	$y = 0.19 * \exp(-0.5 * (\ln(x/25.03)/2.27)^2)$	25	65
		P. halepensis	0.572^{*}	$y = 0.22 * \exp(-0.5 * ((x - 127.74)/169.52)^2)$	128	206
		P. lentiscus	0.585^{**}	$y = 0.35 * \exp(-0.5 * (\ln(x/36.57)/1.42)^2)$	37	70
Root	Cu	*J. oxycedrus*	0.368^{*}	$y = 0.11 * \exp(-0.5 * (\ln(x/25.38)/1.63)^2)$	25	54
		P. halepensis	0.652^{**}	$y = 0.24 * \exp(-0.5 * ((x - 165.7)/105.5)^{1.11})$	165	190
		R. alaternus	0.512^{*}	$y = 0.47 * \exp(-0.5 * (\ln(x/12.63)/0.38)^2)$	13	16
	Ni	*P. halepensis*	0.392^{*}	$y = 0.18 * \exp(-0.5 * ((x - 493.3)/571.2)^2)$	495	756
	Zn	*J. oxycedrus*	0.323^{*}	$y = 0.11 * \exp(-0.5 * (\ln(x/107.1)/3.65)^2)$	107	628
		P. halepensis	0.630^{**}	$y = 0.19 * \exp(-0.5 * (\ln(x/511.1)/2.19)^2)$	512	1462
		P. lentiscus	0.377^{*}	$y = 0.1344 * \exp(-0.5 * (\ln(x/650.5)/2.11)^2)$	650	1705
		R. alaternus	0.394^{*}	$y = 0.050/(1 + ((x - 549.56)/523.9)^2)$	549	724

PT corresponds to heavy metal concentration in shoots and roots at the onset of the decrease in dry matter production, and PT10 corresponds to the tissue concentration resulting in a 10% reduction in the estimated maximum biomass ($n = 14$–15). Only significant models are shown. Asterisks denote significant differences at the 0.05 (one) and 0.01 (two) levels.
[a] Values for phytotoxicity thresholds are in μg g^{-1}.

in roots ranged from 13 μg g^{-1} in *R. alaternus* to 165 μg g^{-1} in *P. halepensis*. Critical Zn concentrations in shoots ranged from 25 μg g^{-1} in *J. oxycedrus* to 128 μg g^{-1} in *P. halepensis* (Fig. 2). In roots, *J. oxycedrus* was more sensitive to Zn than the others species; it showed the lowest PT and PT10 values. The critical Zn concentration was similar in *P. halepensis*, *P. lentiscus* and *R. alaternus* (between 512 and 650 μg g^{-1}), but PT10 values suggested that *R. alaternus* was more sensitive to increases in Zn root concentration than the other two species. Nickel application showed a significant phytotoxicity threshold only in *P. halepensis* roots (495 μg g^{-1}).

4. Discussion

Copper, nickel and zinc toxicity has been studied in several woody species (Balsberg Påhlsson, 1989; Miller and Cumming, 2000; Reichman et al., 2001), but to our knowledge, it has not previously been studied in the species evaluated in the present experiment.

Cu and Zn concentrations in seedling shoots exposed to the lowest heavy-metal doses could be considered either deficient (Kabata-Pendias and Pendias, 1992) or critical (Marschner, 1995) in vascular plants, but little information is available on the optimal concentration for the species studied (Boardman et al., 1997). The maximum heavy metal concentration recorded in seedlings exposed to high doses was similar to that found in other works with woody species both in hydroponic cultures (Arduini et al., 1995; Reichman et al., 2001) and in pot trials with forest soil (Kukkola et al., 2000), but it was low when compared with the maxima found in native grass species used in restoration (Paschke et al., 2000; Gonnelli et al., 2001; Paschke and Redente, 2002).

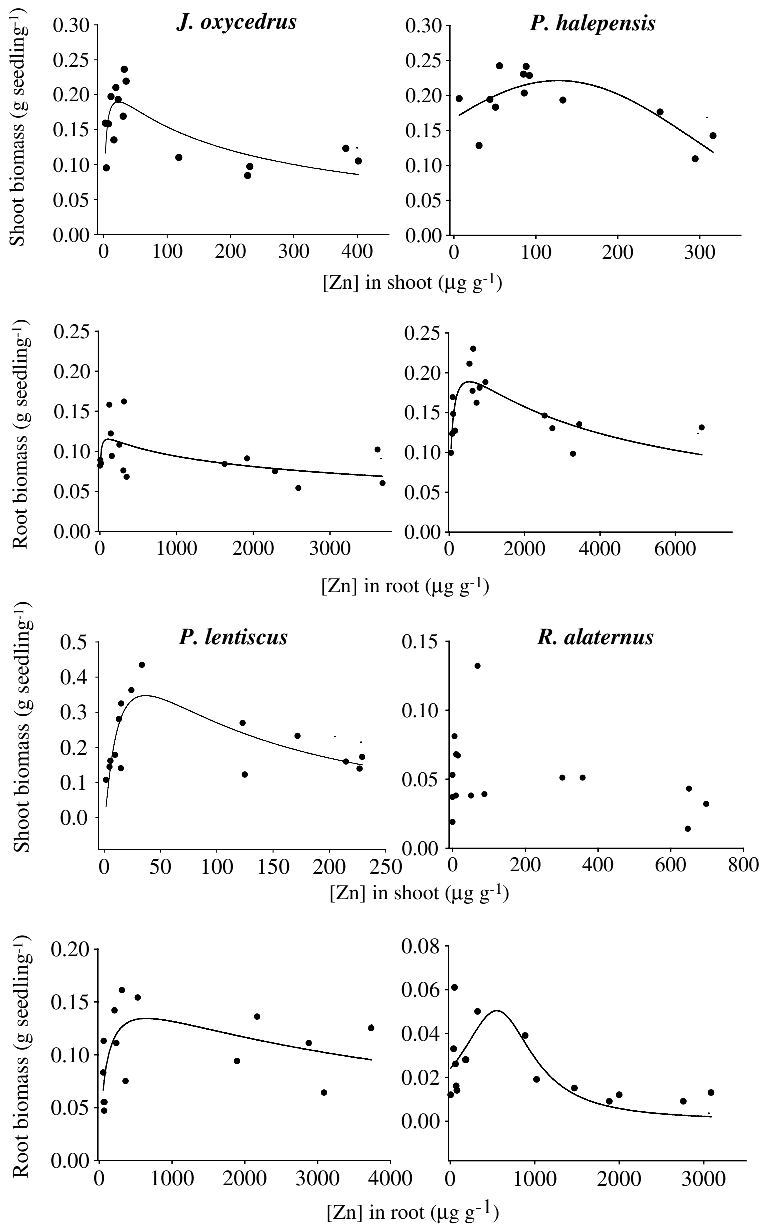

Fig. 2. Relationships between Zn concentration and biomass accumulation in shoots and roots of four Mediterranean woody species. Each point represents one seedling treated with either 0.073, 25 or 100 μM Zn. No function could be adjusted to the observed data in *R. alaternus* shoots (see Table 4). Dotted lines represent the confidence intervals (95%). Note different *X* and *Y* scales.

Rhamnus alaternus shoots exposed to a 100 μM Zn heavy metal concentration exceeded the values considered to be phytotoxic (Balsberg Påhlsson, 1989; Kabata-Pendias and Pendias, 1992). The value attained by these seedlings (531 ± 83 μg g^{-1} Zn) was higher than the tolerable concentration for livestock grazing (300–500 μg g^{-1} Zn; Chaney, 1989).

Heavy metal accumulation was always higher in roots than in shoots, especially in *P. lentiscus* and *P. halepensis*, which showed a lower heavy-metal translocation ability than *J. oxycedrus* and *R. alaternus*. The specific response mechanisms of these species to heavy metal are still unknown. Decreased metal uptake at the plasma membrane level by either deposition on cell wall components or chelate secretion is the most common mechanism of plant adaptation to metal toxicity (Briat and Lebrun, 1999). Nevertheless, it has been suggested that neither the heavy metal accumulation ability nor the distribution of heavy metals within the plant is consistently correlated with plant tolerance (Reichman et al., 2001; Greger, 2004).

Heavy metal application caused a decrease in root uptake efficiency (estimated as the heavy metal concentration ratio between root and nutrient solution) in all species, probably due to an excess of heavy metal concentration per unit of root absorption area and the subsequent saturation in the root tissues (Greger, 2004). Uptake and translocation were also dependent on the type of metal. Usually, Cu transport to aboveground fractions is restricted (Arduini et al., 1996), and in our study, with the exception of *R. alaternus*, root systems were effective barriers to metal translocation. The higher Zn uptake and transport observed in all species, as compared with Cu and Ni, have been observed elsewhere (Kabata-Pendias and Pendias, 1992; Chojnacka et al., 2005).

Overall growth in the four species was low when the lowest metal doses were applied, probably due to weak Cu and Zn deficiencies (see above). Intermediate doses of heavy metals probably improved the nutritional status and thus promoted biomass accumulation. This effect has been observed in other studies (Paschke et al., 2000; Paschke and Redente, 2002; Reichman, 2002) and could reflect a typical dose–response behavior of essential heavy metals in plants (Shaw et al., 2004). Growth stimulation at low levels of toxic elements may also reflect behavioral plasticity to chemical stress (Kabata-Pendias and Pendias, 1992; Arduini et al., 1994).

The complexity of the edaphic medium still makes it difficult to generalize on reliable toxic concentrations in different soils (Poschenrieder and Barceló, 2004). Soil pH, organic matter, CEC and texture, among others, determine the solubility and speciation of heavy metals in the soil and, thus, their availability to plants (Alloway, 1995). Whether or not a critical soil solution is reached in a contaminated soil will depend heavily on these soil conditions. It is known that seedlings may respond to heavy metal enrichment differently in hydroponic cultures than in field experiments (Stoltz and Greger, 2002), but soilless experiments permit the isolation of particular factors to establish phytotoxicity thresholds (Schmidt, 1997; Reichman, 2002).

It has been suggested that critical concentrations should be defined on the basis of the heavy metal concentration in tissue (Davis and Beckett, 1978; Poschenrieder and Barceló, 2004), rather than on heavy metal availability in soils. We have used fitted models to establish these critical concentrations, but our results must be interpreted carefully, since they show considerable variation. Under our experimental conditions, critical Zn root and shoot concentrations were similar to those found in other studies on phytotoxicity in woody plants. Hartley et al. (1999) found ca. 40% reductions in root and shoot biomass in Scots pine seedlings exposed to heavy metals for three months, when root and shoot Zn concentrations were 750 and 300 $\mu g\ g^{-1}$, respectively. Shoot biomass in the seedlings of three Australian species decreased for foliar concentrations ranging from 70 to 370 $\mu g\ g^{-1}$ Zn (Reichman et al., 2001), showing root concentrations similar to those found in roots of *P. halepensis* and *P. lentiscus* in the present experiment. Root sensitivity to Zn, as estimated by PT10, decreased in the following order: *J. oxycedrus* > *R. alaternus* > *P. halepensis* > *P. lentiscus*, whereas the ranking for shoots was *J. oxycedrus* > *P. lentiscus* > *P. halepensis* (the relationship was not significant for *R. alaternus*).

Pinus halepensis was the only species sensitive to Ni application. Nickel concentration in roots was similar to values measured in *Pinus sylvestris* seedlings exposed to 85–170 μM Ni (Ahonen-Jonnarth and Finlay, 2001). In both cases the effect of Ni application on seedling biomass was similar: reduction in the aboveground biomass and increase in the root:shoot ratio. Seedlings exposed to intermediate dose of Ni showed higher root growth than seedlings given the lowest application rate. Nickel can be regarded as essential for plant growth (Gerendás et al., 1999); the positive effects of Ni application have been attributed to enhanced permeability across the plasmalemma, leading to a free flow of nutrients (Kukkola et al., 2000; Ahonen-Jonnarth et al., 2004). As we found no negative responses to Ni application, we were unable to define phytotoxicity levels for this element.

The concentrations of Zn and Ni required to inhibit root growth are usually higher than for Cu (Woolhouse, 1983), which has been described as the most toxic heavy metal found in soil solutions (Baker et al., 1994). Reductions in root and shoot biomass accumulation began at relatively low Cu levels and reached PT10 values with low internal Cu increments. The shoot and root Cu concentrations found by Kukkola et al. (2000) in Scots pine grown for three months in Cu and Ni enriched mineral soils, were similar to those found in *P. halepensis* seedlings exposed to 4 μM Cu in our experiment. With the same external supplies, Arduini et al. (1996) found both much higher Cu concentrations in the roots of *Pinus* and *Fraxinus* seedlings grown in hydroponic culture and more evident effects on root length and root biomass than those found in the current experiment. Root sensitivity to Cu, as estimated by PT10 values, was *R. alaternus* > *J. oxycedrus* > *P. halepensis* > *P. lentiscus*, whereas the ranking for shoots was *J. oxycedrus* > *R. alaternus* > *P. halepensis* ≈ *P. lentiscus*.

In contrast with other works, root biomass was more sensitive than root length to heavy metals (Denny and Wilkins, 1987). It should be taken into account that in our experiment the seedlings were grown in 15-cm-deep sand-filled containers and that this could affect root morphology in different ways than in other culture mediums. The decreases in specific root length detected in *P. halepensis* and *R. alaternus* after Ni and Zn application point to increases in root lignification, as reported in other studies (Arduini et al., 1995; Kukkola et al., 2000). The low severity of this effect, as well as the weak changes in root average diameter (data not shown), could result from using entire root systems in our analysis, rather than only lateral or apical roots which may be more sensitive to heavy metals (Arduini et al., 1995; Greger, 2004). Other works have shown inhibition of root elongation in tree species subjected to doses of Cu and Zn similar to those used here (Arduini et al., 1994; Reichman et al., 2001). This has been related to damages in the plasma membrane (Woolhouse, 1983) affecting root elongation and promoting cell wall lignification (Marschner, 1995).

The species tested showed contrasted sensitivity to Cu, Ni and Zn. *Pinus halepensis* and *P. lentiscus* tolerated higher internal metal concentrations before showing negative effects on plant performance, as compared to *J. oxycedrus* and *R. alaternus*. Moreover, *P. lentiscus* and *P. halepensis* can accumulate almost three and five times less Zn and Ni, respectively, in aboveground parts for a given amount of aboveground biomass than *J. oxycedrus* and *R. alaternus*, as a result of the formers' enhanced capacity for metal retention in roots. These results provide criteria for the use of these species for the restoration of contaminated areas, since they also reduce the potential risks of heavy metals entering food chains.

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