



Sensitivity to zinc of Mediterranean woody species important for restoration

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ABSTRACT

Heavy metals have increased in natural woodlands and shrublands over the last several decades as a consequence of anthropogenic activities. However, our knowledge of the effects of these elements on woody species is scarce. In this study, we examined the responses of six Mediterranean woody species to increasing levels of zinc in hydroponic culture and discussed the possible implications for the restoration of contaminated sites. The species used, *Pinus pinea* L., *Pinus pinaster* Ait., *Pinus halepensis* Mill., *Tetraclinis articulata* (Vahl) Mast., *Rhamnus alaternus* L. and *Quercus suber* L. represent a climatic gradient from dry sub-humid to semi-arid conditions. Zinc concentrations in shoots ranged from 53 $\mu\text{g g}^{-1}$ in *Q. suber* to 382 $\mu\text{g g}^{-1}$ in *T. articulata* and were well below the levels found in roots. Zinc inhibited root elongation and root biomass and changed the root length distribution per diameter class, but the magnitude of the effects was species-specific. Only *P. halepensis* and *Q. suber* showed toxicity symptoms in aboveground parts. Species more characteristic from xeric environments (*T. articulata*, *R. alaternus* and *P. halepensis*) were more sensitive to zinc than species from mesic environments (*Q. suber*, *P. pinaster* and *P. pinea*). According to the Zn responses and bioaccumulation, *Q. suber*, *P. pinea* and *P. halepensis* are the best candidates for field trials to test the value of woody species to restore contaminated sites. None of the species tested seemed suitable for phytoremediation.

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1. Introduction

Human activities are contributing to a worldwide rise in the concentrations and bioavailability of zinc (Zn) in natural ecosystems (Kabata-Pendias and Pendias, 1992). Zinc is required at low levels (e.g., 20–100 $\mu\text{g g}^{-1}$ dry weight in leaves) for plant enzymatic activity, maintenance of biomembrane integrity, carbohydrate metabolism and protein synthesis, and it participates in the metabolism of indoleacetic acid (Marschner, 1995; Cakmak, 2000). But at high concentrations (e.g., above 200 $\mu\text{g g}^{-1}$ d.w. in leaves), Zn has toxic effects on plants (Balsberg Pålsson, 1989; Adriano, 2001). High levels of this element can impair root development and elongation rate (Reichman et al., 2001; Fuentes et al., 2007), alter xylem anatomy (Rosolem et al., 2005), unbalance mineral nutrition (Kabata-Pendias and Pendias, 1992), modify biomass accumulation patterns (Menon et al., 2005) and reduce photosynthetic rate and plant growth (Balsberg Pålsson, 1989).

As compared to crop species, studies on the responses of woody species to Zn are relatively scarce. But woody species can be very sensitive to this element, particularly at the seedling stage when they

strongly depend on upper, usually more contaminated, soil layers (Adriano, 2001; Reichman et al., 2001; Fuentes et al., 2007). In addition, information on species response to heavy metals is needed to optimize their use in the restoration of contaminated areas and to assess the potential risks to wildlife upon incorporation of these elements into the food chain (Domínguez et al., 2008).

Metal toxicity thresholds for species used in ecological restoration have great ecological significance, because they can be used to estimate the ability of plants to establish themselves on contaminated sites (Paschke et al., 2000). Studies on the effects of heavy metals on woody species may help to explain current and potential species distributions (Lottermoser et al., 2008) and plant community responses to pollution (Hüttermann et al., 2004).

Habitats where heavy metal availability is high are frequently subject to other sources of stress such as low water or nutrient availability (Larcher, 2003; Brady et al., 2005), precluding the identification of specific morpho-functional traits of heavy-metal-tolerant plants. On the other hand, most studies on heavy metal tolerance have focused on biochemical and physiological mechanisms that specifically involve heavy metal uptake, transport and storage (Prasad, 2004). In comparison, morpho-functional strategies to withstand heavy metals have received much less attention. However, some evidence suggests that plant responses to heavy metals may be similar to those deployed against other sources of stress. For example, plant species adapted to serpentine soils often show lower specific

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leaf area and leaf size, thicker leaves and deeper roots when grown on serpentine soils than on non-serpentine soils (Wierzbicka and Panufnik, 1998; Brady et al., 2005). These traits have also been related to resistance to drought and extreme temperatures (Larcher, 2003). Furthermore, it has been suggested that plant adaptation to limited water and nutrient availability may contribute to the ability to colonize contaminated areas (Poschenrieder and Barceló, 2004; Brady et al., 2005).

To study the sensitivity of Mediterranean woody species to heavy metals, we have quantified morphological responses to increasing doses of Zn and identified thresholds in these responses. The species selected cover a climatic gradient from dry sub-humid to semi-arid Mediterranean conditions. Our hypothesis was that morphological traits that increase species resistance to drought may buffer the toxic effects of Zn and, as a result, xeric species may exhibit more resistance to this element than mesic species.

2. Materials and methods

2.1. Plant material

We used seeds of *Pinus pinea* L. (Stone pine), *Pinus pinaster* Ait. (Maritime pine), *Pinus halepensis* Mill. (Aleppo pine), *Tetraclinis articulata* (Vahl) Mast. (Barbary thuja), *Rhamnus alaternus* L. (Italian buckthorn) and *Quercus suber* L. (Cork oak). Seeds were provided by the Regional Forest Seed Bank (Banc de Llavors Forestals, Generalitat Valenciana). These materials are collected within the Region of Valencia and comply with local regulations (Conselleria de Medio Ambiente, DOCV 2946).

These species cover a wide climatic gradient, from semi-arid to dry sub-humid conditions, with mean annual precipitation to mean annual potential evapotranspiration ratios of 0.2–0.5 and 0.5–0.65, respectively (UNEP, 1997). *Tetraclinis articulata* is a widely distributed tree in N Africa where it covers a total area of 10 000 km² (Nicolás et al., 2004). Its widespread presence in the Iberian Peninsula in pre-historic times has been reduced to one single location. It is more thermophilic and xerophilic than the other species tested and it occurs in semi-arid regions of Spain with average annual precipitation between 150 and 400 mm (Bartolomé et al., 2005). *Pinus halepensis* covers 25 000 km² in its native range and, together with *T. articulata*, is one of the few European tree species capable of withstanding semi-arid conditions. It outcompetes *T. articulata* on north-facing slopes where they coexist (Nicolás et al., 2004). It has been used extensively for forestation over the last century (Maestre and Cortina, 2004) and grows on a wide range of soils (Richardson, 1998). *Rhamnus alaternus* is a shrub to small tree common in open areas and in the understory of *Quercus ilex* L. forests (Masclans, 1981). It is sensitive to freezing and grows on a wide range of soil types, with preference for basic soils. *Pinus pinea* and *P. pinaster* are common tree species under dry sub-humid and humid Mediterranean conditions. *Pinus pinaster* covers 13 000 km² in the Mediterranean basin on various types of soil. *Pinus pinea* covers 3200 km² mostly on sandstone and sandy soils, although it can also be found on dolomite (Richardson, 1998). *Quercus suber* is found in sub-humid regions of the Mediterranean, with average annual precipitation above 600 mm and average annual temperature near 15 °C. It commonly grows on neutral to slightly acidic well-drained soils (Serrasolses et al., 2009).

We used minimum annual precipitation as an indicator of species resistance to drought. Extreme climatic events, such as unusually dry years, have intense effects on plant performance and may control species distribution (Easterling et al., 2000). To our knowledge, climate envelopes have not been defined for the species studied. Thus, we estimated the minimum average annual precipitation for each species by using two different approaches. First, we used ANTHOS database (Anthos Project, 2009), to randomly select 50 locations per species, except for *T. articulata* as only 4 geographical references were

available for this species. Then, we used the New LocClim 1.10 software (FAO Agroclimatic databases, http://www.fao.org/sd/2002/EN1203a_en.htm) to simulate precipitation dynamics and estimate mean minimum precipitation for each location. This software provides a rough estimate of climatic conditions, so we validated these results by comparing the outputs of this exercise with minimum average annual precipitation values obtained from the distribution range of each species as described in Martín et al. (1998) and Bartolomé et al. (2005). This information was not available for *R. alaternus*. The estimates obtained with both approaches were highly correlated ($r=0.98$, $F_{1,3}=124$, $P=0.002$), suggesting that they provide reasonable estimates of species climatic constraints.

2.2. Growing conditions

Seeds from local provenances were sown on silica sand and kept at 25 ± 2 °C until germination. Seedlings with a mean radicle length of 5 cm and visible aboveground parts were transferred to 1 L pots filled with a nutrient solution containing 170 μM NH₄NO₃, 42 μM KH₂PO₄, 88 μM KNO₃, 3.1 μM MgSO₄·7H₂O, 0.27 μM Fe-EDTA, 0.19 μM H₃BO₃, 0.14 μM Mn-EDTA, 0.078 μM Zn-EDTA, 0.047 μM Cu-EDTA and 0.0004 μM (NH₄)₆Mo₇O₂₄ (as described in Arduini et al., 1994). Pots were kept in a growth chamber under controlled conditions of temperature (23 ± 1 °C/ 18 ± 1 °C, day/night) and photoperiod (12 h, 300 μmol m⁻² s⁻¹ photosynthetic photon flux density during the day). The nutrient solution was constantly aerated and renewed three times a week. Seven days after transplanting, the pots received one of five Zn doses with the nutrient solution: 0.078 (hereafter control), 5, 10, 20 and 90 μM zinc as ZnSO₄.

Ion speciation estimates with MINTEQA2 (Allison Geoscience Consultants, Inc. Flowery Branch, Georgia, USA and HydroGeoLogic, Inc. Herndon, Virginia, USA) indicated that Fe and Zn were highly available, as more than 90% of the Fe in all nutrient solutions was present as chelated Fe(III) and 99% of the Zn present was as a free ion (i.e., Zn precipitation and complexation with EDTA was negligible).

We used a randomized block design which included 3 successive blocks in time (starting dates: November 15th 2003 and January 20th 2004 and March 26th 2004). Each block consisted of one pot per zinc dose and species. Each pot contained 4–5 seedlings of the same species that were supported by a perforated polystyrene top.

2.3. Seedling growth and morphology

The second day after seedling transplant, roots were marked 10 mm above the root tip with a permanent marker. Root growth was measured every 2 days and relative growth rate (in week⁻¹) calculated as $RGR = [\ln(L_2) - \ln(L_1)] / [t_2 - t_1]$, where L_1 and L_2 correspond to the root length measured on successive dates t_1 and t_2 .

Three weeks after the onset of Zn application, seedlings were harvested, stems cut above the root collar and the rooting system carefully washed. Roots were digitized by scanning on an A3 flatbed scanner (Epson Expression 1680 Pro, Long Beach, California, USA) fitted with a transparency adaptor at 300 dpi, using an 8-bit greyscale. Images were analyzed with specific software (WinRhizo, Regent Instruments, Québec, Canada) to evaluate average root diameter, total length and length per diameter class. We defined 20 uniform diameter classes from 0 to 4 mm. The root length in each diameter class was normalized to the total root length. Finally, all biomass fractions were dried at 65 °C for 48 h and weighed. Specific root length was estimated as the ratio between total root length and root biomass.

2.4. Root topology

The topology of rooting systems projected onto a two-dimensional plane can be described by three variables: magnitude (μ), total

exterior pathlength (Pe) and altitude (a) (Fitter, 1991, see Fig. 1, Supplementary material).

Magnitude is the number of root tips. Total exterior pathlength is calculated as the sum of all links (i.e., root segments between successive branchings) counted from each of the root tips back to the base of the rooting system. Altitude is the number of links in the longest unique path from the base link to an exterior link (Fitter, 1991).

Using digitized images and the software WinRhizo, we counted the number of root tips and calculated topological indices for the entire root system of each seedling. Then, we estimated expected altitude ($E(a) = 2.65\mu^{0.5}$) and expected total exterior pathlength ($E(Pe) = 2.17\mu^{1.42}$), that is, the value of a and Pe for a randomly branched rooting system of a given μ , following the method suggested by Werner and Smart (1973), as modified by Berntson (1994). Increases in the ratios $a/E(a)$ and $Pe/E(Pe)$ indicate more herringbone-like root systems, whereas decreases in those ratios correspond to dichotomous branching patterns.

2.5. Zinc concentration in plant tissues

Roots and shoots were digested in a heating block at 250 °C with a mixture of concentrated sulphuric acid and hydrogen peroxide (Jones and Case, 1990). Digests were analyzed for Zn by ICP-OES (Perkin Elmer Optima 4300 Inductively Coupled Plasma with Optical Emission Spectrometry). *Olea europaea* leaf standard reference materials (BCR: CRM 062, Commission of the European Communities Bureau of Reference, Brussels) were included in the analyses for quality control. Coefficient of variation ranged from 3.5 to 7.1%.

2.6. Toxicity thresholds

EC is the effective concentration of a toxin that induces a negative effect in the organism under study (Köhl and Lösch, 2004). In our experiment, we estimated EC_{50-L} , EC_{50-RB} and EC_{50-RGR} as the concentration of Zn producing a 50% reduction in root length, root biomass and relative growth rate, respectively. To estimate EC_{50} , average root length, relative growth rate and root biomass per pot were plotted against the external concentration of zinc for each species. The resulting dose–response curves were fitted to the model $y = y_0 + ae^{-bx}$, where y corresponds to the response variable (root length, root biomass and relative growth rate, in cm, mg and week⁻¹, respectively), y_0 = asymptotic value of the response variable at increasing Zn concentrations (same units as y), $y_0 + a$ = value of the response variable when no Zn is available (same units as y), b = the decrease rate in the response variable for a given increase in Zn concentration (in μM^{-1}), and x = Zn concentration (in μM). We tested different models to fit the curves (hyperbolic decay with 2 and 3 parameters, $y = ae^{-bx}$ and $y = y_0 + ae^{-bx}$, respectively, and exponential decay with 2 and 3 parameters, $y = ab/(b+x)$ and $y = y_0 + ab/(b+x)$, respectively). The negative exponential with 3 parameters provided the best fit for all species ($R^2 > 0.81$ and $P < 0.05$). Finally, we calculated the effective concentration (EC) in (μM) for the three variables (see Fig. 2, Supplementary material for an example on EC estimation).

2.7. Statistical analyses

Seedlings growing in a single container for a given block and Zn dose were not independent, and thus they could not be treated as true replicates. We used an unreplicated randomized block design to analyze our data. Differences in morphological and topological traits between seedlings subjected to various Zn1 doses were analyzed using univariate analysis of variance (ANOVA) with Zn dose and block as fixed factors.

Treatment effects on EC_{50} indices were evaluated using univariate ANOVA with species and block as fixed factors. Root biomass was transformed using base-10 logarithm prior to analysis to homogenize the variance. When significant differences between treatments were found, means were compared pairwise by using Tukey's HSD test. All analyses were performed using the SPSS v.15.0 statistical package (SPSS Inc., Chicago, USA).

3. Results

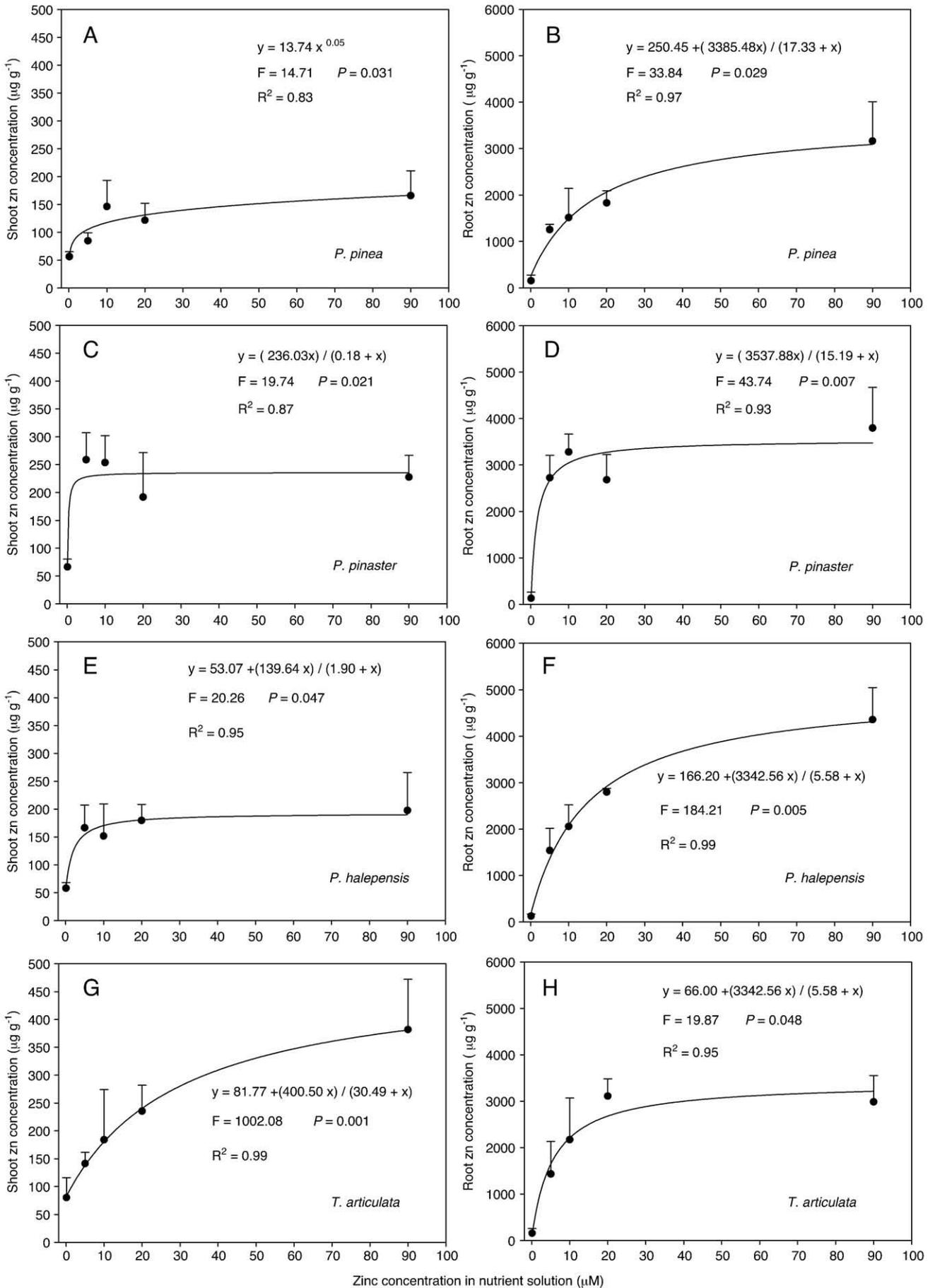
3.1. Zinc bioaccumulation

At the highest application rate, zinc accumulation in shoots ranged from 53 $\mu g g^{-1}$ in *Quercus suber* to 382 $\mu g g^{-1}$ in *T. articulata* (Fig. 1). Zinc concentration was, on average, one order of magnitude higher in roots than in shoots. *Q. suber* showed the highest contrast between root and shoot concentration of zinc and *T. articulata* the lowest. The shape of the curve describing the relationship between Zn availability and plant tissue concentration was dependent on the species and the tissue. *Rhamnus alaternus* and *P. pinaster* showed a sharp increase in Zn in roots and shoots at low application rates, approaching a saturation level at 5 μM . In contrast, Zn concentration above and belowground in *P. pinea* and *Q. suber* increased gradually with increasing application rate. *Quercus suber* was the only species showing gradual increases in Zn concentration over the whole range of Zn availability tested, with no apparent signs of saturation. This observation, together with the low levels of Zn found in roots suggest that this species was the best Zn excluder of the whole set of species. In contrast to the other species, Zn accumulation followed different patterns for shoots and roots in *P. halepensis* and *T. articulata*.

3.2. Biomass accumulation and root morphology

Foliar biomass accumulation decreased in response to Zn in *P. halepensis* and *Q. suber* (Table 1). Roots of all species exposed to Zn doses above 5 μM became brown soon after the onset of Zn application, and root biomass accumulation decreased significantly with increasing Zn dose in *P. pinea*, *P. halepensis*, *T. articulata*, *R. alaternus* and *Q. suber* at relatively low Zn levels; in contrast, root biomass accumulation was not affected by Zn in *P. pinaster*. We found no differences in specific root length (SRL) in *T. articulata* and *Q. suber* (Table 1). In contrast, *Rhamnus alaternus* and all pine species showed a decrease in SRL between 30 and 50% as compared to control seedlings at application rates higher than 5 μM . Relative growth rate (RGR) of the main root was reduced in response to Zn, and it was virtually zero in all species, at application rates above 10–20 μM Zn (Table 1). At low Zn doses root elongation rate decreased sharply in *R. alaternus* and *T. articulata*, whereas the decrease was gradual in the other species. Despite that block had a significant effect on several morphological traits: foliar biomass (*P. pinaster*, *P. halepensis*, *T. articulata* and *Q. suber*), root biomass (*P. pinaster*, *P. halepensis* and *Q. suber*), SRL (*P. pinaster* and *Q. suber*) and RGR (*P. pinea*), the effect of Zn was similar across all blocks (see Fig. 3, Supplementary material for an example on main root RGR).

Root length per diameter class was dependent on species and Zn dose (Fig. 2). Roots in the 0.4–0.6 mm diameter class accounted for more than 40, 50 and 70% of the total root length in control seedlings of *P. pinaster*, *P. halepensis* and *T. articulata*, respectively. A similar pattern was found for *R. alaternus*, but in this species maximum relative root length corresponded to smaller root diameter classes. Unlike the other species, root length in *P. pinea* was evenly distributed between four diameter classes ranging from 0.2 to 1 mm. Finally, in *Q. suber*, root length per diameter class gradually decreased with increasing diameter, and no class showed relative root length accumulation above 25%. Zinc increased the relative abundance of thick roots in *P. pinea* and, especially, in *R. alaternus* and *P. halepensis*.



Zinc concentration in nutrient solution (µM)

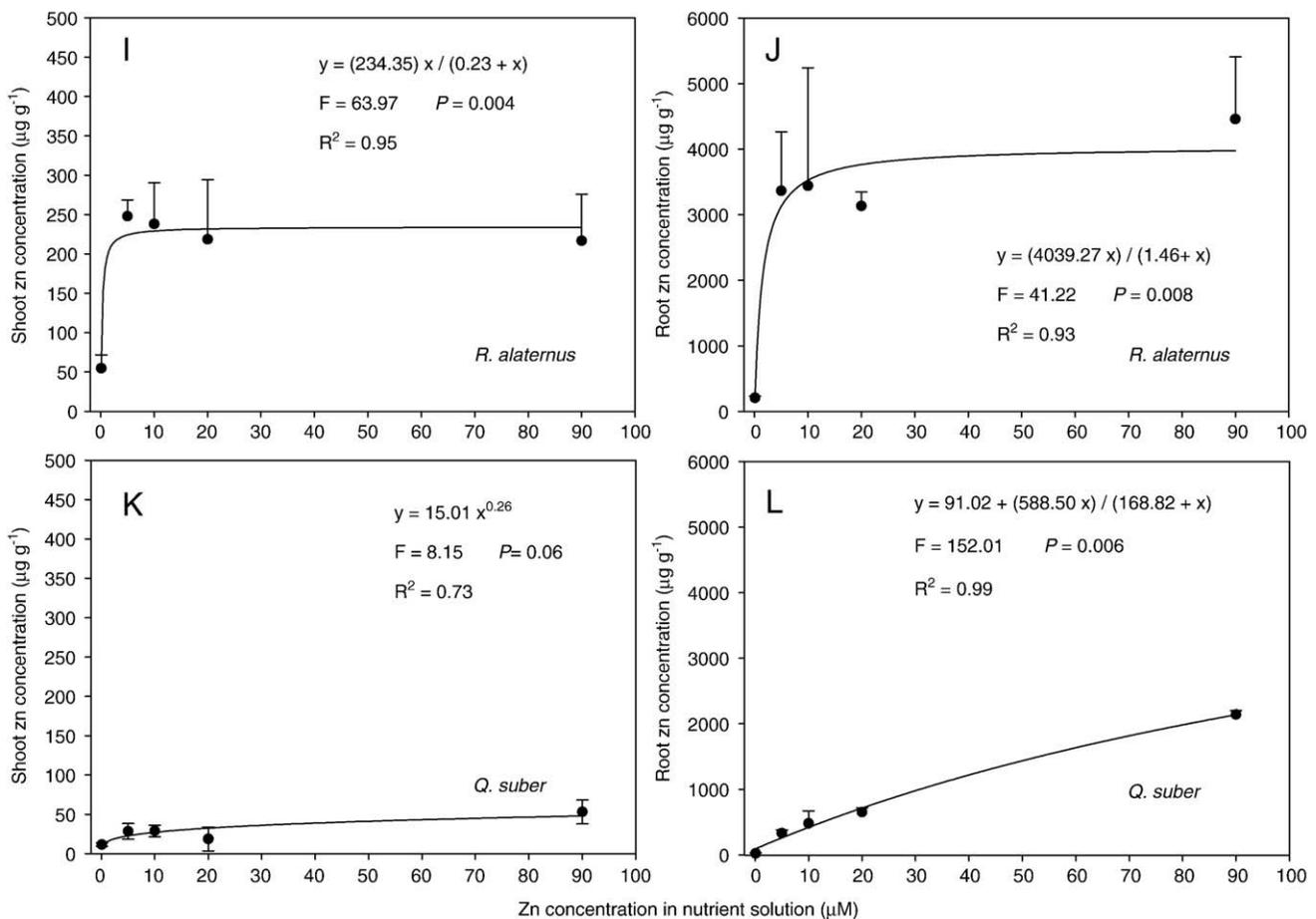


Fig. 1. Effects of Zn application on Zn concentration in shoots (A, C, E, G, I, K) and roots (B, D, F, H, J, L) of *Pinus pinea* (A, B), *Pinus pinaster* (C, D), *Pinus halepensis* (E, F), *Tetraclinis articulata* (G, H), *Rhamnus alaternus* (I, J) and *Quercus suber* (K, L). Note the different scales used for root and shoot. Models describing the relationships between Zn concentration and Zn availability are shown.

In contrast, *T. articulata* showed the opposite trend. Finally, root length distribution was not affected by increasing Zn in *P. pinaster*, and *Q. suber*. Average root diameter was not significantly affected by Zn but plants growing in consecutive blocks showed differences in average root diameter (*P. pinea*, *P. halepensis* and *Q. suber*) (Fig. 3A, Table 2).

3.3. Root topology

Root system magnitude ranged from 4 to 145 in control seedlings of *T. articulata* and *Q. suber*, respectively. This variable decreased significantly in *P. pinaster*, *R. alaternus* and *Q. suber* at application rates above 10–20 μM Zn and was not significantly affected by Zn in the other species tested (Fig. 3B and Appendix A). Plants growing in consecutive blocks showed differences in and root system magnitude (*P. pinaster*, *R. alaternus* and *Q. suber*, Table 2). All species showed herringbone-like root systems that did not change after exposure to increasing Zn doses (data not shown).

3.4. Toxicity thresholds

We calculated the external Zn concentration that produces a 50% reduction in root biomass accumulation ($\text{EC}_{50}\text{-RB}$) for those species and blocks where the response curve leveled off (Table 3). Thus, $\text{EC}_{50}\text{-RB}$ was not calculated for any block of *P. pinea*, *P. pinaster*, and one block of *Q. suber* and *R. alaternus*. Toxicity thresholds estimated using the index $\text{EC}_{50}\text{-RB}$ were higher than those estimated with indices

based on root length and relative growth rate. Both $\text{EC}_{50}\text{-L}$ and $\text{EC}_{50}\text{-RGR}$ identified *R. alaternus* and *T. articulata* as the most sensitive species to Zn and *Q. suber* as the most tolerant.

We found a positive relationship between $\text{EC}_{50}\text{-L}$ index and the mean minimum annual precipitation in the geographical distribution range of each species (Fig. 4). Results were similar when mean maximum annual precipitation and $\text{EC}_{50}\text{-RGR}$ were used (data not shown).

4. Discussion

4.1. Zinc uptake and bioaccumulation

Zinc accumulation in shoots and roots increased with increasing Zn concentration in the growing medium. However, Zn was strongly retained belowground, as the concentrations of this element were substantially higher in roots than in shoots. High Zn accumulation in roots has been observed in other studies (Paschke et al., 2000; Reichman et al., 2001; Fuentes et al., 2007), and may result from metal binding to negatively charged binding sites in the cell wall (Greger, 2004) and restricted transport to shoots (Utriainen et al., 1997; Pulford and Watson, 2003).

Maximum Zn concentrations belowground were observed in *P. halepensis* and *R. alaternus* (4358 and $4559 \mu\text{g g}^{-1}$, respectively) and were similar to those found in woody species grown in hydroponic culture (e.g., $3444 \mu\text{g g}^{-1}$ to $5700 \mu\text{g g}^{-1}$; Reichman et al., 2001; Fuentes et al., 2007). Zinc concentrations in roots showed a non-linear

Table 1

Morphological traits of woody seedlings exposed to increasing doses of Zn in the growing solution. SRL: specific root length. Means, standard deviation of $n = 3$ blocks and results of a univariate ANOVA are shown. Different letters indicate significant differences at $P < 0.05$ (Tukey-HSD test). In title row t: treatment; b: block.

Zinc application rate (μM)	0.078	5	10	20	90	$F_{t,8}$	P_t	$F_{b,8}$	P_b
Foliar biomass (mg)									
<i>P. pinea</i>	147 ± 10	158 ± 5	158 ± 28	139 ± 23	142 ± 13	0.72	0.603	0.44	0.657
<i>P. pinaster</i>	37 ± 8	40 ± 4	45 ± 17	49 ± 13	41 ± 12	1.55	0.280	14.60	0.002
<i>P. halepensis</i>	22 ± 3a	19 ± 4ab	17 ± 5ab	19 ± 6ab	4 ± 6b	5.06	0.025	23.62	<0.001
<i>T. articulata</i>	9 ± 2	10 ± 3	9 ± 1	8 ± 1	8 ± 1	1.02	0.451	8.99	0.009
<i>R. alaternus</i>	7 ± 2	7 ± 1	5 ± 1	6 ± 0	5 ± 0	2.76	0.103	2.33	0.159
<i>Q. suber</i>	249 ± 91ab	231 ± 100ab	297 ± 111a	199 ± 104ab	178 ± 122b	4.17	0.041	32.93	<0.001
Root biomass (mg)									
<i>P. pinea</i>	45 ± 6a	40 ± 7ab	36 ± 6ab	28 ± 2b	28 ± 6b	6.26	0.014	2.06	0.190
<i>P. pinaster</i>	13 ± 3	12 ± 2	11 ± 3	10 ± 4	8 ± 2	3.50	0.062	7.12	0.017
<i>P. halepensis</i>	9 ± 4a	7 ± 2ab	5 ± 2ab	5 ± 2ab	3 ± 1b	6.60	0.012	8.23	0.009
<i>T. articulata</i>	5 ± 1a	3 ± 1ab	2 ± 0ab	2 ± 0b	1 ± 0b	6.89	0.011	2.04	0.192
<i>R. alaternus</i>	3 ± 1a	2 ± 0ab	2 ± 0ab	1 ± 0b	1 ± 0b	6.69	0.011	1.20	0.350
<i>Q. suber</i>	763 ± 245a	545 ± 322bc	682 ± 184ab	541 ± 280bc	385 ± 266c	10.69	0.003	58.16	<0.001
Specific root length (cm mg^{-1})									
<i>P. pinea</i>	1.3 ± 0.3a	0.8 ± 0.2b	0.9 ± 0.2b	0.7 ± 0.0b	0.6 ± 0.1b	8.56	0.005	1.39	0.302
<i>P. pinaster</i>	2.5 ± 0.4a	2.0 ± 0.2b	1.7 ± 0.3b	1.2 ± 0.2c	1.3 ± 0.2c	49.57	<0.001	20.40	0.001
<i>P. halepensis</i>	2.9 ± 0.4a	2.2 ± 0.2ab	1.4 ± 0.3b	1.6 ± 0.3b	2.0 ± 0.3b	12.11	0.002	1.21	0.346
<i>T. articulata</i>	3.1 ± 0.1	2.5 ± 0.8	2.8 ± 0.7	3.5 ± 0.3	5.1 ± 1.9	2.06	0.178	0.076	0.927
<i>R. alaternus</i>	5.8 ± 0.7a	4.2 ± 1.0ab	3.0 ± 1.1b	3.8 ± 0.8ab	4.4 ± 0.3ab	4.44	0.035	1.19	0.354
<i>Q. suber</i>	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	1.63	0.258	5.44	0.032
Relative growth rate in main root length (week^{-1}) × 10									
<i>P. pinea</i>	5.3 ± 0.5a	3.5 ± 0.9b	1.7 ± 0.5c	0.5 ± 0.0d	0.1 ± 0.1d	87.73	<0.001	5.24	0.035
<i>P. pinaster</i>	4.9 ± 1.7a	4.0 ± 0.7a	1.0 ± 0.2b	0.3 ± 0.1b	0.2 ± 0.1b	18.69	<0.001	0.57	0.589
<i>P. halepensis</i>	4.6 ± 0.5a	2.4 ± 1.1b	0.2 ± 0.1c	0.2 ± 0.1c	0.0 ± 0.0c	36.83	<0.001	0.52	0.615
<i>T. articulata</i>	5.4 ± 0.4a	0.8 ± 0.4b	0.7 ± 0.1b	0.2 ± 0.1b	0.1 ± 0.1b	216.14	<0.001	1.97	0.202
<i>R. alaternus</i>	4.0 ± 1.2a	0.6 ± 0.4b	0.2 ± 0.1b	0.1 ± 0.1b	0.2 ± 0.1b	19.19	<0.001	0.13	0.877
<i>Q. suber</i>	3.0 ± 0.7a	3.2 ± 0.7a	2.0 ± 0.7a	1.7 ± 0.2ab	0.1 ± 0.1b	10.70	0.003	0.10	0.901

response to Zn availability, reaching an asymptote within the range of Zn concentrations used in this study in all species but *Q. suber*. This type of curve has been described in the literature (Reichman et al., 2001; Greger, 2004) and may reflect the saturation of metal uptake (Nyquist and Greger, 2007). Indeed, Brunner et al. (2008) found that Zn moves from the cell walls into the lumen after reaching an equilibrium or saturation. When metal concentration is low, ionic competition may be negligible, and uptake efficiency may be high. But, as the Zn concentration in the growing medium increases, competition among Zn ions may follow, thus decreasing uptake efficiency (Greger, 2004). In addition, kinetic studies have found gradual decreases in metal absorption efficiency owing to induced saturation of metal transporters (Hart et al., 1998). Finally, the direct toxic effect of metal may contribute to hinder its uptake (Nyquist and Greger, 2007). *Quercus suber* was the only species showing an almost linear increase in belowground Zn concentrations over the range of Zn doses tested. In addition, maximum Zn concentrations in roots of *Q. suber* ($2141 \mu\text{g g}^{-1}$) were relatively low compared to the other species tested and other studies using hydroponics. For example, Reichman et al. (2001) found that Zn concentration in roots of *Eucalyptus camaldulensis* and *Acacia holosericea* ranged from $4000 \mu\text{g g}^{-1}$ to $6000 \mu\text{g g}^{-1}$ when external Zn concentration was $100 \mu\text{M}$. Four weeks after exposing *Salix fragilis* to $76.5 \mu\text{M}$ of Zn, the Zn concentration in roots was $3370 \mu\text{g g}^{-1}$ (Dos Santos Utmazian et al., 2007). Thus, results suggest that at the early seedling stage, *Q. suber* may behave as a powerful Zn excluder.

Zinc concentrations in shoots remained relatively constant for Zn concentrations above $5 \mu\text{M}$ in all species but *T. articulata*. The range of maximum Zn translocation aboveground was relatively narrow, ranging from 5% in *Q. suber* to 20% in *P. pinea*, *P. pinaster*, *P. halepensis* and *R. alaternus*. Weak increases in Zn concentrations in shoots in response to an increased external concentration of Zn have been attributed to

controlled Zn transport, i.e., regulation of metal translocation and they may reflect the outcome of a suite of mechanisms allowing tolerance to this heavy metal (Denny and Wilkins, 1987; Utriainen et al., 1997). *Tetraclinis articulata* differed from the others species in that Zn concentrations in shoots reached no asymptote with increasing Zn dose. This may reflect uncontrolled or unrestricted Zn transport, as has been observed in other species, and may result in severe toxicity and death (Denny and Wilkins, 1987).

Toxicity limits for Zn depend on plant species, genotype and growth stage (Kabata-Pendias and Pendias, 1992). Most species may be sensitive to Zn when its concentration in aboveground parts ranges between 200 and $300 \mu\text{g g}^{-1}$ (Balsberg Pahlsson, 1989). In our study, three species (*T. articulata*, *P. pinaster* and *R. alaternus*) reached Zn concentration values in leaves and needles that can be considered critical (382 , 235 and $217 \mu\text{g g}^{-1}$, respectively). Nevertheless, none of the six species studied showed visual symptoms of Zn toxicity, such as chlorosis or necrosis.

4.2. Biomass accumulation and root morphology

Shoot growth was not sensitive to Zn in most species, save for *P. halepensis* and *Q. suber*. This finding contrasts with longer-term studies showing decreases in aboveground biomass accumulation in response to this element. But we may note that our experiment lasted for only three weeks and thus it was shorter than other experiments reported in the literature (Balsberg Pahlsson, 1989; Reichman et al., 2001; Fuentes et al., 2007). Root growth was more sensitive to Zn than shoot growth, as observed in other studies (Paschke et al., 2000). Elongation of the main root was completely arrested at the highest Zn doses in all species. Inhibition of root elongation is a common early indicator of heavy metal toxicity, which may be a result of impaired cell elongation (Woolhouse, 1983). This may also have favoured the

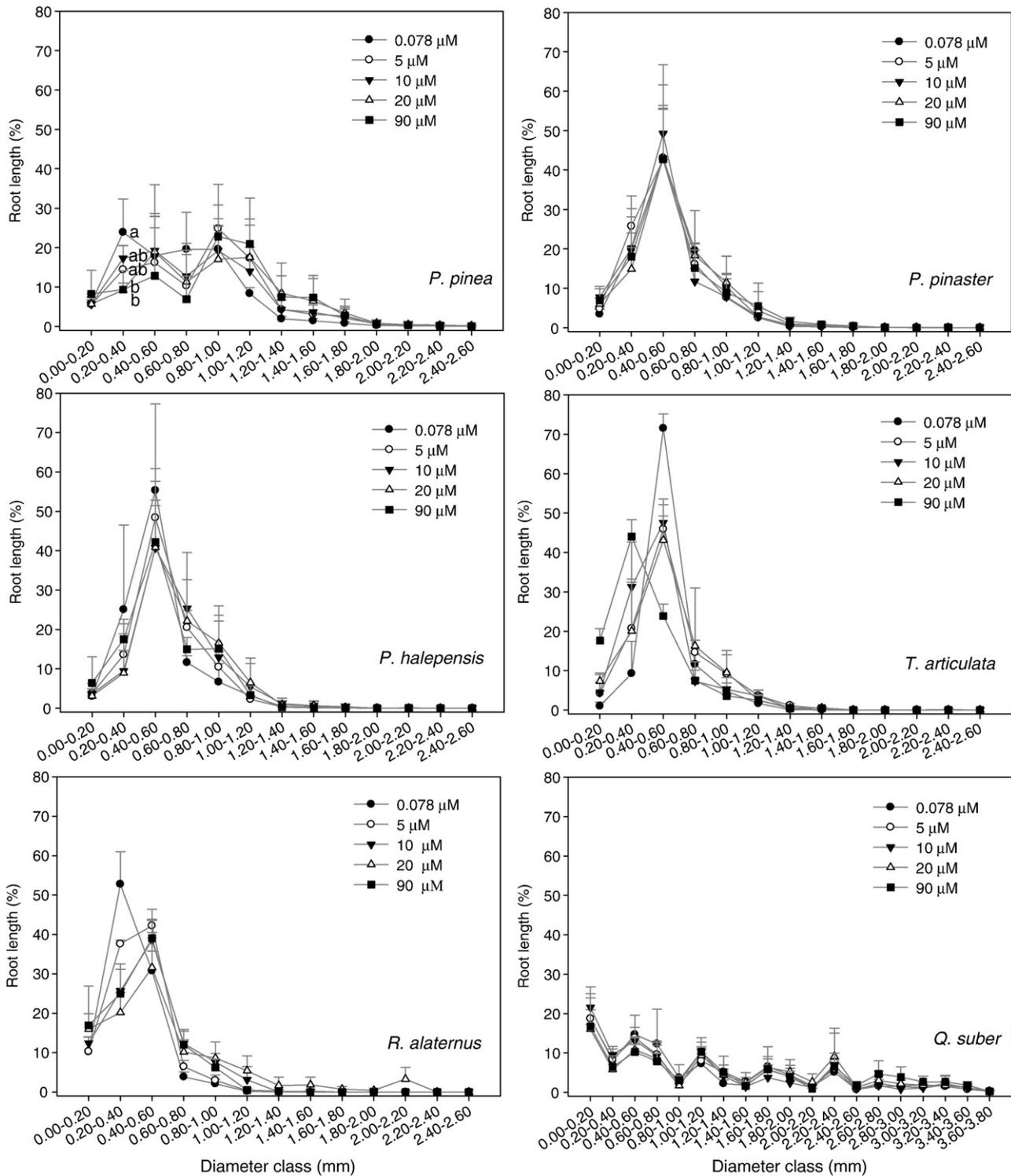


Fig. 2. Root length distribution per diameter class. Means \pm 0.5 standard deviation of $n = 3$ blocks are shown. Different letters indicate significant differences at $P < 0.05$ (Tukey-HSD test). Note the different X-scale used for *Q. suber*.

increase in the proportion of roots of higher diameter observed in *P. pinea*, *P. halepensis* and *R. alaternus*, a phenomenon described elsewhere (Zobel et al., 2007). Changes in root growth patterns may impair the capacity of seedlings to explore the soil for water and nutrients, as fast root elongation after germination is a key factor in the survival and early growth of Mediterranean seedlings (Padilla and Pugnaire, 2007).

Seedlings from different blocks differed in various morphological and topological variables for some species. The causes of such variability are difficult to unveil. Seedlings in different blocks germinated in

different times and may have experimented slightly different growing conditions, despite that they were grown in a controlled chamber. Unfortunately, it is not possible to analyze the interaction between treatment and block in an unreplicated randomized block design.

The control seedlings showed a different pattern of root length distribution per diameter class. In *P. pinea* and *Q. suber* the proportions of thick roots were higher than in the other species. For example, roots thicker than 2 mm represented 15% of the total root length in *Q. suber*. According to our results, Zn can increase, decrease or have no

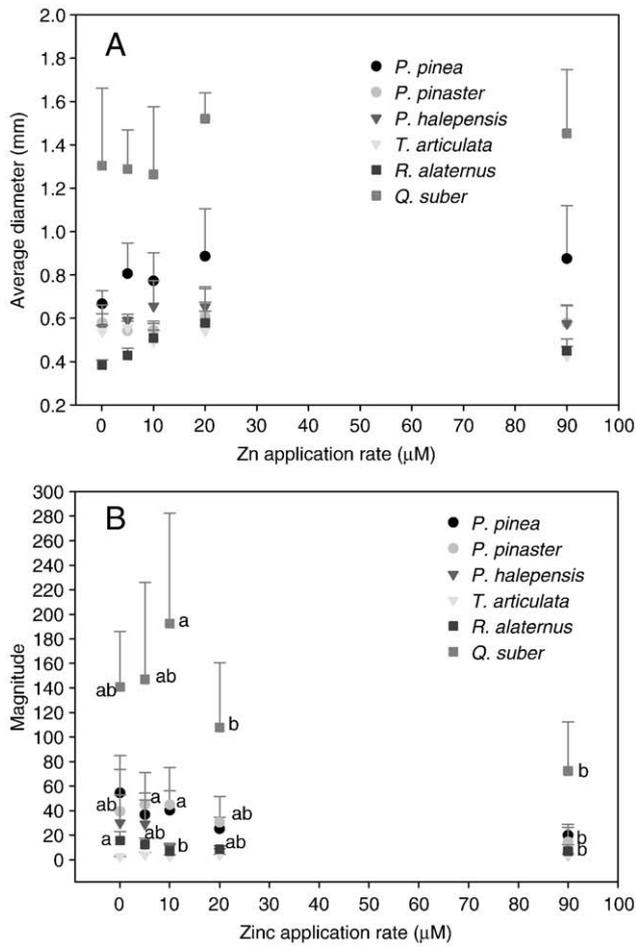


Fig. 3. Average root diameter (A) and number of root tips (magnitude) of the rooting system (B) of seedlings of six Mediterranean woody species grown under different Zn application rates. Symbols and bars represent means \pm 0.5 standard deviation of $n = 3$ blocks. Different letters indicate significant differences at $P < 0.05$ (Tukey-HSD test).

significant effect on root length distribution per diameter class depending on the species. Changes in root length distribution per diameter class have not been evaluated in studies on heavy metal toxicity. Our results emphasize the importance of evaluating this trait, as species that did not differ in their sensitivity to heavy metal availability in terms of root and shoot biomass, and other commonly measured response variables did, however, show strongly contrasted

Table 2

Results of a univariate ANOVA for average root diameter and root system magnitude. Different letters indicate significant differences at $P < 0.05$ (Tukey-HSD test). In title row t: treatment; b: block.

	$F_{t,4,8}$	P_t	$F_{b,2,8}$	P_b
<i>Average root diameter</i>				
<i>Pinus pinea</i>	2.53	0.123	11.89	0.004
<i>Pinus pinaster</i>	0.60	0.693	1.68	0.245
<i>Pinus halepensis</i>	1.79	0.224	5.75	0.028
<i>Tetraclinis articulata</i>	2.97	0.099	1.903	0.218
<i>Rhamnus alaternus</i>	2.42	0.134	0.835	0.468
<i>Quercus suber</i>	2.08	0.175	15.22	0.002
<i>Root system magnitude</i>				
<i>Pinus pinea</i>	1.82	0.218	1.40	0.300
<i>Pinus pinaster</i>	4.93	0.027	30.38	<0.001
<i>Pinus halepensis</i>	3.71	0.058	3.17	0.097
<i>Tetraclinis articulata</i>	1.02	0.458	0.915	0.481
<i>Rhamnus alaternus</i>	5.30	0.022	13.69	0.003
<i>Quercus suber</i>	7.03	0.010	28.87	<0.001

Table 3

Toxicity threshold for seedlings of six Mediterranean woody species subjected to increasing Zn concentration in the nutrient solution. Dose–response curves were fitted to the model: $y = y_0 + ae^{-bx}$. EC_{50} -RB, EC_{50} -length and EC_{50} -RGR were calculated as the Zn concentration in the growing media resulting in a 50% reduction in seedling root biomass, root length and relative root growth rate, respectively. Means, standard deviation of $n = 3$ blocks and results of univariate ANOVA are shown. Different letters indicate significant differences between species at $P < 0.05$ (Tukey-HSD test).

Species	EC_{50} -RB (μ M)	EC_{50} -L (μ M)	EC_{50} -RGR (μ M)
<i>Pinus pinea</i>	>90	$10.0 \pm 4.1ab$	$6.3 \pm 1.7a$
<i>Pinus pinaster</i>	>90	$13.6 \pm 3.6ab$	$5.3 \pm 0.2a$
<i>Pinus halepensis</i>	36.3 ± 32.5	$4.2 \pm 2.1a$	$4.3 \pm 1.2a$
<i>Tetraclinis articulata</i>	11.8 ± 3.0	$4.0 \pm 3.2a$	$1.8 \pm 1.0a$
<i>Rhamnus alaternus</i>	15.7 ± 12.1	$3.7 \pm 1.1a$	$1.8 \pm 0.7a$
<i>Quercus suber</i>	30.0 ± 16.5	$19.8 \pm 9.7b$	$19.2 \pm 7.0b$
Ft	$F_{3,4} = 0.76$	$F_{5,10} = 5.05$	$F_{5,10} = 13.45$
P	0.573	0.014	<0.001
Fb	$F_{2,4} = 0.94$	$F_{2,10} = 0.56$	$F_{2,10} = 1.52$
P	0.463	0.586	0.265

responses in root abundance per diameter class. Changes in root length per diameter class may affect seedling performance because this trait is related to root longevity, carbon balances and seedling ability to capture and transport soil resources (Pregitzer et al., 1998; Eissenstat et al., 2000).

Other morphological changes in response to heavy metals such as decreased diameter of xylem elements and root elongation may impair species ability to withstand drought (Barceló and Poschenrieder, 1990). In turn, adaptive traits to withstand drought could affect seedling resistance to heavy metal toxicity. Some authors have pointed out the importance of pre-adaptation to drought for colonizing metal-rich soils (Poschenrieder and Barceló, 2004). However, to our knowledge this topic has received little attention in the literature.

4.3. Toxicity thresholds

Under the experimental conditions of the current study, EC_{50} -RB was not suitable as a predictor of Zn toxicity, as this value could not be estimated for all species. In addition, Zn may induce root suberization and lignification (Barceló and Poschenrieder, 1990), eventually outbalancing the changes in root biomass resulting from lower growth rates. Increased suberization would lead to over-estimations of EC_{50} -RB. Both EC_{50} -L and EC_{50} -RGR showed similar results and may

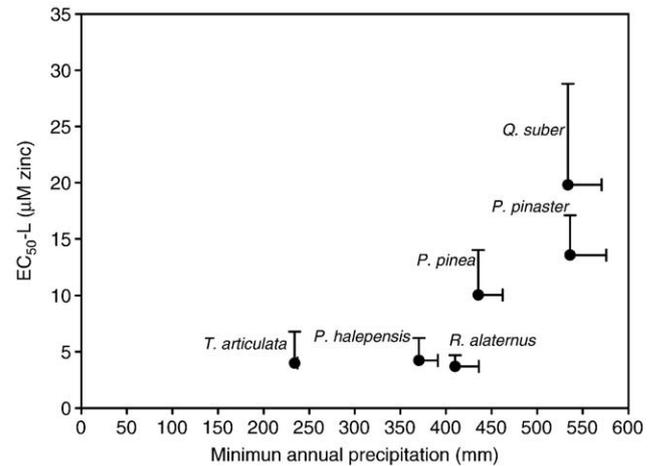


Fig. 4. Relationship between the EC_{50} -L and mean minimum annual precipitation within the geographical distribution of 6 woody Mediterranean species. Bars represent 0.5 standard deviations of $n = 3$ blocks (for EC_{50} -L data), and 50 replicated sites chosen randomly from the Anthos database (for rainfall data), except for *Tetraclinis articulata*, where only 4 populations are available in Spain. EC_{50} -L (in μ M) was estimated as the zinc concentration resulting in a 50% reduction in seedling root length.

be recommended as important metrics to assess toxicity when the response of entire rooting system to Zn does not differ from that of the main root, as was the case in the current experiment. In those cases where Zn may have contrasted effects on different parts of the rooting system, we would recommend the use of EC₅₀-L as it provides an evaluation of the whole rooting system. In addition, EC₅₀-L and EC₅₀-RGR do not reflect changes in root diameter and root tissue density.

The most sensitive species to zinc, *T. articulata*, differed from the most resistant, *Q. suber*, in several trait responses: higher Zn accumulation and translocation, sharp decrease in root elongation rate at relatively low doses of Zn and increased root length in small diameter roots. In addition, by the end of the experiment, *T. articulata* seedlings were substantially smaller than *Q. suber* seedlings. Thus, effective concentrations, such as those provided by the indices EC₅₀-L and EC₅₀-RGR, suggest that *T. articulata*, *R. alaternus* and *P. halepensis*, species with lower requirements of rainfall, are more sensitive to Zn than species from more mesic areas, such as *P. pinea*, *P. pinaster* and *Q. suber*. We note that our results are based on a small set of species, and we did not control for phylogenetic factors, seed size or seed provenance. But the strength of the relationship suggests further studies on this topic are needed.

4.4. Zinc toxicity and ecological restoration

The use of plants to remove, destroy or sequester heavy metals has gained considerable attention in the last decades. Revegetation of contaminated sites must be based on the selection of appropriate species both to prevent the risk of heavy metals entering the food chain and to optimize seedling survival and growth (Pulford and Watson, 2003). In this context, target species should be metal-tolerant and restrict metal uptake and translocation aboveground, particularly to edible parts. Under the experimental conditions of the present study, *Q. suber* and *P. pinea* can be recommended for restoring plant cover in contaminated sites in mesic areas. It is worth noting that these species can thrive in moderately acidic soils (Richardson, 1998; Serrasolses et al., 2009), but may contribute to decrease soil pH (Richardson, 1998), thereby increasing the mobilization of heavy metals. This must be monitored because increasing leaching could increase the heavy metal content in groundwater (Domínguez et al., 2008; Tack and Vandecasteele, 2008). In more xeric environments, *P. halepensis* may be the best choice, owing to the low aboveground Zn accumulation observed. In contrast, *T. articulata* was very sensitive to Zn and accumulated substantial quantities of this element aboveground increasing the risk of entering Zn the food chain. Thus, this species may be unsuitable for the restoration of contaminated sites. On the other hand, plants may be used for harvesting metals from contaminated sites (i.e., phytoremediation). In this case, high aboveground uptake is desired. *Pinus pinaster* in mesic areas and *T. articulata* in xeric areas would be the two species selected among the ones tested in the current study. However, phytoaccumulation ranged from 0.08 to 0.09% of dry weight in *T. articulata* and *P. pinaster*, respectively. These concentrations were low compared with 1%, the threshold for defining a plant as Zn hyperaccumulator (Prasad, 2004).

5. Conclusions

The morpho-physiological responses of Mediterranean woody species to Zn were strongly species-specific. *Quercus suber* was the more resistant species, probably due to its ability to exclude Zn at the early seedlings stage, whereas *T. articulata* was the most sensitive. Species showing higher Zn uptake and translocation rates also showed stronger morphological responses to this heavy metal. Surprisingly, species resistance to Zn was positively related to minimum annual precipitation of the species habitats providing a compelling stimulus for future research.

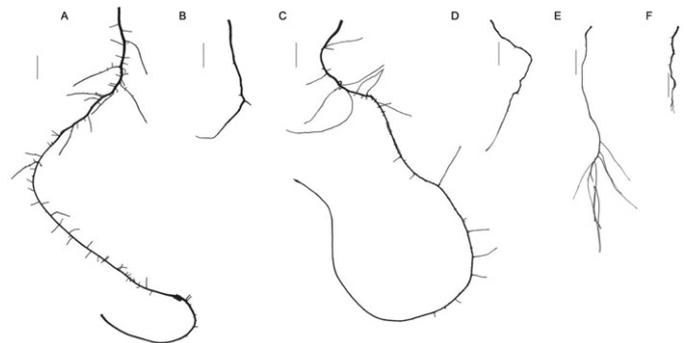
Of the species tested, *Q. suber* and *P. pinea* in mesic environments and *P. halepensis* in xeric environments are the best suited to withstand Zn, and can be recommended for further field trials; but none of the species tested showed a strong potential for phytoremediation.

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Appendix A

Example of scanned images of rooting systems of *Pinus halepensis* (A, B), *Pinus pinaster* (C, D) and *Rhamnus alaternus* (E, F). Images A, C and E correspond to control seedlings. Seedlings B, D and F were exposed to a nutrient solution containing 90 µM Zn. Vertical bars are 1 cm long.



Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scitotenv.2009.12.045.

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