

Alleviation of Zn toxicity by low water availability

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Heavy metal contamination and drought are expected to increase in large areas worldwide. However, their combined effect on plant performance has been scantily analyzed. This study examines the effect of Zn supply at different water availabilities on morpho-physiological traits of *Quercus suber* L. in order to analyze the combined effects of both stresses. Seedlings were treated with four levels of zinc from 3 to 150 μM and exposed to low watering (LW) or high watering (HW) frequency in hydroponic culture, using a growth chamber. Under both watering regimes, Zn concentration in leaves and roots increased with Zn increment in nutrient solution. Nevertheless, at the highest Zn doses, Zn tissue concentrations were almost twice in HW than in LW seedlings. Functional traits as leaf photosynthetic rate and root hydraulic conductivity, and morphological traits as root length and root biomass decreased significantly in response to Zn supply. Auxin levels increased with Zn concentrations, suggesting the involvement of this phytohormone in the seedling response to this element. LW seedlings exposed to 150 μM Zn showed higher root length and root biomass than HW seedlings exposed to the same Zn dose. Our results suggest that low water availability could mitigate Zn toxicity by limiting internal accumulation. Morphological traits involved in the response to both stresses probably contributed to this response.

Introduction

In nature, plants are commonly exposed to a combination of stress factors. Multiple stresses may affect plants in ways that are hardly predictable from studies of single stress factors, as synergistic and antagonistic interactions are widespread (Nilsen and Orcutt 1996, Larcher 2003). Water is the most limiting factor for plant productivity worldwide and several studies have evaluated the combined effect of drought and other sources

of stress such as extreme temperature and radiation (Valdadares and Pearcy 1997, Aranda et al. 2005, Gimeno et al. 2008). In contrast, the combined effect of drought and heavy metal contamination has received less attention, despite the potential interaction between both stress factors (Poschenrieder and Barceló 2004, Brady et al. 2005, Sardans and Peñuelas 2007). Several studies have shown that the effect of heavy metals on plant performance may be modulated by other sources of

Abbreviations – HPFM, high pressure flow meter method; HW, high watering; IAA, indole-3-acetic acid; ICP-OES, inductively coupled plasma optical emission spectrometry; IWUE, instantaneous water use efficiency; LW, low watering; SPE, solid phase extraction; SMC, substrate moisture content; SRL, specific root length.

stress as sulfur and proton concentration during rainfall, heat and frost (Kukkola et al. 1997, Öncel et al. 2000).

Among heavy metals, Zn is an essential micronutrient for higher plants and it is required for several physiological processes (Marschner 1995, Cakmak 2000, Broadley et al. 2007). Zinc abundance is increasing in natural systems as a consequence of human activities (Kabata-Pendias and Pendias 1992, Fuentes et al. 2007a, Domínguez et al., 2008). When Zn concentration exceeds a critical level, it becomes toxic (Woolhouse 1983, Balsberg Pålsson 1989). Symptoms of Zn toxicity include alteration in biomass partitioning (Ruano et al. 1988), morphological changes in root system (Fuentes et al. 2007b, Disante et al. 2010), reductions in photosynthetic rate and chlorophyll measurements (Di Baccio et al. 2009, Disante et al. 2011), unbalance mineral nutrition (Kabata-Pendias and Pendias 1992, Monnet et al., 2001) and modifications in xylem anatomy (Kasim 2007). These changes may affect plant capacity to uptake and transport water to the above ground parts (Barceló and Poschenrieder, 1990, Kasim 2007).

High levels of Zn and low water availability may induce similar responses in vascular plants, including stomatal closure and decreased CO₂ uptake, reduced chlorophyll fluorescence and foliar area, and activation of defense mechanisms against oxidative damage (Vaillant et al. 2005, Valladares et al. 2005). Conversely, plants may exhibit contrasted responses to Zn toxicity and drought. While reduced root length and root biomass have been described in seedlings exposed to Zn (Woolhouse 1983), adaptive mechanisms in response to water limitation include deeper root systems and increased proportion of biomass allocated belowground (Nicotra et al. 2002).

The interaction between Zn and drought may result in synergistic or antagonistic responses. Thus, small increases in Zn availability (20 mg l⁻¹ of Zn) promoted growth in water-stressed soybeans (Gadallah 2000). Similarly, heavy metal-enriched sewage sludge induced a positive effect on morpho-physiological traits of seedlings subjected to drought (Pascual et al. 2004, Fuentes et al. 2007a, Santala and Ryser 2009). Finally, in a previous study we observed that exposition of *Quercus suber* seedlings to Zn delayed the effects of a short drought (Disante et al. 2011). High Zn concentrations could hamper seedling capacity to absorb and transport water and compromise seedling ability to withstand drought due to reduction of root length and biomass (Disante et al. 2010). Limited water availability may also predispose leaves to cellular damage and photoinhibition, altering the carbon balance (Werner et al. 2001, Vilagrosa et al. 2010). Finally,

drought may impair uptake and acropetal translocation of nutrients, including Zn (Hu and Schmidhalter 2005).

Despite the potential interaction between Zn and drought, studies dealing with the simultaneous effect of both stress factors are scarce, and they frequently show conflictive results (Balsberg Pålsson 1989). The objective of this study is to evaluate the combined effect of Zn supply and water availability on plant performance, and contribute to unveil the interactions between both sources of stress. The underlying hypothesis is that morpho-physiological responses to drought may mitigate the effects of high Zn availability. For this study we used cork oak (*Quercus suber*) seedlings, a drought-resistant tree widely distributed in the western Mediterranean basin. Cork oak woodlands produce valuable goods and services, including cork, and they are protected by the European Union (Habitat directive 92/43/EEC; Pausas et al. 2009). In addition, we performed this experiment with young seedlings because this stage is one of the most sensitive for plant capacity to resist stress conditions (Vallejo et al. 2000; Vilagrosa et al. 2003a). To account for this objective, we analyzed plant physiological status, root capacity to supply water to leaves, and phytohormonal response in several Zn supply rates under two levels of water availability.

Materials and methods

Plant material and growing conditions

We cultivated *Quercus suber* seedlings from acorns. Seeds from local origin were supplied by the Forest Service Seed Bank (Banco de Semillas El Serranillo, Ministerio de Medio Ambiente y Medio Rural y Marino). *Quercus suber* acorns were sown in silica sand and kept at 24±2°C. Two weeks after germination, when the radicle was approximately 2 cm long, acorns were transplanted into 5-cm diameter and 30-cm length tubes filled with fine, medium and coarse silica sand (the size of sand ranged from 0.5 to 2.2 mm) in a proportion corresponding to 1:2:3 by volume, respectively. The substrate was tested in the laboratory before the onset of the experiment to ensure proper drainage. The bottom of the tubes was closed with a 1 × 1 mm mesh, which prevented substrate loss. Tubes were placed in a growth chamber under controlled conditions of temperature (22±1°C/18±1°C, day/night), 12-h photoperiod (600 μmol m⁻² s⁻¹ of photosynthetic photon flux density during the day) and 50% relative humidity. During this period, seedlings were irrigated with a nutrient solution containing 500 μM KCl, 435 μM NaNO₃, 300 μM MgSO₄, 45 μM NaH₂PO₄, 300 μM CaCl₂, 0.37 μM FeSO₄, 0.012 μM CuSO₄, 1.6 μM H₃BO₃, 0.006 μM

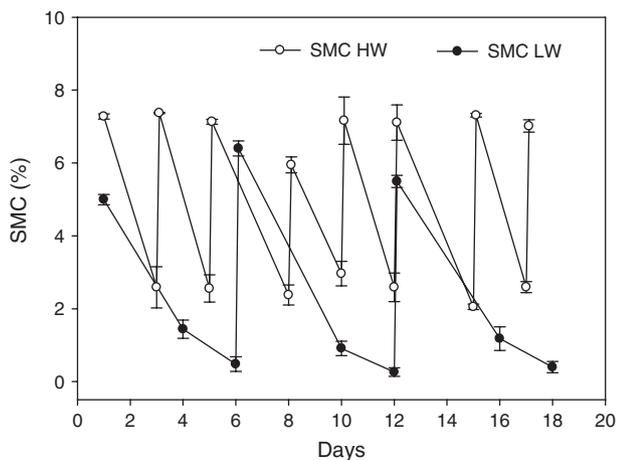


Fig. 1. Changes in substrate moisture content (SMC) for HW and LW seedlings during 18 consecutive days. HW and LW correspond to high and low watering frequency, respectively. Means and standard errors of $n = 6-7$ tubes per treatment are shown.

KI, $0.6 \mu\text{M MnSO}_4$ and $0.0004 \mu\text{M (NH}_4)_6\text{Mo}_7\text{O}_{24}$. This solution was a modification of the one used by Arduini et al. (1994) to simulate forest soil conditions.

Experimental design

Eight weeks after germination, we added ZnSO_4 to the nutrient solution to achieve a Zn concentration of 3 (Control), 10, 50 and $150 \mu\text{M}$, corresponding to those commonly found in uncontaminated and contaminated soils (Knight et al. 1997; Kabata-Pendias and Pendias 1992, González et al. 2011).

We applied two irrigation regimes using this solution: high watering (HW) and low watering (LW). HW seedlings were irrigated three times a week (Monday, Wednesday and Friday), whereas LW seedlings were irrigated every 5 days. During the experiment, seedlings were irrigated with 60 ml of nutrient solution. Substrate moisture content (SMC) was estimated by using measurements of water content by means of gravimetric method. SMC for HW and LW treatments ranged from $2.0 \pm 0.1\%$ to $7.4 \pm 0.1\%$ and from $0.3 \pm 0.1\%$ to $6.40 \pm 0.2\%$ respectively. The SMC during 18 consecutive days is shown in Fig. 1 as an example of SMC dynamics.

Each Zn level (hereafter treatments) was replicated 10 times, i.e. 10 seedlings per Zn treatments and irrigation level. Ion speciation calculations for solutions containing different quantities of Zn were conducted using MINTEQA2 software (Allison Geoscience Consultants, Inc. Flowery Branch, Georgia and HydroGeoLogic, Inc. Herndon, Virginia). The concentrations in mol l^{-1} of all anions and cations present in the nutrient solution (described

earlier) were entered in MINTEQA2 software to estimate the equilibrium composition of the experimental solution. pH was fixed at 5.5 (the pH of the nutrient solution was adjusted to this value) and quartz was added as the mineral present in the equilibrium.

Calculations showed that 98% of Fe was present as Fe (II) and 81% of Zn was present as free ion and 18% as ZnNO_3^+ . Both elements were highly available for plants, as only small precipitations of PO_4^{3-} (0.2%) and Mn^{2+} (16.2%) were found by chemical equilibrium estimations using MINTEQA2 (see Appendix S1). Leachates obtained immediately after watering showed that Zn concentration in the solution was always close to the intended concentration, independently of water availability and plant uptake. For 3 (Control), 10, 50 and $150 \mu\text{M}$ Zn concentrations were 4.2 ± 0.4 , 10.3 ± 3.7 , 47.6 ± 8.7 ; $150.2 \pm 12.9 \mu\text{M}$ Zn and 2.8 ± 0.4 , 7.8 ± 1.3 , 46.8 ± 6.8 , $162.9 \pm 16.3 \mu\text{M}$ Zn for HW and LW seedlings, respectively. Twelve weeks after the onset of the treatments, when seedlings were 5 months old, we assessed their physiological status, and measured hydraulic conductivity and indole-3-acetic acid (IAA) concentration. Finally, we quantified their morphological traits.

Gas exchange and chlorophyll fluorescence

Physiological status was assessed 5 months after the onset of the experiment on 5 seedlings per treatment level (i.e. 40 seedlings in total). Gas exchange was analyzed with a Li-Cor 6400 portable infrared gas analyzer system (Li-Cor Inc. Lincoln, Nebraska). We determined photosynthetic rate (A), stomatal conductance (g_s) and transpiration rate (E) in one fully expanded leaf per seedling. From these data, we calculated the instantaneous water use efficiency (IWUE). We maintained gas flow rate at 350 ml min^{-1} , CO_2 concentration at 400 ppm and a photon flux density of $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$ during the course of the measurements. Previous determinations of light response curves showed that a value of $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$ was sufficient to saturate the PSII photosystem in seedlings of this species. Measurements were conducted early in the morning (09:00–11:00 h solar time).

A portable fluorometer (pulse-amplitude modulated photosynthesis yield analyzer, PAM-2010 Walz, Effeltrich, Germany) was used to measure chlorophyll fluorescence from intact leaves. In dark adapted leaves, we measured the minimum fluorescence yield (F_0) under weak red light and the maximum fluorescence (F_m) after a saturating pulse of white light. Maximal PSII photochemical efficiency ($F_v:F_m$)

was calculated as $F_v:F_m = (F_m - F_0)/F_m$ according to Genty et al. (1989).

Root hydraulic conductance

Four to five seedlings per Zn dose and irrigation regime were used to measure water transport capacity of complete and intact root systems by means of a High Pressure Flowmeter (HPFM, Dynamax Inc., Houston, TX). Measurements were carried out at the same time when gas exchange variables were measured. The night before the measurements, seedlings were watered to pot capacity, which is the amount of water remaining in a pot after irrigation and visible drainage had ceased. Root hydraulic conductance (K_R) measurements were carried out early in the morning. Root systems were kept in their substrate and perfused with distilled and degassed ultra-pure water filtered through a 0.2- μm water filtration membrane using the HPFM, Dynamax Inc. connected to the stump. K_R was measured in a transient way within a range of 0–0.5 MPa pressure at a constant increment rate of 3–5 kPa s^{-1} and the flow rate (F) was recorded every 3 s as described in Tyree et al. (1995). K_R ($\text{kg m s}^{-1} \text{MPa}^{-1}$) was calculated as the slope of ΔF (kg s^{-1}) versus ΔP (MPa) as $K_R = dF/dP$. K_R was corrected for variations in water temperature. Morphological variables were used to normalize root hydraulic conductance (K_R) on root surface area basis (K_{R-RA} ; $\text{kg m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$).

Seedling morphology

Five months after the onset of the experiment, seedlings were harvested, stems cut above the root collar and the rooting system carefully washed. Roots and leaves were digitized by scanning on an A3 flatbed scanner (Epson Expression 1680 Pro, Long Beach, CA) fitted with a transparency adaptor at 300 dpi, using an 8-bit grayscale. Images were analyzed with specific software (WINRHIZO, Regent Instruments, Québec, Canada) to evaluate projected leaf area, total root length and total root surface area. All biomass fractions were dried at 65°C for 48 h, and then weighed. Specific root length (SRL) was calculated as the ratio between total root length and root biomass.

Zn concentration in plant tissues

Dry roots and leaves were digested in a heating block at 250°C with a mixture of sulfuric acid and hydrogen peroxide (Jones and Case 1990). Digests were analyzed for Zn by inductively coupled plasma optical emission spectrometry (ICP-OES); Perkin Elmer Optima 4300 (Perkin Elmer Instruments, Edge Wood, FL).

Olea europaea leaf standard reference material (BCR: CRM 062, Commission of the European Communities Bureau of Reference, Brussels) was digested and analyzed for quality control.

Quantification of IAA

Frozen leaves (10 mg) were homogenized in 0.5 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing 0.02% diethyldithiocarbamic acid as an antioxidant and 500 pg $^{13}\text{C}_6$ -IAA as an internal standard, using the Retsch vibration mill (Retsch MM 200 mixer mill, Retsch GmbH, Haan, Germany) and a 3-mm tungsten carbide bead at a frequency of 30 Hz for 3 min. The pH was adjusted to 2.7 with 1 M HCl, and the samples were purified by solid phase extraction (SPE) and derivatized as described Andersen et al. (2008). Finally, samples were dissolved in 30 μl heptane and analyzed by gas chromatography–selected reaction monitoring–mass spectrometry (GC–SRM–MS) as described in Edlund et al. 1995.

Data analysis

Treatment effects on physiological and morphological variables were evaluated using two-way ANOVA. When the interaction term was significant, one-way ANOVA and Student's *t*-test were used to evaluate the effect of Zn at each watering level, and the effect of increased water availability at each Zn level, respectively. Root biomass and IWUE were raised to a power of 0.5 and root Zn concentration was transformed using natural logarithm prior to analysis, to homogenize the variance. Factor levels were compared pairwise (Tukey's HSD test) when ANOVA showed a significant treatment effect.

We used regression analysis to evaluate the significance of the relationship between K_{R-RA} and A and g_s . All analyses were performed using the SPSS v.15.0 statistical package (SPSS Inc., Chicago, IL).

Results

Zn bioaccumulation

Foliar Zn concentration increased in HW and LW seedlings with increasing Zn concentration in the growing medium (Fig. 2A). The magnitude of the increase was dependent on water availability (Table 1, Table 2). Thus, at the highest Zn dose, its concentration was two-fold higher in leaves of HW seedlings than in leaves of LW seedlings. The Zn bioaccumulation in roots was affected by the increase in Zn load and by the interaction between both factors (Fig 2B, Table 1).

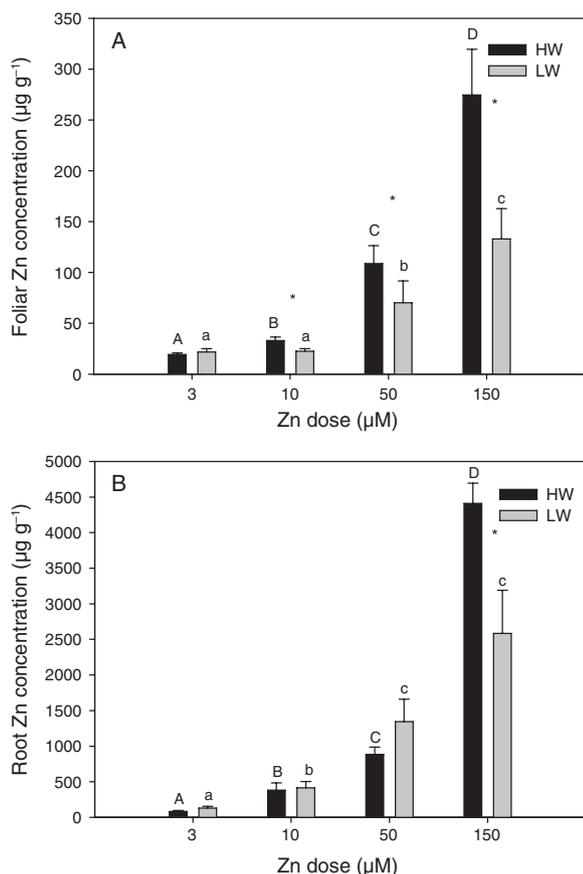


Fig. 2. Foliar (A) and root (B) Zn concentration of *Quercus suber* seedlings growing at four Zn doses and two contrasting water availability levels (HW and LW for high and low watering frequency, respectively). Bars are means and standard errors of $n = 5$ seedlings. Note the different scale used for leaves and roots. Note that data distribution along the X-axis is not proportional. Different capital letters and lowercase letters indicate significant differences at $P < 0.05$ for HW seedlings and LW seedling respectively (Tukey's HSD test). Asterisks indicate significant differences at $P < 0.05$ between seedlings HW and LW exposed to the same Zn dose (Student's t -test).

Morphological response

Foliar biomass was not affected by Zn application, water availability or the interaction between both (Tables 1 and 3). On the contrary, root biomass showed a significant effect of the increase in Zn load, and the interaction between Zn application and water availability. While root biomass in HW seedlings decreased with increasing Zn dose, LW seedlings showed the opposite trend (Table 2). At the highest Zn dose, root biomass accumulation was 33% lower in HW seedlings than in LW seedlings. This response was similar for root length, but changes in root length in LW seedlings with increasing Zn concentration were not statistically significant (Table 2). We found a marginally significant trend toward lower

specific root length as Zn dose increased at any watering level (Tables 1 and 3).

Physiological response

Maximum PSII photochemical efficiency ($F_v:F_m$) ranged between 0.65 and 0.72. $F_v:F_m$ was significantly decreased by Zn but not by water availability (Table 1). The interaction between Zn and watering regime was marginally significant as the effect of drought on this variable decreased as Zn dose increased above 3 μM Zn (Table 1, Appendix S2). In HW seedlings, $F_v:F_m$ decreased with the increase of Zn while F_o showed the opposite trend (Table 2). No significant effect of Zn application in $F_v:F_m$ and F_o in LW seedlings was found.

Zinc application, water availability and their interaction significantly affected gas exchange (Table 1, Fig. 3A and 3B). HW seedlings receiving 3 μM Zn, had 2.8 and 3.9 times higher net photosynthetic rate (A) and stomatal conductance (g_s) than LW seedlings, respectively. g_s and A decreased with increasing Zn dose in HW (Table 2). In contrast, A in LW seedlings was weakly affected by Zn application, and g_s showed a unimodal response to increase in Zn load (Table 2). IWUE increased as Zn dose increased and, in contrast to other variables, this effect was independent of the irrigation regime (see Appendix S3).

Hydraulic conductance

Root hydraulic conductance on a root area basis (K_{R-RA}) was sensitive to Zn doses, watering regime and their interaction (Fig. 4, Table 1). K_{R-RA} gradually decreased as Zn availability increased in HW seedlings, whereas K_{R-RA} was not affected by Zn doses in LW seedlings (Fig. 4, Table 2). K_{R-RA} was significantly higher in HW seedlings than in LW seedlings at low Zn availability, but this difference disappeared at Zn concentrations of 50 μM and higher (Appendix S4). Root hydraulic conductance per unit of leaf area and root hydraulic conductance per unit of root length showed similar trends (data not shown).

IAA concentration

Foliar IAA concentration was significantly affected by the increase in Zn load, and marginally affected by the interaction between Zn application and water availability (Fig. 5, Table 1). This was the result of a substantial increase in IAA in HW seedlings receiving 150 μM of Zn compared to HW seedlings receiving lower Zn doses and LW seedlings receiving any Zn dose (Table 2).

Table 1. Results of two-way ANOVA to evaluate the effects of Zn and water availability (w) on morpho-physiological traits of *Quercus suber* seedlings. Foliar Zn, foliar Zn concentration; root Zn, root Zn concentration; $F_v:F_m$, predawn maximum photochemical efficiency; F_o , minimum fluorescence; A, photosynthesis; g_s , stomatal conductance; K_{R-RA} , root hydraulic conductance per root area; IAA, auxin concentration in leaves.

Variables	F_{Zn}	P_{Zn}	F_w	P_w	$F_{Zn \times w}$	$P_{Zn \times w}$
Foliar Zn	$F_{3,26} = 105.34$	<0.001	$F_{1,26} = 12.78$	0.001	$F_{3,26} = 3.34$	0.034
Root Zn	$F_{3,26} = 46.67$	<0.001	$F_{1,26} = 0.01$	0.920	$F_{3,26} = 2.86$	0.056
Foliar biomass	$F_{3,48} = 1.95$	0.135	$F_{1,48} = 0.01$	0.905	$F_{1,48} = 2.00$	0.127
Root biomass	$F_{3,50} = 2.95$	0.041	$F_{1,50} = 0.94$	0.337	$F_{3,50} = 5.11$	0.004
Root length	$F_{3,48} = 0.25$	0.858	$F_{1,48} = 0.02$	0.870	$F_{3,48} = 3.29$	0.028
SRL	$F_{3,45} = 2.44$	0.076	$F_{1,45} = 0.93$	0.339	$F_{3,45} = 0.13$	0.943
$F_v:F_m$	$F_{3,30} = 5.09$	0.006	$F_{1,30} = 2.14$	0.154	$F_{3,30} = 2.36$	0.091
F_o	$F_{3,30} = 2.66$	0.066	$F_{1,30} = 1.18$	0.287	$F_{3,30} = 2.85$	0.054
A	$F_{3,30} = 3.74$	0.021	$F_{1,30} = 32.52$	<0.001	$F_{3,30} = 3.97$	0.017
g_s	$F_{3,30} = 4.04$	0.016	$F_{1,30} = 31.35$	<0.001	$F_{3,30} = 4.00$	0.017
IWUE	$F_{3,30} = 2.86$	0.054	$F_{1,30} = 2.71$	0.111	$F_{3,30} = 0.40$	0.756
K_{R-RA}	$F_{3,32} = 2.96$	0.047	$F_{1,32} = 22.76$	<0.001	$F_{3,32} = 6.96$	0.001
IAA	$F_{3,23} = 3.58$	0.029	$F_{1,23} = 0.08$	0.773	$F_{3,23} = 2.84$	0.060

Table 2. Results of one-way ANOVA to evaluate the effects of Zn on morpho-physiological traits of *Quercus suber* seedlings subjected to high and low watering frequency (HW and LW, respectively). Foliar Zn, foliar Zn concentration; root Zn, root Zn concentration; $F_v:F_m$, predawn maximum photochemical efficiency; F_o , minimum fluorescence; A, photosynthesis; g_s , stomatal conductance; K_{R-RA} , root hydraulic conductance per root area; IAA, auxin concentration in leaves.

Variables	F_{HW}	P_{HW}	F_{LW}	P_{LW}
Foliar Zn	$F_{3,13} = 92.33$	<0.001	$F_{3,13} = 31.51$	<0.001
Root Zn	$F_{3,14} = 99.27$	<0.001	$F_{3,14} = 35.55$	<0.001
Root biomass	$F_{3,23} = 4.23$	0.016	$F_{3,27} = 2.93$	0.052
Root length	$F_{3,22} = 2.57$	0.080	$F_{3,26} = 1.35$	0.281
$F_v:F_m$	$F_{3,15} = 6.66$	0.004	$F_{3,15} = 0.86$	0.485
F_o	$F_{3,15} = 4.88$	0.015	$F_{3,15} = 0.31$	0.819
A	$F_{3,16} = 5.02$	0.012	$F_{3,15} = 2.46$	0.106
g_s	$F_{3,16} = 4.61$	0.017	$F_{3,15} = 3.34$	0.050
K_{R-RA}	$F_{3,15} = 7.30$	0.003	$F_{3,18} = 0.63$	0.602
IAA	$F_{3,11} = 4.39$	0.029	$F_{3,12} = 0.90$	0.468

Discussion

In Mediterranean type ecosystems, water stress is the main constraint to plant performance (Di Castri 1973, Rhizopoulou and Mitrakos 1990). In these areas, prolonged summer drought, low water storage capacity of forest soils and high summer temperatures could amplify the effects of other sources of stress such as heavy metals, high temperatures or high radiation levels. In addition, the deleterious impact of both sources of stress may be enlarged in the future as a consequence of climate change and increased inputs of heavy metals (IPCC 2001, Micó et al. 2006, Sardans and Peñuelas 2007). Results of this study show that water availability and heavy metal contamination may indeed interact in complex ways, and they suggest that the combined

effects of both sources of stress may not be additive simply.

Zn effects on seedling performance

Zinc concentration in leaves of seedlings exposed to 3 μM Zn was similar to concentrations found in 1-year-old *Quercus suber* (Robert et al. 1996). Foliar Zn concentration reached critical levels (e.g. 200 $\mu\text{g g}^{-1}$ DW; Balsberg Pålsson 1989) only after exposition to 150 μM Zn. Maximum Zn concentration belowground in HW seedlings was 1.7 higher than in LW seedlings. Similar values of Zn bioaccumulation have been found in other 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. 2007b).

Aboveground biomass accumulation showed no evidence of Zn toxicity. Similar response had been observed previously (Disante et al. 2011). This could be a consequence of time span of the experiment and low Zn concentration in shoots, as belowground biomass accumulation and ecophysiological status (as leaf photosynthetic rate) were affected by Zn application. In addition, stem elongation in oaks follows successive flushes (Johnson et al. 2001). Thus, we might observe changes in shoot biomass in response to the increase in Zn load after a new flush of growth. Reductions in aboveground biomass accumulation and chlorosis in seedlings exposed to high levels of Zn have been reported in other woody species in experiments of different time span (Reichman et al. 2001, Fuentes et al. 2007b, Disante et al. 2010).

On the contrary, belowground parts were more sensitive to increase in Zn load than aboveground parts as reflected by the reduction in root biomass and the

Table 3. Morphological attributes of 5-months-old *Quercus suber* seedlings grown under a range of Zn supply rates and contrasted irrigation regimes. HW and LW correspond to high and low watering frequency, respectively. Means and standard errors of n=6–7 seedlings are shown. Different capital letters and lowercase letters indicate significant differences at $P < 0.05$ for HW seedlings and LW seedling respectively (Tukey's HSD test). Asterisks indicate significant differences at $P < 0.05$ between seedlings HW and LW exposed to the same Zn dose (Student's t -test).

	Zn application rate (μM)							
	3		10		50		150	
	HW	LW	HW	LW	HW	LW	HW	LW
Foliar biomass (g)	0.47±0.10	0.45±0.05	0.50±0.05	0.56±0.05	0.71±0.09	0.54±0.03	0.48±0.08	0.64±0.01
Root biomass (g)	2.43±0.33 ^{AB}	2.02±0.15	2.93±0.33 ^B	2.43±0.15	2.86±0.28 ^B	2.35±0.16	1.75±0.18 ^A	2.59±0.13*
Root length (cm)	1479±119	1306±123	1283±180	1199±105	1398±217	1193±94	976±105	1482±132*
Specific root length (cm g^{-1})	593±72	660±42	481±70	493±41	494±42	549±76	560±42	578±48

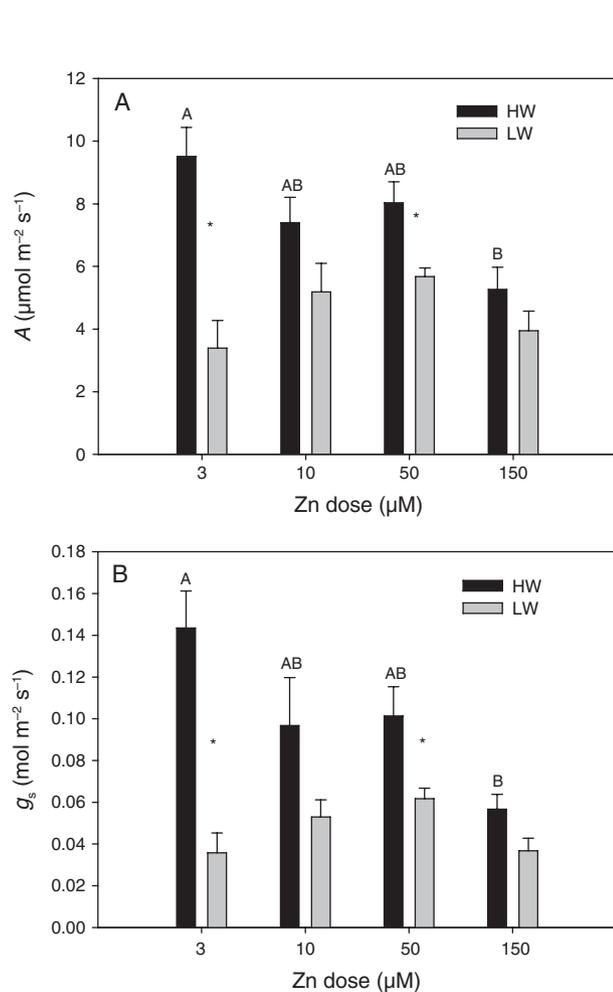


Fig. 3. Photosynthesis (A) and stomatal conductance (B) of *Quercus suber* seedlings exposed to different levels of Zn availability and contrasting watering frequency. Bars are means and standard errors of n=5 seedlings. Note that data distribution along the X-axis is not proportional. Different capital letters and lowercase letters indicate significant differences at $P < 0.05$ for HW seedlings and LW seedling respectively (Tukey's HSD test). Asterisks indicate significant differences at $P < 0.05$ between seedlings HW and LW exposed to the same Zn dose (Student's t -test).

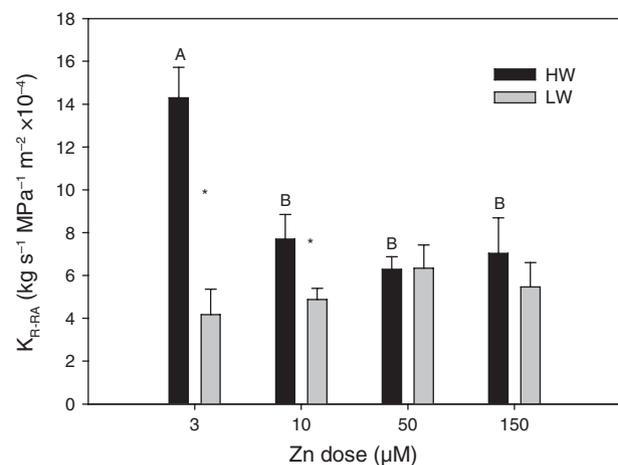


Fig. 4. Specific hydraulic conductance per root surface area in *Quercus suber* seedlings exposed to different levels of Zn availability and contrasting watering frequency. Bars are means and standard errors of n=4–5 seedlings. Note that data distribution along the X-axis is not proportional. Different capital letters and lowercase letters indicate significant differences at $P < 0.05$ for HW seedlings and LW seedling respectively (Tukey's HSD test). Asterisks indicate significant differences at $P < 0.05$ between seedlings HW and LW exposed to the same Zn dose (Student's t -test).

marginally significant decrease in root length of well watered seedlings receiving increasing doses of heavy metal. The reduction in root biomass accumulation is a common and quick response to high Zn availability (Ruano et al. 1988). Indeed Zn concentration in roots was up to 10 times higher than in shoots. This may be a result of the strong control of Zn translocation of *Quercus suber*, possibly excluder species (Disante et al. 2010).

Functionality of *Quercus suber* leaves was negatively affected by the increase in Zn load. Previous studies suggested that Zn increases plant sensitivity to photoinhibition (Joshi and Mohanty 2004, Mateos-Naranjo et al. 2008). The decline observed in $F_v:F_m$ may be due to damage in the reaction center and downregulation processes (Osmond et al., 1999). In our experiment,

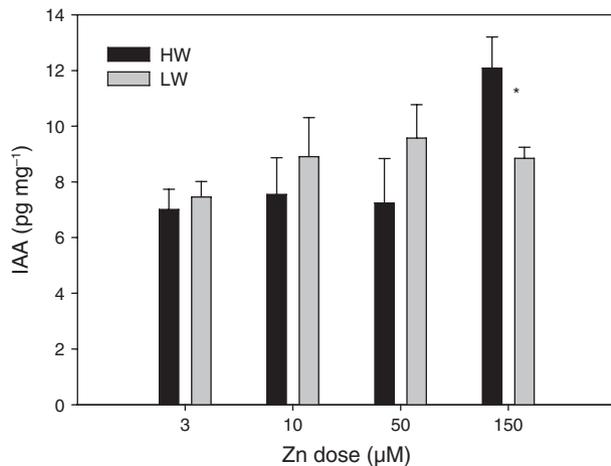


Fig. 5. Indole-3-acetic acid (IAA) concentration in leaves of *Quercus suber* seedlings exposed to different levels of Zn availability and contrasting watering frequency. Bars are means and standard errors of $n=3-4$ seedlings. Note that data distribution along the X-axis is not proportional. Different capital letters and lowercase letters indicate significant differences at $P < 0.05$ for HW seedlings and LW seedling respectively (Tukey's HSD test). Asterisks indicate significant differences at $P < 0.05$ between seedlings HW and LW exposed to the same Zn dose (Student's t -test).

this reduction was caused by a weak increase in F_o that can be induced by damages to the PSII reaction center (Moustakas et al. 1997), including reductions in the energy transfer from the antennae to the reaction center associated with chronic photoinhibition (Ralph and Burchett 1998, Popovic et al. 2003). Similarly, we observed a reduction in A and g_s in seedlings exposed to Zn application, as described in previous works (Myśliwa-Kurdziel et al. 2004, Disante et al. 2011). Impaired carbon fixation may partly reflect photoinhibition and photon damage, but also the reduction in g_s .

Another direct effect over g_s may be associated with the decrease in water transport capacity in roots affected by increment of this heavy metal. Supporting this hypothesis, we found a reduction in hydraulic conductance of roots and a positive relationship between K_{R-RA} and gas exchange rates ($R^2 = 0.64$, $F = 10.66$, $P = 0.0171$; and $R^2 = 0.73$, $F = 15.99$, $P = 0.0071$ for A and g_s , respectively). These results are in agreement with observations on stomatal sensitivity to decreasing K_{R-RA} , and the prevention of xylem cavitation (Brodrribb and Holbrook 2003, Vilagrosa et al. 2003b, Otieno et al. 2007). The negative effect of the increase in Zn load altering root morphology supports our observations in root functionality (i.e. decreases of K_{R-RA}). Heavy metals may modify root hydraulic conductance by different mechanisms, but studies on this topic are relatively scarce (Poschenrieder and Barceló 2004). Thus, previous

studies found that Hg can block aquaporins in cell membranes (Lee et al. 2005), and Zn and Cd may foster the deposition of phenolic compounds and the reduction in vessel size (Fuhrer 1982, Poschenrieder et al. 1989). Independently of the mechanisms involved, the reduction of K_{R-RA} and the concomitant decrease in A and g_s , together with changes in root biomass accumulation and root morphology, suggest that Zn may hamper the ability of *Quercus suber* seedlings to access and transport soil resources to aboveground parts. The development of extended rooting system is important to ensure plant survival and early growth in Mediterranean conditions, and especially for seedling establishment in areas subjected to intense droughts (Padilla and Pugnaire 2007, León et al. 2011).

Hormonal response to heavy metals is another poorly studied response, especially in woody species. We found a substantial increase in IAA in leaves of well-watered seedlings as Zn concentration increased. Auxins are mainly synthesized in shoots, and then translocated and accumulated in roots (Marschner 1995, Ljung et al. 2005). This hormone is involved in several processes related to the coordination of root and shoot growth and development, participating in processes such as cell elongation, formation and differentiation of vascular tissue, and in the shaping of root architecture (Mattsson et al. 2003, Aloni et al. 2006, Robert and Friml 2009). Our results show that the increase in Zn load may elicit alterations in endogenous auxin levels and suggest that this could promote the observed alterations in root morphology and functionality. Indeed, other studies have found changes in auxin accumulation after Cu exposition, which was related to morphological changes as the formation of lateral roots (Lequeux et al. 2010, Petó et al., 2011).

Combined effects of Zn and water availability

We found no significant effect of the increase in Zn load on aboveground biomass accumulation in HW seedlings, whereas aboveground biomass showed a marginally significant increase with Zn application in LW seedlings. Several studies have found increases in plant growth in response to small amounts of available Zn (Gadallah 2000, Fuentes et al. 2007b). In contrast, belowground biomass increased and decreased in LW and HW seedlings, respectively, as Zn availability increased. The marginal increase in root biomass in LW seedlings was probably because of the combination of small changes in root diameter (data not shown) and root length (Table 3): 5% and 12% increase, respectively, in seedlings exposed to 150 μM Zn compared to seedlings exposed to 3 μM Zn. We may note that root biomass

and root length were 1.5 times higher in LW seedlings than in HW seedlings when both sets received 150 μM Zn. A study combining Zn and salinity found similar results, as Zn application reduced root length and root biomass accumulation in unstressed seedlings, but it increased root growth in seedlings subjected to saline stress (Gadallah and Ramadan 1997).

Quercus suber displays intermediate tolerance to drought (Ramírez-Valiente et al. 2009). In our experiment, we did not test intense and extended drought that could probably affect root anatomy. Our objective was to study how the reduction in the frequency of irrigation influences seedling response to different Zn doses. This may explain why contrasted watering frequency did not induce changes in root length or root biomass in seedlings receiving 3 μM Zn. Data in Table 1 show that the effects of Zn and watering frequency on several seedling traits depend on the levels of the other factors. The interaction between drought and Zn points out at a synergistic or antagonistic effect of the two factors. Table 2 and Table 3 show that Zn had contrary effects on seedlings depending on the watering level. Despite that the effect of Zn on root architecture (i.e. root biomass) was marginally significant in LW seedlings ($P = 0.052$), we consider this effect to be important and worth discussing. Besides, low watering induced a marginal decrease in root average diameter compared to HW (Appendix S5). Results of a two-way ANOVA showed the lack of a significant interaction between Zn and drought, and indicate that roots of seedlings receiving low irrigation were thinner than HW seedlings. In other words, LW induced changes in root morphology.

Physiological response to increasing Zn availability also differed in LW and HW seedlings. Low irrigation frequency decreased gas exchange rates, and probably limited Zn uptake and accumulation. This may explain the lower foliar and root Zn concentrations found in LW seedlings compared with HW seedlings, and may be partially responsible for the smaller effect of Zn doses observed in the former seedlings. Zinc is incorporated by specific transporters (Lasat et al. 2000, Takahashi et al. 2012) and to our knowledge there is no report about the effect of water availability on these transporters. But drought generally reduces nutrient transport because it impairs transpiratory flow and active transport (Hu and Schmidhalter 2005).

All physiological traits measured in *Quercus suber* decreased as Zn concentration increased in HW seedlings, whereas most traits showed a unimodal response, albeit not statistically significant, to increasing Zn in LW seedlings. Stomatal conductance and carbon assimilation rates of LW seedlings receiving the lowest Zn corresponded to values obtained in *Quercus suber*

seedling exposed to mild drought (Disante et al. 2011), but they were not further reduced by the increase in Zn load. As a consequence, physiological status of LW and HW seedlings strongly differed in seedlings receiving the lowest doses of Zn, but differences gradually disappeared as Zn availability increased, or Zn concentration in roots increased above nearly 1000 $\mu\text{g g}^{-1}$ (Appendix S2). When water was limiting, the addition of 50 μM Zn increased A and g_s by 67% and 73% as compared with 3 μM Zn, respectively. Moreover, in the last case this increment was significant. These results are in agreement with observed trends in morphological variables.

In the same way, K_{R-RA} showed a similar behavior as gas exchange rates. K_{R-RA} decreased with increasing Zn concentration in LW seedlings and increased by 30% after adding 50 μM Zn. This may be a consequence of the morphological changes occurring in LW plants subjected to increased Zn availability. As a result, K_{R-RA} values converged in HW and LW seedlings at root Zn concentration above nearly 1000 $\mu\text{g g}^{-1}$ (see Appendix S4). Similar results were observed when we analyzed K_R on a leaf area and on a root length basis. Limitations to water flow through xylem vessels can increase the risk of cavitation by increasing water tension from roots to leaves (Sperry et al. 2002). However, some limitation to water flow may increase IWUE of plants by regulating water losses. This response would be beneficial to maintain a positive water balance, especially for plants living in water limited ecosystems (Vilagrosa et al. 2003a, Hernández et al. 2010).

The effect of heavy metals on IWUE has been scarcely studied, but an increase in IWUE can alleviate the effects of drought. We found that the increase in Zn load enhanced carbon assimilation per unit of water loss independently of the irrigation regime. This is remarkable, as A and g_s behaved in quite different ways in both groups of seedlings.

To what extent was the differential morphophysiological response of LW seedlings to Zn a simple consequence of reduced Zn uptake? The relationships between root Zn concentration, on the one hand, and g_s and K_R , on the other, did not overlap in LW and HW seedlings (see Appendix S4). These results suggest that the response of seedlings to low water availability influence the response of *Quercus suber* seedlings to Zn application.

Conclusion

Our results show that the combination of the increase in Zn load and moderate water stress did not induce a synergistic response in *Quercus suber* seedlings, supporting our initial hypothesis. The combination of

both stress factors may contribute to alleviate their negative impact. On the one hand, low water availability prevented Zn accumulation, and probably buffered its negative effects. On the other hand, when water was limiting, intermediate Zn concentrations may have contributed to the improvement of traits as water use efficiency and root growth, buffering the effects of water limitation. Low water availability expanded the range of Zn concentrations where this heavy metal may have a positive effect on plant performance, shifting the toxicity level up. Our results suggest that legal and practical regulations of Zn levels should take into account the water regime of each zone.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Results obtained from MINTEQ2A.

Appendix S2. Results of Student's *t*-test to evaluate the effects of drought on morpho-physiological traits of *Quercus suber* seedlings subjected to increasing Zn concentration.

Appendix S3. Instantaneous water use efficiency of *Quercus suber* seedlings exposed to different levels of Zn availability and contrasting watering frequency.

Appendix S4. Relationship between g_s and root Zn concentration, and between K_{R-RA} and root Zn concentration in *Quercus suber* seedlings exposed to different levels of Zn availability and contrasting watering frequency.

Appendix S5. Average root diameter of 5-month-old *Quercus suber* seedlings grown under a range of Zn concentrations and contrasted irrigation regimes.