



Alleviation of Zn toxicity by low water availability

Journal:	<i>Physiologia Plantarum</i>
Manuscript ID:	PPL-2013-00011.R2
Manuscript Type:	Regular manuscript - Ecophysiology, stress and adaptation
Date Submitted by the Author:	n/a
Complete List of Authors:	Disante, Karen; Departamento de Ecología. Universidad de Alicante, Ap 99, 03080 Alicante., ; Instituto Multidisciplinar para el Estudio del Medio "Ramon Margalef" (IMEM). Universidad de Alicante, Ap 99, 03080 Alicante., Cortina, Jordi; Departamento de Ecología. Universidad de Alicante, Ap 99, 03080 Alicante., ; Instituto Multidisciplinar para el Estudio del Medio "Ramon Margalef" (IMEM). Universidad de Alicante, Ap 99, 03080 Alicante., Vilagrosa, Alberto; Fundación Centro de Estudios Ambientales del Mediterráneo (CEAM), Joint Research Unit University of Alicante-CEAM, PO Box 99, 03080 Alicante., Fuentes, David; Fundación Centro de Estudios Ambientales del Mediterráneo (CEAM), Joint Research Unit University of Alicante-CEAM, PO Box 99, 03080 Alicante., Hernández, Encarni; Departamento de Ecología. Universidad de Alicante, PO Box 99, 03080 Alicante., Ljung, Karin; Umeå Plant Science Center, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83 Umeå.,
Key Words:	heavy metal, drought, Zn application, auxin, Quercus suber, multiple stresses

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6 3 Karen B. Disante^{a,b*}, Jordi Cortina^{a,b}, Alberto Vilagrosa^c, David Fuentes^c, Encarni I. Hernández^b, Karin
7 4 Ljung^c

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9 5

10 6 ^a Instituto Multidisciplinar para el Estudio del Medio “Ramon Margalef” (IMEM). Universidad de
11 7 Alicante, PO Box 99, 03080 Alicante, Spain

12 8 ^b Departamento de Ecología. Universidad de Alicante, PO Box 99, 03080 Alicante, Spain

13 9 ^c Fundación Centro de Estudios Ambientales del Mediterráneo (CEAM), Joint Research Unit
14 10 University of Alicante-CEAM, PO Box 99, 03080 Alicante, Spain.

15 11 ^d Umeå Plant Science Center, Department of Forest Genetics and Plant Physiology, Swedish
16 12 University of Agricultural Sciences, SE-901 83 Umeå, Sweden

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18 14 * Corresponding author: kb.disante@ua.es; Tel.: + 34 965909564; fax: + 34 965 909 825.

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17 Abstract

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

34 Key words: heavy metal, drought, Zn application, auxin, *Quercus suber*, multiple stresses

36 Introduction

38 In nature, plants are commonly exposed to a combination of stress factors. Multiple stresses may
39 affect plants in ways that are hardly predictable from studies of single stress factors, as synergistic and
40 antagonistic interactions are widespread (Nilsen and Orcutt 1996, Larcher 2003). Water is the most
41 limiting factor for plant productivity worldwide and several studies have evaluated the combined
42 effect of drought and other sources of stress such as extreme temperature and radiation (Valladares
43 and Percy 1997, Aranda et al. 2005, Gimeno et al. 2008). In contrast, the combined effect of drought
44 and heavy metal contamination has received less attention, despite the potential interaction between
45 both stress factors (Poschenrieder and Barceló 2004, Brady et al. 2005; Sardans and Peñuelas 2007).
46 Several studies have shown that the effect of heavy metals on plant performance may be modulated by
47 other sources of stress as sulphur and proton concentration in rainfall, heat and frost (Kukkola et al.
48 1997, Öncel et al. 2000).

50 Among heavy metals, Zn is an essential micronutrient for higher plants and it is required for several
51 physiological processes (Marschner 1995, Cakmak 2000, Broadley et al. 2007). Zinc abundance is
52 increasing in natural systems as a consequence of human activities (Kabata-Pendias and Pendias 1992,
53 Fuentes et al. 2007a; Dominguez et al. 2008). When Zn concentration exceeds a critical level, it

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3 54 becomes toxic (Balsberg Pålsson 1989, Woolhouse 1983). Symptoms of Zn toxicity include
4 55 alteration on biomass partitioning (Ruano et al. 1988), morphological changes in root system (Fuentes
5 56 et al. 2007b, Disante et al. 2010), reductions in photosynthetic rate and chlorophyll measurements (Di
6 57 Baccio et al. 2009, Disante et al. 2011), unbalance mineral nutrition (Kabata-Pendias and Pendias
7 58 1992, Monet et al. 2001) and modifications in xylem anatomy (Kasim 2007). These changes may
8 59 affect plant capacity to uptake and transport water to above ground parts (Barceló and Poschenrieder
9 60 1990, Kasim 2007).

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15 62 High levels of Zn and low water availability may induce similar responses in vascular plants,
16 63 including stomatal closure and decreased CO₂ uptake, reduced chlorophyll fluorescence and foliar
17 64 area, and activation of defence mechanisms against oxidative damage (Vaillant et al. 2005, Valladares
18 65 et al. 2005). Conversely, plants may exhibit contrasted responses to Zn toxicity and drought. While
19 66 reduced root length and root biomass have been described in seedlings exposed to Zn (Wolhouse
20 67 1983), adaptive mechanisms in response to water limitation include deeper root systems and increased
21 68 proportion of biomass allocated belowground (Nicotra et al. 2002).

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26 69 The interaction between Zn and drought may result in synergistic or antagonistic responses. Thus,
27 70 small increases in Zn availability (20 mg l⁻¹ of Zn) promoted growth in water-stressed soybeans
28 71 (Gadallah 2000). Similarly, heavy metal-enriched sewage sludge induced a positive effect on morpho-
29 72 physiological traits of seedlings subjected to drought (Pascual et al. 2004, Fuentes et al. 2007a; Santala
30 73 and Ryser 2009). Finally, in a previous study we observed that exposition of *Quercus suber* seedlings
31 74 to Zn delayed the effects of a short drought (Disante et al. 2011). High Zn concentrations could
32 75 hamper seedling capacity to absorb and transport water and compromise seedling ability to withstand
33 76 drought due to reduction of root length and biomass (Disante et al. 2010). Limited water availability
34 77 may also predispose leaves to cellular damage and photoinhibition, altering the carbon balance
35 78 (Werner et al. 2001, Vilagrosa et al. 2010). Finally, drought may impair uptake and acropetal
36 79 translocation of nutrients, including Zn (Hu and Schmidhalter 2005).

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44 81 Despite the potential interaction between Zn and drought, studies dealing with the simultaneous effect
45 82 of both stress factors are scarce, and they frequently show conflictive results (Balsberg Pålsson
46 83 1989). The objective of this study is to evaluate the combined effect of Zn supply and water
47 84 availability on plant performance, and contribute to unveil the interactions between both sources of
48 85 stress. The underlying hypothesis is that morpho-physiological responses to drought may mitigate the
49 86 effects of high Zn availability. For this study we used cork oak (*Quercus suber* L.) seedlings, a
50 87 drought-resistant tree widely distributed in the western Mediterranean basin. Cork oak woodlands
51 88 produce valuable goods and services, including cork, and they are protected by the European Union
52 89 (Habitat directive 92/43/EEC; (Pausas et al. 2009)). In addition, we performed this experiment with
53 90 young seedlings because this stage is one of the most sensitive for plant capacity to resist stress

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3 91 conditions (Vallejo et al. 2000, Vilagrosa et al. 2003a). To account for this objective, we analyzed
4 92 plant physiological status, root capacity to supply water to leaves, and phytohormonal response in
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6 93 several Zn supply rates under two levels of water availability.
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10 96 Materials and methods

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16 100 We cultivated *Q. suber* seedlings from acorns. Seeds from local origin were supplied by the Forest
17 101 Service Seed Bank (Banco de Semillas El Serranillo, Ministerio de Medio Ambiente y Medio Rural y
18 102 Marino). *Quercus suber* acorns were sown in silica sand and kept at $24 \pm 2^\circ\text{C}$. Two weeks after
19 103 germination, when the radicle was approximately 2 cm long, acorns were transplanted into 5 cm
20 104 diameter and 30 cm length tubes filled with fine, medium and coarse silica sand (the size of sand
21 105 ranged from 0.5 to 2.2 mm) in a proportion corresponding to 1:2:3 by volume, respectively. The
22 106 substrate was tested in the lab before the onset of the experiment to ensure proper drainage. The
23 107 bottom of the tubes was closed with a 1 x 1 mm mesh, which prevented substrate loss. Tubes were
24 108 placed in a growth chamber under controlled conditions of temperature ($22 \pm 1^\circ\text{C}/18 \pm 1^\circ\text{C}$, day/night),
25 109 12 h photoperiod ($600 \mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetic photon flux density during the day) and 50%
26 110 relative humidity. During this period, seedlings were irrigated with a nutrient solution containing 500
27 111 μM KCl, 435 μM NaNO_3 , 300 μM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 45 μM NaH_2PO_4 , 300 μM CaCl_2 , 0.37 μM
28 112 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.012 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.6 μM H_3BO_3 , 0.006 μM KI, 0.6 μM $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.0004
29 113 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$. This solution was a modification of the one used by Arduini et al. (1994) to
30 114 simulate forest soil conditions.
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33 116 Experimental design

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35 118 Eight weeks after germination, we added ZnSO_4 to the nutrient solution to achieve a Zn concentration
36 119 of 3 (Control), 10, 50 and 150 μM , corresponding to those commonly found in uncontaminated and
37 120 contaminated soils (Knight et al. 1997, Kabata-Pendias and Pendias 1992, González et al. 2011).
38 121 We applied two irrigation regimes using this solution: high watering (HW) and low watering (LW).
39 122 HW seedlings were irrigated three times a week (Monday, Wednesday and Friday), whereas LW
40 123 seedlings were irrigated every 5 days. During the experiment, seedlings were irrigated with 60 ml of
41 124 nutrient solution. Substrate moisture content (SMC) was estimated by measurements of water content
42 125 by gravimetric method. SMC for HW and LW treatments ranged from $2.0 \pm 0.1\%$ to $7.4 \pm 0.1\%$ and
43 126 from $0.3 \pm 0.1\%$ to $6.40 \pm 0.2\%$ respectively. The moisture content (SMC) for the substrate during 18
44 127 consecutive days is shown in Fig. 1 as example of SMC dynamics.
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3 128 Each Zn level (hereafter treatments) was replicated 10 times, i.e., 10 seedlings per Zn treatments and
4 129 irrigation level. Ion speciation calculations for solutions containing different amounts of Zn were
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6 130 conducted using MINTEQA2 software (Allison Geoscience Consultants, Inc. Flowery Branch,
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8 131 Georgia, USA and HydroGeoLogic, Inc. Herndon, Virginia, USA). The concentration in mol/L of all
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10 132 anions and cations present in the nutrient solution (described above) were entered in MINTEQA2
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12 133 software to estimate the equilibrium composition of the experimental solution. pH was fixed at 5.5
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14 134 (the pH of the nutrient solution was adjusted to this value) and quartz was added as the mineral present
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16 135 in the equilibrium.
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18 136 Calculations showed that 98% of Fe was present as Fe (II) and 81% of Zn was present as free ion and
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20 137 18% as ZnNO_3^{3+} . Both elements were highly available for plants, as only small precipitations of PO_4^{-3}
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22 138 (0.2%) and Mn^{+2} (16.2%) were found by chemical equilibrium estimations using MINTEQA2 (see
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24 139 Appendix S1 in Supporting information). Leachates obtained immediately after watering showed that
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26 140 Zn concentration in the solution was always close to the intended concentration, independently of
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28 141 water availability and plant uptake. For 3 (Control), 10, 50 and 150 μM Zn were: 4.2 ± 0.4 , 10.3 ± 3.7 ,
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30 142 47.6 ± 8.7 ; 150.2 ± 12.9 μM Zn and 2.8 ± 0.4 , 7.8 ± 1.3 , 46.8 ± 6.8 , 162.9 ± 16.3 μM Zn for HW and
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32 143 LW seedlings, respectively. Twelve weeks after the onset of the treatments, when seedlings were 5-
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34 144 months-old, we assessed their physiological status, and measured hydraulic conductivity and IAA
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36 145 concentration. Finally, we quantified their morphological traits.
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39 147 Gas exchange and chlorophyll fluorescence

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43 149 Physiological status was assessed 5 months after the onset of the experiment on 5 seedlings per
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45 150 treatment level (i.e. 40 seedlings in total). Gas exchange was analysed with a Li-Cor 6400 portable
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47 151 infrared gas analyser system (Li-Cor Inc. Lincoln, Nebraska, USA). We determined photosynthetic
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49 152 rate (A), stomatal conductance (g_s) and transpiration rate (E) in one fully expanded leaf per seedling.
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51 153 From these data, we calculated the instantaneous water use efficiency (IWUE). We maintained gas
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53 154 flow rate at 350 mL min^{-1} , CO_2 concentration at 400 ppm and a photon flux density of $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$
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55 155 1 during the course of the measurements. Previous determinations of light response curves showed that
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57 156 a value of $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$ was sufficient to saturate the PSII photosystem in seedlings of this species.
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59 157 Measurements were conducted early in the morning (09:00–11:00 h solar time).
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61 158 A portable fluorometer (pulse-amplitude modulated photosynthesis yield analyzer, PAM-2010 Walz,
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63 159 Effeltrich, Germany) was used to measure chlorophyll fluorescence from intact leaves. In dark adapted
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65 160 leaves, we measured the minimum fluorescence yield (F_0) under weak red light and the maximum
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67 161 fluorescence (F_m) after a saturating pulse of white light. Maximal PSII photochemical efficiency
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69 162 ($F_v:F_m$) was calculated as $F_v:F_m = (F_m - F_0) / F_m$ according to Genty et al. (1989)
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72 164 Root hydraulic conductance

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4 166 Four to five seedlings per Zn dose and irrigation regime were used to measure water transport capacity
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6 167 of complete and intact root systems by means of a High Pressure Flowmeter (HPFM, Dynamax Inc.,
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8 168 Houston, USA). Measures were carried out at the same time that gas exchange variables were
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10 169 measured. The night before the measurements, seedlings were watered to pot capacity, which is the
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12 170 amount of water remaining in a pot after irrigation and visible drainage had ceased. Root hydraulic
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14 171 conductance (K_R) measurements were carried out early in the morning. Root systems were kept in
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16 172 their substrate and perfused with distilled and de-gassed ultra-pure water filtered through a 0.2 μm
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18 173 water filtration membrane using the high pressure flow meter method (HPFM, Dynamax, USA)
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20 174 connected to the stump. K_R was measured in a transient way within a range of 0–0.5 MPa pressure at
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22 175 a constant increment rate of 3–5 kPa s^{-1} and the flow rate (F) was recorded every 3 s as described in
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24 176 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
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26 177 $K_R = dF/dP$. K_R was corrected for variations in water temperature. Morphological variables were used
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28 178 to normalize root hydraulic conductance (K_R) on root surface area basis (K_{R-RA} ; $\text{Kg m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$).
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31 Seedling morphology

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33 182 Five months after the onset of the experiment, seedlings were harvested, stems cut above the root
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35 183 collar and the rooting system carefully washed. Roots and leaves were digitized by scanning on an A3
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37 184 flatbed scanner (Epson Expression 1680 Pro, Long Beach, California, USA) fitted with a transparency
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39 185 adaptor at 300 dpi, using an 8-bit greyscale. Images were analyzed with specific software (WinRhizo,
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41 186 Regent Instruments, Québec, Canada) to evaluate projected leaf area, total root length and total root
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43 187 surface area. All biomass fractions were dried at 65°C for 48 h, and then weighed. Specific root length
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45 188 (SRL) was calculated as the ratio between total root length and root biomass.
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48 Zn concentration in plant tissues

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50 192 Dry roots and leaves were digested in a heating block at 250°C with a mixture of sulphuric acid and
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52 193 hydrogen peroxide (Jones and Case 1990). Digests were analyzed for Zn by ICP-OES (Perkin Elmer
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54 194 Optima 4300 Inductively Coupled Plasma Optical Emission Spectrometry). *Olea europaea* L. leaf
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56 195 standard reference material (BCR: CRM 062, Commission of the European Communities Bureau of
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58 196 Reference, Brussels) was digested and analysed for quality control.
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59 Quantification of indole-3-acetic-acid (IAA)

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61 200 Frozen leaves (10 mg) were homogenised in 0.5 ml of 0.05 M sodium phosphate buffer (pH 7.0)
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63 201 containing 0.02% diethyldithiocarbamic acid as an antioxidant and 500 pg^{13}C_6 -IAA as an internal

202 standard, using the Retsch vibration mill (Retsch MM 200 mixer mill, Retsch GmbH, Haan, Germany)
203 and a 3-mm tungstencarbide bead at a frequency of 30 Hz for 3 min. The pH was adjusted to 2.7 with 1
204 M HCl, and the samples were purified by solid phase extraction (SPE) and derivatized as described
205 Andersen et al. (2008). Finally, samples were dissolved in 30 μ l heptane and analyzed by GC-SRM-
206 MS (gas chromatography - selected reaction monitoring - mass spectrometry) as described in Edlund
207 et al. 1995.

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209 Data analysis

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211 Treatment effects on physiological and morphological variables were evaluated using two-way
212 ANOVA. When the interaction term was significant, one-way ANOVA and Student-t were used to
213 evaluate the effect of Zn at each watering level, and the effect of increased water availability at each
214 Zn level, respectively. Root biomass and instantaneous water use efficiency were raised to a power of
215 0.5 and root Zn concentration was transformed using natural logarithm prior to analysis, to
216 homogenize the variance. Factor levels were compared pairwise (Tukey's HSD test) when ANOVA
217 showed a significant treatment effect.

218 We used regression analysis to evaluate the significance of the relationship between K_R and A and g_s .
219 All analyses were performed using the SPSS v.15.0 statistical package (SPSS Inc., Chicago, Illinois,
220 USA).

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222 Results

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224 Zn bioaccumulation

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226 Foliar Zn concentration increased in HW and LW seedlings with increasing Zn concentration in the
227 growing medium (Fig. 2A). The magnitude of the increase was dependent on water availability (Table
228 1, Table 2). Thus, at the highest Zn dose, its concentration was two-fold higher in leaves of HW
229 seedlings than in leaves of LW seedlings. The Zn bioaccumulation in roots was affected by the
230 increase in Zn load and by the interaction between both factors (Fig 2B, Table 1).

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232 Morphological response

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234 Foliar biomass was not affected by Zn application, water availability or the interaction between both
235 (Table 1 and 3). On the contrary, root biomass showed a significant effect of the increase in Zn load,
236 and the interaction between Zn application and water availability. While root biomass in HW
237 seedlings decreased with increasing Zn dose, LW seedlings showed the opposite trend (Table 2). At
238 the highest Zn dose, root biomass accumulation was 33% lower in HW seedlings than in LW

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3 239 seedlings. This response was similar for root length, but changes in root length in LW seedlings with
4 240 increasing Zn concentration were not statistically significant (Table 2). We found a marginally
5 241 significant trend towards lower specific root length as Zn dose increased at any watering level (Table
6 242 1 and 3).
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10 244 Physiological response

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13 246 Maximum PSII photochemical efficiency ($F_v:F_m$) ranged between 0.65 and 0.72. $F_v:F_m$ was
14 247 significantly decreased by Zn but not by water availability (Table 1). The interaction between Zn and
15 248 watering regime was marginally significant as the effect of drought on this variable decreased as Zn
16 249 dose increased above 3 μM Zn (Table 1, Appendix S2 in Supporting Information). In HW seedlings,
17 250 $F_v:F_m$ decreased with the increase of Zn while F_o showed the opposite trend (Table 2). We found no
18 251 significant effect of Zn application in $F_v:F_m$ and F_o in LW seedlings.
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24 253 Zinc application, water availability and their interaction significantly affected gas exchange (Table 1,
25 254 Fig. 3A and 3B). HW seedlings receiving 3 μM Zn, had 2.8 and 3.9 times higher net photosynthetic
26 255 rate (A) and stomatal conductance (g_s) than LW seedlings, respectively. Stomatal conductance and A
27 256 decreased with increasing Zn dose in HW (Table 2). In contrast, A in LW seedlings was weakly
28 257 affected by Zn application, and g_s showed a unimodal response to increase in Zn load (Table 2).
29 258 Instantaneous water-use-efficiency (IWUE) increased as Zn dose increased and, in contrast to other
30 259 variables, this effect was independent of the irrigation regime (see Appendix S3 in Supporting
31 260 Information).
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36 262 Hydraulic conductance

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39 264 Root hydraulic conductance in a root area basis (K_{R-RA}) was sensitive to Zn doses, watering regime
40 265 and their interaction (Fig. 4, Table 1). K_{R-RA} gradually decreased as Zn availability increased in HW
41 266 seedlings, whereas K_{R-RA} was not affected by Zn doses in LW seedlings (Fig. 4, Table 2). K_{R-RA} was
42 267 significantly higher in HW seedlings than in LW seedlings at low Zn availability, but this difference
43 268 disappeared at Zn concentrations of 50 μM and higher (Appendix S4 in Supporting Information). Root
44 269 hydraulic conductance per unit of leaf area and root hydraulic conductance per unit of root length
45 270 showed similar trends (data not shown).
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52 272 IAA concentration

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55 274 Foliar IAA concentration was significantly affected by the increase in Zn load, and marginally
56 275 affected by the interaction between Zn application and water availability (Fig. 5, Table 1). This was
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276 the result of a substantial increase in IAA in HW seedlings receiving 150 μM of Zn compared to HW
277 seedlings receiving lower Zn doses and LW seedlings receiving any Zn dose (Table 2).

278

279 Discussion

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281 In Mediterranean type ecosystems, water stress is the main constraint to plant performance (Di Castri
282 1973, Rhizopoulou and Mitrakos 1990). In these areas, prolonged summer drought, low water storage
283 capacity of forest soils and high summer temperatures could amplify the effects of other sources of
284 stress such as heavy metals, high temperatures or high radiation levels. In addition, the deleterious
285 impact of both sources of stress may be enlarged in the future as a consequence of climate change and
286 increased inputs of heavy metals (Sardans and Peñuelas 2007, Micó et al. 2006, IPCC 2001). Results
287 of the present study show that water availability and heavy metal contamination may indeed interact in
288 complex ways, and they suggest that the combined effects of both sources of stress may not be
289 additive simply.

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291 Zn effects on seedling performance

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293 Zinc concentration in leaves of seedlings exposed to 3 μM Zn was similar to concentrations found in
294 1-year-old *Q. suber* (Robert et al., 1996). Foliar Zn concentration reached critical levels (e.g., 200 μg
295 $\text{g}^{-1}\text{d.w.}$; Balsberg Pahlsson, 1989) only after exposition to 150 μM Zn. Maximum Zn concentration
296 belowground in HW seedlings was 1.7 higher than in LW seedlings. Similar values of Zn
297 bioaccumulation have been found in other woody species grown in hydroponic culture (e.g., 3444 μg
298 g^{-1} to 5700 $\mu\text{g g}^{-1}$; Reichman et al., 2001; Fuentes et al., 2007b).

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

309 On the contrary, belowground parts were more sensitive to increase in Zn load than aboveground parts
310 as reflected by the reduction in root biomass and the marginally significant decrease in root length of
311 well watered seedlings receiving increasing doses of heavy metal. The reduction in root biomass
312 accumulation is a common and quick response to high Zn availability (Ruano et al. 1988). Indeed Zn

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3 313 concentration in roots was up to ten times higher than in shoots. This may be a result of the strong
4 314 control of Zn translocation of *Q. suber*, possibly excluder species (Disante et al. 2010).

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7 316 Functionality of *Q. suber* leaves was negatively affected by the increase in Zn load. Previous studies
8 317 suggested that Zn increases plant sensitivity to photoinhibition (Joshi and Mohanty 2004, Mateos-
9 318 Naranjo et al. 2008). The decline observed in $F_v:F_m$ may be due to damage in the reaction centre and
10 319 down-regulation processes (Osmond et al. 1999). In our experiment, this reduction was caused by a
11 320 weak increase in F_o that can be induced by damages to the PSII reaction centre (Moustakas et al.
12 321 1997), including reductions in the energy transfer from the antennae to the reaction centre associated
13 322 with chronic photoinhibition (Ralph and Burchett 1998, Popovic et al. 2003). Similarly, we observed a
14 323 reduction in A and g_s in seedlings exposed to Zn application, as described in previous works (Myśliwa-
15 324 Kurdziel et al. 2004, Disante et al. 2011). Impaired carbon fixation may partly reflect photoinhibition
16 325 and photon damage, but also the reduction in g_s .

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18 327 Another direct effect over g_s may be associated with the decrease in water transport capacity in roots
19 328 affected by increment of this heavy metal. Supporting this hypothesis, we found a reduction in
20 329 hydraulic conductance of roots and a positive relationship between K_R and gas exchange rates ($R^2=$
21 330 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
22 331 are in agreement with observations on stomata sensitivity to decreasing K_R , and the prevention of
23 332 xylem cavitation (Brodribb and Holbrook 2003, Vilagrosa et al. 2003b, Otieno et al. 2007). The
24 333 negative effect of the increase in Zn load altering root morphology supports our observations in root
25 334 functionality (i.e. decreases of K_R). Heavy metals may modify root hydraulic conductance by different
26 335 mechanisms, but studies on this topic are relatively scarce (Poschenrieder and Barceló 2004). Thus,
27 336 previous studies found that Hg can block aquaporins in cell membranes (Lee et al. 2005), and Zn and
28 337 Cd may foster the deposition of phenolic compounds and the reduction in vessel size (Fuhrer 1982,
29 338 Poschenrieder et al. 1989). Independently of the mechanisms involved, the reduction of K_R and the
30 339 concomitant decrease in A and g_s , together with changes in root biomass accumulation and root
31 340 morphology, suggest that Zn may hamper the ability of *Q. suber* seedlings to access and transport soil
32 341 resources to aboveground parts. The development of extended rooting system is important to ensure
33 342 plant survival and early growth in Mediterranean conditions, and especially for seedling establishment
34 343 in areas subjected to intense droughts (Padilla and Pugnaire, 2007, León et al. 2011).

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37 346 Hormonal response to heavy metals is another response poorly studied, especially in woody species.
38 347 We found a substantial increase in IAA in leaves of well watered seedlings as Zn concentration
39 348 increased. Auxins are mainly synthesized in shoots, and then translocated and accumulated in roots
40 349 (Marschner 1995, Ljung et al. 2005) This hormone is involved in several process related to the

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3 350 coordination of root and shoot growth and development, participating in processes as cell elongation,
4 351 formation and differentiation of vascular tissue, and in the shaping of root architecture (Mattsson et al.
5 352 2003; Aloni et al. 2006, Robert and Friml 2009). Our results show that the increase in Zn load may
6 353 elicit alterations in endogenous auxin levels and suggest that this could promote the observed
7 354 alterations in root morphology and functionality. Indeed, other studies have found changes in auxin
8 355 accumulation after Cu exposition, which was related to morphological changes as the formation of
9 356 lateral roots (Lequeux et al. 2010, Petó et al. 2011).

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14 358 Combined effects of Zn and water availability

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18 360 We found no significant effect of the increase in Zn load on aboveground biomass accumulation in
19 361 HW seedlings, whereas aboveground biomass showed a marginally significant increase with Zn
20 362 application in LW seedlings. Several studies have found increases in plant growth in response to small
21 363 amounts of available Zn (Gadallah 2000, Fuentes et al. 2007b). In contrast, belowground biomass
22 364 increased and decreased in LW and HW seedlings, respectively, as Zn availability increased. The
23 365 marginal increase in root biomass in LW seedlings was probably due to the combination of small
24 366 changes in root diameter (data not shown) and root length (Table 3): 5% and 12% increase,
25 367 respectively, in seedlings exposed to 150 μM Zn compared to seedlings exposed to 3 μM Zn. We may
26 368 note that root biomass and root length were 1.5 times higher in LW seedlings than in HW seedlings
27 369 when both sets received 150 μM Zn. A study combining Zn and salinity found similar results, as Zn
28 370 application reduced root length and root biomass accumulation in unstressed seedlings, but it increased
29 371 root growth in seedlings subjected to saline stress (Gadallah and Ramadan 1997).

30 372 *Quercus suber* displays intermediate tolerance to drought (Ramírez-Valiente et al. 2009). In our
31 373 experiment, we did not test intense and extended drought that could probably affect root anatomy. Our
32 374 objective was to study how the reduction in the frequency of irrigation influences seedling response to
33 375 different Zn doses. This may explain why contrasted watering frequency did not induce changes in
34 376 root length or root biomass in seedlings receiving 3 μM Zn. Data on Table 1 show that the effects of
35 377 Zn and watering frequency on several seedling traits depend on the levels of the other factors. The
36 378 interaction between drought and Zn points out at a synergistic or antagonistic effect of the two factors.
37 379 Table 2 and Table 3 show that Zn had opposite effects on seedlings depending on the watering level.
38 380 Despite that the effect of Zn on root architecture, (i.e., root biomass) was marginally significant in LW
39 381 seedlings ($P=0.052$), we consider this effect to be important and worth discussing. Besides, low
40 382 watering induced a marginal decrease in root average diameter compared to HW (Appendix S5 in
41 383 Supporting information). Results of a two-way ANOVA showed the lack of a significant interaction
42 384 between Zn and drought, and indicate that roots of seedlings receiving low irrigation were thinner than
43 385 HW seedlings. In other words, LW induced changes in root morphology.

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3 387 Physiological response to increasing Zn availability also differed in LW and HW seedlings. Low
4 388 irrigation frequency decreased gas exchange rates, and probably limited Zn uptake and accumulation.
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6 389 This may explain the lower foliar and root Zn concentrations found in LW seedlings compared with
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8 390 HW seedlings, and may be partially responsible for the smaller effect of Zn doses observed in the
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10 391 former seedlings. Zinc is incorporated by specific transporters (Lasat et al. 2000, Takahashi et al.
11 392 2012) and to our knowledge there is no report about the effect of water availability on these
12 393 transporters. But drought generally reduces nutrient transport because it impairs transpiratory flow and
13 394 active transport (Hu and Schmidhalter 2005).
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16 396 All physiological traits measured in *Q. suber* decreased as Zn concentration increased in HW
17 397 seedlings, whereas most traits showed a unimodal response, albeit not statistically significant, to
18 398 increasing Zn in LW seedlings. Stomatal conductance and carbon assimilation rates of LW seedlings
19 399 receiving the lowest Zn corresponded to a values obtained in *Q. suber* seedling exposed to mild
20 400 drought (Disante et al. 2011), but they were not further reduced by the increase in Zn load. As a
21 401 consequence, physiological status of LW and HW seedlings strongly differed in seedlings receiving
22 402 the lowest doses of Zn, but differences gradually disappeared as Zn availability increased, or Zn
23 403 concentration in roots increased above ca. 1000 $\mu\text{g g}^{-1}$ (Appendix S2). When water was limiting, the
24 404 addition of 50 μM Zn increased A and g_s in a 67% and 73% as compared with 3 μM Zn, respectively.
25 405 Moreover, in the last case this increment was significant. These results are in agreement with observed
26 406 trends in morphological variables.
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29 408 In the same way, K_{R-RA} showed a similar behaviour as gas exchange rates. K_{R-RA} decreased with
30 409 increasing Zn concentration in LW seedlings and increases a 30% after 50 μM Zn. This may be a
31 410 consequence of the morphological changes occurring in LW plants subjected to increased Zn
32 411 availability. As a result, K_{R-RA} values converged in HW and LW seedlings at root Zn concentration
33 412 above ca. 1000 μg^{-1} (see Appendix S4 in Supporting Information). Similar results were observed when
34 413 we analysed K_R on a leaf area and a root length basis. Limitations to water flow through xylem vessels
35 414 can increase the risk of cavitation by increasing water tension from roots to leaves (Sperry et al. 2002).
36 415 However, some limitation to water flow may increase IWUE of plants by regulating water losses. This
37 416 response would be beneficial to maintain a positive water balance, especially for plants living in water
38 417 limited ecosystems (Vilagrosa et al. 2003a, Hernández et al. 2010).

39 418 The effect of heavy metals on IWUE has been scarcely studied, but an increase in IWUE can alleviate
40 419 the effects of drought. We found that the increase in Zn load enhanced carbon assimilation per unit of
41 420 water loss independently of the irrigation regime. This is remarkable, as A and g_s behaved in quite
42 421 different ways in both groups of seedlings.

43 422 To what extent was the differential morpho-physiological response of LW seedlings to Zn a simple
44 423 consequence of reduced Zn uptake? The relationships between root Zn concentration, on the one hand,
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424 and g_s and K_R , on the other, did not overlap in LW and HW seedlings (see Appendix S4 in Supporting
425 Information). These results suggest that the response of seedlings to low water availability influence
426 the response of *Q. suber* seedlings to Zn application.
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For Peer Review

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3 429 Conclusion

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6 431 Our results demonstrate that the combination of the increase in Zn load and moderate water stress did
7 432 not induce a synergistic response in *Q. suber* seedlings, supporting our initial hypothesis. The
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9 433 combination of both stress factors, may contribute to alleviate their negative impact. On the one hand,
10 434 low water availability prevented Zn accumulation, and probably buffered its negative effects. On the
11 435 other hand, when water was limiting, intermediate Zn concentrations may have contributed to the
12 436 improvement of traits as water use efficiency and root growth, buffering the effects of water
13 437 limitation. Low water availability expanded the range of Zn concentrations where this heavy metal
14 438 may have a positive effect on plant performance, shifting the toxicity level up. Our results suggest that
15 439 legal and practical regulations of Zn levels should take into account the water regime of each zone.

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17 441 Acknowledgements

18 442

19 443 We thank Jose Huesca for their assistance in the lab, Banco de Semillas El Serranillo for the seeds
20 444 used in this experiment and the University of Alicante for an FPU (Formacion del Profesorado
21 445 Universitario) grant awarded to K. Disante. This research was funded by the Spanish Ministry of
22 446 Science and Innovation (Project GRACCIE, Programa Consolider-Ingenio 2010 (CSD 2007-00067)
23 447 and SURVIVE (CGL-2011-30531-CO2-02)) and Generalitat Valenciana (FEEDBACKS-
24 448 PROMETEO/2009/006). E.I. Hernández thanks the University of Alicante for her FPU research
25 449 fellowship. CEAM is supported by Generalitat Valenciana.

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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, SRL: specific root length, $F_v:F_m$: predawn maximum photochemical efficiency, F_o : minimum fluorescence, A : photosynthesis; g_s : stomatal conductance, $IWUE$: instantaneous water use efficiency, K_{R-RA} : root hydraulic conductance per root area, IAA: auxin concentration in leaves.

Variables	F_{Zn}	P_{Zn}	F_w	P_w	F_{Znxw}	P_{Znxw}
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

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 showed.

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 (t-Student).

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

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3 Figure 1. Changes in substrate moisture content (SMC) for HW and LW seedlings during 18
4 consecutive days. HW and LW correspond to high and low watering frequency, respectively.
5 Means and standard errors of N= 6-7 tubes per treatment are shown.
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9 Figure 2. Foliar (A) and root (B) Zn concentration of *Quercus suber* seedlings growing at four
10 Zn doses and two contrasting water availability levels (HW and LW for high and low watering
11 frequency, respectively). Bars are means and standard errors of N=5 seedlings. Note the
12 different scale used for leaves and roots. Note that data distribution along the X-axis is not
13 proportional.
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16 Different capital letters and lowercase letters indicate significant differences at $P < 0.05$ for HW
17 seedlings and LW seedling respectively (Tukey's HSD test). Asterisks indicate significant
18 differences at $P < 0.05$ between seedlings HW and LW exposed to the same Zn dose (t-
19 Student).
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26 Figure 3. Photosynthesis (A) and stomatal conductance (B) of *Quercus suber* seedlings exposed
27 to different levels of Zn availability and contrasting watering frequency. Bars are means and
28 standard errors of N=5 seedlings. Note that data distribution along the X-axis is not
29 proportional.
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32 Different capital letters and lowercase letters indicate significant differences at $P < 0.05$ for HW
33 seedlings and LW seedling respectively (Tukey's HSD test). Asterisks indicate significant
34 differences at $P < 0.05$ between seedlings HW and LW exposed to the same Zn dose (t-
35 Student).
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41 Figure 4. Specific hydraulic conductance per root surface area in *Quercus suber* seedlings
42 exposed to different levels of Zn availability and contrasting watering frequency. Bars are
43 means and standard errors of N=4-5 seedlings. Note that data distribution along the X-axis is
44 not proportional.
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47 Different capital letters and lowercase letters indicate significant differences at $P < 0.05$ for HW
48 seedlings and LW seedling respectively (Tukey's HSD test). Asterisks indicate significant
49 differences at $P < 0.05$ between seedlings HW and LW exposed to the same Zn dose (t-
50 Student).
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56 Figure 5. Indole-3-acetic acid (IAA) concentration in leaves of *Quercus suber* seedlings
57 exposed to different levels of Zn availability and contrasting watering frequency. Bars are
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3 means and standard errors of N=3-4 seedlings. Note that data distribution along the X-axis is
4 not proportional.

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6 Different capital letters and lowercase letters indicate significant differences at $P < 0.05$ for HW
7 seedlings and LW seedling respectively (Tukey's HSD test). Asterisks indicate significant
8 differences at $P < 0.05$ between seedlings HW and LW exposed to the same Zn dose (t-
9 Student).
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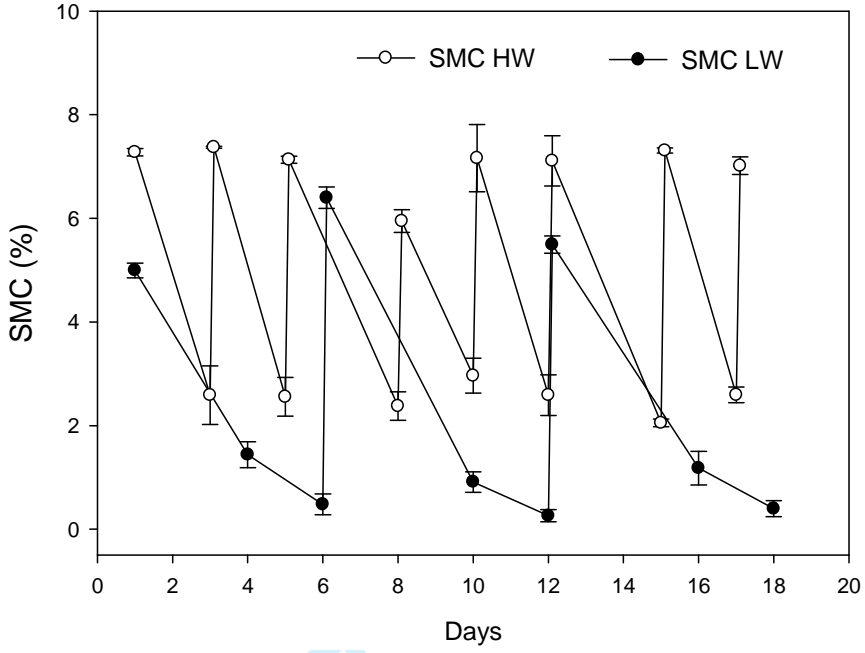


Figure 1

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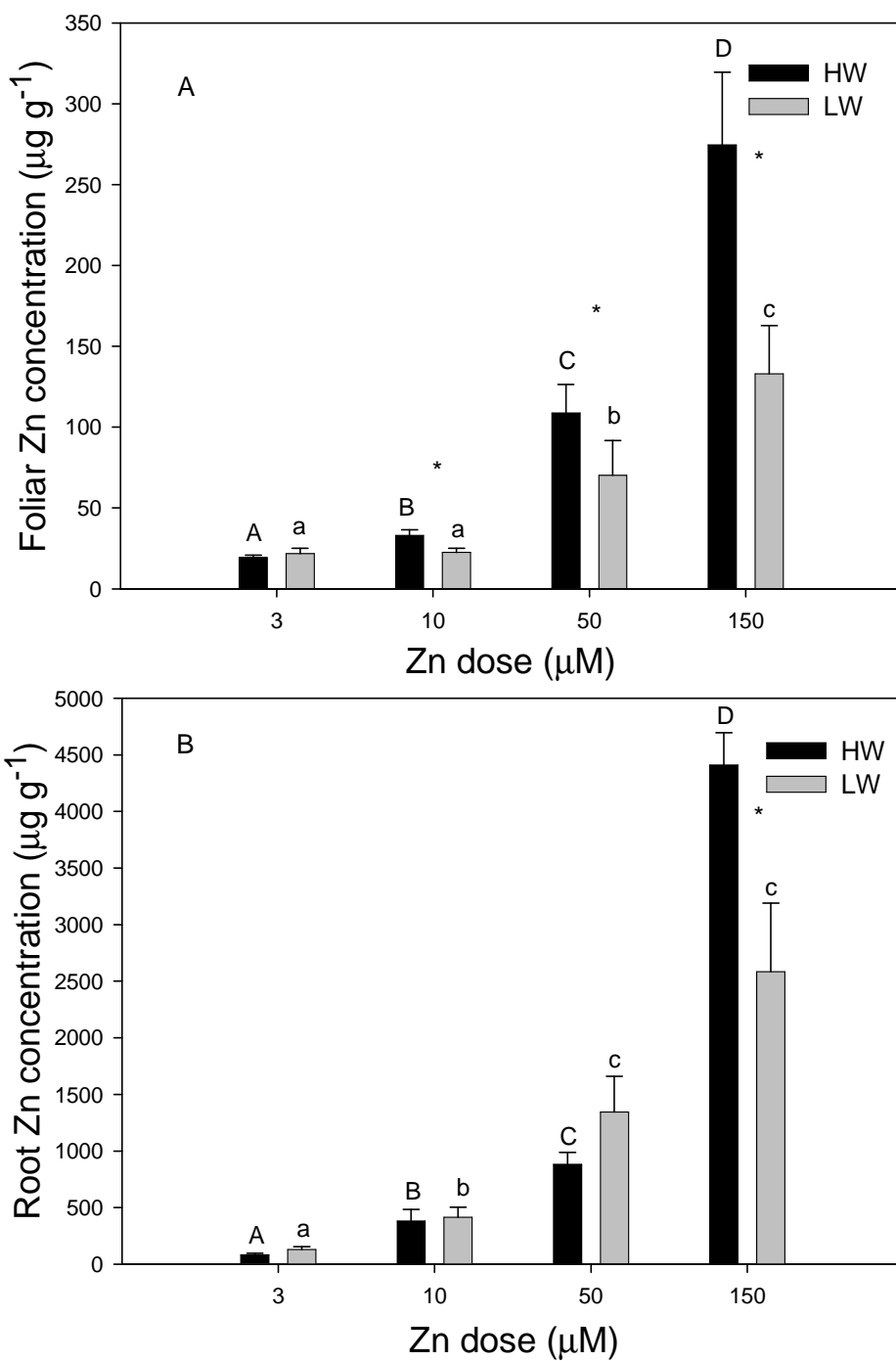


Figure 2.

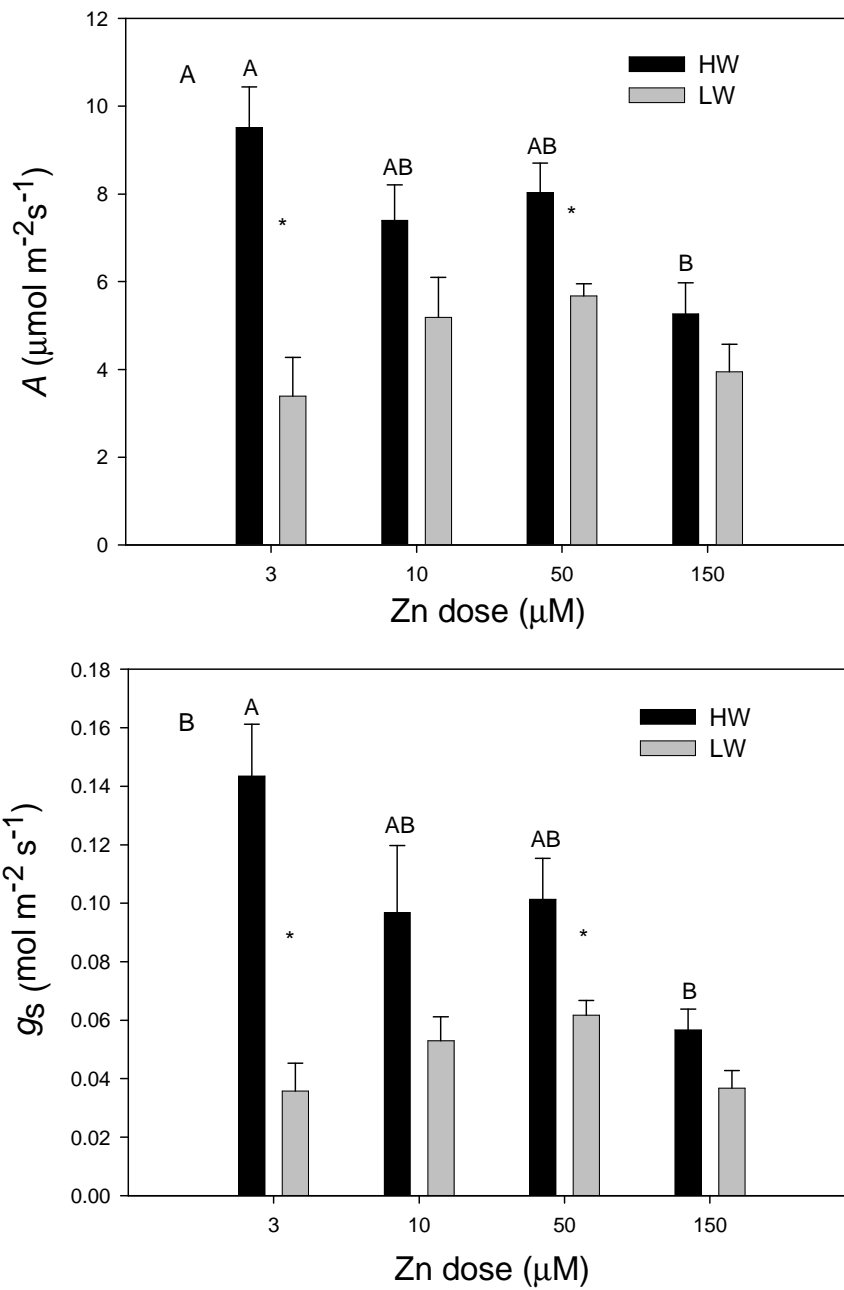


Figure 3

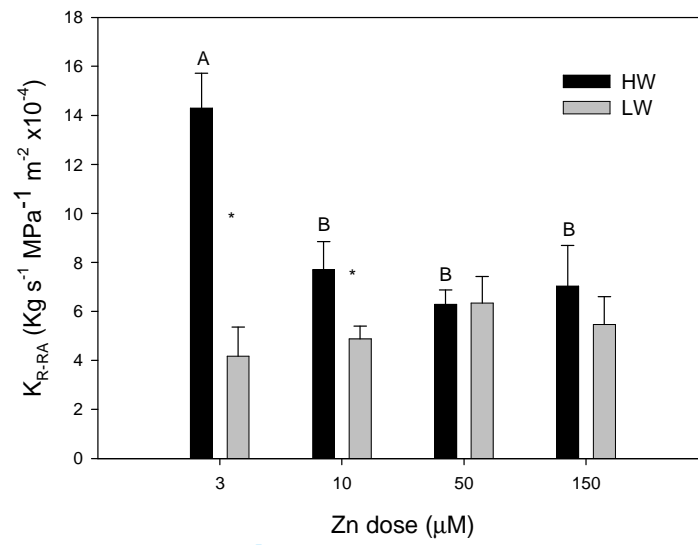


Figure 4

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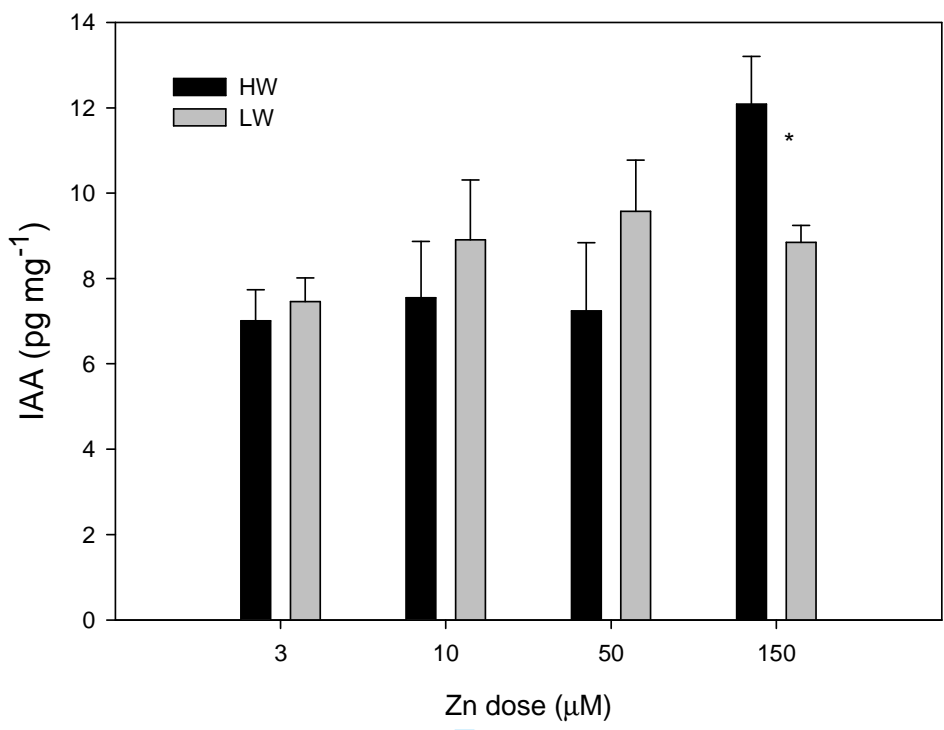


Figure 5

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