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RESEARCH ARTICLE

Effects of mercury on the germination and growth of *Quercus ilex* L. seedlings

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Abstract

While it is well-known that the toxicity of mercury for plants is related to its bioavailability in the environment in which the plant lives, few studies have addressed Hg effects under controlled conditions of life-limiting available Hg concentrations. This study examines the effects of Hg on the holm oak (*Quercus ilex* L.) exposed to medium-high available Hg concentrations. Holm oak seeds were sown in a perlite substrate and grown in the presence of a nutrient solution containing 0, 5, 25, or 50 μM Hg. The variables determined as outcome measures were impacts on germination, growth, and nutrient accumulation along with Hg concentration in leaves, stems, and roots at different growth stages. Our findings suggest no overall detrimental effects of the metal on germination, nutrient accumulation, and plant growth, although root morphology was clearly modified. Mercury accumulation in the plant varied according to time, organ, Hg treatment dose, and plant growth stage. When comparing Hg build-up in the different organs, highest concentrations of the metal were detected in the roots, followed by the leaves and stems. The Hg accumulation pattern was positively correlated with time and Hg dose, whereas negative correlation was observed with growth stage. The impacts of all these factors on Hg accumulation were not additive pointing to interesting interaction effects that should be explored in future work.

Keywords Holm oak · Growth stage · Mercury accumulation · Nutrient accumulation · Interaction effects

Introduction

Mercury (Hg) is a toxic heavy metal that naturally occurs in the environment mostly as deposits of mercuric sulphide. On the Earth's surface, its presence is fairly scarce, except in places where geological anomalies have occurred generating high concentrations. Examples of these places are Almadén (Ciudad Real, Spain), Mieres (Asturias, Spain), or Idrija (Slovenia) where total Hg concentrations in soil of up to 1650–10,200 mg kg^{-1} have been detected (Fernández-Martínez et al. 2005; Millán et al. 2006; Tomiyasu et al. 2017). Despite these high soil concentrations of the metal, plants are able to survive and reproduce in such environments (Higueras et al. 2004; Millán et al. 2006; Moreno-Jiménez et al. 2006; Sierra et al. 2012; Millán et al. 2012; Miklavcic et al. 2013; Millán et al. 2014; Fernández-Martínez et al. 2015). This is because Hg toxicity is limited as a consequence of its low bioavailability. However, where Hg mining activities have been intense, high bioavailable Hg concentrations really limit the living conditions of plants, as shown in experiments conducted on herbaceous and shrubby plants (Cho and Park 2000; Li et al. 2005; Israr et al. 2006; Moreno-Jiménez et al. 2007, 2013; Cardoso et al. 2015; Marrugo-Negrete et al. 2016). It thus seems of particular interest to examine plant tolerance to concentrations of bioavailable Hg that could pose a risk for life.

The present study was designed to assess the behavior of an arboreal species subjected to medium-high available Hg concentrations. Few studies have assessed the effects of medium-high Hg levels on tree species under controlled conditions, although Hg accumulation in plants has been a topic of research for several decades (Sengar et al. 2010). In this regard, our experimental design offers some novelty. Firstly, seeds were sown from the beginning in a substrate contaminated

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with the metal, which is usual in germination studies but not in studies involving seedlings. This approach should offer information regarding the difficulties encountered by the plant to reproduce naturally in Hg-polluted sites. Secondly, Hg accumulation in leaves and stems was determined according to growth stage. Prior studies have shown that plants can accumulate Hg over time (Rasmussen 1995; Rodríguez-Alonso et al. 2014), and this made us consider that the given growth stage could also influence Hg build-up.

For our study, we looked for a tree species that grows in abundance and occurs naturally in places highly polluted with mercury. As the study area, we chose the Almadén mining region, which is the place in the world with the higher presence of Hg and also renowned for its Hg exploitation (Higueras et al. 2006). As the arboreal species, we selected the holm oak (*Quercus ilex* subsp. *ballota* (Desf.) Samp.), which is the main tree species in the Almadén mining district. Moreover, the holm oak is widespread in the Mediterranean region (Spain, Italy, Greece, Morocco, Algeria, Syria, etc.).

To date, the effects of Hg on the holm oak have been scarcely addressed. Mercury concentrations in holm oak leaves have been determined by some authors (Sardans and Peñuelas 2006; Higueras et al. 2017). There is also information about mercury distributions in plants (Villadóniga et al. 2009; Rodríguez-Alonso et al. 2014). However, all these investigations have been based on observational studies and are somewhat incomplete with respect to the influence of Hg on the holm oak. In prior work (Rodríguez-Alonso et al. 2017), we compared holm oak populations from Almadén to those from the surrounding areas and were unable to find populations especially adapted to Hg in Almadén. In the present study, we examined the biological response of the holm oak to mercury, with the objectives of assessing (i) how Hg affects the germination of seeds, seedling growth, and nutrient uptake; ii) mercury distributions according to the organs of the seedlings and growth stage; and iii) changes over time in plant Hg concentrations.

Materials and methods

Sampling sites and acorn collection

A field sampling effort was designed to collect six lots of *Q. ilex* subsp. *ballota* seeds from six different sites in the Almadén mining region and areas close by. For this study, we only used five lots as they have shown similar Hg-tolerance (Rodríguez-Alonso et al. 2017). Supplementary Table 1 shows the locations and a general description of the selected sampling sites.

According to Jiménez et al. (1996), all sampling sites belong to the 11th provenance region for the distribution of *Q. ilex* subsp. *ballota* in Spain. All populations are adapted to the same climate, and therefore, it was assumed they would also respond similarly to the greenhouse conditions established.

At each sampling site, 3-5 holm oaks with abundant seed production were chosen. From these trees, we collected large, healthy-looking acorns. The mean number of acorns collected was 742 ± 126 per sampling site. Acorns were stored until sowing in a wet, cold environment. More details of seed storage can be found in Rodríguez-Alonso et al. (2017).

To determine mean seed weight of the seed lots, 50 seeds per lot were weighed. Mean seed weight was 6.6 ± 1.1 g. Mean Hg concentration in the seeds was 0.016 ± 0.007 mg kg^{-1}(d.w.). Prior to sowing, all the acorns were checked to discard those drilled by insects, misshapen, or dehydrated, as suggested by Zazo et al. (2000). The dehydration condition of the seeds was assessed through a gravimetric test described by these last authors (Zazo et al. 2000).

Culture methods

The experiment ran for 93 days (March 28–June 28). The acorns were sown in 24-cell forestry seedling trays filled with a perlite substrate in a greenhouse. Ninety-six acorns were sown per treatment and lot, as two acorns per cell. When two acorns germinated in the same cell, only one of them was kept. Seeds and seedlings were watered with a nutrient solution prepared as described elsewhere (Moreno-Jiménez et al. 2007). Mercury was added to this solution as $HgCl_2$ at the concentrations 0 (control), 5, 25, and 50 μM. These doses were selected on the basis of results reported for soils of the Almadén mining area in which the available Hg concentrations were the highest (Millán et al. 2006; Millán et al. 2011). Once the seeds were sown, they were watered in the forestry trays placed in large standard trays by filling the latter with the respective nutrient solution. Solutions were prepared fresh weekly.

Germination

All the seeds that germinated were counted and two variables calculated: the number of seeds germinated as a germination percentage, and the speed of germination index (SGI) as the germination rate. Both variables were determined throughout the study period (93 days). As the seeds were slightly buried in the perlite substrate, germination was determined as the number of shoots that emerged over the surface. Two or more shoots from the same seed were counted as one. SGI was calculated following Maguire's formula (1962), which takes into account how many seeds germinate per day. The faster the seeds germinate, the higher the SGI.

Plant sampling

Seedlings were sampled 15 days and 68 days after their emergence (Day 0). Sample sizes were 2–4 seedlings and 10–16 seedlings per combination (treatment × lot) in the first and second sampling sessions, respectively. In all samplings, plants were divided into two portions: roots and shoots. In the second session (68 days), the above-ground, or aerial, parts of the plants were separated into three height levels corresponding to growth stages (Fig. 1). Not all plants reached the third growth stage.

After sampling, the roots and above-ground portions of each plant were weighed and measured one by one to determine fresh weights and lengths or heights. The leaves were separated from the stem and then both washed separately to remove external contamination. For above-ground organs, the washing procedure consisted of washing twice in deionized water in an ultrasonic bath for 10 min per cycle. Roots were washed in deionized water for 10 s. All plant matter was dried at room temperature until a constant weight was obtained. A composite sample of the same plant fractions (roots; first–growth stage stem; first–growth stage leaves, etc.) was prepared per each combination (treatment × lot). This was needed to analyze the Hg and nutrient concentration of the fractions. Thus, each fraction of each combination was counted as only one datum, but each datum arose from the mix of several holm oak seedlings. Dry weight was determined by weighing those composite samples. Finally, the samples were ground by an IKA A-10 mill for chemical analysis.

Chemical element determinations: macronutrients, micronutrients, and mercury

To determine the macronutrient (Ca and K) and micronutrient (Cu, Fe, Mg, Mn, and Zn) concentration following the classification of Mengel et al. (2001), the plant samples were digested according to the acid digestion method (Lozano Rodríguez et al. 1995) under pressure in a microwave MARS 5 (CEM Corporation). Concentrations of Ca, Cu, Fe, Mg, Mn, and Zn were measured by atomic absorption spectroscopy (Perkin Elmer AAnalyst 700). Potassium was determined by flame emission spectroscopy (Perkin Elmer AAnalyst 700).

The Hg concentrations of all plant samples were determined in an atomic absorption spectrophotometer especially designed for Hg determination (Advance Mercury Analyzer, AMA-254, LECO Instruments). Leaves and stems were directly assayed in the AMA, while roots needed prior digesting, as described above for nutrient concentrations.

Certified reference material (BCR-060, IRMM) was used to determine the accuracy of the measurements.

Data analysis

Statistical analysis of data was performed using the software package SPSS for Windows (version 14.0, IBM, Armonk, NY). Germination percentages were compared through logistic regression analysis, while germination rates were compared by one-way ANOVA (post-hoc Tukey's test). The Mann-Whitney *U* test was used to determine differences in

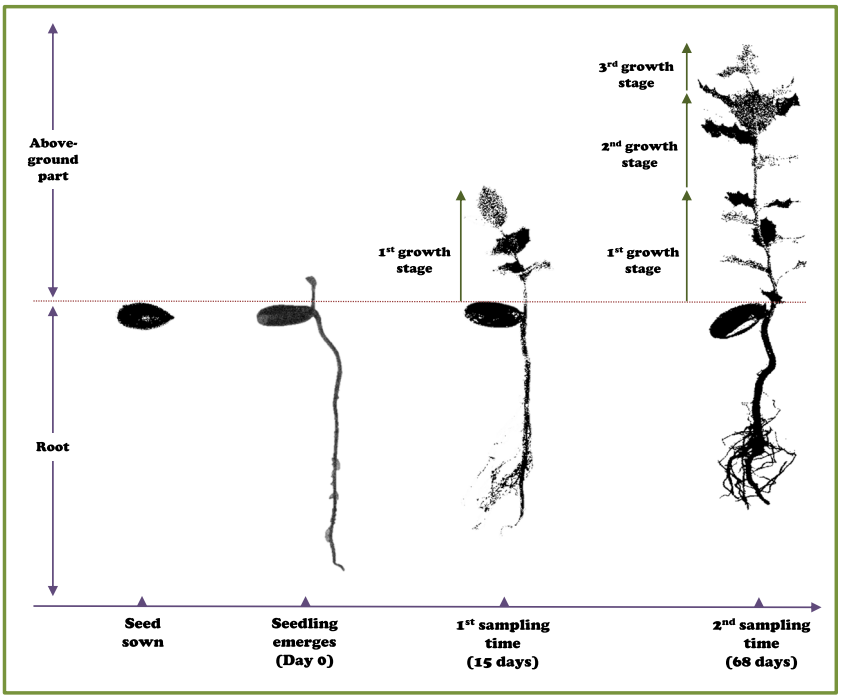

Fig. 1. Time points and growth stages of the seedlings defined for the study

fresh weight, height, and dry weight of the plants, Hg concentrations, and nutrient uptake. This test was used only when either the assumptions in ANOVA (normality and homoscedasticity) could not be accepted or the sample size was too small ($n < 12$). The normality was assessed by the Shapiro-Wilk test and homoscedasticity by the Levene test. Significance was set at $\alpha = 0.05$.

All data in the text, figures, and tables are expressed as means ± standard deviation.

Results and discussion

Effect of mercury on seedling germination

In all treatments, maximum seedling emergence was recorded from the 18th–36th day after sowing (Supplementary Figure 1). During this period, 86 ± 2% of the seeds emerged without significant differences observed among treatments (≤ 5%).

Both germination percentages and rates were not significantly affected by the Hg treatments, as can be seen in Fig. 2. This was not expected according to the findings reported in the literature. In effect, reduced germination percentages in response to Hg treatment have been described for several species of Hyacinthaceae (Street et al. 2007), *Arabidopsis thaliana* (Li et al. 2005), *Brassica* spp. (Ling et al. 2010), and *Zea mays* (Deng et al. 2016). Impacts on germination rates have been less studied but some authors have linked low germination rates to the presence of diesel oil (Bona et al. 2011), arsenic (Li et al. 2007), or Hg (Cardoso et al. 2015) in soil. Here, most differences in germination were detected when comparing the control treatment with the remaining

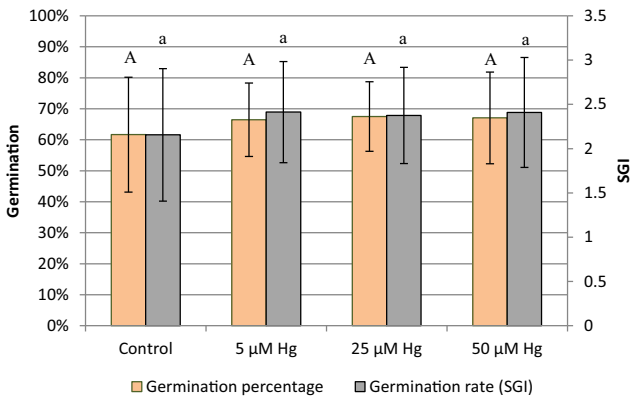

Fig. 2. Germination percentages (n = 480 acorns per treatment) and germination rates (n = 5 seed lots per treatment) recorded for each mercury treatment level. Germination rate was determined as the Speed of Germination Index (SGI). Values represent mean ± S.D. Different letters indicate significant differences between the treatments ($\alpha = 0.05$)

treatments, as the former returned the lowest germination percentages and rates (Fig. 2). As possible explanations for our unexpected results, we propose, first, that toxic elements in low levels can stimulate germination (Kranner and Colville 2011) and, secondly, that the Hg treatments examined were not sufficiently toxic in the perlite substrate employed. Moreover, mercury could have acted as a disinfectant for the acorns, since the metal could affect soil pathogens that cause damping off effects and this could improve germination values.

Effects of mercury on plant growth

Biomass and height

Biometric variables were overall affected by the Hg treatments at the second sampling time. However, treatments did not equally affect all the variables measured (Fig. 3).

The root system was the plant fraction showing most sensitivity. All the Hg treatments increased root fresh weight, especially the 25 μM dose (Fig. 3a). Root length was greatest for the 5 μM Hg treatment. Improved plant growth due to Hg has been reported in studies that have assessed the effects of low Hg concentrations (Israr et al. 2006; Ren et al. 2014). However, more studies have linked reduced biomass to the presence of Hg (Suszcynsky and Shann 1995; Cho and Park 2000; Moreno-Jiménez et al. 2007; Zornoza et al. 2010; Marrugo-Negrete et al. 2016). It should be noted that root fresh weight and length did not behave equally with increasing Hg dose. Thus, in response to treatment, root length was affected while biomass remained unaffected. This means that the reduction in root length was offset by greater seedling development within the tray cells. This effect is comparable with the effect of the holes in the bottom of a simple plant pot: when the root tips are naturally pruned as they dry out, new tips develop inside the pot. In a pot like this, air is toxic to the roots. In our experiment, Hg will be the factor that limits growth, and the cell will act as a refuge for the roots. Thus, to explain our results, we propose that perlite interacts chemically with Hg, and because of this interaction, both the availability and the detrimental effects of Hg will be reduced. In effect, perlite is not really inert as it can accumulate Hg over time (Moreno-Jiménez et al. 2007).

In response to all the Hg treatments, the above-ground parts of the plants (Fig. 3b) showed significant improvements in terms of both fresh weight and height compared with the control treatment. No significant differences were observed among the Hg treatments.

Finally, no significant changes in the biomass distribution of the plants were produced in response to the Hg treatments (Fig. 3c). This figure also shows that the Hg treatments led to overall improved development of the seedlings, which is inconsistent with the literature data, as already mentioned.

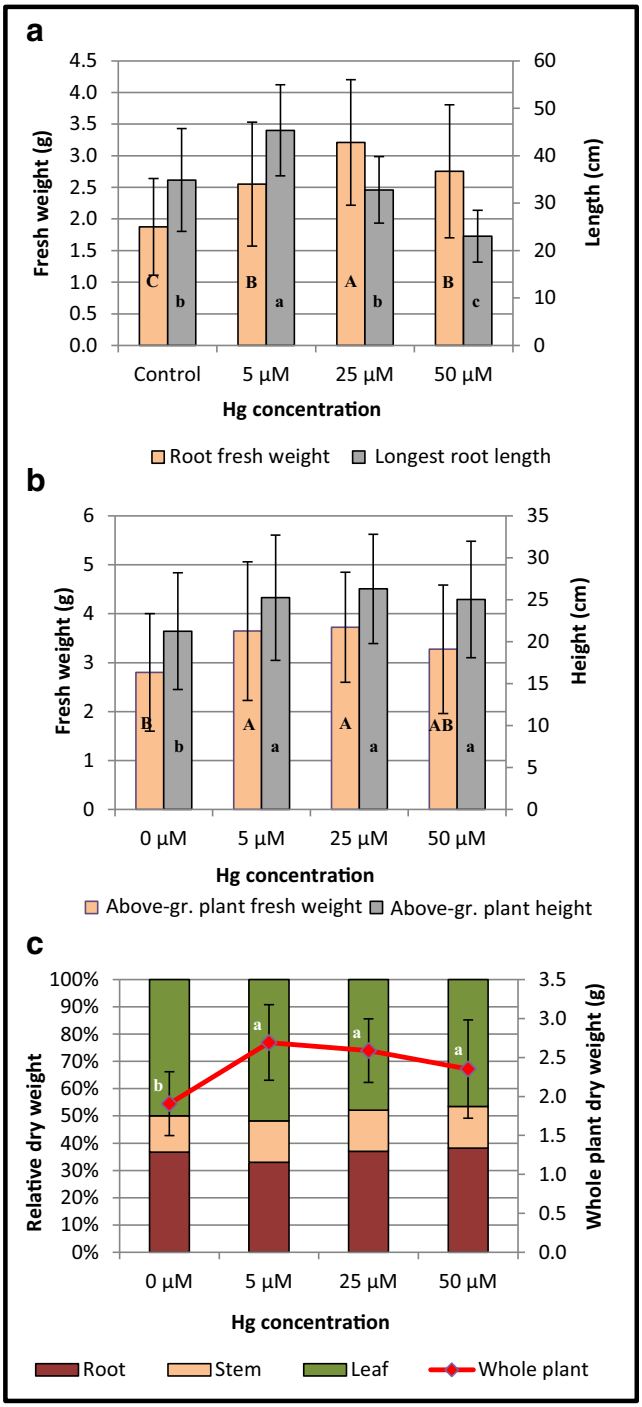

Fig. 3. Effects of the different Hg treatments on biometric variables: a) root fresh weight and longest root length; b) above-ground part fresh weight and height; c) relative dry weights recorded for each tissue and whole plant dry weight. Values are means ± SD (n = 78 – 93 seedlings per treatment). Different letters within the same coloured bars indicate significant differences between the treatments at α= 0.01

However, as the Hg concentration increased in the solutions, these beneficial effects on total biomass were reduced. Thus, the plants showed slight sensitivity to the Hg concentration, as would be expected.

Phenotypic signs of mercury toxicity

No seedlings died during the experiment. Only two plants, one exposed to 25 μM Hg and one to 50 μM Hg, showed symptoms of poor health. These symptoms were chlorosis of mature leaves representing less than 1% of the plants grown in both conditions. We do not interpret this as a representative result, and consider there were not harmful effects of Hg on the holm oak seedlings.

Mercury accumulation in plant

Mercury distribution

In all treatments except the control, Hg build-up in the 68-day seedlings mainly took place in the roots (≥ 97%) (Table 1). Beauford et al. (1977) reported similarly high root levels of Hg and noted that exposure to different levels of the metal did not modify the proportion of Hg retained in roots relative to shoots. Overall, we observed the following rank order of Hg distribution (Table 1): roots >> leaves > stem. This distribution pattern is similar to that reported for other species (Millhollen et al. 2006; Sierra et al. 2009; Millán et al. 2012; Marrugo-Negrete et al. 2016).

Mercury accumulation by growth stage

The greenhouse environment along with the hydroponic culture provided excellent conditions for the growth of the seedlings, and these conditions determined their 3-fold increase in height in a short time (68 days after emergence). In Table 1, we provide Hg concentrations recorded in the different plant organs at the different growth stages. These results are discussed in the following sections.

Mercury accumulation in leaves versus stems At all treatment levels, leaves showed higher Hg concentrations than stems in the older (first and second) growth stages (see Table 1). Higher Hg concentrations in leaves compared with stems have been reported in observational studies carried out on the same species (Villadóniga et al. 2009; Rodríguez-Alonso et al. 2014). Remarkably, however, we detected no significant differences between both organs at the third growth stage. If both organs equally accumulate Hg at this newest stage of growth, the factor time could be responsible for the different Hg accumulation capacity of the two organs. Namely, both organs could accumulate similar amounts of Hg as they start to grow, while over time, leaf tissue seems to accumulate a higher Hg concentration than the stem. Although, the surface-to-weight ratio could also affect the Hg accumulation process in these organs. Being the surface-to-weight ratio higher in leaves than in stems, the ability of the leaves to take gaseous Hg would be

Table 1 Mercury distributions in 68-day holm oak seedlings subjected to three treatment levels (5, 25, and 50 μM Hg). The seedlings were divided into three tissues: leaves, stem, and roots. Leaves and stems were further separated according to the corresponding growth stages (first, second, and third). $n = 5$ lots, mean ± SD, Hg concentrations in dry weight

Treatment	Growth stage	Leaves (mg kg^{-1})		Stem (mg kg^{-1})		Roots (mg kg^{-1})
0 μM Hg	First	1.47 ± 0.23	A d *	0.59 ± 0.15	B d	
	Second	1.45 ± 0.24	A d *	0.64 ± 0.21	AB d	
	Third	1.27 ± 0.33	A d	0.97 ± 0.27	A c	
	Mean	1.46 ± 0.16		0.61 ± 0.19		0.48 ± 0.08 c
		(74.4%)		(8.0%)		(17.6%)
5 μM Hg	First	4.58 ± 0.79	A c *	3.12 ± 0.51	A c	
	Second	3.51 ± 0.56	B c *	2.09 ± 0.28	B c	
	Third	2.73 ± 0.55	B c	2.17 ± 0.65	B b	
	Mean	4.05 ± 0.43		2.81 ± 0.62		290 ± 35 b
		(2.1%)		(0.4%)		(97.5%)
25 μM Hg	First	16.50 ± 3.25	A b *	12.40 ± 2.17	A b	
	Second	8.74 ± 1.50	B b *	4.80 ± 0.71	B b	
	Third	6.12 ± 1.94	C b	6.22 ± 3.42	B a	
	Mean	12.40 ± 1.45		10.31 ± 2.01		814 ± 119 a
		(1.9%)		(0.5%)		(97.6%)
50 μM Hg	First	31.85 ± 11.27	A a	25.53 ± 3.12	A a	
	Second	13.43 ± 1.73	B a *	7.67 ± 0.91	B a	
	Third	10.06 ± 2.25	C a	7.25 ± 1.27	B a	
	Mean	20.37 ± 2.59		19.39 ± 3.44		1014 ± 96 a
		(2.3%)		(0.7%)		(97.0%)

Row indicated as mean: (i) upper values are mean mercury concentrations per tissue considering the dry weight of growth stage, (ii) lower values are the relative mean mercury contents of each tissue

Different capital letters indicate significant differences between growth stages for the same treatment and tissue ($\alpha = 0.05$). Different small letters indicate significant differences between treatments for the same growth stage and tissue ($\alpha = 0.05$). * indicates significant differences between leaves and stems for the same growth and treatment ($\alpha = 0.05$)

also greater, and thus, the leaves will probably concentrate more total Hg over time owing to their shape.

To confirm the relationship observed between the Hg concentrations of leaf and stem, we conducted a correlation analysis (Supplementary Figure 2). This analysis revealed strong positive linear correlation (Spearman's rank correlation = 0.96 at 0.01 level) between Hg concentrations in both organs. Consistently, in prior work, we were able to relate Hg concentrations in the stem and bark also in the holm oak (Rodríguez-Alonso et al. 2014).

Mercury accumulation by growth stage In the control treatment, no significant difference in Hg concentrations among growth stages was detected in both stems and leaves (Table 1). However, when the seedlings were grown in the presence of a 5 μM Hg solution, Hg concentrations in the first growth stage were significantly higher than in the second and third stages, which showed similar concentrations of Hg. This difference between the first and remaining stages was also observed for the 25 μM or 50 μM Hg solutions in the stems, while in the leaves, Hg concentrations were dependent on growth stage. Consequently, Hg accumulation was more influenced by growth stage in leaf than stem.

As growth stage is related to the factor time, every plant organ generated at a particular growth stage, whether aboveground, leaf, or stem, will be exposed to Hg over a given time period. In effect, many authors such as Rasmussen (1995) or Rodríguez-Alonso et al. (2014) have reported that Hg accumulation varies as a function of time. Accordingly, we would expect greater Hg accumulation in the older plant organs (first growth stage) than the younger organs (second followed by third growth stages). However, our findings reveal much greater differences in Hg accumulation between the first and the second growth stages in the seedlings subjected to 25 and 50 μM Hg solutions compared with the lower treatment levels (0 and 5 μM Hg). Thus, using the higher Hg doses, extensive Hg accumulation seems to take place. This phenomenon can be clearly seen in Table 1 and Supplementary Figure 3. Our study design differs from others in which seedlings are often exposed to pollutants after sprouting and this makes it difficult to compare our results with those of others. Nevertheless, some studies have assessed Hg accumulation in plant organs at different time points. For example, Millhollen et al. (2006) detected no peaks in leaf Hg accumulation during plant growth, though they did find that the younger leaves accumulated significantly less Hg than the older ones. In addition,

Ericksen et al. (2003) reported increased leaf Hg levels over time but did not observe intensive Hg build-up at a given time point. In both studies, treatments consisted of low Hg levels (Millhollen's group used 6, 14, and 30 ng m^{-3} in air and < 0.01, 0.09, and 0.9 μg g^{-1} in soil, while Ericksen et al. used 12.3 μg g^{-1} in soil) and this could explain the differences with our study. We propose the following hypotheses: (i) periods of intense Hg accumulation in above-ground organs are a symptom of stress induced by high Hg doses, requiring that plants redistribute the Hg accumulated among all tissues; (ii) the comparable Hg concentrations detected here in the second and third stages of growth suggest that this stress is relatively controlled by the plants after their initial growth; (iii) as intense Hg build-up occurred in some of the treatments and at given growth stages, it seems there is interaction between both factors; and finally, (iv) as reported, the first growth stage can highly influence Hg accumulation in above-ground organs, but it remains unknown if the factors involved are really the first stage of the plant's life or first growth in the growing season. Further research is needed to address these hypotheses.

Mercury accumulation by treatment level The impacts of the different Hg treatments are shown in Table 1. On the whole, at a given growth stage, both leaves and stems showed increasing Hg concentrations with increasing exposure dose of Hg. However, differences between treatments were reduced as the growth stage advanced such that the effect of treatment level on above-ground organ Hg concentrations was lower for the latest growth stages.

Mercury accumulation according to sampling time

Our experimental design was such that we could compare above-ground organ Hg concentrations across different time points and development stages of the seedlings (Day 15 and Day 68; Fig. 1). The samples compared were as follows: (i) whole above-ground mass of the 15-day seedlings; (ii) only the first growth of the 68-day seedlings, showing similarly developed leaves and stem as (i); and iii) whole above-ground mass of the 68-day seedlings. The first two samples served to monitor Hg accumulation during the initial growth of the seedlings from the first to the second sampling time points. Thus, no significant differences were noted between Hg concentrations recorded in the 15-day seedlings versus the first growth portion of the 68-day seedlings (Fig. 4a) at the higher treatment levels (≥ 25 μM Hg). In contrast, significant Hg increases were produced at the 5 μM Hg and control treatment levels. In summary, the results of this experiment revealed that Hg accumulation in above-ground organ did not increase over time in all cases. This finding contrasts with Hg accumulation observed as a function of time in several studies in conditions of long-term exposure to low levels of Hg

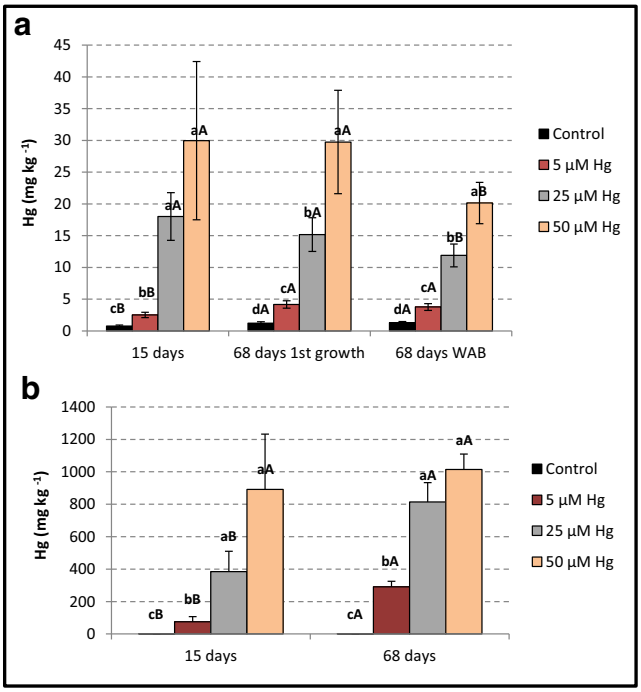

Fig. 4. Mercury concentrations (dry weight) recorded in the above-ground part (A) and roots (B) according to the Hg treatment level at two sampling times: 15 and 68 days post seed emergence. In figure A, at the 68 day sampling time, mercury accumulation is shown for the 1st growth stage and for the whole above-ground mass (WAB). Values are means ± SD (n = 5 lots per treatment). Different capital letters indicate significant differences between the samplings for the same treatment (α = 0.05). Different small letters indicate significant differences between treatments for the same sampling time (α = 0.05).

(Rasmussen 1995; Ericksen et al. 2003; Sardans and Peñuelas 2006; Higueras et al. 2012; Rodríguez-Alonso et al. 2014; Hutnik et al. 2014). Millhollen et al. (2006) and Assad et al. (2016) obtained similar results even over shorter time periods (< 10 weeks). Thus, our observations seem to indicate that when subjecting seedlings to high Hg doses (in our case ≥ 25 μM Hg), rather than the age of the above-ground organ, the most influential factor in Hg accumulation seems to be the treatment dose. Growth stage, however, can also influence this pattern, as discussed in the "Mercury accumulation by growth stage" section. Finally, we speculate that longer experimental times than the present could have a greater impact on Hg accumulation.

By comparing Hg concentrations between the first growth stage and the whole above-ground mass of the 68-day seedlings (Fig. 4a), we noted that as the plant grows, total Hg concentrations in above-ground biomass diminish. This was detected as less Hg per biomass unit in the last stages of growth (second and third) than the initial growth stage (Table 1) and is in line with the findings of Panda et al. (1992).

In the roots, Hg concentrations increased significantly but differently with rising Hg solution concentrations (Fig. 4b) both in the 15-day and 68-day seedlings. At the first sampling,

Table 2 Macronutrient (Ca and K) and micronutrient (Cu, Fe, Mg, Mn, and Zn) accumulation in 68-day seedlings, separated into the three fractions leaf, stem, and root (n = 5 lots, mean ± SD)

Tissue	Hg treatment	Ca	Fe	K	Cu	Mg	Mn	Zn
Leaf	Control	3503 ± 385a	32.6 ± 8.4a	11,140 ± 567a	4.4 ± 1.0a	1314 ± 157a	609 ± 79a	13.1 ± 1.4a
	5 μM	3303 ± 189a	31.2 ± 5.3a	11,774 ± 1245a	5.7 ± 1.8a	1311 ± 111a	553 ± 101a	11.2 ± 0.6b
	25 μM	3226 ± 296a	28.1 ± 3.8a	11,508 ± 419a	5.3 ± 3.1a	1317 ± 79a	512 ± 64a	12.1 ± 2.4ab
	50 μM	2656 ± 178b	23.2 ± 6.5a	12,050 ± 611a	4.5 ± 0.5a	1208 ± 135a	360 ± 62b	11.7 ± 2.0ab
Stem	Control	4154 ± 881a	18.6 ± 6.8a	12,326 ± 534a	5.1 ± 1.4a	1085 ± 157a	266 ± 54a	12.3 ± 2.3a
	5 μM	3877 ± 870a	20.0 ± 5.8a	12,832 ± 1561a	6.2 ± 2.7a	1081 ± 229a	269 ± 54a	10.2 ± 0.8a
	25 μM	3525 ± 966a	18.6 ± 13.6a	13,091 ± 1096a	6.1 ± 2.9a	1062 ± 169a	273 ± 56a	10.2 ± 2.7a
	50 μM	3823 ± 816a	20.8 ± 16.3a	14,479 ± 1551a	5.5 ± 0.7a	1279 ± 187a	246 ± 31a	10.6 ± 3.5a
Root	Control	2124 ± 405a	502 ± 109a	11,633 ± 1316ab	6.2 ± 1.3a	1527 ± 185a	132 ± 20a	20.3 ± 3.5a
	5 μM	2319 ± 305a	490 ± 42a	12,183 ± 901a	5.7 ± 0.5a	1550 ± 266a	155 ± 13a	21.4 ± 1.9a
	25 μM	2555 ± 344a	468 ± 50a	11,694 ± 331a	5.1 ± 0.4a	1532 ± 95a	160 ± 22a	16.9 ± 3.9ab
	50 μM	2369 ± 451a	318 ± 64b	10,035 ± 827b	4.5 ± 1.1a	1378 ± 157a	148 ± 25a	13.0 ± 3.2b

Data are in mg kg^{-1} (d.w.). Different small letters indicate significant differences between treatments within the same tissue (α = 0.05)

root Hg concentrations linearly increased as the Hg exposure dose increased. However, in the second sampling effort, the variation shown by root Hg concentrations best fitted a hyperbolic function. This suggests a saturation event, such that a threshold Hg concentration would be reached in the root, beyond which the root system would not be able to accumulate more mercury. This hyperbolic behavior has been described by Esteban et al. (2008), although it may also be inferred from the findings of other studies (Suszcynsky and Shann 1995; Moreno-Jiménez et al. 2007; Marrugo-Negrete et al. 2016). Of note is that this threshold Hg concentration will be specific to the holm oak and our experimental conditions.

Despite differences according to the Hg treatments, Hg concentrations in the roots increased significantly over time (Fig. 4b). In the 0–25 μM Hg solutions, roots significantly concentrated more Hg from one sampling time to the other. Nevertheless, at 50 μM Hg, roots rapidly (15 days from emergence) accumulated values close to 1000 mg kg^{-1} (d.w.), and this did not vary significantly over time. This confirms that levels of around 1000 mg kg^{-1} are the maximum Hg concentrations accumulating in the roots of the holm oak in our study. Finally, in the long term, our results suggest that root Hg concentrations could equalize over time despite the different treatments. It should be noted that over 53 days (from the first to second sampling time), Hg concentrations in roots exposed to 5 μM Hg increased 3.8 times, while for the 25 μM Hg and 50 μM Hg treatments, these values increased by only 2.1-fold and 1.1-fold, respectively. In the 0–25 μM Hg solutions, roots significantly concentrated more Hg from one sampling time to the other.

Nutrient accumulation

Macronutrient (Ca and K) and micronutrient (Cu, Fe, Mg, Mn, and Zn) concentrations (nutrient classification following Mengel et al. (2001)) were determined in the 68-day seedlings. All plant organs took up nutrients though accumulation patterns differed according to the micronutrients or

Table 3 Macronutrient (Ca and K) and micronutrient (Cu, Fe, Mg, Mn, and Zn) accumulation in the first two growth stages of above-ground portions of 68-day seedlings (n = 2 lots, mean ± SD)

Hg treatment	Growth stage	Ca	Fe	K	Cu	Mg	Mn	Zn
Control	First	4457 ± 541	27.1 ± 2.9	10,869 ± 714	4.3 ± 0.5	1264 ± 124	470 ± 73	13.3 ± 1.9
	Second	2940 ± 284	27.3 ± 2.6	11,169 ± 816	5.5 ± 0.8	1263 ± 92	481 ± 31	13.0 ± 1.0
5 μM	First	4423 ± 280	30.3 ± 2.3	11,993 ± 544	7.0 ± 0.7	1311 ± 69	432 ± 20	10.6 ± 1.0
	Second	2895 ± 213	24.2 ± 6.2	11,953 ± 570	8.4 ± 0.9	1175 ± 60	438 ± 21	11.4 ± 0.6
25 μM	First	3879 ± 380	31.8 ± 3.7	11,998 ± 747	7.2 ± 0.6	1316 ± 106	390 ± 30	13.0 ± 1.1
	Second	2685 ± 194	24.0 ± 2.3	11,800 ± 656	7.8 ± 0.4	1236 ± 60	479 ± 25	14.8 ± 1.4
50 μM	First	3660 ± 337	22.5 ± 3.6	11,993 ± 565	4.9 ± 0.8	1320 ± 80	298 ± 22	12.0 ± 0.9
	Second	2225 ± 154	23.8 ± 5.3	12,916 ± 763	5.0 ± 0.7	1063 ± 65	298 ± 15	12.3 ± 1.3

Data are in mg kg^{-1} (d.w.)

macronutrients considered (Table 2). Thus, Fe and Zn mainly accumulated in roots, Mn mainly in leaves, and Ca slightly more in stems. Only in the case of the 50 μM Hg treatment were the concentrations of some nutrients significantly reduced (Ca-leaves; Mn-leaves; Fe-roots; K-roots, Zn-roots). In fact, in 66.7% of cases, in response to the Hg treatments, nutrient accumulation was higher than for the control treatment. Accordingly, nutrient concentration was not overall significantly affected by the Hg treatments. This finding is inconsistent with the results of similar studies in which the presence of Hg in the growth substrate was found to lead to diminished nutrient concentration (Godbold 1991; Gupta and Chandra 1998; Moreno-Jiménez et al. 2007; Rodríguez et al. 2009; Sierra et al. 2012).

As we assumed that Hg accumulation by the seedlings would be influenced by growth stage, we also expected the nutrient accumulation pattern to show some dependence on growth stage. According to Rodríguez et al. (2009), Hg can replace some nutritional elements such as Mg, Zn, and Mn. To test this rationale, leaf and stem from seedlings derived from two seed lots (2 and 5; Supplementary Table 1) were compared between growth stages 1 and 2, as the sample size for the third growth stage was insufficient. Also, since nutrient accumulation patterns were similar, we combined results for the leaf and stem. Our data (Table 3) indicate a significant difference only between both growth stages in terms of Ca accumulation. Thus, in the initial growth stage, on average, 153% more Ca was accumulated than in the second one regardless of the treatment level. No differences in the other nutrients (Cu, Fe, K, Mg, Mn, and Zn) were produced between the two growth stages or among the Hg treatment levels. Consequently, nutrient accumulation in leaf and stem was stable across both growth stages and independent from Hg accumulation.

Conclusions

The findings of this study indicate that holm oak germination patterns were not significantly affected by the Hg treatments applied. Accordingly, germination in sites containing high soil concentrations of this pollutant would not be expected to be a problem for the holm oak.

The Hg treatments tested (5, 25, and 50 μM Hg) did not impair seedling growth, kill any seedlings, provoke toxicity symptoms, or significantly affect nutrient accumulation, and thus did not affect holm oak seedling development in general. Indeed, in response to the presence of 5 and 25 μM Hg, some growth parameters were improved (fresh weight and plant height). However, when exposed to the higher available Hg concentrations (50 μM Hg), some measures of seedling development such as nutrient accumulation or root length were slightly affected.

The holm oak seedlings accumulated Hg mainly in their roots (97%), followed by leaves and stems. Mercury concentrations in leaf and stem showed positive and high significant correlation in response to all three Hg treatment levels. The build-up of Hg in the above-ground portion of the seedlings varied as a function of time, treatment level, plant organ, and growth stage. In general, Hg concentrations increased with increasing time and treatment level, whereas concentrations decreased with growth stage. However, we were able to detect complex interaction effects between these factors, as the impacts of some prevailed over others. In roots, Hg concentrations increased with increases in both time and treatment level. However, these root Hg concentrations were close to 1000 mg kg^{-1} when the seedlings were exposed to the 50 μM Hg solution at both sampling times (15 and 68 days). This dose of Hg will limit the capacity of the root system to concentrate Hg, although longer-term studies are needed to confirm this hypothesis.

As an overall conclusion, this study shows that levels ≥ 25 μM Hg and growth stage can lead to Hg accumulation variations in holm oak seedlings. Neither of these factors has been taken into account in prior studies.

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