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ORIGINAL PAPER

Effects of zinc and lead on seed germination of *Helichrysum microphyllum* subsp. *tyrrhenicum*, a metal-tolerant plant

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Abstract
The seed germination of *Helichrysum microphyllum* subsp. *tyrrhenicum*, an endemic species of Sardinia and Corsica with metal tolerance capability, was evaluated against zinc (Zn; 0, 250, 500 and 1000 mg/L) and lead (Pb; 0, 25, 50 and 100 mg/L) stress. Seeds were collected in three localities: (a) inside a mine dump highly polluted with these metals, (b) outside but close to this area and (c) far from the metal-polluted site. Germination responses were assessed at 10, 15 and 20 °C, and the percentage, time of germination and mortality of seedlings were evaluated. The taxon showed a high capacity to germinate under Zn and Pb stress, and the germination was never completely inhibited; however, the germination decreased with increasing Zn concentrations, but not under Pb stress. Moreover, the seeds from specimens growing in mining sites appeared to be less affected by Zn stress than seeds coming from the other localities. A successful survival of seedlings during the first days of their development under metal stress and under controlled conditions was detected. Our study suggests that this species may give an important contribution to future phytoremediation programs on mining sites, in which it could be spread by seeds in order to form a vegetation cover.

Keywords Germination test · Heavy metals · Mediterranean area · Mine areas · Phytoremediation

Introduction

Throughout its history, Sardinia has been characterized by a long-standing mining heritage, in particular from the pre-Roman time to the 90's of the twentieth century (Boni et al. 1999; Aversa et al. 2002; Frau et al. 2015). Mining activities were spread all over the island, but the most important and richest ores were located in the Sulcis-Iglesiente region (south-western Sardinia), where the main extracted metals were zinc (Zn) and lead (Pb) (Bechstädt and Boni 1994).

Metals dispersion is one of the main issues in these areas, since only very few remediation actions were designed after mine closure and a big amount (70 Mm^3, RAS 2003) of mine waste was left abandoned to weathering and aerial dispersion (Zavattero et al. 2006; Jiménez et al. 2011; Bacchetta et al. 2015). As a consequence, metals pollution still affects fresh water, soils and plants in the surrounding areas (Cidu et al. 2011; Bacchetta et al. 2018; Concas et al. 2015; De Giudici et al. 2017; Medas et al. 2017).

Although mine tailings are known to be poor in nutrients, several pioneer Sardinian endemic species, like *Helichrysum microphyllum* Cambess. subsp. *tyrrhenicum* Bacch., Brullo & Giusso, *Ptilostemon casabonae* (L.) Greuter, *Euphorbia*

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cupanii Guss. ex Bertol., *Limonium merxmulleri* Erben, *Iberis integerrima* Moris, *Echium anchusoides* Bacch., Brullo & Selvi and *Scrophularia canina* subsp. *bicolor* (Sibth. & Sm.) Greuter, are able to grow and develop phytocenoses typical of mining areas. These species create grasslands and garigues, also on incoherent surfaces like mine dumps and tailings (Angiolini et al. 2005; Zavattero et al. 2006).

Several studies showed the applicability of some Sardinian autochthonous species such as *Pistacia lentiscus* L. (Bacchetta et al. 2012, 2015; Concas et al. 2015; De Giudici et al. 2015), *Cistus salviifolius* L. (Jiménez et al. 2005), *Euphorbia cupanii* (Jiménez et al. 2014; Medas et al. 2015), *Juncus acutus* L. (Medas et al. 2017) and *Phragmites australis* (Cav.) Trin. ex Steud (Bacchetta et al. 2015; De Giudici et al. 2017) in phytoremediation actions. In addition to their ability to tolerate heavy metals and survive to stress conditions, their use is recommended because of the autochthonous status. The autochthonous and endemic plants species have a crucial role in phytoremediation (Barbafieri et al. 2011; Doumas et al. 2018; Monaci et al. 2019), indeed, they are better adapted to local climatic conditions than allochthonous taxa and can help to preserve local biodiversity (Concas et al. 2015). Furthermore, autochthonous plants build the basis for primary successions in mine environments, promoting the establishment of a more evolved vegetation (Bacchetta et al. 2007; Ginocchio et al. 2017). The use of these plant species in phytoremediation activities can provide a cost-efficient and long-lasting solution for the rehabilitation of contaminated sites (Wong 2003; Mendez and Maier 2008; Cao et al. 2009; Jiménez et al. 2011; Bacchetta et al. 2015; Concas et al. 2015). Among the plant species mentioned above, recent studies highlighted the metal tolerance capability of *H. microphyllum* subsp. *tyrrhenicum* to Zn, Pb and Cd, suggesting its suitability in phytoremediation activities (Cao et al. 2004; Bacchetta et al. 2017, 2018). These researches showed that this plant species is able to grow on high Zn–Pb polluted substrates, accumulating these heavy metals into roots and limiting the translocation into areal organs. Therefore, the transfer into the food chains is limited, even if this plant species is not used for human alimentary consumption and/or as food for farm animals. Indeed, *H. microphyllum* subsp. *tyrrhenicum* is mainly used for cosmeceutical and pharmaceutical purpose wherein the main extracted product is the essential oil (Leonardi et al. 2013; Antunes Viegas et al. 2014). Moreover, *H. microphyllum* subsp. *tyrrhenicum* is an endemic shrub of Sardinia and Corsica which is well adapted to different ecological conditions of the Mediterranean region (Bacchetta et al. 2003; Angiolini et al. 2005). Because of its wide adaptability to various edaphic and climatic conditions, it grows from sea level up to 1500 m a.s.l. and on many kinds of substrates, especially sandy and muddy soils. It is noteworthy that it can grow spontaneously on many kinds of contaminated substrates, especially on mining dumps and tailings (Bacchetta et al. 2003; Angiolini et al. 2005). It is characteristic of many plant assemblages and, in particular, of the garigue of the association *Dactylo hispanicae-Helichrysetum tyrrhenici*, which represents a successor stage of the Sardinian special series of the "heavy metals-polluted mine substrates" (Bacchetta et al. 2007).

Although some authors reported the capacity of several Sardinian autochthonous plants to grow and survive in contaminated substrates, information is lacking on the ability of their seeds to germinate under heavy metal stress. Elucidating these aspects would allow to better define the limiting ecological conditions during seed germination. This information can be helpful when considering phytoremediation strategies as a final aim. Indeed, seed germination is the first part of a plant's life and it is the most sensitive process where different factors, like environmental ones (abiotic and biotic) and hormonal interaction (Márquez-García et al. 2013; Baskin and Baskin 2014), are involved. In fact, some conditions like temperature, water availability and soil substrate can promote or inhibit germination (Porceddu et al. 2013; Krichen et al. 2014). Another impacting factor could be the high concentration of metals in the soils. Their effects on germination depend on their ability to reach the embryo tissues through the seed coat and on the chemical properties of the involved metal. The seed coat may offer protection from metal stress, but it could also be more permeable during germination. Germination is affected by metals in two ways: by their general toxicity and by the inhibition of water uptake (Kranner and Colville 2011).

Several seed germination studies under metal stress were conducted, in particular on cultivated plant species such as tomato, broccoli and lettuce seeds (Di Salvatore et al. 2008), but only a few studies are available for autochthonous taxa, e.g., *Calamagrostis epigeios* (L.) Roth (Madzhugina et al. 2008), and Mediterranean plant species, e.g., *Atriplex halimus* L., *Salicornia ramosissima* J. Woods (Márquez-García et al. 2013) and *Dorycnium pentaphyllum* Scop. (Lefèvre et al. 2009). With their results, these authors gave an important contribution in suggesting the use of these plants in phytoremediation programmes and in stimulating more research on this topic. As reported by Kranner and Colville (2011) in their review, the seeds of metal-tolerant plants may have a substantially higher threshold for toxicity than non-tolerant ones. In addition, they reported that seeds produced by plants growing on contaminated soil may accumulate high concentrations of metals which may hamper germination.

Given these considerations, this study is focused on the seed germination response of *H. microphyllum* subsp. *tyrrhenicum* (hereafter *H. m.* subsp. *tyrrhenicum*), in order to gain new pieces of knowledge which may be useful for planning more efficient phytoremediation actions. In detail, the aims of this study were to: (1) evaluate the capacity of *H. m.* subsp. *tyrrhenicum* seeds to germinate at different concentrations of Zn and Pb and at temperature of 10, 15 and 20 °C and, (2) detect if the sites of seed collection (i.e., mine contaminated substrates and non-contaminated soils) may cause differences in the seed germination behaviour under different heavy metal stress and temperature conditions. It can be highlighted that previous reported studies on seed germination of *H. m.* subsp. *tyrrhenicum* were aimed at investigating the optimal germination conditions in terms of light, temperature and altitudinal gradient (Picciau et al. 2019). The importance of this experimental research is that this is the first study concerning the germination capability under heavy metal stress proposed for Sardinian native plant species of mine environments. The experiment was carried out in 2016/2017 in the laboratories of the Sardinian Germplasm Bank (Cagliari, Italy).

Materials and methods

Seed lot details

Helichrysum microphyllum subsp. *tyrrhenicum* seeds were collected from three different localities of the Sulcis-Iglesiente region (SW Sardinia) in July 2016, at the time of natural seed dispersal. The sites are listed as follows: (1) Campo Pisano's mine dump (CP), (2) an area outside the mine dump (OUT), approximately 2 km far away from the Campo Pisano dump, which is characterized by the same mineralogical background of CP but without direct mining impact and (3) Campu S'Isca (CSI), an area located at a linear distance of approximately 50 km from Campo Pisano and which is not affected by any kind of anthropic impact. Plants in CP grow on mine waste rich in Zn and Pb, resulting from the flotation process of blende (ZnS) and galena (PbS) which were the main extracted minerals. Other metals and metalloids such as cadmium (Cd), mercury (Hg), arsenic (As), chromium (Cr) and antimony (Sb) are present, whose concentration is, at least, one order of magnitude lower than Zn and Pb (Bacchetta et al. 2012; Medas et al. 2017) and they were not considered in this study. Previous studies (Bacchetta et al. 2017, 2018) showed very high concentration of Zn and Pb in mine waste and in plant specimens of *H. m.* subsp. *tyrrhenicum* that colonize this dump (Table S1, see Online Resource). In OUT, plants grow on soils developed from metamorphic rock (mud schists). The soils and plants of *H. m.* subsp. *tyrrhenicum* growing in this site showed a very high concentration of metals (Table S1) confirming both the same geochemical background of CP and the dispersion of contaminants by aeolic dispersion of fine particles. Conversely, plant specimens of CSI grow on soils developed from granite rock and the content in Zn and Pb are low (Table S1). CP and OUT soils were made of fine grained particles (< 425 μm), where carbon is mainly present as calcite and dolomite (Bacchetta et al. 2015), and the pH is neutral/slightly alkaline in accordance with the carbonatic lithology. CSI soil was made of large grained (> 3 mm) particles which came from the weathering of granite with a slightly acidic pH. Furthermore, the three sampling points are located in the same biogeographical sector and, as a consequence, *H. m.* subsp. *tyrrhenicum* plants growing in these areas are subjected to the same climatic conditions. Table 1 reports the information about the sampled seed lots, such as their geographical coordinates, mean altitude and substrate. Seeds were stored at room conditions (20 °C and 40% of R.H.) for ca. two weeks until the beginning of germination test.

Table 1 Seed lot details

Locality	Mean coordinate (WGS 84)	Mean altitude	Outcropping substrate
Campo Pisano mine dump (CP)	39° 17′ 45″ N–8° 32′ 12″ E	177 m a.s.l.	Zn–Pb mine waste
Outside Campo Pisano (OUT)	39° 17′ 32″ N–8° 32′ 35″ E	208 m a.s.l.	Metamorphic
Campu S'Isca (CSI)	39° 23′ 60″ N–8° 38′ 56″ E	326 m a.s.l.	Granitic

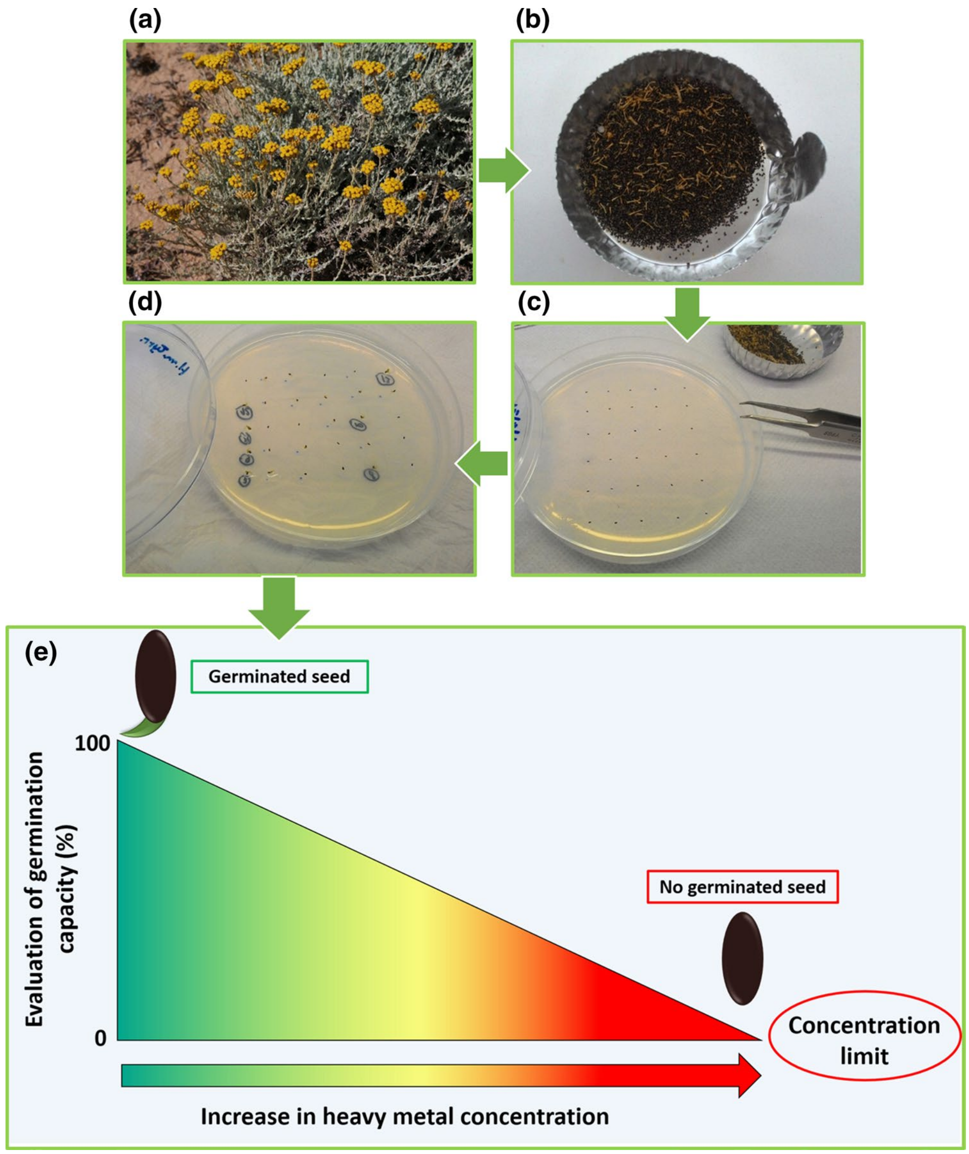

Fig. 1 Sketch of the experimental procedure; **a** *H. m.* subsp. *tyrrhenicum*; **b** seeds after cleaning step; **c** sowing in Agar solution with heavy metal; **d** check of the germinated seeds, **e** evaluation of the concentration limit (i.e., concentration value above which the germination is null)

Experimental conditions and germination assay

Four replicates of 30 seeds each of *H. m.* subsp. *tyrrhenicum* belonging to three different localities were sown on the surface of 1% agar water plus four different Zn and Pb concentrations (0, 250, 500 and 1000 mg/L of Zn and 0, 25, 50 and 100 mg/L of Pb, respectively) in 90-mm diameter plastic Petri dishes and incubated in the light (12 h light/12 h dark) at three constant temperatures (10, 15 and 20 °C) which were chosen within the optimal germination temperatures detected for this taxon (Picciau et al. 2019). The concentrations of the selected metals were applied based on the levels found in the mine dump area (total amount and bioavailable fraction in soil); these concentrations are higher than those used in other studies carried out on other Mediterranean species (e.g., Martínez-Fernández et al. 2011; Márquez-García et al. 2013). The experiments were conducted using growth chambers (model Sanyo MLR-351, SANYO Electric Co., Ltd), equipped with white fluorescent lamps (FL40SS.W/37 70–10 µmol m^{-2} s^{-1}). Germination, defined as visible radicle emergence (> 1 mm), and mortality were monitored three times a week for 90 days. The experimental steps are summarized in Fig. 1. Germination dynamics were analysed for each operating concentration (Zn and Pb) and temperature through: (1) final germination percentage after 90 days, (2) time of the first germination (T_1) and (3) time to reach 50% of germinated seeds (T_{50}). In addition, in order to evaluate the mortality of the young seedlings under the four tested Zn and Pb concentrations, the first 30 germinated seeds per temperature, selected randomly, were maintained under the same conditions and the number of died seedlings was monitored until the end of the germination test.

Statistical analyses

A two-way ANOVA analysis was performed in order to evaluate the effect of the concentrations of Zn and Pb, germination temperatures and provenance of seeds, as well as their interaction, on final germination percentages. In addition, a two-way ANOVA analysis was carried out in order to evaluate if Zn and Pb concentrations and the provenance of seeds had an effect on seedling mortality. Statistically significant differences highlighted by ANOVA were then analysed by a post hoc pairwise comparisons *t* test (Tukey's test). All statistical analyses were performed using R v. 3.0.3 (R Development Core Team 2014). SigmaPlot V11.0 (Systat Software, Inc. 2008) was used to create the artwork.

Table 2 Results of the two-way ANOVA analysis on Zn tests

Source of variation	*DF*	*SS*	*MS*	*F*	*p*
Locality	2	3191.821	1595.910	21.352	< 0.001
Temperature	2	5400.617	2700.309	36.128	< 0.001
Concentration	3	49,137.654	16,379.218	219.141	< 0.001
Locality × temperature	4	508.642	127.160	1.701	> 0.05
Locality × concentration	6	2263.735	377.289	5.048	< 0.001
Temperature × concentration	6	1853.086	308.848	4.132	< 0.001

DF, degrees of freedom; *SS*, sum of square; *MS*, mean square; *F*, F statistic; *p*, *p* value

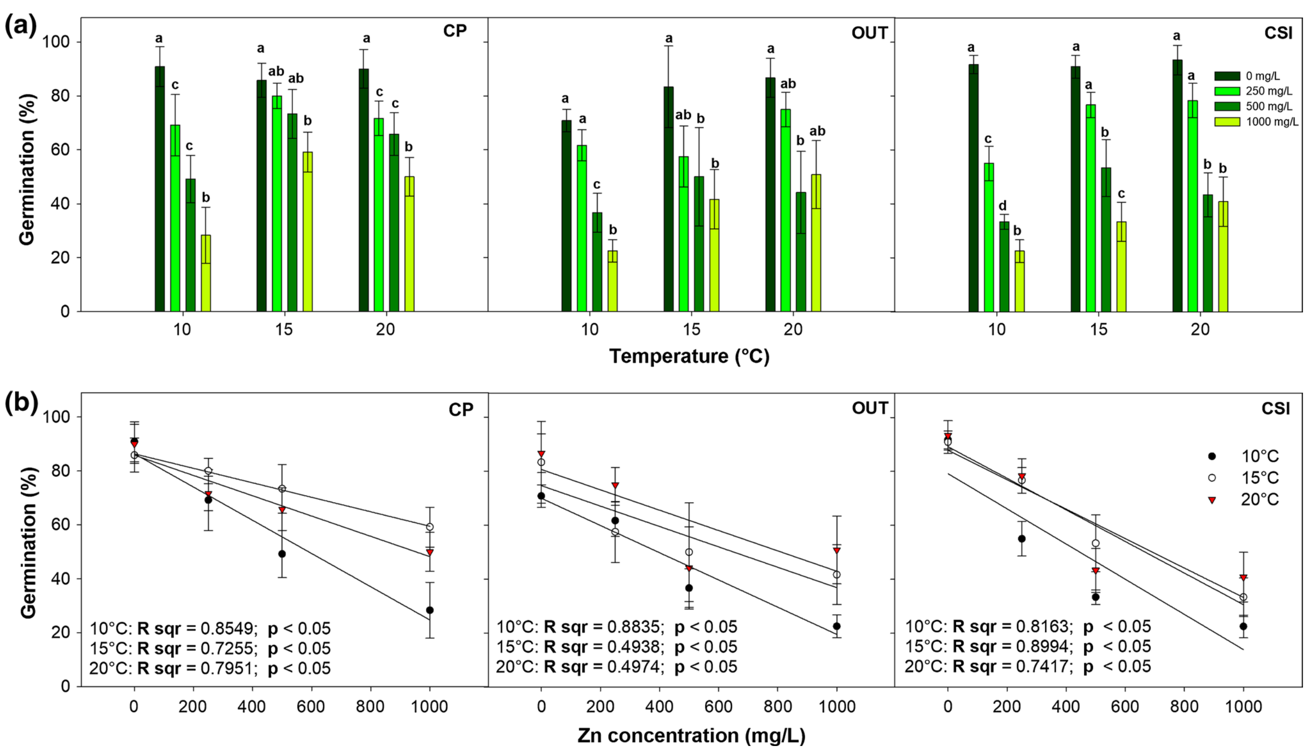

Fig. 2 **a** Final germination (% ± SD) under different Zn concentrations and temperatures; letters indicate statistically significant differences at $p < 0.05$; **b** linear correlation between germination (%) and Zn concentrations in each site; *R* sqr = *R* square; *p* = *p* values

Results and discussion

Seed germination under Zn stress

The ANOVA analysis showed statistically significant differences ($p < 0.001$) among the studied localities, temperatures and concentrations, as well as in the interactions "locality × concentration" and "temperature × concentration" ($p < 0.001$; Table 2). The interaction "locality × temperature" showed no statistical differences ($p > 0.05$; Table 2). The final germination percentage decreased with the increase of the Zn concentration in every locality and at all tested temperatures (Fig. 2a), as observed also in other studies carried out with other species (see Kranner and Colville 2011).

As previously reported, the three studied localities were significantly different ($p < 0.001$) and, according to Tukey's test, CP differed from OUT and CSI ($p < 0.001$), but no statistical differences were detected between OUT and CSI ($p > 0.05$; i.e., localities referable to sites without direct mining impact). At 0 mg/L of Zn, the highest germination percentages were recorded in CSI, followed by CP and OUT. The best germination temperature at this concentration was obtained at 20 °C for all studied populations. At 250 mg/L of Zn, CP showed the highest germination percentage in comparison to the others localities, with the optimal germination temperature being 15 °C. The same behaviour was observed for the other Zn concentrations. CSI was most affected by the increase of the Zn concentration, followed by OUT and CP (Fig. 2a). When considering each locality, differences among concentrations were observed in the germination response at every tested temperature (Fig. 2a). The final germination percentage of seeds from CP was influenced by the Zn concentration; however, 250 and 500 mg/L of Zn affected the germination response in the same way ($p > 0.05$). CSI seeds showed different germination responses at every tested concentration ($p < 0.05$; Fig. 2a).

The results here reported suggested that seeds of *H. m.* subsp. *tyrrhenicum* from the mine dump (CP) appear to be less affected by Zn stress than seeds belonging to the area without direct mining impact (OUT) and those from the area not affected by mining impact (CSI); this aspect could indicate a higher resistance of the population growing on mine waste to metal (Zn) stress condition. Maheshwari and Dubey (2008) observed a trend where seeds of metal-tolerant plants can germinate under high metal concentrations, and essential micronutrients (like Zn) cause damage only at very high ones. This is in agreement with previous studies concerning *H. m.* subsp. *tyrrhenicum* growing on mine waste surfaces (Cao et al. 2004; Bacchetta et al. 2017, 2018; Medas et al. 2018) which elucidate the metal tolerance capability of this taxon and its suitability for revegetation and phytostabilization of Zn and Pb contaminated sites. Germination under metals stress (in particular Zn) was investigated on some Mediterranean species like *Atriplex halimus*, *Salicornia ramossisima* (Márquez-García et al. 2013) and *Bitumaria bituminosa* C.H.Stirt. (Martínez-Fernández et al. 2011), showing a high germination capability. For example, Márquez-García et al. (2013) observed that *A. halimus* seeds were able to germinate under different concentrations of Zn (from 10 to 2000 μM) with percentages close to 100%, whilst in *S. ramosissima* the percentages were lower (close to 60%). The germination percentages reported by the same authors for each tested species were higher than those recorded in this work for CP and OUT. It is noteworthy that the concentrations used in this work are higher than those used in the study by Márquez-García et al. (2013). This hinders a direct comparison between germination capabilities of the different species. Moreover, it is not possible to make a comparison among *A. halimus*, *S. ramosissima* and *H. m.* subsp. *tyrrhenicum* growing on unpolluted sites, since in the study by Márquez-García et al. (2013) specimens growing on uncontaminated sites were not taken into account.

Table 3 Concentration limits of Zn (mg/L) on seed germination in CP, OUT and CSI and linear regression lines

Temperature (°C)	Locality	Concentration limit (mg/L)	Linear regression line
10	CP	1402	$y = -0.06x + 86.30$
	OUT	1386	$y = -0.05x + 70.00$
	CSI	1214	$y = -0.06x + 79.17$
15	CP	3209	$y = -0.03x + 86.33$
	OUT	1975	$y = -0.04x + 74.67$
	CSI	1522	$y = -0.06x + 89.17$
20	CP	2283	$y = -0.04x + 85.83$
	OUT	2140	$y = -0.04x + 80.67$
	CSI	1609	$y = -0.05x + 87.83$

The correlations between the total germination percentage and the Zn concentration were calculated, according to the different temperatures applied. As shown in Fig. 2b, a negative linear correlation between the total germination percentage and the Zn concentrations at the different temperatures was found. The best correlation was recorded at 10 °C in CP and OUT ($R^2 = 0.8549$; $p < 0.05$ and $R^2 = 0.8835$;

$p < 0.05$, respectively), whilst CSI showed the best correlation at 15 °C ($R^2 = 0.8994$; $p < 0.05$).

Considering the values obtained by the linear regression analysis (Fig. 2b) and the corresponding equations (see Table 3), it was possible to estimate the concentration limit of Zn during germination ($[Zn]_{0\%}$), here defined as the concentration value above which the germination percentage was null. As reported in Table 3, this value was calculated for every population and temperature. The highest value of $[Zn]_{0\%}$ was ascribable to CP, followed by OUT and CSI (see Table 3). In detail, it was estimated that the seeds of this taxon may maintain the ability to germinate up to Zn values in the range from 1402 to 3209 mg/L (CP), from 1386 to 2140 mg/L (OUT) and from 1214 to 1609 mg/L (CSI; see Table 3 for details). The results also showed that *H. m.* subsp. *tyrrhenicum* may be able to germinate at very high concentrations in every tested locality, in particular in the mine dump one. Once again, specimens of this taxon growing on mine waste surfaces seem to be more adapted to metal pollution in comparison to unpolluted one. Hence, the results suggest the suitability of using the seeds of *H. m.* subsp. *tyrrhenicum* growing on mine waste surfaces as a source of plants for phytoremediation and revegetation action of these areas. A previous study by Kranner and Colville (2011) also suggested using the seeds harvested from plants growing on polluted sites as a principal source to be used in phytoremediation actions. Moreover, the opportunity of evaluating the concentration limit of a specific heavy metal during germination can allow a preliminary assessment of the suitability of a certain taxon. Therefore, as far as the choice of the plant species to be applied is considered, these parameters should be taken into account, along with the autochthony of the species, metal tolerance and translocation to aerial organs. In the broader perspective to design a revegetation project for these areas, a self-sustainable plant canopy is a favourable property; hence, plant species with high germination capability must be chosen.

Table 4 Two-way ANOVA analysis on Pb tests

Source of variation	*DF*	*SS*	*MS*	*F*	*p*
Locality	2	543.673	271.836	4.798	< 0.05
Temperature	2	722.377	361.188	6.374	< 0.05
Concentration	3	2924.306	974.769	17.203	< 0.001
Locality × temperature	4	123.457	30.864	0.545	> 0.05
Locality × concentration	6	1043.981	173.997	3.071	< 0.05
Temperature × concentration	6	568.981	94.830	1.674	> 0.05

DF, degrees of freedom; *SS*, sum of square; *MS*, mean square; *F*, F statistic; *p*, *p* value

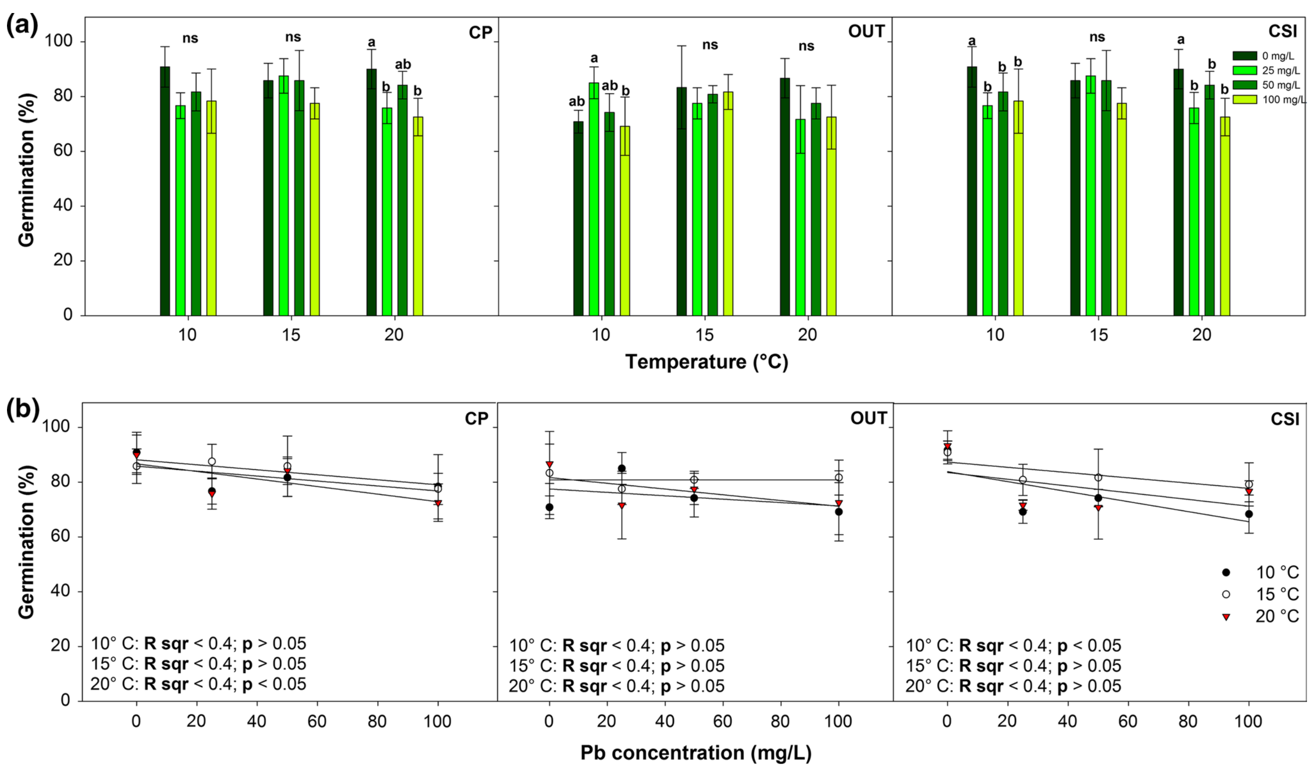

Fig. 3 **a** Final germination (% ± SD) under different Pb concentrations and temperatures; letters indicate statistically significant differences at $p < 0.05$; **b** linear correlation between germination (%) and Pb concentrations in each site; *R* sqr = *R* square; *p* = *p* values

As far as the time of germination is concerned, Table S2 (see Online Resource) shows the time of the first germination (T_1) and the time to reach 50% of germinated seeds (T_{50}) assessed during the germination test under Zn stress. A general increase of both T_1 and T_{50} under increasing Zn concentration was recognized. This behaviour was observed in all localities at each temperature. The seeds of the three localities reached T_{1s} in ca. 15 days and T_{50s} in ca. 30 days after sowing (under all concentrations and temperatures) (Table S2). T_1 and T_{50} should be taken into account when planning phytoremediation activities, indeed plants with high germination rate should be a desirable character for phytoremediation. However, the germination tests here presented were the first case of application for Sardinian species within the mine context, which hinders a comparison with other species and/or similar studies.

Seed germination under Pb stress

The ANOVA analysis (Table 4) showed statistically significant differences among the tested localities, temperatures and concentrations ($p < 0.05$, $p < 0.05$ and $p < 0.001$, respectively). Statistically significant differences were highlighted in the interaction "locality × concentration" ($p < 0.05$), but none were observed in the interactions "temperature × concentration" and "locality × temperature" ($p > 0.05$; Table 4).

The increase of the Pb concentration generated a slight drop in germination percentages in every tested locality at all temperatures, but with some statistical differences (Fig. 3a) and, in general, the final germination reached values ≥ 70% at each tested condition. At 0 mg/L of Pb, the highest germination percentage was recorded in CSI, followed by CP and OUT. At 25, 50 and 100 mg/L of Pb, CP showed the best germination percentages, in particular at 15 °C. CSI was the locality which was most affected by the increment of Pb concentration, followed by OUT and CP, even if the percentages in CSI and OUT were very similar (Fig. 3a). Seeds of specimens growing on mine waste surface appeared more adapted to Pb stress (CP) than those of the unpolluted localities.

The decrease in the germination of seeds treated with Pb was observed in some plant species, for example in *Triticum aestivum* L. and *Heliantus annuus* L. (Jadia and Fulekar 2008; Yang et al. 2010). It is known that in many species the seed coat may have an important role in the defence against abiotic stress and that the interspecific variation in the seed coat morphology may affect the permeability to different metals, including Pb (Wierzbicka and Obidzinska 1998). For example, Seregin and Kozhevnikova (2005), in their study on the seed coat permeability to lead of *Zea mays*, showed that Pb was restricted to the cells of the seed coat and it was not translocated into the endosperm and scutellum cells. The high germination detected in this study for *H. m.* subsp. *tyrrhenicum* under Pb stress leads us to think that the seed coat may have physical and/or chemical properties that control the permeability to Pb. However, further research is recommended to affirm this notion. Anyway, the germination response of *H. m.* subsp. *tyrrhenicum* detected for the two metals under study was comparably high as long as the concentration range 0–100 mg/L is considered, whilst at the very high Zn concentrations, a detrimental effect was noticed.

Unlike those detected under Zn stress, the values of R^2 and p values suggested the absence of a linear correlation between the final germination (%) and the Pb concentration

Table 5 Mortality percentages at the end of germination test (i.e., 90 days after sowing) calculated on seeds germinated under different concentrations of Zn and Pb

Locality	Zn (mg/L)	Mortality (%)				Pb (mg/L)	Mortality (%)			
		10 °C	15 °C	20 °C	Mean (± SD)		10 °C	15 °C	20 °C	Mean (± SD)
CP	0	0	40	0	13 ± 23[a]	0	0	40	0	13 ± 23[a]
	250	0	10	37	16 ± 19[a]	25	10	10	27	16 ± 10[a]
	500	0	0	20	7 ± 12[a]	50	20	37	30	29 ± 8[a]
	1000	0	0	63	21 ± 36[a]	100	13	17	17	16 ± 2[a]
OUT	0	0	0	10	3 ± 6[a]	0	0	0	10	3 ± 6[a]
	250	7	57	67	43 ± 32[a]	25	30	20	53	34 ± 17[b]
	500	23	53	37	38 ± 15[a]	50	37	47	70	51 ± 17[b]
	1000	0	13	23	12 ± 12[a]	100	50	27	37	38 ± 12[b]
CSI	0	0	0	17	6 ± 10[a]	0	0	0	17	6 ± 10[a]
	250	13	3	10	9 ± 5[a]	25	7	70	60	46 ± 34[a]
	500	7	10	7	8 ± 2[a]	50	20	67	33	40 ± 24[a]
	1000	0	3	17	7 ± 9[a]	100	3	33	40	26 ± 20[a]

Different letters indicate statistically significant differences at $p < 0.05$

in each locality (Fig. 3b). The absence of this correlation did not allow to determine the Pb concentration limits as done for the Zn, suggesting and reinforcing what said previously that probably Pb does not interfere on the germination processes sensu stricto, at least for Pb concentrations up to 100 mg/L.

As far as the time of germination is concerned, the T_1 and T_{50} values of Pb are reported in Table S3 (see Online Resource). In general, T_{1s} were reached in ca. 10 days and T_{50s} in ca. 20 days after sowing at all concentrations and temperatures tested.

Seedling mortality under Zn and Pb stress

The statistical analysis showed no statistically significant differences as regards seedling mortality under Zn stress, both for the locality and the concentration factor, as well as for their interaction ($p > 0.05$). The ANOVA analysis highlighted statistically significant differences among Pb concentrations ($p < 0.05$), but none among localities and in the interaction between concentration and locality ($p > 0.05$). In CP and CSI, the ANOVA showed no statistically significant differences ($p > 0.05$), whilst statistically significant differences were detected in OUT. By analysing the latter with the Tukey's test, statistical differences ($p < 0.05$) in seedling mortality without (0 mg/L) and with (from 25 to 100 mg/L) Pb stress were found (Table 5).

These results showed a successful survival of *H. m.* subsp. *tyrrhenicum* seedlings during the first day of their development under metal stress and under controlled conditions, determined by their low mortality, apparently with a better response in the Zn tests as compared to the Pb ones. The recorded seedling mortality could be partially linked to the toxicity of metals. As previously cited, Zn is an essential nutrient and has important functions in the metabolism of plants, but it becomes toxic at high concentrations (Kabata-Pendias 2011; Márquez-García et al. 2013). Consequently, *H. m.* subsp. *tyrrhenicum* may catch a certain amount as nutrient, also suitable for the embryo, and the remaining amount may be softened by tolerance mechanisms. This is in agreement with the capability of the plants of this taxon to tolerate high concentration of Zn and Pb in the context of the abandoned mining sites (Cao et al. 2004; Bacchetta et al. 2017, 2018); in fact, this plant appears to be highly resistant to both heavy metals at several phenological stages including, as shown in this work, the first basic phase of the growth of any plant (i.e., seed germination). The evaluation of the mortality of earlier seedling can offer important information about the yield of growing in a potential phytoremediation project.

Practical implication of this study and future perspective

The seed germination tests carried out under Zn and Pb stress gave further information about the metal tolerance of *H. m.* subsp. *tyrrhenicum*, adding pieces of knowledge about metal-tolerant plants and for their practical use in real context. In details, the results of this study confirm that *H. m.* subsp. *tyrrhenicum* may give an important contribution to the development of future phytostabilization activities, suggesting the possibility to use its seeds as an important source of plants for the rehabilitation of contaminated soils. Above its high germination capability and its metal tolerance, *H. m.* subsp. *tyrrhenicum* gives other practical advantages: it is a perennial species and it must be preferred to herbaceous, annual and biennial ones; indeed, the use of long life plant species reduces the release of pollutant into soils and can guarantee the presence of a long-term vegetation canopy on contaminated soils; the canopy will prevent the dispersion of the pollutants in the surrounding area through wind dispersion and water erosion; it can influence the soil's retention and can help to rehabilitate the vegetation cover. Hence, the propagation by seeds of this plant species on mine waste surface would be extremely cheaper than the planting of already adult specimens.

In a broader perspective, the results here presented permit to suggest that the plant species growing on mine substrates may likely be better adapted to metal stress. For this reason, the careful selection of seeds in terms of provenance and the evaluation of the real metal stress on the seeds are of crucial importance when planning phytoremediation activities.

The methodology proposed in this work could be applied to several species growing in many abandoned mine sites and as far as the appropriate policies/best practices for phytoremediation of abandoned mine lands is concerned, the results suggest that the germination test under metal stress could be carried out as a preliminary evaluation about the metal tolerance capability of a certain plant species as well as the potential use in phytoremediation.

Finally, the proposed method can be applied to other investigation concerning the germination of *H. m.* subsp. *tyrrhenicum*'s seeds under other heavy metals and metalloids (Cd, Hg, As) as well as on seeds of other autochthonous plant species (i.e., *C. salviifolius*, *E. cupanii*, *P. lentiscus*). Furthermore, studies about *H. m.* subsp.

tyrrhenicum of abandoned mine sites are in progress, in details a phytoremediation laboratory experiment and an environmental mineralogical investigation concerning biomineral formation in the soil–plant system are taking place.

Conclusion

The present study demonstrates that the seeds of *Helichrysum microphyllum* subsp. *tyrrhenicum* are able to germinate under Zn and Pb stress and that the germination process is never completely inhibited. The results suggest that high concentrations of Zn lead to a reduction in seed germination, in particular in unadapted populations, whilst Pb at concentrations up to 100 mg/L appear not to interfere in the germination processes. Moreover, the seeds of this plant species seem to maintain the germination capability at very high metal concentration tested, especially for specimens growing inside the mine dump and early seedlings showed a successful survival during the first day of their development under metal stress.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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