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Adaptation to lead in a Pb/Zn-mine population of *Marrubium cuneatum*

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ABSTRACT

Marrubium cuneatum is an endemic Iranian plant species, which can be found in lead-contaminated environments. This study aimed to investigate the effects of lead on the growth and germination in two *M. cuneatum* populations, from metalliferous, Pb-contaminated and uncontaminated soil, respectively. Soil and plant samples of the Tang-e Douzan Pb-Zn mine and Morghab Spring (non-contaminated site) were collected and analysed. Germination and growth were recorded under increasing Pb exposure in the non-metallicolous (NM) and the metallicolous (M) local *M. cuneatum* populations. Germination percentage (GP), mean germination time (MGT), germination index (GI), healthy plant percentage (HPP), seedling death (SD), fresh weight (FW) and dry weight (DW), were measured. Primary assessments showed toxic levels of lead in soil samples of the Tang-e Douzan mine and in leaves of plants growing at the mine site. Germination experiments showed that the germination parameters were stimulated by low concentrations of Pb in both populations, but inhibited by high concentrations. However, the adverse effects of Pb at high concentrations on GP, GI, and HPP were significantly less in the M population. The mortality of germinating seeds (SD) was consistently associated with fungal infection, which gradually decreased with increasing Pb exposure, but to the same degree in both populations. Growth experiments clearly demonstrated Pb hypertolerance in the M population. In the NM population, seedling growth is more sensitive to excess Pb than seed germination, compared to the M population. The M population has high biomass and a relatively high Pb accumulation in its shoots, which suggests it is a suitable candidate for phyto-extraction of Pb.

1. Introduction

Anthropogenic activities, such as mining, generate a large amount of waste or residue materials, which causes severe pollution of the surrounding areas and has detrimental effects on living organisms (Oker-efor et al., 2020). The mining residues (tailing dams, deposits, and slag) are considered to be important sources of heavy metals and metalloids contamination (Espinosa-Reyes et al., 2014).

Heavy metals (HM) are elements of which the atomic number and density are higher than 20 and 5 g/cm³, respectively (Vidican et al., 2020). HM such as Fe, Zn, Cu, Mn, Co, and Ni are essential micronutrients for plants, but toxic when present in excess, such as comparatively low levels of non-essential HM, such as Hg, Cd, and Pb usually are (Palma et al., 2013). Certain plants can tolerate high concentrations of heavy metals. Most metal-hypertolerant species restrict the uptake and / or root-to-shoot transport of heavy metals (Baker, 1981). However, at least part of their hyper-tolerance is usually based on their

superior capacity to sequester heavy metals through chelation and subcellular compartmentalization, usually mainly in root cell vacuoles (Hall, 2002).

A minority of metal-hypertolerant species, called 'hyper-accumulators', accumulate and sequester metals at extremely high concentrations in their leaves, rather than their roots (Baker and Brooks, 1989). Hyperaccumulators with high metal concentrations in their shoots often have low biomass and slow growth, and are, therefore, often unsuitable for use in 'phyto-extraction', i.e., the extraction of metals from contaminated soils through harvesting above-ground metal-accumulating plant parts (Cunningham et al., 1995). Instead, the use of plants that accumulate less metal, but with high biomass production potential, compared to hyperaccumulators, has been considered to be more effective (Ahmad et al., 2018; Garbisu et al., 2002; Hernández-Allica et al., 2008; Kumar et al., 1995; Tlustoš et al., 2006; Tong et al., 2004).

In recent years, much attention has been paid to the dangers of heavy

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metals, such as lead, which has led to extensive research on plants native to metalliferous (metal-enriched) soils (Danh et al., 2009; Fitz and Wenzel, 2002). Lead is a highly phytotoxic metal, potentially causing a wide range of harmful effects, from germination to yield formation. Furthermore, lead can accumulate in plant tissue and thus enter the human food chain (Kumar et al., 2020). However, lead toxicity depends on the concentration and duration of exposure. Toxic levels of Pb disturb the plant water and nutritional status, cause oxidative damage, impair cell membranes and cause stomatal closure. Moreover, Pb induces conformational changes in the photosynthetic apparatus and can reduce the biosynthesis of chlorophylls, resulting in retardation of the carbon metabolism and plant growth (Zulfiqar et al., 2019).

Pb toxicity obstructs the germination process and early plant growth and has adverse effects on the physiology and morphology of seeds. It has been suggested that lead can inhibit seed germination and seedling development through preventing the mobilization of the seed's starch reserve, inhibiting the activities of enzymes involved in carbohydrate metabolism, such as α -amylase, β -amylase, acid phosphatases, thus retarding the emergence of the radicle (Mohamed, 2011; Seneviratne et al., 2019). Contamination of the soil with Pb disturbs the osmoregulation and changes the seed protein profile regulation of seeds. Lead also interferes with plant growth regulators and the antioxidant defense system in early seedling growth. Numerous reports have shown Pb-imposed germination inhibition in different plants (Zulfiqar et al., 2019). Permanent exposure to heavy metals has driven the evolution of metal resistance in metallophytes (plants that are specifically adapted to heavy metal-rich soils) (Ernst, 2000). Pb hypertolerance has been demonstrated in metallicolous populations of a number of facultative metallophytes (Brown and Brinkmann, 1992; Mahdavian et al., 2016).

There are several naturally and anthropogenically metal-contaminated (metalliferous) soils in Iran; however, there is little information on their flora's and the concentrations of trace elements in the local plant species (Ghaderian and Baker, 2007). The Tang-e Douzan Zn-Pb mining area is located in a mountainous area in central Iran, near Fereydoonshahr, 175 km west of Isfahan. To identify species with beneficial properties for the phytoremediation of metal-polluted soils, in a previous study we analyzed 69 plant species and several soil samples around the Tang-e Douzan Pb-Zn mine (Hesami et al., 2018). Eventually, *Marrubium cuneatum* was selected as a potentially suitable species for further research, due to its high shoot biomass, and a relatively high lead accumulation in its shoot (Salehi-Eskandari, unpublished).

The genus *Marrubium* L. belongs to the Lamiaceae family, and accommodates approximately 30 species, indigenous in Europe, the Mediterranean area, and Asia (Mabberley, 1997). *Marrubium cuneatum* Russell, is one of the native Iranian *Marrubium* species, on which only few data can be found in the scientific literature. *Marrubium cuneatum* is native to Iran, Iraq, Turkey, Palestine and Lebanon-Syria (İçen et al., 2021). It is distributed in the Irano-Turanian and the Mediterranean phyto-geographical regions of Iran. It usually is a perennial herbaceous and suffruticose species (Kharazian and Hashemi, 2017). It grows in steppe, which has an elevation range of 1870–2450 m asl in the Zagros mountains (Kharazian and Hashemi, 2017), with an annual precipitation of 300–600 mm.

The seed germination test is often used to evaluate hazardous effects and the toxicity of organic and inorganic compounds (Di Salvatore et al., 2008). Also, the first step in phytoremediation research and its application is plant reproduction. The metallicolous population of *M. cuneatum* has been suggested to have phytoremediation potential in soils contaminated with Pb (Hesami et al., 2018). However, the ability of *M. cuneatum* to germinate and grow under Pb contaminated conditions and its capacity for Pb remediation have not been studied. Therefore, in this study, we evaluated the Pb concentrations in the rhizosphere environment and the leaves of *M. cuneatum* at and around the Tang-e Douzan Pb-Zn mine. The germination percentage (GP), germination index (GI), mean germination time (MGT), seedling death (SD), and healthy plant percentage (HPP) under different concentrations of lead

were compared in a laboratory experiment in a metallicolous (M) and a nearby non-metallicolous (NM) population of *M. cuneatum*. In addition, the effects of Pb on root and shoot growth, the relative water content (RWC) of leaves, and the root and shoot Pb concentrations in 60-d old plants were compared in an additional experiment.

2. Materials and methods

2.1. Plant and soil sampling and analysis

The Tang-e Douzan Zn-Pb mine is located near Fereydoonshahr city, at 49° 57' 7" E and 33° 2' 39" N, 175 km west of Isfahan, central Iran, at 2800–2850 m asl elevation. The average annual rainfall in Tang-e Douzan is 546.4 mm, of which 41 % occurs in winter, 31 % in autumn, and 24 % in spring. Minimum and maximum temperatures in winter and summer are –25 and 34.6 °C, respectively. In this study, soil and plant samples were collected from four different sites around (by 300 m) the Tang-e Douzan mining area, located east, west, south, and north of the excavated location (contaminated sites). Also, soil and plant samples were taken at a non-contaminated site, in the eastern part of Morghab Spring (50° 49' E, 32° 51' N; 2190 m asl). Soil samples (0–20 cm depth) were taken only near the roots of the *M. cuneatum*. Soil samples (0–20 cm) were ground, sieved through a 80-mesh-screen, and dried in an oven at 70 °C. Then a 0.5-g subsample was digested in 10 ml of a 3:1 mixture of concentrated HCl (37 %) and HNO₃ (65 %). Subsequently, each digest was filtered and collected into a volumetric flask, and made up to volume with distilled water (10 mL). Finally, the total Pb concentrations were determined by atomic absorption spectrophotometry (AAS; Shimadzu AA-6200). Exchangeable Pb was determined based on the ammonium acetate method (Sparks et al., 1996) by mixing 5-g subsample with 50 ml ammonium acetate (1 mol/L, pH 7.0) on an electric stirrer for 2 h. Then, the filtrate was acidified with HNO₃ (0.2 %) to determine Pb by AAS. Water-soluble Pb was determined in a 1:10 mixture of soil and distilled water after shaking in a rotary shaker for 24 h. Then, the solution was centrifuged at 3000 rpm and the supernatant was filtered. Finally, AAS was used to measure the concentration of Pb.

Four randomly selected individual plants of *M. cuneatum* with flowers and seeds, at least 10–15 m away from each other, were harvested at each site. To measure the Pb concentrations in *M. cuneatum* leaves, the leaf samples were washed with distilled water twice and then oven-dried at 70 °C, to constant weight. Then dry leaves were powdered, and 0.1-g subsamples were digested at 120 °C for 1 h in a mixture of H₂O₂ (30 %), HCl (37 %), and HNO₃ (65 %) (1:3:6, v/v/v). Then, the digests were cooled and filtered through a moisturized filter paper (Whatman No. 40), and made up to 10 ml with distilled water (Salehi-Eskandari et al., 2022). The resulting solutions were assayed for lead by AAS.

2.2. Seed collection, treatments, and germination characteristics

Approximately 1000 mature fruits of *M. cuneatum* were randomly collected around the Tang-e Douzan mine and at Morghab spring (M and NM populations, respectively). Fruits (consisting of 4 nutlets with 1 seed each) were broken with a tweezer and the seeds were isolated and used in the germination experiments. The seeds were sterilized in 5 % Na-hypochlorite solution for 7 min and then rinsed three times with distilled water. Preliminary experiments, including pretreatments with different concentrations of gibberellic acid and low temperatures, were done to determine the best germination conditions. To accelerate seed germination, based on the results obtained from pilot experiments (data not shown), the sterilized seeds were placed in 750 mg/L gibberellic acid solution for 24 h and then exposed to 4 °C for 2 weeks. Lead effects on seed germination parameters were assessed with lead (II) nitrate [Pb(NO₃)₂], supplied at concentrations of 0, 5, 10, 25, 50, 100, 150, and 200 mg/L in Petri dishes. Three replicates with 40 seeds each, per Pb treatment level, were incubated at 25 ± 2 °C. Experiments were

performed in the dark, because it appeared in pilot experiments that light did not affect germination. Seeds were considered to be germinated when the radicle emerged from the seed by one mm.

The germination percentage (GP), mean germination time (MGT), germination index (GI), seedling death (SD), and healthy plant percentage (HPP) were calculated according to the germination counts during 14 days (after 14 days, there was no more germination in pilot experiments).

The germination percentage (GP) was calculated as:

$$GP(\%) = \frac{n}{N} \times 100,$$

In which n is the number of germinated seeds, and N is the total number of seeds (Salehi-Eskandari et al., 2017b). GP only shows the final germination percentage and does not indicate the rate of germination, but the MGT provides a precise estimate of the germination time (Kader, 2005). The mean germination time (MGT) was calculated as:

$$MGT = \frac{\sum D \times n}{\sum n},$$

In which n is the number of seeds germinated on day D , and D is the number of days after the start of the experiment (Salehi-Eskandari et al., 2017b). The germination index (GI) was calculated as:

$$GI = G1/T1 + G2/T2 + \dots + Gn/Tn,$$

In which $G1, G2, \dots, Gn$ are the number of germinated seeds on the first count, second count, and so on until the last count (n), respectively, and $T1, T2, \dots, Tn$, in which T represents the number of days between sowing and the first count, between sowing and the second count, and so on until the last count (n), respectively (Dastanpoor et al., 2013).

Germinated seeds, i.e., seeds without fungus, were transferred to new Petri dishes, and their percentage was calculated as healthy plant percentage (HPP), and infected ones, i.e. those with fungus, were kept in the same Petri dishes, and their percentage was calculated as seedling death percentage (SD):

$$SD = \frac{Dn}{N} \times 100,$$

in which Dn is the number of germinated seeds with fungus, and N is the total number of seeds; and healthy plant percentage as:

$$HPP = \frac{HPn}{N} \times 100,$$

in which HPn is the number of healthy plants, and N is the total number of seeds.

2.3. Effects of Pb on growth parameters and concentrations Pb in root and shoot

After germination, healthy seedlings were grown in a greenhouse with a 16 h photoperiod (light intensity $200 \mu\text{mol m}^{-2} \text{s}^{-1}$), day/night temperature of 25/20 °C, and regularly watered with a modified half-strength Hoagland's nutrition solution containing 3 mM KNO_3 , 2 mM $\text{Ca}(\text{NO}_3)_2$, 1 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 0.5 mM MgSO_4 , 20 μM $\text{Fe}(\text{Na})\text{-EDTA}$, 1 μM KCl , 25 μM H_3BO_3 , 2 μM MnSO_4 , 2 μM ZnSO_4 , 0.1 μM CuSO_4 , 0.1 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, with pH adjusted to 5.8–6.0. After 60 days plants of uniform size having about 12 cm height were transferred to hydroponics (450 ml light-proof vessels, 2 plants per vessel) with different concentrations of Pb (0, 10, 50, 100, 150 and 200 mg L^{-1}), added as $\text{Pb}(\text{NO}_3)_2$ with three replicates per Pb concentration. The solutions were aerated continuously and replaced by freshly prepared ones every three days during the 14-day treatments.

2.4. Plant growth and Pb accumulation

After harvesting, plants were separated into shoot and root portions and rinsed several times in deionized water and fresh weight (FW) was

measured. Dry weight, SDW (shoot dry weight), and RDW (root dry weight) were measured after drying at 70 °C for 48 h in an oven. The relative water content (RWC) was calculated as: $\text{RWC}(\%) = [(\text{FW}-\text{DW}) / (\text{TW}-\text{DW})] \times 100$, in which FW, DW and TW are fresh weight, dry weight and turgid weight, respectively (TW was determined as the fresh mass after saturating the samples with water for 4 h (Bandurska, 2000)). Lead concentrations were determined in roots and shoots after preparing the plant material as above, and Pb translocation factors (TFs) were calculated as the Pb concentration in shoots divided by that in roots (Salehi-Eskandari et al., 2017a).

2.5. Statistical analysis

The experiment was performed in a fully factorial design with three replicates. Using SPSS 18, data were analysed through one-way or two-way ANOVA. Data were tested for normal distribution using Kolmogorov-Smirnov test and for homogeneity of variances with Levene's test. To compare individual means of Pb treatment levels within each population, Duncan's test was used with a significance threshold of $P < 0.05$. For comparing the two populations at each level of Pb treatment, a t -test was applied.

3. Results

3.1. Effect of lead on germination

The germination percentage (GP) was significantly increased in both populations (N and NM) at 10 mg/L of Pb. The highest Pb treatment level (200 mg/L) significantly decreased GP in both populations, but more strongly in the NM population (22.9 %) than in the M one (6.7 %; Fig. 1a; Table 1, $P < 0.001$ for population \times Pb interaction).

In the NM population, the lowest (4.9 days) and highest (6.75 days) MGT were observed at 50 and 200 mg/L Pb concentrations, respectively. Similar to the NM population, the highest MGT (7.27 days) was measured at 200 mg/L of Pb for the M population, but the lowest MGT (5.00 days) was recorded at 100 mg/L of Pb. In addition, the controls were not significantly different in MGT (6 and 5.95 days for NM and M, respectively). Furthermore, a significant decrease compared to control in MGT was recorded in the range of 25 to 100 mg/L Pb for NM and M (Fig. 1B; Table 1, $P < 0.05$ for population \times Pb interaction).

The highest GI, in both populations, was recorded at the 25-mg/L Pb treatment. However, in the higher than 25- and 100-mg/L treatments, a concentration-dependent decrease of GI was observed in both NM and M. The M population consistently showed higher GI values in the 50-, 100-, 150- and 200 mg/L Pb concentrations by 15.8 %, 23.1 %, 15.4 %, 13.5 %, respectively, compared to the same treatments in NM population (Fig. 1C; Table 1, $P < 0.001$ for population \times Pb interaction).

The seedling death rate (SD) owing to fungal infection gradually decreased with increasing Pb exposure in both populations, except for the 5-mg/L Pb treatment level (Fig. 2A). However, the effects of population and of the population \times Pb interaction were both insignificant (Table 1).

The percentage of healthy seedlings at the end of the experiment (HPP) was significantly higher in the M than in the NM population, except for the 0, 5, 10, and 25 mg/L Pb concentrations (Fig. 2B). The healthy plants percentage was highest at the 10- and 25-mg/L Pb concentrations in both populations, and then decreased at 50-, 100-, 150- and 200-mg/L Pb concentrations, but more so in NM (29.4 %, 34.1 %, 30.3 % and 27.7 %) than in M (3.8 %, 8.9 %, 8.9 % and 1 %; Table 1, $P < 0.01$ for population \times Pb interaction).

3.2. Plant growth and lead accumulation

The population \times Pb interaction for SDW and RDW was significant, showing that the populations responded distinctly to the Pb treatment (Table 1). Increasing Pb concentrations in the nutrient solution

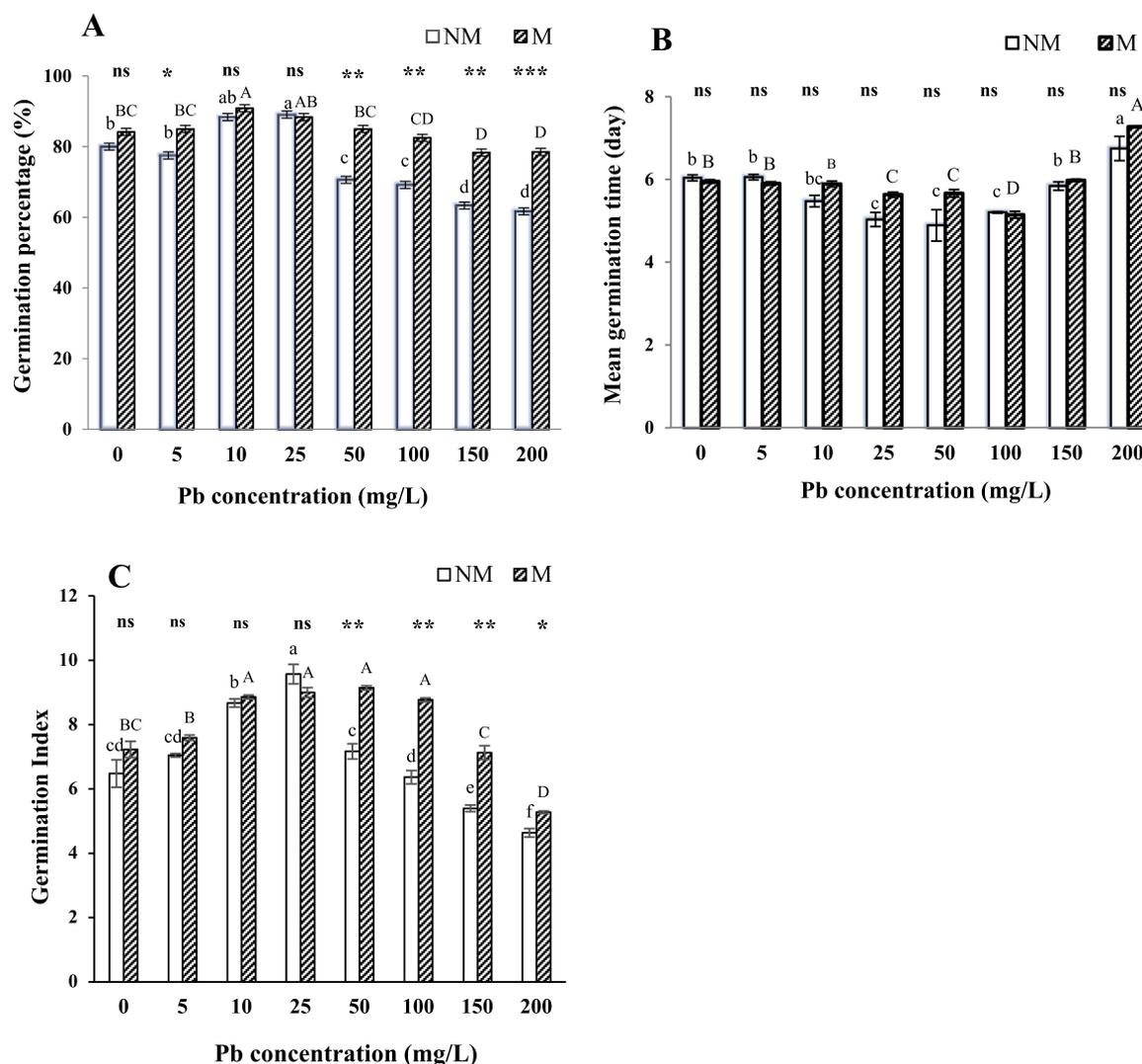


Fig. 1. Effect of lead on germination percentage (A), mean germination time (B) and germination index (C) in *Marrubium cuneatum* metallicolous (M) and non-metallicolous (NM) populations (mean \pm SE, $n = 3$). Significant differences between treatment means according to Duncan's test ($P < 0.05$) are indicated by different letters (uppercase letters for metallicolous (M) and lowercase letters for non-metallicolous (NM) populations). Significant differences between NM or M in each treatment were based on t -test (** $= P < 0.001$, ** $= P < 0.01$, * $= P < 0.05$ and ns $P > 0.05$).

gradually decreased the shoot and root dry weights (SDW and RDW) of the non-metallicolous (NM) population (by 30.9 % and 39.6 % at 200 mg/L), but SDW and RDW of the metallicolous (M) population were not significantly affected, except for RDW at the 200-mg/L Pb concentration (Fig 3A and B).

The relative water content (RWC) gradually decreased with the Pb exposure level in both populations (Fig. 4). RWC in the metallicolous and the non-metallicolous populations was similar, except in 200-mg/L treatments, where RWC was higher in the metallicolous population than in the non-metallicolous one, resulting in a significant population \times Pb interaction (Table 1, $P < 0.001$ for population \times Pb interaction).

The Pb concentration \times population interaction was significant ($P < 0.01$) only for the Pb concentration in the shoot, because of an irregularly increasing difference between populations with increasing Pb exposure. The Pb concentrations in shoots and roots increased with increasing Pb concentrations in the nutrient solution by a factor of 11.9 and 7.5 in shoots and 4.4 and 3.9 in roots of non-metallicolous and metallicolous populations respectively (200 mg/L in comparison with the 10 mg/L treatment.; Fig. 5A and B). The root Pb concentrations of both populations were similar at all the Pb treatments, but the shoot Pb concentrations were significantly higher in the metallicolous population

than in the non-metallicolous one.

3.3. Lead concentration in soil and plant material at Tang-e Douzan mine

The total, exchangeable and soluble concentrations of Pb at the four sampling sites around the Tang-e Douzan mine are shown in Table 2. The average total concentration of lead in the soil samples taken around the mine was 902 mg/kg. Among all sites, the samples taken west of the mine had the highest concentration of total lead (1968 mg/kg). In the Morghab spring soil samples Pb was undetectable. The highest exchangeable and soluble fractions of Pb were also observed in the soil samples taken west of the mine. The average amount of soluble and exchangeable lead in soil samples taken from the four mine sites was between 2.1 and 4.7 % of total Pb, respectively.

The lead concentrations in leaf samples of 16 *M. cuneatum* plants, collected from the mine sites, are presented in Table 3. The average concentration of Pb in leaves of *M. cuneatum* was approximately 120 mg/kg. The leaves sampled west of mine had the highest mean Pb concentration (166.2 ± 19.2 mg/kg, mean \pm sd).

Table 1

Two-way analysis of variance of the effect of Pb and population and their interaction on germination in *Marrubium cuneatum*.

Source of variance	Df	F ratio				
		GP	MGT	GI	SD	HPP
Pb	7	38.3***	33.95***	108.2***	19.9***	15.0***
population	1	118.8***	14.2**	102.2***	1.63ns	78.1***
population × Pb	7	7.8***	3.1*	13.9***	0.04 ^{ns}	4.3**
Error	32					

Source of variance	Df	F ratio				
		SDW	RDW	RWC	Pb.R	Pb.S
Pb	4	25.1***	20.9***	53.7***	516.7***	411.7***
population	1	89.4***	22.1***	15.8**	0.72ns	68.1***
population × Pb	4	22.9***	5.3**	8.23***	0.61 ^{ns}	5.9**
Error	20					

GP, germination percentage; MGT, mean germination time; GI, germination index; SD, seedling death; HPP, healthy plants percentage; SDW, shoot dry weight; RDW, root dry weight; RWC, relative water content; Pb.R, concentration of Pb in root; Pb.S, concentration of Pb in shoot. ns, non-significant; *, ** and ***, significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

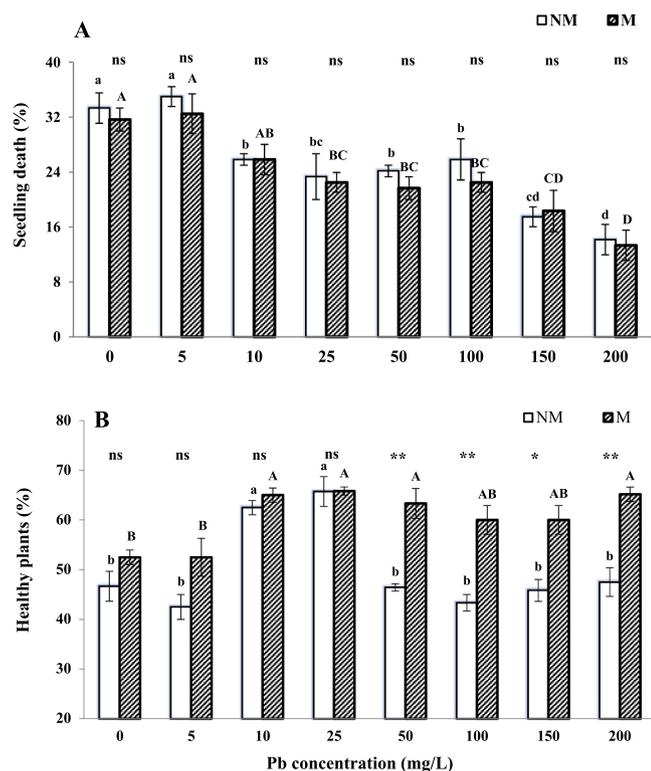


Fig. 2. Effect of lead on seedling death percentage (A) and healthy plants percentage (B) in *Marrubium cuneatum* metallicolous (M) and non-metallicolous (NM) populations (mean ± SE, $n = 3$). Significant differences between treatment means according to Duncan's test ($P < 0.05$) are indicated by different letters (uppercase letters for metallicolous (M) and lowercase letters for non-metallicolous (NM) populations). Significant differences between NM or M in each treatment were based on t -test (***) = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$ and ns $P > 0.05$).

4. Discussion

In this study, in order to evaluate the potential effectiveness of metallicolous *M. cuneatum* as a Pb-resistant plant in the

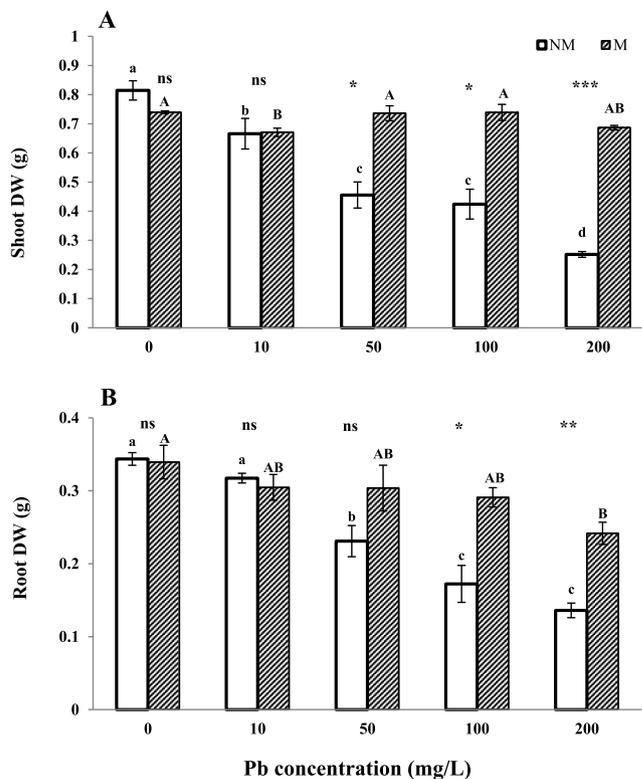


Fig. 3. Effect of Pb on shoot (A) and root (B) dry weight of *Marrubium cuneatum* metallicolous (M) and non-metallicolous (NM) populations (mean ± SE, $n = 3$). Significant differences between treatment means according to Duncan's test ($P < 0.05$) are indicated by different letters (uppercase letters for metallicolous (M) and lowercase letters for non-metallicolous (NM) populations). Significant differences between NM or M in each treatment were based on t -test (***) = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$ and ns $P > 0.05$).

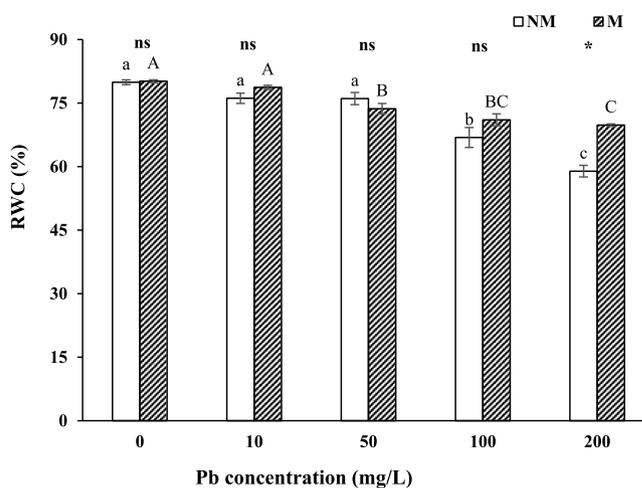


Fig. 4. Effect of Pb on the relative water content (RWC) of *Marrubium cuneatum* metallicolous (M) and non-metallicolous (NM) populations (mean ± SE, $n = 3$). Significant differences between treatment means according to Duncan's test ($P < 0.05$) are indicated by different letters (uppercase letters for metallicolous (M) and lowercase letters for non-metallicolous (NM) populations). Significant differences between NM or M in each treatment were based on t -test (***) = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$ and ns $P > 0.05$).

phytoremediation process, we measured the concentration of lead in soil samples and leaves, collected from sites adjacent to the Tang-e Douzan Pb-Zn mine, and determined shoot and root dry weight (SDW and RDW) and Pb accumulation in *M. cuneatum* populations treated with Pb

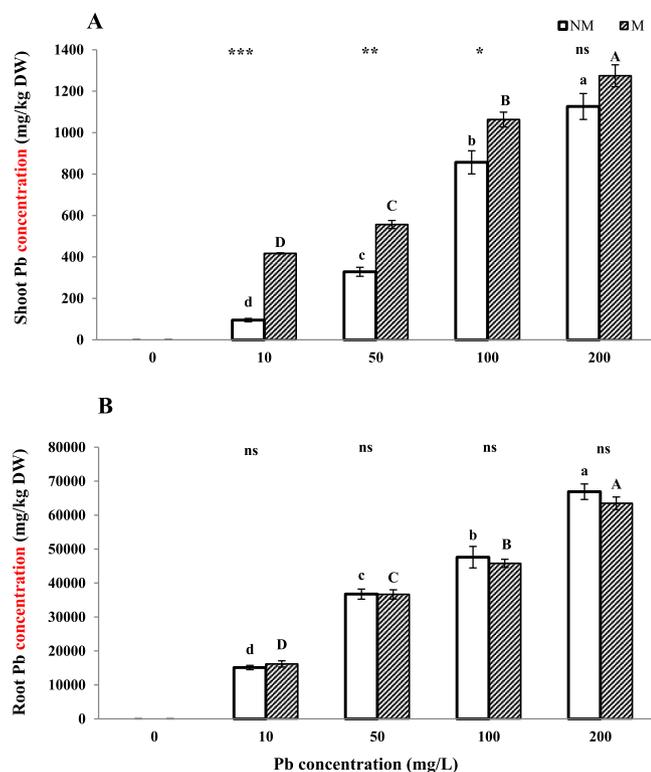


Fig. 5. Shoot (A) and root (B) Pb concentrations *Marrubium cuneatum* metallicolous (M) and non-metallicolous (NM) populations (mean ± SE, n = 3). Significant differences between treatment means according to Duncan’s test (P < 0.05) are indicated by different letters (uppercase letters for metallicolous (M) and lowercase letters for non-metallicolous (NM) populations). Significant differences between NM or M in each treatment were based on t-test (*** = P < 0.001, ** = P < 0.01, * = P < 0.05 and ns P > 0.05).

Table 2

Total, exchangeable and water-soluble concentrations of Pb in soils of sampling sites.

Site	Mean total Pb (mg/kg)	Mean Exchangeable Pb (mg/kg)	Mean water-soluble Pb (mg/kg)
South	213	20	4
North	225	25	9
West	1968	63	40
East	1202	59	21
Mean	902	42	19

Table 3

Lead concentrations in shoots of the *Marrubium cuneatum* metallicolous population of the Tang-e Douzan lead-zinc mine, Iran.

sample	Site	Pb (mg/kg)	sample	Site	Pb (mg/kg)
1	South	89.6	9	West	166.7
2	South	89.3	10	West	149.3
3	South	104.7	11	West	197.5
4	South	67.5	12	West	151.5
5	North	128.4	13	East	114.4
6	North	85.6	14	East	137.6
7	North	56.5	15	East	139.4
8	North	106.7	16	East	145.3
			Mean		120.63

concentrations for 14 days under laboratory conditions. Seed germination and seedling health characteristics were also evaluated. The results demonstrated that the soil near the roots of the *M. cuneatum* at the Tang-e Douzan mining sites is highly contaminated with lead (as a total

fraction), when compared with the global average (27 mg/kg) reported by Kabata-Pendias (2000). Maximum contamination (1968 mg/kg) was recorded in the soil samples from the partly unvegetated western part of the mining site, i.e., around 70 times more than the global average. Maximum exchangeable and soluble fractions of Pb were observed in the soil samples collected west of mine. It is obvious that high Pb total soil concentrations may be largely insoluble, and thus not immediately bioavailable (Ievinsh, 2023; Pollard et al., 2002). Of course, although the soil at the mine site is doubtlessly metal-toxic, it cannot be claimed beforehand that Pb contributes to the soil toxicity at the site, because the ore is ‘poly-metallic’, and since the toxicity of Pb depends strongly on soil pH and the degree of Ca saturation of the soil’s cation exchange complex (CEC) (Brown and Brinkmann, 1992; Rieuwerts et al., 1998).

Concerning the better germination under high Pb exposure of the M population compared to the NM one, it may be relevant that Pb can interfere with enzymes involved in seed germination, such as amylase and protease (Sengar et al., 2008). Moreover, under Pb toxicity, the mobilization of stored food can be expected to be reduced, potentially leading to a disruption of the cellular osmoregulation, inhibition of proteolytic activities, and reduction in radicle formation which can lead to inhibition of germination and seedling development (Cokkizgin and Cokkizgin, 2010). Our data indeed seem to confirm that Pb hyper-tolerance may become already evident during the germination process, in spite of the fact that the Pb concentrations in the seeds, if any, must have been higher in the (field-collected) M seeds than in the NM ones. More precisely, the stronger inhibition of germination in NM seeds under high Pb exposure suggests that ungerminated seeds, at least those from the NM population, must have taken up Pb in toxic amounts before their germination was observed, which is not self-evident. Further research is required to resolve this issue.

In this study, high Pb concentrations delayed germination, increasing MGT in both populations; this has also been reported for other species (Cokkizgin and Cokkizgin, 2010). These effects might be related to Pb interference with the water balance of the germinating seed (Poschenrieder and Barceló, 1999). In addition, it has been shown that lead reduces the activities of enzymes involved in carbohydrate metabolism, possibly through a reduction of seed water uptake (Mohamed, 2011; Seneviratne et al., 2019).

Similar to GP, the GI was reduced at high lead concentrations (150 and 200 mg/L) in both populations, but particularly in the NM one. Similarly, lead treatment also reduced GI in *Medicago arborea* (Bezini et al., 2019). Despite the inhibitory effects of Pb at high concentrations on GI in both populations, our data indicate a stimulatory effect of Pb at low concentrations (10 to 100 mg/L for M and 5 to 25 mg/L for NM). Similarly, Kang et al. (2009) showed that Pb concentrations less than 50 mg/L increased the germination rate in wheat. This could be due to the beneficial effect of mild stress, called ‘hormesis’ (Rojas-Loria et al., 2012), and may be related to the production of reactive oxygen species (ROS), which play a dual role in plants. It is known that overproduction of ROS under severe stresses induces oxidative stress, but it can also act as a signal to upregulate stress responses (Yuan et al., 2013). Therefore, it is possible that Pb-generated ROS could have had a stimulating effect on germination under mild exposure.

Pb reduced seedling death (SD) mediated by fungal infection in both *M. cuneatum* populations. The effectiveness of heavy metals in reducing fungal contamination of plants and seedling mortality has been reported previously. For example, wheat seedlings pre-exposed to Cd²⁺ experienced less fungal infection compared to untreated seedlings (Mitra et al., 2004). In another report, selenium treatment inhibited fungal infections in Indian Mustard (Hanson et al., 2003). It has been suggested that metal accumulation by plants confers protection against fungal infection (Ghaderian and Baker, 2007). The toxic effect of heavy metals on fungal growth might relate to changes in membrane permeability and ROS-induced oxidative stress (Morkunas et al., 2018), owing to metals that are adsorbed to the seed coat (Moreira et al., 2020). In any case, in our study seedling survival was equally increased by Pb exposure in both

populations, suggesting that the Pb tolerance of the seedling was unimportant regarding its chance to survive fungal attack.

Shoot and root dry weights (SDW and RDW) of the NM population decreased with increasing concentration of Pb in the nutrient solution, while the SDW and RDW of the M population only decreased at the highest treatment concentrations of Pb. Also, in the highest Pb (200 mg/L) treatments, the relative water content (RWC) was more decreased in the NM population than in the M population. The populations had similar root-internal Pb concentrations. However, the Pb concentrations in the shoot were always higher in the metallicolous population than in the non-metallicolous one. This clearly proves that the metallicolous population is more resistant to Pb than the non-metallicolous population. Hypertolerance and hyperaccumulation are created by local adaptive processes that result in specific strategies which distinguish metallicolous from non-metallicolous ecotypes within the same genetic unit (Manara et al., 2020). Pb is extremely toxic compared to most heavy metals, especially for shoots, and all plant species studied thus far appear to transport only small amounts to the shoot (Gupta et al., 2013; Zhou et al., 2016), which could explain that Pb toxicity is primarily manifested in the root, in agreement with our results. Reduced root growth, owing to inhibition of cell division or cell elongation could conceivably represent a direct Pb effect. It is also conceivable that Pb phytotoxicity could indirectly rely on Pb interference with the uptake of essential nutrients, such as Fe, Mg, Zn, Cu, Mn and P, or with the plant water balance (Hakeem et al., 2019; Singh et al., 2015). In any case, our growth experiments demonstrate Pb hypertolerance in the M population, compared to the NM one, which indicates Pb toxicity at the mine site, indeed. It is possible that the Pb hypertolerance observed in the mine population might merely represent a pleiotropic by-product ("co-tolerance") of a hypertolerance mechanism for another metal (e. g., Zn). In general, combined hypertolerances to different metals ("multiple tolerance") seem to be completely, or at least largely, genetically independent, or when not, apparent pleiotropic hypertolerances have been found to be fairly inconsiderable, thus far (Schat and Vooijs, 1997; Schat et al., 1996). However, further genetic studies are definitely required to evaluate the potential genetic correlations, if any, of Pb hypertolerance with other metal hypertolerances, at least Zn hypertolerance, in the Tang-e Douzan population of *M. cuneatum*.

The use of organisms in biological remediation is a promising and sustainable approach to soil remediation. To remediate soil that is metal-polluted, either plants (phytoremediation) or microbes (microbial remediation) are employed. It is generally considered as a 'green' and cost-effective approach (Chibuike and Obiora, 2014; Zulfiqar et al., 2019). In our study, we observed considerable foliar Pb concentrations in the M population of *M. cuneatum*, both at the mine site (Table 2), and in Pb-amended nutrient solution, with Pb concentrations in leaves up to 10 times higher than at the mine site (Fig. 5). Although the species cannot be classified as a (facultative) Pb hyperaccumulator, since its foliar Pb concentrations are far below the nominal threshold for hyperaccumulation (Van der Ent et al., 2013), they are certainly above average, even higher than in Zn/Cd-hyperaccumulating *Noccaea* species, growing in comparable circumstances (Koubová et al., 2016). It can thus be concluded that *M. cuneatum*, in particular the metallicolous population under study here, is a relatively good foliar accumulator of Pb and, therefore, in view of its high biomass productivity and superior Pb (hyper-)tolerance, a potential candidate for Pb phyto-remediation (Hesami et al., 2018), the more so in view of its relatively high biomass (about 80 cm plant height, with about 400 g shoot fresh weight per plant) and abundant seed production (more than 100 seeds per axis).

5. Conclusion

Marrubium cuneatum is an almost unknown facultative metallophyte. Our results clearly demonstrated that the metallicolous (M) population germinated more completely and grew faster at high Pb concentrations, while Pb moderated the seedling death (SD) mediated by fungal

infection to the same extent in both the metallicolous and the non-metallicolous population. There was clearly Pb hypertolerance in M, estimated from the capacity to grow and to maintain a positive water balance during the established phase of the life cycle. In general, it can be concluded that metallicolous *M. cuneatum* is potentially suitable for the phytoremediation of Pb-contaminated soils. In addition, as a facultative metallophyte with evidently population-specific Pb hypertolerance, it might also be useful in further analyses of the physiology and genetics of Pb hypertolerance in metallophytes.

CRedit authorship contribution statement

Behrooz Salehi-Eskandari: Writing – review & editing, Writing – original draft, Software, Methodology. **Reza Hesami:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation. **Azam Salimi:** Visualization, Project administration. **Henk Schat:** Writing – review & editing, Writing – original draft, Software, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest. We confirm that the manuscript: "Possible genetic adaptation regarding germination capacity in a Pb/Zn-mine population of *Marrubium cuneatum*" complies to the Ethical Rules applicable for this journal. We certify that the submission is an original work is not under review at any other publication.

Data availability

Data will be made available on request.

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