

Physiological responses to lead and PEG-simulated drought stress in metallicolous and non-metallicolous *Matthiola* (Brassicaceae) species from Iran

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Abstract

Aims

Plants growing on quarry tailings at the Irankouh Pb/Zn mine encounter both drought stress and high levels of Pb. To better understand role of drought and Pb in plant adaptation to Pb/Zn quarry tailings, we compared effects of drought stress (simulated by polyethylene glycol - PEG) and Pb, individually and in concert, to determine how these stressors affected two plant species: the metallicolous species *Matthiola flavida* and the non-metallicolous *M. incana*.

Methods

Plants were exposed to Pb ($\text{Pb}(\text{NO}_3)_2$) and three levels of PEG (0, -0.5, and -0.75 MPa) in a complete factorial design.

Results

Lead had non-significant effects on growth and oxidative stress but enhanced levels of osmoprotectants and phenol compounds in the metallicolous *M. flavida*, whereas in the non-metallicolous *M. incana* Pb had non-significant or toxic effects on the same variables (except for the osmoprotectants proline and glycine betaine, and anthocyanins). In contrast to *M. incana*, the metallicolous species was hypertolerant of Pb, showing strongly reduced root-to-shoot translocation and enhanced Pb accumulation in the root, especially when under drought stress.

Conclusion

We conclude that enhanced Pb accumulation in the root and reduced translocation to the shoot, particularly when under high PEG exposure in the metallicolous species, reduced toxic effects of Pb in the shoot. This was aided by the accumulation of reducing sugars and phenolic compounds as well as greater catalase activity.

Introduction

Mine quarry wastes are a challenging plant growth substrate because they create simultaneous stresses: these often include drought stress as well as stress from elevated soil metal levels (Ernst, 1988; Mansfield et al., 2014). Cotolerance, which allows a plant to withstand simultaneous stresses, has been widely studied (de Silva et al., 2012; Nabi et al., 2019; Von Wettberg et al., 2014) but not often in the context of the stresses created by mine quarry wastes. Although tolerance and adaptation to high soil concentrations of heavy metals by plants is metal-specific, and usually those plants cannot tolerate more than one or a few heavy metals (Boyd and Rajakaruna, 2013; Konečná et al., 2020; Singh et al., 2016), the

stimulation of general stress response pathways by heavy metals may promote cotolerance of other stresses such as drought (Von Wettberg et al., 2014; Selby et al., 2014; Selby and Willis, 2018).

One of the most abundant and toxic heavy metals is Pb, which can have lethal effects on plants, animals, and humans (Fahr et al., 2015; Li et al., 2016; Wani et al., 2015). Lead pollution stems from its widespread uses in industrial activities, including in metal mining/smelting and burning of coal; in energy production (Pb in gasoline, emissions from power plants, and use in batteries); from agricultural activities (as ingredient/contaminant in pesticides and fertilizers); as ingredient in ammunition, explosives, and Pb-containing dyes; and its presence in sewage sludge and wastewaters (Gupta et al., 2013; Kumar et al., 2012; Mahdavian et al., 2016).

Lead is a non-essential element for plants and produces many phytotoxic effects, including harm to morphological, biochemical, and physiological processes (Pourrut et al., 2013; Zulfiqar et al., 2019). Some plant species, termed metallophytes by Baker (1987), can survive and reproduce on the intensely metal-enriched soils found on metal mine sites. Quarry tailings present around a mining area often are severely polluted by heavy metals (such as Pb) and are characterized by other stresses such as lack of water holding capacity, deficiencies of essential nutrients, and low amounts of soil organic matter, and as a consequence are often poorly vegetated (Ghasemi et al., 2018; Mansfield et al., 2014).

The Irankouh Pb/Zn mine is located in the semiarid region of Central Iran at 51°42' E and 32°30' N. Therefore, plants in this mine area encounter not only heavy metal stress but also drought stress. Drought stress reduces plant growth by affecting various physiological and biochemical processes, such as water relations, nutrient uptake, leaf chlorophyll levels, photosynthesis, water content, and others (Farooq et al., 2012; Okunlola et al., 2017). Plants can ameliorate drought stress through osmotic adjustment, which is promoted by the production and accumulation of soluble carbohydrates, sucrose, proline, glycine betaine, and other solutes that help maintain cell turgor (Cvikrová et al., 2013; Salehi-Eskandari et al., 2017). Drought stress, like other stresses, stimulates reactive oxygen species (ROS) generation, which induces oxidative damage to cellular components and photoinhibition (Agrawal et al., 2016; Ashraf and Harris, 2013; Cruz de Carvalho, 2008; You and Chan, 2015). To protect from oxidative stress, plants possess antioxidant scavenger systems which can be classified into two broad categories: antioxidant enzymes, such as catalase (CAT), superoxide dismutases (SOD), and ascorbate peroxidase (APX), and non-enzymatic antioxidants such as glutathione, ascorbate, proline, flavonoids, phenolics, α -tocopherol, and carotenoids (Kisa et al., 2016; Salehi-Eskandari et al., 2017).

Matthiola flavida Boiss. (Brassicaceae) is a perennial herb that grows on dry rocky hills and sandy plains in Iran, Pakistan, Kashmir, Afghanistan, and Turcomania (Mohtadi, 2014; Sarwar and Qaiser, 2012). It has been suggested to be useful for phytoremediation of soils contaminated with Pb (Mohtadi et al., 2012a, b). In addition, by adding Fe (Heidari Dehno and Mohtadi, 2018) and the synthetic and biodegradable chelator (S,S)-N,N'-ethylenediamine disuccinic acid (EDDS), hydroponically grown *M. flavida* accumulates even more Pb in its shoot tissue (Mohtadi et al., 2013). Despite the generally lethal effect of Pb on plants, Pb hypertolerance mechanisms, and the combined effects of drought stress with Pb stress on plant

physiology, have not been well studied. Therefore, to better understand these effects and particularly their interactions, we studied Pb stress and drought stress induced by polyethylene glycol (PEG) in the metalicolous species *Matthiola flavida* and the congeneric non-metallicolous *M. incana* (L.) R.Br. The species were compared for Pb uptake and translocation, plant growth (dry weight [DW] biomass and relative water content [RWC]), pigments (carotenoids, chlorophylls a and b), osmotic molecules (proline, glycine betaine, reducing sugars), phenolic compounds (anthocyanin, total phenols), and activities of two antioxidant enzymes, catalase (CAT) and ascorbate peroxidase (APX).

Materials And Methods

Plant materials and treatment

About 5000 seeds of the metalicolous *Matthiola flavida* were collected from a population of about 300 plants growing at the Irankouh Pb/Zn mining site, and seeds of a non-metallicolous species, *M. incana*, were provided from a commercial supplier (Pakan Bazr Co., Isfahan, Iran). *Matthiola incana* was selected as a comparative non-metallicolous species because it has a close phylogenetic position within the genus to *M. flavida* (Jaén-Molina et al., 2009). Seeds of both species were surface-sterilized using 1% (v/v) sodium hypochlorite (bleach) for 15 min, rinsed twice with distilled water, and germinated on Perlite wetted with deionized water. After three weeks, seedlings were transferred to hydroponic culture in 450 ml light-proof vessels (two plants per vessel) containing a modified one-fourth-strength Hoagland's solution composed of 1.5 mM $\text{Ca}(\text{NO}_3)_2$, 0.75 mM MgSO_4 , 1.25 mM KNO_3 , 0.28 mM KH_2PO_4 , 10 μM $\text{Fe}(\text{Na})\text{-EDTA}$, 1 μM ZnSO_4 , 0.5 μM CuSO_4 , 5 μM MnSO_4 , 25 μM H_3BO_3 , 0.1 μM Na_2MoO_4 , and 50 μM KCl . Final pH was adjusted to 5.8 and nutrient solutions were renewed twice a week. Experiments were performed in a greenhouse with a day/night temperature of 25/20°C and 16 h photoperiod (light intensity 200 $\mu\text{Em}^{-2} \text{s}^{-1}$).

Because the species differed in Pb tolerance, the Pb-added treatment for each species was selected as the concentration of Pb to which exposure induced Pb toxicity symptoms (reduced growth, chlorosis, etc.) for that species. Based on results of prior experiments, 75 mg L^{-1} and 150 mg L^{-1} were selected as the concentrations of $\text{Pb}(\text{NO}_3)_2$ sufficient to produce toxicity symptoms for the non-metallicolous *M. incana* and the metalicolous *M. flavida*, respectively. After 45 days for *M. incana*, and 60 days for *M. flavida*, half of the vessels for each species received nutrient solution amended with $\text{Pb}(\text{NO}_3)_2$. To avoid precipitation of Pb phosphate and formation of Pb-EDTA resulting from Pb replacing Fe from the EDTA complex in the Pb-added solutions, the KH_2PO_4 and $\text{Fe}(\text{Na})\text{-EDTA}$ were removed. They were also removed from the nutrient solutions of the no Pb-added treatments to make the solutions as similar to one another as possible.

After another 7 days, all pots were additionally modified with 0%, 13.8%, or 23.3% of polyethylene glycol 6000 (PEG, w/v), equivalent to osmotic potentials of 0, -0.25, and -0.75 MPa, respectively (Money, 1989). To avoid osmotic shock, PEG was added in 0.25 MPa steps at 6 h intervals until the desired

concentration was reached. Nutrient solutions were aerated constantly and replaced every three days during the 14 day treatment period.

Each combination of the Pb and drought stress treatments had six pots (replicates) for each species. Three replicates were harvested for measurements of plant growth and Pb accumulation, and the other three were used to provide plant material for measuring physiological parameters.

Plant growth and Pb accumulation

After harvesting, plants were separated into root and shoot portions and rinsed three times in deionized water, fresh weight (FW) was measured, and dry weight (RDW, root dry weight, and SDW, shoot dry weight) was measured after drying samples at 70°C for 48 h in an oven. Plant water content (WC) was calculated as percent water from the FW and dry weight data. Lead concentrations were determined in shoots and roots by digesting 0.1 g of dried ground plant material in a 1/4 (v/v) mixture of 37% (v/v) HCl and 65% (v/v) HNO₃, and Pb was determined using a flame atomic absorption spectrophotometer (AAS, Shimadzu model 6200). Pb translocation factors (TFs) were calculated as Pb concentration in shoots divided by Pb concentration in roots (Salehi-Eskandari et al., 2018).

Photosynthetic pigments measurement

The youngest mature leaves (4-5th leaf from the apex) of plants in replicates used for physiological measurements were extracted in 80% (v/v) acetone. Total chlorophyll, as well as chlorophyll a, chlorophyll b, and carotenoids contents, were measured by spectrophotometry according to Lichtenthaler and Wellburn (1983).

Proline, glycine betaine, and total reducing sugar measurements

Free proline was extracted from fresh leaves from replicates used for physiological measurements and determined according to Bates et al. (1973). Glycine betaine was extracted from powdered dried leaves and content calculated following the method of Grieve and Grattan (1983). The amount of total reducing sugars (RS) was measured in fresh leaves as defined in Somogyi (1952).

Measurement of anthocyanins and total phenolic contents

Anthocyanins were extracted from fresh leaves from replicates used for physiological measurements with acidified methanol (methanol: HCl, 99:1 v/v). Anthocyanins were measured spectrophotometrically and contents calculated using an extinction coefficient of 33,000 mol⁻¹ cm⁻¹ (Wanger, 1979). Total phenolic contents were determined using Folin–Ciocalteu reagent according to the method of Velioglu et al. (1998).

Enzyme assays

One gram of fresh leaf samples was homogenized in 3 ml of cold 50 mM potassium phosphate buffer (pH 7.8), including 0.2 mM EDTA and 2% (w/v) polyvinylpyrrolidone (PVP), in an ice bath. The homogenate was centrifuged at 13,000 rpm at 4 °C for 20 min and the supernatant used for enzyme activity assays. CAT activity was determined and expressed using the UV spectrophotometric method of Aebi (1984). Ascorbate peroxidase activity was determined based on the method of Asada and Takahashi (1987).

Statistical analysis

The experiment used a randomized complete block design with three replications for plant growth and Pb accumulation measurements, and three for physiological parameters. Data analysis was accomplished using SPSS (version 16, for Windows; SPSS Inc., Chicago, IL, USA). Data were analyzed using three-way ANOVA, with Pb, PEG, and Species as fixed main factors. Individual means were compared using Duncan's test with $P < 0.05$ as a significance threshold. Since our main goal is to compare responses of the metallicolous *M. flavida* against those of the non-metallicolous *M. incana*, we will focus mostly on the Species factor and its two- and three-way interactions with Pb and PEG.

Results

Plant growth and shoot relative water content

The Species factor was significant for all growth measures (Table 1), as *M. incana* plants were larger than *M. flavida* plants and had greater relative water contents (Fig. 1). PEG significantly reduced all growth measures (Fig. 1, Table 1). The species responded similarly to PEG for RDW, as the PEG*Species interaction was not significant (Table 1), but the PEG*Species interaction was significant for SDW and RWC (Table 1). The significant SDW interaction is explained by the relative stability of SDW for *M. flavida* as PEG concentrations increased (Fig. 1D), whereas SDW declined for *M. incana* as PEG concentrations increased (Fig. 1C). For RWC the significant interaction was due to an opposite pattern, with RWC declining in *M. flavida* as PEG concentrations increased (Fig. 1F), whereas RWC was more stable as PEG concentrations increased for *M. incana* (Fig. 1E).

Table 1

Significance of the effects of the main factors Pb, PEG, and Species, and their interactions, on root dry weight (RDW), shoot dry weight (SDW), relative water content (RWC), Pb concentration in roots (Pb R) and shoots (Pb S), Pb root-to-shoot translocation (Pb S/R), total chlorophyll (Total chl), chlorophyll a/b ratio (Chl a/b), carotenoids (Car), proline (Pro), glycine betaine (GB), reducing sugar (RS), anthocyanins (Anth), total phenolic concentrations (Phenol), catalase (CAT), and ascorbate peroxidase (APX).

	Species	PEG	Pb	Species*PEG	Species*Pb	PEG*Pb	Species*PEG*Pb
RDW	***	***	**	NS	**	NS	NS
SDW	***	***	**	**	***	NS	NS
RWC	***	***	NS	***	NS	NS	NS
Pb R	***	**	***	**	***	**	**
Pb S	***	**	***	**	***	**	**
Pb S/R	***	NS	***	*	***	NS	*
Total chl	NS	***	***	*	NS	*	NS
Chl a/b	***	***	NS	***	NS	**	NS
Car	**	***	***	*	**	NS	*
Pro	NS	***	***	***	***	**	**
GB	NS	***	***	*	NS	NS	NS
RS	***	***	***	NS	**	NS	*
Anth	***	***	NS	**	NS	*	*
Phenol	***	**	NS	***	NS	NS	NS
CAT	*	*	**	*	**	NS	NS
APX	***	NS	NS	NS	NS	NS	NS
Significance was determined using three-way ANOVA, with Pb, PEG, and Species as main factors (*** P < 0.001, ** P < 0.01 and * P < 0.05, NS = not significant).							

The main factor of Pb was significant for SDW and RDW but not RWC (Table 1). The Species*Pb interaction for RDW and SDW was significant because for *M. incana* there were significant adverse effects of Pb on RDW and SDW whereas for *M. flavida* Pb had no effect (Fig. 1A-D). The Species*Pb interaction for RWC was not significant because there were no effects of Pb on *M. incana* and for *M. flavida* Pb had a positive effect on RWC only in the - 0.25 MPa treatment (Fig. 1E, F).

The Pb*PEG interaction and the Species*PEG*Pb interaction were insignificant for RDW, SDW, and RWC (Table 1), showing that the species responded similarly to combined Pb and PEG treatments.

Lead concentrations

Responses to Pb were relatively complex, with significant main effects and interactions for Pb in shoots, Pb in roots, and the Pb shoot/root ratio with the exceptions of non-significance for Pb shoot/root ratio for PEG and the PEG*Pb interaction (Table 1). Lead concentrations in the metalicolous *M. flavida* were consistently greater in roots (20.6-, 23.1-, 30.1-fold) and shoots (10.5-, 11-, 9.4-fold) at PEG treatments of 0, -0.25, and -0.75 MPa compared to those in the non-metallicolous *M. incana*, resulting in an approximately 2.4-fold difference in the root-to-shoot translocation factor between the species (Fig. 2 C, D). The concentration of root Pb, and the Pb root-to-shoot translocation factor, were marginally but significantly ($P < 0.05$) affected by PEG in *M. flavida* (Fig. 2 B, D) yet were unaffected by PEG in *M. incana* (Fig. 2 A, C), resulting in a significant Species*PEG interaction for Pb R and Pb S/R but not for PEG*Pb for the Pb S/R variable (Table 1).

Photosynthetic pigments

Total chlorophyll concentration did not significantly differ between species (the Species factor was non-significant) but was greatly reduced by both PEG and Pb (Table 1, Fig. 3). Lead decreased total chlorophyll in *M. incana* (except in the -0.75 MPa treatment) and strongly decreased it for all three PEG treatments in *M. flavida*, resulting in a significant PEG*Pb interaction (Table 1). The Species*PEG*Pb interaction was insignificant for total chlorophyll (Table 1).

The species differed significantly in chlorophyll a/b ratio (the Species factor was highly significant: Table 1) with greater values for *M. incana* compared to *M. flavida* at all PEG treatments (Fig. 1 C, D). The main factor of Pb did not significantly affect chlorophyll a/b ratio, but PEG significantly affected chlorophyll a/b ratio and there was a significant Species*PEG interaction (Table 1). The interaction is explained by PEG significantly decreasing the chlorophyll a/b ratio in *M. incana* (Fig. 3 C) but having little effect on *M. flavida* (Fig. 3 D). Lead treatment increased the chlorophyll a/b ratio only for the control treatment in both species (Fig. 3 C, D), resulting in a significant PEG*Pb interaction (Table 1). The Species*PEG*Pb interaction was insignificant (Table 1).

The effects of PEG and Pb on carotenoid concentration mostly followed the patterns observed for total chlorophyll. However, the Species*PEG*Pb interaction was significant (Table 1) which was due to alleviation of the Pb effect by PEG treatments in *M. incana* (Fig. 3 E).

Proline, glycine betaine, and total reducing sugars

Proline concentrations were significantly enhanced by PEG in both species (Fig. 4 A, B), resulting in a significant main effect of PEG (Table 1). However, the significant Species*PEG interaction (Table 1) showed that the degree of enhancement differed between species, with *M. incana* responding more strongly than *M. flavida* as PEG water potential decreased (Fig. 4 A, B). Lead enhanced proline concentrations in both species, resulting in a significant Pb main effect (Table 1), but did not affect proline concentrations in the control treatment in both species and the -0.25 MPa PEG treatment in *M. flavida* (Fig. 4 B). Thus, the Species*Pb interaction and the Species*PEG*Pb interaction were significant (Table 1).

Glycine betaine (GB) concentrations were similar in both species, resulting in an insignificant Species main effect (Table 1). The PEG main effect was also significant (Table 1) with concentrations increasing in both species as PEG water potential decreased (Fig. 4 C, D). There was also a significant Species*PEG interaction (Table 1), as GB was increased by PEG in both species but the increase was greater for *M. incana* than in *M. flavida* for the most extreme (-0.75 MPa) drought treatment (Fig. 4 C, D).

Reducing sugar (RS) concentrations were significantly affected by all three main factors (Table 1). The Species factor was significant because *M. incana* RS concentrations were greater than in *M. flavida* (Fig. 4 E, F). PEG generally increased RS concentrations for both species (Fig. 4 E, F), as did Pb (Fig. 4 E, F), but there were significant Species*Pb and Species*PEG*Pb interactions (Table 1). The significant interactions stemmed from increased RS concentrations due to PEG treatment in both species, but they increased in different concentration-dependent ways between species (Fig. 4 E, F). Lead increased RS concentrations for all PEG treatments for *M. flavida* (Fig. 4 F), but only did so for the control PEG treatment in *M. incana* (Fig. 4 E). The magnitude of the Pb effect was generally greater for *M. flavida*, particularly in the -0.75 MPa PEG treatment (Fig. 4 E), which resulted in a significant Species*PEG*Pb interaction (Table 1).

Anthocyanins and total phenolic contents

Anthocyanins concentrations were significantly affected by the Species and PEG main factors but not by Pb (Table 1). Anthocyanins concentrations were much greater in *M. flavida* than *M. incana* but generally increased with decreasing PEG water potential in both species (Fig. 5 A, B). Anthocyanins concentrations in *M. incana* were relatively similar across PEG treatments (Fig. 5 A) yet they increased relative to the control for *M. flavida* in the -0.25 and -0.75 MPa PEG water potential treatments (Fig. 5 B), resulting in a significant Species*PEG interaction (Table 1). The significant Species*PEG*Pb interaction (Table 1) showed that while Pb as a main factor was not significant, the species differed in how the combination of PEG and Pb affected anthocyanins. This difference was most evident in the highest PEG treatment, for which *M. incana* showed a greater increase in anthocyanins when treated with Pb than *M. flavida* (Fig. 5 A, B).

Similar to anthocyanins, total phenolic contents were significantly affected by the Species and PEG main factors but not by Pb (Table 1). Total phenolic contents also were much greater in *M. flavida* than *M. incana* and generally increased with decreasing PEG water potential (Fig. 5 C, D). There was a significant Species*PEG interaction (Table 1), which resulted from PEG increasing total phenolic contents in different concentration-dependent ways. In *M. incana*, values for both the -0.25 and -0.75 MPa treatments were greatly increased relative to those for the control treatment (Fig. 5 C), whereas for *M. flavida* there was a more stepwise increase in total phenolic contents as PEG water potentials decreased (Fig. 5 D). Unlike anthocyanin concentrations, other interactions were not significant for total phenolic contents (Table 1).

Antioxidant enzymes

CAT activity was significantly (but relatively weakly) affected by Species and PEG but more strongly by Pb (Table 1). There also were significant Species*PEG and Species*Pb interactions (Table 1). The

significant Species*PEG interaction resulted from CAT activity showing no significant changes for the PEG treatments in *M. incana*, but in *M. flavida* CAT activity increased in the -0.75 MPa PEG treatment (Fig. 6 B). The significant Species*Pb interaction stemmed from PEG treatments with Pb consistently decreasing CAT activity compared to the same treatments without PEG (except in the control treatment) for *M. incana* (Fig. 6 B). In contrast, for *M. flavida* PEG treatments with Pb added to the nutrient solution enhanced CAT activity except in the control treatment (Fig. 6 A).

The other examined antioxidant enzyme, APX, was highly affected by the Species factor but not by other factors or factor interactions (Table 1). APX activity was generally much greater in *M. flavida* than in *M. incana* regardless of PEG or Pb treatments (Fig. 6 C, D).

Discussion

Drought stress in arid and semiarid habitats can severely reduce plant growth, change plant chemical composition, and reduce seed quantity and plant survival (Osakabe et al., 2014; Zhang et al., 2018). A common adverse effect of drought stress on plants is reduction of fresh and dry biomass due to decreased numbers and sizes of leaves, which results from reduced plant water potential, increased oxidative stress, and limited photosynthesis (Sharma et al., 2020; Wang et al., 2018). These growth responses help reduce water loss from plants under drought stress (Anjum et al., 2011; Farooq et al., 2009; Hajjhashemi and Sofo, 2018). Indeed, any factor that stimulates drought resistance in plants will increase fitness under conditions of low water availability. Our results showed that growth indices (including RDW, SDW, and RWC) decreased gradually with increased PEG concentration in both species. The highly significant Species*Pb interaction for SDW stemmed from the high level of Pb tolerance by *M. flavida*. In the presence of Pb, growth indices for the metallicolous *M. flavida* generally were unchanged by PEG treatments compared to the same PEG treatments without Pb, except that RWC was increased for *M. flavida* in the - 0.25 MPa PEG with Pb treatment (Fig. 1). In contrast, Pb consistently decreased growth indices in the non-metallicolous species (*M. incana*) compared to the same PEG treatments without Pb, except for RWC (Fig. 1).

Mohtadi et al. (2012b) reported *M. flavida* as a Pb hyperaccumulator that grows on metalliferous soils and can contain $> 1 \text{ mg g}^{-1}$ (0.1% dry weight) Pb in the shoot without suffering phytotoxic effects (Rascio and Navari-Izzo, 2011). In our results (Fig. 2), Pb concentration was consistently greater in *M. flavida* than in *M. incana*. In both species Pb concentration in the root was several-fold higher than in the shoot, showing that most Pb was absorbed by roots but excluded from transport to shoots. Restricting metal to roots has been reported as a mechanism to avoid Pb toxicity in several plant species (Ginn et al., 2008; Krzesłowska et al., 2010; Mahdavian et al., 2016; Zheng et al., 2012). On the other hand, root Pb concentration was significantly increased by PEG, and the Pb translocation factor (Fig. 2C, D) was reduced in the - 0.75 MPa PEG treatment in the metallicolous *M. flavida*, but not in *M. incana*. In *M. incana* there were adverse additive effects of Pb and PEG treatments on growth parameters (Fig. 1). Phytotoxicity of Pb can be due to its interference with plant water balance and nutrient uptake (Ekmekçi et al., 2009; Hakeem et al., 2019) and can result in reduced cell division and elongation (Dalla Vecchia et

al., 2005; Mondal et al., 2015). De Silva et al. (2012) observed similar responses to heavy metal toxicity and drought stress, which they measured by xylem conductivity properties. They concluded that responses of plants to heavy metal toxicity were identical to responses to drought, as heavy metal toxicity reduces water uptake and transport capacity in plants.

Why *M. flavida* fell so short of hyperaccumulation levels in hydroponic cultivation needs additional study. It will be important to investigate if the plants will eventually hyperaccumulate Pb if grown for longer than the 14-day period we used in the current study. Our results indicate that *M. flavida* preferentially accumulates Pb in the root and acts as an excluder, despite being able to translocate and detoxify Pb in the shoot. It is unclear if, given more time than in our 14-day experiment, it can reach hyperaccumulation levels for Pb as previously shown by Mohtadi et al. (2012a), despite the low TF we documented in this study. For most metals, hyperaccumulators have $TF > 1$. Sequestration of toxic metals in hyperaccumulator leaves has been suggested as a mechanism for protecting sensitive roots from metal toxicity, and $TF > 1$ has been proposed as a defining feature of hyperaccumulation (Kazakou et al., 2008). Lead is extremely toxic compared to most metals, especially for shoots, and all plants restrict translocation of Pb (Gupta et al., 2013; Huang et al., 2012). Our study shows that even the metallophyte and documented hyperaccumulator *M. flavida* (Mohtadi et al. 2012a) can have a low TF (~ 0.15) and the ability to restrict translocation even further under Pb stress.

PEG and Pb had adverse additive effects on photosynthetic pigments in both species (Fig. 3). Drought stress can enhance degradation of photosynthetic pigments because of oxidative stress (Basal et al., 2020; Hajihashemi and Sofo, 2018). Lead prevents the function of chlorophyll synthase by displacing beneficial divalent ions (Mg^{2+} , Fe^{2+}), thereby inducing both ion deficiency and elevated ROS production (Kumar and Prasad, 2018; Li et al., 2016). Lead significantly increased the chlorophyll a/b ratio in the control (0 MPa) PEG treatment for both species, suggesting that chlorophyll b appears to be more sensitive to Pb than chlorophyll a (Stiborova et al., 1986; Vodnik et al., 1999).

Accumulation of solutes such as soluble sugars, glycine betaine, proline, etc. under water stress not only helps maintain turgor pressure but also protects the quaternary structure of macromolecules from the adverse effects of ROS (Cechin et al., 2006; Miri and Armin, 2013). Measured solute concentrations were increased by PEG but were more strongly enhanced by Pb in both species (Fig. 4), except for reducing sugars, which only increased in response to combinations of Pb and PEG in the metallophilous *M. flavida*. Accumulation of solutes due to Pb stress contributes to an osmotic balance that allows a plant to minimize sufficient storage reserves to support basal metabolism under Pb-stressed conditions (Hakeem et al., 2019).

Phenolic compounds (including anthocyanins) have carboxyl and hydroxyl groups that bind heavy metals and are responsible for ROS scavenging in stressed plants (Kisa et al., 2016; Michalak, 2006). Phenolic compounds were increased by PEG treatments in both species (Fig. 5). Lead also increased phenolics in the metallophilous *M. flavida* but only in the -0.75 MPa PEG treatment, which could be due to

the greater Pb tolerance of *M. flavida* in comparison to the non-metallicolous *M. incana* (Houda et al., 2016).

CAT and APX are scavengers of H_2O_2 , which is produced in peroxisomes, chloroplasts, and the cytosol (Sekmen et al., 2014). Activities of CAT and APX (Fig. 6) increased with increasing PEG exposure in the absence and presence of Pb in *M. flavida*, but *M. incana* showed differing responses to Pb, with lower CAT activity in treatments of PEG without Pb compared to those treatments with Pb. A similar result was reported for the Cd/Zn accumulator *Sedum alfredii*, which had increased CAT and APX activity in roots exposed to Pb (Huang et al., 2012). ROS have a central role in response to stresses, both in signaling and through direct deleterious effects, and there are intimate tolerance responses in reaction to metals and drought. In a recent study involving overexpression of Glutathione s-transferase, Srivastava et al. (2019) observed a dual role for that gene in detoxification of heavy metals and in producing glutathione peroxidase (GPX)-like activity, both of which are essential for heavy metal and drought stress tolerance. Indeed, we suggest that other antioxidant enzymes also act in coordinated response to different stresses.

Finally, the regulatory networks that act in response to simultaneous stresses could have additive effects. For example, metal contamination in combination with drought may aggravate water stress or alleviate drought symptoms by induction of hydraulic safety (de Silva et al., 2012).

Conclusions

Growth responses showed that the metallicolous species *M. flavida* displayed greater Pb tolerance than the non-metallicolous species *M. incana* and consistently showed greater Pb accumulation in both roots and shoots. A possible reason for the superior Pb tolerance of *M. flavida* could be a reduced translocation factor (TF) under drought stress, which would decrease the Pb concentration in aerial parts of *M. flavida*. On the other hand, Pb could partly inhibit the negative effect of drought stress in the metallicolous *M. flavida* through accumulation of greater amounts of reducing sugars, phenolic compounds, and induction of CAT activity. Joint response elements, such as common and specific transcription factors, or response sequences, need to be determined in both species to identify interactive pathways that address different environmental abiotic stresses.

Declarations

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Figures

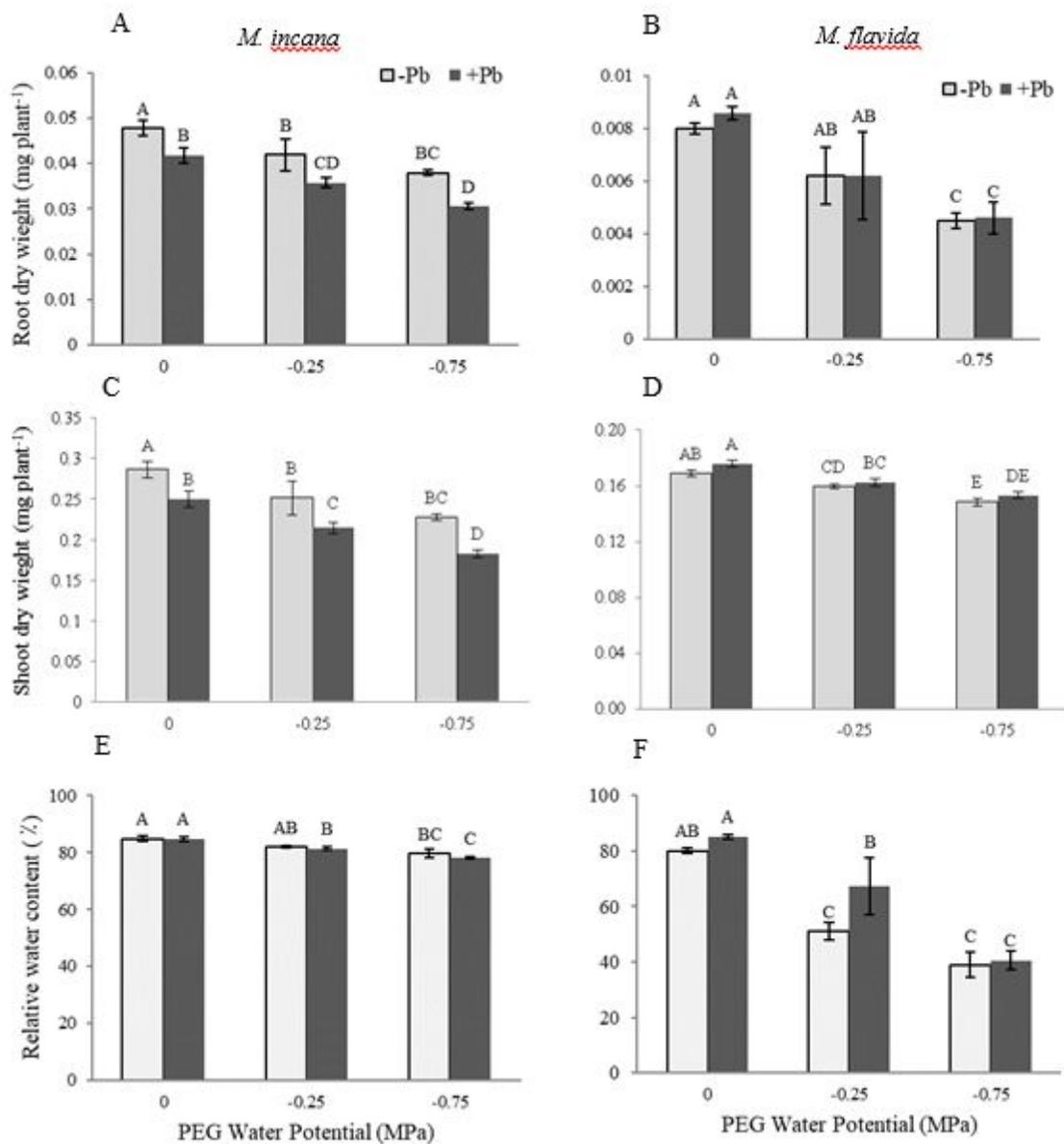


Figure 1

Effect of drought stress induced by PEG on root dry weight, shoot dry weight, and relative water content (%) in *Matthiola incana* (A, C, E) and *M. flavida* (B, D, F), exposed to no-Pb added (gray bars) or Pb added as Pb(NO₃)₂ (black bars) treatments for 14 days. Significant differences between means (P < 0.05) are indicated by different letters.

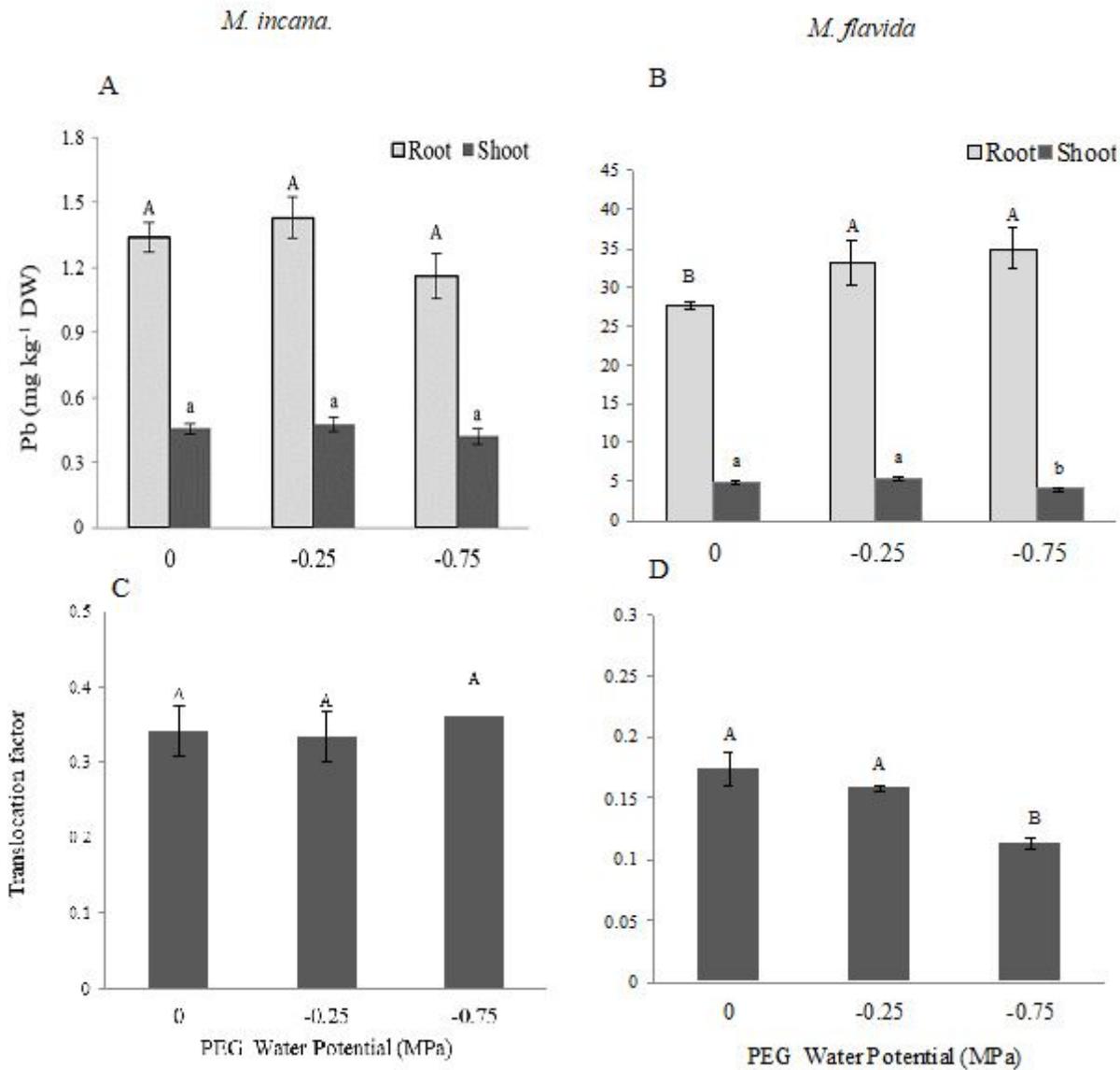


Figure 2

Effect of drought stress induced by PEG on Pb concentration in roots (gray bars in A, B) and shoots (black bars in A, B) and Pb translocation factors (C, D) in *Matthiola incana* and *M. flavida* exposed to the Pb-added treatment for 14 days. Significant differences between means ($P < 0.05$) are indicated by different letters.

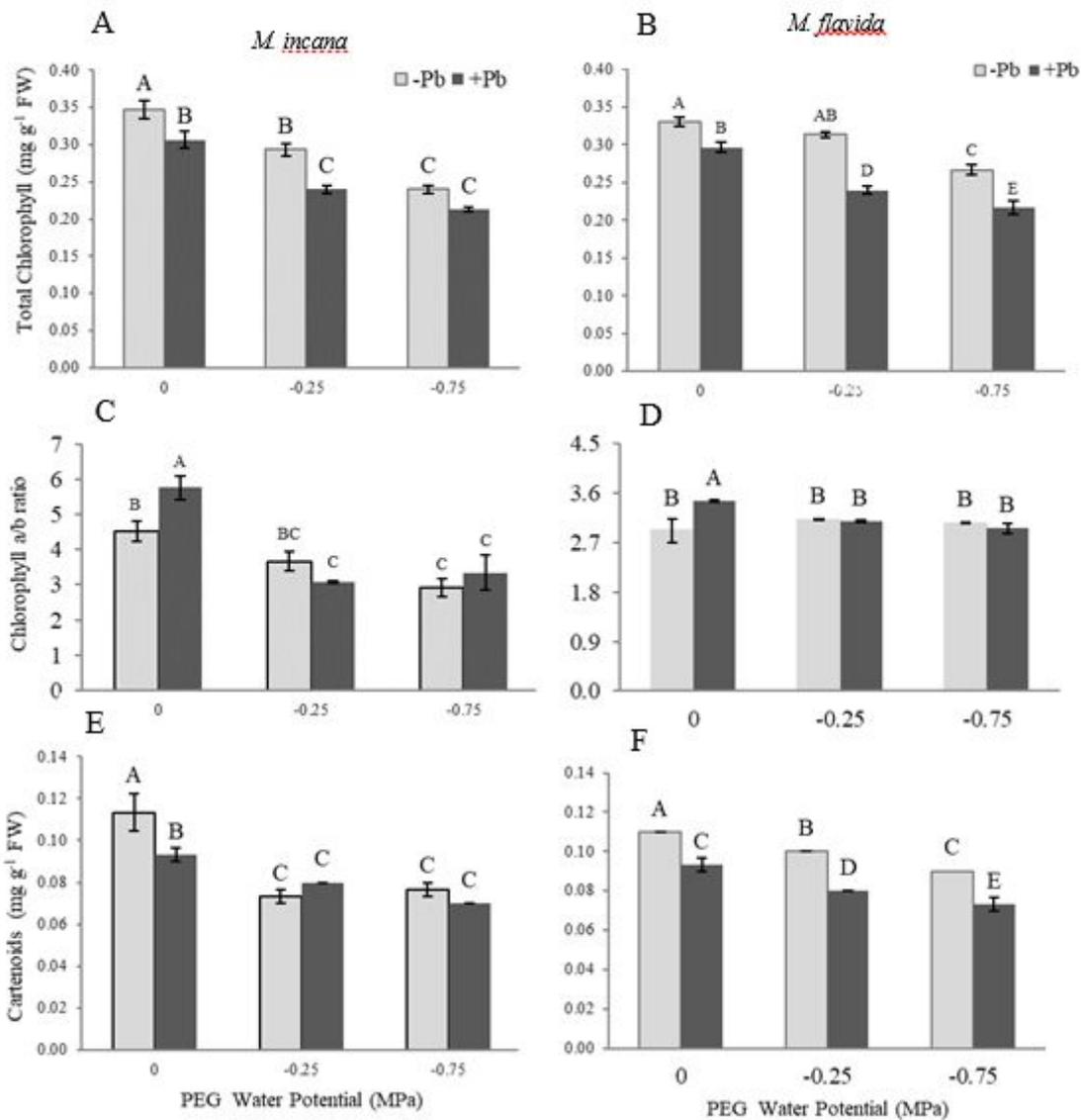


Figure 3

Effect of drought stress induced by PEG on concentration of total chlorophyll (A, B), chlorophyll a/b ratio (C, D), and concentration of carotenoids (E, F) in *Matthiola incana* and *M. flavida* exposed to no Pb-added (gray bars) or Pb-added as Pb(NO₃)₂ (black bars) treatments for 14 days. Significant differences between means (P < 0.05) are indicated by different letters.

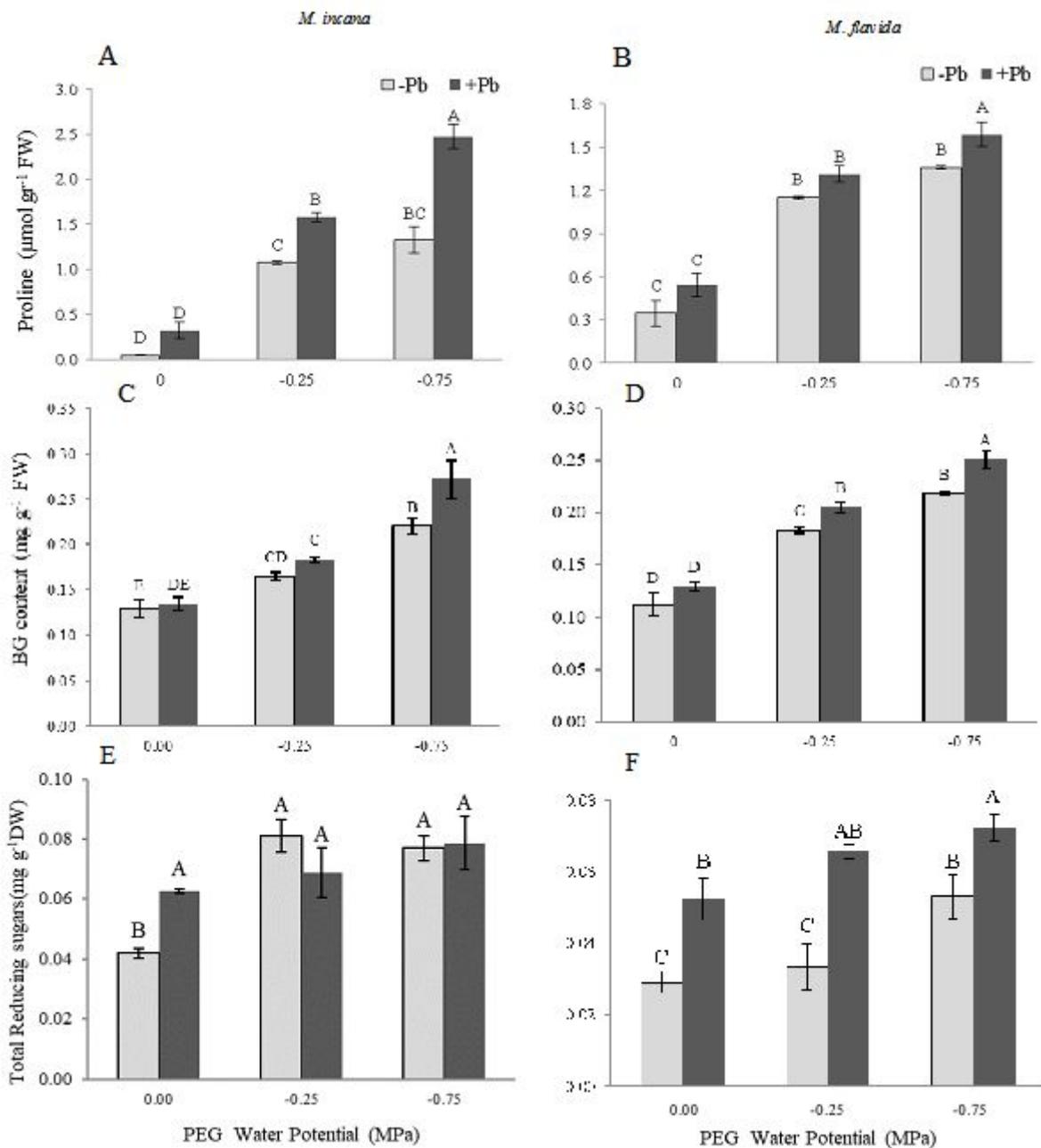


Figure 4

Effect of drought stress induced by PEG on proline, glycine betaine (GB), and Total Reducing Sugars (TRS) concentrations in *Matthiola incana* (A, C, E) and *M. flavida* (B, D, F) exposed to no Pb-added (gray bars) or Pb-added as $\text{Pb}(\text{NO}_3)_2$ (black bars) treatments for 14 days. Significant differences between means ($P < 0.05$) are indicated by different letters.

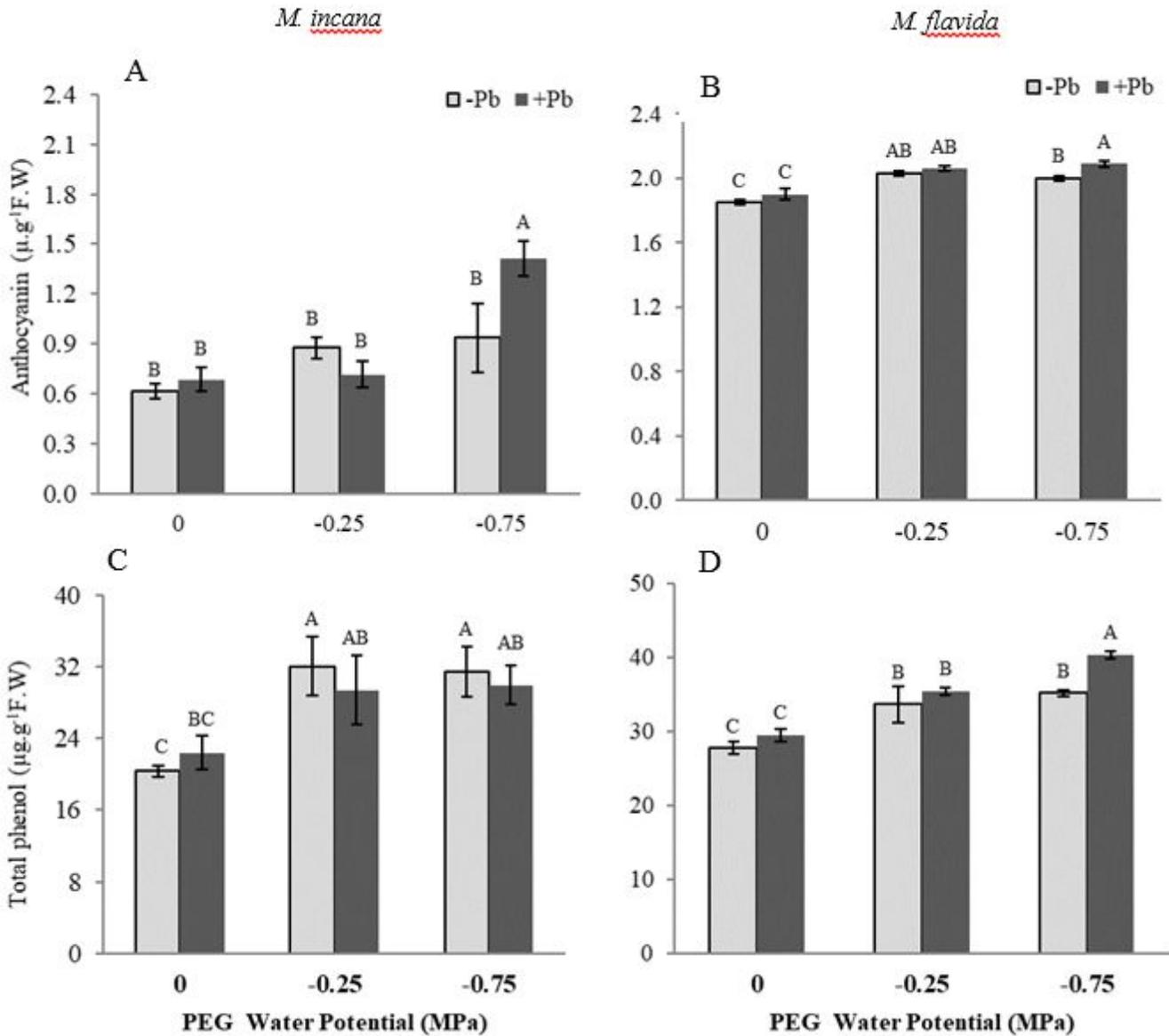


Figure 5

Effect of drought stress induced by PEG on anthocyanins and total phenolic contents in *Matthiola incana* (A, C) and *M. flavida* (B, D) exposed to no Pb-added (gray bars) or Pb-added as $\text{Pb}(\text{NO}_3)_2$ (black bars) treatments for 14 days. Significant differences between means ($P < 0.05$) are indicated by different letters.

M. incana

M. flavida

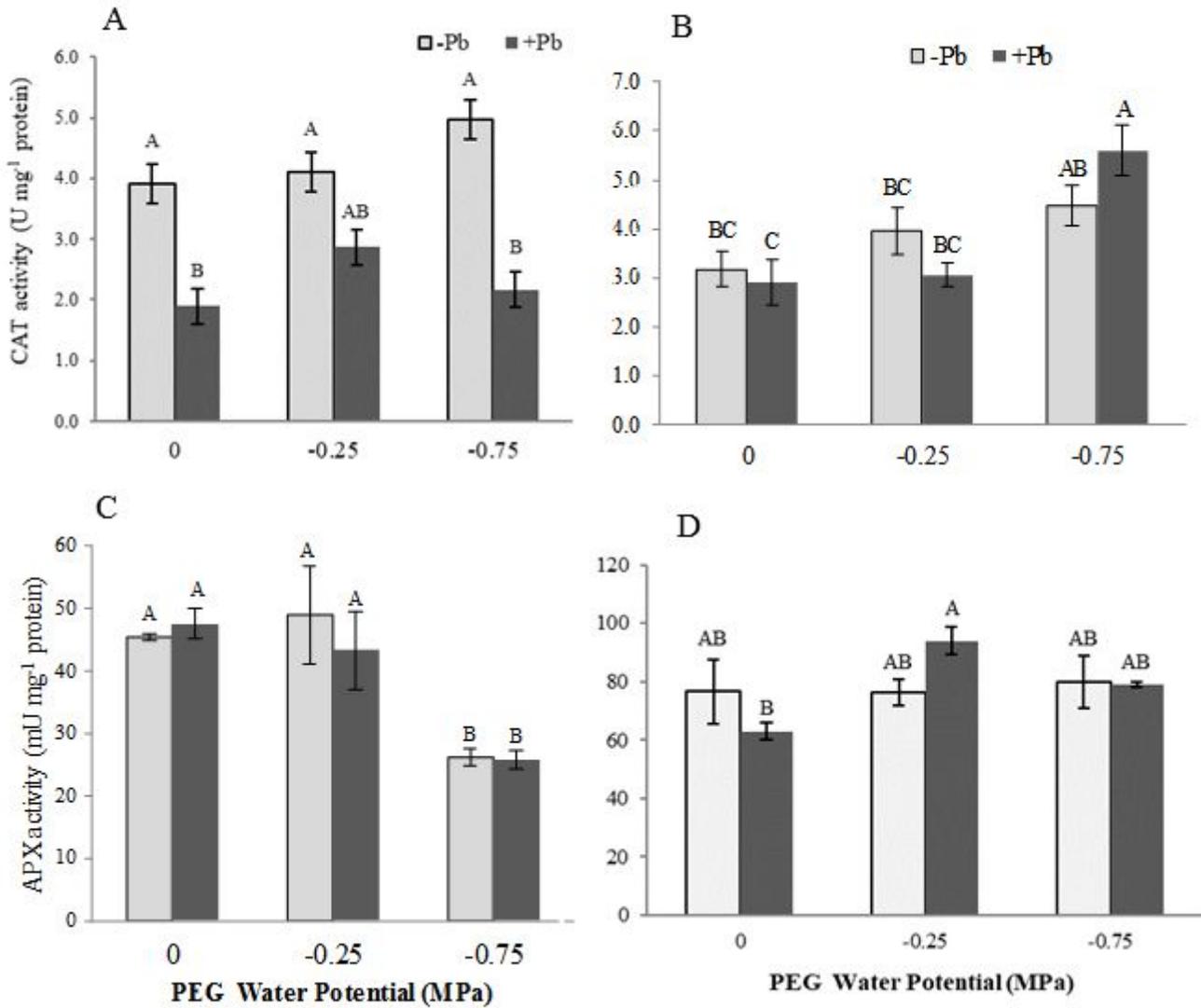


Figure 6

Effect of drought stress induced by PEG on activities of catalase (CAT) and ascorbate peroxidase (APX) in *Matthiola incana* and *M. flavida* exposed to no Pb-added (gray bars) or Pb-added as $Pb(NO_3)_2$ (black bars) treatments for 14 days. Significant differences between means ($P < 0.05$) are indicated by different letters.