DECIPHERING THE PHYTOTOXICITY OF FIVE METAL/METALLOID ELEMENTS TO *LOLIUM PERENNE* L.

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Abstract. Heavy metals and metalloids (metal/loids) contamination are widespread in the biosphere of Earth. Rehabilitation of contaminated environment thus becomes a hot spot in environmental science. In the present study, the ryegrass *Lolium perenne* L. was applied to test its tolerance to five phytotoxic metal/loids, cadmium (Cd), lead (Pb), zinc (Zn), stibium (Sb), and arsenic (As) in 5% Hoagland's solutions. In our study, biomass of ryegrass, metal/loids accumulation amount, effects on chloroplasts, effects on cellular damage, and oxidative resistance were investigated successively. Cd10 and Zn10 treatments trigger the oxidation resistance associated with maintaining general homeostasis of macronutrients and chlorophyll content. Pb exposure causes leaf chlorosis and the inability of regulating antioxidants. Pb, Sb, and As exposure showed the higher translocation and membrane damage in shoots. Neighbor-joining clustering reveals that Pb10, Sb10, and As10 treatments could cluster as one class. For Pb, Sb, and As are transition elements, they may share the same P-style transporters and cause analogous biochemical responses. Our results make up the research gap related to uptake and phytotoxicity of metals and metalloids in plants.
Keywords: *metal/loids, tolerance, phytotoxicity, RuBPCase, macronutrients*

Introduction

Heavy metal/loids (HMs) pollution is an environmental problem of global concern, threatening the health of the ecosystem and even human beings. The levels of HMs such as Cd, Pb, Zn, Sb, and As in nature are usually very low. Intense anthropogenic activities including ore mining, industrial production, fertilization, and pesticide application have accelerated the release of such mutually connected pollutants into the environment (Norini et al., 2019). Cd content in the Pb/Zn tailing is about 46.3 mg kg^{-1} (Hale et al., 2012). The contents of As and Cd in fertilizers and pesticides are 65.89~70.00 mg kg^{-1} and 1.34~1.56 mg kg^{-1}, respectively, and both are the main source of Cd and As pollution for paddy soils (Lin et al., 2021). Sb and Cd are usually coexistent in the wastewater from the textile printing and dyeing industry, causing an accumulation of 1.0~118 mg kg^{-1} and 0.5~3.0 mg kg^{-1} in the Taipu river basin in East China, respectively (Xu et al., 2021). These pollutants can be transported to air, streams, and soils, resulting in trophic transfer and accumulation in the food web (Meeinkuirt et al., 2013).

The phytotoxicity of HMs has received considerable attention. Among these HMs mentioned above, Cd is the most toxic trace element without any known physiological function for plants. Plants are sensitive to Cd even at relatively low doses due to its high bioavailability. The divalent metal transporter probably helps the transmembrane transport of Cd, which probably disturbs the intracellular macronutrients homeostasis. For non-tolerant plants, Cd interferes with water balance, mineral homeostasis, and plant

growth (Rizwan et al., 2019; You et al., 2021). However, tolerant plants (*Elsholtzia argyi*, *Sedum alfredii*, and *Robinia pseudoacacia*) are not affected by a certain dosage of Cd, i.e. maintenance of chlorophyll content and photosynthesis-related enzyme activity (RuBPCase), as well as high accumulation of Cd in tissues (Li et al., 2014; Dezhban et al., 2015; Lin et al., 2020). Considering the fact of Cd pollutions in different contexts, i.e. the case of Cd accompanied by divalent cations, or the case of Cd coupled with metalloid Sb and As (Norini et al., 2019), it is crucial to distinguish the plant tolerance and phytotoxicity when exposed to the different coexistent element.

As one of the main chill season pasture species grown worldwide, a Cd tolerant plant, *L. perenne* is a good candidate for phytotoxicity study replying to heavy metal stress, because this plant species has developed roots, grows rapidly, tolerates many metals, and has other tolerant characteristics (Bai et al., 2014). As demonstrated, *L. perenne* has great potential to be used for phytoremediation of Pb (Fan et al., 2020), Zn (Lambrechts et al., 2011), Cd (Li et al., 2020b), and even multi-metal compounds (Lopareva-Pohu et al., 2011; Bai et al., 2014).

By reviewing the literature, we selected the metal/loids concentration that have toxic and physiological effects on plants. For example, studies revealed that a typical effect of Cd ion on lettuce yield at 5 to 10 mg L^{-1} concentration (Abu-Shahba et al., 2022); Park et al. (2016) studied the absorption of arsenic in *arabidopsis thaliana* at concentrations ranging from 5 to 10 mg L^{-1}; Sooksawat et al. (2013) studied the remediation potential of charophytes for heavy metals Pb and Zn at concentrations of 5 mg L^{-1} and 10 mg L^{-1}; Metallic antimony at a concentration of 20 mM was used to study the effect of antimony uptake by rice seedlings (Long et al., 2020). It could be concluded that the commonly used metal/metalloid dosage of 10 mg L^{-1} in hydroponic experiment is effective and suitable. Adopting a uniform concentration will be more conducive to contrast the toxicity from different metal/metalloids.

The expression of heavy metal tolerance predominantly reflects on physiological and biochemical processes. Leaf chlorosis is the first obvious symptom of HMs stress that is disturbing photosynthesis, showing chlorophyll deficiency and enzyme inactivation. Similar to other plant species, *L. perenne* shows oxidative stress as indicated by the generation of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and electrolyte leakage (EL), representing membrane permeability change and oxidative damage of cells (Jia et al., 2020). Plants also possess various strategies to alleviate heavy metals stress, including ROS-scavenging by regulating antioxidant enzyme activities (Siddiqui et al., 2020). The antioxidative components such as catalase (CAT) and Peroxidase (POD) are involved in converting reactive H_2O_2 and O_2 to benign H_2O. In addition, superoxide dismutase (SOD) quenches $O_2^{\cdot-}$ and converts it to H_2O_2 and O_2. These are the main mechanisms for plants adapting to abiotic stresses from heavy metal/loids.

In this study, hydroponic experiments were conducted to test heavy metal tolerance, phytotoxicity, and biochemical interactions of *L. perenne* in response to mono Cd, Pb, Zn, Sb, and As stresses. The objective was to determine the ability of ryegrass to extract five metal/loids from hydroponic solutions and to assess and cluster plant tolerance to metals and metalloids.

Materials and Methods

Experiments setup

Seeds of *L. perenne* were surface sterilized in NaClO solution (0.1%) for 15 min and then were washed with distilled water. The seeds were incubated in sterile water for two days to promote germination. An equal number of seeds (20 seeds) were sown in Petri dishes (D = 9 cm) and kept in the dark for three days at 28 ± 2 °C. Seedlings of uniform length (10 cm) were transferred to 100 ml glass cups (20 plants per cup) containing about 5% Hoagland medium of 170 μM KNO_3, 288 μM $Ca(NO_3)_2$, 60 μM $NH_4H_2PO_4$, 200 μM $MgSO_4$, 24 μM NaFe- EDTA, 0.3 μM H_3BO_3, 0.06 μM $HMoO_4$, 6 μM $MnCl_2$, 0.16 μM $CuSO_4$, and 0.38 μM $ZnSO_4$ in deionized water and pH 7.0 ± 0.1 (Yu et al., 2020) and then grown in a growth chamber at a 16/8 photoperiod at 18 ± 2 °C and 70% relative humidity. The nutrient media was replaced every three days.

$CdCl_2$, $Pb(NO_3)_2$, $Zn(NO_3)_2$, $NaSbO_2$ and $NaAsO_2$ were dissolved with diluted acid, then adjusted pH with HCl and NaOH and gradually diluted by 5% Hoagland solution for later use. After 10 days of culture, seedlings were treated with nutrient medium (CK), Cd10, Pb10, Zn10, Sb10, As10 (10mg L^{-1}). Twenty-one days later, after the plants were harvested and seedlings were appropriately washed and then separated into shoots and roots. One part of the harvested seedlings was oven-dried at 90 °C to constant weight for elemental analysis and the other part was frozen in liquid N_2 and kept at -80 °C for biochemical analysis. The hydroponic cups were arranged in a simple randomized design with 5 replicates.

Assessment of heavy metals tolerance

The R/S ratio representing the root growth compared to the shoot growth by *Eq(1)* (Siddiqui et al., 2020). The tolerance index (TI) is dry weight of the treated plants compared to the dry weight of the control plants *Eq(2)* (Bai et al., 2014). Oven-dried plant samples (1 g) were digested with $HClO_4$-HNO_3 (3:7). Heavy metal contents and macronutrients were determined with ICP (Leeman, Prodigy, the USA). Metal contents were expressed as mg kg^{-1} DW. the translocation factor (TF) representing metal concentration in shoots compared to metal concentration in roots *Eq(3)* (Yu et al., 2020).

$$\text{R/S ratio = dry weight of roots/dry weight of shoots} \qquad \text{(Eq.1)}$$

$$\text{TI=dry weight of the treated plants/dry weight} \qquad \text{(Eq.2)}$$

$$\text{TF=metal concentration in shoots /metal concentration in roots} \qquad \text{(Eq.3)}$$

Determination of photosynthetic pigment and RuBPCase activity

Photosynthetic pigments were extracted with 80% acetone and absorbance at 663, 645, and 470 nm were determined by using the UV–vis spectrophotometer, and the contents of Chl *a*, *b* and carotenoid were calculated according to literature (Vernay et al., 2007). RuBPCase activity was determined using Plant RuBPCase ELISA kit (Faye, Jiangsu Science and Technology Limited Company) according to manual.

Determination of electrolyte leakage, H₂O₂ and MDA

Electrolyte leakage (EL) was determined following the method of Rizwan et al. (2019). Briefly, shoots were cut into pieces and immersed in 20 mL deionized water while shaking for 30 min. EL was measured with a conductivity meter, *Eq(4)*. H_2O_2 was extracted from plant tissues as described by Siddiqui et al. (2020), and H_2O_2 content was read at 390 nm and expressed as nmol g^{-1} FW. The amount of malondialdehyde (MDA) was determined following the method of (Lucas et al., 2019) and expressed as nmol g^{-1} FW.

$$EL\,(\%) = (EL_1 - EL_0)/(EL_2 - EL_0) \times 100 \tag{Eq.4}$$

where,
EL_1 was initial conductivity before boiling. EL_2 was the final conductivity after boiling. EL_0 was the conductivity of deionized water.

Determination of antioxidant enzyme activity

We quantified CAT activity using spectrophotometer at 240 nm (Hu et al., 2020). The reaction mixture comprised of 40 μL enzyme extract and 9.96 ml of H_2O_2 in phosphate buffer (pH 7.0). Changes in absorbance were followed for 60 s. POD activity and SOD activity were measured according to Jia et al. (2020). POD activity was measured using guaiacol (0.05 M; 1 mL) as a substrate. The reaction mixture contains 100 μL crude enzyme extract, 1 mL of 9.8 M H_2O_2, 2.9 mL of 0.05 M PBS and 3 mL ultrapure water. The reaction mixture was kept at 37 °C for 5 min in a water bath (without shaking), and then analyzed by spectrophotometry at 470 nm. SOD activity was determined using the method of nitroblue tetrazolium (NBT) photoreduction. Briefly, crude enzyme solution samples (0.1 mL) were mixed with PBS buffer (0.05 M; 1.5 mL), methionine (130 mM; 0.3 mL), NBT (750 μM; 0.3 mL), riboflavin (20 μM; 0.3 mL), and EDTA-Na2 (100 μM; 0.3 mL). The mixtures were centrifuged (RCF=10,451×g) for 15 min. Supernatants were analyzed by spectrophotometry at 560 nm wavelength.

Statistical analysis

Data were analyzed using the SPSS 19.0 software package. Significant differences between treatments were tested using Duncan's multiple comparison method ($p < 0.05$). The relationships between plant parameters and different treatments were analyzed by principal component analysis (PCA) using origin 2022. All results in the present study were expressed as mean values ± standard deviations (n = 3).

Results

Heavy metal tolerance and uptake

As seen in *Fig. 1a*, the root biomass of *L. perenne* did not differ significantly between treatments (P > 0.05), while Cd applied slightly increased shoot biomass of *L. perenne*, but the difference was not significant, the calculated TI from *Eq(2)* was 1.12. In contrast, other treatments significantly decreased shoot biomass. Compared with the control, the Arsenic exposure (As10) significantly increased R/S ratio (P < 0.05) and reached the maximum amount of 0.57. The other elements did not have a marked effect on R/S ratio (P > 0.05).
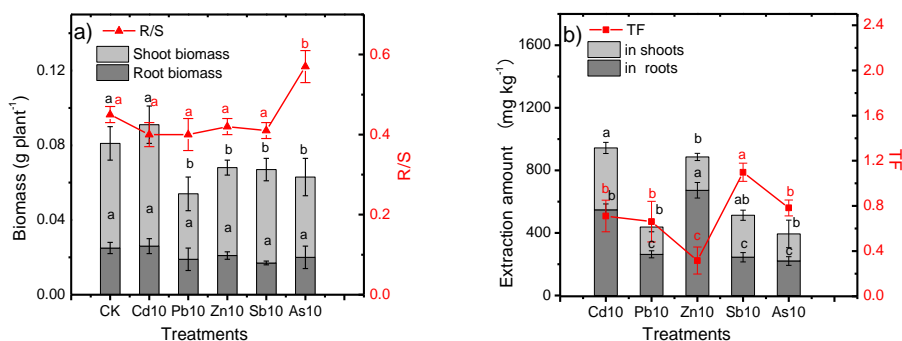
Figure 1. Plant growth, R/S ratio (a), heavy metal contents and TF (b) of Lolium perenne in response to different metal stress. Different lowercase letters within treatments indicate a significant difference at P < 0.05 according to Duncan's test

The accumulation of metal/loids by *L. perenne* were different. *Fig. 1b* indicated that the mean content of heavy metal in plants were ranked in the order of $Cd_{plant} > Zn_{plant} > Sb_{plant} > Pb_{plant} > As_{plant}$. In particular, Cd content in ryegrass shoots reached the highest amount of 395.33 mg kg^{-1}, whereas in roots Zn content reached the highest amount of 672.81 mg kg^{-1}. The nonessential element Pb induced a low accumulation of 263.48 mg kg^{-1} in roots and 174.14 mg kg^{-1} in shoots, which almost draw near to that of metalloids (Sb and As) exposure. TF from *Eq(3)* used to represent the heavy metals translocation abilities to shoots in plants was shown in *Fig. 1b*. The highest TF of 1.10 at Sb10 treatments was observed, followed by another metalloid As treatments which reached the TF of 0.78, but Sb10 and As10 showed no significant difference in TF (P > 0.05).

Effects on chloroplasts

As shown in *Fig. 2a*, application of Zn at 10 mg L^{-1} increased chlorophyll b content compared with the CK, but there was no significant difference. Meanwhile, Cd10, Pb10, Sb10, and As10 generally induced the chlorophyll *a*, chlorophyll *b*, or carotenoid decreased significantly (P<0.05). Especially referred to Pb10, Sb10, and As10 exposure, the Chl *a*/Chl *b* ratio decreased remarkly. As for carotenoid content, Pb10 exposure induced a sharp decrease to the lowest value of 0.69 mg g^{-1} FW, establishing that the Pb seriously damaged photosynthesis pigments in *L. perenne*.
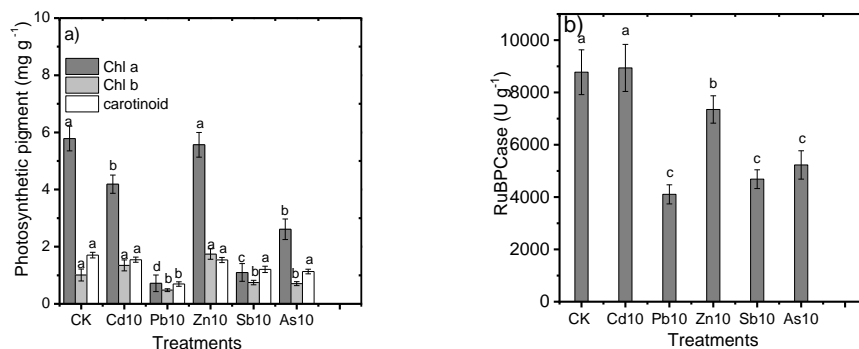

Figure 2. Chlorophyll a, chlorophyll b, and carotenoid contents (a) and RuBPCase activity (b) in shoots of L. perenne after 21days of treatment periods. According to Duncan's test, different lowercase letters within treatments indicate a significant difference in values (P < 0.05)

RuBPCase was the richest enzyme in the chloroplast. *Fig. 2b* indicated that RuBPCase activity was significantly decreased under all treatments except with Cd10 treatments. The Pb10, Sb10, and As10 exposure resulted in similar trends to those of the total chlorophyll and induced the RuBPCase activity decreasing by 53.22%, 46.11%, and 40.40%, respectively. Meanwhile, Zn10 exposure resulted in a relatively less decrease of 16.24% compared with the CK.

Cellular damage

As for EL in shoots, application of Sb10 and As10 to *L. perenne* triggered a significant increase by 59.83% and 184.78% compared with the CK, while Zn10 treatments induced a decrease in EL from *Eq(4)* by 25.16% (P<0.05). It can be observed that Cd10 and Pb10 stress showed no significant effects on EL (P > 0.05).

Similar trends were observed in H_2O_2 content in *L. perenne* shoots. H_2O_2, is a potential source of highly reactive hydroxyl radical promoted by oxidative damage, which is toxic due to its high permeability across the membrane. As shown in *Fig. 3b*, metalloids of As and Sb triggered a significant increase in H_2O_2 content by 102.30% and 98.47%, but Zn exposure induced a significant decrease in H_2O_2 (P<0.05), whereas Cd10 and Pb10 showed no significant effects to H_2O_2 (P > 0.05).
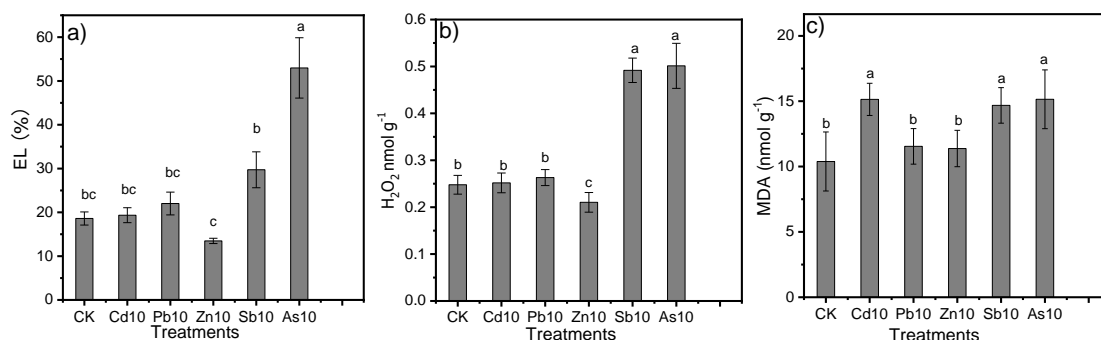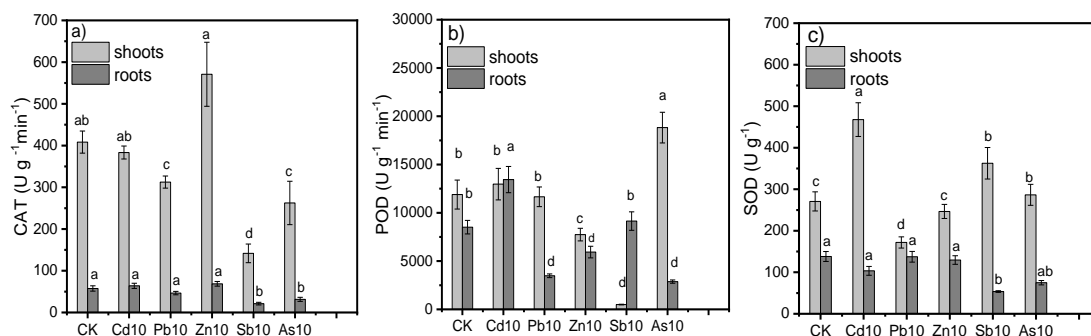

Figure 3. Electrolyte leakage (a), H_2O_2 content (b), and MAD content (c) in shoots of Lolium perenne. Different lowercase letters within treatments indicate a significant difference in values, according to Duncan's test (P < 0.05)

Lipid peroxidation is generally considered as a biochemical marker for the ROS mediated injury stress indicator. Malondialdehyde content is widly used to represent lipid peroxidation levels. *Fig. 3c* showed that the Cd10, Sb10, and As10 stress increased MDA contents significantly by 38.89%, 29.62%, and 37.04% in *L. perenne* shoots compared with the CK (p < 0.05). However, obvious variations in the MDA content at Pb10 and Zn10 exposure were not observed (P > 0.05).

Oxidation resistance

Fig. 4a showed that Cd10 treatments had no effects on *L. perenne* in both shoots and roots. But Zn10 exposure triggered CAT increase in shoots by 37.5%, whereas the significant decrease of CAT activity in shoots triggered by Pb, Sb, and As stress was observed, which certified their severe damage to antioxidant enzymes in *L. perenne*.

Figure 4. *Catalase (CAT) activity (a), POD activity (b), and SOD activity (c) in L. perenne shoots and roots. Different lowercase letters within treatments indicate a significant difference in values, according to Duncan's test (P < 0.05)*

POD was another antioxidant enzyme which averagely distributed in roots and shoots. *Fig. 4b* showed that Cd10 significantly increased POD activity in roots, while Zn10 significantly decreased POD activity in shoots and roots, and Pb10 significantly decreased POD activity in roots. As10 treatments increased this enzyme activity by 53.06% in shoots but decreased it by 68.24% in roots. Conversely, Sb10 treatments decreased it in shoots by 95.63% but increased it in roots by 8.23%.

SOD is distributed more in shoots than in roots. Interestingly, Cd, Pb, and Zn exposure have no effects on SOD activity in roots, while Cd10 significantly increased SOD activity in shoots, Zn10 and Pb10 significantly decreased SOD activity in shoots ($P < 0.05$). In contrast, *Fig. 4c* showed that metalloids Sb and As exposure increased SOD activity in shoots but Sb exposure decreased SOD activity in roots significantly ($P < 0.05$).

Effects on macronutrients homeostasis

The effect of HMs exposure on macronutrients of K, Ca, Na, Mg, Fe, and P assimilation in *L. perenne* were tabulated in *Table 1*. After Cd exposure, the uptake of all the macronutrients of K, Ca, Na, Mg, and Fe significantly increased in shoots by 60%, 81%, 44%, 49%, and 71%, respectively, while in roots, the content of K, Mg, Fe, and P significantly increased by 47%, 38%, 104%, and 35%, respectively, compared with the CK. In Zn exposed shoot, the content of K, Ca, and Fe significantly increased by 11%, 72%, and 99%, respectively, while, the content of Ca, and Na in roots decreased significantly compared with the CK.

After exposure to Pb10, Sb10, and As10 treatments, the shoot P content significantly decreased by 41%, 66%, and 62%, respectively. The decreasing trend of P is consistent with the level of heavy metals accumulation in shoots. In the Pb10 treatments, the content of Na and Mg in shoots significantly decreased by 29% and 40%, while in roots, the content of Ca, Mg, Fe, and P significantly increased by 55%, 106%, 60%, and 107%. As to Sb10 treatments, all the content of macronutrients in shoots were almost significantly ($P<0.05$) decreased, whereas the content of K, Mg, Fe, and P significantly increased by 183%, 48%, 38%, and 66% in roots, respectively. In the As10 treatments, the content of Ca and Fe significantly increased by double 67% in shoots, while, in roots significantly increased by 28% and 52%, and there is no effect on the content of K, Mg, and P in roots.

Table 1. *Effect of heavy metals on macronutrient contents in shoots and roots of L. perenne*

Treatments	K	Ca	Na	Mg	Fe	P
Macronutrients in shoots (mg g $^{-1}$)						
CK	9.77 ± 0.64^c	4.77 ± 0.42^c	4.37 ± 0.47^b	3.86 ± 0.24^b	1.03 ± 0.15^b	6.66 ± 0.54^a
Cd10	15.62 ± 0.76^a	8.63 ± 1.25^a	6.28 ± 0.59^a	5.75 ± 0.32^a	1.76 ± 0.11^a	7.25 ± 0.42^a
Pb10	8.15 ± 0.92^c	4.39 ± 0.25^c	3.10 ± 0.41^c	2.33 ± 0.19^c	0.98 ± 0.14^b	3.92 ± 0.27^b
Zn10	10.83 ± 0.90^b	8.22 ± 0.56^a	4.30 ± 0.44^b	3.52 ± 0.25^b	2.05 ± 0.17^a	4.19 ± 0.31^b
Sb10	5.67 ± 0.86^d	3.42 ± 0.26^d	2.64 ± 0.25^c	1.74 ± 0.14^c	0.82 ± 0.12^b	2.28 ± 0.19^c
As10	7.86 ± 0.85^{cd}	7.95 ± 0.86^{ab}	4.70 ± 0.42^b	3.79 ± 0.22^b	1.72 ± 0.17^a	2.54 ± 0.22^c
Macronutrients in roots (mg g $^{-1}$)						
CK	2.29 ± 0.15^c	4.78 ± 0.39^c	2.41 ± 0.14^a	1.34 ± 0.10^c	1.37 ± 0.18^b	2.98 ± 0.23^c
Cd10	3.36 ± 0.21^b	5.04 ± 0.42^c	2.95 ± 0.18^a	1.85 ± 0.14^b	2.80 ± 0.24^a	4.03 ± 0.51^b
Pb10	3.80 ± 0.21^b	7.40 ± 1.03^a	2.33 ± 0.10^a	2.76 ± 0.21^a	2.17 ± 0.19^a	6.17 ± 0.84^a
Zn10	2.09 ± 0.16^c	3.18 ± 0.31^d	1.80 ± 0.09^b	1.35 ± 0.15^c	1.38 ± 0.18^b	2.73 ± 0.31^c
Sb10	6.49 ± 0.37^a	4.92 ± 0.39^c	2.86 ± 0.29^a	1.99 ± 0.18^b	1.89 ± 0.21^a	4.90 ± 0.59^b
As10	2.23 ± 0.18^c	6.12 ± 0.56^b	1.53 ± 0.17^b	1.44 ± 0.11^c	2.08 ± 0.17^a	2.88 ± 0.32^c

The data represented an average of three replicates (±SD). Different letters define the level of significance of the difference between treatments at *p=0.05*

Principal component analysis (PCA)

Based on the typical physiological effects and macronutrient altering as variate factors, we carried out principal component analysis and cluster analysis for six treatment groups, as shown in *Fig. 5*. The Pb, Sb and As treatments had positive effects on macronutrients in roots, as well as negative effects on macronutrients in shoots. On the contrary, the Cd and Zn treatments had positive effects on macronutrients in shoots, but negative effects on macronutrients in roots. The Cd and Zn exposure had highest effects on RuBPCase and Chl a, while the Pb, Sb and As treatments had the greater H_2O_2 and EL (*Fig. 5a*). Experiments resulted that the exposure of Cd and Zn induced analogous elements flux and antioxidation. In Pb exposure, the severe damage in chlorophyll and cell membrane is similar to the behaviors triggered by metalloid Sb and As exposure. Six groups were divided into three cluster (*Fig. 5b*). Cluster one consists of Cd, Zn, and CK treatments, representing tolerant metal class. Cluster two consists of Pb and Sb, representing non-tolerant metalloid class. Cluster three consists of As, representing tolerant metalloid class.
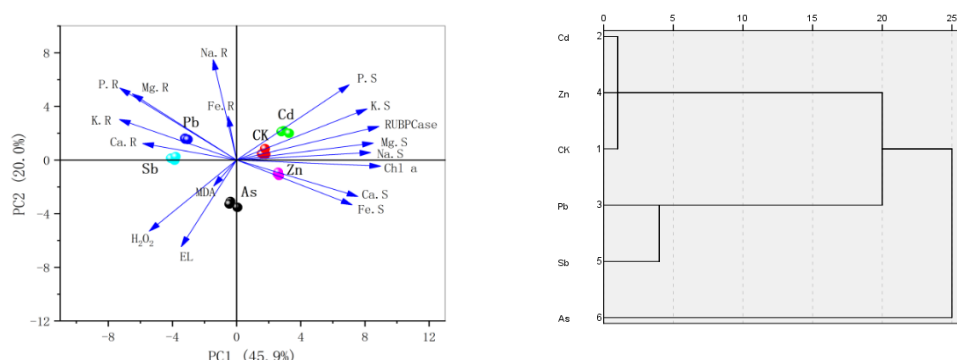
Figure 5. *Principal cpmponent analysis plot (PCA) (a) and neighbor-joining clustering tree plot (b) based on the 17 variate factors from experimental data of Chl a, RuBPcase, EL, MDA, H2O2, macronutrients content in shoots (K.S, Ca.S, Na.S, Mg.S, Fe.S, and P.S) and in roots (K.R, Ca.R, Na.R, Mg.R, Fe.R, and P.R) from different treatment groups (Cd, Pb, Zn, Sb, and As exposure to ryegrass, including CK group)*

Discussion

Cd is highly bioavailable. Generally, plants are sensitive to Cd at concentrations higher than 10~50 μg/plant. Cd exposure has been found to cause oxidative stress in rice (Bari et al., 2021), maize (Rizwan et al., 2019), alfalfa (Fang et al., 2019), and mustard (Chowardhara et al., 2019). Interestingly, *L. perenne* is certified as uniquely adaptable to cadmium stress which was reported for its Cd tolerance and accumulation (Li et al., 2020a). In our experiments, *L. perenne* was promoted in growth parameters by assimilation of more nutrients and increasing POD, SOD, and lipid peroxidation under the $10 \, mg \, L^{-1}$ of Cd treatments. The mechanism is assumed that Cd stimulated *L. perenne* increasing nutrient assimilation in a low ions intensity medium, production of antioxidants, and activation of other biochemical processes.

Zn is essential as a central atom for many enzymes (e. g., Cu/Zn-SOD, and Fe-Zn-regulated transporters), and also serves an important function in the transcription of many genes (Nawrot et al., 2021). High Zn can cause osmoregulatory disturbances and may also cause cytotoxic effects in the presence of hydrogen peroxide (Rizwan et al., 2019). Electrolyte leakage is generally considered as an indirect measure of cell membrane permeability on various plant tissues, which may also cause by injury of membrane components (Kiamarsi et al., 2020). In our study, Zn10 treatments prevented plants from oxidized by decreasing both electrolyte leakage and H_2O_2. Zn10 exposure also triggered CAT increased by 37.5%. CAT is a critical antioxidant enzyme for the detoxification of H_2O_2 to H_2O and O_2 (Daud et al., 2013). It is indicated that Zn10 induced the effective resistance to metal oxidation, which represents low toxicity to *L. perenne*.

On the contrary, another divalent metal Pb exposure significantly decreased the biomass of *L. perenne* and induced chlorosis of shoot. Chlorosis is the first visible symptom of metal phytotoxicity and is closely related to chlorophyll content (Hu et al., 2020). Wheeler and Power (1995) reported that in the presence of heavy metals, less tolerant plant species decrease their chlorophyll content both in absolute terms and relative to carotenoid contents, and/or alter the Chl a/Chl b ratio, which is verified by Pb10 exposure on *L. perenne*. In addition, Pb10 stress generally induced CAT, POD, and SOD to decrease in shoots or roots. Pb transport to other parts of the cell possibly through pleiotropic drug resistance (PDR), mitochondrial inner membrane protein and ATP-binding cassette (ABC) transporters (Lopareva-Pohu, 2011). These transporters, known as P-type pumps, play a key role in trace metals mobilization across the plasma membrane. It is concluded that Pb10 stress damaged chloroplast function and antioxidation system resulting in serious phytotoxicity (Dezhban et al., 2015).

With Sb and As exposure, the TF are higher than those of other cationic metals exposure, describing the higher affinity in *L. perenne* shoots to metalloids. The Sb and As stress increased malondialdehyde contents in shoots compared to the CK, which was evidenced non-enzymatic anti oxide response of lipid peroxidation (Jia et al., 2020). It could be thought that metalloid elements Sb and As accumulation in shoots disturbed membrane structure which is verified by increasing electrolyte leakage and H_2O_2. Sb10 inhibited macronutrients transportation and resulted in shoots malnutrition.

Not like the down-regulated macronutrients in shoots induced by Sb exposed, in As treatments, the content of macronutrients even increased in Ca, Fe content. It is reported that plants tend to increase R/S to get more nutrients to withstand adverse impacts (Siddiqui et al., 2020), which may be the strategy for *L. perenne* to detoxify metalloid As. Babula et al. (2008) reviewed that the similarity of As to essential element P predestinated

its high uptake due to the possibility to replace essential elements was also verified in our work. As a result, *L. perenne* tends higher accumulation in contrast Arsenic to Antimony.

There are reasons to believe that the Pb transporter was involved in metals accumulation and nutrients homeostasis is more like those of metalloid. Transport of metals across plasma membrane is essential for plant development and signal transduction between biochemical processes. Cd, Zn, and Fe share the same transporters, such as ZIP, CDF, and OsHMAs (You et al., 2021). Whereas metalloids of Sb, Pb, and As uptake in the plant are by the aquaporins, P type pumps and phosphate transporters (Cao et al., 2021).

Conclusion

Our work was designed to distinguish the tolerance mechanism of ryegrass to five metal/loids. From the angle of metal/loids uptake, metal/loids translocation, effect on physiology, and effect on macronurients homeostasis, we compared the ryegrass responses to Cd, Pb, Zn, Sb and As exposure. Neighbor-joining clustering tree analysis based on 17 variate factors indicated that ryegrass is not only tolerant to divalent cationic cadmium, but also has adaptive strategies to metalloid As. Meanwhile, the response of ryegrass to Pb exposure is classified into the behavior induced by metalloids. However, the plant tolerance to metal/loids in practicality may be affected by various environmental factors. Therefore, *Lolium perenne* exposed to composite pollution, higher or lower metal/loids concentrations, and even exogenous amendments could be considered in the future study.

Statements. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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