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Antioxidant response by alfalfa (*Medicago sativa* L.) to Pb pollution – A study to value the feasibility of soil phytoremediation

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Abstract: With the surrounding environment of Inner Mongolia lead (Pb) ore as the research background, the germination and physio-biochemical effects of Pb stress on alfalfa were discussed to employ this species for the remediation of Pb contaminated soil. Research has shown that a low Pb stress concentration could improve the biological resistance of alfalfa seeds, while a high Pb stress concentration cannot be tolerated. Interestingly, when the Pb concentration was 5 mg/L, the germination rate of the seed was promoted, and the chlorophyll content was especially increased. As the Pb content and stress increased, the amount of malondialdehyde (MDA), H_2O_2, catalase (CAT) increased; while the root cell viability, chlorophyll and soluble protein content decreased. In consequence, alfalfa was tolerant to Pb stress of 5 mg/L, inversely, its growth was inhibited at levels higher than 5 mg/L, and it was poisoned at 500 mg/L. Based on the principal component analysis (PCA), the H_2O_2, O_2^-, chlorophyll total, chlorophyll a, CAT and proline content explicitly reflected the change in the physiology on the alfalfa and its tolerance under Pb stress.

Keywords: detoxification mechanism; physio-biochemical functions; principal component analysis

China is a lead (Pb) producing country having the second largest Pb reserves in the world. According to the China Mineral Resources Report (Li et al. 2019), by the end of 2018, the total amount of lead metals in China was 92.1631 million tonnes, and there were 2 347 lead-zinc deposits in China, mainly in Yunnan, Inner Mongolia, Gansu and Qinghai. During mineral exploitation, waste water discharge, waste rock, and tailings stacking and leaching cause a large amount of heavy metal accumulation in the mining area and surrounding soil, which not only destroys the vegetation, but also causes heavy metal pollution, induces collapses and other geological disasters (Jamal et al. 2018; Kasemodel et al. 2019). Pb was listed as a second priority hazardous substance in the U.S. Agency for Toxic Substances & Disease Registry. The sedimentary nature, persistence and irreversibility of Pb in the soil make it difficult

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to effectively remove from contaminated soil, thus, causing persistent harm to the soil ecological environment. Furthermore, Pb is not a basic element of plant metabolism, therefore, when it enters into the cells, it causes a series of negative effects, including obstructing the plant nutrient absorption, damaging the light, system and membrane integrity, thus interfering with metabolism which accelerates the plants necrosis and ageing (Yang et al. 2017; Ashraf et al. 2020).

At present, true Pb-hyperaccumulator plants have not been identified, considering the maximum lead transfer, most of the studies have adopted fast-growing plants to increase the biomass (Zaier et al. 2010). As an excellent perennial legume herbage, alfalfa (*Medicago sativa* L.) has high a nutritional and economic value and can be mowed repeatedly. It is widely cultivated in Inner Mongolia because it is rich in proteins, minerals, vitamins and carotene, and can be used as poultry feed. Furthermore, it is consumed by human beings as a therapy which can reduce the cholesterol and blood fats, regulate the immunity, is an antioxidant, and can prevent ageing (Liu 2010). Inner Mongolia has more than 100 lead mining enterprises, thus, the surrounding land is contaminated with lead to varying degrees. Pb toxicity results in changes in the catalytic activities of various enzymes, increases the membrane permeability, decreases the photosynthetic pigment contents, and is a disturbance in the mineral nutrient balance (Wang et al. 2012). However, the effects of Pb stress on the physiology and biochemistry of alfalfa should be understood before its application in reducing heavy metals in soil.

In this study, the growth and development of alfalfa were analysed under different Pb concentrations to determine its root cell activity, reactive oxygen species (ROS), malondialdehyde (MDA), antioxidant enzyme, osmotic adjustable substance, chlorophyll and Pb accumulation characteristics. The growth and the physiological response of alfalfa under Pb stress were investigated to explore the feasibility using it in remediating lead-contaminated soil.

MATERIAL AND METHODS

Experimental materials and treatment. The alfalfa seeds (Golden empress, America) were surface-sterilised in 5% $HClO_4$. Forty (40) plump seeds were selected and placed into a culture dish. The concentration of Pb^{2+} was set as 0 (control group, CK), 5, 10, 50, 100, 250 and 500 mg/L. Each concentration was added into the culture dish with a 1/2 Hoagland solution. The seeds were cultured in a full-automatic illumination incubator, under the conditions of 20 ± 1 °C, 16 : 8 h (day : night photoperiod) and an illumination intensity of 1 800 Lux. Each group of concentrations was set via three repetitions, with a 10-day culture cycle.

Measurement of the seed germination. The calculation formulas for the germination rate (G_r), germination potential (G_e), germination index (G_i), vigour index (V_i), root and stem length inhibition index (I_i) are as follows:

$$G_r = G_n/S_n \times 100\% \quad (1)$$

G_n – number of germinated seeds;
S_n – total seeds.

$$G_e = G_4/S_n \times 100\% \quad (2)$$

G_4 – number of germinated seeds on the 4th day.

$$G_i = \Sigma(G_t/D_t) \quad (3)$$

G_t – number of germinated seeds on day t;
D_t – germination days.

$$V_i = G_i \times S \quad (4)$$

G_i – germination index;
S – length of the bud.

$$I_i = [(L_{ck} - L_d)/L_{ck} \times 100\%] \quad (5)$$

L_{ck} – length of the control group;
L_d – length of the sample.

Plant physiological determination. Evans blue spectrophotometry was used to identify the loss of the root cell viability based on previous studies (Baker & Mock 1994); the production of O_2^- and H_2O_2 in the alfalfa was determined according to previous studies (Yin et al. 2010); the cell lipid peroxidation was observed by measuring the MDA content according to Tobita's method (Dionisiosese & Tobita 1998); the superoxide dismutase (SOD) activity was detected by the nitro-blue tetrazolium dye method (Rao et al. 1996); the catalase (CAT) activity was detected by monitoring the decrease in the absorbance due to hydrogen peroxide at 240 nm as described in previous studies (Rao et al. 1996); the proline content was detected according to the method as described in earlier studies (Bates et al. 1973); the soluble pro-

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tein content was detected by using the Coomassie Brilliant Blue G-250 method (Bradford 1976); the Pb content in the sample was determined by atomic absorption spectrophotometry (Analytik Jena AG, Germany, ZEEnit·700P) (Dias et al. 2019; Ashraf et al. 2020); the chlorophyll content was detected by using the ethanol extraction spectrophotometry method as described by Ding et al. (2016).

Statistical analysis. All the data in this study were processed and drawn using SPSS (Ver. 26.0) and Origin 2018 software, respectively. A one-way analysis of variance (ANOVA) in the SPSS (Ver. 26.0) software was used to treat the growth and physiological indices of the alfalfa seedlings under stress, where the error values in the repeated tests were eliminated.

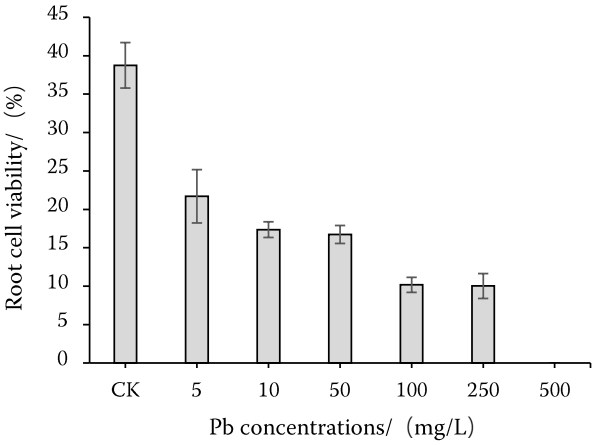

Figure 1. Effects of the Pb on the root cell activity and root length inhibition index at different concentrations
Values are means ± SD (n = 3)

RESULTS

Seed germination and plant growth. It was found that the 5–10 mg/L Pb stress promoted the germination of the seeds, interestingly, at 500 mg/L, the seeds did not germinate. The maximum germination potential was 87% under the stress of the different concentrations of Pb. The seed germination index increased after the first drop in the different Pb concentrations. The seed vigour index changed with an increasing concentration, but then decreased at 500 mg/L. With the increase in the Pb concentration, the root cell viability at 5 mg/L was decreased to 21.7% compared to the blank. On the contrary, the root length inhibition index progressively increased, where increased suddenly to 63.19% at 100 mg/L (Table 1). When the Pb^{2+} concentration was 500 mg/L, there was no root formation, the activity of the seed root cells could not be measured (Figure 1).

O_2^- and H_2O_2 contents. The results showed that under the Pb concentrations of 5 mg/L to 500 mg/L, there was a slight accumulation of ROS (both O_2^- and H_2O_2, etc.) in the alfalfa, with the trend in the variation of the H_2O_2 and O_2^- content likened to that of the controls. However, when the Pb concentration exceeded 100 mg/L, both the O_2^- and H_2O_2 contents increased significantly, reaching maximum values of 1.20 μmol/g FW and 12.31 μmol/g Fresh weight (FW) at 500 mg/L, respectively (Figure 2).

Leaf chlorophyll content. With the increase in the Pb concentration, the chlorophyll a, chlorophyll b, and total chlorophyll of the alfalfa leaves all showed a tendency to firstly increase and then decrease. At 10 mg/L, the peak values were 0.69, 0.21 and 0.89 mg/g FW, respectively. Nevertheless, when the treatment concentration was 500 mg/L, no leaf formation was observed in the alfalfa due to poisoning by heavy metals, thus, the test could not be carried out (Figure 3).

Table 1. Effect of the Pb on the seed germination and growth of the alfalfa

Pb content (mg/L)	Germination rate	Germination potential	Germination index	Vigour index	Root inhibition index	Stem inhibition index
	(%)					
CK	85.0 ± 9.1^{a}	80.0 ± 10.3^{ab}	69.7 ± 5.2^{a}	332.3 ± 27.5^{a}	0^{f}	0^{e}
5	85.3 ± 10.2^{a}	85.8 ± 8.4^{a}	71.3 ± 8.6^{a}	346.9 ± 20.5^{a}	–9.1±1.2^{f}	13.6 ± 0.9^{d}
10	85.80 ± 7.3^{a}	87. 7 ± 5.9^{a}	74.4 ± 7.3^{a}	322.7 ± 19.1^{a}	10.1 ± 1.0^{e}	19.7 ± 1.5^{c}
50	87.5 ± 6.3^{a}	80.0 ± 10.8^{ab}	67.3 ± 4.4^{a}	230.3 ± 16.6^{b}	35.6 ± 2.9^{d}	21.4 ± 2.1^{c}
100	85.0 ± 1.2^{ab}	77.0 ± 13.6^{ab}	66.1 ± 3.9^{a}	122.9 ± 8.8^{c}	60.0 ± 7.1^{c}	63.2 ± 0.9^{b}
250	76.9 ± 5.7^{b}	69.4 ± 8.1^{ab}	50.5 ± 5.2^{b}	25.3 ± 4.6^{d}	89.0 ± 10.6^{b}	65.9 ± 3.0^{b}
500	66.6 ± 10.0^{b}	61.3 ± 13.9^{ab}	42.5 ± 3.2^{b}	14.9 ± 2.7^{d}	100^{a}	100^{a}

CK – control; data are means ± SD, n = 3; different letters indicate a statistical difference in the same column (P < 0.05)

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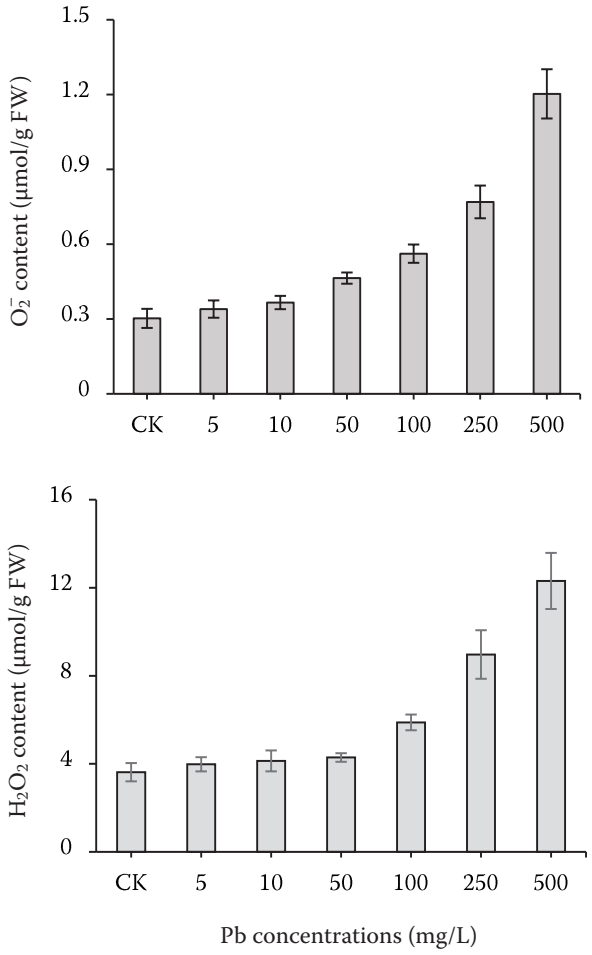

Figure 2. Effects of the Pb on reactive oxygen species (ROS) content at different concentrations

Degree of lipid peroxidation. The MDA content is one of the important indices for measuring the degree of membrane lipid peroxidation. With the increase in the Pb concentration, the MDA content also increased, and the lowest MDA content in the control was 20.87 μmol/g FW. Nonetheless, when the Pb concentration reached 500 mg/L, the maximum MDA was 68.95 μmol/g FW (Figure 4).

Pb determination in the seedlings. The Pb accumulation in the alfalfa seedlings significantly increased with the increase in the Pb content in the medium, as the surge point was at 250 mg/L under which the content was 20.37 mg/g dry weight (DW), where the maximum accumulation value was observed at 500 mg/L under which the content was 24.40 mg/g DW (Figure 4).

Antioxidant enzyme activities. The SOD activity was gradually enhanced at a low concentration level, reaching a peak of 298 U/g FW at 50 mg/L. Meanwhile, with an increase in the concentration beyond the plant tolerance, the SOD activity was inhibited and exhibited a downward trend, reaching a minimum value of 260 U/g FW at 500 mg/L. With an increase in the stress concentration, the CAT activity also increased reaching a maximum of 93 U/g FW at 500 mg/L, which was an increase of 55.91%, compared to the control (Figure 5A, B).

Proline and soluble protein contents. With an increase in the Pb concentration, the proline content in the alfalfa leaves increased gradually, reaching a peak value of 75.88 μg/g FW at 100 mg/L, which

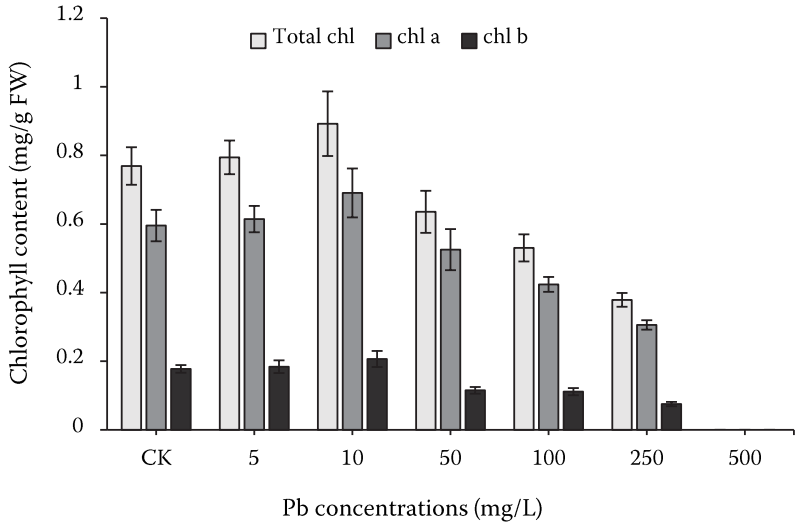

Figure 3. Effects of the Pb on the chlorophyll content at different concentrations
Total chl – total chlorophyll; chl a – chlorophyll a; chl b – chlorophyll b

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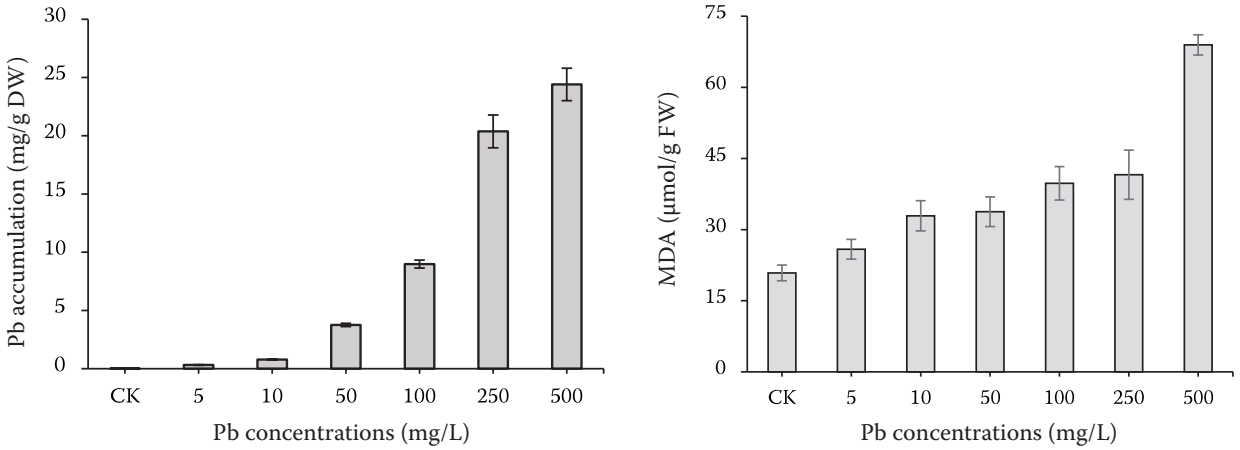

Figure 4. Pb accumulation and malondialdehyde (MDA) content

was an increase of 85.96% compared to the control. Subsequently, the proline content gradually decreased to 41.06 μg/g FW at 500 mg/L. Interestingly, under the Pb stress, the protein content of the alfalfa decreased, where, at 500 mg/L, it decreased 67.95 % compared to the control (Figure 5C, D).

Principal component analysis. It can be seen from Table 2 that the cumulative contribution value of components 1 and 2 was 95.21%, which well reflect the levels of the Pb resistance of the alfalfa. Combined with Table 3, we can conclude that the characteristic value of principal component 1 was composed of five

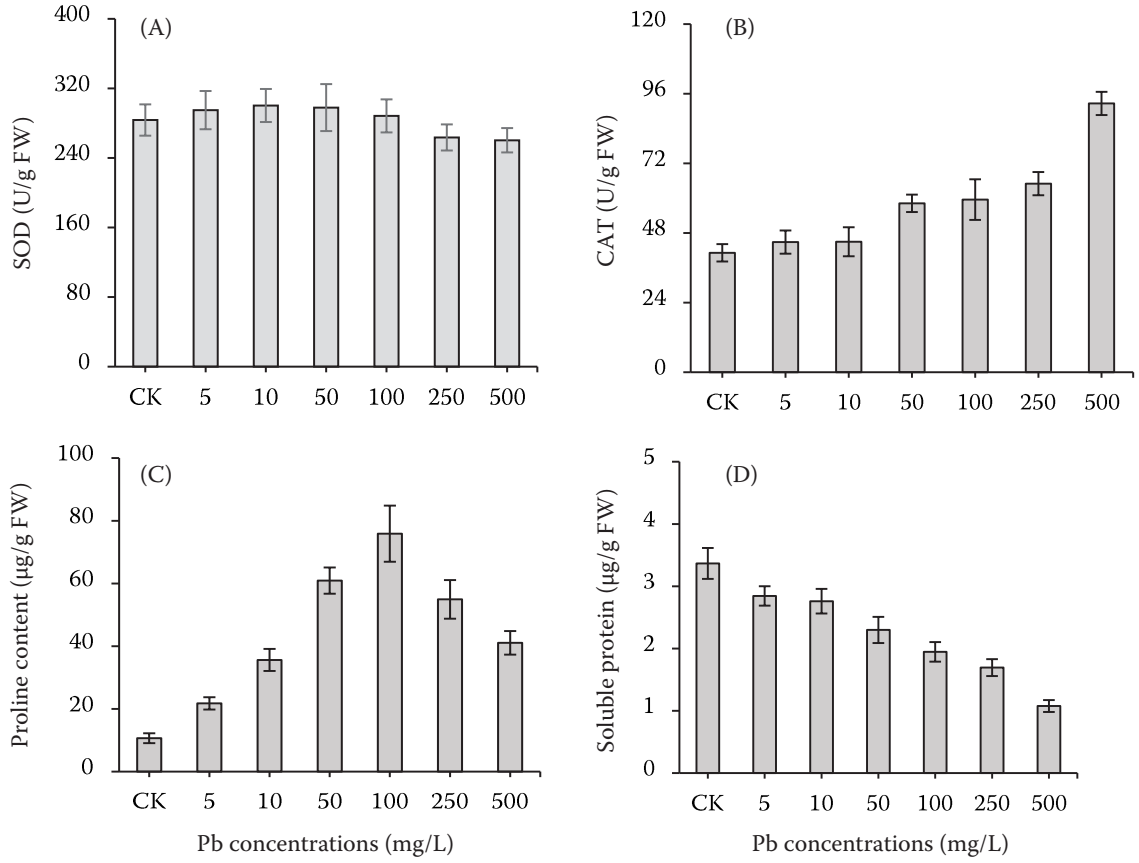

Figure 5. Changes in the superoxide dismutase (SOD) (A); catalase (CAT) (B); proline (C), and soluble protein (D) under Pb stress

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Table 2. The correlation of the growth indices under Pb stress

Element	Initial eigenvalue			Extracting eigenvalue		
	eigenvalue	contribution ratio	accumulate contribution ratio	eigenvalue	contribution ratio	accumulate contribution ratio
		(%)			(%)	
1	10.2	84.89	84.89	10.2	84.89	84.89
2	1.2	10.32	95.21	1.2	10.32	95.21
3	0.35	2.95	98.16			
4	0.18	1.51	99.67			
5	0.03	0.23	99.91			
6	0.01	0.09	100.00			

indices including H_2O_2, O_2^-, the total chlorophyll, chlorophyll a and CAT, and represented 84.89% of the information of the comprehensive index. As the supplementary ingredient of principal component 1, principal component 2 represented 10.32% of the information of the comprehensive index, and the major contributor was the proline content. In general, the total chlorophyll, chlorophyll a, H_2O_2, O_2^-, CAT and proline content can reflect the regularity of the physiological change and Pb tolerance of the alfalfa under the Pb stress.

DISCUSSION

On the whole, alfalfa seed germination and root growth showed significant growth stimulation response under low Pb concentrations and exhibited a certain tolerance. With a Pb stress concentration above 100 mg/L, the toxicity was more obvious, thus, the Pb resistance of the alfalfa decreased greatly, leading to serious growth inhibition and a significant reduction in the biomass, the same inhibition was found in Pb stressed *Brassica juncea* L. (Zaier et al. 2010).

It is known that roots are the major plant organs to come into contact with heavy metals during the nutrient absorption (Kolahi et al. 2020). This study found that the Pb content in the seedlings increased with an increase in the stress concentration and time, and the toxicity was similarly enhanced. With an increase in the Pb stress concentration, the root cell activity was decreased, and the root inhibition index was increased. Research has shown that due to the restriction of the root hair growth caused by heavy

Table 3. Growth index factor coefficient under Pb stress

Index	Component factor coefficient		Component score coefficient	
	1	2	1	2
A	0.854	–0.422	0.084	–0.34
B	–0.977	–0.174	–0.096	–0.14
C	–0.989	–0.108	–0.097	–0.087
D	0.983	0.115	0.096	0.093
E	0.98	0.151	0.096	0.121
F	0.972	0.008	0.095	0.007
G	–0.958	0.034	–0.094	0.027
H	–0.973	–0.084	–0.095	–0.068
I	0.831	0.455	0.082	0.366
J	–0.982	0.007	–0.096	0.006
K	–0.443	0.844	–0.044	0.681
L	0.969	–0.238	0.095	–0.192

A – root vitality; B – H_2O_2; C – O_2^-; D – total chlorophyll; E – chlorophyll a; F – chlorophyll b; G – malondialdehyde; H – Pb content; I – superoxide dismutase (SOD); J – catalase (CAT); K – proline content; L – soluble protein

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metals, the root surface area was reduced, the water absorption was reduced, and the plant's absorption and transformation of nutrients was seriously affected, resulting in hypoplasia (Gouia et al. 2000).

It is noteworthy that the stability of the plant cell membrane is the premise and basis for maintaining a normal physiological function. As the major product of lipid peroxidation, MDA plays a significant role in measuring the stability of membrane (Zhang et al. 2020a). In this study, with an increase in the Pb stress concentration, the MDA content in the alfalfa increased. The heavy metal stress damaged the cell membrane permeability, MDA as a peroxide, can cross-link with nucleic acids, amino acids, proteins and other active substances to form insoluble compound deposits. Hence, MDA affects the normal life activities and permeability of the cells, resulting in an increase in the cell membrane permeability and cell membrane lipid peroxidation (Ohkawa et al. 1979). This indicated that the production and scavenging system of the reactive oxygen species in the alfalfa were destroyed under such stress, and the accumulated reactive oxygen species caused membrane peroxidation.

The role of ROS in plants is a double-edged sword, composed of an indispensable redox agent in plant life activities (Baxter et al. 2014); however, excessive ROS during environmental stresses can pose a threat to plants, destroying the plant redox balance, causing cell membrane peroxidation and cell structure oxidative damage, ultimately leading to the death of the cells (Gill & Tuteja 2010). SOD, CAT and other enzymes are coordinated to form an antioxidant system, which can effectively eliminate the excessive ROS produced by the plants and prevent membrane peroxidation and other damages associated with ROS. Plant cells are able to reduce the potential harmful effects and damage caused by free radicals by developing various antioxidant defence systems. Previous studies have shown that within a certain Pb concentration range, the Pb treatment could improve the SOD and CAT activities in maize leaves, thus maintaining the ROS balance (Benavides et al. 2005). The experiments showed that the defence system of antioxidant enzymes played a scavenging role in the scavenged reactive oxygen species, maintaining the balance of the ROS metabolism system in cells, in order to maintain its normal level in alfalfa. However, under a Pb stress concentration of 100 mg/L, the antioxidant enzyme activity was inhibited, the ROS clearance effect was decreased, and the ROS content in the alfalfa was significantly increased, which damaged the cell membrane and interfered with the metabolic ability of the plants. Interestingly, the same phenomenon was found in dwarf bamboo under high Pb soil stress concentrations (Cai et al. 2021).

When plants are poisoned by heavy metals, the production of a large amount of proline synthesis is one of the important mechanisms for plants to respond to heavy metal stress. Protein is related to nutrient cycling, the metabolism, growth and development of plants, and its content change plays an indicator role in the plant's strength under stress (Cao et al. 2019; Dias et al. 2019). The proline content firstly increased and then decreased with an increase in the Pb stress concentration. Under low concentrations of Pb stress, proline may be involved in regulating the intracellular permeability and scavenging the reactive oxygen radicals, reducing the damage to the Pb to alfalfa cells (Ahmad et al. 2015). Nevertheless, when the Pb stress concentration exceeded a certain level, the proline content decreased, which destroyed the internal structure of the cells and led to an imbalance in the plant osmotic regulation, thus producing toxic effects on the alfalfa, causing a disorder in the cell metabolism. The structural and functional changes of the proteins under Pb stress, including protein denaturation and fracture, as well as protein inhibition, are related to the decrease of protein content. This work also verified the idea that the soluble protein content gradually decreased with an increase in the stress concentration.

The chlorophyll contents are the most direct manifestation of the photosynthesis ability and growth of plants. Studies have shown that chlorophyll in the alfalfa under low stress concentrations showed an increasing trend , but with an increase in the stress concentration beyond its resistance and tolerance point, it leads to a decrease in the chlorophyll content and causes changes to the chloroplast structure and causes chlorophyll decomposition due to oxidation (Liu et al. 2008). This is not conducive for plant photosynthesis, as it reduces the energy production, thus causing heavy metal poisoning symptoms (Zhang et al. 2020b).

CONCLUSION

The lead stress affected most of the alfalfa attributes, the overall growth, root vitality and soluble protein were suppressed by the Pb-induced ROS, the MDA

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and Pb excessively accumulated. In response, the CAT, SOD and proline contents increased to protect the alfalfa from further damage. It was interesting that the seed germination and pigment system were promoted at low Pb concentrations (below 10 mg/L), but were inhibited at high stress concentrations (overtake 50 mg/L). From this, it seemed that the alfalfa could maintain normal growth and metabolism at low concentrations. Combined with the principal component analysis (PCA), the physiological characteristics and lead-resistance of alfalfa can be characterised by the H_2O_2, O_2^-, total chlorophyll, chlorophyll a, CAT and proline contents. These findings are important for the better understanding of the physiological mechanisms in alfalfa under Pb stress.

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