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Interactive Effects of Cd and Pb on the Photosynthesis Efficiency and Antioxidant Defense System of *Capsicum annuum L*

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Abstract

In this study, the interactive effect of Cd and Pb on the growth of *Capsicum annuum L.* was studied through pot experiments, and the indicators of photosynthesis efficiency (PE) and antioxidant defense system (ADS) were measured at different plant ages. Single Pb stress on PE and ADS was stronger than single Cd stress at the first month. Both the PE and ADS response showed a significant decrease under the combined stress of Cd and Pb, which was primarily dependent on the Pb concentration. With increasing plant age, the PE and response of non-enzymatic ADS exhibited dramatic decreases under Cd and/or Pb stress, and the activities of enzymatic ADS showed increases to some extent. The factorial analysis showed that Cd and Pb had an interactive effect to reduce PE, while slightly enhanced the activities of enzymatic ADS. Those results are useful to explore the interaction between Cd and Pb in the combined stress and understand their accumulation in the plants.

Keywords Heavy metal stress · Photosynthesis efficiency · Antioxidant defense system · *Capsicum annuum L.*

Farmland soil contamination by various heavy metals derived from smelting activities is a global environmental problem and has attracted the attention of many scientists and policy makers, owing to its potential threat for human health through food chain (Fang et al. 2014; Liu et al. 2019; Zhou et al. 2020; Wang et al. 2021). Among various heavy metals, cadmium (Cd) and lead (Pb) are two major metallic environmental pollutants, which are not essential elements for plant metabolism and can be absorbed and transported by plants (Xu et al. 2009). The excessive accumulation of heavy metals in cells can induce a wide range of negative effects on the plants (Le et al. 2013). In previous studies, such toxic effects of Cd and Pb on plants have been well-documented (Alamri et al. 2018; Pinto et al. 2017). It has been verified that under heavy metal stress, the excessive production of reactive oxygen species (ROS) is a ubiquitous and initial biochemical process in plants (Lin and Aarts 2012). ROS can damage organelles such as nuclei, mitochondria and chloroplasts in plant cells, and then alter the photosynthesis efficiency (PE) and disturb the redox homeostasis (Nagajyoti et al. 2010).

The antioxidant defense system (ADS) in plants is responsive to the oxidative stress induced by heavy metals (Gill and Tuteja 2010). The non-enzymatic and enzymatic antioxidants of the ADS can work in coordination to maintain the redox balance by eliminating the ROS to avoid oxidative damage in plant tissues (Zagorchev et al. 2013). The non-enzymatic antioxidants in plants, such as proline, carotenoids, glutathione and ascorbate, can directly mitigate the oxidative damage induced by excessive ROS in cells through reduction reaction (Chen et al. 2017). Plants have also developed some enzymatic antioxidants in the ADS, which can eliminate ROS by activating various antioxidant enzymes such as catalase (CAT), peroxidase (POD), superoxide dismutase (SOD) and guaiacol peroxidase (Dias et al. 2019).

Due to anthropogenic activities such as mining and smelting of ores, metalworking industries and agricultural practices, Cd and Pb are usually present in the soil simultaneously (Marrugo-Negrete et al. 2017). Compared with exposure to single Cd or Pb, the exposure to co-existing Cd and Pb may have distinctly different effects on the growth of plants (Huang et al. 2015). However, there is

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little information about the oxidative damage caused by the interactive effect of different heavy metals to plants and the corresponding response mechanism.

In this study, the stress of single Cd or Pb and their combination on *Capsicum annuum L.* was investigated by pot experiments, and the response of PE and ADS to heavy metal stress was determined at different plant ages. Then, a factorial analysis was performed to reveal the interaction of Cd and Pb in the combined stress to *Capsicum annuum L.* Therefore, the general aims of this study include: (1) detecting the Cd and Pb stress on the PE and activity of ADS; (2) exploring the interaction of Cd and Pb in the combined stress; (3) dissecting the response of ADS in *Capsicum annuum L.* to heavy metal stress at different plant ages.

Materials and Methods

The tested soil was collected from Xianning City, Hubei Province (29° 93′ N; 114° 41′ E). The measured pH and soil organic matter (SOM) of tested soil is 5.61 (water:soil = 2.5:1, ml:g) and 29.48 g/kg, respectively. The cation exchange capacity is 9.81 cmol/kg. And the total content of Pb and Cd is 17.53 and 0.17 mg/kg, respectively. The samples were air-dried and sieved to 2 mm, and then artificially contaminated with $Pb(NO_3)_2$ and $Cd(NO_3)_2$ solutions with Cd at 0, 0.3, 0.6 and 3 mg/kg and Pb at 0, 125, 250 and 500 mg/kg or their combination. As a result, there were 16 soil treatments with different Cd and Pb concentrations. The Pb and Cd concentration in the aged soil was digestion with HNO_3–HCl–HF (a volume/volume ratio of 6:2:2) and was analyzed with a flame atomic absorption spectrometry (AA240FS, Varian, America). A standard soil samples (GBW07403, GSS-3, Institute of Geophysical and Geochemical Exploration, Chinese Academy of Geological Sciences) were analyzed simultaneously as reference for the quality control. The limit of quantitation (LOQ) of Pb and Cd was 0.5 and 0.06 mg/L; and the standard lines of Pb and Cd was established in the range of 1 ~ 20 mg/L and 0.1 ~ 2 mg/L with the correlation coefficient $R^2 > 0.995$ and 0.996, respectively. The measured heavy metals concentration was summarized in Table 1. Before the beginning of pot experiments, the soil samples were added with ultrapure water to keep about 80% field capacity and aged for three months at 25.0°C to ensure that the chemical reaction between heavy metals and soil particles reached a thermodynamic equilibrium.

Capsicum annuum L. was used to study the effect of the single or combined stress of Cd and Pb on the growth of plants. The seeds were purchased from the Anhui First Seed Co. Ltd and sown in pots filled with the wet peat-based soil. Then, 30-day-old seedlings with 6–10 leaves were transplanted into a round pot with 5 kg aged soil, with four replicates for each soil treatment. The *Capsicum annuum L.* plants were cultivated and maintained in the greenhouse under conventional water management to avoid water stress.

At the first and fourth month after transplantation, the PE and antioxidative capacity of the plants were monitored. The net photosynthetic rate (NPR), stomatal conductance (SC) and intercellular carbon dioxide concentration (ICDC) were determined by the portable photosynthetic apparatus (Li-6400XT, LI-COR, America) and used to identify the PE (Zhang et al. 2020). The accumulation of free proline (Pro) and the enzyme activities of CAT, POD and SOD were determined with the kit developed by Nanjing Jiancheng Bioengineering Research Institute using the colorimetric method (Cary 60-G6860A, Agilent, America) to indicate the response of ADS in plants to heavy metal stress (Bagheri et al. 2021). Pro content was estimated according to the method of Bates et al. (1973). CAT activity was measured of consumption of H_2O_2 according to the method of Aebi (1984). POD activity was determined according to the method of Chance and Maehly (1955). SOD activity was determined according to the method of Beauchamp and Fridovich (1971). LOD was 0.5 and 0.2 U/mL for Pro and CAT, 0.5 and 5.0 U/mL for POD and SOD, respectively. The range of measurement was located between 0.5 ~ 20 U/mL for Pro, 0.2 ~ 24.8 U/mL for CAT, 0.5 ~ 300 U/mL for POD, 5.0–122.1 U/mL for SOD and the R^2 of standard curve were higher than 0.99.

The experimental data were presented as the average value of four replicates for all heavy metal treatments. Differences between control and treated groups were analyzed by one-way ANOVA test followed by Tukey's post hoc test ($p < 0.05$), which was carried out with the software SPSS. The normality of data was checked by Anderson–Darling

Table 1 Total Cd/Pb concentration in the aged soil

Measured Cd/Pb concentration (mg/kg)		Cd treatment (mg/kg)			
		0	0.3	0.6	3
Pb treatment (mg/kg)	0	0.17 ± 0.02/17.53 ± 0.53	0.48 ± 0.16/17.89 ± 0.70	0.67 ± 0.03/18.46 ± 0.39	2.33 ± 0.22/17.42 ± 1.83
	125	0.13 ± 0.01/139.31 ± 5.55	0.39 ± 0.02/136.81 ± 3.52	0.63 ± 0.02/135.88 ± 4.24	2.19 ± 0.17/141.63 ± 5.44
	250	0.13 ± 0.01/252.38 ± 6.15	0.39 ± 0.03/252.75 ± 10.40	0.59 ± 0.05/265.75 ± 1.59	2.27 ± 0.36/264.94 ± 5.13
	500	0.15 ± 0.02/511.94 ± 2.19	0.37 ± 0.04/512.50 ± 11.58	0.60 ± 0.04/495.25 ± 6.17	2.40 ± 0.17/526.31 ± 7.59

test. Factorial analysis was performed to identify the interaction of Cd and Pb with the software Minitab 17.1.

Results and Discussion

The effect of single Cd or Pb stress on PE of *Capsicum annuum L* was determined at the first and fourth months of stress. The measured indicators (NPR, SC and ICDC) of PE are presented in Fig. 1a–c and Fig. 1d–f under Cd and Pb stress, respectively. It can be observed that the effect of Cd on the PE of *Capsicum annuum L.* was significantly different from that of Pb in a concentration-dependent manner.

At the first month of stress, with increasing heavy metal concentration, NPR and ICDC showed no obvious change under Cd stress, while dramatically decreased under Pb stress (Fig. 1a and c). The value of SC decreased obviously under both Cd and Pb stress, with Pb showing a much stronger effect than Cd (Fig. 1b). With the increase in plant age, the values of SC and ICDC decreased at all heavy metal concentrations, indicating a decrease in the PE of *Capsicum annuum L.* Similar phenomena could be found in previous studies, which can be attributed the larger area of leaves (Chapin et al. 1993; Khudsar et al. 2008). With increasing concentration of heavy metals, the value of NPR and SC increased under Cd stress, while that of ICDC exhibited no consistent change. Under Pb stress, the concentration effect on PE could be ignored, except for the treatment of 125 mg/kg Pb, which resulted in much higher values of NPR and SC than other treatments (Fig. 1e and f).

To explore the influence of Cd or Pb stress on the ADS of *Capsicum annuum L.*, the Pro content and the activities of antioxidant enzymes such as SOD, POD and CAT were determined (Fig. 2). The changes in the Pro content and SOD enzyme activity were remarkably similar between Cd stress and Pb stress, while the CAT and POD enzyme activities were slightly higher under Cd stress than those under Pb stress.

At the first month of stress, with increasing heavy metal concentration, the Pro content showed obvious increases under both Cd and Pb stress, but the increase induced by Cd stress was much lower than that induced by Pb stress. For the enzyme activities of CAT and POD, consistent trends could be hardly found depending on Cd and Pb concentration (Fig. 2b and c). At different concentrations of Cd and Pb, the SOD activity of *Capsicum annuum L.* showed almost no response to heavy metal stress (Fig. 2d), and a similar phenomenon was observed at the fourth month of stress

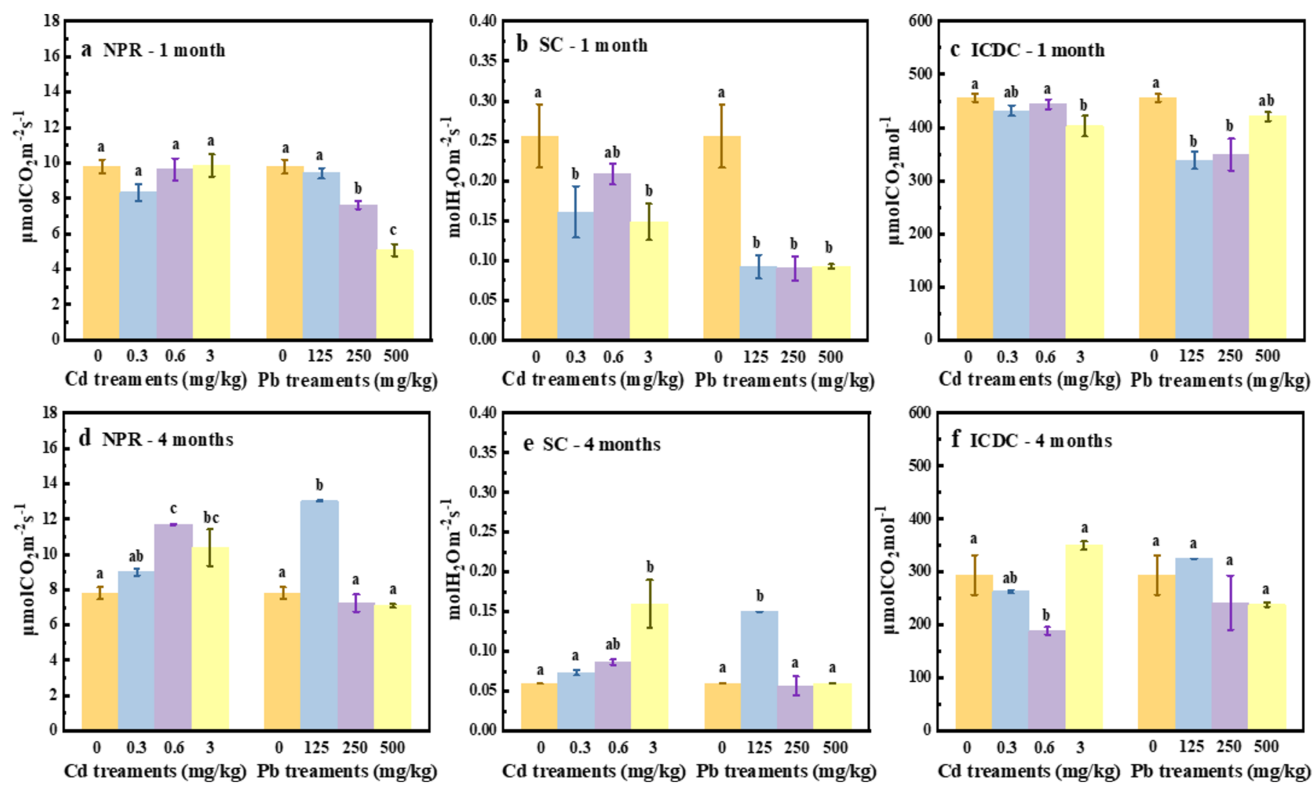

Fig. 1 Effect of single Cd or Pb stress on the photosynthesis efficiency of *Capsicum annuum* L. with increasing heavy metal concentration and plant age. **a** and **d** NPR, **b** and **e**: SC, **c** and **f** ICDC. **a**, **b** and **c** 1 month, **d**, **e** and **f** 4 months. Values with different letters are significantly different at $p < 0.05$ (Tukey test)

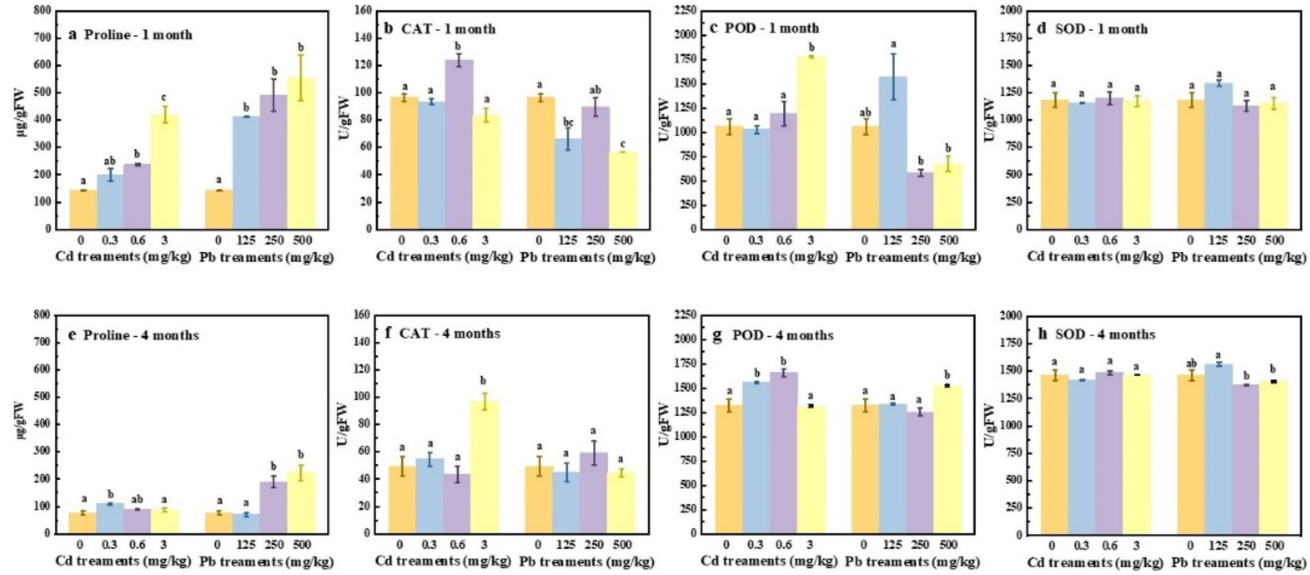

Fig. 2 Effect of single Cd or Pb stress on the antioxidant defense system of *Capsicum annuum* L. with increasing heavy metal concentration and plant age. **a** and **e** content of Pro, **b** and **f** activity of CAT, **c** and **g** activity of POD, **d** and **h** activity of SOD. **a–d** 1 month, **e–f** 4 months. Values with different letters are significantly different at $p<0.05$ (Tukey test)

(Fig. 2h). Besides, the SOD activity at the fourth month was very approximate to the value at first month. These results indicated that the SOD activity is very stable and is hardly affected by the type and concentration of heavy metals and plant age. At the fourth month of stress, the Pro content (Fig. 2e) and CAT activity (Fig. 2f) became much lower than those in the plant at the first month of stress, while the POD activity was much higher (Fig. 2g). Compared with those at first month of stress, single Cd or Pb stress showed no significant concentration effect on the enzyme activity of plants at the fourth month of stress, except for the Pro content under Pb stress and the CAT activity under Cd stress, which increased with increasing heavy metal concentrations.

The change in the PE (NPR, SC and ICDC) of *Capsicum annuum L.* under the combined Cd and Pb stress was expressed with the ratios of the data measured in the experimental group to those measured in the control group and the results are summarized in Table 2. NPR and SC were much more strongly affected by the combined stress of Cd and Pb than single heavy metal stress with increasing Pb concentration, especially at high Pb concentrations. However, no significant changes in the effect of the combined stress were identified with increasing Cd concentration, indicating that the combined heavy metal stress was dominated by Pb. For ICDC, most ratios approximated to 1.0, indicating that ICDC is hardly affected by the combined heavy metal stress. The PE at the fourth month of stress was much higher than that at the first month of stress. All values of NPR and SC were higher than 1.0 under single Cd stress, while were lower under single Pb stress except for the treatment of 125 mg/kg. Under the combined stress of Cd and Pb, most values of NPR, SC and ICDC were higher than 1.0, suggesting that the combined stress of Cd and Pb could enhance the PE at the fourth month of stress.

In order to investigate the interaction between Cd and Pb in the combined stress to *Capsicum annuum L.*, a factorial analysis of PE was carried out. The results are shown in Fig. 3 and Table 3. The interactive effect of Cd and Pb on the PE of *Capsicum annuum L.* was similar at the first and fourth month of stress. As shown in Fig. 3, single Pb stress resulted in a much wider data point distribution for both NPR and SC than single Cd stress, while the data point distribution for ICDC was similar between the two stresses, suggesting that Pb has a stronger effect on PE than Cd. The data point distribution of Cd was different from that of Pb depending on the heavy metal concentration, implying that there is a strong interaction between Cd and Pb in the combined stress. The η^2 obtained from the factorial analysis followed an obvious decreasing order in magnitude of Pb > Cd × Pb > Cd at the first month of stress and Cd × Pb > Pb > Cd at the fourth month of stress (Table 3). These results indicated that the heavy metal stress on the PE of *Capsicum annuum L.* was primarily controlled by Pb at the first month of stress, while was strengthened by the interaction between Cd and Pb at the fourth month of stress.

The effect of the combined stress of Cd and Pb on the ADS of *Capsicum annuum L.* was evaluated with the ratios of the data measured in the experimental group to those measured in the control group, and the results are presented in Table 4. Under the combined stress of Cd and Pb, the

Table 2 Ratios of the data measured in the experimental group to those measured in the control group for NPR, SC and ICDC of *Capsicum annuum L.* under the combined stress of Cd and Pb

Treatment (mg/kg)		1st Month of stress			4th Month of stress		
Cd	Pb	NPR[a]	SC[a]	ICDC[a]	NPR[a]	SC[a]	ICDC[a]
0	125	0.96 ± 0.03	0.36 ± 0.06[c]	0.75 ± 0.04[b]	1.67 ± 0.00[c]	2.57 ± 0.01[c]	1.11 ± 0.00
0	250	0.78 ± 0.02	0.35 ± 0.06[c]	0.77 ± 0.07[b]	0.93 ± 0.06	0.96 ± 0.20	0.82 ± 0.17
0	500	0.52 ± 0.03[c]	0.36 ± 0.01[c]	0.92 ± 0.02	0.91 ± 0.01	0.94 ± 0.00	0.81 ± 0.01
0.3	0	0.85 ± 0.05	0.63 ± 0.13	0.95 ± 0.02	1.15 ± 0.02	1.26 ± 0.05	0.89 ± 0.01
0.3	125	0.92 ± 0.04	0.59 ± 0.08[b]	0.88 ± 0.05	1.77 ± 0.09[c]	3.32 ± 0.02[c]	1.20 ± 0.02
0.3	250	0.48 ± 0.12[c]	0.38 ± 0.08[c]	0.98 ± 0.01	1.01 ± 0.01	1.18 ± 0.19	0.92 ± 0.12
0.3	500	0.55 ± 0.11 [c]	0.42 ± 0.06[c]	0.94 ± 0.01	0.96 ± 0.02	1.01 ± 0.01	0.86 ± 0.00
0.6	0	0.98 ± 0.06	0.81 ± 0.05	0.97 ± 0.02	1.50 ± 0.00[c]	1.49 ± 0.04	0.64 ± 0.02
0.6	125	0.88 ± 0.04	0.39 ± 0.05[c]	0.81 ± 0.03	1.32 ± 0.01[c]	1.86 ± 0.00	1.10 ± 0.00
0.6	250	0.52 ± 0.09[c]	0.49 ± 0.01[b]	1.00 ± 0.03	1.02 ± 0.01	1.39 ± 0.03	1.03 ± 0.01
0.6	500	0.46 ± 0.12[c]	0.39 ± 0.07[c]	0.97 ± 0.02	0.93 ± 0.01	1.90 ± 0.01	1.17 ± 0.00
3	0	1.01 ± 0.06	0.58 ± 0.09	0.89 ± 0.04	1.32 ± 0.13[c]	2.69 ± 0.53[c]	1.19 ± 0.03[b]
3	125	0.81 ± 0.06	0.29 ± 0.07[c]	0.67 ± 0.08[c]	0.90 ± 0.01	1.62 ± 0.00	1.24 ± 0.00
3	250	0.48 ± 0.10[c]	0.37 ± 0.06[c]	0.95 ± 0.04	1.37 ± 0.01[c]	2.36 ± 0.00[c]	1.12 ± 0.00
3	500	0.52 ± 0.05[c]	0.43 ± 0.08[c]	0.79 ± 0.04	0.83 ± 0.01	1.23 ± 0.02	1.06 ± 0.01

[a]NPR, SC and ICDC are shown in terms of the ratio of data measured in experimental groups to those measured in the control group (Mean ± SE)

[b]Significant difference compared with control (ANOVA) $p < 0.05$

[c]Significant difference compared with control (ANOVA) $p < 0.01$

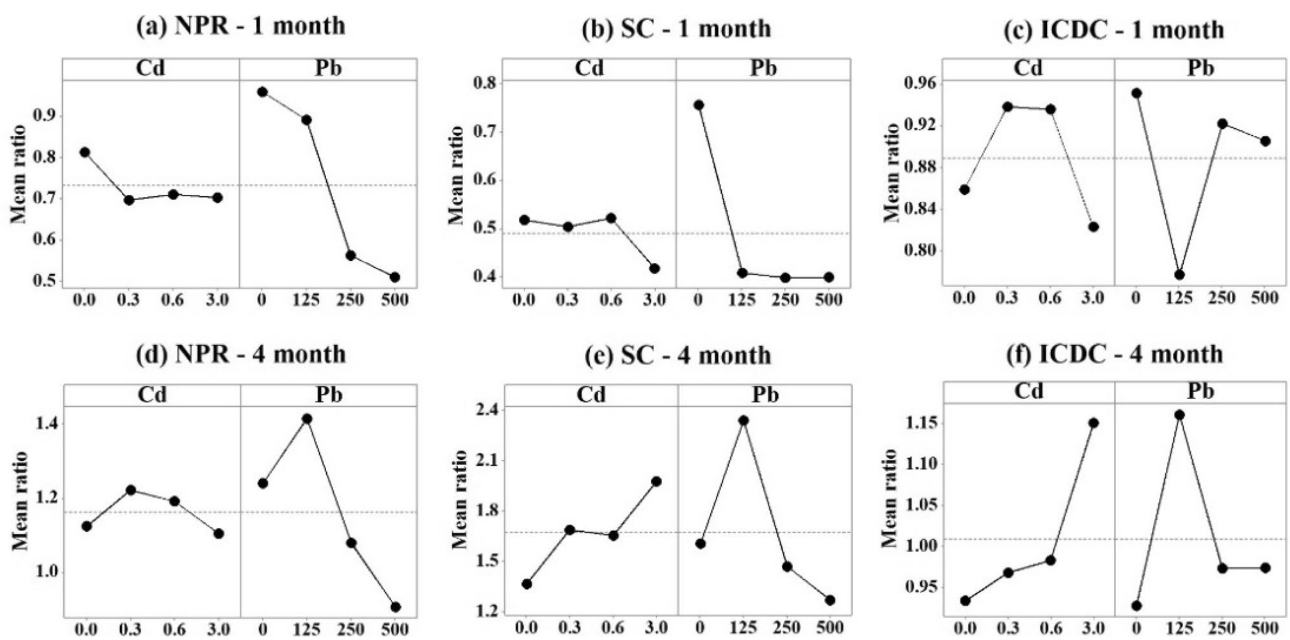

Fig. 3 Combined effects of Cd and Pb at different concentrations on the photosynthesis efficiency of *Capsicum annuum L.* with increasing heavy metal concentration and plant age. **a** and **d** NPR, **b** and **e** SC, **c** and **f** ICDC. **a**, **b** and **c** 1 month of stress, **d**, **e** and **f** 4 months of stress. Values with different letters are significantly different at $p < 0.05$ (Tukey test)

content of Pro was similar to that under single Pb stress but much higher than that under single Cd stress; and the enzyme activities of CAT, POD and SOD approximated to the values under single Pb stress but lower than those under single Cd stress. These results indicated that under the combined heavy metal stress, the activities of antioxidant enzymes are predominately determined by the Pb concentration. Compared with the values at the first month of

Table 3 Factorial analysis of the factors influencing the photosynthetic efficiency

Factor		1st Month of stress			4th Month of stress		
		Mean effects	*p* value	η^2	Mean effects	*p* value	η^2
NPR	Cd	1.967	0.058	0.045	3.328	0.002	0.053
	Pb	9.883	0.000	0.684	10.051	0.000	0.416
	Cd*Pb	1.904	0.066	0.099	8.514	0.000	0.437
SC	Cd	1.005	0.322	0.020	2.956	0.006	0.072
	Pb	7.890	0.000	0.462	6.904	0.000	0.308
	Cd*Pb	4.962	0.000	0.340	6.660	0.000	0.467
ICDC	Cd	5.071	0.000	0.169	4.036	0.000	0.189
	Pb	7.883	0.000	0.379	4.479	0.000	0.225
	Cd*Pb	5.251	0.000	0.305	4.070	0.000	0.344

Table 4 Ratios of the data measured in the experimental group to those measured in the control group for Pro, CAT, POD and SOD in the leaves of *Capsicum annuum L.* under the combined stress of Cd and Pb

Treatment (mg/kg)		1st Month of stress				4th Month of stress			
Cd	Pb	Pro	CAT	POD	SOD	Pro	CAT	POD	SOD
0	125	2.92 ± 0.01^c	0.69 ± 0.08^c	1.48 ± 0.22^b	1.13 ± 0.02	0.92 ± 0.08	0.91 ± 0.13	1.01 ± 0.01	1.07 ± 0.01
0	250	3.49 ± 0.41^c	0.93 ± 0.07	0.55 ± 0.04	0.96 ± 0.04	2.48 ± 0.29	1.19 ± 0.18	0.95 ± 0.03	0.94 ± 0.01
0	500	3.92 ± 0.60^c	0.58 ± 0.00^c	0.64 ± 0.07	0.97 ± 0.05	2.93 ± 0.36^b	0.90 ± 0.06	1.15 ± 0.01	0.96 ± 0.01
0.3	0	1.41 ± 0.15	0.97 ± 0.02	0.97 ± 0.04	0.98 ± 0.00	1.44 ± 0.03	1.10 ± 0.10	1.18 ± 0.01	0.97 ± 0.00
0.3	125	3.24 ± 0.06^c	0.60 ± 0.01^c	0.90 ± 0.01	1.06 ± 0.01	1.30 ± 0.19	3.16 ± 0.04	1.02 ± 0.07	0.93 ± 0.01
0.3	250	3.12 ± 0.03^c	1.31 ± 0.02^c	0.74 ± 0.02	0.85 ± 0.02	2.53 ± 0.71	1.24 ± 0.06	1.04 ± 0.02	0.94 ± 0.01
0.3	500	4.91 ± 0.03^c	0.53 ± 0.05^c	0.69 ± 0.05	0.87 ± 0.02	4.59 ± 0.67^c	1.02 ± 0.07	1.10 ± 0.05	0.95 ± 0.01
0.6	0	1.68 ± 0.02	1.29 ± 0.05^c	1.13 ± 0.11	1.01 ± 0.05	1.17 ± 0.01	0.88 ± 0.12	1.25 ± 0.03^c	1.02 ± 0.01
0.6	125	3.63 ± 0.35^c	0.65 ± 0.01^c	0.84 ± 0.11	1.05 ± 0.01	1.67 ± 0.02	2.09 ± 0.15^c	1.03 ± 0.01	0.99 ± 0.01
0.6	250	3.52 ± 0.14^c	0.41 ± 0.00^c	0.90 ± 0.06	0.87 ± 0.02	2.09 ± 0.44	1.21 ± 0.04	1.29 ± 0.01^c	0.92 ± 0.01^b
0.6	500	4.37 ± 0.20^c	0.50 ± 0.03^c	0.80 ± 0.07	0.97 ± 0.06	4.81 ± 0.20^c	1.40 ± 0.06	1.19 ± 0.02	0.88 ± 0.01^c
3	0	2.97 ± 0.21^c	0.86 ± 0.04	1.68 ± 0.01^c	0.99 ± 0.04	1.14 ± 0.08	1.96 ± 0.12	1.00 ± 0.01	1.00 ± 0.00
3	125	3.57 ± 0.36^c	0.56 ± 0.04^c	0.94 ± 0.14	0.79 ± 0.03	1.29 ± 0.07	1.45 ± 0.07	1.13 ± 0.03	0.87 ± 0.01^c
3	250	2.89 ± 0.30^c	0.41 ± 0.06^c	0.79 ± 0.01	0.83 ± 0.04	1.88 ± 0.40	1.46 ± 0.14	1.37 ± 0.02^c	0.86 ± 0.00^c
3	500	4.34 ± 0.54^c	0.44 ± 0.04^c	0.73 ± 0.02	0.80 ± 0.03	1.79 ± 0.11	1.54 ± 0.18	1.43 ± 0.04^c	0.78 ± 0.02^c

[b]Significant difference compared with control (ANOVA) $p<0.05$

[c]Significant difference compared with control (ANOVA) $p<0.01$

stress, the Pro content decreased while the enzyme activity of CAT and POD increased for most treatments at the fourth month of stress under the combined stress of Cd and Pb. The change in the enzyme activity of SOD was not significant for all treatments.

A factorial analysis was also carried out to explore the interactive effect of Cd and Pb on the ADS and the results are shown in Fig. 4 and Table 5. Under the combined stress of Cd and Pb, the Pro content and SOD enzyme activity of *Capsicum annuum L.* showed no significant variations between the first and fourth month of stress, while there were slight differences in CAT and POD activities. The range of data point distribution of Cd stress was much smaller than that of Pb stress (Fig. 4a and e), and Cd stress exhibited similar or slightly less significant effects on CAT, POD and SOC enzyme activities than Pb stress. These results implied that the enzyme activity in the plant is more strongly affected by Pb than by Cd. The effect of Cd concentration on the enzyme activity of ADS was different from that of Pb concentration, indicating an obvious interaction between Cd and Pb in the combined heavy metal stress. As shown in Table 5, the η^2 of the factorial analysis followed the order of Pb > Cd × Pb > Cd at the first month of stress, except for the SOD activity, whose values were in a relatively narrow range. The η^2 for the Pro content and SOD activity at the fourth month of stress had a similar order to that at the first month of stress, while that for CAT and POD activities followed the order of Cd × Pb > Pb > Cd. Therefore, it can be concluded

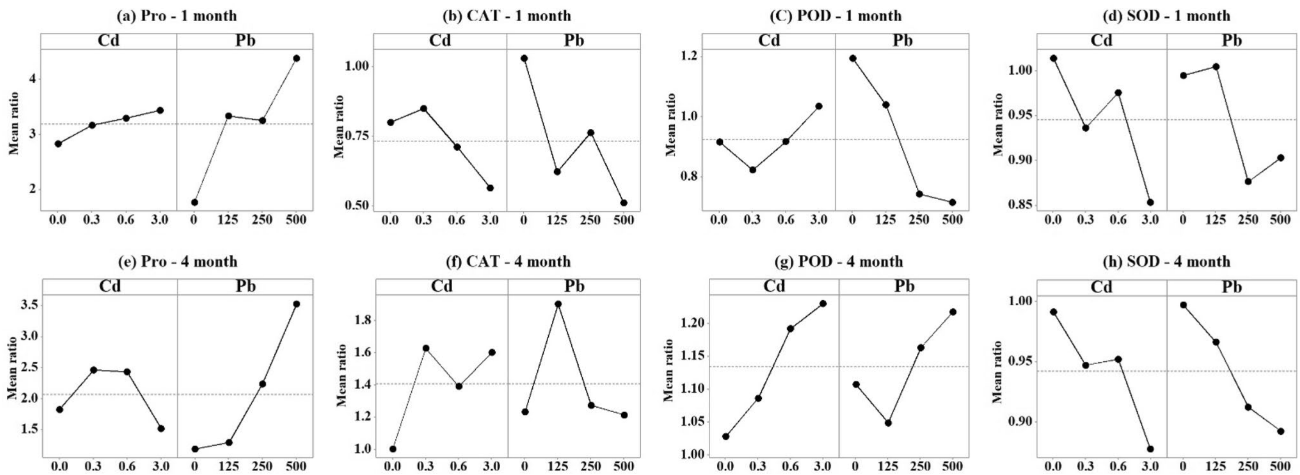

Fig. 4 Effects of combined Cd and Pb stress at different concentrations on the antioxidant system of *Capsicum annuum* L. with increasing heavy metal concentration and plant age. **a** and **e** content of Pro, **b** and **f** activity of CAT, **c** and **g** activity of POD, **d** and **h** activity of SOD. **a–d**: 1 month of stress, **d–h** 4 months of stress. Values with different letters are significantly different at $p < 0.05$ (Tukey test)

Table 5 Factorial analysis of factors influencing the antioxidative capacity

Factor		1st month of stress			4th month of stress		
		Mean effects	*p* value	η^2	Mean effects	*p* value	η^2
Pro	Cd	2.316	0.027	0.043	3.417	0.002	0.085
	Pb	10.051	0.000	0.704	10.014	0.000	0.583
	Cd*Pb	2.900	0.007	0.120	3.856	0.001	0.189
CAT	Cd	1.196	0.241	0.043	7.533	0.000	0.181
	Pb	5.700	0.000	0.444	8.703	0.000	0.237
	Cd*Pb	2.117	0.042	0.200	10.051	0.000	0.506
POD	Cd	2.410	0.022	0.054	4.458	0.000	0.225
	Pb	7.952	0.000	0.413	3.995	0.000	0.187
	Cd*Pb	5.717	0.000	0.375	4.047	0.000	0.344
SOD	Cd	5.514	0.000	0.290	8.347	0.000	0.290
	Pb	5.219	0.000	0.263	9.222	0.000	0.350
	Cd*Pb	3.293	0.002	0.231	5.997	0.000	0.259

that the Pro content is mainly controlled by Pb stress, and the effect of heavy metal stress on ADS could be enhanced by the interaction between Cd and Pb.

Heavy metal pollution of soil is a widespread environmental problem, which has a significant biological effect on the growth of crops (Lin and Aarts 2012; Srivastava et al. 2014). Under heavy metal stress, active metal cations can be adsorbed by the plant through the cation channels, and then affect the enzyme activity by interacting with each other (Riaz et al. 2021). In the present study, the effects of single and combined stress of Cd and Pb on PE (NPR, SC and ICDC; Figs. 1 and 3) and the enzyme activities of ADS (Pro, CAT, POD and SOD; Figs. 2 and 4) were comprehensively investigated. The ADS of plants primarily mitigates the damage of the tissues, which is more sensitive to injury than PE under the stress of heavy metals (Yan et al. 2007). The response mechanism of *Capsicum annuum L.* to heavy metal stress is illustrated by Fig. 5 and then discussed in detail in the following text.

Heavy metal stress can quickly cause oxidative damage in plant tissues through the excessive production of ROS such as H_2O_2, O_2^-, OH^- and $^1O_2$, which can disturb the homeostasis of ADS and lead to the accumulation of ROS in plants (Nagajyoti et al. 2010). The non-enzymatic and enzymatic ADS of plants will respond to the heavy metal stress to eliminate the ROS so as to avoid the oxidative damage. In this study, Pro was one of the most important antioxidants of non-enzymatic ADS, which can eliminate O_2^- and OH^- in cells (Hayat et al. 2012). With increasing heavy metal concentration, the Pro content increased dramatically, and at high heavy metal treatment (3 mg/kg Cd and 500 mg/kg Pb), it was increased by nearly three folds relative to that

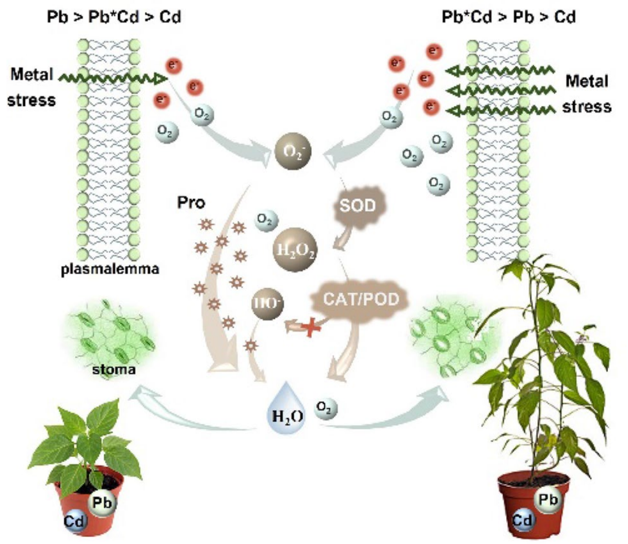

Fig. 5 Scheme for the response mechanism of ADS in *Capsicum annuum L.* to the combined stress of Pb and Cd

in the control group (Fig. 3). The SOD and CAT enzymes of the enzymatic ADS can directly reduce the ROS in the plant. It should be noted that the O_2 produced from photosynthesis can accept an electron to form O_2^-, which has relatively high oxidative toxicity to plant cells (Fig. 5). Two O_2^- can be catalyzed by SOD to produce one H_2O_2 and one O_2 in the disproportionation reaction. Then, H_2O_2 is quickly decomposed to H_2O and O_2 under the catalysis of CAT and POD (Gill and Tuteja 2010; Mittler 2002). These reactions can minimize the formation of OH^-. With increasing heavy metal concentration, the enzyme activities of CAT, POD and SOC in the plant were maintained at relatively high levels at the first month of stress.

With the increase in plant age, the adaptability of plants to heavy metal stress would gradually increase and the homeostasis of ADS would be enhanced, which can be reflected by changes in the content of antioxidants and the activities of antioxidant enzymes. The Pro content decreased remarkably at the fourth month of stress compared with that at the first month of stress. No obvious tendency was observed for the change in CAT enzyme activity, whose values were in a relatively narrow range. There were obvious increases in the enzyme activity of SOD for all treatments and POD under combined stress at high heavy metal treatment. These results indicate that at the first month of stress, non-enzymatic reaction of antioxidants is the main response mechanism for the elimination of ROS, while at the fourth month of stress, the reduction of ROS is predominantly controlled by the catalytic reaction of the antioxidant enzymes.

Under single heavy metal stress, Pb exerted a much stronger effect on PE and a slightly stronger effect on ADS than Cd. Under the combined stress of Cd and Pb, the PE and response of ADS showed remarkable decreases in a concentration-dependent manner. The factor analysis indicated that they are primarily controlled by Pb concentration. The non-enzymatic reaction of antioxidants such as Pro is the main response mechanism to mitigate the oxidative damage in cells induced by ROS. At the fourth month of stress, the PE increased, while the Pro (non-enzymatic ADS) content decreased dramatically under both single and combined heavy metal stress. In addition, the effect of heavy metal stress on the activity of enzymatic ADS was somewhat enhanced by the interaction between Cd and Pb. The findings of the present study may help to explore the interaction between Cd and Pb in the combined stress and understand their accumulation in plants. The reduction of ROS is predominantly controlled by the catalytic reaction of various antioxidant enzymes such as CAT, POD and SOD.

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