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Phytotoxicity of cadmium on peroxidation, superoxide dismutase, catalase and peroxidase activities in growing peanut (*Arachis hypogaea* L.)

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A pot experiment treated with cadmium (Cd) was conducted to evaluate the physiological and yield responses of peanut to cadmium in different growth stages. The results indicated that the peanut treated with cadmium level of 12 mg/kg did not cause obvious visible toxic symptoms, while the antioxidant enzymes activities concluding superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) showed significant decrease in tested growth stages. The content of total chlorophyll decreased significantly in the growth stages ($P < 0.05$). The results indicated that Cd destroyed the balance of free radical metabolisms, which resulted in increasing malondialdehyde (MDA) content and the relative cell membrane permeability (RMP). The kernel yield and kernel rate per pot showed significant decrease under cadmium stress ($P < 0.05$). The varieties FengHua3, HuaYu20 and Luhua 12 showed more sensitive than the other varieties. The results indicated that the MDA, total chlorophyll content and RMP may be more sensitive or indicative than the others under Cadmium stress.

Key words: Peanut (*Arachis hypogaea* L.), cadmium, phytotoxicity, physiological mechanism.

INTRODUCTION

Of all the heavy metals, cadmium (Cd) is one of the most toxic heavy metals and a non-essential element that is present in the soil naturally and from anthropogenic sources, including atmospheric deposition, application of sewage sludge and manures, irrigation water, and in fertilizers and soil amendments (Mclaughlin et al., 2000; Sanità di Toppi and Gabbrielli, 1999). It has been demonstrated that Cd induces the formation of reactive oxygen species (ROS), which can damage biological molecules (DNA, RNA and proteins) and membranes by inducing lipid peroxidation and stimulate chlorophyll degradation (Unyayar et al., 2006). Protective enzymes include catalase (CAT, E.C. 1.11.1.6), peroxidases (POD, E.C.1.11.1.7), and superoxide dismutase (SOD, E.C. 1.15.1.1), while several molecules such as glutathione, ascorbate and carotenoids provide non-enzymatic protection (Kuo and Kao, 2004; Lombardi and Sebastiani, 2005).

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Abbreviations: SOD, Superoxide dismutase; **CAT,** catalase; **POD,** peroxidase; **MDA,** malondialdehyde; **Chl,** chlorophyll; **RMP,** relative cell membrane permeability.

Accumulation of H_2O_2 is prevented in the cell by CAT, POD, or by the ascorbate-glutathione cycle, where ascorbate peroxidase (APX) reduces it to H_2O (Das et al., 1997). Given the above cited mechanisms utilized by plants to detoxify ROS, it is important to establish what effect enzyme activity has on this detoxification process.

Peanut (*Arachis hypogaea* L.) is one of the major oil seed and economic crops. There are some previous studies on peanut under Cd stress. For instance, peanuts take up Cd mainly through the true root system rather than pod wall, and significant differences exist between cultivars of peanut in term of Cd accumulation in kernels from analysis of plants growing under field condition (Bell et al., 1997; Mclaughlin et al., 2000; Dinakar et al., 2008), a combination of soil amendments (lime, zinc, and organic matter additions) reduced phyto-available Cd and Cd uptake ($P<0.05$) by peanut plants (Bell et al., 2001). Most previous studies were carried out based on Cd uptake mainly (Wang and Zhang, 2008; Vecchia et al., 2005). However the metabolism of ROS, changes in oxidative status, and antioxidant responses involved in peanut under Cd stress were poorly reported. The purpose of this study was to examine the effects of soil Cd on the yield, oxidative stress, and antioxidant response of peanut (*A. hypogaea* L.) in different growth stages in pot experiment. Information from this study will be helpful to identify toxic critical values of Cd in soil based on physiological response.

MATERIALS AND METHODS

Plant material and treatment

Peanut varieties, including HuaYu22, LuHua14, FengHua3, HuaYu20, HuaYu23 and LuHua12 were collected from the Shandong Peanut Research Institute in Qingdao city, Shandong Province, PR China. Soil sample was collected from the farmland located on the Shandong Academy of Agricultural Sciences then air-dried, ground and sieved through 3 mm mesh sieve before use. Properties of the tested soil used in pot experiment were as follows, pH 7.08, available nitrogen 45.5 mg/Kg, available phosphorus 17.0 mg/Kg, available potassium 176 mg/Kg, organic matter 19.5 g/Kg, total cadmium 0.06 mg/Kg. Soil sample was mixed with $CdCl_2$ solutions at rate of 12 mg Cd^{2+} kg^{-1}dry soil in the treatment group. Soluble fertilizers (100 mg N, 150 mg P_2O_5, and 100 mg K_2O kg^{-1} dry soil) were applied at the same time. After sufficient mixing, soil was transferred into plastic pots and incubated for two weeks before the next step. Each variety of treatment and control was performed in 10 replications, respectively. Peanut seeds were sown in each pot and the pots were watered daily. The functional leaves of the main stem were taken in pod-setting stage, plump pod stage, and harvest stage, respectively. The yield per pot was measured in the harvest stage.

Enzyme assay

Enzyme extraction

Enzymes were extracted according to the modified method of Cho and Seo (2005). The fresh leaves from each treatment were homogenized in a pestle and mortar with 0.05 M sodium phosphate buffer (pH 7.5) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 12,000 g for 20 min and the supernatant was used for analyzing SOD, POD and CAT. The above steps were carried out at 4°C.

SOD assay

SOD activity was detected according to the modified method of Zhou et al. (2003). Reaction mixtures contained 81 mL of 14.5 mM methionine, 3 mL of 2.25 mM nitroblue tetrazolium chloride (NBT), 3 mL of 3 µM EDTA-Na_2 and 3 mL of 60 µM riboflavin, and these reagents above were prepared with 0.05 M sodium phosphate buffer (pH 7.8) except riboflavin which was prepared with deionized water. After adding 50 µL enzyme extract to 3 mL reaction mixtures in tubes, the reaction started by placing the tubes into illumination incubator for a duration of 30 min. The reaction was finished by keeping the tubes in the dark for 10 min. The absorbance was recorded at 560 nm using a spectrophotometer. One unit of SOD enzyme activity was defined as the quantity of SOD required to produce a 50% reduction of NBT under experimental conditions and the specific enzyme activity was expressed as units per g fresh weight of leaves (FW).

POD assay

The reaction mixtures, containing 75 mL of 100 mM sodium phosphate buffer (pH 6.0), 28.5 µL H_2O_2 (30%) and 42 µL guaiacol, were prepared immediately before use. 1 mL enzyme extract was then added to 3 mL reaction mixtures. Increasing in absorbance was measured at 470 nm at 1 min intervals up to 3 min using spectrophotometer. One unit of POD enzyme activity was defined as absorbance changes at 470 nm per minute. Enzyme specific activity was expressed as units per g FW (Xu et al., 2008).

CAT assay

CAT activity was determined by spectrophotometer in reaction mixture containing 1.5 mL of 0.05 M sodium phosphate buffer (pH 7.8), 1 mL deionized water and 0.3 mL of 0.1 M H_2O_2 prepared immediately before use, then 0.2 mL enzyme extract was added. CAT activity was measured by monitoring the decrease in absorbance at 240 nm as a consequence of H_2O_2 consumption. One unit of CAT enzyme activity was defined as changes at 240 nm per minute. Enzyme specific activity was expressed as units per g FW (Xu et al., 2008).

Total chlorophyll (Chl) assay

The fresh leaves from each treatment was homogenized in 5 mL of 80% acetone at 4°C, and added 15 mL acetone to a total of 20 mL in each tube. Tubes were stored in the dark at 4°C for 48 h prior to spectrophotometer measurements. Pigmentation of the sample was centrifuged (4000 g) for determination. The absorbance was measured at 470, 646 and 663 nm with spectrophotometer, respectively. Chlorophyll content was calculated according to Zhang and Zhai (2003).

Lipid peroxidation assay

The level of lipid peroxidation was expressed as the malondialdehyde (MDA) content in µM per g FW according to the method of Zhang et al. (2005). The fresh leaves from each treatment were homogenized in 5 mL of 10% trichloroacetic acid (TCA) with a pestle and mortar. Homogenates were centrifuged at 4000 g for

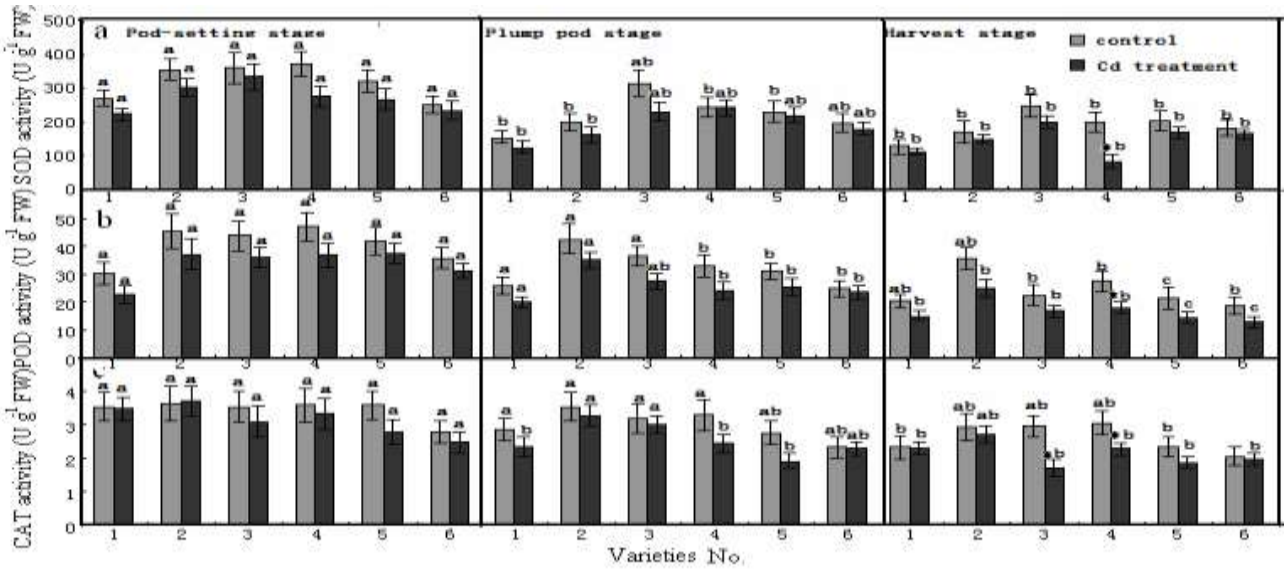

Figure 1. Effects of Cd treatment on activities of SOD, POD, and CAT in peanut leaves of different growth stages.

10 min. To each 2 mL aliquot of the supernatant, 2 mL of 0.6% thiobarbituric acid (TBA) in 10% TCA was added. The mixtures were heated at 100°C for 15 min and then quickly cooled in an ice bath. After centrifugation at 10,000 g for 10 min, the absorbance of the supernatant was recorded at 532 nm and 450 nm. The fresh leaves from each treatment were taken 0.2 g mesophyll sample from the 5th leaf which is fully expanded, then shred the sample and put it into a 20ml scale test tube, added 15 mL distilled water, put the test tube into the vacuum desiccator and pumped for 20 min. The conductance value of the maceration extract was tested by the electric conductivity meter. After that, the test tube was put into the boiling water to heat for 40 min and then test the conductance value again. The cell membrane permeability was expressed as relative electric conductivity (the conductance value of the osmotic electrolyte before boiled/ the conductance value of the osmotic electrolyte after boiled×100%).

Statistical analysis

Experimental results presented are the mean values of the experiments done in triplicates. Statistical analyses were performed with SPSS13.0 statistical program (SPSS Inc., Chicago, USA). The data was statistically analyzed using a two tailed T-test to comparepaired means between Cd treatment and control at 5 and 1% level of probability. Differences between growth stages were evaluated for significance by using Duncan's multiple range test (DMRT) ($P < 0.05$).

RESULTS

Effects of cadmium on the activities of antioxidant enzymes

The activities changes of antioxidant enzymes including SOD, POD, and CAT in different growth stages under cadmium stress were shown in Figure 1. Antioxidant enzymes activities in treatment samples generally were lower than in the control samples in different growth stages, and different among varieties, whereas it showed no significant difference compared to the control in growth stages, except that SOD, POD and CAT of the variety HuaYu20 and CAT of the variety FengHua3 under cadmium stress in harvest stage showed significant decrease to 45, 61, 78 and 62% of the control, respectively ($P < 0.05$, Figure 1). Antioxidant enzymes activities showed significant decrease in tested growth stages between control and treatment ($P < 0.05$, Figure 1), while the treatment did not significantly decrease slope compared to the control.

Effects of Cadmium on the content of total chlorophyll content

Table 1 showed the content of total chlorophyll content under cadmium stress in the growth stages. From the data in Table 1, we can see that the content of total chlorophyll decreased significantly in the cadmium treatment samples compared to the control and gradually decreased in the growth stages ($P < 0.05$, Table 1). In the pod-setting stage, the decrease rate of total chlorophyll content under cadmium stress showed no difference among varieties, about decreasing to 75%. However, in the harvest stage, the decrease rates showed difference among varieties ($P < 0.05$, Table 1). For instance, the content of variety HuaYu22 under cadmium decreased to 72% compared to the control, while the variety HuaYu20 decreased to 53%. The slope changes of the regression equation in the growth stages under cadmium stress showed difference among varieties. The variety FengHua3, HuaYu20 and LuHua12 showed significant decrease compared to the control ($P < 0.05$, Table 1), while the others showed slight increase.

Table 1. Total chlorophyll content of peanut in different growth stages under Cd stress (mg/g).

Varieties		Growth stage			Parameters of the regression equation		
		Pod-setting stage	Plump pod stage	Harvest stage	a	b	R^2
1	Control	5.72±0.41	5.29±0.51	4.01±0.41	-0.86	6.72	0.924
	Treatment	4.14±0.38*	3.08±0.45**	2.87±0.21*	-0.64	5.3	0.998
2	Control	4.79±0.46	4.3±0.51	3.39±0.37	-0.69	5.55	0.971
	Treatment	3.53±0.35*	2.77±0.31*	2.32±0.22*	-0.61	4.41	0.977
3	Control	6.15±0.55	4.92±0.53	4.32±0.22	-0.91	6.96	0.961
	Treatment	4.83±0.43*	3.08±0.54*	2.46±0.31**	-1.18*	5.83	0.929
4	Control	4.88±0.25	4.21±0.34	3.78±0.41	-0.55	5.39	0.986
	Treatment	3.68±0.33*	3.3±0.21*	1.98±0.25**	-0.85**	4.68	0.906
5	Control	4.53±0.49	3.78±0.41	2.93±0.32	-0.79	5.34	0.999
	Treatment	3.34±0.36*	2.75±0.35*	2.05±0.24*	-0.65	4.33	0.997
6	Control	4.77±0.48	4.47±0.45	3.06±0.29	-0.85	5.8	0.875
	Treatment	3.66±0.33*	2.82±0.31*	1.64±0.11**	-1.01*	4.72	0.991

Value is the mean of three individual triplicates±SD. *and**indicate the value that differs significantly from the control at $P < 0.05$ and $P < 0.01$ by T-test, respectively.

Effects of cadmium on the lipid peroxidation

MDA contents in peanut leaves of different growth stages are shown in Table 2. MDA contents increased gradually in different growth stage. MDA contents under cadmium stress generally increased compared to the control, especially the variety FengHua3, HuaYu20 and LuHua12 which MDA contents increased significant by 19, 11 and 18% compared to the control in harvest stage. The slope changes of the regression equation in the growth stages under cadmium stress did not show significant difference compared to the control, except for the variety FengHua3 and LuHua12 ($P< 0.05$, Table 2). Figure 2 showed the relative cell membrane permeability (RMP) of tested peanut leaves in harvest stage. The relative cell membrane permeability of peanut leaves under Cd stress increased significantly compared to the control ($P < 0.05$), and the variety HuaYu22 and LuHua12 increased to 43and 64% compared to the control, respectively (Figure 2).

Effects of cadmium on the yields

The yields of the tested varieties were shown in Table 3. The pot yield and pods number per pot in the Cadmium treatment generally showed no significant difference compared to the control, whereas the kernel yield and kernel rate per pot showed significant decrease under cadmium stress ($P < 0.05$, Table 3). Kernel yields among tested varieties decreased to the range from 58 to 85%. The variety HuaYu20 decreased the most to 58%, and the variety HuaYu22 decreased the least to 85%. The kernel rate in the average of the tested varieties decreased from 71.5 to 59% under cadmium stress. The most changes variety in kernel rate is the variety HuaYu20 which decreased from 72.3 to 52.9%, and the variety HuaYu22 decreased the least from 71.9 to 63.1%.

The component and cluster analysis under cadmium treatment in harvest stage

Principal component analysis of physiological change index in peanut leaves under Cd treatment in harvest stage was performed with SPSS13.0. In Table 4, we can see that the indexes of MDA, chlorophyll and RMP played important role in the first principle component, and the antioxidant enzymes played in the other two principal components. The cluster analysis result of the tested

varieties performed through the physiological and yield change index under Cd treatment in harvest stage were shown in Table 5. We can see that the varieties FengHua3, HuaYu20 and Luhua12 showed more sensitive than the other varieties.

DISCUSSION

Response of the antioxidant enzymatic system in peanut of Cd stress

In this study, there were no visible toxic symptoms in the

Table 2. MDA content in leaves of peanut in growth stages (μmol/gFW).

Varieties		Growth stage			Parameters of regression equation		
		Pod-setting stage	Plump pod stage	Harvest stage	a	b	R^2
1	Control	2.72±0.35	3.56±0.28	4.36±0.45	0.82	1.96	0.999
	Treatment	3.15±0.32	3.82±0.43	4.76±0.36	0.81	1.91	0.958
2	Control	3.63±0.38	4.46±0.36	5.35±0.47	0.86	2.76	0.999
	Treatment	3.71±0.25	4.89±0.39	5.56±0.55	0.93	2.87	0.975
3	Control	3.57±0.31	3.92±0.25	4.32±0.28	0.38	3.19	0.998
	Treatment	3.85±0.39	4.41±0.33	5.13±0.32*	0.64**	3.18	0.995
4	Control	3.06±0.39	3.93±0.44	4.69±0.29	0.81	2.26	0.999
	Treatment	3.57±0.42	4.45±0.51	5.21±0.36*	0.82	2.77	0.998
5	Control	2.68±0.35	3.43±0.48	4.35±0.36	0.84	1.82	0.997
	Treatment	3.03±0.41	3.72±0.42	4.68±0.45	0.83	2.29	0.982
6	Control	2.64±0.25	3.46±0.34	3.92±0.26	0.64	2.06	0.974
	Treatment	3.06±0.32	4.02±0.41	4.63±0.37*	0.79*	2.33	0.984

Value is the mean of three individual triplicates ± SD. *, **value that differs significantly from the control at $P < 0.05$ and $P < 0.01$ by T-test, respectively.

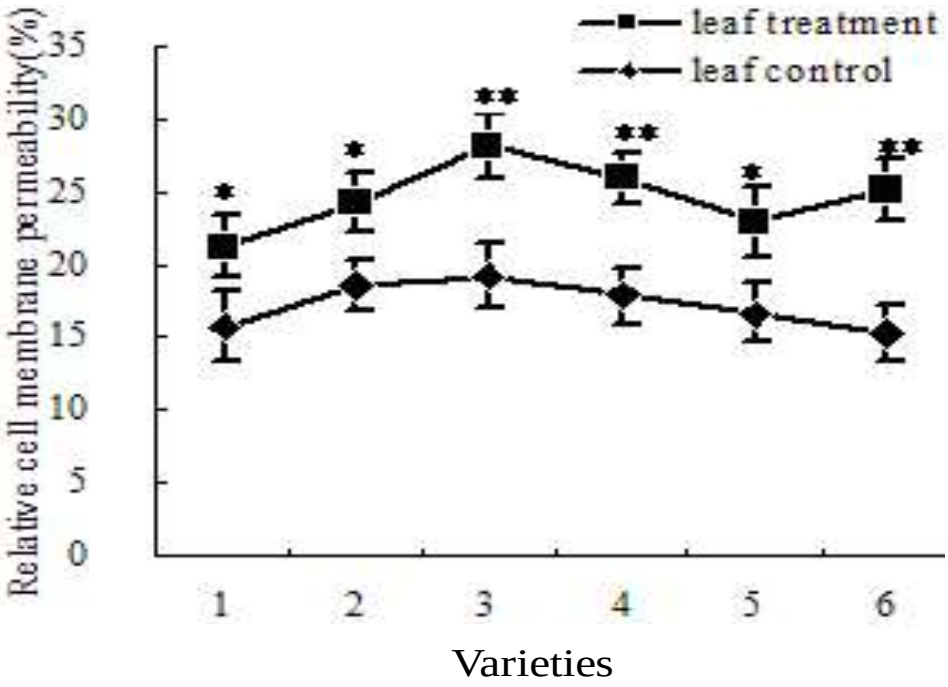

Figure 2. Effects of Cd treatment on the relative cell membrane permeability in leaves of peanut in harvest stage. *, **Value that differs significantly from the control at $P < 0.05$ and $P < 0.01$ by T-test, respectively.

peanut (data not shown), however a decrease of the antioxidant enzymes activities in leaves were observed at the Cd treatment of 12 mg/kg in tested growth stages (Figure 1). This may be attributed to the Cd treatment level of 12 mg/kg which has reached the high threshold values for inhibition of the antioxidant enzymatic system, resulting in the cooperative function of these antioxidant enzymes destroyed. Our results showed that the activity

Table 3. Effects of Cd treatment on the yield of peanut.

Item		Variety no.					
		1	2	3	4	5	6
Pot yield/g	Control	65.4±4.2	67.6±3.7	65.5±3.2	67.9±4.7	70.1±5.3	64.9±7.5
	Treatment	60.4±3.3	58.7±5.1	55.3±3.8*	59.1±4.9*	68.5±4.7	61.6±5.7
Kernel yield/g	Control	47.1±3.1	48.6±4.1	46.5±5.3	49.1±6.4	49.7±5.3	46.3±3.5
	Treatment	38.1±2.1	36.6±3.3*	31.1±3.6*	31.2±4.3**	42.1±3.8	35.7±3*
Pods number	Control	32.3±3.2	25±2.6	23.6±2.5	37.3±5.4	31.3±3.4	31±3.4
	Treatment	31.7±2.5	27.3±3.1	26±3.9	33.2±4.7	28±2.7	33.7±4.7
Kernel rate/%	Control	71.9±2.9	71.9±2.1	70.9±3.2	72.3±3.7	71.1±2.4	71.4±2.6
	Treatment	63.1±2.6*	62.4±2.2*	56.2±2**	52.9±2.5**	61.5±1.7*	57.9±3**

Value is the mean of three individual triplicates ± SD. *, **indicate the value that differs significantly from the control at $P < 0.05$ and $P < 0.01$ by T-test, respectively.

Table 4. Component Matrix of physiological change index in leaves of peanut under Cd treatment in harvest stage.

Component	MDA	RMP	Chl	SOD	POD	CAT
1	-0.784	-0.694	0.916	0.166	0.742	0.567
2	0.165	0.651	-0.325	-0.548	0.645	0.022
3	0.587	0.298	0.025	0.726	0.077	0.721

Table 5. Cluster analysis of the tested varieties under Cd treatment.

Cluster	Variety	Cd sensitivity
I	1, 2, 5	Less
II	3, 4, 6	More

of SOD in leaves of plump pop stage and harvest stage exhibited significant decrease compared to pop-setting stage ($P < 0.05$, Figure 1a). In addition, SOD activity under Cd treatment among varieties except variety HuaYu20 was not significantly decreased. This may be attributed to the excess production of superoxide, resulting in the destruction of existing enzyme pools or decreased expression of genes encoding SOD.

Our results showed that the POD and CAT activities in harvest stage decreased significantly compared to the pod-setting stage ($P < 0.05$, Figure 1b and c). What's more, both of them of the variety HuaYu20 treated with Cd showed significant decrease compared to the control. However, the CAT activity of the variety LuHua14 treated with Cd were a slight drop or raise in the different growth stages. This may indicate that the antioxidant enzymatic system respond differently among varieties. It is possible that oxidative stress caused by low-level Cd in plants led to increased expression and activities of antioxidant enzymes such as POD and CAT, while they were reduced by high-level Cd treatment.

Response of total chlorophyll content and yield of peanut to Cd stress

It is evident from this study as well as earlier investigations (Xu et al., 2008) that cadmium can alter both chlorophyll biosynthesis by inhibiting protochlorophyllide reductase and the photosynthetic electron transport by inhibiting the water-splitting enzyme located at the oxidizing site of photosystem II (Van and Clijsters, 1990a). The variety FengHua3, HuaYu20 and Luhua12 showed significant decrease compared to the control, while the others showed slight increase ($P < 0.05$, Table 1). Photosynthesis was hinder under Cd stress, which can result in declined significantly peanut yield ($P < 0.05$, Table 3). The pot yield and pods number per pot in the cadmium treatment generally showed no significant difference compared to the control, whereas the kernel yield and kernel rate per pot showed significant decrease under cadmium stress ($P < 0.05$, Table 3), and the variety HuaYu22, LuHua14 and HuaYu23 showed more Cd-tolerance than the others (Table 3). The results indicated that plump pop stage may be the main stage of Cd effects on peanut yield and the degree of Cd-tolerance was different among varieties.

Effects of cadmium on the lipid peroxidation

MDA content is commonly considered as a general indicator of lipid peroxidation as well as stress level (Chaoui et al., 1997). In this study, MDA content in leaves under Cd stress almost increased compared to the control and the degree of the increase were different among varieties (Table 2). Moreover, the relative cell membrane permeability of peanut leaves in harvest stage increased significantly compared to the control ($P<0.05$, Figure 2), especially variety FengHua3, HuaYu20 and Luhua12 ($P<0.01$, Figure 2). It is probable that coopera-tive function of the antioxidant enzymes was destroyed (Figure 1), which resulted in increasing the MDA content which contributed to destroy the cell membrane and increase the relative cell membrane permeability (Figure 2).

Conclusion

In this study, the peanut treated with cadmium level of 12 mg/kg did not cause obvious visible toxic symptoms, while the physiological and yield responses indicated that Cd inhibited the peanut yield and destroyed the balance of free radical metabolisms, which resulted in decreasing the synthesis of cholorophyl and yield, and increasing the degree of membrance lipid peroxidation. In addition, the plump pop stage may be the main stage of Cd effects on peanut yield and the degree of Cd-tolerance was different among varieties. The varieties FengHua3, HuaYu20 and LuHua12 showed more sensitive than the other varieties. Our results indicated that the MDA, total chlorophyll content and the relative cell membrane permeability may be more sensitive or indicative than the others.

Conflict of interest

Authors did not declare any conflict of interest.

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