Alleviative effects of exogenous AsA on Cd intimidation of rape seedlings

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Abstract

In the study, rape seedlings treated by AsA of different concentration(0.1. 1.0. 2.5. 5.0mmol·L^{-1}) were used to determine the biomass, chlorophyll and carotenoid content, malondialdehyde and proline content, SOD activity, POD activity, CAT activity, deoxidide AsA content in leaves.The results indicated that the 25mg·L^{-1}Cd treatment damage the growth of seedlings,chl.a and b content, carotenoid content, SOD, POD, CAT activity decrease significantly, MDA content increase.Alleviative effects of 0.1mmol·L^{-1}AsA on Cd intimidation is best, rape seedlings biomass, chlorophyll and carotenoid content, SOD activity, POD activity, CAT activity increase significantly,MDA content decrease. 2.5. 5.0mmol·L^{-1} AsA has done nothing to alleviate Cd intimidation, they do some damage to rape seedlings instead.

Key words: AsA; rape seedling; Cd intimidation; alleviation

1. Introduction

Cadmium(Cd) is one of the most toxic non-essential elements with high mobility. Cadmium directly or indirectly inhibits main physiological processes, such as photosynthesis, water relations, gas exchange, respiration and nitrogen assimilation, etc. Cd disturbs mineral nutrition of plant and even causes plant death. According to statistics, farmland contaminated by Cd has already reached $1.09\times10^4 hm^2$ (Qin and Wu, 1997). And this data even tends to go up. Oilseed rape has become an increasingly important crop in China and the world. It is also the exclusive oil crop in winter in China. Many developed countries like Astralia and America has already begun to promote oilseed production, which lead to dramatic competition in the international market. Accordingly, it is necessary to study how to alleviate Cd intimidation on oilseed rape and enhance its Cd endurance so as to improve the yield consequently. On the other hand, it was reported that many species or genotypes of *Brassica*, such as Indian Mustard, have strong Cd absoption(Salt and Prince et al., 1995),. Thus it is important to taking steps to enhance the growth and biomass of rape in order to improve the elimination of Cd in soil.

The Cd injury is also probably attributed to the alternation of oxidant level in the plants, as it has been observed that Cd caused the occurrence of activated oxygen and symptoms of oxidative injury. The presence of high concentration of active oxygen species(AOS), including superoxide radical($O_2^{\cdot}$), hydroxyl radical(OH) and hydrogen peroxide(H_2O_2), causes oxidative damage (Xiao and Zhang, 2004). Correspondingly plants will be induced to develop a defence and scavenging enzymes of active oxygen. One of the protective mechanisms is the enzymatic antioxidant system, which involves the sequential and simultaneous action of a number of enzymes including superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT). Endogenesis antioxidant-glutathione(GSH) and AsA play important roles in scavenging enzymes of active oxygen. It has been revealed that chloroplast depends on the circulation of AsA-GSH to resist the contamination from AOS(Zhang and Lei, et al., 2000). Therefore, it is significant to determine the change in AsA-induced enzymatic antioxidant system, such as SOD, POD and CAT activities, and the malondialdehyde (MDA) concentration, a general indicator of lipid peroxidation.

Previous research most indicated the damage mechanism of cadmium on oilseed rape, and research on how to alleviate cadmium intimidation has been taken into, but just some traditional measure were studied, and there are no reports on imposing antioxidant. In our previous study we found oilrape seedlings'growth were significantly inhibited when exposed to Cd stress. The experiment reported in this paper was undertaken to study the alleviative effects of extrinsic AsA on *Brassica napu*s grown in container intimidated by 25 mg·L^{-1} Cd, and filtrate one AsA concentration which has the most effective alleviation.

2. Materials and methods

Cultivar Qinyou 7 was used in this experiment. The seeds were first surface sterilized in 2% H_2O_2 for 10 min, rinsed many times with deionized water. Later they were dipped in distilled water for 6h and then germinated on silicon sand. When seedlings grew the first leaf(16 days old), they were selected for uniformity and transplanted onto the container(24.5cm×35.5cm) containing 6L hydroponic solution. These containers were covered with a foam board with 24 evenly spaced holes. In each hole one seedling was located. Seedlings grew in the greenhouse in Nanjing Aricultral University. In the first 6 days, seedlings were grown in 1/4 Hoagland solution and in 1/2 Hoagland solution for the next 6 days, and in normal on the 13th day. The composition of the basic nutrient solution was (mg·L^{-1}):KH_2PO_4 136.09; KNO_3 505.50; $Ca(NO_3)_2$ 1180.75; $MgSO_4$ 240.74; H_3BO_3 2.86; $MnCl_2{\cdot}4H_2O$ 1.81; $CuSO_4{\cdot}5H_2O$ 0.08; $(NH_4)_2MoO_4{\cdot}H_2O$ 0.02; $ZnSO_4{\cdot}7H_2O$ 0.22;

Fe-EDTA 26.40. The solutions were continuously aerated with an aquarium air pump and changed every 2d, and the solutions pH was adjusted every day to 6.0±0.5 with NaOH or HCl, as required. On the 18th day, Cd was added to the hydroponic medium as $CdCl_2$, 2d later, AsA was applied on seedlings leaves every 2d for 5 times, to form 5 concentrations: 0, 0.1, 1, 2.5, 5.0 mmol·L^{-1}. Seedlings grew 12d after AsA treatment and then all the indexes were determined.

The trial was arranged in a randomized design with 6 treatments (Table 1); each treatment was replicated three times for statistical purposes.

Table1 Cd and L-Ascorbic acid concentrations in each treatment

Treatment	Cd concentration(mg·L^{-1})	L-Ascorbic acid(mmol·L^{-1})
T_1	0	0
T_2	25	0
T_3	25	0.1
T_4	25	1.0
T_5	25	2.5
T_6	25	5.0

The upper second fully expanded leaves were sampled for analysis. The samples were washed with distilled water.

2.1. Biomass determination

Eighteen plants(6 plants per replicated) of each treatment were harvested and washed thoroughly with distilled water, wiped off water and separated into roots and shoots, then weighed fresh weight. After that, samples were killed at 105°C for 30 min, then dried at 80°C for 24h and weighed.

2.2. Chlorophyll and carotenoid content determination

Chlorophyll and carotenoid was extracted by homogenizing 0.1g fresh leaves in 10ml solution(ethanol/acetone=1/1). After being placed in dark for 10h, chlorophyll and carotenoid content was analyzed spectrophotometrically on the solution supernatant at 470,645,652nm.

2.3. Malondialdehyde determination

Lipid peroxidation was measured as the amount of MDA determined by the thiobarbituric acid(TBA) reaction as described by Shijie Zhao (1994). Leaf discs(0.6g) were homogenized in 6ml of 5%(w/v) trichloroacetic acid(TCA). The homogenate was centrifuged at 10000×*g* for 20 min. To 2ml of the resulting supernatant, 2ml 0.67%(w/v) TBA was added. The mixture was boiled for 30 min and then quickly cooled. The contents were centrifuged at 10000×*g* for10 min and the absorbance was measured at 450,532,600 nm. The concentration of MDA was calculated using an extinction coefficient of 155mM/ (L·cm).

2.4. Proline determination

2.5 Enzyme preparations and assays

Extracts for determination of CAT, SOD and POD activities were prepared from 0.5g of leaf discs homogenized under ice-cold conditions in 5ml of extraction buffer, containing 50mM phosphate buffer(Ph7.0), 1mM EDTA, 1g PVP, and 0.5(v/v) Triton X-100 at 4°C. Homogenates were centrifuged at 10000×g for 20 min controlling temperature during -4°C to 4°C and the supernatant fraction was used for assays.

Catalase (CAT) activity was determined in homogenates by measuring the decrease in absorption at 240nm(an extinction coefficient of 0.036$mm^{-1}cm^{-1}$) in a reaction medium containing 50mm potassium phosphate buffer(pH7.0) and 0.075%(w/v)H_2O_2 (Chance and Maehly, 1955). The pseudo-first order reaction constant (k′=k×[CAT]) of the decrease in H_2O_2 absorption was determined and the catalase content in pmol/mg protein was calculated (k=4.7×107/M per s.)

Superoxide dismutase (SOD) activity was assayed by using the photochemical nitroblue tetrazolium(NBT) method. In this assay, 1 unit of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50%.

Peroxidase (POD) activity was measured with guaiacol as the substrate in a total volume of 3ml. The reaction mixture consisted of 0.1mm acetum buffer (pH5.4), 0.25%(v/v) guaiacol, 0.075%(w/v) H_2O_2 and enzyme extract. Increase in the absorbance due to oxidation of guaiacol(E=25.5mm-1cm-1) was measured at 470nm. Enzyme activity was calculated in terms of μmol of guaiacol oxidized $min^{-1}g^{-1}$ fresh weight at 25±2°C.

2.6 Statistics

All data presented are the mean values. The measurement was done with three replicates on all parametres.The data were analyzed by one-way ANOVA inserted in the graphic program Origin. Letters were used to identify the levels of significance in the differences between treatments on the figures: a, b, c indicate significant difference at 5% probability level; A, B, C at 1% probability level.

3. Results and analyze

3.1 Growth characteristics of oil rape seedlings under treatments

Addition of 25mg·kg^{-1}(T_2) Cd in nutrient solution produced significant decrease in biomass. Compared with CK, plant dry weight (DW) and shoot DW decreased by 14.16% and 17.19%, respectively. However, Cd did not effect the root significantly. There was little difference between T_1 and T_2 treatments.

T_3(0.1mM AsA) treatment significantly increased the biomass compared to T_2. Plant DW showed a 36.97% increase, even heavier than that of T_1. It also showed in Table 2 that the shoot was affected more than the root. However, T_4(1.0mM AsA), T_5(2.5mM AsA) and T_6(5.0mM AsA) caused no significant increase compared to T_2 and cause significant decrease relative to T_1 instead.

Table 2 Effects of L-AsA on plant characteristics of Cd treated rape seedlings

Treatment	Plant DW (g/plant)	Shoot DW (g/plant)	Root DW (g/plant)
T_1	0.438bAB	0.349bB	0.089abAB
T_2	0.376cBC	0.289cBC	0.087aAB
T_3	0.515aA	0.426aA	0.089abA
T_4	0.308dC	0.238dC	0.070cB
T_5	0.375cBC	0.298cBC	0.077bcAB
T_6	0.391bcBC	0.296cBC	0.085abAB

Note: Those marked with a,b,c indicate significant difference at 5% probability level; those marked with A,B,C indicate significant difference at 1% probability level; The same below.

3.2 Leaf pigment contents

As shown in Table 3, the plants exposed to 25mg·kg-1(T_2) Cd showed statistically significant decrease in chlorophyll content and car content relative to T_1.

Table 3 Effects of L-AsA on chlorophyll and carotenoid of Cd treated rape seedlings

Treatment	Chla contents mg/g	Chlb contents mg/g	Chl contents mg/g	The ratio of chla and chlb	Car contents mg/g
T_1	1.061aA	0.397aA	1.458aA	2.857	0.239aA
T_2	0.415cdCD	0.106cB	0.521eE	3.919	0.119bB
T_3	0.904bB	0.285bA	1.189bB	3.240	0.246aA
T_4	0.322dD	0.086cB	0.407fF	3.762	0.084cB
T_5	0.513cC	0.140cB	0.653cC	3.660	0.121bB
T_6	0.466cC	0.117cB	0.583dD	3.982	0.111bB

The alleviative effect of T_3 treatment on leaf pigment is significant. T_3 increased leaf chlorophyll a, chlorophyll b and carotenoids by 117.83%,168.87% and 106.72%, respectively, compared to T_2. It was found that T_5, T_6 slightly prevented the decrease in leaf pigment, but not significant while T_4 even showed a decrease.

Chlorophyll a/b ratio accompanied all above changes. It is scales whether a leaf is intimidated or not. In comparison to T_1, The rise of the ratio reflected chlorophyll b decrease more than chlorophyll a under Cd stress. However, T_3 treatment could improve this ratio. This ratio gradually decreased with the increase of AsA concentrations.

3.3 MDA content

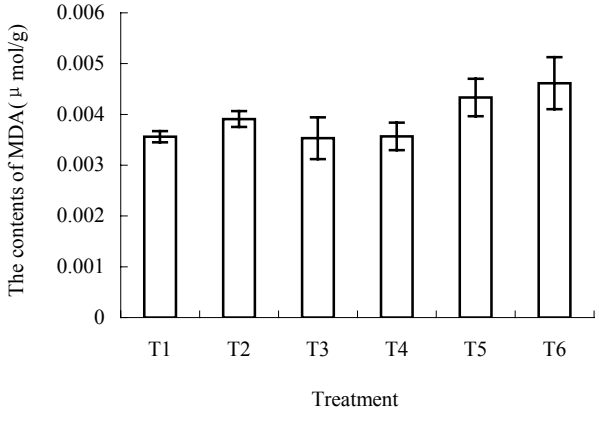

Fig.1 Effects of L-AsA on the contents of MDA in leaf of rape seedlings treated by Cd

Oxidative stress due to the existence of the nonredox heavy metal can be demonstrated by MDA content. MDA level is considered as an essential parameter in order to determine membrane injury. The oxidative damage in membrane, caused by mass accumulation of active oxygen species(AOS) such as superoxide radical ($O_2^{-}\cdot$) and hydrogen peroxide(H_2O_2), produces increase in MDA.

It is shown in Fig.1 that lipid peroxidation measures as MDA content was markedly raised by 9.74% over T_1 with T_2. T_3 and T_4 AsA restrained the increase and produced 9.67% and 8.77% decrease respectively instead, compared to T_2. However, with the increasing of AsA concentration, a large enhancement in MDA content in rape seedlings was observed in T_5 (10.85%), and significant increase (18.06%) was caused by T_6.

3.4 Proline content

Proline has been the subject of numerous reviews over the last 20 years. As a highly water soluble amino acid, proline may has dual function: (i) Proline accumulation is a common metabolic responses of plants to adversity; (ii) Proline is a adaption symbol to adversity and closely related to convers-succession-resistant capability of the plants (Bian and Chen, 1988). Proline protects membranes and proteins against the adverse effects of high concentrations of inorganic ions. Proline may also function as a protein-compatible hydrotrope and as a hydroxyl radical scavenger(Kaevi and Hong, 1995; Smirnoff and Cumbes,1989).

As shown in Fig.2, proline content in 25mg·kg^{-1} Cd treated seedlings (T_2) was raised 15.71% over T_1. Applying of 0.1mM AsA (T_3) produced a 15.40% increase in proline compared to T_2. With the increase of the applied AsA, proline content in seedlings decreased significantly.

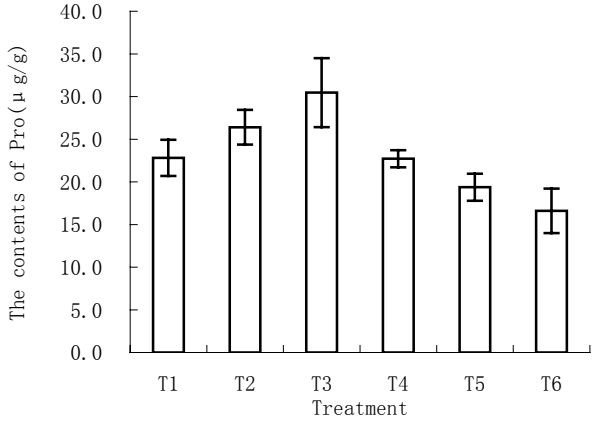

Fig.2 Effects of L-Ascorbic acid on the contents of Pro in leaf of rape seedlings treated by Cd

3.5 Enzymes involved in detoxification of AOS

3.5.1 CAT activity

Seedlings exposed to 25 mg·kg^{-1} Cd showed a significant decrease in CAT activity (Fig.3). The activity of CAT under T_3 treatment was significantly higher than that under T_2. There were no significant increase in T_4, T_5 and T_6 compared to T_2.

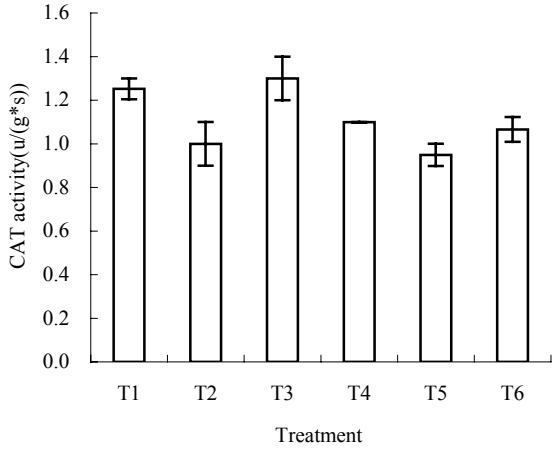

Fig.3 Effects of L-AsA on CAT activity in leaf of rape seedlings treated by Cd

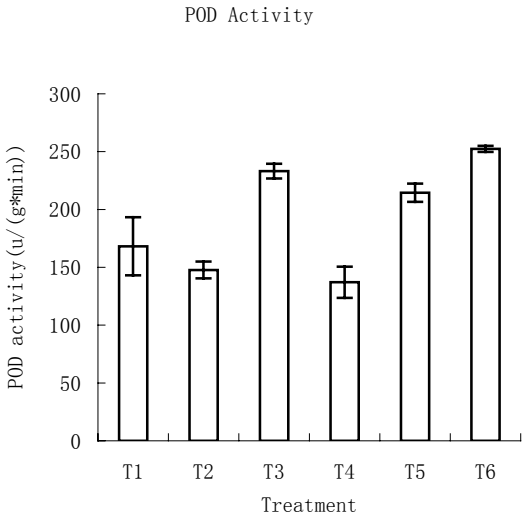

Fig.4 Effects of L-AsA on POD activity in leaf of rape seedlings treated by Cd

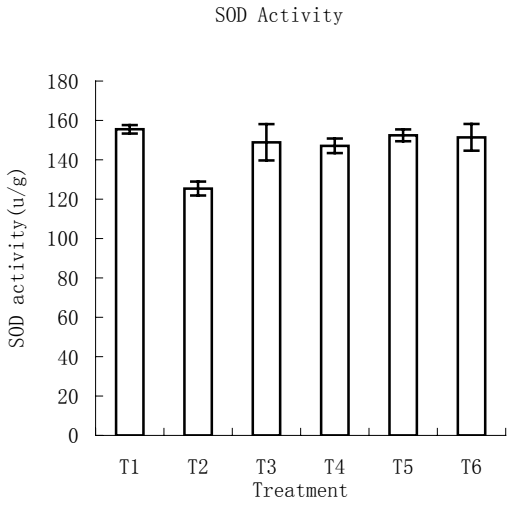

Fig.5 Effects of L-AsA on SOD activity in leaf of rape seedlings treated by Cd

3.5.2 POD activity

It is obtained from Fig.4 that significant increase in POD activity under T_3 treatment was observed compared with T_2, while there was no significant change under T_4 treatment. However, the increase of POD activity at higher AsA levels (T_5 and T_6) remained and was as muchu as SOD activity, that can be conferred that the damage of the high AsA level excitated the increase of POD activity.

3.5.3 SOD activity

Significant decrease in the activities of SOD was observed in seedlings to 25mg • kg^{-1} Cd ion when compared with T_1(Fig.5). Heavy metal ions decrease and the synthesis of isoenzyme was of a hindrance in plants to Cd adversity. Seedlings treated by AsA of each concentration(T_3,T_4,T_5,T_6) all showed significant increases in the activity of SOD compared to T_2. However, the AsA-induced changes in SOD activity was much smaller than that of POD and CAT, and no significant difference was observed between each AsA treatment.

4. Discussion

In the present study, the seedlings exposed to 25 mg • kg^{-1} Cd ion showed significant decrease in biomass accumulation, leaf pigment contents and increase in chlorophyll a/b ratio, MDA (Jiang and Zhou, 2006). The activity of the enzymatic antioxidant system(SOD, CAT, POD) decreased. It proved the previous finding that there is severe damage of the toxic element-Cd on plant growth at high concentration. Abiotic stress of Cd cause molecular damage to plant cells either directly or indirectly though the formation of AOS. This suggests that 25 mg • kg^{-1} Cd ion directly or indirectly leads to production of superoxide radicals, resulting in increasing lipid peroxidation(MDA), and oxidative stress in oilrape seedlings. Although cadium is a kind of nonredox heavy metal, it can induces the occurrence of activated oxygen in tissues (Gallego and Benavides, et al., 1986, Hendry and Baker, 1992).

At AsA concentration, 0.1mM as used in this work, a significant increase in biomass and leaf pigment contents with corresponding decrease in chlorophyll a/b ratio compared to T2. Measurement of MDA levels is routinely used as an index of lipid peroxidation under stress conditions. MDA concentration decreased significantly when Cd treated seedlings were subjected to 0.1 mM AsA treatment.

To mitivated and repair the damage initiated by oxygen, plants have developed a complex antioxidant system. The primary components of this system include free radical scavengers such as carotenoids, ascorbate, glutathione and tocopherols, as well as enzymes such as SOD, POD and CAT.

When the effects of 0.1mM AsA on antioxidant enzymes on Cd intimidation seedlings was studied, it was found that all of them were significantly increased. Endogenetic proline can also be a hydroxyl radical scavenger when plants expose to stess (Smirnoff and Cumbes, 1989). There was a 15.40% increase in 0.1mM AsA treated seedlings. From the change of all these indexes, it is implied that 0.1mM AsA enhanced the fastmess of oilrape seedlings, could alleviate the Cd intimidation.

Along with the increase of the AsA concentration, there were no significant change in biomass and chlorophyll content. On the contrary, MDA content was in markedly climbing direction. After 2.5, 5.0mm AsA used for the second time, burning speckle can be observed. It indicates that high AsA concentration cannot alleviate the Cd intimidation and damage the leaves instead in seedlings. The significant increase in POD activity in 1.0, 2.5, 5.0mM AsA treated seedlings were observed. It may a kind of self-protect response to leaf damage by high concentration AsA in these seedlings. However, in this study, SOD was

least affected by AsA. It may be suggested that SOD activity is probably compensated for by other isoperoxidases.

The results suggest that AsA can alleviate the Cd intimidation, and 0.1mM behaved the best alleviative effects. On the other hand, the higher concentration AsA was applied, the alleviative effect was worse. AsA with the excess concentration of 1.0mM has no alleviative effects and probably damage to rape seedlings. At the same time, in this study, AsA was applied on seedlings leaves every 2d for 5 times, whether that produced accumulating contamination need to be validated. Therefore, further research is needed on concentration and applying method in order to apply AsA to agriculture production properly.

References

BianYM, ChenSY, LiuSK, XieMY. (1988). Effects of HF on praline of some plants. Plant Physiology Communications **6**:19-21

Chance B, Maehly A C. (1955). Assays of catalase and peroxidase. In:Colowick S P, Kapalan N O(eds). Methods of Enzymology(VolII). New York: Academic Press, 764–775

Fridovich I. (1978). The biology of oxygen radical. Science **201**: 875-880.

Gallego S M,Benavides M P,Tomaro ML. (1986). Effect of heavy metal ion excess on sunflower leaves:evidence for involvement of oxidative stress. Plant Sci **121**: 151-159

Hendry G A F,Baker A J M,Swart C F. (1992). Cadmium tolerance and toxicity, oxygen radical processes and molecular damage in cadmium tolerant and cadmium-sensitive clones of Holcus lanatus L. Acta Bot Need **41**: 271-281.

Jiang HD, Zhou Q, Li N, Sun XF. (2006). Effect of Cd on the growth and physiological characteristics of rape seedling. Chinese journal of oil crop science **28**(1):39-43.

Kavi Kishor PB,Hong ZL,Miao GH,Hu C-AA,Verma DPS. (1995). Over expression of 1-pyrroline-5-carboxy late synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol **108**: 1387-1394.

Qin TC, Wu YS. (1997). Effects of cadmium, lead single and combination pollution on the contents of abscrobic acid in B rassica chinensis L.Chinese Journal of Ecology **16** (3): 31-34.

Ren A, Gao YB, Liu S. (2000). Effects of Cr, Cd and Pb on free praline content etc in leaves of Brassica Chinese L. Chin J Appl Environ Biol **6**(2):112-116.

Salt D, Prince R C, Pickering I J, et al. (1995). Mechanisms of cadium mobility and accumulation in Indian mustard. Plant Physiol. **109**: 1427-1433.

Smirnoff N, Cumbes QJ. (1989). Hydroxyl radical scavenging activity of compatible solutes. Phytochem. **28**(4): 1057-1060.

Tang ZC. (1989). The accumulation of free praline and its roles in water-stressed sorghum seedlings. Acta Phutophysiol Sinica. **15**: 105.

Wu FB, Zhang GP, Dominy P. (2003) Four barley genotypes respond differently to cadmium: lipid per-oxidation and activities of antioxidant capacity. Envir Exp Bot. **50**: 67-77.

Wu XH, Fu BL. (2005). Effects of different concentrations of cadmium on growth and antioxidant system in medicago sativa L. cv. Journal of Natural Science of Heilonjiang University. **22**(3):364-366.

Xiao YP, Zhang GP. (2004). Cadmium - induced Oxidative Stress and its Alleviation in Triticeae Crops. Barley Science. **1**:23-26.

Zhang YX, Lei XB, Yan JY. (2000). Effects of cadmium, nickel and mercury on the peroxidase isozymes of hordeum vulgar. Acta Agriculturae Boreali-Sinica **15**: 27-31.

Zhao SJ, Xu CC, Zou Q, et al. (1994). The improvement of measuring method of MDA, Plant Physiology Communications, **30**(3): 207-210.