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, 5.0 mmol·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 25 mg·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 0.1 mmol·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, 5.0 mmol·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 1.09×10⁴ hm² (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 Australia 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 absorption (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 (O₂⁻), hydroxyl radical (OH) and hydrogen peroxide (H₂O₂), 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 napus 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% H₂O₂ 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 (24.5 cm × 35.5 cm) 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 Agricultural 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): KH₂PO₄ 136.09; KNO₃ 505.50; Ca(NO₃)₂ 1180.75; MgSO₄ 240.74; H₃BO₃ 2.86; MnCl₂·4H₂O 1.81; CuSO₄·5H₂O 0.08; (NH₄)₂MoO₄·H₂O 0.02; ZnSO₄·7H₂O 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 CdCl₂, 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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cd concentration(mg·L⁻¹)</th>
<th>L-Ascorbic acid(mmol·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₁</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T₂</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>T₃</td>
<td>25</td>
<td>0.1</td>
</tr>
<tr>
<td>T₄</td>
<td>25</td>
<td>1.0</td>
</tr>
<tr>
<td>T₅</td>
<td>25</td>
<td>2.5</td>
</tr>
<tr>
<td>T₆</td>
<td>25</td>
<td>5.0</td>
</tr>
</tbody>
</table>

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 24 h 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 10 h, chlorophyll and carotenoid content was analyzed spectrophotometrically on the solution supernatant at 470, 645, 652 nm.

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 for 10 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 0.036mm⁻¹cm⁻¹) in a reaction medium containing 50mm potassium phosphate buffer (pH7.0) and 0.075%(w/v) H₂O₂ (Chance and Maehly, 1955). The pseudo-first order reaction constant (k′=k×[CAT]) of the decrease in H₂O₂ 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 photo reduction 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) H₂O₂ and enzyme extract. Increase in the absorbance due to oxidation of guaiacol (E=25.5mm·L·cm⁻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 parameters. 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\(^{-1}\) (T2) 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 T1 and T2 treatments.

T3 (0.1mM AsA) treatment significantly increased the biomass compared to T2. Plant DW showed a 36.97% increase, even heavier than that of T1. It also showed in Table 2 that the shoot was affected more than the root. However, T4 (1.0mM AsA), T5 (2.5mM AsA) and T6 (5.0mM AsA) caused no significant increase compared to T2 and cause significant decrease relative to T1 instead.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plant DW (g/plant)</th>
<th>Shoot DW (g/plant)</th>
<th>Root DW (g/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>0.438bAB</td>
<td>0.349B</td>
<td>0.089abAB</td>
</tr>
<tr>
<td>T2</td>
<td>0.376cBC</td>
<td>0.289cBC</td>
<td>0.087aAB</td>
</tr>
<tr>
<td>T3</td>
<td>0.515aA</td>
<td>0.426aA</td>
<td>0.089aA</td>
</tr>
<tr>
<td>T4</td>
<td>0.308dC</td>
<td>0.238dC</td>
<td>0.070cB</td>
</tr>
<tr>
<td>T5</td>
<td>0.375cBC</td>
<td>0.298cBC</td>
<td>0.077cAB</td>
</tr>
<tr>
<td>T6</td>
<td>0.391bcBC</td>
<td>0.296cBC</td>
<td>0.085abAB</td>
</tr>
</tbody>
</table>

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\(^{-1}\) (T2) Cd showed statistically significant decrease in chlorophyll content and car content relative to T1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chla contents (mg/g)</th>
<th>Chlb contents (mg/g)</th>
<th>Chl contents (mg/g)</th>
<th>The ratio of chla and chlb</th>
<th>Car contents (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>1.061aA</td>
<td>0.397aA</td>
<td>1.458aA</td>
<td>2.857</td>
<td>0.239aA</td>
</tr>
<tr>
<td>T2</td>
<td>0.415cdCD</td>
<td>0.106cB</td>
<td>0.521cE</td>
<td>3.919</td>
<td>0.119bB</td>
</tr>
<tr>
<td>T3</td>
<td>0.904bB</td>
<td>0.285aA</td>
<td>1.189bB</td>
<td>3.240</td>
<td>0.246aA</td>
</tr>
<tr>
<td>T4</td>
<td>0.322dD</td>
<td>0.086cB</td>
<td>0.407fF</td>
<td>3.762</td>
<td>0.084cB</td>
</tr>
<tr>
<td>T5</td>
<td>0.513cC</td>
<td>0.140cB</td>
<td>0.653cC</td>
<td>3.660</td>
<td>0.121bB</td>
</tr>
<tr>
<td>T6</td>
<td>0.466cC</td>
<td>0.117cB</td>
<td>0.583dD</td>
<td>3.982</td>
<td>0.111bB</td>
</tr>
</tbody>
</table>

The alleviative effect of T3 treatment on leaf pigment is significant. T3 increased leaf chlorophyll a, chlorophyll b and carotenoids by 117.83%,168.87% and 106.72%, respectively, compared to T2. It was found that T5, T6 slightly prevented the decrease in leaf pigment, but not significant while T4 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 T1, The rise of the ratio reflected chlorophyll b decrease more than chlorophyll a under Cd stress. However, T3 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](image)
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$_2^-$) and hydrogen peroxide (H$_2$O$_2$), produces increase in MDA.

It is shown in Fig. 1 that lipid peroxidation measures as MDA content was markedly raised by 9.74% over T1 with T2. T3 and T4 AsA restrained the increase and produced 9.67% and 8.77% decrease respectively instead, compared to T2. However, with the increasing of AsA concentration, a large enhancement in MDA content in rape seedlings was observed in T5 (10.85%), and significant increase (18.06%) was caused by T6.

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 (T2) was raised 15.71% over T1. Applying of 0.1mM AsA (T3) produced a 15.40% increase in proline compared to T2. With the increase of the applied AsA, proline content in seedlings decreased significantly.

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 T3 treatment was significantly higher than that under T2. There were no significant increase in T4, T5 and T6 compared to T2.
3.5.2 POD activity

It is obtained from Fig.4 that significant increase in POD activity under T3 treatment was observed compared with T2, while there was no significant change under T4 treatment. However, the increase of POD activity at higher AsA levels (T5 and T6) remained and was as much as much as SOD activity, that can be conferred that the damage of the high AsA level excited 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 T1 (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 (T3, T4, T5, T6) all showed significant increases in the activity of SOD compared to T2. 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 cadmium 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 fastness 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