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5-Aminolevolinic acid mitigates the cadmium-induced changes in *Brassica napus* as revealed by the biochemical and ultra-structural evaluation of roots

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ABSTRACT

In the present study, the ameliorating effects of 5-aminolevulinic acid (ALA) under cadmium (Cd) stress conditions were studied with special emphasis on root morphology and ultra-structure in oilseed rape. For this purpose, plants were treated hydroponically at three different Cd levels (0, 100, 500 μM) and foliar spray of ALA with three concentrations (0, 12.5, 25 mg/l) simultaneously. The results showed that foliar application of ALA improved the plant growth, root morphology and reduced the reactive oxygen species and malondialdehyde contents in roots under Cd stress conditions. The higher concentration of Cd (500 μM) decreased the activities of antioxidants enzymes like catalase (CAT), superoxide dismutase (SOD), peroxidase (POD) and glutathione reductase (GR) and also reduced the oxidized glutathione and total glutathione contents in roots. Application of ALA at 25 mg/l dosage significantly enhanced the antioxidant activities e.g. APX, SOD, POD, and GSH contents under Cd stress. The microscopic micrographs showed that application of exogenous ALA improved the cell structure under Cd toxicity. A whole cell with developed nucleus, nuclear membrane, smooth cell wall, continuous endoplasmic reticulum, and well shaped mitochondria was observed under the combine application of ALA and Cd. These results suggest that, application of ALA helped the plants to improve root growth, root antioxidant enzymes, and ultra-structural changes in root tip cells under fifteen days Cd-induced stress.

1. Introduction

Cadmium (Cd) has become a serious environmental threat due to wide use of pesticides, herbicides, fertilizers and expansion of industrialization (Folgar et al., 2009). Cd is most toxic element in the environment because even at low concentrations it is very toxic to living cells and that's why it is considered as carcinogen in human (Stohs et al., 2000). Absorption and accumulation of Cd caused the deficiency of Fe (II) in the roots because Cd inhibited the root Fe (III) reductase (Alcantara et al., 1994). Elevated levels of Cd can suppress the plant growth in terms of fresh and dry biomass (Daud et al., 2009). The relative decrease in root dry weights under Cd stress has been investigated in cotton (Bachir et al., 2004) and in wheat seedlings (Jalil et al., 1994). Cd increased the production of reactive oxygen species (ROS) and showed various toxic levels in plant cells; ultimately inhibit growth of plant by suppressing root elongation (Stohs and Bagchi 1995; Arisi et al., 2000; Schutzendubel et al., 2001). The toxicity of heavy metals can reduce surface area for water absorption by inhibiting the growth of root hairs (Gouia et al., 2000).

Heavy metals can damage the plant membrane and cell organelles; impair the growth and metabolic activities through the generation of ROS and free radicals in the plant cells (Sinha et al., 2009). Cd can cause the oxidative stress in the plant cells by production of superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (^-OH) (Hendry et al., 1992). Toxic species can cause lipid oxidation, membrane damage and inactivation of the enzymes due to reactions with proteins, lipids and nucleic acids. Absence of effective mechanisms which scavenge or remove these free radicals can cause degradation of protein, DNA breakage and cell death by lipid peroxidation (Thian and Li, 2006). In the plants there are a number of antioxidant systems which protect the plants from oxidative damage (Hasan et al., 2009). These antioxidants systems are comprised of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) (Dazy et al., 2009). Plants scavenge the oxidative stress by using enzymatic (including SOD, CAT and APX) and non-enzymatic antioxidants (ascorbate and glutathione) (Asada, 1999; Shah

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et al., 2001). Cd can change the anatomic and structural features of the cells which considered as a worst effect of Cd (Vitoria et al., 2004; Kupper et al., 2000). Liu et al. (1992) and Shah and Dubey (1995) observed the low mitotic index, cell division, cell proliferation and chromosomal aberrations in the various crops under the Cd stress. Moreover, an increase in the number of nucleoli and vacuoles, condensed cytoplasm, reduction in mitochondria, plasmolysis, enlarged vacuoles, disorganized chloroplast and ruptured nuclear envelope were found in root and leaf cells in different crops under the Cd stress (Liu and Kottke, 2004; Liu et al., 1995; Stoyanova and Chakalova, 1990).

Plant growth regulators (PGRs) play an important role in physiology of plants which include uptake of nutrients, stomatal movement, photosynthesis and activities of different enzymes under the different stress conditions (Hayat and Ahmad, 2007; Zhou and Leul, 1998, 1999). In recent years PGRs are use to increase the tolerance of plants against stress conditions (El-Tayeb, 2005; Zhang et al., 2006; Leul and Zhou, 1999). ALA has synergetic effect on the antioxidant enzymes and it can improve all enzymes activities significantly under the salinity stress (Naeem et al., 2011). Nishihara et al. (2003) reported that ALA regulates the activities of reduced glutathione and ascorbate in spinach. Liu et al. (2011) found that ALA at 0.1–10 mg/l can improve the GSH a total glutathione contents and GSH/GSSG ratio under water-deficit stress. Zhang et al. (2006) reported that ALA provides protection against oxidative damage of membranes. Foliar application of ALA can increase the length of chloroplast under the salinity stress in *Brassica napus* plants and it can reduce the oxidative stress and improve the cell structure (Naeem et al., 2012).

Oilseed rape (*B. napus* L.) is grown throughout the world for edible oil production. *Brassica* species are generally considered tolerant to heavy metals due to fast growth, higher biomass and ability of heavy metal absorption (Momoh and Zhou, 2001; Meng et al., 2009). Under heavy metals stress environment, *Brassica* plants employ different strategies against the metal toxicity through specific physiological mechanisms (Papazoglou et al., 2005). However, as far as we know, there are few reports about assessing the effects of exogenous application of ALA on oilseed rape (*B. napus* L.) under Cd stress conditions. Therefore, in the present study, the effects of exogenous ALA regarding the Cd-induced morphological, biochemical and ultra-structural changes in roots of *B. napus* L. cv. ZS 758 were studied.

2. Materials and methods

2.1. Plant material

Seeds of *B. napus* L. cv. ZS 758 were obtained from the College of Agriculture and Biotechnology, Zhejiang University and were sown in plastic pots (170 mm × 220 mm) filled with peat soil. After 30 days of sowing, morphologically uniform seedlings were selected and plugged these seedlings into plate holes on plastic pots (five plants per pot) containing a half strength nutrient solution (Arnon and Hoagland, 1940), aerated continuously with an air pump, in the green house. The pH of solution was adjusted to 6.0. The light intensity was in the range of 250–350 μmol m^{-2} s^{-1}, temperature was maintained at 16–20 °C and the relative humidity was approximately 55%–60%. Each treatment was replicated four times. The nutrient solution was renewed every five days. After acclimatization period of two weeks, solutions were adjusted to desired cadmium concentrations (0, 100 and 500 μM Cd) and plants were sprayed simultaneously with an aqueous solution of ALA (Cosmo Oil Co. Ltd., Tokyo, Japan) at the concentrations of (0, 12.5 and 25 mg/l ALA). The lower as well as the upper leaf surfaces were sprayed until wetted with a hand-held atomizer, as it was reported that absorption by the lower leaf surface was rapid and effective (Hull et al., 1975). Plants sprayed with distilled water served as control. After fifteen days of treatment, root samples for morphological, biochemical and microscopic studies were taken according to the procedure described below.

2.2. Morphological parameters

For measurement of plant biomass, six plants per treatment were weighed immediately after being harvested and then placed into an oven at 80 °C. The dried samples were weighed immediately after the removal from the oven until biomass become stable (Momoh and Zhou, 2001). Root surface area, volume, diameter and number of root tips of selected plants were determined using root automatism scan apparatus (MIN MAC, STD1600$^+$), equipped with WinRHIZO software offered by Regent Instruments Co. Average values of these four plants were considered as one replicate.

2.3. Analysis of lipid peroxidation and reactive oxygen species (ROS)

The oxidative damage to lipids was determined according to Zhou and Leul (1998) as lipid peroxidation in terms of malondialdehyde (MDA) production. Fresh roots (0.1 g) were homogenized and extracted in 10 ml of 0.25% TBA made in 10% trichloroacetic acid (TCA) then extract was heated at 95 °C for 30 min and cooled on ice. The samples were centrifuged at 5000 g for 10 min. The absorbance was measured at 532 nm. Correction of non specific turbidity was made by subtracting the absorbance value taken at 600 nm. The level of MDA was expressed as μmol g^{-1} fresh weight by using extinction coefficient of 155 mM cm^{-1}. For determination of hydrogen peroxide (H_2O_2) contents, roots (100 mg) were extracted with 5.0 ml of TCA (0.1%, w/v) in an ice bath, and the homogenate was centrifuged at 12,000 g for 15 min (Velikova et al., 2000). In 0.5 ml of the supernatant, 0.5 ml of phosphate buffer (pH 7.0) and 1.0 ml of potassium iodide (1 M) were added. The absorbance of the mixture was measured at 390 nm. H_2O_2 content was determined using extinction coefficient of 0.28 μM cm^{-1} and expressed as nmol g^{-1} FW.

Superoxide radical ($O_2^{\bullet-}$) was determined according to Jiang and Zhang (2001) method with some modifications. The sample of fresh roots (300 mg) was homogenized in 3 ml of 65 mM potassium phosphate buffer (pH 7.8) and then homogenate was centrifuged at 5000 g for 10 min at 4 °C. After that the supernatant (1 ml) was mixed with 0.9 ml of 65 mM potassium phosphate buffer (pH 7.8) and 0.1 ml of 10 mM hydroxylamine hydrochloride, and then incubated it at 25 °C for 24 h. After incubation 1 ml of 17 mM sulfanilamide and 1 ml of 7 mM a-naphthylamine was mixed in 1 ml solution for further 20 min at 25 °C. After incubation, n-butanol in the same volume was added and centrifuged at 1500 g for 5 min. The absorbance in the supernatant was read at 530 nm. Standard curve was used to calculate the generation rate of $O_2^{\bullet-}$. For estimation of extra-cellular hydroxyl radicals ($^{\bullet}$OH), 50 mg roots were incubated in 1 ml of 10 mM Na-phosphate buffer pH 7.4 consisting 15 mM 2-deoxy-D-ribose (SRL, Mumbai) at 37 °C for 2 h (Halliwell et al., 1987). Following incubation an aliquot of 0.7 ml from the above mixture were added to reaction mixture containing 3 ml of 0.5% (w/v) thiobirbuteric acid (TBA, Hi Media, Mumbai, 1% stock solution made in 5 mM NaOH) and 1 ml glacial acetic acid, heated at 100 °C in a water bath for 30 min and cooled down to 41 °C for 10 min before measurement. The absorbance of MDA was measured at 532 nm and concentration was calculated using an extinction coefficient (ε=155 mM cm^{-1}) and expressed in mmol g^{-1} FW.

2.4. Enzyme activities measurement

For enzyme activity, roots (0.6 g) were homogenized in 8 ml of 50 mM potassium phosphate buffer (pH 7.8) under ice cold conditions. Homogenate was centrifuged at 10,000 g for 20 min at 4 °C and the supernatant was used for the determination of the following enzyme activities. Total soluble protein content was determined by using the method of Bradford (1976) and bovine serum albumin was used as a standard. The assay for ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured in a reaction mixture of 3 ml containing 100 mM phosphate (pH 7), 0.1 mM EDTA-Na_2, 0.3 mM ascorbic acid, 0.06 mM H_2O_2 and 100 μl enzyme extract. The change in absorption was taken at 290 nm 30 s after addition of H_2O_2 (Nakano and Asada, 1981). Catalase (CAT, EC 1.11.1.6) activity was measured according to Aebi (1984) with the use of H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) for 1 min at A_{240} in 3 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA-Na_2, 10 mM H_2O_2 and 100 μl enzyme extract. Glutathione reductase (GR, EC 1.6.4.2) activity was assayed by Jiang and Zhang (2002) with the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM cm^{-1}) for 1 min. The reaction mixture was contained of 50 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA-Na_2, 0.15 mM NADPH, 0.5 mM GSSG and 100 μl enzyme extract in a 1 ml volume. The reaction was started by using NADPH. Total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined with the method of Zhang et al. (2008) following the inhibition of photochemical reduction due to nitro blue tetrazolium (NBT). The reaction mixture was comprised of 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 100 μl of enzyme extract in a 3-ml volume. One unit of SOD activity was measured as the amount of enzyme required to cause 50% inhibition of the NBT reduction measured at 560 nm. Peroxidase (POD, EC 1.11.1.7) activity was assayed by Zhou and Leul (1999) with some modifications. The reactant mixture was contained of 50 mM