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Ultrastructural, metabolic and proteomic changes in leaves of upland cotton in response to cadmium stress

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HIGHLIGHTS

- Cd deposited more in roots than leaves.
- Leaf's physiology and ultramorphology did not drastically alter.
- ROS-scavenging enzymes were active in leaves.
- ROS-combating and mitochondrial respiration related proteins were upregulated.
- Methionine synthase, involved in lignification process, was also upregulated.

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ABSTRACT

Present study explores physiological, biochemical and proteomic changes in leaves of upland cotton (ZMS-49) using 500 μM cadmium (Cd) along with control. Leaves' biomass and chlorophyll pigments decreased at 500 μM Cd. Cd contents in roots were higher than leaves. Levels of ROS (O_{2^-} and H_2O_2) both *in vivo* and *in vitro* and MDA contents were significantly increased. Chlorophyll parameters (F_0, F_m, F'_m and F_v/F_m), total soluble protein contents and APX showed a decline at 500 μM Cd. SOD, CAT and POD and GR activities significantly enhanced. Less ultrastructural alterations in leaves under Cd stress could be observed. Scanning micrographs at 500 μM Cd possessed less number of stomata as well as near absence of closed stomata. Cd could be located in cell wall, vacuoles and intracellular spaces. Important upregulated proteins were methionine synthase, ribulose 1,5-bisphosphate carboxylase, apoplastic anionic guaiacol peroxidase, glyceraldehydes-3-phosphate dehydrogenase (chloroplastic isoform) and ATP synthase D chain, (mitochondrial). Important downregulated proteins were seed storage proteins (vicilin and legumin), molecular chaperones (hsp70, chaperonin-60 alpha subunit; putative protein disulfide isomerase), ATP-dependent Clp protease, ribulose-1,5-bisphophate carboxylase/oxygenase large subunit. Increase in the activities of ROS-scavenging enzymes, less ultrastructural modification, Cd-deposition in dead parts of cells as well as active regulation of different proteins showed Cd-resistant nature of ZMS-49.

1. Introduction

Cadmium (Cd) is a toxic pollutant, which mainly comes from different sources such as pesticides, mining and chemical fertilizers (Daud et al., 2013a) into environment. In comparison with animals, plants are more vulnerable to Cd stress (Daud et al., 2013b). In plants, it influences several physiological events such as water uptake, nutrient assimilation, photosynthesis and respiration (Lage-Pinto et al., 2008). At ultramorphological level, a number of sub-cellular alterations in roots and leaves under Cd stress have also been documented by Daud et al. (2009a) and Daud et al. (2009b). Cd can be deposited in different subcellular organelles. Knowledge about its deposition and distribution in cellular compartments is gained through energy-dispersive X-ray analyses (EDX) and electron energy loss spectroscopy (EELS) attached with transmission and scanning electron microscopy.

Cd also disturbs the biochemistry of plant cells by causing stressful conditions (Lannig et al., 2006). Resultantly, reactive

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; (EDX), energy-dispersive X-ray analysis; H_2O_2, hydrogen peroxide; OH^-, hydroxyl radical; MDA, malondialdehyde; POD, guaiacol peroxidase; ROS, reactive oxygen species; SEM, scanning electron microscopy; SOD, superoxide dismutase; O_2^-, superoxide radical; TEM, transmission electron microscopy.

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oxygen species (ROS) of toxic nature are generated (Ahsan et al., 2009). To avoid or to reduce their production, various mechanisms regarding its absorption and uptake, sequestration and synthesizing antioxidant molecules (Jin et al., 2008) become active. Important one is the activation of antioxidant defense mechanism, which is mainly comprised of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) etc. Alternatively, Cd greatly disturbs the expression levels of various proteins. How various proteins are up and down-regulated can be better understood through proteomic studies. These studies are based on the systematic analysis and documentation of expressed proteins and their study at the functional level (Nwugo and Huerta, 2011).

Present study was designed to explore physiological, biochemical and proteomic changes in leaves of upland cotton induced by Cd stress. This is first comprehensive study by employing physiological, ultramorphological, biochemical and proteomic approaches in order to have a deeper look about various mechanisms being active in cotton leaves under Cd stress.

2. Materials and methods

2.1. Plant materials and growth conditions

An upland cotton cultivar (ZMS-49, kindly provided by Chinese Academy of Agricultural Sciences), was used in the present growth chamber experiment. Uniform grade matured seeds were surface sterilized by first immersing in 70% ethanol for 3 min and then in 0.1% $HgCl_2$ for 8–10 min. They were washed with ddH_2O for three times and soaked overnight in dH_2O. Sterilized seeds were sown in a mixture of peat and vermiculite (7:3, v:v) and grown in a growth chamber for ten days. Complete dark conditions were provided for three days and for seven days 16 h photoperiod of 50 μmol m^{-2} s^{-1} under white fluorescent light was provided. At eleventh day, uniformly grown seedlings were washed thoroughly with care and were grown in modified Hoagland solution for four hours for acclimatization. Then seedlings were transferred to fresh medium having 500 μM Cd as $CdCl_2{\cdot}2.5H_2O$. Medium without Cd salt acted as control. Seedlings were grown in the Cd stressful conditions for 24 h. At the end of stress period, seedlings were divided into roots, stems and leaves. Leaves were subjected to various physiological, ultrastructural, biochemical, and proteomic studies. For each study, separate independent experiments were run. There were kept three replications per each level. Throughout experiment 28 ± 2 °C culture temperature as well as 60% relative humidity were maintained.

2.2. Measurements of biomass, photosynthetic pigments and Cd contents

After 24 h Cd stress, leaves were subjected to biomass, photosynthetic pigments and Cd analyses. For each measurement, there were kept three replications with different number of plants. Regarding fresh and dry biomasses, three plants per replication were taken. In order to quantify photosynthetic pigments, 0.1 g fresh leaves/replication was used to determine the chlorophyll pigment composition. 80% acetone was used to extract the pigments, which were determined spectrophotometerically using the method of Ahammed et al. (2012).

For Cd contents analysis in both roots and leaves, the number of plants per replication was fifteen. Seedlings were washed with tap and distilled water thrice, respectively. Roots were immersed in 20 mM EDTA-Na_2 for 15 min in order to remove adhering metals and were washed with dH_2O for three- four times. After that, seedlings were blotted dry to remove the excessive water and were separated into roots and leaves, oven dried at 80 °C for 48 h, and then ground into powder. 0.2 g of each root and shoot samples were digested with a mixture of 5 ml of HNO_3 + 1 ml of $HClO_4$. The resultant solutions were diluted to 25 ml using 2% HNO_3 and then filtered. The concentrations of Cd in the filtrate were determined using inductively coupled plasma atomic emission spectroscope (ICP-AES, IRIS/AP optical emission spectrometer, Thermo Jarrel Ash, San Jose, CA) following standard procedures.

2.3. In situ tissue localization of reactive oxygen species

In situ localization of ROS (i.e. $O_2^{\cdot-}$ and H_2O_2) was done according to Bernstein et al. (2010) with some modifications. Briefly, leaves were detached from the stem and washed for three times in ddH_2O and were subjected to staining for both superoxide and hydrogen peroxide. For superoxide localization, NBT was used. Leaves were gently vacuum-infiltrated (5 min) with 0.01% NBT solution and incubated in the dark in the same solution for 2 h at 30 °C under very slow shaking. To determine that this staining was due to the formation of $O_2^{\cdot-}$, 10 mM $MnCl_2$ was added together with NBT as a negative control. After staining for 2 h, chlorophyll was removed from the tissue by boiling the segments in a 9:1 solution of 99% ethanol and glycerin for 10 min. Leaves were observed for the presence of blue spots, which show the presence of superoxide.

For H_2O_2 detection in leaves, 3, 3-diaminobenzidine (DAB) was used as staining agent. Leaves were gently vacuum-infiltrated with 1 mg ml^{-1} DAB solution for 10 min and incubated in the dark in the same solution for 4 h at 30 °C under very slow shaking. As a negative control, 200 μM diphenyleneiodonium (DPI) was added together with DAB. After staining, the chlorophyll was removed from the leaf tissue by boiling the segments in 95% ethanol for 10 min.

Brown spots were searched, which are the characteristics of the presence of H_2O_2. Three replications per treatment were done for each type of localization studies and stained segments were photographed with digital camera to obtain images.

2.4. Quantification of MDA contents and ROS

0.5 g leaves materials of young seedlings of ZMS-49 were used for the determination of lipid peroxidation and reactive oxygen species. Lipid peroxidation was estimated in terms of malondialdehyde (MDA) contents and was determined as 2-thiobarbituric acid (TBA) reactive substances following the method of Zhou and Leul (1998).

For determination of hydrogen peroxide (H_2O_2) content, 0.5 g leaves were crushed with 5.0 ml of TCA (0.1%, w/v) in an ice cold conditions, and the homogenate was centrifuged at 14,000 g for 20 min (Velikova et al., 2000). In total 4 ml reaction mixture, there was added 1 ml supernatant, 1 ml phosphate buffer (pH 7.8) and 2 ml potassium iodide (1 M) and the absorbance was read at 390 nm. H_2O_2 content was determined using an extinction coefficient of 0.28 μM cm^{-1} and expressed as μmol g^{-1} FW.

Superoxide radical ($O_2^{\cdot-}$) content was determined according to Jiang and Zhang (2002) method with some modifications. Leaves samples (0.5 g) were homogenized in 3 ml of 50 mM potassium phosphate buffer (pH 7.8) and then homogenate was centrifuged at 10 000 g for 10 min at 4 °C. 1 ml of supernatant was mixed with 0.9 ml of 50 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 sulphanilamide 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 of supernatant was noted at 530 nm in spectrophotometer. Standard curve was used to calculate the production rate of $O_2^{\cdot-}$ in the leaves.