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Castasterone confers copper stress tolerance by regulating antioxidant enzyme responses, antioxidants, and amino acid balance in *B. juncea* seedlings

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ABSTRACT

The aim of the present study was to explore the effect of exogenous application of castasterone (CS) on physiologic and biochemical responses in *Brassica juncea* seedlings under copper (Cu) stress. Seeds were pre-soaked in different concentrations of CS and grown for 7 days under various levels of Cu. The exposure of *B. juncea* to higher levels of Cu led to decrease of morphologic parameters, with partial recovery of length and fresh weight in the CS pre-treated seedlings. Metal content was high in both roots and shoots under Cu exposure while the CS pre-treatment reduced the metal uptake. Accumulation of hydrogen peroxide (H_2O_2) and superoxide anion radical (O_2^-) were chosen as stress biomarker and higher levels of H_2O_2 (88.89%) and O_2^- (62.11%) showed the oxidative stress in metal treated *B. juncea* seedlings, however, CS pre-treatment reduced ROS accumulation in Cu-exposed seedlings. The Cu exposures lead to enhance the plant's enzymatic and non-enzymatic antioxidant system. It was observed that enzymatic activities of ascorbate peroxidase (APOX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), glutathione perxoidase (GPOX) and gultrathione-s-transferase increased while activity of monodehydroascorbate reductase (MDHAR) decreased under Cu stress. The pre-treatment with CS positively affected the activities of enzymes. RT-PCR analysis showed that mRNA transcript levels were correlated with total enzymatic activity of DHAR, GR, GST and GSH. Increase in the gene expression of *DHAR* (1.85 folds), *GR* (3.24 folds), *GST-1* (2.00 folds) and *GSH-S* (3.18 folds) was noticed with CS pre-treatment. Overall, the present study shows that Cu exposure induced severe oxidative stress in *B. juncea* plants and exogenous application of CS improved antioxidative defense system by modulating the ascorbate-glutathione cycle and amino acid metabolism.

1. Introduction

At lower concentrations, copper (Cu) is an essential micronutrient as it participates in various biological functions being a constituent of several enzymes and proteins. But a slight increase in the level of Cu in soil induces phytotoxic effects on plants. Being a redox active metal, it catalyzes the production of free radicals especially reactive oxygen species (ROS) like superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2) *via* Haber-Weiss and Fenton reactions. The ROS accumulation results in oxidative stress which damages the vital cellular components like DNA, proteins and lipids, and ultimately hampers the growth of plants (Poonam et al., 2014). To cope with this oxidative stress, plants have evolved complex antioxidant system comprising of both enzymatic and non-enzymatic antioxidants (Dat et al., 2000). Ascorbate-glutathione cycle plays an important role in quenching of ROS. Key antioxidant enzymes belonging to ascorbate-glutathione cycle are SOD, CAT, APX, DHAR and MDHAR. SOD dismutates $O_2^{\bullet-}$ to H_2O_2, which afterwards is scavenged by APX and CAT (Gill and Tuteja, 2010). Non-enzymatic antioxidants comprises various compounds like glutathione (GSH), ascorbic acid (AA), phenolic compounds and amino acids, these compounds show strong antioxidant capacity required to ameliorate the harmful effects of ROS (Gill and Tuteja, 2010). The mechanisms of Cu induced oxidative stress and antioxidant system need further investigation. The change in gene

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expression of various antioxidant enzymes provides a better understanding of enhancement in the activity of enzymes. It has been reported that overexpression of genes in plants helps in the improvement in stress tolerance (Fernandez-Garcia et al., 2014). There are no reports on gene expression on enzymes under the influence of Cu.

Various secondary metabolites and plant growth regulators also participates in the defense of plants. Brassinosteroids (BRs) are plant steroidal hormones with polyhydroxylated sterol structure resembling to animal steroid and show high activity even at low concentrations (Clouse, 2011). They are recognized to control various aspects of plant growth and development such as cell elongation, xylem differentiation, regulation of gene expression, protein synthesis, photosynthesis *etc.* (Clouse, 2011). Brassinosteroids are also well recognized for its role in abiotic stress tolerance including drought, salinity, temperature and heavy metal stress by increasing the activities of antioxidant enzymes and level of antioxidants (Kumar et al., 2010; Saini et al., 2015). Recent studies using genetic and biochemical techniques had identified BRs receptor, some key signaling molecule and gene expression related to BRs. Although much has been learned about role of different BRs in enhancing plant tolerance, the role of CS is not fully explored in this direction. CS is a C-28 BR and was isolated for the first time in 1982 from the insect gall of chestnut by Yokota et al. (1982). It has strong biological activity and plays an important role in growth and development of plants.

Brassica juncea L. *(B. juncea)* is an oil seed crop grown widely in India. It is known for its hyper accumulation potential for various heavy metals including Cu. Bearing in mind the reports pointing towards the toxicity produced by copper and considering the protective role of BRs against wide range of stresses; and importance of antioxidant system in plant defense, the present study was designed. This study explores variety of physiological, biochemical parameters and gene expression pattern associated with antioxidant system in *B. juncea* exposed to various levels of Cu. It also evaluates that whether pre-soaking of seeds with CS influences the antioxidant pool and associated glutathione enzymes under Cu stress in *B. juncea* seedlings, and also whether it affects the tolerance of *B. juncea* to this metal.

2. Materials and methods

2.1. Plant material, growth conditions and treatments

B. juncea L. (cv. RLC-1) seeds, used in present study, were procured from Punjab Agriculture University, Ludhiana, Punjab, India. Seeds were surface sterilized with 0.5% (v/v) sodium hypochlorite and then rinsed with double distilled water repeatedly. Cu in the form of copper sulfate ($CuSO_4{\cdot}5H_2O$) was used. A concentration-response curve with *B. juncea* was prepared for workable concentration of Cu. IC_{50} of 0.5 mM of Cu was found to inhibit the germination and seedling growth significantly but not completely. Two more concentrations one below IC_{50} (0.25 mM) and one above IC_{50} (0.75 mM) were also used in the present study. The steroid concentration was selected on the basis of preliminary experiments. In autoclaved 90 mm-Petri plates lined with Whatman No. 1 filter paper, 20 seeds were sown and placed in seed germinator under controlled conditions of 25 ± 0.5 °C, 16 h photoperiod for 7 days. Seeds were soaked in different concentrations of CS (0, 10^{-11} M, 10^{-9} M and 10^{-7} M) for 8 h. Various concentrations of Cu ions were applied to seeds by adding 3 ml of solution to Petri plates. 1 ml of test solution of Cu was added every alternate day. Control with only distilled water was also maintained. On the 7th day, 10 seedlings were randomly selected from each Petri plate and the root, shoot length, and fresh weight were recorded. Each experiment was repeated twice with three replicates.

2.2. Determination of Cu content

After harvesting, seedlings were washed with deionized water followed by washing in 0.5 M EDTA and blotted on blotting paper to remove extra water. The seedlings were then separated into roots and shoots and dried in hot air oven for 24 h at 80 °C. Dried seedlings parts (1 g) were digested with solution of HNO_3 and $HClO_4$ (2:1 ratio) using the method given by Allen et al. (1976). The mixture was placed over hot plate at the temperature 90–95 °C until the clear solution was left. This solution was cooled and diluted with double distilled water (DDW) to make the final volume of 100 ml. The samples were then analyzed in triplicate for Cu content using atomic absorption spectroscopy (Agilent 240FS AAS).

2.3. Reactive oxygen species analysis

The hydrogen peroxide content was estimated using method proposed by Jana and Choudhuri (1981). Fresh seedlings (1 g) were homogenized in 50 mM phosphate buffer (pH 6.5) followed by centrifugation at 6000 rpm for 20 min at 4 °C. The obtained extract was mixed with 0.1% titanium sulfate in 20% (v/v) H_2SO_4 and centrifuged at 6000 rpm for 15 min. The absorbance of yellow colored supernatant was observed at 410 nm·H_2O_2 content was calculated using extinction coefficient 0.28 $\mu mol^{-1}\ cm^{-1}$. The superoxide anion content was determined following the method of Wu et al. (2010) with some modifications. 1 g of seedlings were homogenized in phosphate buffer (50 mM, pH 7.8) and centrifuged for 15 min at 4 °C at 13,000 rpm. 0.5 ml of supernatant was mixed with 0.5 ml of phosphate buffer and 0.1 ml of Hydroxylamine hydrochloride and reaction mixture was incubated at 25 °C for 30 min. After incubation, 1 ml of incubated solution was mixed with 1 ml of 3-aminobenzene and 1 ml of 1-napthylamine. The reaction mixture was again incubated at 25 °C for 30 min. The absorbance was noted at 530 nm and superoxide anion content was calculated from standard curve prepared using $NaNO_2$.

2.4. Localization of hydrogen peroxide production

A fluorescent dye 2′7′-dichlorofluorescein diacetate (2′7′-DCF-DA) was used to monitor the production rate of H_2O_2 in 7 days old seedlings of *B. juncea* (Murata et al., 2001). Intact roots of *B. juncea* seedlings were stained by submerging the roots in 10 μM fluorescent dye for 15 min and water mounted sections were observed under confocal laser scanning microscope (Nikon A1R confocal laser scanning microscope, CLSM, Japan) at excitation wavelength of 495 nm and emission wavelength of 530 nm. Intensity of green color in roots represents the rate of H_2O_2 production, thus more green color means more H_2O_2 production.

2.5. Determination of antioxidant enzyme activities

Shoots of 7 days old seedlings (1 g) were homogenized in 50 mM of potassium phosphate buffer (PPB, pH 7.0) with addition of 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM EDTA, 0.5% triton-X and 2% PVP using pre-chilled pestle and mortar. In case of monodehydroascorbate reductase activity (MDHAR), 1 g of seedlings was homogenized in Tris-HCl buffer (50 mM, pH 7.6) containing 2.5 mM ascorbic acid. The extract was centrifuged at 13,000 rpm for 20 min and obtained supernatant was used to measure activities of enzymes. The protein content in the enzyme extract was calculated according to Bradford (1976) using BSA (bovine serum albumin) as the standard.

Ascorbate peroxidase activity (APX; E.C 1.11.1.11) was performed by using the method of Nakano and Asada (1981). The reaction mixture (3 ml) contained 50 mM PPB (pH 7.0), 1 mM H_2O_2, 0.5 mM ascorbate and enzyme extract. The decrease in optical density was noticed at 290 nm within 1 min and specific activity (μmol units per minute per milligram protein) was calculated using extinction coefficient 2.8 $mM^{-1}\ cm^{-1}$. Glutathione reductase (GR; E.C. 1.6.4.2) activity was measured by following the method proposed by Carlberg and Mannervik (1975). Reaction mixture (3 ml) contained 50 mM PPB (pH