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Oxidative damages induced by short-term exposure to cadmium in bean plants: Protective role of salicylic acid

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ABSTRACT

The role of salicylic acid (SA) in alleviating cadmium (Cd) toxicity was investigated in a hydroponic cultivation system. Short-term exposure of bean (*Phaseolus vulgaris*) plants to 20 μM Cd inhibited biomass production and intensively increased accumulation of Cd in both roots and leaves. At leaf level, Cd significantly decreased mineral ions, chlorophyll and carotenoids concentrations. Concomitantly, Cd enhanced electrolyte leakage, H_2O_2 content and lipid peroxidation as indicated by malondialdehyde (MDA) accumulation. SA pretreatment decreased the uptake and the transport of Cd, alleviated the Cd-induced inhibition of nutrient absorption and led to a significant increase of chlorophyll and carotenoid content. SA application alleviated the oxidative damages as evidenced by the lowered H_2O_2 and MDA content. SA particularly induced an increase in both CAT and APX activities accompanied by a significant reduction in SOD and POD activities. As important antioxidants, ascorbate and glutathione contents in bean leaves exposed to cadmium were significantly decreased by SA treatment. These results reveal the potentiating effect of salicylic acid in regulating cadmium induced oxidative stress in bean plants.

1. Introduction

Cadmium (Cd) is an important environmental pollutant and a potent toxicant to organisms, including plants (Harminder et al., 2008). Its toxic symptoms in plants can be linked to changes in morphological, physiological and biochemical processes, leading to growth perturbation and cell death (Seregin and Ivanov, 2001).

At the morphological level, an excessive amount of Cd causes stunted growth, root browning, leaf epinasty, chlorosis and necroses (Benavides et al., 2005). At cell level, intoxication by Cd induces oxidative stress as evidenced by enhanced lipid peroxidation, hydrogen peroxide (H_2O_2) generation and ion leakage (Smeets et al., 2005). Cd can enhance prooxidant status by decreasing the antioxidant glutathione (GSH) pool, activating calcium-dependent systems and affecting iron-mediated processes (Pinto et al., 2003). It can also disrupt photosynthetic electron chain, leading to oxygen radical production (Asada and Takahashi, 1987). Cd toxicity may result from the binding of metal to sulphydryl groups in proteins, leading to inhibition of activity or disruption of structure (Benavides et al., 2005; Romero-Puertas et al., 2004). Metal interaction with ligand groups of enzymes largely defines its toxicity and the inhibition of enzymes may be due to masking of catalytically-active groups (Das et al., 1997). To survive against Cd toxicity, the plants have evolved several protective mechanisms. One of them is the response of antioxidant enzymatic system, which involves the sequential and simultaneous action of a number of enzymes such as SOD, CAT, POD and APX and non-enzymatic scavengers such as glutathione (GSH), ascorbate (AsA), carotenoids (Cars) and α-tocopherol, that are responsible for scavenging excessively-accumulated reactive oxygen species (ROS) in plants under stress conditions (Shah et al., 2001). Among these defenses, SODs are a group of enzymes that accelerate the conversion of superoxide radicals to H_2O_2 (Fernández-Ocaña et al., 2011). CAT is one of the main H_2O_2-scavenging enzymes that dismutates H_2O_2 into H_2O and O_2 (Corpas et al., 1999). PODs are enzymes that catalyze the H_2O_2-dependent oxidation of a wide variety of substrates, mainly phenolics (Kawano, 2003). GSH, a disulfide reducer, plays a central role in protecting plants from ROS. It protects thiols of enzymes, regenerates AsA and reacts with singlet oxygen, H_2O_2 and hydroxyl radicals (Foyer, 1993). AsA is an important antioxidant that reacts not only with H_2O_2, but also with O_2^-, OH^- and lipid hydroperoxides (Ramachandra et al., 2004). Depending on metal concentrations, Cd can either inhibit or stimulate the activities of these antioxidants before visible symptoms of toxicity appear (Correa et al., 2006).

Salicylic acid (SA), a naturally occurring plant hormone, influences various physiological and biochemical functions in plants, acts as an important signaling molecule and has diverse effects on tolerance to

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; Cars, carotenoids; CAT, catalase; Chl, chlorophyll; GSH, glutathione; MDA, malondialdehyde; POD, guaiacol peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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biotic stress (Horvath et al., 2007). Its role in plant tolerance to abiotic stresses such as drought (Munne-Bosch and Penuelas, 2003), chilling (Janda et al., 1999; Kang and Saltveit, 2002), heavy metals (Belkhadi et al., 2010), heat (Larkindale and Knight, 2002) and osmotic stress (Borsani et al., 2001) has also been reported. In addition, SA could contribute to maintaining cellular redox homeostasis through the regulation of antioxidant enzyme activity (Slaymaker et al., 2002) and induction of the alternative respiratory pathway (Moore et al., 2002) as well as to regulating gene expression by inducing an RNA-dependent RNA polymerase that is important for post transcriptional gene silencing (Xie et al., 2001). Therefore the present study was undertaken to determine the physiological and biochemical changes in bean plant pretreated with SA during Cd-induced stress and to investigate the possible mediatory role of SA in protecting plants from Cd-induced oxidative stress.

2. Material and methods

2.1. Plant material and growth conditions

Bean seeds (*Phaseolus vulgaris*) were disinfected with 1% NaOCl for 5 min, then washed thoroughly with distilled water and germinated between wet paper towels at 24 °C in the dark. Four days after, obtained seedlings were transferred into plastic beakers (6 L capacity, 6 plants per beaker) filled with nutrient solution containing: 1.0 mM $MgSO_4$, 2.5 mM $Ca(NO_3)_2$, 1.0 mM KH_2PO_4, 2.0 mM KNO_3, 2.0 mM NH_4Cl, 50 μM EDTA–Fe–K, 30 μM H_3BO_3, 10 μM MnSO4, 1.0 μM $ZnSO_4$, 1.0 μM $CuSO_4$ and 30 μM $(NH4)_6Mo7O_{24}$. After an initial growth period of 7 days in different SA concentrations (10, 50 and 100 μM), treatments were performed by adding 20 μM $CdCl_2$ to the nutrient solution. Hence, eight treatments of five replicates each were established, including a control (neither SA-pretreated nor Cd-stressed), Cd-stressed only, SA-treated only and SA–Cd-stressed (pretreatment with SA and then Cd-stressed). Plants were grown in a growth chamber with a 16-hour photoperiod and a 25 °C/20 °C regime, irradiance of 150 μmol m^{-2} s^{-1} and 65–75% relative humidity. The nutrient solution was buffered to pH 5.5 with HCl/KOH, aerated and changed twice per week. After 3 days of Cd-treatment, all plant organs were harvested, thoroughly washed with water, soaked in 20 mM EDTA for 15 min to remove adsorbed metals on the root surfaces and rinsed with distilled water. For biochemical analyses, primary leaves were harvested and immediately stored in nitrogen liquid. In each treatment group, five plants were examined for biochemical analysis.

2.2. Plant growth parameters and water absorption capacity (WAC)

Samples were oven-dried at 90 °C for 15 min, kept at 70 °C for 24 h to obtain a constant weight and weighted for dry biomass. Root and leaf water absorption capacity (WAC) was calculated using the following formula;

$$\text{Water Absorption Capacity } (\%) = 100 \times (\text{FW} - \text{DW})/\text{FW}$$

where FW and DW are fresh weight and dry weight of the plant materials, respectively.

2.3. Determination of ion concentrations

Dry plant material was powdered and wet-digested in acid mixture (HNO_3:$HClO_4$, 3:1, v/v) at 100 °C. Ion concentrations were estimated by atomic absorption spectrophotometry (Perkin-Elmer, AAnalyst 300) using an air–acetylene flame. The translocation factor (TF) was calculated as TF = 100 × [Cd] leaf/[Cd] root (Ait et al., 2002).

2.4. Determination of chlorophyll and carotenoid concentrations

Leaf chlorophyll was extracted from 100 mg FW with 80% chilled acetone and estimated by the method of Arnon (1949). Carotenoid concentration in the same extract was calculated by the formula given by McKinney (1941).

2.5. Determination of antioxidative enzyme activities

Frozen leaf tissue (0.4 g) was homogenized in 4 mL ice-cold extraction buffer (50 mM potassium phosphate, pH 7.0, 4% PVP 40) using a pre-chilled mortar and pestle. The homogenate was squeezed through a nylon mesh and centrifuged for 30 min at 14,000 *g* at 4 °C. The supernatant was used for assays of the activities of SOD, CAT, POD and APX. All spectrophotometric analyses were conducted at 25 °C.

The activity of SOD (EC1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) following the method of Beauchamp and Fridovich (1971). The reaction mixture (1 mL) included 50 mM phosphate buffer (pH 7.4), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and 100 μL enzyme extract. The reaction was allowed to proceed for 15 min illuminated with fluorescent tubes. Absorbance of the reaction mixture was read at 560 nm. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of photochemical reduction of NBT. SOD activity was expressed as U mg^{-1} protein.

CAT (EC1.11.1.6) activity was assayed by the decomposition of hydrogen peroxide according to Aebi (1984). The reaction mixture (1 mL) consisted of 100 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1% H_2O_2 and 100 μL enzyme extract. The decrease of H_2O_2 was monitored at 240 nm and quantified by its molar extinction coefficient ($E = 39.4$ mM^{-1} cm^{-1}). CAT activity was expressed as μmol H_2O_2 min^{-1} mg^{-1} protein.

The activity of POD (EC1.11.1.7) was determined in terms of oxidation of guaiacol by measuring increase in absorbance at 470 nm (Chance and Maehly, 1955). The reaction solution (1 mL) was composed of 25 mM phosphate buffer (pH 7.0), 0.05% guaiacol, 1.0 mM H_2O_2 and 0.1 mM EDTA and 100 μL enzyme extract. The reaction was started by addition of H_2O_2 and the increase in absorbance was recorded at 470 nm ($E = 26.6$ mM^{-1} cm^{-1}). POD activity was expressed as U mg^{-1} protein.

APX (EC1.11.1.1) activity was determined by the method of Nakano and Asada (1981). The reaction mixture (2 mL) contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.3 mM ascorbate, 0.1 mM H_2O_2 and 100 μL enzyme extract. The reaction was initiated by addition of H_2O_2 and the oxidation rate of ascorbic acid was estimated by following the decrease in absorbance at 290 nm. Activity of APX was calculated by using the molar extinction coefficient for ascorbate ($E = 2.8$ mM^{-1} cm^{-1}). Activity was expressed as U mg^{-1} protein.

2.6. Determination of antioxidative compound concentrations

Glutathione (GSH) concentration was determined by the method of Ellman (1959) based on the development of a yellow color when 5,5-dithiobis-2-nitrobenzoic acid (DTNB) was added to compounds containing sulfhydryl groups. 500 μL tissue homogenate in phosphate buffer was added to 3 mL 4% (v/v) sulfosalicylic acid. The mixture was centrifuged at 3000 *g* for 15 min. Then, 500 μL supernatant were taken and added to Ellman's reagent. The absorbance was measured at 412 nm after 10 min of reaction. Total GSH concentration was expressed as μmol g^{-1}.

Ascorbate (AsA) concentration was determined spectrophotometrically by using dinitrophenyl-hydrazine according to Mukherjee and Choudhuri (1983). The assay is based on the reduction of Fe^{3+} to Fe^{2+} by AsA. Briefly, leaf samples were powdered in liquid nitrogen and extracted in 6% (w/v) trichloroacetic acid (TCA), 2% (w/v)