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Regulation of growth and antioxidant enzyme activities by 28-homobrassinolide in seedlings of *Raphanus sativus* L. under cadmium stress

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28-Homobrassinolide (28-HBL), a brassinosteroid is reported to play significant role in diverse physiological processes. It induces a range of cellular and adaptive responses to a range of environmental stresses. Cadmium (Cd) is a non-essential metal which alters various physiological processes and generates ROS, which can oxidize biological macromolecules and causes oxidative stress. This stress is generally overcome by the internal antioxidative defense system and stress shielding phytohormones. In this study, effect of 28-HBL was studied on growth and activities of antioxidant enzymes in known hyperaccumulator *Raphanus sativus* L. (radish) seedlings grown under cadmium (Cd) metal stress. To determine the influence of 28-HBL (0, 10^{-11}, 10^{-9}, 10^{-7} M) in radish seedlings subjected to Cd (0, 0.5, 1.0, 1.5 mM) stress, the activities of antioxidant enzymes (APOX, CAT, GR, POD and SOD) were analyzed. In addition, length and biomass of radish seedlings was also recorded. Cd toxicity resulted in reduced length, biomass, protein content and activities of antioxidant enzymes. 28-HBL treatments lowered the Cd toxicity by enhancing the activities of antioxidant enzymes, biomass and seedling length. The present study thus suggests a possible role of 28-HBL in amelioration of metal stress by regulating the activities of antioxidant enzymes in radish.

Keywords: 28-Homobrassinolide, Antioxidant enzymes, *Raphanus sativus*, Cd metal stress

Heavy metals toxicity is a worldwide problem of rising significance for ecological, evolutionary, and environmental rationale. Cadmium (Cd) is a relatively rare non-essential element and one of the most toxic environmental and industrial pollutants for animal and plants[1,2]. It is a byproduct of the mining and smelting, and is used in electroplating, nickel-cadmium batteries, PVC plastics, paint pigments, cigarettes and commercial fertilizers[3]. It is well documented that Cd phytotoxicity leads to reduction in the yield, seed germination, growth and development and inhibition of other plant metabolic activities such as respiration, photosynthesis, water relations and gas exchange[1,4,5]. Cd metal toxicity is mediated by the formation of reactive oxygen species (ROS)[6,7] and by the catalysis of the Haber-Weiss reaction[8]. ROS are highly toxic and can oxidize biological macromolecules such as nucleic acids, proteins and lipids, thereby disturbing the membrane permeability[9,10] and resulting in oxidative stress.

Several plant hormones like abscisic acid, ethylene, jasmonates and brassinosteroids (BRs) play a determinant role in plant defence signaling pathways, implicating oxidative stress[11]. However, BRs are unique in their activities for not only regulating the diverse physiological and morphogenetic responses in plants, but also having a significant role in amelioration of various biotic and abiotic stresses at nanomolar to micromolar concentrations[12,13].

Earlier, 28-homobrassinolide (28-HBL) has been reported to combat heavy metals stress in *Zea mays*[14]. Keeping in the view, the wide occurrence and economic importance of *Raphanus sativus* and the presence of BRs in this plant, in the present investigation, we have studied the role of 28-HBL in amelioration of Cd metal stress in *R. sativus* L. (Pusa Chetaki) seedlings. The study reports the regulation of growth and antioxidant enzyme activities like ascorbate peroxidase (APOX), catalase (CAT), glutathione reducatse (GR), guaiacol peroxidase

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Abbreviations: APOX; ascorbate peroxidase; BRs; brassinosteroids; CAT; catalase; Cd; cadmium; DHAR; dehydroascorbate reductase; GSH; glutathione; GR; glutathione reductase; GSSG; glutathione disulphide; 28-HBL; 28-homobrassinolide; MDHAR; mono-dehydroascorbate reductase; NBT; nitrobluetetrazolium; ROS; reactive oxygen species; POD; guaiacol peroxidase; SOD; superoxide dismutase.

(POD) and superoxide dismutase (SOD) and protein content by 28-HBL in *R. sativus* L. seedlings under Cd metal stress.

Materials and Methods

Plant material and growth conditions

Seeds of *Raphanus sativus* L. (Pusa Chetaki) were procured from Punjab Agriculture University, Ludhiana, India and surface sterilized with 0.4% sodium hypochlorite for 15 min, followed by repeated rinses in sterile distilled water. The surface sterilized seeds were then germinated on Whatman No. 1 filter paper lined autoclaved glass petri dishes (10 cm diameter, 20 seeds/dish) containing different concentrations of Cd (0, 0.5, 1.0 and 1.5 mM) and 28-HBL (0, 10^{-7}, 10^{-9} and 10^{-11} M) alone or in combinations. The experiment was conducted under controlled conditions (25 ± 5°C, 16 h photoperiod, 175 µmol m^{-2} s^{-1} light intensity) and repeated twice with 5 replications for each treatment.

Growth analysis

Seven days old seedlings were harvested and roots and shoots were separated. Seedling growth in terms of root and shoot length was recorded. Twenty seedlings per petridish were used for the determination of morphological parameters (root/shoot length) and fresh biomass. The seedlings were oven-dried at 80°C for 24 h to determine their dry weights.

Biochemical analysis

Preparation of leaf extracts

The leaf extracts were prepared to estimate the activities of antioxidant enzymes and the protein content. The cotyledonary leaves were homogenized in 50 mM phosphate buffer [pH 7.0, 1 mM EDTA, 1 mM PMSF, 0.5% (v/v) Triton X-100 and 2% (w/v) polyvinylpyrrolidone (PVP-30)] in a pre-chilled mortar and pestle. For estimation of APOX, 0.5 mM ascorbate was added to the extraction buffer. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was further used for biochemical analysis.

Protein quantification

Total protein content of seedlings treated with concentrations of Cd (0, 0.5, 1.0 and 1.5 mM) and 28-HBL (0, 10^{-7}, 10^{-9} and 10^{-11} M) alone or in combinations was quantified by following the method of Bradford[15] using bovine serum albumin as a standard.

Antioxidant enzyme activities

The ascorbate peroxidase (APOX, EC 1.11.1.11) activity was determined spectrophotometrically as described previously[16]. Three ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 1.0 mM H_2O_2 and 100 µl enzyme extract. The H_2O_2-dependent oxidation of ascorbate was followed by monitoring the decrease in absorbance at 290 nm using the extinction coefficient 2.8 $mM^{-1}cm^{-1}$. The reaction was carried out for 3 min at 25°C.

Catalase (CAT, EC 1.11.1.6) activity was assayed by measuring the initial rate of H_2O_2 disappearance[17]. Three ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 100 µl enzyme extract. The decrease in H_2O_2 was followed as decline in optical density at 240 nm at 25°C.

Glutathione reductase (GR, EC 1.6.4.2) activity was determined by using the previously described method[18]. Three ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.6), 1 mM GSSG, 0.5 mM EDTA, 0.1 mM NADPH and 100 µl enzymes extract. The reaction was initiated by addition of 0.1 mM NADPH at 25°C. The GR activity was determined by the oxidation of NADPH at 340 nm with extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

Guaiacol peroxidase (POD, EC 1.11.1.7) activity was assayed using the method of Sànchez[19] with slight modifications. Three ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 12.3 mM H_2O_2 and 100 µl enzyme extract. The POD activity was determined by measuring the absorbance at 436 nm and using an extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT[20]. For total SOD assay, 3.0 ml reaction mixture contained 50 mM sodium carbonate (pH 10.2), 24 µM NBT, 0.1 mM EDTA, 1 mM hydroxylamine, 0.03% (v/v) Triton X-100 and 70 µl enzyme extract. The absorbance was recorded at 560 nm for 2 min.

Statistical analysis

Five replicates (each containing 20 seedlings) for each treatment were maintained. The data were subjected to one-way analysis of variance (SPSS 10.0.0) and expressed as the mean ± standard error of five replications. The significance of difference between the control and treatments was set at $p \leq 0.05$.

Results

Morphological parameters

Cd metal stress resulted in decreased root and shoot length as well as fresh and dry biomass of radish seedlings. However, supplementation of Cd solutions with 28-HBL considerably reduced the repressing effects of Cd on seedling's length and biomass. The root and shoot length decreased significantly with increased Cd concentrations. Root length (Fig. 1A) was lowest (0.433 ± 0.080 cm) at 1.5 mM Cd as compared to distilled water (9.333 ± 0.400 cm) and improved significantly by the application of 10^{-7} M (16.22 ± 0.597 cm), 10^{-9} M (15.37 ± 0.426 cm) and 10^{-11} M (15.2 ± 0.295 cm) 28-HBL alone (Fig. 1). When 10^{-7} M 28-HBL was supplemented with 0.5 mM Cd solution, root length (12.25 ± 0.381 cm) was significantly enhanced as compared to control (seedlings treated with 0.5 mM Cd alone) (2.3 ± 0.170 cm) (Fig. 1).

A similar trend was observed when effect of 28-HBL was studied on shoot length of the seedlings (Fig. 1B). Furthermore, fresh biomass (Fig. 2A) of the seedlings was observed to be lowest (0.106 ± 0.003 g) at 1.5 mM Cd as compared to control (1.23 ± 0.005 g). However, 28-HBL supplementation enhanced fresh biomass significantly. When 10^{-7} M 28-HBL was added to 1.5 mM Cd metal, fresh biomass was elevated (1.52 ± 0.040 g) as compared to 1.5 mM Cd metal treatment alone (Fig. 2A). A similar trend was observed for dry biomass of seedlings under various treatments (Fig. 2B). A promoting effect of 28-HBL on root/shoot length and biomass of radish seedlings under Cd stress was noticed.

Biochemical parameters

The significant effects of 28-HBL treatments were observed on the biochemical parameters in radish shoots. The total protein content and activities of antioxidant enzymes increased with increasing concentrations of 28-HBL under Cd metal stress. The observations on Cd stressed shoots revealed that protein content decreased with increased concentrations of Cd metal (Fig. 3). Minimal protein content was observed in case of shoots treated with 1.5 mM Cd (7.144 ± 1.788 mg g^{-1} FW) as compared to control (19.49 ± 0.797 mg g^{-1} FW) (Fig. 3). Shoots treated with 28-HBL alone showed significant increase in soluble protein content (Fig. 3) in comparison to untreated shoots. The treatment of shoots with 10^{-7} M of 28-HBL resulted in significantly enhanced protein content (22.36

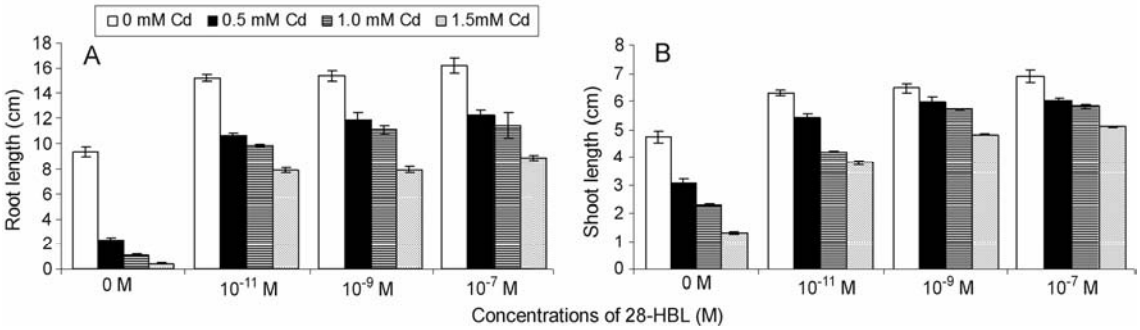

Fig. 1—Effect of 28-HBL on root length (A) and shoot length (B) in 7-days old *Raphaus sativus* seedlings under Cd metal stress [Bar represents the SE (n = 100)]

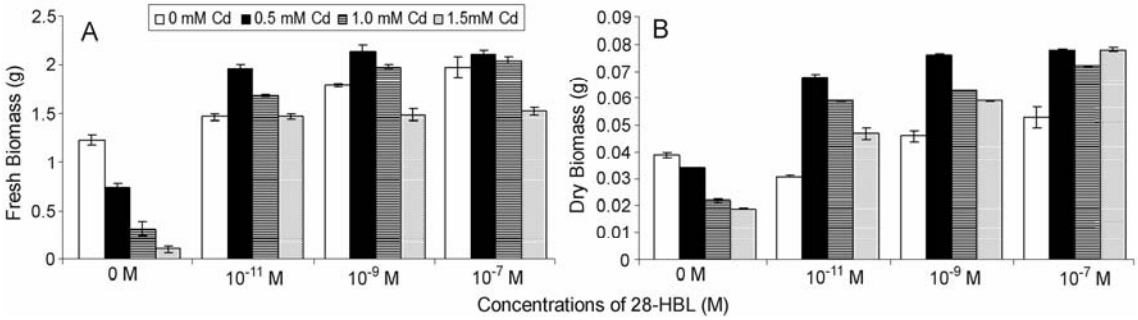

Fig. 2—Effect of 28-HBL on fresh biomass (A) and dry biomass (B) in 7-days old *R. sativus* seedlings under Cd metal stress [Bar represents the SE (n = 100)]

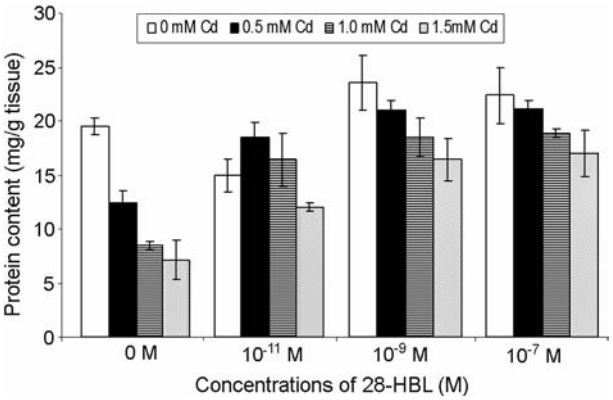

Fig. 3—Effect of 28-HBL on protein content in 7-days old *R. sativus* seedlings under Cd metal stress [Bar represents the SE (n = 100)]

± 3.130 mg g^{-1} FW) when compared to the control (19.49 ± 0.797 mg g^{-1} FW). The protein content (Fig. 3) was significantly higher in the shoots treated with Cd supplemented with 28-HBL than Cd alone.

APOX activity was increased with increased concentrations of Cd as compared to control (Fig. 4A). It was lowest (0.052 ± 0.007 mol UA mg $protein^{-1}$) in 1.5 mM Cd treated seedlings as compared to control (0.186 ± 0.004 mol UA mg $protein^{-1}$). The activity was further enhanced by applications of different concentrations of 28-HBL under Cd stress. The maximum APOX activity was observed in case of shoots, treated with 0.5 mM of Cd solution supplemented with 10^{-7} M 28-HBL (0.196 ± 0.008 mol UA mg $protein^{-1}$).

A similar trend was observed when effect of 28-HBL was studied on the activities of CAT under Cd metal stress (Fig. 4B). CAT activity (Fig. 4B) was lowest at 0.5 mM Cd (33.48 ± 1.211 mol UA mg $protein^{-1}$) when compared to control (53.29 ± 4.000 mol UA mg $protein^{-1}$). It was maximum (60.01 ± 1.566 mol UA mg $protein^{-1}$) in shoots treated with 10^{-7} M 28-HBL supplemented with 0.5 mM of Cd solution (33.48 ± 1.211 mol UA mg $protein^{-1}$).

Similarly, SOD activity (Fig. 4C) increased significantly under 0.5 mM (6.64 ± 0.173 mol UA mg $protein^{-1}$) and 1.0 mM (7.72 ± 0.562 mol UA mg $protein^{-1}$) Cd metal concentrations in radish shoots as compared to control (5.2 ± 0.073 mol UA mg $protein^{-1}$). 28-HBL alone was not able to alleviate the decreased levels of SOD activity, but on supplementation with Cd metal solutions resulted in increased levels of SOD activity (Fig. 4C). SOD activity was reported to be maximum in case of shoots treated with 10^{-7} M 28-HBL supplemented with 0.5 mM Cd (11.21 ± 0.487 mol UA mg $protein^{-1}$) when compared to 0.5 mM Cd alone (6.64 ± 0.173 mol UA mg $protein^{-1}$).

Conversely, GR activity declined under Cd stress (Fig. 4D) and it was found to increase significantly by application of different concentrations of 28-HBL under Cd stress. POD activity in seedlings (Fig. 4E) also increased under Cd stress upto 1.0 mM Cd and further no significant change was observed when supplemented with 28-HBL. POD activity was increased at 1.0 mM Cd (0.310 ± 0.012 mol UA mg $protein^{-1}$) when compared to control (0.210 ± 0.002 mol UA mg $protein^{-1}$). Application of 28-HBL did not improve the POD activity significantly under Cd metal stress. It was observed that 28-HBL helped in ameliorating the stress in radish plants by regulating the activities of antioxidant enzymes.

Discussion

The present study revealed that exogenous application of Cd concentrations caused stress to radish seedlings, as reflected in significant inhibition of root and shoot growth as well as in changes at protein levels and activities of antioxidant enzyme. Cd phytotoxicity can be accounted due to interference with metabolic processes in plants, such as protein structure disruption or displacement of essential elements[21-23]. In present study, decrease in root and shoot length (Figs 1A, B) as well as biomass (Figs 2A, B) was observed under Cd stress. However, supplementation of 28-HBL improved the seedling growth and biomass (Figs 1,2) under Cd stress in radish seedlings.

Earlier studies revealed that 28-HBL treatments enhanced the activities of enzymes and protein concentration of *Brassica juncea* seedlings under Zn metal stress[14,24]. 28-HBL application may increase cell division, reduces chromosomal aberrations and alters the membrane permeability. Also, BRs have been reported to increase the ATPase activity in Azuki bean epicotyls and maize roots, leading to proton extrusion, and induced cell wall relaxation[25,26]. It is proposed that BRs-induced changes are mediated through the repression and/or depression of specific genes[27]. In *Chlorella vulgaris,* BRs are reported to increase DNA, RNA and protein contents[28]. Similarly, in this study, protein content was found to be significantly increased with 28-HBL treatments to stressed seedlings (Fig. 3).

In response to biotic/abiotic stresses, an array of ROS like superoxide radical, H_2O_2, hydroxyl radical, peroxy radicals, alkoxy radicals, and singlet oxygen

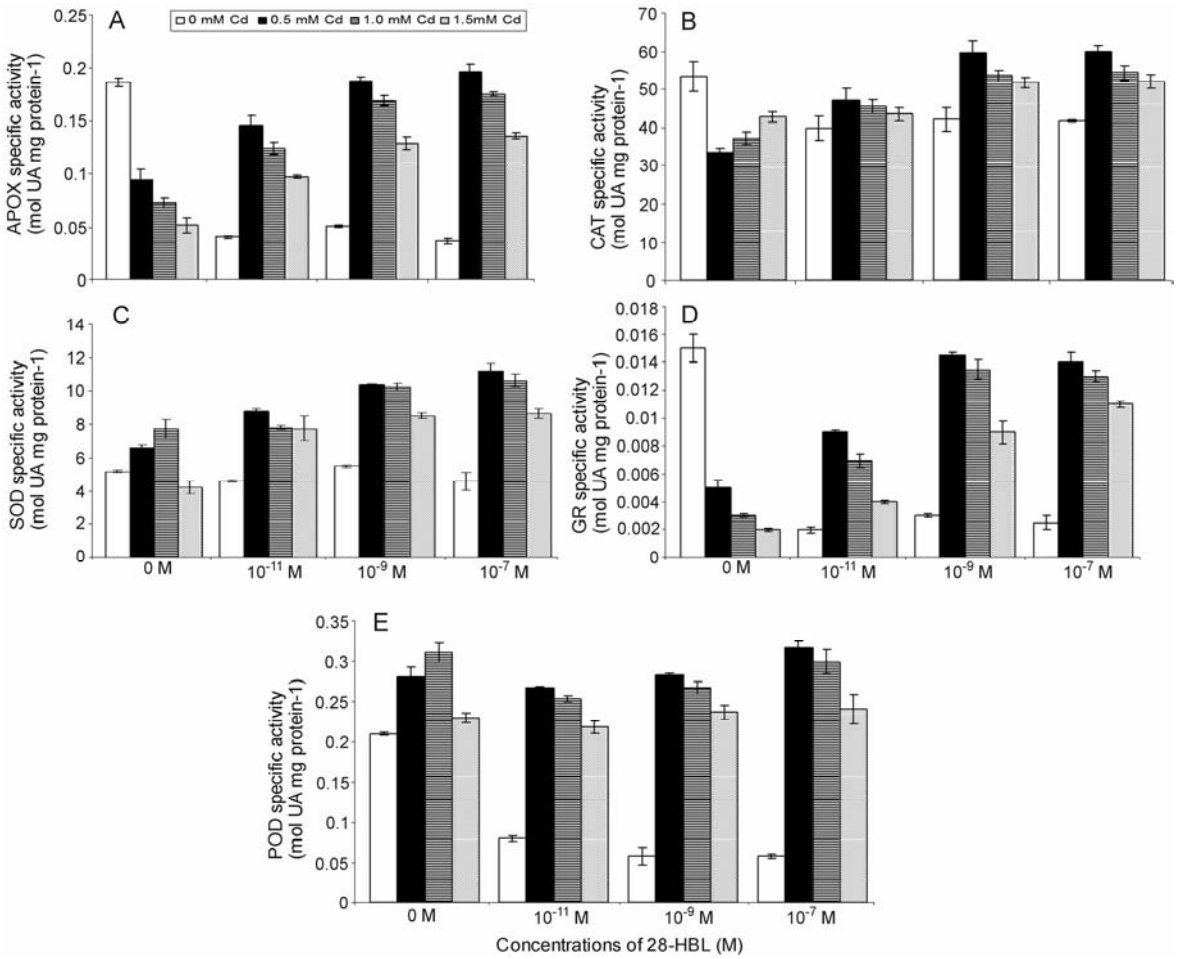

Fig. 4—Effect of 28-HBL on specific activity (mol UA mg protein $^{-1}$) in APOX (A), CAT (B), SOD (C), GR (D), and POD (E) of 7-days old *R. sativus* seedlings under Cd metal stress [Bar represents the SE (n = 1000]

etc. are generated in plant cells[29]. Cd metal stress also leads to their production and thus causes oxidative damage, leading to membrane destruction which in turn affects the levels of antioxidants and antioxidant enzymes[3,22]. The ROS are potentially harmful molecules that cause severe perturbations of various physiological functions[7,10]. Plants have evolved an extensive system of antioxidant enzymes and antioxidants to scavenge these ROS, which include SOD, CAT, POD, APOX, GR, DHAR (dehydroascorbate reductase), MDHAR (mono-dehydroascorbate reductase) and glutathione, ascorbate, tocopherols, carotenoids etc.[30,31]. The increased Cd metal concentrations in the plant tissues cause induction/deduction in the activities of antioxidant enzymes either as a result of *de novo* protein synthesis or by the activation of enzymes already present in plant cells[21]. In the present study, Cd-induced oxidative stress resulted in enhancing the activities of CAT, GR and SOD, whereas the activities of APOX and POD were declined (Figs 4 A-E).

Previous reports have shown that exogenous application of BRs altered antioxidant enzyme activities in plants[32]. The elevated H_2O_2 levels resulting from enhanced NADPH oxidase activity are involved in the BRs-induced stress tolerance in *Cucumis sativus*[33]. BRs (sterols) can modulate the activity of proteins and other enzymes within the membrane by affecting either protein conformation (functionality) or protein activity by direct protein–sterol interactions[34]. 28-HBL-treated seedlings might be scavenging ROS more effectively than those treated with metal alone[35].

In current study, in the presence of 28-HBL the specific activities of APOX (Fig. 4A), CAT (Fig. 4B), SOD (Fig. 4C) and GR (Fig. 4D) and were elevated under Cd stress, indicating that these antioxidant enzymes might play a role in 28-HBL-induced detoxifying processes to overcome Cd phytotoxicity.

This was in accordance with the previous reports, where exogenous application of BRs has been reported to change antioxidant enzyme activities[14,36,37]. In the present investigation, POD activity (Fig. 4E) was increased under Cd stress and further no significant change was observed when supplemented with 28-HBL. However, 28-HBL enhanced APOX activities under Cd stress (Fig. 4A). Both POD and APOX are involved in removal of H_2O_2. Accordingly, it can be concluded that 28-HBL enhanced the activities of APOX and didn't affect POD activity. This was in accordance with the prior reports in *Zea mays* and *Vigna radiata* respectively[14,38].

Conclusion

The influence of 28-HBL on antioxidant enzymes, biomass and seedlings length was more prominent under metal stress, suggesting that 28-HBL-treated seedlings were less affected by Cd than the untreated seedlings. Also, 28-HBL-induced elevated levels of antioxidant enzymes increased the tolerance of radish seedlings to Cd stress. However, the molecular mechanisms involved in stress protection remain to be explored.

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