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Seed Presoaking Treatment of 28-Homobrassinolide Modulates Antioxidative Defence System of *Brassica juncea* L. under Zinc Metal Stress

Priya Arora • Renu Bhardwaj* • Mukesh Kumar Kanwar

Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar-143005, India

Corresponding author: * **renubhardwaj82@gmail.com** or **renu_bhardwaj@rediffmail.com**

ABSTRACT

In the present study, the effects of seed presoaking treatment of 28-homobrassinolide (28-HBL; 0, 10^{-6}, 10^{-8} and 10^{-10} M) were investigated on growth (shoot length and number of leaves), protein content and activities of antioxidative enzymes (superoxide dismutase, catalase, guaiacol peroxidase, glutathione reductase, ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase in leaves of 30 days-old *Brassica juncea* L. plants treated with different concentrations of zinc metal. It was observed that treatment of different concentrations (0, 0.5, 1.0, 1.5 and 2.0 mM) of zinc alone decreased the shoot length and number of leaves and regulated the activities of enzymes and protein content of plants. However, seed-presoaking with 28-HBL improved the growth and stimulated the activities of antioxidative enzymes and protein content in leaves of *B. juncea* plants thus indicating the stress-ameliorative properties of 28-HBL.

Keywords: brassinosteroids, heavy metal stress, reactive oxygen species

Abbreviations: 24-EBL, 24-epibrassinolide; **28-HBL**, 28-homobrassinolide; **ANOVA**, analysis of variance; **APOX**, ascorbate peroxidase; **BR**, brassinosteroid; **CAT**, catalase; **DHAR**, dehydroascorbate reductase; **FW**, fresh weight; **GR**, glutathione reductase; **MDHAR**, monodehydroascorbate reductase; **POD**, guaiacol peroxidase; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase

INTRODUCTION

Brassinosteroids (BRs) play important roles in regulating plant growth and development. They are widely distributed in the plant kingdom and are active at very low concentrations ranging from nano- to micromolar levels (Mussig 2005). BRs are known to improve plant tolerance to a wide array of abiotic (drought, chilling, pesticides, salinity, heavy metals) and biotic stresses (bacteria, virus, fungi) (Krishna 2003; Pullman *et al*. 2003). BR-induced stress tolerance is associated with increased accumulation of reactive oxygen species (ROS) which in turn is regulated by genes involved in plant stress response pathways (Xia *et al*. 2010). These steroids are common plant-produced compounds with antioxidative potential (Haubrick and Assmann 2006). They also help to overcome stress by regulating the activities of various antioxidative enzymes (Liu *et al*. 2009).

Although heavy metals are essential as micronutrients for plants, but they are toxic at higher concentrations. Metals like Zn, Ni, Cu, Mo, Mn, Co, Cr, etc., when present in high concentrations in soil, show potential toxic effects on overall growth and metabolism of plants (Schutzendubel and Polle 2002; Hall and Williams 2003; Agrawal and Shaheen 2007). Heavy metals can create a major ecological crisis since they are non-degradable and often accumulate in plant parts, get biologically magnified through various trophic levels leading to deleterious biological effects (Jaleel *et al*. 2009). Heavy metal stress results in the production of ROS which leads to the activation of defence mechanisms in terms of antioxidative enzymes and non-enzymatic molecules. Generation of ROS such as superoxide, H_2O_2 and hydroxyl molecules cause rapid damage of cellular membrane and various cell components by triggering off a chain reaction (Imlay 2003; Jaleel *et al*. 2006). Though numerous reports have confirmed the potential of plant hormones to synergistically improve crop performance under normal conditions, very little information is available on the influence of BRs under heavy metal stress. *Brassica juncea* L., an amphidiploid species, is grown as an oilseed crop in India. It is also known as Indian mustard or mustard greens or leaf mustard. Indian mustard and its oil have been used as a topical treatment for rheumatisms and arthritis. Although, oilseed *Brassicas* are grown over 15% arable land in India but their productivity is considerably hindered by various biotic and abiotic stresses (Shah 2002; Yusuf *et al*. 2010) like chilling, drought, pesticide and heavy metals etc. Keeping in mind the stress-protective properties of BRs, the present study was undertaken to observe the effects of 28-homobrassinolide (28-HBL) on growth, protein content and activities of antioxidative enzymes in 30-days old *B. juncea* L. plants under Zn metal stress.

MATERIALS AND METHODS

To unravel the role of BRs in modulation of plant responses to oxidative burst produced due to heavy metal toxicity, a seasonal field experiment was performed. The seeds of *B. juncea* L. cv. 'PBR 91' (certified) used in the present study were procured from the Department of Plant Breeding, Punjab Agriculture University, Ludhiana, India. This cultivar was chosen among various varieties of *B. juncea* since it shows stability for most of the important yield-contributing characters. Seeds of *B. juncea* were surface sterilized with 0.01% $HgCl_2$, washed and rinsed three times with double distilled water. These surface-sterilized seeds were soaked for 8 hrs in different concentrations of 28-HBL (Sigma-Aldrich) (0, 10^{-6}, 10^{-8} and 10^{-10} M). The experimental pots were arranged in triplicates in the Botanical Garden of the University. Different concentrations of zinc metal in the form of $ZnSO_4{\cdot}7H_2O$ (0, 0.5, 1.0, 1.5 and 2.0 mM) were added to the pots. All the chemicals were procured from Sigma-Aldrich. The soil used for the present

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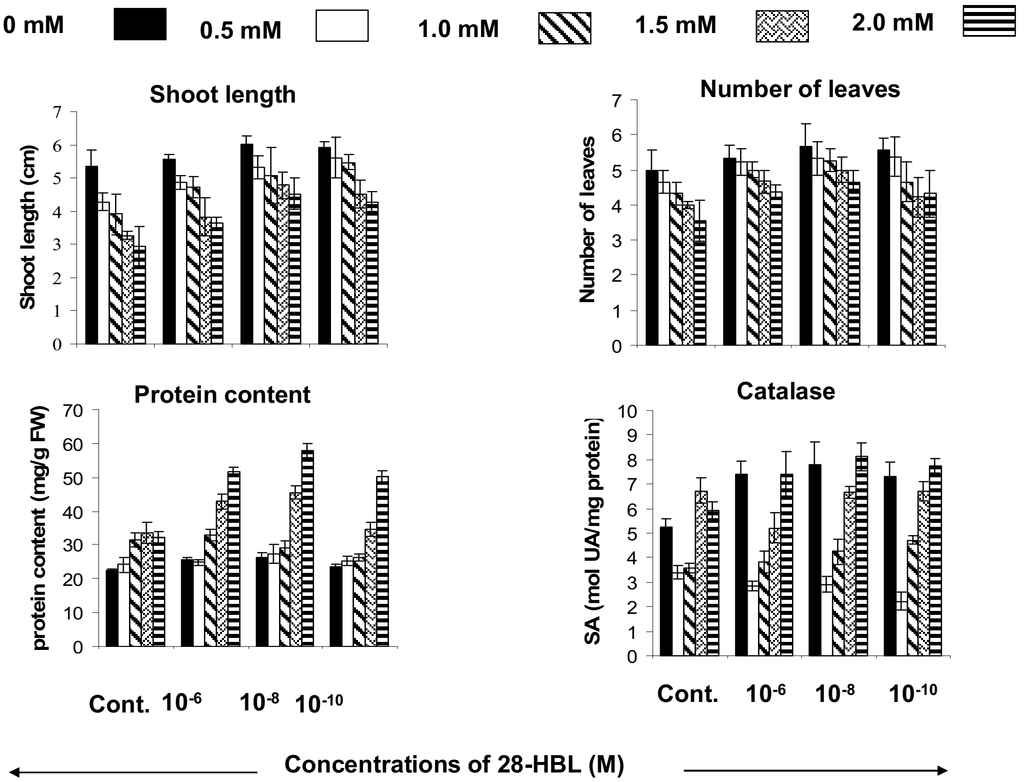

Fig. 1 Effect of 28-HBL on shoot length, number of leaves, protein content and catalase activity in 30-d old *B. juncea* plants under Zn metal stress. Bars represent SE (n=3).

study was prepared using garden soil, silt and cow dung manure in the ratio of 2: 1: 1. The seeds pre-treated with 28-HBL for 8 hrs were sown in the pots containing different concentrations of zinc (Zn) metal. The pots were kept under natural seasonal conditions.

Morphological parameters

On the 30th day, the plants were analyzed for morphological parameters viz. shoot length and number of leaves.

Biochemical parameters

For estimation of protein content and antioxidative enzyme activities viz. superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APOX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), 0.5 g of leaf tissue was homogenized in a pre-chilled pestle and mortar with 5.0 ml of 100 mM potassium phosphate buffer (pH 7.0) under ice-cold conditions. The homogenate was centrifuged at 4°C for 20 min at 15,000 × *g* and the supernatant was used for determining protein content and activities of SOD, CAT, POD, APOX, GR, MDHAR and DHAR.

Protein content was determined following the method of Lowry *et al.* (1951). The activity of SOD was determined by monitoring its ability to inhibit photochemical reduction of nitrobluetetrazolium (NBT) at 540 nm (Kono 1978). CAT activity was determined by following the initial rate of disappearance of H_2O_2 at 240 nm (Aebi 1983). POD activity was determined according to Putter (1974). The activities of APOX and GR were measured by the method of Nakano and Asada (1981) and Carlberg and Mannervik (1975) respectively. MDHAR and DHAR activities were determined according to Hossain *et al.* (1984) and Dalton *et al.* (1986), respectively.

Statistical analysis

All the experiments were performed in triplicates in natural field conditions. The data presented in the graphs are means of three values. The data obtained was statistically analyzed using one-way analysis of variance (ANOVA) and $P \leq 0.05$ were considered significantly different from control (Bailey 1995).

RESULTS

Seed-presoaking treatment with 28-HBL significantly modulated the growth parameters and antioxidative defence system of *B. juncea*. This effect was clearly visible in terms of increased shoot length and number of leaves. The protein content and activities of SOD, CAT, POD, APOX, GR, MDHAR and DHAR were considerably regulated by presoaking of seeds in 28-HBL, both in stressed and non-stressed conditions. The effects of 28-HBL on shoot length and number of leaves of 30 days old plants are shown in **Fig. 1**.

Morphological parameters

The shoot length was reduced significantly from 5.37 cm in untreated control plants to 2.94 cm under increasing concentrations of Zn stress. However, 28-HBL treatment markedly increased the shoot length at 10^{-6}, 10^{-8} and 10^{-10} M concentration. Seed presoaking treatment with 28-HBL considerably reduced the inhibitory effect of Zn on plant growth. The plants given seed presoaking treatment with 10^{-8} M of 28-HBL and treated with 0.5 mM of Zn metal revealed maximum shoot length (5.61 cm) in comparison to metal-treated plants only (4.28 cm). A similar trend was observed for the number of leaves.

Biochemical parameters

The protein content of leaves of 30 days old *B. juncea* plants increased considerably in all treatments of 28-HBL in comparison to untreated control (**Fig. 1**). It was significantly higher (57.91 mg/g FW) in leaves of plants treated with 10^{-8} M of 28-HBL and 0.5 mM of Zn when compared to metal treated plants alone (32.07 mg/g FW). Considerable increase in CAT activity was recorded under the influence of 28-HBL, more significantly at 10^{-8} M concentration of 28-HBL (7.77 mol UA/mg protein). Further, CAT activity got enhanced when metal treatments were given to seeds pre-

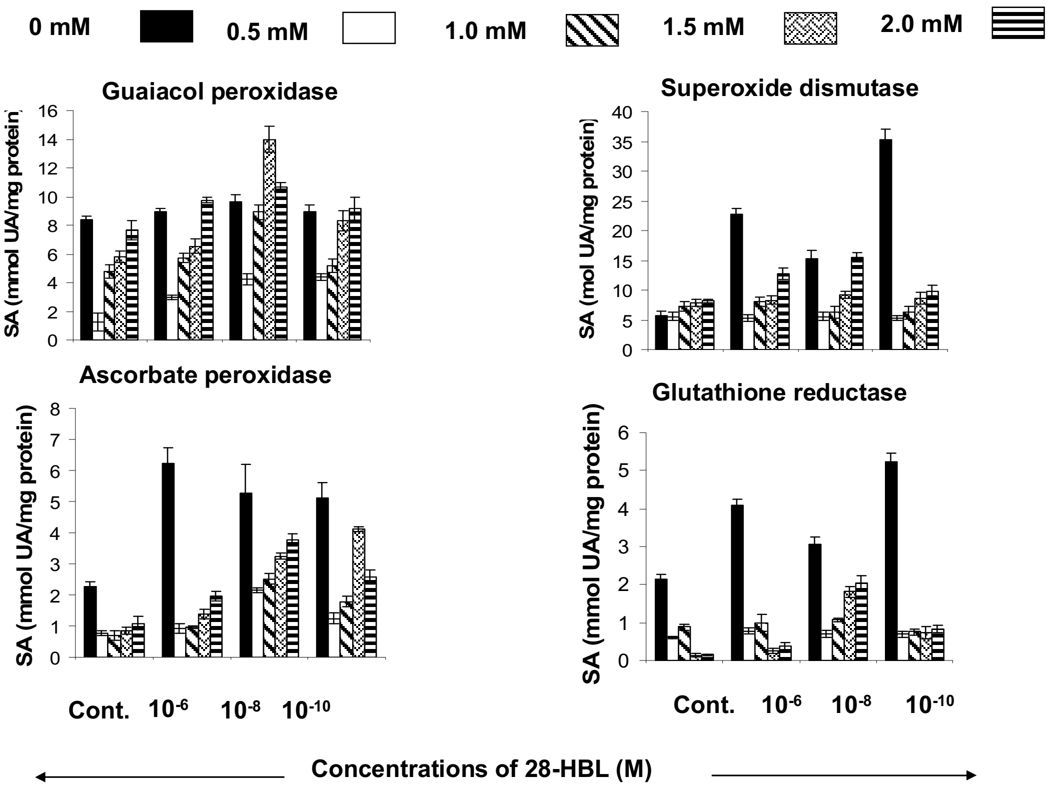

Fig. 2 Effect of 28-HBL on activities of guaiacol peroxidase, superoxide dismutase, ascorbate peroxidase and glutathione reductase in 30-d old *B. juncea* plants under Zn metal stress. Bars represent SE (n=3).

soaked with 28-HBL (10^{-6}, 10^{-8} and 10^{-10} M). Maximum increase in CAT activity (8.12 mol UA/mg protein) was observed in plants treated with 2.0 mM of Zn and given seed presoaking treatment with 10^{-8} M of 28-HBL in comparison to Zn treated plants only (5.93 mol UA/mg protein) (**Fig 1**).

The activity of SOD was considerably enhanced from 5.75 mol UA/mg protein in control leaves to 8.13 mol UA/mg protein under Zn stress. But, the activity of SOD got further enhanced by the treatment of 28-HBL alone, with maximum rise at 10^{-10} M (35.32 mol UA/mg protein). Maximum increase in SOD activity was observed in leaves of plants given seed presoaking treatment with 10^{-8} M of 28-HBL and treated with 2.0 mM of Zn (15.47 mol UA/mg protein) (**Fig. 2**). The activity of POD got increased with seed presoaking treatment of 28-HBL alone (9.67 mmol UA/mg protein at 10^{-8} M) in comparison to untreated control (8.39 mmol UA/mg protein). Maximum activity of POD was observed in leaves of plants given seed presoaking treatment with 10^{-8} M and treated with 1.5 mM of Zn (13.98 mmol UA/mg protein) when compared to metal treated plants only (5.81 mmol UA/mg protein) (**Fig. 2**).

The activities of APOX (2.26 m mol UA/mg protein), MDHAR (0.33 m mol UA/mg protein) and DHAR (0.45 m mol UA/mg protein) in Zn metal treated leaves of *B. juncea* were observed to be highest at 2.0 mM of metal concentration. But for GR, no definite trend was observed with increasing Zn concentrations. 28-HBL pre-treatments to Zn treated plants further enhanced APOX (4.12 m mol UA/mg protein) (**Fig. 2**) and DHAR activities (0.94 m mol UA/mg protein) (**Fig. 3**) that were maximum in plants given treatments with 10^{-10} M of 28-HBL and 1.5 mM and 2.0 mM of metal, respectively. Highest activity of GR was revealed (2.04 m mol UA/mg protein) in leaves of plants raised by seed presoaking treatment of 10^{-8} M of 28-HBL and 2.0 mM of Zn in comparison to same concentration of metal (0.14 m mol UA/mg protein) (**Fig. 2**). However, the activity of MDHAR was relatively less influenced by 28-HBL treatment. Maximum enhancement in MDHAR activity (2.95 m mol UA/mg protein) was observed in plants treated with 2.0 mM of Zn metal and treated with 10^{-6} M of 28-HBL (**Fig. 3**).

DISCUSSION AND CONCLUSION

The present findings revealed that presoaking of seeds in 28-HBL remarkably improved the growth of plants under Zn metal stress. Exogenous application of 28-HBL improved the growth of Zn-stressed *B. juncea* plants by increasing shoot length and number of leaves (**Fig. 1**). Earlier studies also indicated stress-ameliorative properties of BRs. Kartal *et al.* (2009) reported that 28-HBL treatment significantly increased protein content in barley (*Hordeum vulgare*) seedlings while Ali *et al.* (2007) observed that 24-EBL and 28-HBL improved the growth of Al-stressed mung bean (*Vigna radiata*) seedlings by increasing the rate of photosynthesis and carbonic anhydrase activity. Brassinolide (BL) ameliorated the Al toxicity and promoted the growth of mung bean seedlings (Abdullahi *et al.* 2003). These growth promoting effects of BRs in heavy metal stressed *B. juncea* plants can be corrected due to their capacity to regulate cell elongation and divisional activities by activating the cell wall loosening enzymes, which increase the synthesis of cell wall and membrane materials (Khripach *et al.* 2000; Park *et al.* 2010). The cell wall loosening enzymes get activated by H+-ATPases which acidifies the apoplast. Microarray analysis has also shown that BRs treatment up-regulates several additional genes related to cell expansion and cell wall organization, including expansins and pectin-modifying enzymes (Haubrick and Assmann 2006).

28-HBL further improved the plant growth by increasing the protein content (**Fig. 1**). This was possibly due to the well-documented effects of BRs on tanscription and/or translation processes of specific genes related to stress tolerance (Fariduddin *et al.* 2004; Kagale *et al.* 2007). Bajguz (2000) also found that BRs increased DNA, RNA and protein contents of *Chlorella vulgaris* as the number of cells increased in medium.

The observations in the present study revealed that 28-HBL seed presoaking treatment to *B. juncea* plants resulted in increased activities of antioxidative enzymes, which is in accordance with the studies carried out by Mazorra *et al.* (2002), who found enhanced CAT activity in rice (*Oryza sativa*) under the influence of BRs. Heavy metals-generated

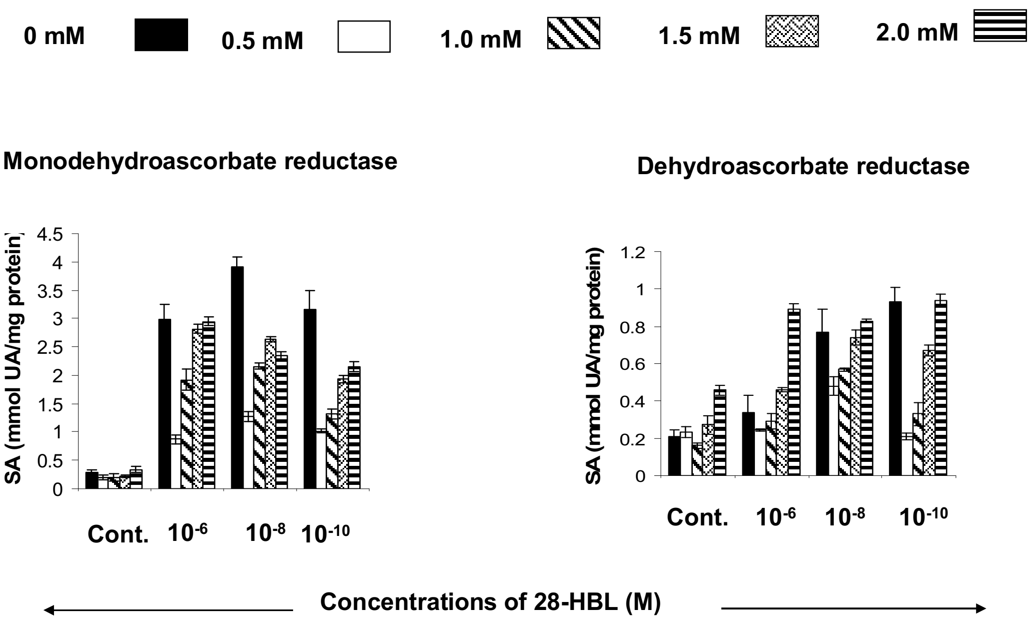

Fig. 3 Effect of 28-HBL on activities of monodehydroascorbate reductase and dehydroascorbate reductase in 30-d old *B. juncea* plants under Zn metal stress. Bars represent SE (n=3).

ROS could thus be alleviated by brassinolide treatments (Almeida *et al*. 2005; Hayat *et al*. 2007). Application of BRs have been shown to involve the major antioxidative enzymes resulting in increased relative water content, nitrate reductase activity, chlorophyll content and photosynthesis and membrane stability under stress conditions (Hayat *et al*. 2007; Kagale *et al*. 2007) (**Figs. 1-3**). As membrane destruction results from ROS induced oxidative damage (McCord 2000), the 28-HBL-treated plants might be scavenging ROS more efficiently than those treated with metal alone. Similarly, Liu *et al*. (2009) demonstrated that 24-EBL treatment significantly enhanced antioxidative enzyme activity and content of antioxidants in *Chorispora bungeana* under chilling stress. BRs application involves the major antioxidative enzymes which result in increased relative water content, nitrate reductase activity, chlorophyll content, photosynthesis and stability of membranes under stress conditions (Hayat *et al*. 2007, 2008).

Our earlier studies also reveal that 24-EBL treatments improved the shoot emergence and plant biomass production in *B. juncea* seedlings and plants under heavy metal stress like Mn and Ni. The mechanism involved in reducing toxicity may be the chelation of metal ions by the ligands. Such ligands include amino acids, organic acids, peptides or polypeptides (Kaur and Bhardwaj 2004; Arora *et al*. 2008a; Sharma *et al*. 2008). Altered activities of antioxidative enzymes further suggest that 24-EBL treated plants were less affected by Cr metal than the untreated plants (Arora *et al*. 2010). Further, our studies on heavy metal stress indicate that 28-HBL ameliorates the Ni toxicity by increasing the activities of antioxidative enzymes like superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, catalase and glutathione reductase (Arora *et al*. 2008b, 2008c; Bhardwaj *et al*. 2008). BRs-regulated stress response occurred possibly as a result of a complex sequence of biochemical reactions such as activation or suppression of key enzymatic reactions, stimulation of protein synthesis and the production of various chemical defence compounds (Qayyum *et al*. 2007). Thus, the present study demonstrated the possibility of BRs to reduce the impact of zinc metal on plant growth by stimulating the activities of key antioxidative enzymes.

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