# Effect of 28-homobrassinolide on growth, zinc metal uptake and antioxidative enzyme activities in *Brassica juncea* L. seedlings

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The present work was undertaken to study the effects of 28-homobrassinolide on growth, zinc metal uptake, antioxidative enzyme [guaiacol peroxidase (EC 1.11.1.7), catalase (EC 1.11.1.6), glutathione reductase (EC 1.6.4.2), ascorbate peroxidase (EC 1.11.1.11), superoxide dismutase (EC 1.15.1.1)] activities and protein content in 7-d-old seedlings of *Brassica juncea* L. treated with Zn metal at different concentrations. 28-homobrassinolide at $10^{-9}$ M concentration lowered Zn uptake and bioconcentration factor. Different concentrations of Zn-metal treatment alone decreased the enzyme activities and protein concentration of seedlings. However, pre-sowing treatments of 28-homobrassinolide lowered the uptake of metal and enhanced the activities of enzymes and protein concentration of seedlings.

**Key words:** antioxidative enzymes, 28-homobrassinolide, brassinosteroids, zinc toxicity

**Efeitos do 28-homobrassinolídeo sobre o crescimento, absorção de zinco e atividades de enzimas antioxidativas em plântulas de *Brassica juncea* L.**: O presente estudo foi conduzido para estudarem-se os efeitos do 28-homobrassinolídeo sobre o crescimento, a absorção de zinco e a atividade de enzimas antioxidativas [peroxidases do guaiacol (EC 1.11.1.7), catalase (EC 1.11.1.6), redutase da glutationa, (EC 1.6.4.2), peroxidase do ascorbate (EC 1.11.1.11) e dismutase do superóxido (EC 1.15.1.1)] e concentração de proteínas em plântulas de *Brassica juncea* L. tratadas com Zn em diferentes concentrações, aos sete dias de idade. O brassinolídeo, à concentração de $10^{-9}$ M, reduziu a absorção de Zn e o fator de bioconcentração. O Zn, quando aplicado em diferentes concentrações, isoladamente, acarretou decréscimos nas atividades das enzimas e na concentração de proteínas. Contudo, o efeito do 28-homobrassinolídeo, aplicado antes da semeadura, reduziu a absorção do metal e fez aumentar as atividades das enzimas antioxidativas e a concentração de proteínas nas plântulas.

**Palavras-chave:** 28-homobrassinolídeo, brassinosteróides, enzimas antioxidativas, toxidez por zinco

## INTRODUCTION

Plants need relatively small amounts of metals for their growth and soils harbor these metal ions either naturally or as a consequence of contamination. Soil contamination with heavy metals is now a worldwide problem, leading to agricultural losses and hazardous health effects as metals enter the food chain. Zinc is one of the micronutrients essential for plant growth but is toxic to plants at higher concentrations and can retard plant growth and disrupt various essential physiological processes (Cakmak and Marschner, 1993; Salt et al., 1995). An excess of Zn is indicated by a decrease in growth and development, metabolic activity and an induction of oxidative damage in various plant species (Panda et al., 2003). The oxidative stress results in generation of toxic free radicals in plants (Asada and Takahashi, 1987). Heavy metals accelerate generation of reactive oxygen species (ROS) that have the capacity to

initiate lipid peroxidation and degrade proteins, lipids and nucleic acids (Halliwell and Gutteridge, 1999). Plants respond to heavy metals by changes in the levels of antioxidants and antioxidative enzymes (Noctor and Foyer, 1998; Pandey et al., 2005). Several hormones are implicated in modulating the plant responses to oxidative stress, including ethylene (Vahala et al., 2003), abscisic acid (Kovtun et al., 2000), salicylic acid (SA) (Metwally et al., 2003) and brassinosteroids (BRs) (Cao et al., 2005).

Brassinosteroids are a class of plant polyhydroxysteroids that are ubiquitously distributed in the plant kingdom. These compounds, when applied to plants, improve their quality and yield. They have been further explored for stress-protective properties in plants against a number of stresses like chilling (Dhaubhadel et al., 1999), salt (Ozdemir et al., 2004), heat (Dhaubhadel et al., 2002) and heavy metals (Bajguz, 2000; Janeczko et al., 2005). However, it is unclear whether BRs are involved in the modulation of plant responses to oxidative stresses. The influence of BRs on the response of the antioxidative enzymes of plants under stress conditions has been studied recently (Cao et al., 2005; Hayat et al., 2007). The available data shows that the changes induced in the activity of antioxidative enzymes by BRs differed with plant species and with stress conditions (Ozdemir et al., 2004; Almeida et al., 2005; Hayat et al., 2007).

*Brassica juncea*, a known hyperaccumulator, is capable of accumulating high levels of heavy metals in its shoots. The present study was undertaken to observe the growth, uptake of Zn metal and activities of antioxidative enzymes under the influence of 28-homobrassinolide (28-homoBR).

## MATERIAL AND METHODS

The seeds of *Brassica juncea* L. cv. PBR 91 (certified) used in the present investigation were procured from the Department of Plant Breeding, Punjab Agriculture University, Ludhiana, India. They were surface sterilized with 0.01% $HgCl_2$. The sterilized seeds were soaked for 8 h in different concentrations of 28-homoBL (0, $10^{-7}$, $10^{-9}$ and $10^{-11}$M). The 28-homoBL-treated seeds were germinated in Petri dishes lined with *Whatman No. 1* filter paper (25 ± 0.5ºC, 16 h light and 8 h dark period) containing different concentrations (0, 25, 50 and 100 mg $L^{-1}$) of Zn metal. Zn was given in the form of $ZnSO_4.7H_2O$ dissolved in distilled water. Growth parameters (shoot and root length), Zn ions uptake, activities of enzymes and protein concentration were determined in 7-d-old seedlings. Seedlings were then dried at 80ºC for 24 h. The dried material was digested using the digestion mixture ($H_2SO_4$/$HNO_3$/$HClO_4$ in 1:5:1 ratio) according to the method of Allen et al. (1976). Uptake of Zn metal was determined using an atomic absorption spectrophotometer (Model AA-6200, Shimadzu, Japan). The bioconcentration factor (BCF) was calculated as the ratio of: [trace element concentration in plant tissues (mg $kg^{-1}$) at harvest] / [initial concentration of the element in the applied external solution (mg $L^{-1}$)].

Shoot tissues (1 g) were homogenized in 3 mL of 100 mM potassium phosphate buffer at pH 7.0 containing 1% insoluble polyvinylpyrolidone using an ice-chilled pestle and mortar. The homogenates were centrifuged at 15,000 *g* for 20 min at 4°C and supernatants were collected and used for assessing the activity of antioxidative enzymes [guaiacol peroxidase (POD; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6), glutathione reductase (GR; EC 1.6.4.2), ascorbate peroxidase (APX; EC 1.11.1.11), superoxide dismutase (SOD; EC 1.15.1.1)] and protein concentration. These assays were determined in different homogenates prepared from different treatments of heavy metal, 28-homoBL alone and heavy metal and 28-homoBL together. Three replicates of each treatment were taken.

Peroxidase was estimated according to Putter (1974). Activity was assayed in a reaction mixture containing 50 µL guaiacol (20 mM), 30 µL $H_2O_2$ (12.3 mM), 3 mL phosphate buffer (100 mM, pH 7.0) and 100 µL of enzyme extract. The rate of formation of oxidized guaiacol was followed spectrophotometrically at 436 nm. Enzyme activity was calculated using the extinction coefficient of 25 $mM^{-1}$ $cm^{-1}$. The POD activity was expressed as the amount of enzyme catalyzing the formation of 1 µmol guaiacol dehydrogenation products (GDHP) $min^{-1}$ $g^{-1}$ tissue. Catalase activity was determined according to the method of Aebi (1974). The rate of decomposition of $H_2O_2$ was followed by decrease in absorbance at 240 nm in a reaction mixture containing 1.5 mL K-phosphate buffer (100 mM, pH 7.0), 1.2 mL $H_2O_2$ (150 mM) and 300 µL of enzyme extract. Enzyme activity was determined using the extinction coefficient of 6.93 x $10^{-3}$ $mM^{-1}$ $cm^{-1}$. The activity of GR was measured using the method of Carlberg and Mannervik (1975) following the oxidation of

NADPH at 340 nm in a reaction mixture containing 1.8 mL K-phosphate buffer (50 mM, pH 7.6), 300 µL EDTA (3 mM), NADPH (0.1 mM), oxidized glutathione (GSSG) (1 mM) and 150 µL enzyme extract. Enzyme activity was determined using the extinction coefficient of 6.22 $mM^{-1}$ $cm^{-1}$, and was calculated as the amount of enzyme required to oxidize 1 µmol of NADPH $min^{-1}$ $g^{-1}$ tissue. The APX activity was estimated according to the method of Nakano and Asada (1981) following the decrease in absorbance at 290 nm in a reaction mixture containing 1.5 mL K-phosphate buffer (100 mM, pH 7.0), 300 µL ascorbate (5 mM), 600 µL $H_2O_2$ (0.5 mM) and 600 µL of enzyme extract. Enzyme activity was determined using the extinction coefficient of 2.8 $mM^{-1}$ $cm^{-1}$, and was calculated as the amount of enzyme required to oxidize 1 µmol of ascorbate $min^{-1}$ $g^{-1}$ tissue. The SOD activity was estimated according to Kono (1978) by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) dye by superoxide radicals, which are generated by the autooxidation of hydroxylamine hydrochloride. The reduction of NBT was followed by an absorbance increase at 540 nm in reaction mixture containing 1.3 mL Na-carbonate buffer (50 mM, pH 10.0), 500 µL NBT (96 µM), and 100 µL Triton X-100 (0.6%). The reaction was initiated by addition of 100 µL hydroxylamine-HCl (20 mM, pH 6.0); 2 min later 70 µL of enzyme sample were added. The enzyme activity was calculated as the SOD concentration inhibiting reduction of NBT by 50%.

Protein estimation was done following the method of Lowry et al. (1951). The amount of protein was expressed as mg $g^{-1}$ tissue.

The data obtained was statistically analyzed using one-way ANOVA (Bailey, 1995). Data were presented as means ± SE.

## RESULTS

Shoot and root length of seedlings decreased with increasing concentrations of Zn alone as compared to control seedlings (distilled water). However, when 28-homoBL pre-sowing treatments (0, $10^{-7}$, $10^{-9}$ and $10^{-11}$ M) were given, both shoot and root length were improved compared to control (distilled water and heavy metal) plants (Figure 1). With 28-homoBL alone, $10^{-7}$ M concentration revealed maximum increase in shoot length (6.00 ± 0.24 cm) as compared to Zn metal-treated seedlings (4.76 ± 0.28 cm). In roots, $10^{-7}$ M 28-homoBL treatment induced maximum increase in their length (6.16 ± 0.17 cm) as compared to control (4.80 ± 0.28 cm). Maximum increase in shoot length (4.06 ± 0.39 cm) and root length (5.26 ± 0.26 cm) was observed in seedlings treated with $10^{-9}$ M 28-homoBL under the stress of 50 mg Zn $L^{-1}$ and 25 mg Zn $L^{-1}$, respectively, when compared to the control (3.01 ± 0.28 cm) (Figure 1).

Seedlings treated with Zn (alone) at different concentrations showed an enhanced uptake of metal with increasing Zn concentrations. The maximum uptake was observed in seedlings treated with 100 mg Zn $L^{-1}$ alone (2.74 ± 0.40 mg $g^{-1}$ DW). The seed-pre-sowing application of 28-homoBL at different concentrations ($10^{-7}$, $10^{-9}$ and $10^{-11}$ M) significantly lowered the Zn uptake in seedlings when compared with the control individuals treated with

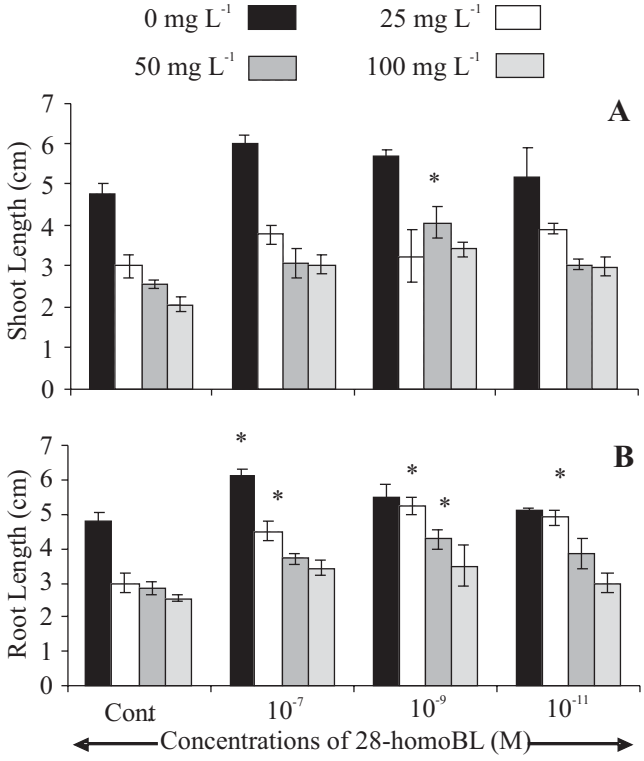


**Figure 1.** Effect of 28-homobrassiniolode (28-homoBL) on shoot (**A**) and root (**B**) length of 7-d-old *Brassica juncea* seedlings under zinc metal stress. Bars represent the SE ($n$ = 3) and asterisks indicate statistically significant differences from control seedlings at $P < 0.05$.

Zn alone. In distilled water- and 28-homoBL-treated seedlings Zn was not detected. Maximum reduction in Zn uptake (0.80 ± 0.48 mg $g^{-1}$ DW) was observed in seedlings treated with 28-homoBL at $10^{-9}$ M under the stress of 25 mg Zn $L^{-1}$ as compared to Zn-treated seedlings without 28-homoBL application (1.67 ± 0.136 mg $g^{-1}$ DW). Similarly BCF decreased on supplying 28-homoBL to Zn-stressed seedlings. Minimum BCF (20.80 ± 0.87) was observed in seedlings treated with 28-homoBL ($10^{-11}$ M) under the stress of 100 mg Zn $L^{-1}$ as compared to heavy metal-treated seedlings (27.38 ± 0.40) (Figure 2).

The decreased activities of antioxidative enzymes under Zn stress was increased by the treatment of 28-homoBL. Maximum increase in POD activity (5.66 ± 0.52 mmol GDHP $min^{-1}$ $mg^{-1}$ protein) was observed in seedlings treated with $10^{-11}$ M 28-homoBL under 100 mg Zn $L^{-1}$ as compared to heavy metal-treated seedlings only (1.45 ± 0.22 mmol GDHP $min^{-1}$ $mg^{-1}$ protein). Similarly, the activity of CAT was also increased by the treatment with 28-homoBL in Zn-stressed seedlings. Maximum CAT activity (9.12 ± 0.52 mol $H_2O_2$ $min^{-1}$ $mg^{-1}$ protein) was observed in seedlings treated with $10^{-11}$ M 28-homoBL under the effect of 50 mg Zn $L^{-1}$ as compared to control plants (2.77 ± 0.05 mol $H_2O_2$ $min^{-1}$ $mg^{-1}$ protein) (Figure 3).

Glutathione reductase showed maximum activity (14.17 ± 2.23 mmol NADPH $min^{-1}$ mg $protein^{-1}$) in seedlings treated with $10^{-11}$ M 28-homoBL under the stress of 25 mg Zn $L^{-1}$ as compared to control plants (5.29 ± 0.23 mmol NADPH $min^{-1}$ $mg^{-1}$ protein). Similar trends were observed for APX and SOD activities. Maximum APX activity (20.37 ± 2.58 mmol ascorbate $min^{-1}$ $mg^{-1}$ protein) was observed in seedlings treated with $10^{-11}$ M 28-homoBL under 50 mg Zn $L^{-1}$ when compared to the control (3.76 ± 0.14 mmol ascorbate $min^{-1}$ $mg^{-1}$ protein). Similarly maximum increase in SOD activity (15.10 ± 0.52 U $min^{-1}$ $mg^{-1}$ protein) was observed seedlings treated with $10^{-7}$ M 28-homoBL under the Zn metal stress of 100 mg $L^{-1}$ while the control plants exhibited 5.52 ± 0.27 U $min^{-1}$ $mg^{-1}$ protein.

The protein concentration decreased due to metal treatment, particularly at the highest Zn concentration. In contrast, 28-homoBL led to increases in protein levels, although such an increase was relatively smaller for the seedlings treated with the highest Zn concentration (Figure 3).

## DISCUSSION

The plants acclimate the negative effects of heavy metals and show specific and non-specific responses, which lead to tolerance to the stress (Hall, 2002). The general stress response reactions involving plant growth regulators are of prime importance in stress protection and tolerance mechanisms. Brassinosteroids play an essential role in plant growth and development and are involved in the modulation of stress responses. Enhanced resistance of BR-treated plants to extreme temperature, salt, pathogens and environmental stresses (heavy metals) has been reported by Krishna (2003). The present study revealed the effect of 28-homoBL in reducing the toxic effect of Zn metal treatments by improving seedling growth, lowering metal uptake and BCF. A survey of the literature on metal uptake under the influence of brassinosteroids reveals scanty information. The few reports available suggest that BRs play a significant role in ion uptake. Khripach et al. (1999) studied the accumulation of heavy metals under the

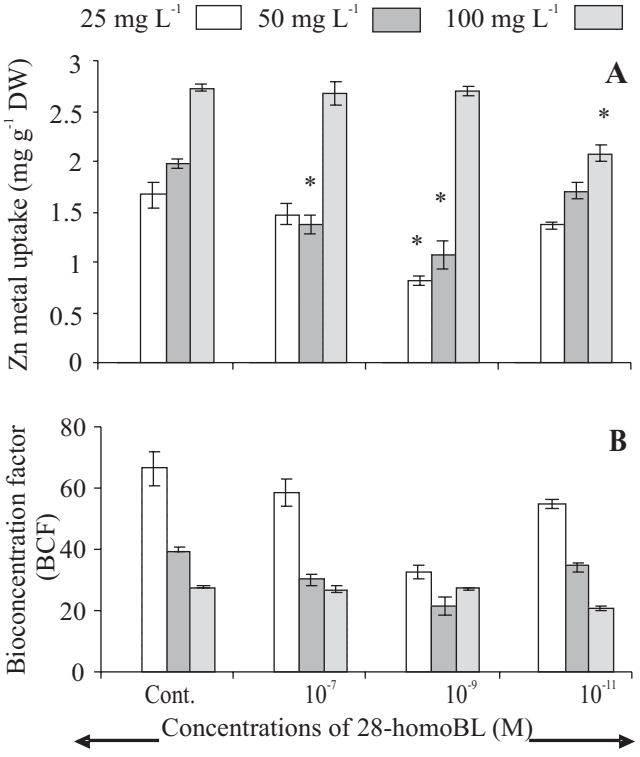


**Figure 2**. Zinc concentration (mg $g^{-1}$ DW) (A) and bioconcentration factor (B) of 7-d-old *Brassica juncea* seedlings under the influence of 28-homobrassiniolode (28-homoBL). See further details in legend to Figure 1.

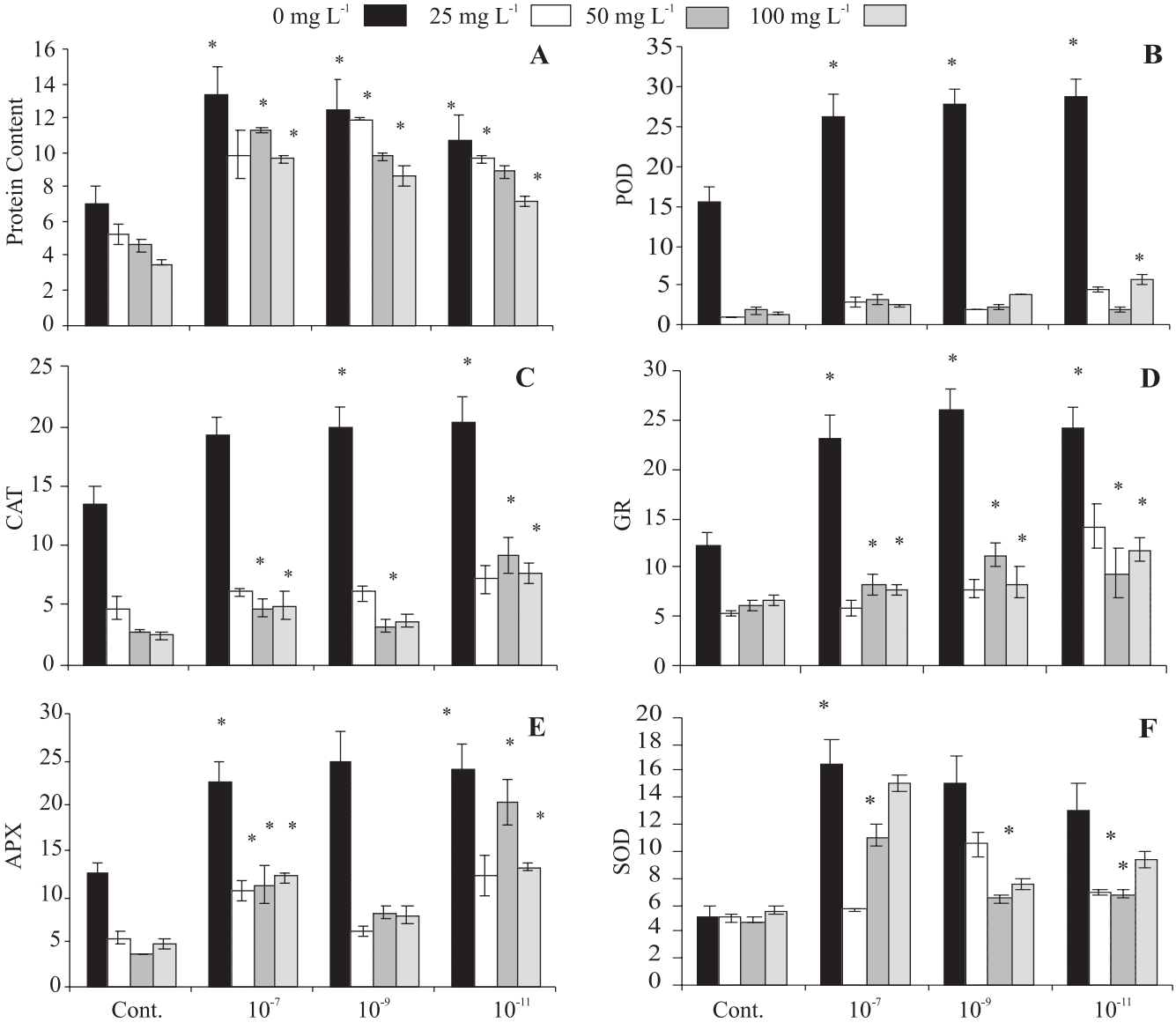


**Figure 3.** Effect of 28-homoBL on protein content (A) and specific activity (mmole UA mg protein-1) of POD (B), CAT (C), GR (D), APX (E) and SOD (F) under Zn metal stress. Bars represent the SE. (* Indicate statistically significant differences from control at $P < 0.05$).

influence of BRs, for different agricultural plants such as barley, tomato, radish and sugar beet. It was found that the application of 24-epibrassinolide significantly reduced the metal absorption as compared to controls. Similarly, Bajguz (2000) observed that 24-epibrassinolide at the concentration range of $10^{-6}$–$10^{-4}$ M in combination with heavy metals blocked metal accumulation in algal cells. Bilkisu et al. (2003) reported that brassinolide during Al-related stress stimulated growth in *Phaseolus aureus*. The application of epibrassinolide on winter rape plants under Cd stress showed stress-protective effects (Janeczko et al., 2005).

The present finding revealed that 28-homoBL seed-pre-sowing treatments enhanced protein content in 7-d-old *B. juncea* seedlings under Zn metals stress conditions as compared to control. Earlier reports indicated that 24-epibrassinolide-treated seedlings of *B. napus* showed maximum resistance to lethal heat treatments compared to control seedlings and this was found to correlate with higher levels of heat-shock

proteins and corresponding mRNA during heat stress (Dhaubhadel et al., 1999, 2002; Kagale et al., 2007). The observations in the present study revealed that homobrassinolide applications to *B. juncea* seedlings resulted in increased activity of antioxidative enzymes, which is consistent with Mazorra et al. (2002), who found enhanced CAT activity in rice under the influence of BRs. Similarly, Saglam-Cag (2007) revealed that POD activity showed the greatest effect when 0.1 μM 24-epibrassinolide was applied to wheat plants. He observed that POD activity increased 2.4, 2.9 and 2.4 fold in $10^{-3}$, $10^{-1}$ and 10 μM concentrations of 24-epibrassinolide, respectively. A few other earlier reports also suggested that epibrassinolide treatments increased the resistance of cucumber seedlings by enhancing activities of antioxidant enzymes under the effect of pesticides (Xia et al., 2006). The studies by Hayat et al. (2007) also revealed that the activities of antioxidative enzymes (CAT, POD and SOD) increased in roots and aerial parts of *B. juncea* plants when 28-homoBL foliar treatments were given. Similar reports were found by Nunez et al. (2003) who revealed that the application of BRs caused the activation of antioxidative enzymes under water and salt stresses and increased SOD and proline contents under NaCl stress.

Our earlier studies showed that BRs have the ability to lower metal uptake in plants. This may be due to the binding of BRs with membrane proteins that enhance metabolic activities by detoxifying the heavy metal stresses (Kaur and Bhardwaj, 2004). Brassinosteroids protect the plants from toxic action of ROS either by directly acting on them or indirectly by regulating the enzymatic and non-enzymatic systems of plants. Heavy metals-generated ROS could thus be alleviated by brassinolide treatments (Almeida et al., 2005; Hayat et al., 2007). Application of BRs has 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 (Nunez et al., 2003; Ozdemir et al., 2004; Janeczko et al., 2005; Hayat et al., 2007; Kagale et al., 2007). These beneficial effects led to higher leaf area, biomass production, grain yield and yield-related parameters in the treated plants. Increased water uptake, membrane stability and higher carbon dioxide and nitrogen assimilation rates under stress seemed to be related to homobrassinolide-induced stress tolerance (Hayat et al., 2007; Kagale et al., 2007).

## CONCLUSION

The present study shows that, although zinc metal is essential for normal plant growth and physiological processes, above threshold concentrations the metal is toxic and may result in growth inhibition and altered metabolic processes. The observations of the present study clearly indicate heavy metal stress-protective properties of BRs in *Brassica juncea* plants. Stress-ameliorative properties of BRs are clearly demonstrated by better growth, reduced uptake of metal ions, and antioxidative enzymes in seedlings to which various treatments of heavy metals and brassinolides were applied. It points to the possibility of BR-regulated stress-protection in plants but extensive studies are still needed on various aspects related to stress.

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