RESEARCH ARTICLE

24-epibrassinolide regulated diminution of Cr metal toxicity in *Brassica juncea* L. plants

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ABSTRACT

The present work was undertaken to study the effects of 24-epibrassinolide (0, 10^{-10}, 10^{-8} and 10^{-6} M) on growth (shoot length and number of leaves), protein content and activities of antioxidative enzymes [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), monodehydroascorbate reductase (EC 1.1.5.4) and dehydroascorbate reductase (EC 1.8.5.1)] in leaves of 60 days-old *Brassica juncea* L. plants treated with different concentrations of Cr metal. It was observed that treatment of different concentrations (0, 0.5, 1.0, 1.5 and 2.0 mM) of Cr metal alone decreased the shoot length and number of leaves and regulated the enzyme activities and protein concentration of plants. However, seed-presoaking treatments of 24-epibrassinolide improved the growth and stimulated the activities of antioxidant enzymes and protein content in leaves of *B. juncea* plants thus indicating the stress-ameliorative properties of 24-epibrassinolide.

Key words: antioxidative enzymes, brassinosteroids, metal toxicity

Abbreviations: 24-epiBL, 24-epibrassinolide; CAT, catalase; POD, guaiacol peroxidase; SOD, superoxide dismutase; APOX, ascorbate peroxidase; GR, Glutathione reductase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase.

INTRODUCTION

The hormones regulate the pace of growth of individual part and integrate them to produce the form that we recognize as a plant (Davies, 1995) and metabolism provides the power and building blocks for plant life. Until quite recently, plant growth and development was considered to be regulated only by five groups of hormones, namely auxins, gibberellins, cytokinins, ethylene and abscisic acid. However, there are compelling evidences for considering brassinosteroids, a group of steroidal substances first isolated from the pollen of *Brassica napus*, as the sixth group of plant hormones. Since the isolation and identification of brassinolide from the pollen of rape as the first bioactive steroid with high plant growth-promoting activities, brassinosteroids (BRs) have received increasing impetus as a new class of phytohormone (Davis, 1995; Kauschmann et al., 1996; Clouse and Sasse, 1998; Khripach et al., 1999). Various studies have shown that BRs are essential for plant growth and development, and are actively involved in many physiological processes (Clouse et al., 1996; Steber and McCourt, 2001). BRs have pleotropic effects and can induce a broad array of cellular responses including stem elongation, leaf bending and epinasty, pollen tube growth, induction of ethylene biosynthesis, proton pump activation, xylem differentiation, and regulation of gene expression (Clouse and Sasse, 1998; Li and Chory, 1999; Hu et al., 2000; Arteca and Arteca, 2001; Dhaubhadel et al., 2002). Use of BRs has also been examined in agricultural production. Several studies have established that BRs influence seed germination, plant growth, nitrogen fixation, senescence, leaf abscission and enhanced tolerance against drought and cold stress, heavy metal stress, salt stress and diseases (Khripach et al., 2000; Anuradha and Rao, 2001; Nakashita et al., 2003; Yu et al., 2004). As a consequence, extensive research has been undertaken to develop BRs as plant growth regulators for

agricultural production (Ikekawa and Zhao, 1991; Sasse, 2003; Ozdimer et al., 2004).

Though heavy metals are essential as micronutrients for plants, but they are toxic at higher concentrations (Dalton et al., 1988). Heavy metal stress leads to the production of reactive oxygen species (ROS). These include superoxide radical, hydrogen peroxide, hydroxyl radical in various plant species (Marschner, 1995). These ROS have the ability to initiate lipid peroxidation and degrade proteins and nucleic acid. To scavenge these ROS, plants are occupied with strong antioxidative defence system comprising of antioxidants such as glutathione, vitamin C, polyphenols, flavanoids etc. and antioxidative enzymes such as superoxide dismutases, catalases, guaiacol peroxidases, glutathione reductases, ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase (Salin, 1988). Brassinosteroids help to overcome stress by regulating the activities of these antioxidative enzymes (Ozdimer et al., 2004; Liu et al., 2009).

Although improved plant growth has been shown in field trials using BRs, the physiological basis of these effects is still poorly understood. Therefore, the present study was undertaken to observe the influence of 24-epibrassinolide on growth, protein content and activities of antioxidative enzymes under Cr metal stress.

MATERIALS AND METHODS

The seeds of *Brassica juncea* L. cv. 'PBR 91' (certified) used in the present investigation were obtained from the Department of Plant Breeding, Punjab Agriculture University, Ludhiana, India. Seeds were surface sterilized with 0.01% $HgCl_2$, washed and rinsed thrice with double distilled water. These surface sterilized seeds were soaked for 8 hrs in different concentrations of 24-epiBL (0, 10^{-10}, 10^{-8} and 10^{-6} M). The earthern pots to be used for the experiment were arranged in triplicates in the Botanical Garden of the University. Different concentrations of chromium metal in the form of K_2CrO_4 (0, 0.5, 1.0, 1.5 and 2.0 mM) were added in the pots containing approximately 5 Kg soil per pot.

All the chemicals were procured from Sigma-Aldrich. The soil used for the present study was prepared using garden soil, silt and cow dung manure in the ratio of 2: 1: 1. The seeds treated with 24-epiBL for 8 hrs were sown in the earthern pots that contained different concentrations of Cr metal. The earthern pots were kept in natural seasonal conditions. Field management followed normal agricultural practices. On 60th day, the plants were analyzed for morphological parameters viz. shoot length and number of leaves. Experiment was repeated twice. For estimation of protein content and antioxidant enzyme activities viz. SOD, CAT, GR, POD, APOX, MDHAR and DHAR, 0.5 g leaf tissue was homogenized in pre-chilled pestle and mortar with 5 ml of 100 mM potassium phosphate buffer (pH 7.0) under ice-cold conditions. The homogenate was centrifuged at 4^{o}C for 20 min at 15,000 *g* and the supernatants was used for determining protein content and activities SOD, CAT, POD, APOX, GR, MDHAR and DHAR spectrophotometrically (Shimadzu 2202).

The activity of SOD was determined by monitoring its ability to inhibit photochemical reduction of nitrobluetetrazolium (NBT) at 540 nm (Kono, 1978). POD activity was determined according to Putter (1974). CAT activity was determined by following the initial rate of disappearance of H_2O_2 at 240 nm (Aebi, 1983). 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. Protein content was determined following the method of Lowry et al., (1951).

Statistical Analysis: All the experiments were performed in triplicates taking three replicas of each 24-epiBL treatment, under 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 comparison of P-value at 0.05 was considered significantly different from control (Bailey, 1995).

RESULTS

The observations made on morphological parameters indicated that the treatments of 24-epiBL significantly affected plant growth. Application of 24-epiBL alone increased shoot length and number of leaves when compared with untreated control plants alone. 10^{-8} M concentration of 24-epiBL alone was found to be most effective in increasing all morphological parameters of plants (shoot length and number of leaves). It was noted that shoot length of plants and number of leaves decreased with the increase in concentration of Cr and maximum decline in growth was observed in 2.0 mM of Cr treated plants.

However, supplementation of 24-epiBL with Cr metal solution considerably reduced the inhibitory effect of Cr thereby

revealing improved growth. Maximum increase in shoot length and number of leaves under the influence of brassinosteroids treatments was observed in *B. juncea* plants treated with 10^{-8} M concentration of 24-epiBL in combination with 0.5 mM of Cr as compared to plants treated with Cr alone (Figure 1).

The studies done on biochemical constituents also revealed significant effects of BRs treatments. The protein content of leaves of 60 days old plants increased considerably in all treatments of 24-epiBL in comparison to untreated control (Figure 1).

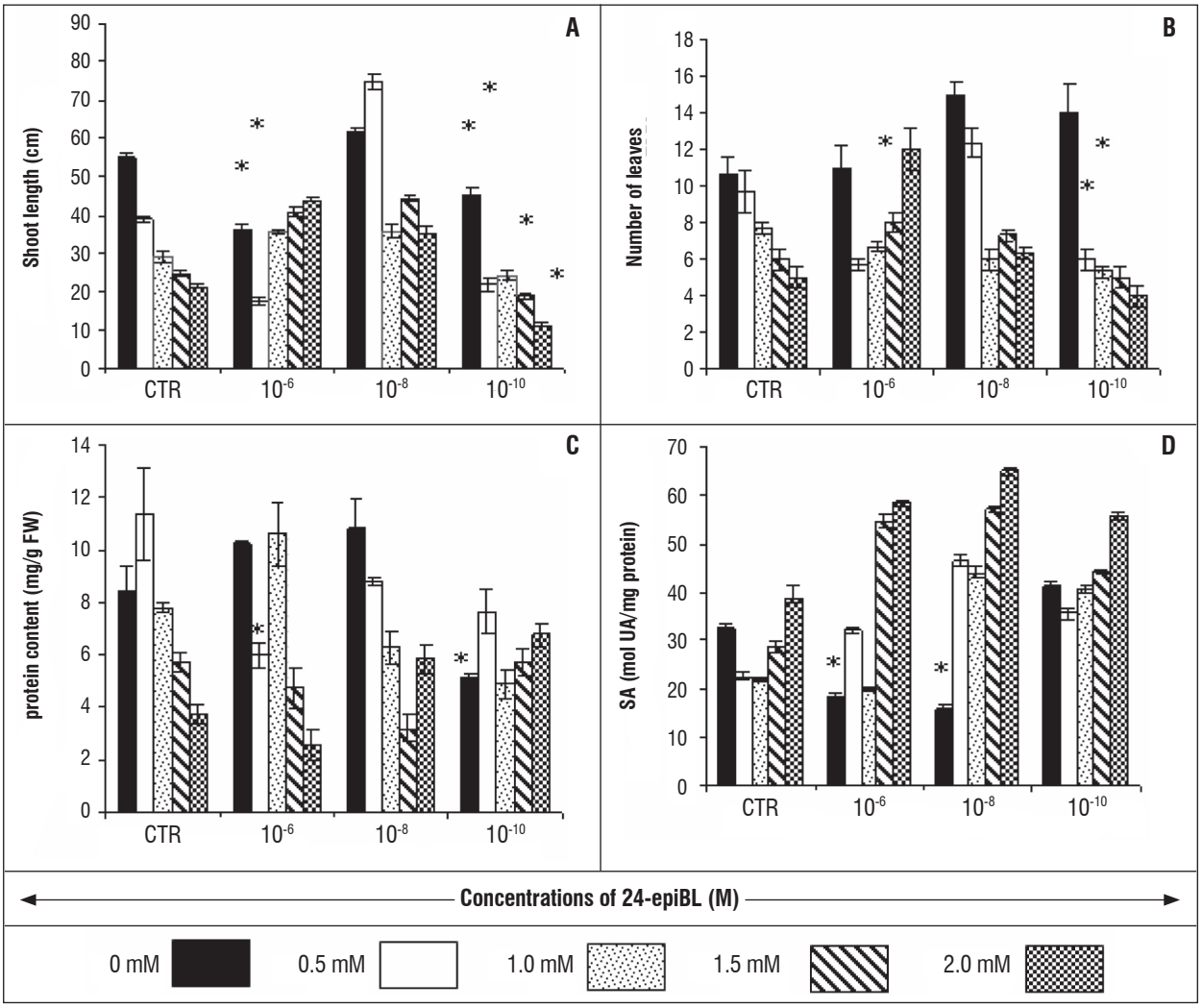

Figure 1. Effect of 24-epiBL on shoot length, number of leaves, protein content and specific activity of catalase on leaves of 60-days old *Brassica juncea* L. plants under Cr metal stress. Bars represent the SE (n=3) and asterisks indicate statistically significant differences from control treatments at $P<0.05$.

The treatment of 10^{-8} M of 24-epiBL resulted in maximum protein content (10.84 mg g^{-1} FW) in leaves of B. *juncea* plants. The protein content of leaves under heavy metal stress was found to increase significantly in all treatments of 24-epiBL. Maximum protein content (10.61 mg g^{-1} FW) was observed in case of leaves of plants treated with 10^{-8} M of 24-epiBL supplemented with 1.0 mM of Cr metal as compared to 1.0 mM Cr treated plants (7.806 mg g^{-1} FW). The activities of antioxidative enzymes (CAT, POD, SOD, APOX, GR, MDHAR and DHAR) were also enhanced in leaves of plants treated with 24-epiBL alone, 10^{-8} M concentration being the most effective. The activities of antioxidative enzymes were also enhanced in plant leaves by the application 24-epiBL supplemented Cr metal solutions. The CAT activity in leaves of *B. juncea* plants increased with increasing concentration of Cr metal and maximum increase was observed in case of 2.0 mM Cr treated leaves (38.64 mol

UA mg^{-1} protein). CAT activity showed maximum enhancement (65.03 mol UA mg^{-1} protein) in case of plant leaves treated with 10^{-8} M 24-epiBL supplemented with 2.0 mM of Cr metal. The activity of POD increased with Cr metal stress. 24-epiBL treatments to Cr-stressed plant leaves further enhanced the POD activity that was maximum (75.05 mmol UA mg^{-1} protein) in leaves of plants treated with 10^{-8} M 24-epiBL supplemented with 1.5 mM of Cr metal concentration. The activity of SOD was alleviated by 10^{-8} M of 24-epiBL supplemented with 1.0 mM of Cr (93.2 mol UA mg^{-1} protein) when compared to 1.0 mM of Cr metal (34.36 mol UA mg^{-1} protein). The activities of APOX and GR were found to increase with increase in concentrations of Cr metal. However, the treatments of 24-epiBL to Cr stressed plant leaves further enhanced the APOX and GR activities. Maximum enhancement in the activity of APOX (93.73 mmol UA mg^{-1} protein) and GR (39.64 mmol UA mg^{-1} protein) was observed in case of leaves of plants treated with 10^{-8} and 10^{-10} M respectively of 24-epiBL supplemented with 1.0 and 1.5 mM respectively of Cr metal concentrations (Figure 2). Similarly, the activities of MDHAR and DHAR were also found to increase with increase in concentrations of Cr metal. However, the treatments of 24-epiBL to plant leaves under Cr metal stress further enhanced the MDHAR and DHAR activities. Maximum enhancement in the activity of MDHAR (25.29 mmol UA mg^{-1} protein) and DHAR (88.37 mmol UA mg^{-1} protein) was observed in case of leaves of plants treated with 10^{-10} and 10^{-8} M respectively of 24-epiBL supplemented with 1.5 mM of Cr metal (Figure 3).

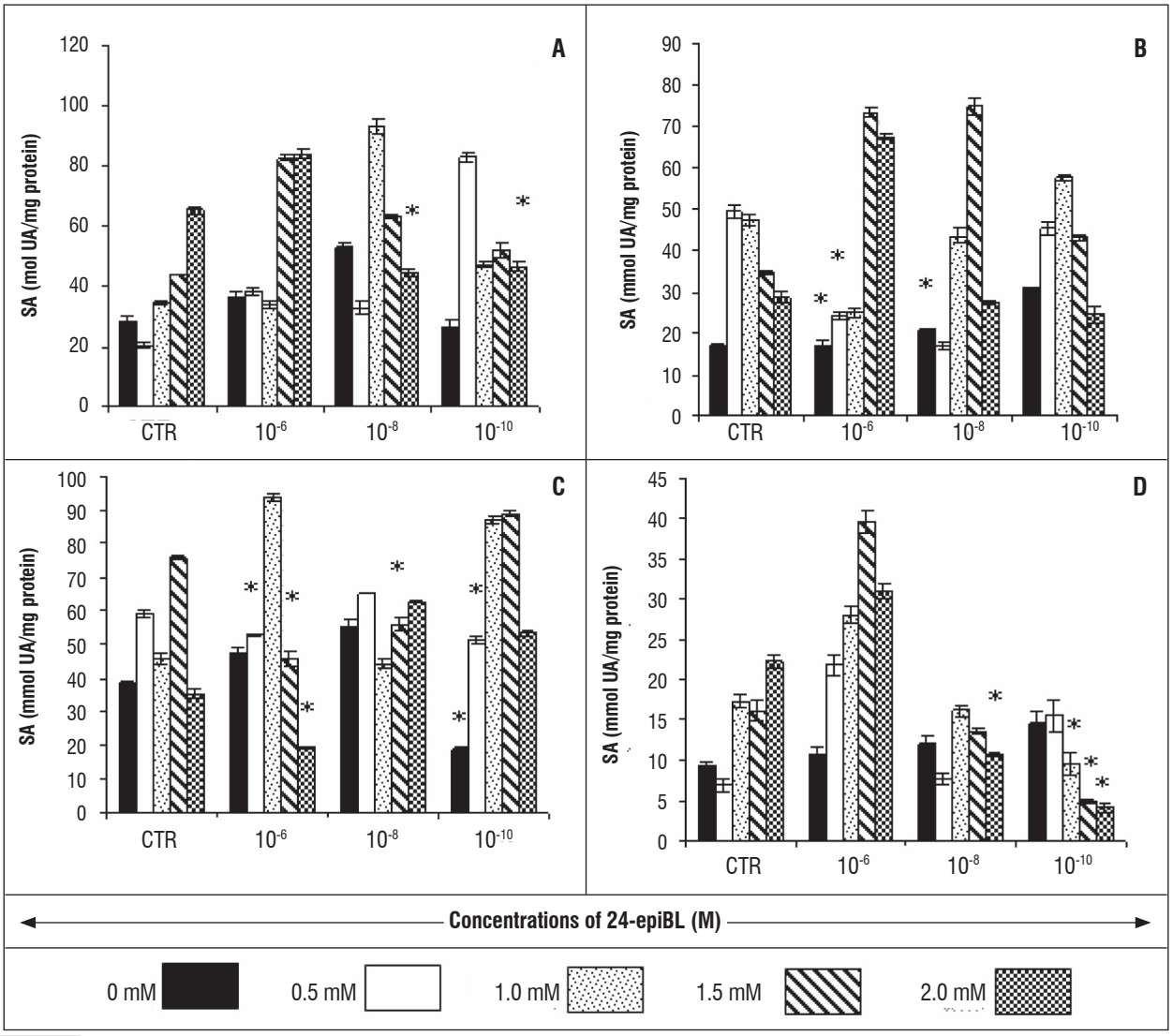

Figure 2. Effect of 24-epiBL on specific activities of superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase and glutathione reductase on leaves of 60-days old *Brassica juncea* L. plants under Cr metal stress. Bars represent the SE (n=3) and asterisks indicate statistically significant differences from control treatments at $P<0.05$.

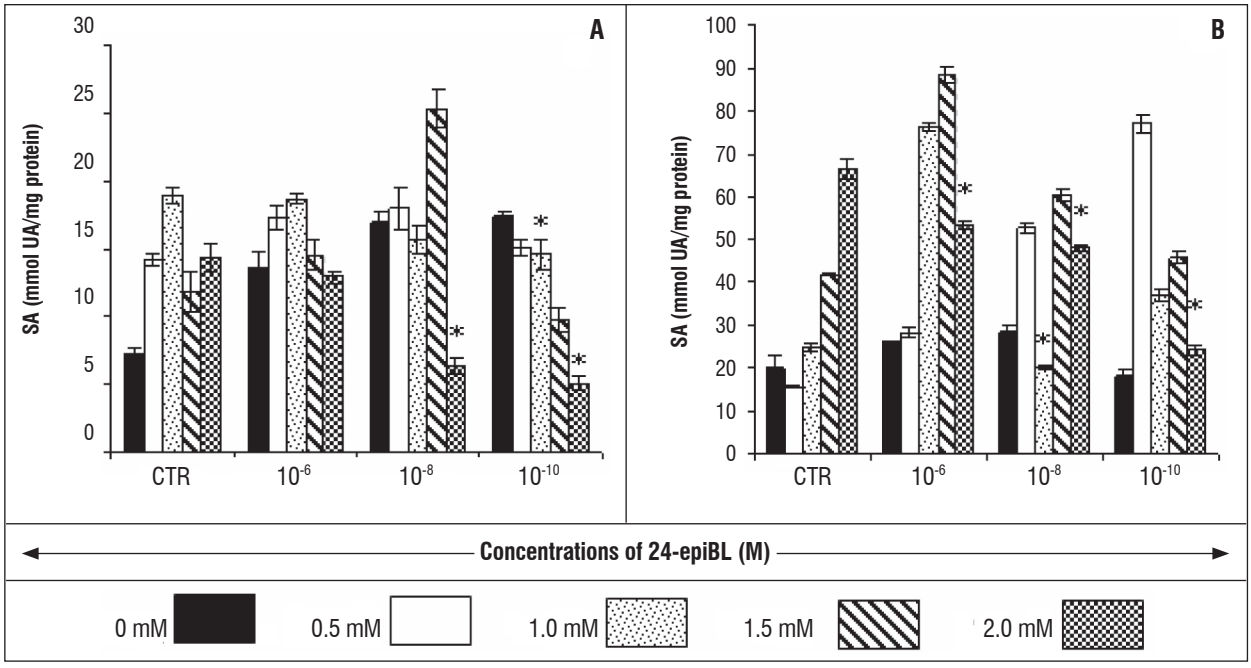

Figure 3. Effect of 24-epiBL on specific activities of monodehydroascorbate reductase and dehydroascorbate reductase on leaves of 60-days old *Brassica juncea* L. plants under Cr metal stress. Bars represent the SE (n=3) and asterisks indicate statistically significant differences from control treatments at $P<0.05$.

DISCUSSION

The present experiment revealed that the application of 24-epiBL improved the seed vigor in terms of shoot length and number of leaves, increased protein content and regulated the activities of enzymes of Asada-Halliwell pathway viz. SOD, APOX, GR, MDHAR, DHAR and water-water cycle enzyme activities i.e. POD and CAT under Cr metal stress (Figure 4).

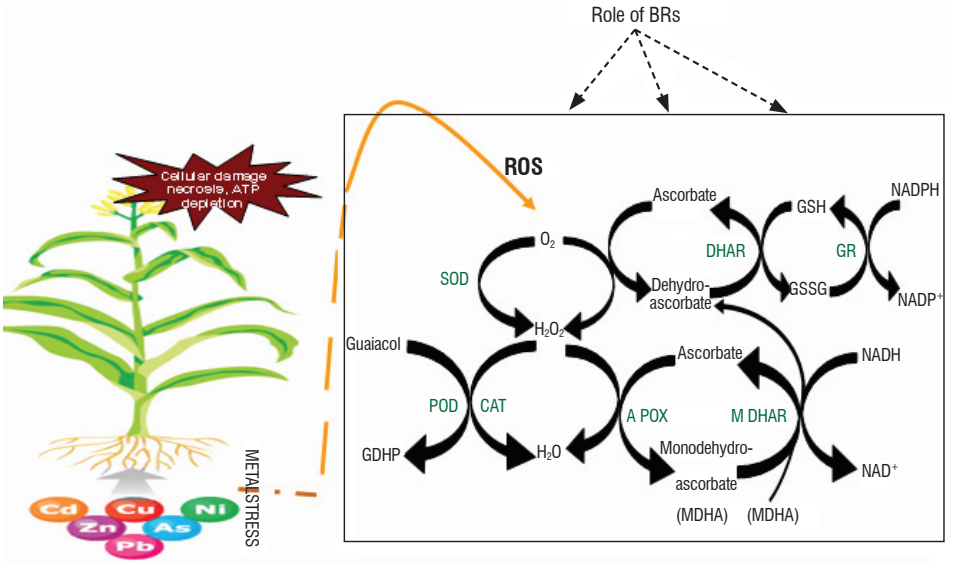

Figure 4. Brassinosteroids-regulated Asada-Halliwell pathway in plants (modified after Arora *et al*., 2002; Mittler, 2002; Shaw *et al*., 2004). CAT, catalase; POD, guaiacol peroxidase; SOD, superoxide dismutase; APOX, scorbate peroxidase; GR, Glutathione reductase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase.

The primary effect of BRs is promoting cell elongation and division. They are highly effective in stimulating growth in young vegetative tissues. Brassinosteroids promoted elongation of mung bean, soybean, pea epicotyls, bean, sunflower and cucumber hypocotyls, *Arabidopsis* peduncles and *Hordeum vulgare* seedlings (Gregory, 1982; Clouse et al., 1992; Yu et al., 2004; Clouse, 2008 and Kartal et al., 2009). Hypocotyl elongation of Packchoi (*Brassica chinensis*) by brassinosteroids was reported (Wang et al., 1993) and application to apical 3-mm region gave the best effect. Use of an ATPase inhibitor i.e. dicyclohexyl carbodiimide has revealed that elongation induced by BRs involve ATPases (Katsumi, 1985). Since other plant growth regulators such as GAs and auxins also promote cell elongation, the regulation of some auxin-regulated genes such as SAUR, GH1 and JCW was studied for response to BR (Clouse et al., 1992). This experiment showed that although GH1 and SAUR genes are upregulated by BR, the kinetics of regulation was very different when compared to their regulation by auxin. (Clouse and Sasse, 1998). This was the first molecular evidence suggesting that BRs are likely to have an independent mode of action in cell elongation. Oh and Clouse (1998), using *Petunia hybrida* mesophyll protoplasts, reported that BRs promoted the frequency of cell divisions and also accelerated the time of first cell division. By well-designed experiments, Arteca and Arteca (2001) proved that the exaggerated growth induced by brassinolide in *Arabidopsis thaliana* was independent of other known phytohormones.

Environmental stresses frequently cause membrane damage, decrease hydrolytic enzyme activity and increased lipid peroxidation. It may stimulate formation of ROS, such as O_2^- H_2O_2, and $OH^{\cdot}$ radicals. To neutralize the toxicity of ROS, plants are equipped with enzymatic (e.g. monodehydroascorbate reductase, dehydroascorbate reductase, superoxide dismutase, glutathione reductase, ascorbate peroxidase, guaiacol peroxidase and catalase) and non-enzymatic (e.g. glutathione, glycine-betains, ascorbate, proline, sorbitol and mannitol) defence system to operate (Mittler, 2002). Among ROS, superoxide radical ($O_2^{\cdot -}$) is dismutated by SOD enzyme into H_2O_2 and is further scavenged by CAT and various peroxidases like APOX and POD. GR, MDHAR and DHAR also play a key role by reducing H_2O_2 to water through the ascorbate-glutathione cycle (Noctor and Foyer, 1998). It is widely accepted that ROS are responsible for various stress-induced damages to macromolecules and ultimately to cellular structures (Halliwell and Gutteridge, 1999). Consequently, the role of antioxidative enzymes, such as SOD, APOX, GR, MDHAR, DHAR, CAT and POD become very important. The level of these enzymes is increased by the action of 24-epiBL to overcome the stress generated by Cr and to enhance the resistance capacity of plants (Figure 1-3). This data is consistent with the studies that exogenous BRs treatment is effective in ameliorating stressful conditions rather than in optimal conditions (Sasse, 1997, Bhardwaj et al., 2007, Sharma and Bhardwaj, 2007b; Arora et al., 2008, Hayat et al., 2009). Similarly, Sharma et al., (2007) reported that pre-sowing treatments of 28-homobrassinolide lowered the uptake of metal and enhanced the activities of antioxidative enzymes and protein concentration of 7-d old *B. juncea* seedlings under zinc metal stress. Moreover, Khripach et al., (1999) suggested that BRs affected the biosynthesis of enzymes by involving gene expression and hence the effect of BRs on cell membrane. So the difference in alteration of antioxidative enzyme activities may suggest that 24-epiBL treated plants were less affected by Cr metal stress than the untreated plants. Therefore, the present study would be useful in providing an insight into the stress-ameliorative properties of BRs in *Brassica juncea*.

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