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Presoaking Seed Treatment of 24-epiBL Modulates Cr Stress in *Brassica juncea* L.

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ABSTRACT

The present work was undertaken to study the effects of 24-epibrassinolide on growth, protein content and 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)] activities in leaves of 30 days-old *Brassica juncea* L. plants treated with different concentrations of chromium (Cr). Different concentrations of Cr alone decreased the enzyme activities and protein concentration of plants. However, pre-sowing treatments of 24-epibrassinolide improved the growth and enhanced the activities of antioxidative enzymes and protein content in leaves of *B. juncea* plants.

Keywords: antioxidant enzymes, 24-epibrassinolide, brassinosteroids, chromium toxicity
Abbreviations: 24-epiBL, 24-epibrassinolide; **ANOVA**, analysis of variance; **APOX**, Ascorbate peroxidase; **CAT**, Catalase; **Cr**, chromium; **DHAR**, Dehydroascorbate reductase; **GR**, Glutathione reductase; **MDHAR**, Monodehydroascorbate reductase; **POD**, Guaiacol peroxidase; **ROS**, reactive oxygen species; **SOD**, Superoxide dismutase

INTRODUCTION

Chromium (Cr) compounds are discharged in solid, liquid, and gaseous wastes into the environment, resulting in significant adverse ecological and biological effects (Kabata-Pendias and Pendias 2001). Out of various forms of chromium, Cr (VI) is considered to be the most toxic. Morphological symptoms of Cr toxicity in plants are stunted growth, reduced root system, curled and discolored leaves (Pratt, 1966), leaf chlorosis, narrow leaves (Hunter and Vergnano 1953), chlorotic bands on cereals (Kabata-Pendias and Pendias 1992) and yield reduction (Parr and Taylor 1982). Some plants may exhibit purpling of basal tissues or brownish-red leaves containing small necrotic areas.

Cr, like other metals, induces oxidative stress by generating free radicals and toxic reactive oxygen species (ROS) (Hegedus *et al*. 2001; Arvind and Prasad 2003). These ROS are partially reduced forms of atmospheric oxygen and under normal conditions their production in cells is low and tightly controlled (Dat *et al.* 2000). Heavy metal toxicity enhances the production of ROS up to 30-fold (Mittler 2002). These species react with lipids, proteins, pigments and nucleic acids and cause lipid peroxidation, membrane damage and inactivation of enzymes and thus affect the cell viability. One of the mechanisms that make a plant species tolerant to heavy metal stress is the presence of a strong antioxidant defence system (Pandey *et al*. 2005; Qureshi *et al*. 2005). The deleterious effects resulting from the cellular oxidative state may be alleviated by the enzymatic and non-enzymatic antioxidant machinery of the plant (Halliwell 1999) which include superoxide dismutase (SOD), ascorbate peroxidase (APOX), catalase (CAT), guaiacol peroxidase (POD), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) as enzymatic form and non-enzymatic antioxidants like ascorbic acid, α-tocopherol and glutathione.

Brassinosteroids (BRs) are a large group of plant steroid hormones present universally in plants (Bajguz and Tretyn 2003). Brassinosteroids have been first isolated from *Brassica* species by Grove *et al.* (1979) and till date more than 70 types of BRs have been reported from a large number of plant species. 24-epibrassinolide (24-epiBL) is one of the most active forms of BRs, which is also available commercially. These chemicals elicit a wide range of physiological responses in plants, including stem elongation, pollen tube growth, leaf bending and epinasty, root growth inhibition, induced synthesis of ethylene, activation of proton pump, xylem differentiation, synthesis of nucleic acids and proteins, activation of enzymes and photosynthesis (Sasse 2003; Yu *et al*. 2004; Hayat *et al*. 2007; Clouse 2008). BRs are known to promote adventitious shoot regeneration from segments of cauliflower hypocotyls (Sasaki 2002) and improve embryogenic tissue initiation in conifers and rice (Pullman *et al.* 2003). They are widely spread in plants including dicots, monocots, gymnosperms, ferns and algae, and exist in all parts of the plant (Khripach *et al*. 2000; Rao *et al*. 2002). They are mainly produced in pollens and also present in seeds, stems, young leaves and buds but in less amounts (Fujioka *et al*. 1998). BRs, which are known to be produced in roots, have also been detected in relatively higher amounts in crown tumors produced by *Agrobacterium tumefaciens*, which is commonly present in soil. Several studies have demonstrated that BRs influence plant growth, seed germination, nitrogen fixation, senescence, leaf abscission, and enhanced tolerance against various abiotic and biotic stresses (Ali *et al*. 2008; Kartal *et al*. 2009). These steroids have been reported to improve plant resistance against various environmental stresses like thermal stress (Kurepin *et al*. 2008), salinity (Ali *et al*. 2007), chilling injury (Liu *et al*. 2009) and heavy metal stress (Nunez *et al*. 2003; Arora *et al*. 2008; Hayat *et al*. 2010). In Belarus and Russia, 24-epiBL is the most active ingredient of the plant growth promoter named as Epin (Moiseev 1998), which has been officially registered since 1992. It is recommended for treatment of agricultural plants such as potato, tomato, pepper, barley and cucumber (State Chemical Commission of Belarus 1998; State Chemical Commission of Russian Federation 1999). Another chemical namely Tian-

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fengsu, has been developed in China as the result of a joint research project between Japanese and Chinese scientists (Ikekawa and Zhao 1991). This preparation is mainly a mixture of natural 24-epiBL and its unnatural 22S and 23S isomer. Tianfengsu is widely used in China to increase the yield of crops such as wheat, rice, maize, cotton and vegetables (Ikekawa and Zhao 1991). Taking into consideration the stress-ameliorative properties of BRs, the present work was undertaken to observe the effect of 24-epiBL on the antioxidant defence system of *Brassica juncea* L. plants under chromium metal stress.

MATERIALS AND METHODS

Study material

The seeds of *B. juncea* L. cv. 'PBR 91' (certified) used in the present investigation were obtained from the Department of Plant Breeding, Punjab Agriculture University, Ludhiana, India. This cultivar was chosen from among the various varieties of *B. juncea*, viz. 'PBR 91', 'PBR 97' and 'Pusa Agrani' since it shows stability for most of the important yield contributing characters as well as seed yield under the existing conditions in the area of study.

Raising of plants in field

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 clay pots used for the experiment were arranged in triplicate 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 to 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 pots (24 cm diameter) that contained different concentrations of Cr metal. The pots were kept in natural seasonal conditions. Irrigation was applied on every second day to achieve soil water field capacity level.

Estimation of morphological parameters

On the 30th day, plants were analyzed for morphological parameters viz. shoot length and number of leaves. Fifteen plants per treatment were used to determine these parameters. Experiment was repeated twice. Observations were made on 30th day as this represents the growth period before anthesis.

Estimation of protein content and antioxidant enzyme activities

1) Preparation of plant extracts

For estimation of protein content and antioxidant enzyme activities *viz*. CAT, SOD, 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°C for 20 min at 15,000 × *g* and the supernatants were used for determining protein content and activities of SOD, CAT, POD, APOX, GR, MDHAR and DHAR.

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 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 comparisons of P-value at 0.05 were considered significantly different from control (Bailey 1995).

RESULTS

The metabolism of *B. juncea* was significantly affected by seed-presoaking treatment of 24-epiBL. This effect was observed in terms of altered morphological parameters and antioxidant enzyme activities.

Plant growth

The effect of 24-epiBL on shoot length and number of leaves of 30 days old plants are shown in **Fig. 1**. The shoot length was reduced significantly from 3.8 cm in untreated control plants to 2.5 cm under increasing concentrations of Cr stress. However, 24-epiBL treatment markedly increased the shoot length at 10^{-10}, 10^{-8} and 10^{-6} M concentration. Supplementation of Cr metal solution with 24-epiBL considerably reduced the inhibitory effect of Cr on plant growth. The shoot length (6.0 cm) of plants treated with 10^{-8} M of 24-epiBL supplemented with 0.5 mM of Cr metal solution was maximum in comparison to metal treated plants (3.73 cm). Similarly, the number of leaves of plants decreased as the concentration of metal increased and this decrease was maximum (4.0) in case of plants treated with 2 mM of Cr. But, treatment with 24-epiBL improved the number of leaves at 10^{-10}, 10^{-8} and 10^{-6} M alone and in combination with Cr metal. Whereas, supplementation of 10^{-8} M 24-epiBL with 0.5 mM Cr metal revealed an increase (6.33) when compared to metal treated plants (5.0). In addition to these effects Cr metal stress also resulted in reduced root system and purpling of leaves containing small necrotic areas.

Protein content and antioxidant enzyme activities

The protein content of leaves of 30 days old plants increased considerably in all treatments of 24-epiBL in comparison to untreated control (**Fig. 1**). It was significantly

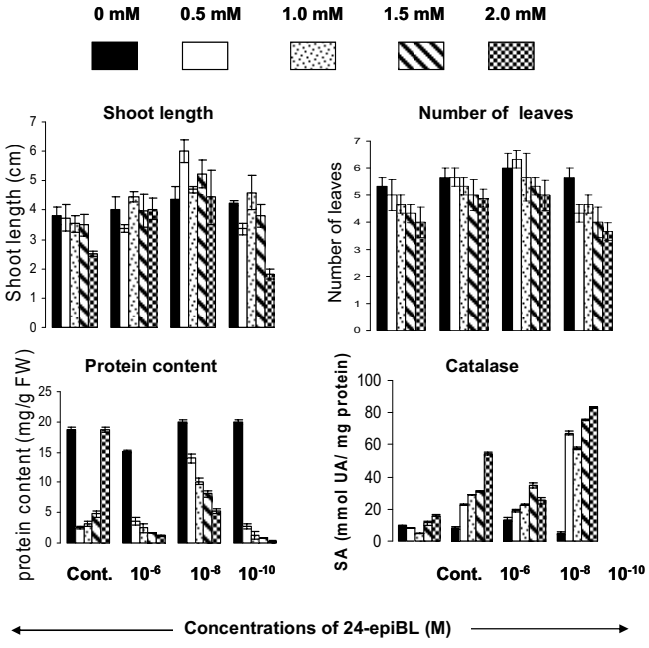

Fig. 1 Effect of 24-epiBL on shoot length, number of leaves, protein content and catalase activity in 30-d old *B. juncea* plants under Cr stress. Bars represent SE (n=3)

higher (13.99 mg gFW^{-1}) in the leaves of plants treated with 10^{-8} M of 24-epiBL supplemented with 0.5 mM of Cr when compared to metal treated plants (2.51 mg gFW^{-1}). Remarkable decrease in SOD activity was recorded under the influence of 24-epiBL, more significantly (6.82 mol UA mg^{-1} protein) at 10^{-10} M concentration of 24-epiBL. However, SOD activity got enhanced when metal treatments were supplemented with 24-epiBL (10^{-10}, 10^{-8} and 10^{-6} M). Maximum increase in SOD activity (60.17 mol UA mg^{-1} protein) was observed in plants treated with 0.5 mM of Cr in combination with 10^{-6} M concentration of 24-epiBL in comparison with metal treated plants (9.25 mol UA mg^{-1} protein) (**Fig. 1**). On the other hand, guaiacol peroxidase and catalase activities were enhanced considerably from 6.7 mmol UA mg^{-1} protein (POD) and 9.69 mol UA mg^{-1} protein (CAT) in control seedlings to 14.39 mmol UA mg^{-1} protein (POD) and 16.06 mol UA mg^{-1} protein (CAT) under Cr stress. POD and CAT activity further got enhanced by the treatment of 24-epiBL alone, with maximum rise at 10^{-8} M concentration of 24-epiBL 14.19 mol UA mg^{-1} protein for POD and 13.59 mol UA mg^{-1} protein for CAT. Maximum increase in activity of POD (26.32 mmol UA mg^{-1} protein) and CAT (83.16 mol UA mg^{-1} protein) was observed in leaves of plants treated with 10^{-10} M of 24-epiBL in combination with 2 mM of Cr (**Fig. 2**). The APOX activity got increased from 16.2 mmol UA mg^{-1} protein (control) to 37.82 mmol UA mg^{-1} protein (Cr stress). Considerable increase was recorded in APOX activity under the influence of 24-epiBL alone at 10^{-6} M (23.83 mmol UA mg^{-1} protein). This activity revealed further increase when Cr metal stress was supplemented with 24-epiBL. Maximum enhanced activity of APOX (11.87 mmol UA mg^{-1} protein) was observed for plants treated with 0.5 mM Cr in combination with 10^{-10} M of 24-epiBL (**Fig. 2**). Contrary to the enhancement of APOX activity under Cr stress, GR showed decrease from 11.57 mmol UA mg^{-1} protein in control to 10.33 mmol UA mg^{-1} protein in Cr-stressed plants. The GR activity was not considerably increased under the influence of 24-epiBL alone. However, the combination of Cr metal and 24-epiBL showed restoration of GR activity (11.91 mmol UA mg^{-1} protein) significantly in plants given treatments of Cr metal and 24-epiBL in combination (10^{-6} M of 24-epiBL with 2.0 mM of Cr) (**Fig. 2**). MDHAR and DHAR activities got increased from 5.005 mmol UA mg^{-1} protein and 5.935 mmol UA mg^{-1} protein, respectively in control untreated plants to 7.2 mmol UA mg^{-1} protein (MDHAR) and 6.58 mmol UA mg $protein^{-1}$ (DHAR) in metal treated plants. But, there was not a significant increase in the activities of MDHAR and DHAR under the effect of 24-epiBL alone. However, Cr metal supplemented with 24-epiBL helped in restoring MDHAR and DHAR activities. Maximum activity of MDHAR (19.44 mmol UA mg $protein^{-1}$) and DHAR (6.391 mmol UA mg^{-1} protein) was observed in leaves of plants treated with 10^{-6} and 10^{-10} M of 24-epiBL, respectively in combination with 2.0 mM Cr metal (**Fig. 3**).

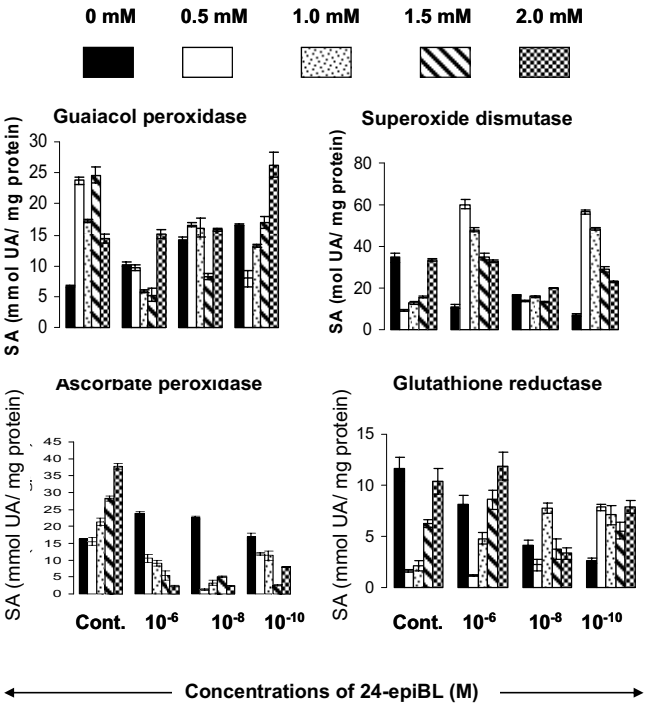

Fig. 2 Effect of 24-epiBL on activities of guaiacol peroxidase, superoxide dismutase, ascorbate peroxidase and glutathione reductase in 30-d old *B. juncea* plants under Cr stress. Bars represent SE (n=3)

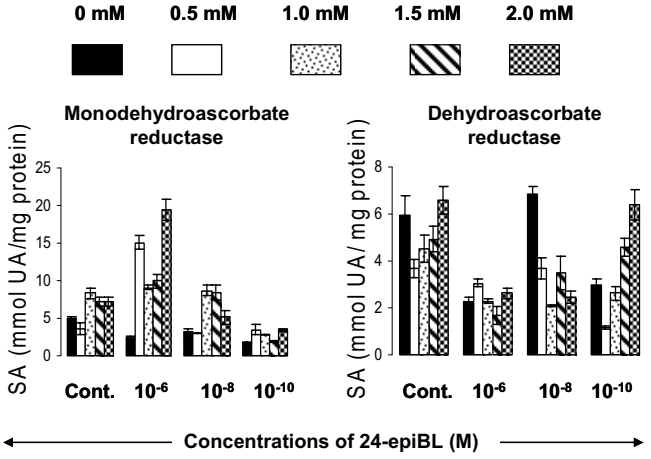

Fig. 3 Effect of 24-epiBL on activities of monodehydroascorbate reductase and dehydroascorbate reductase in 30-d old *B. juncea* plants under Cr stress. Bars represent SE (n=3)

DISCUSSION AND CONCLUSION

The present finding revealed that 24-epiBL seed-presoaking treatments remarkably improved the growth of plants under Cr metal stress. It was observed that application of 24-epiBL improved the growth of Cr-stressed *B. juncea* plants in terms of increased shoot length and number of leaves (**Fig. 1**). Earlier studies also indicated stress-ameliorative properties of BRs. Kartal *et al.* (2009) reported that HBR treatment significantly increased protein content in barley seedlings. Ali *et al.* (2007) also observed that 24-epiBL and 28-homoBL improved the growth of Al-stressed mung bean seedlings by increasing the rate of photosynthesis and carbonic anhydrase activity. Brassinolide (BL) ameliorated the Al toxicity and promoted the growth of mungbean seedlings (Abdullahi *et al*. 2003). The studies on BRs report that they 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). The cell wall loosening enzymes get activated by H+-ATPases which acidifies the apoplast. The involvement of BRs in enhancing the growth of seedlings might be taking place through activation of H+-ATPase (Haubrick and Assman 2006).

24-epiBL 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 24-epiBL applications to *B. juncea* plants resulted in increased activities of antioxidant enzymes, which is consistent with Mazorra *et al*. (2002), who found enhanced CAT activity in rice under the influence of BRs. Heavy metals-generated ROS could thus be alleviated by brassinolide treatments (Almeida *et al*. 2005; Hayat *et al*. 2007). Application of

Table 1 Effect of 24-epiBL in different physiological and biochemical events in various plant species.

Plant species	Physiological and biochemical events	References
Oryza sativa	Promotion of growth, enhanced levels of nucleic acids and proteins under salt stress	Anuradha and Rao 2001
Vigna radiata L. Wilczek	Improved and enhanced growth, photosynthesis and activities of antioxidant enzymes (catalase, superoxide dismutase and peroxidase) under aluminium stress	Ali *et. al.* 2008
Brassica napus and *Lycopersicon esculentum*	Improved thermotolerance. Higher accumulation of heat-shock proteins (hsp 100, hsp 90, hsp 70 and low molecular weight hsps)	Dhaubhadel *et al*. 1999, 2002
Chorispora bungeana	24-epiBL treatment significantly enhanced antioxidant enzyme activity and content of antioxidants under chilling stress	Liu *et al*. 2009

BRs have been shown to involve the major antioxidant 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) (**Table 1**). As membrane destruction results from ROS induced oxidative damage (McCord 2000), the 24-epiBL treated plants might be scavenging ROS more efficiently than the plants treated with metal alone. Similarly, Liu *et al*. (2009) demonstrated that EBR treatment significantly enhanced antioxidant enzyme activity and content of antioxidants in *Chorispora bungeana* under chilling stress.

Our earlier studies had also shown that 24-epiBL treatments improved the shoot emergence and plant biomass production in *Brassica 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.* 2008; Sharma and Bhardwaj 2008). Altered activities of antioxidant enzymes further suggest that 24-epiBL treated plants were less affected by Cr metal than the untreated plants. Further, our studies on heavy metal stress indicate that 28-HomoBL ameliorates the Ni toxicity by increasing the activities of antioxidant enzymes like superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, catalase and glutathione reductase (Bhardwaj *et al.* 2008). The present study on the similar lines demonstrated the possibility of BRs to reduce the impact of chromium metals also on plant growth by stimulating the activities of key antioxidant enzymes.

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