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Role of 24-Epibrassinolide in Amelioration of High Temperature Stress through Antioxidant Defense System in *Brassica juncea* L.

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ABSTRACT

The present work has been undertaken to study the role of different concentrations of 24-epibrassinolide (24-EBL) (10^{-10}, 10^{-8} and 10^{-6} M) on growth, antioxidant enzyme [catalase (CAT), ascorbate peroxidase (APOX), superoxide dismutase (SOD)] activities and total protein content in 10-days-old seedlings of *Brassica juncea* L. exposed to heat stress for 5 h daily for three consecutive days. Heat treatment lowered total protein content of *B. juncea* seedlings. The seedlings treated with different concentrations of 24-EBL showed better growth and enhanced protein content than the control. Similarly, the activities of SOD, CAT, APOX, PPO, and auxinases were enhanced by the application of different concentrations of 24-epiBL. APOX activity was enhanced maximum with 10^{-10} M 24-epiBL while 10^{-8} M was most effective for maximum enhancement in the activity of CAT and SOD.

Keywords: brassinosteroids, *Brassica juncea* heat shock protein, reactive oxygen species

INTRODUCTION

High temperature is one of the most important environmental stresses that adversely affect plant growth and development thereby limiting plant productivity. It causes heat injury, which results into degradation of proteins, lipids and bring changes in metabolism. These changes cause break down of cell products and membranes etc. Plant responds and adapt to high temperature by several physiological, biochemical and molecular responses. They tolerate high temperature through the synthesis of heat shock proteins (HSPs) that either directly or indirectly governs levels of osmolytes, components of cell detoxification mechanisms and factors that regulate membrane fluidity. Levels of reactive oxygen species (ROS) are immensely high under stressed conditions (Alscher *et al*. 2002). There are increasing evidences that high temperature increased the generation of Reactive oxygen species (ROS) such as superoxide ($O^{\cdot}_2$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) which have greater toxicity potentials on biomolecules and biomembranes in plants. Plants have antioxidant enzymes to scavenge the increased generation of ROS, which includes SOD (superoxide dismutase), CAT (catalase), APOX (ascorbate peroxidase), DHAR (dehydroascorbate reductases) and MDHAR (monodehydroascorbate). Plant growth regulators like auxins, gibberellins, ethylene jasmonates, and salicylic acid have been reported to play stress protective role in plants (Cao *et al*. 2005). Recent studies indicate stress ameliorative properties of brassinosteroids (BRs), which are an important group of plant steroids. (Dhaubhadel *et al.* 2002; Sirhindi *et al.* 2009) observed that BRs play a vital role in protecting the translation machinery and inducing HSPs under thermal stress. Similarly, Kumar *et al*. (2010) observed in 10-day-old H_2O_2-treated *Brassica juncea* L. seedlings, that low temperature causing reduction in seed germination and seedling growth but treatment of H_2O_2 alone or in combination with BRs makes the plant more adaptable to chilling stress. BRs have been reported to provide protection to plant from heat (Zhu *et al.* 1996), drought (Li and van Staden 1998), chilling (Kumar *et al.* 2010) and metal stress (Sharma *et al.* 2010) by enhancing antioxidant defense system and make the plant resistant to variety of environmental stresses (Krishna 2003). One mechanism that may be involved in resistance to many types of stresses by escalating activity of enzymes involved in antioxidant defense system. Keeping this in mind, the aim of present experiment was to study the effect of high temperature on seedling growth and role of 24-epiBL in amelioration of high temperature stress by regulating the activity of various enzymes involved in antioxidant defense system along with Polyphenol Oxidase and auxinases in *B. juncea*.

MATERIALS AND METHODS

Seeds of *B. juncea* L. cv. 'RCM 619' were procured from the Department of Plant Breeding, Punjab Agriculture University, Ludhiana, India. This is an improved variety of brassica launched recently for farmers of Punjab. This is resistant certified variety having more seed production as compare to other varieties. Seeds were surface sterilized with 0.01% $HgCl_2$ and rinsed 5-6 times with double distilled water. The sterilized seeds were soaked for 8 h in different concentrations of 24-epiBL (Sigma-Aldrich. USA) (0, 10^{-6}, 10^{-8} and 10^{-10} M). The treated seeds were propagated in triplicate in Petri dishes under lab conditions at 20 ± 2°C and 16-h photoperiod while 7-days old seedling were exposed to 40°C for 5 h daily for three days. After 40°C heat shock treatment, seedlings were transferred to normal lab conditions. Plants were sampled on the 10^{th} day after sowing for measuring morphological parameters (root length and shoot length) and the activity of different antioxidant enzymes.

Preparation of enzyme extract

The enzyme extract of 10-days-old seedlings was prepared by homogenizing 1 g fresh plant material in 3 ml of 100 mM phosphate buffer (pH 7.0) for the estimation of total protein content and antioxidant enzyme activities.

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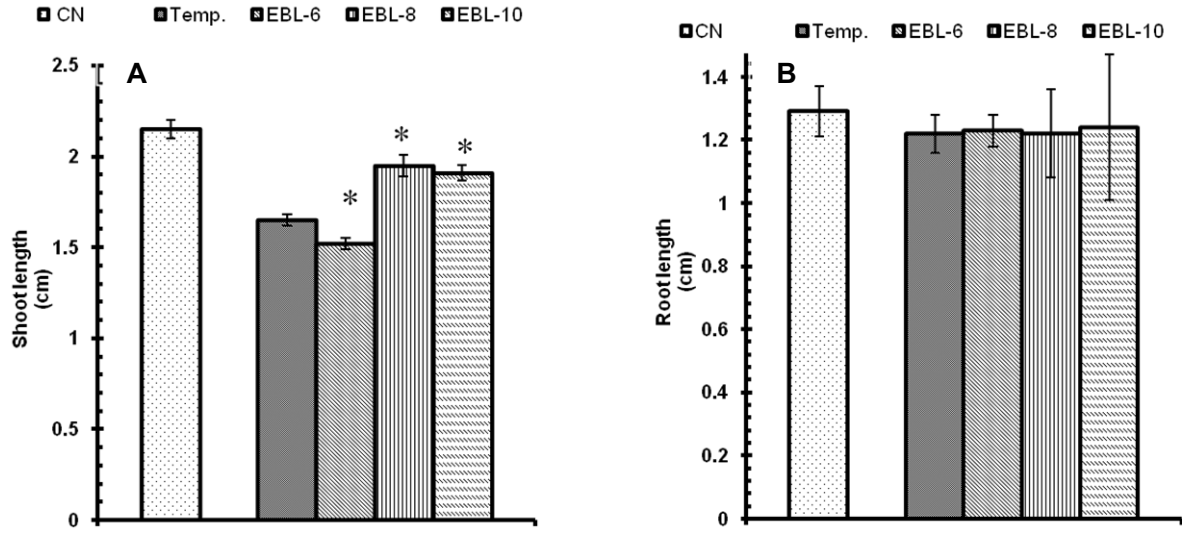

Fig. 1 Effect of temperature stress on (**A**) shoot length (cm) and (**B**) root length (cm) of 10-days-old seedlings of *Brassica juncea* L. treated with different concentrations of 24-epibrassinolide (epiBL) (10^{-6}, 10^{-8}, 10^{-10} M). Values represent mean ± standard error (SE). * Shows significance difference at $P > 0.05$.

Enzyme activity

SOD activity was estimated by recording the increase in absorbance of superoxide nitro blue tetrazolium at 540 nm following the method of Kono (1978). CAT activity was measured according to Aebi (1983). 3 ml reaction mixture containing 1.5 ml of 100 mM phosphate buffer (pH 7.0) and 0.05 ml of 75 mM H_2O_2, 0.05 ml enzyme extract and distilled water was used. Reaction was started by adding H_2O_2 and CAT activity was measured as decrease in absorbance at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed. Activity of APOX was measured following the method of Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm. The reaction mixture contains 100 mM phosphate buffer (pH 7.0), 5 mM ascorbate, 5 mM H_2O_2 and the enzyme extract. PPO activity was measured using the method of Bastin and Unlaer (1972). The reaction mixture contain of 60 mM phosphate buffer (pH 7.0) and 0.01 M chlorogenic acid. The reaction mixture was incubated at 30 ± 2°C for 1 h. Absorbance was measured at 430 nm. IAAO activity was measured according to the method of Gordon and Weber (1951). The reaction mixture contained Salkowski reagent and 0.01% IAA solution. Enzyme extract was incubated at 40°C for 20 min in the dark. Then mixture was cooled and absorbance was taken at 535 nm. Total protein content was determined following the method of Lowry *et al*. (1951)

Statistical analysis

One way analysis of variance (ANOVA) was performed in order to compare the mean of all measured characteristics of 24-epiBL (10^{-6}, 10^{-8}, 10^{-10} M) treated, control and temperature treated seedlings. Significant differences between the means were evaluated by an honestly significant difference (HSD) test.

RESULTS

Better initial plant growth was observed in all 24-epiBL treated seeds at 20 ± 2°C than control seedlings without 24-epiBL treatment. Root length (**Fig. 1B**) was inhibited by 10^{-6} M 24-epiBL (1.11 ± 0.89 cm) to a greater extent than that of temperature stress (1.22 ± 0.33 cm) than control seedlings (1.29 ± 0.88 cm). Temperature stress inhibited shoot length (**Fig. 1A**) of 10-day-old seedlings, but this was ameliorated by 24-epiBL. 10^{-8} M was the most effective concentration for this amelioration (1.95 ± 0.95 cm). Highest total protein (**Fig. 2A**) content (20 ± 1.98 mg g^{-1} FW) was observed in 10-day-old seedlings than in temperature-treated seedlings (13.29 ± 0.99 mg g^{-1} FW). 24-epiBL helped the seedlings overcome temperature stress and 10^{-8} M was the most effective concentration for temperature amelioration (15.408 ± 0.99 mg g^{-1} FW). The activities of antioxidant enzymes (SOD, CAT, APOX) were enhanced in *B. juncea* seedlings, treated with 24-epiBL exposed to temperature stress. APOX activity (**Fig. 2D**) was significantly enhanced at 10^{-10} M 24-epiBL (26.66 ± 0.67 µmol U mg^{-1} protein). 10^{-8} M 24-epiBL was the best concentration for enhancement of CAT (**Fig. 2C**) and SOD (**Fig. 2B**) activities, 9.34 ± 0.54 and 2.13 ± 0.97 mol U mg^{-1} protein, respectively in seedlings that were exposed to temperature stress. The activity of auxinases (**Fig. 3B**) was maximum at 10^{-6} M 24-epiBL (0.090 ± 0.032) although all the 24-epiBL treatments showed significantly enhanced IAAO activity compared to control seedlings and seedling exposed to temperature stress. On the other hand, PPO activity (**Fig. 3A**) was enhanced in all 24-epiBL treatments, significantly so at 10^{-8} M of 24-epiBL (104.1 ± 6.28 mol U mg^{-1} protein). Exposure to temperature stress ameliorated the antioxidant enzyme system, which was further enhanced by treating the seedlings with different concentrations of 24-epiBL. However, the concentration of 24-epiBL that could ameliorate different enzymes and make the plant more resistant to temperature stress varied from enzyme to enzyme.

DISCUSSION

Preliminary studies have demonstrated that EBR treatment improved heat tolerance of wheat leaf cells (Kulaeva *et al.* 1991), and bromegrass suspension cells (Wilen *et al*. 1995). In the present study, *B. juncea* seedlings showed susceptibility to temperature stress. After temperature stress treatment to seedlings, growth (e.g., shoot and root length) was significantly inhibited. High temperature stress decreased the germination rate and shoot length of tomato seedlings as reported by Singh and Shono (2005) and in barley and radish seeds by Cavusoglu and Kabar (2007). Our previous studies also illustrated that BR protects plants starting from their germination till maturity by up and down regulation of various non-enzymatic and enzymatic activities at the cellular level (Sirhindi *et al.* 2009). BRs also enhanced the germination rate of seeds and the growth after direct sowing in submerged paddy pots at low temperature (Bajguz and Hayat 2008). Protein synthesis was maintained in BR-treated leaves at 43°C at levels similar to those at 23°C (Kulaeva *et al.* 1991), whereas in untreated leaves it decreased 2.5-fold at 43°C compared to samples at the control temperature. Sirhindi *et al.* (2009) and Kumar *et al.* (2010) observed that 24-epiBL treatment helped to mitigate stress by regulating activities of antioxidant enzymes. High tem-

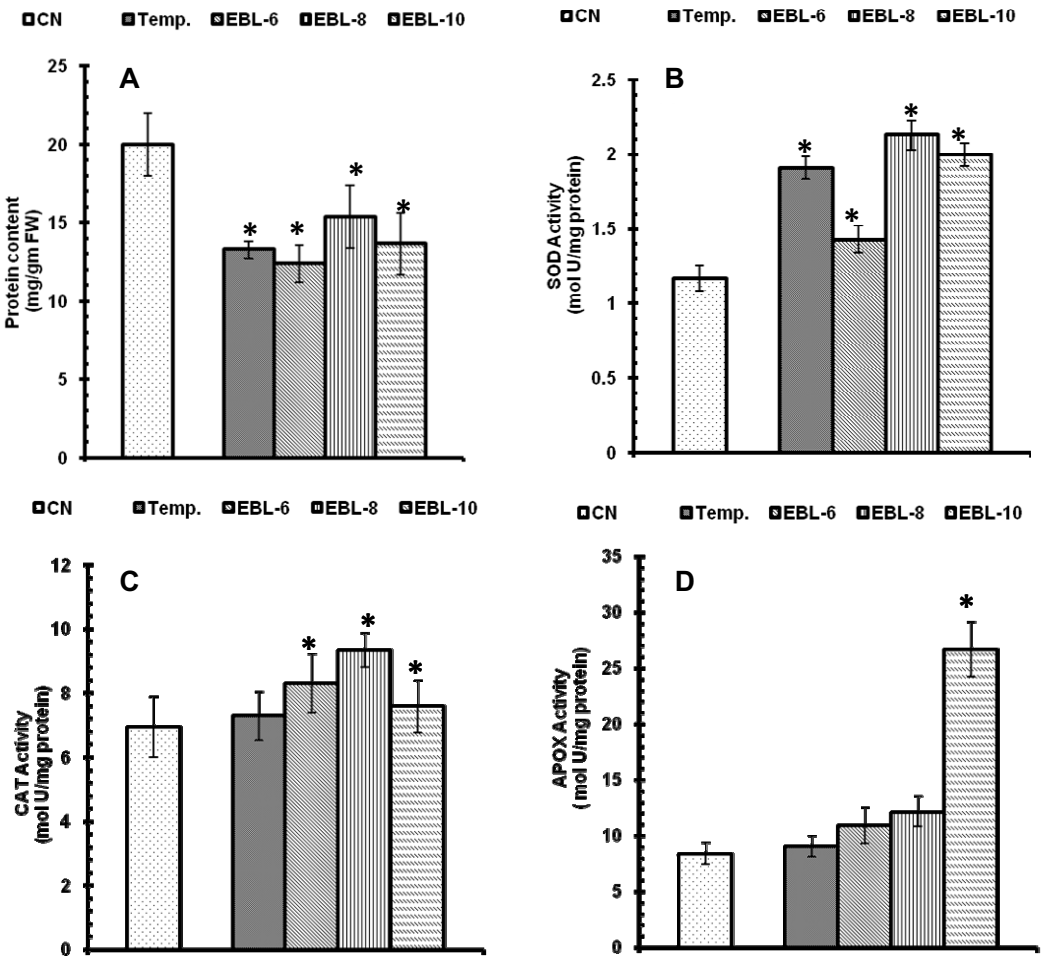

Fig. 2 (**A**) Total protein (mg mg^{-1} FW), (**B**) activity of SOD (mol U mg^{-1} protein), (**C**) activity of CAT (mol U mg^{-1} protein), and (**D**) APOX (mol U mg^{-1} protein) of 10-days-old seedlings of *Brassica juncea* L. exposed to temperature stress (40°C) and different concentrations of 24-epiBL (10^{-6}, 10^{-8}, 10^{-10} M). Values represent mean ± standard error (SE). * Shows significance difference at $P > 0.05$.

perature stress often causes membrane damage, decrease hydrolytic enzyme activities and increases the lipid peroxidation level. It may stimulate the formation of ROS, such as OH^-, H_2O_2, and $O_2^{\cdot-}$ radicals. Among ROS, superoxide radical ($O_2^{\cdot-}$) is dismutated by SOD in H_2O_2 and is further scavenged by CAT and various peroxides. APOX and GR also play a key role by reducing H_2O_2 to water through the Ascorbate-glutathione cycle (Noctor and Foyer 1998). The level of CAT, SOD and APOX were increased by the application of 24-epiBL to overcome the stress generated by high temperature and to boost the resistance capacity of *B. juncea* plants. Various studies indicated that 24-epiBL treatment enhanced the antioxidant enzyme activities under different abiotic stresses (Dhaudhadel *et al.* 2002; Bhardwaj *et al.* 2007; Arora *et al.* 2008; Sharma *et al.* 2010). The present investigation also revealed the induction of oxidative stress and ROS production during temperature stress treatment in *B. juncea* seedlings. Reports are also available indicating that a mild increase in temperature enhanced the growth of plant, but when this temperature exceeded a permissible limit, it increased the production of ROS. The effects of high temperature stress in BR-treated and -untreated *Brassica napus* and tomato seedlings were examined at the expression level of total protein synthesis, which increased in BR-treated seedlings (Dhaubhadel *et al.* 1999). In the present investigation total protein content increased quantitatively in 24-epiBL-treated and untreated seedlings. In the present study SOD and CAT activity were enhanced in temperature- and BR-treated seedlings. 10^{-8} M 24-epiBL was best in ameliorating temperature stress. APOX activity was the same in temperature-treated and control seedlings. However, this activity was enhanced when seedlings exposed to temperature stress were treated with 24-epiBL, with 10^{-8} and 10^{-10} M 24-epiBL showing significant differences in APOX activity compared to control seedlings. The results shown in the present study proved that 24-epiBL enhances APOX activity at a very dilute concentration. Bhardwaj *et al.* (2007) reported that exogenous treatment is effective in stressful rather than in optimal conditions. The effect of 24-epiBL and MH5 (polyhydroxylated spirostanic analogue of brassinosteroids) was analyzed by Mazorra *et al.* (2002) on CAT, APOX and SOD activity in tomato leaf discs at 25-40°C; BRs altered the activity of these enzymes, suggesting a possible role of 24-epiBL and MH5 in the reduction of cell damage produced by heat stress. Although PPO is not considered a component of the antioxidant defense system, it plays an important role to control oxidative stresses. The activity of PPO was increased under stress (Zhukova *et al.* 1996). PPO activity was enhanced by the application of 24-epiBL in the present study. Darbyshire (1971) reported that the activity of IAA Oxidase increased under water stress in pea plants. The present work also shows an increase in IAA Oxidase activity under the influence of 24-epiBL in seedlings subjected to temperature stress.

In conclusion, the present study reveals that temperature is an important environmental factor responsible for normal functioning of plant growth and metabolism but an elevated degree of temperature beyond a certain limit cause growth inhibition and damages the cell and ultimately leads to necrosis. 24-epiBL showed stress-ameliorative properties in *B. juncea* seedlings exposed to temperature stress by improving seedling growth and enhancing protein content and activities of CAT, SOD, APOX, PPO, IAA Oxidase. This study culminates to the role of BR as an anti-stress agent for protecting plants exposed to various stresses although ex-

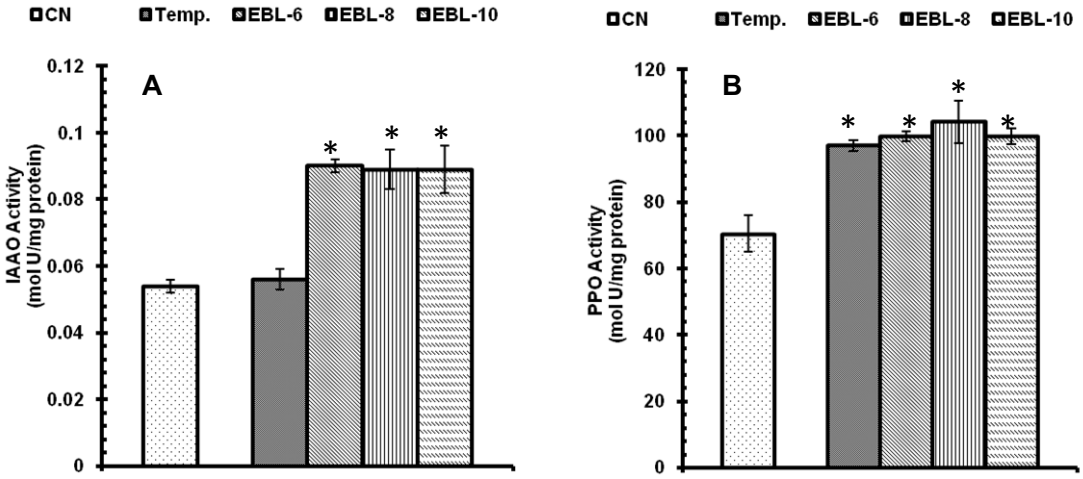

Fig. 3 (**A**) Activity of IAAO (mol U mg^{-1} protein), (**B**) activity of PPO (mol U mg^{-1} protein) of 10-days-old seedlings of *Brassica juncea* L. exposed to temperature stress (40°C) and different concentrations of 24-epiBL (10^{-6}, 10^{-8}, 10^{-10} M). Values represent mean ± standard error (SE). * Shows significance difference at $P > 0.05$.

tensive studies are still needed on various aspects related to stress and the role of BRs in regulating them.

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