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RESILIENCE OF GROWTH AND TEMPERATURE TOLERANCE BY 24-EPIBRASSINOLIDE IN MUSTARD (*Brassica juncea*)

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ABSTRACT

Brassinosteroids (BRs), natural steroids in plant kingdom are well-known to regulate essential processes of plants' growth and development. Besides this, BRs have been well acknowledged to induce tolerance towards various environmental stress conditions by mitigating the reactive oxygen species (ROS) in order to maintain cellular homeostasis. Present study was carried out to investigate the effect of exogenously applied different concentrations (0, 10^{-6}, 10^{-9}, 10^{-12} M) of 24-epibrassinolide (EBL) on *Brassica juncea* seedlings subjected to low temperature (5°C) under laboratory conditions in 2020-21 at Plant Physiology laboratory, Department of Botany, Punjabi University Patiala-147002, Punjab, India. Low temperature stress reduced the shoot and root lengths, fresh and dry weight, but supplementation of EBL considerably augmented these morphological parameters. Further under low temperature condition, an increase in H_2O_2 and MDA content was observed, but their toxic effect was alleviated with EBL supplementation. The content of non-enzymatic antioxidants such as carotenoids and vitamins increased with EBL application which proved beneficial in mitigating the deleterious effects of low temperature. Overall, the seed treatment with EBL extended plant's potential to overcome toxic effects imposed by low temperature stress by enhancing ability of antioxidant defense system to greater horizons thus paving the way towards use of eco-friendly approaches in inculcating stress tolerance in plants.

(Key words:Low temperature stress, reactive oxygen species, antioxidants, 24- epibrassinolide)

INTRODUCTION

Growth and development of plants rely essentially on the plant growth hormones as these are the substances when added in minute quantity alter the growth of plant generally by stimulating or reducing part of the natural growth regulation (Deotale *et al.,* 2019). Among the phytohormones, Brassinosteroids (BRs) are polyhydroxylated plant steroid hormones that can stimulate plant tolerance to variety of abiotic stress conditions including low and high temperature, drought and salinity (Harpreet *et al.,* 2014; Serna *et al.,* 2015; Sirhindi *et al.,* 2017; Ahmad *et al.,* 2018a). BRs protect the plants throughout their developmental processes by regulating the several metabolites (Geetika *et al.,* 2014).

Temperature is the chief environmental factor that affects plant growth and development. Every plant requires a specific temperature range to thrive well and enjoy their genetic potential to the fullest. This temperature range is usually represented by minimum, optimum and maximum values within which the biological processes are governed normally. However, any fluctuation in the favorable temperature would put a halt on the operation of physiological reactions as the plants cannot escape from the unprecedented changes in the temperature being sessile organisms. In order to endure in this dynamic temperature regime, plants must be able to sense these transitory fluctuations in the temperature and adjust their physiology actively in order to flourish well even in harsh environment (Kaur *et al.,* 2017;Planas-Riverola *et al.,* 2019). Low temperature stress causes over-production of reactive oxygen species (ROS), thereby distressing the cellular homeostasis. These ROS are responsible for lipid peroxidation and DNA modifications that lead to the irretrievable metabolic, structural dysfunction and ends in cell death (Yin *et al.,* 2016). In order to cope with these ROS and maintain redox homeostasis, the inbuilt antioxidant defense system of plants acts as frontline to shield them. The high efficiency of these antioxidants can alleviate the oxidative damage under abiotic stress (Ahmad *et al.,* 2018b).

Mitigation of the conferred abiotic stress requires hormonized regulation of various enzymatic reactions with external signals as well as with endogenous chemicals and this synchrony is inseparably linked with phytohormones (Pacifici *et al.,* 2015). Therefore, BRs could be an essential index for adaptive mechanism in the adverse circumstances.In recent years more attention on the carotenoids group of pigments has been focused in understanding their function, mainly as antioxidants. The "core" structural component of carotenoids is a polyene backbone consisting of a sequence of conjugated C=C bonds. This feature is primarily responsible for the ability

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of many of these compounds to interact with free radicals and thus act as effective antioxidant (Young and Lowe, 2018). Vitamin A, C and E comprises of the non- enzymatic antioxidant defense system. Vitamin C (ascorbic acid) acts as a key substrate for the detoxification of reactive oxygen entities (Qian *et al.,*2014). Additionally, ascorbic acid also acts as a cofactor for prolyl hydroxylase which hydroxylates proline residues in the cell wall required for cell division and cell expansion (Raut *et al.,* 2020). Tocopherols can capably quench singlet oxygen, scavenge various radicals, particularly the lipid peroxy radicals, and thus terminate lipid peroxidation chain reactions (Schneider, 2005). These vitamins are not only known for protecting plant cells from free radicals but also for offering essential nutrients for plant growth (Blesseena *et al.,* 2020).

Owing to the numerous benefits of BRs that it offers to the plants, the present research was conducted to explore its functions under low temperature. The present study was conducted to appraise the protective role of EBL in *B. juncea* exposed to low temperature because mustard seeds contain 30% to 48% oil content and it is a vital seed crop that accounts for almost 20-22% of entire oilseeds produced in the country (Raut *et al.,* 2021)

MATERIALS AND METHODS

Plant material and growth treatments

For the present study, *B. juncea* (L.) cv. RLC-3 seeds were procured from Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, India. The experimental work was performed in 2020-2021. Seeds were sterilized by washing with sodium hypochlorite (0.5%) for 15 minutes followed by rinsing in double-distilled water (DDW) for 4-5 times. Afterwards, sterilized seeds were dipped in different concentrations of EBL (0, 10^{-6}, 10^{-9}, 10^{-12} M) for 8 hours. The seeds were allowed to germinate on Whatman filter paper No. 1 lined in sterile petri-plates for three days. Petri plates were placed in seed germinator under controlled conditions those were set at 25 °C, 16/8 hours light/dark periods, uniform light fall of 200 PAR (Photosynthetically Active Radiation) $m^{-2}s^{-1}$ and 70% humidity. Germinated seeds were then shifted to brown germination paper by following the Cigar Roll method. 8 days old seedlings were exposed to 5°C temperature shock up to three consecutive days for 5 hours daily. All these treatments and their duration were selected on the basis of preliminary studies that were previously conducted in laboratory.

Growth measurement

Random sampling was done to select ten seedlings from each treatment on 10^{th} day for shoot and root length, measured with the ruler (cm). Fresh and dry weights of fifteen seedlings were measured with an electronic laboratory weighing machine.

Quantification of Hydrogen peroxide (H_2O_2) content

To estimate H_2O_2 content, the procedure of Velikova *et al.* (2000) was followed. Extract was prepared by crushing 500 mg of fresh plant material in 0.1% trichloroacetic acid followed by centrifugation. Supernatant was dissolved in 0.5 ml of 10 mM potassium phosphate buffer, 1 ml of potassium iodide and absorbance was taken at 390 nm. Known concentrations were used for preparation of standard curve. μg g^{-1} FW expressed the results.

Estimation of Malondialdehyde (MDA) content

MDA was determined according to Heath and Packer(1968). Known volume (1 ml) of extract was added to 2 ml of a reaction solution consisting 20% (v/v) trichloroacetic acid and 0.5% (v/v) thiobarbituric acid. The same was kept in water bath for 30 minutes at 95 °C, later kept in ice water bath for cooling followed by centrifugation for 10 minutes at 10,000 x g. Absorbance was taken at 450 nm, 532 nm and 600 nm.

Detection of carotenoids content

According to the method of Lichtenthaler (1987) Carotenoids estimation was done. Known weight (200 mg) of plant material was crushed in 80% acetone. Centrifugation was done at 3000 x g and supernatant was collected. Optical density was recorded at 645 nm, 663 nm and 470 nm.

Determination of Vitamin A, C and E content

Vitamin A was determined according to Bayfield and cole (1980) method. Plant tissue was homogenized in 2N KOH and heated gently for 20 minutes at 60°C. Add 20 ml of water to it and mixed properly. Using separating funnel extraction was done with Petroleum Ether. Sodium sulphate was added to remove the moisture of the sample. Ether extract was evaporated at 60°C till dryness and then dissolved dried residues in 1 ml of Chloroform and 2 ml of TCA. Absorbance was recorded at 620 nm.

According to the method of Chinoy *et al.* (1976) Vitamin C was estimated. 2 ml of plant extract was prepared. 8 ml of 2, 6-dichlorophenol indophenols dye was added to plant extract. The absorbance was recorded at 530 nm.

Estimation of Vitamin E was done with Rosenberg (1992) method. Sample was mixed gradually with 0.1 N sulphuric acid and incubated at room temperature for overnight. To the 1.5 ml of tissue extract, 1.5 ml of xylene was added and centrifuged. 1.0 ml of xylene was separated and mixed with 1.0 ml of 2, 2-dipyridyl. Absorbance was noted at 460 nm. Then 0.33 ml of $FeCl_3$ was added. After 15 minutes, test was read at 520 nm.

Data analysis

One-way analysis of variance (ANOVA) was done with comparison of mean differences by Tukey's test using Prism Software 7. Data taken for calculations were the mean of three replicates (n = 3) and comparisons of p values\0.05 were considered significant and different from control.

RESULTS AND DISCUSSION

Growth parameters

Seedlings exposed to temperature stress showed significant decrease in almost all growth aspects like seed

germination (Table 1), shoot length and root length, fresh weight and dry weight of seedlings as shown in Figure 1. Priming with all concentrations mentioned above of EBL augmented the seed germination significantly under stress or without stress condition as compared to analogous control. 10^{-9} M EBL ameliorated the seed germination by 17% under normal conditions and 47%in seedlings treated with 5°C temperature shock in comparison to respective controls. Seedlings treated with EBL overcame the low temperature stress and supported the seedling growth.

Exogenous application of EBL increased the shoot and root length at all concentrations. Best results were at 10^{-9} M EBL for shoot length where 19% enhancement was recorded and root length was ameliorated up to maximum with10^{-12} M EBL supplementation by 11% over control. Further, shoot and root length got affected under cold stress and decreased by 12% and 7% over their controls respectively. But pre-sowing soaking treatment of EBL to seedlings exposed to temperature shock improved the shoot as well root length. Where, 10^{-9} M EBL showed considerable enhancement by 25% in shoot length and10^{-12} M EBL concentration augmented root length by 25% in comparison to corresponding controls, respectively.

Seedlings raised under low temperature had reduction in fresh and dry weight. Pre-sowing soaking of seeds with EBL (10^{-6}, 10^{-9},10^{-12}M) exposed to low temperature enhanced the fresh weight noticeably and maximum enhancement was shown by 10^{-9} M EBL treatment and it was 9% more as compared to control. Dry weight of seedlings also showed amelioration with EBL treatments in comparison to control whether there was stress or not. All aforementioned EBL concentrations enhanced the dry weight and maximum amplification was reported under 10^{-9} M EBL by 21% under normal conditions and by 30% under stress conditions, with respect to corresponding controls (Figure 1).

EBL decreased H_2O_2 and MDA content

An increase in hydrogen peroxide and malondialdehyde content was observed under low temperature which marks the onset of oxidative stress. Table 1 depicts that H_2O_2 content was decreased with age in seedlings on exogenous application of EBL. All EBL concentrations significantly decreased the H_2O_2 content on 10^{th} day of growth but it was 10^{-12} M concentration that showed best results by 37% decline in its content as compared to control under cold stress. Moreover, the EBL treatment also decreased the MDA content too significantly in seedlings those were under stress as well as in seedlings those were raised under room temperature. In case of MDA also it was 10^{-12} M EBL appliance that reduced its content up to minimum by 32% over that of control under room temperature and by 69% as compared to control treated with stress alone (Table 1).

Carotenoids content

The results of influence of temperature stress on carotenoids shown in (Figure 2) revealed that direct exposure of seedlings to low temperature resulted in increase in carotenoids content up to 8% in comparison to control on 10^{th} day of growth. Whereas different concentrations of EBL ameliorated the carotenoids content significantly under stress or normal conditions and maximum augmentation was seen under 10^{-9} M EBL supplementation by17% as compare to control under room temperature while 7% enhancement was observed in the seedlings subjected to low temperature, with the treatment of 10^{-9} M EBL as compared to corresponding controls, respectively.

Vitamins

Under cold stress conditions, Vitamin A and E content was declined by 20% and 30% in comparison to their analogous controls whereas vitamin C content was enhanced under stress by 53% over its control (Figure 2). Further, the significant increase in vitamin A, vitamin C and vitamin E content was observed in the seedlings treated with various concentrations of EBL under normal and stress environment. However for different vitamins, EBL behaved in a dose dependent manner.For vitamin A, 10^{-12} M EBL appliances enhanced the content by 99% under room temperature and 97% under cold stress as compared to respective control. Whereas, 10^{-9}M EBL increased the vitamin C content by 73% under normal and 31% under stress condition in comparison to analogous control. Further, the content of vitamin E was ameliorated up to maximum by 10^{-6} M EBL under normal conditions by 8% and under cold stress conditions by 18% over respective controls.

Temperature is a pivotal abiotic factor that regulates almost all the developmental processes in plants from germination up to senescence. These processes of growth and development involve several biochemical reactions which require optimum temperature for their efficient operation (Zhang *et al.,* 2020). But due to fluctuating environmental conditions there always occurs variations in temperature beyond the critical threshold for a phase of time that is enough to cause irreversible damages to plant growth and development (Wahid *et al.,* 2007). Plant growth hormones play central role in the ability of plants to adapt to changing environments, by mediating the growth and development.BRs have been reported to wield anti stress effects on plants. Over last few years, remarkable research has been carried out in order to monitor the potential involvement of BRs to persuade resistance against low temperature.

Present study explored the protective role of EBL in shielding the mustard seedlings against low temperature stress as mustard is an economically important crop and it is cultivated mostly under temperate climate (Gopale *et al.,* 2021). Application of EBL enhanced the seedling growth as well as also avoided the harmful effects of low temperature stress. These findings are in accordance with the reports of Krasensky and Jonak (2012) where temperatures stress affected the growth but EBL appliance significantly abridged the injurious effects.H_2O_2 plays dual role in the plants; acts as a signaling molecule at lower concentrations but at higher

concentration leads to the oxidative damage (Quan *et al.,* 2008). In the present study, poor growth performance of the seedlings was reported. Further we ascribed that under low temperature, levels of H_2O_2 rise abruptly marking the onset of oxidative burst which further cause MDA content to rise due to lipid peroxidation. The increased concentration of H_2O_2 during stress conditions agrees with the study of Sharma *et al.*(2014) on *Brassica juncea*. Oxidative burst causes either partial or complete dysfunction of cell membranes that leads to lipid peroxidation of the membranes and finally forms MDA as end product (Kaur *et al.* 2015). In the present study, EBL minimized the production of H_2O_2 concurs with the research of Ahmed *et al.* (2017) and this is directly giving evidence that EBL enhances the ROS scavenging ability of antioxidants in *B. juncea* seedlings that ultimately diminish the lipid peroxidation. Parallel pattern of results was observed in *Chorispora bungeanna* where BR shielded the cells from chilling stress by inhibiting formation of MDA (Liu , 2009).

There are several non-enzymatic antioxidants that play chief role in the detoxification of ROS. Carotenoids are proficient antioxidants protecting plants against the oxidative damage (Stahl and Sies, 2003). Lycopene is an intermediate in biosynthetic pathway of carotenoids and acts as scavenger of ROS. In this study, alleviation in carotenoids content revealed that, exogenous application of EBL plays key role in enhancing the content.Ascorbic acid is very well known to protect organelles and cells from ROS; that over-accumulate during stress (Naz *et al.,* 2016). Ascorbic acid also controls cell division and expansion, acts as a cofactor of many enzymes, modulates the plant sense and implicated in hormone biosynthesis and antioxidants regeneration (Lisko *et al.,* 2014). In our study, there is elevation in the content of ascorbic acid, supporting the survival of mustard seedlings under stress conditions. Vitamin E is considered as a major antioxidant in the biomembranes. It plays an important role in protecting the thylakoid membrane from photo-oxidative damage (Havaux *et al.,* 2005). These two antioxidants have potential to conquer the negative effects of ROS and free radicals. Bothascorbate and tocopherol have metal ion chelation movement inhibiting the formation of ROS. These non-enzymatic antioxidants are up-regulated under initiation of stress.

In conclusion, investigation of the effects of EBL was found to enhance the antioxidant defense system of *B. juncea* (L). to combat the ROS imbalance under low temperature. This study further consolidated the fact that EBL seed priming decreased the extent of damage efficiently at harsh low temperature by reducing the impact of stress on growth and development and allowing the adaptation to environmental changes. Further, molecular studies involving BRs receptor complexes would prove to be helpful in exploring the underlying mechanism of BRs induced stress tolerance in plants.

Thus due to its ameliorative potential in making crops/plants tolerant to harsh environment; EBL may prove to be a good candidate for *B. juncea* (L.) to protect from several stress conditions.

Table 1. 24-Epibrassinolide enhances seed germination and represses oxidative damage by declining H_2O_2 and MDA content in *B. juncea* seedlings under control and cold stress

Sr. No.	Treatments	Seed Germination (%)	H_2O_2 Content (µg g^{-1} FW)	MDA Content (µmol g^{-1} FW)
1.	Control	76.66±6.67^{a}	0.63±0.0^{a}	1.33±0.04^{a}
2.	1 ìM EBL	80.00±3.34^{a}	0.52±0.01^{b}	1.17±0.15^{a}
3.	1 nM EBL	90.00±3.34^{b}	0.63±0.01^{ab}	1.25±0.19^{a}
4.	1 pM EBL	76.66±3.34^{ab}	0.56±0.02^{c}	0.91±0.08^{a}
5.	5°C	56.66±3.34^{c}	0.73±0.01^{d}	3.26±0.16^{b}
6.	5°C +1 ìM EBL	70.00±0.00^{ac}	0.60±0.02^{ac}	1.61±0.02^{ab}
7.	5°C +1 nM EBL	83.33±3.33^{ad}	0.58±0.01^{cd}	1.72±0.02^{ab}
8.	5°C +1 pM EBL	66.66±0.00^{d}	0.46±0.02^{e}	1.02±0.02^{abc}
	F ratio$_{(7.16)}$	25.91 (A)	119 (B)	143.4 (C)

(A) Seed gemination was recorded on 4th day; (B) H_2O_2 was estimated on 10th day by following potassium iodide (KI) oxidation (C) Lipid peroxidation was quantified as malondialdehyde (MDA) levels. Five hundred mg fresh tissue was collected on 10th day, homogenized and used for TBA-based lipid.

Data is represented here with standard deviation (SD) and different letters after SD represent a significant difference as determined by one-way ANOVA and F ratio is simply signifying the ratio of two variances.

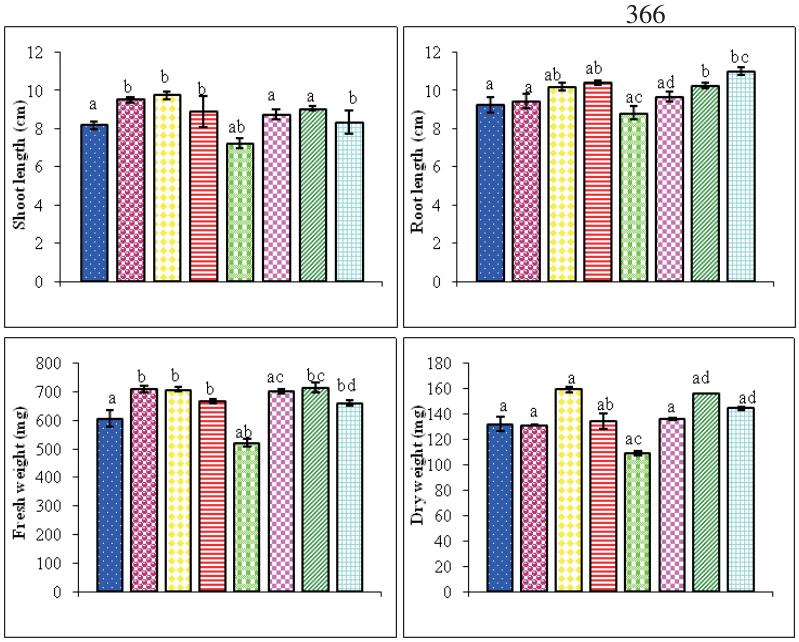
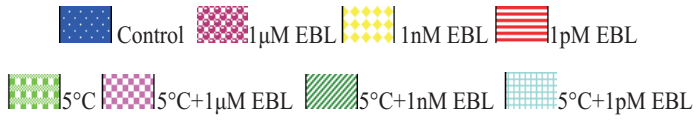


Fig. 1.Effect of 24-Epibrassinolide on growth parameters of *B. juncea*seedlings under room temperature and low temperature stress conditions. Measurement (A) Shoot Length, (B) Root Length (C) Fresh Weight and (D) Dry Weight.10 random seedlings were collected on 10th day to measure these parameters. Data represent the mean of three replicates. Error bars represent standard deviation (SD) and different letters above the bars represent a significant difference as determined by one-way ANOVA.



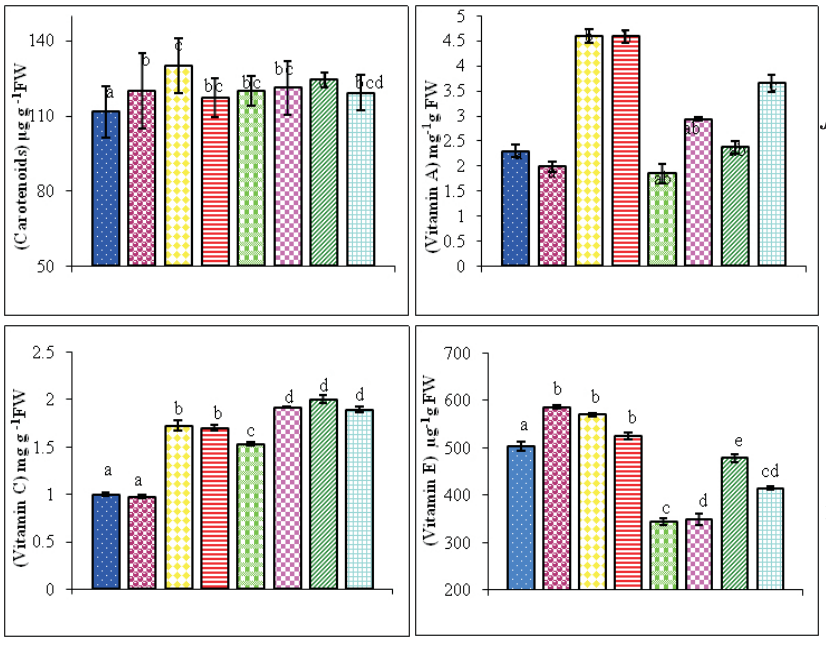
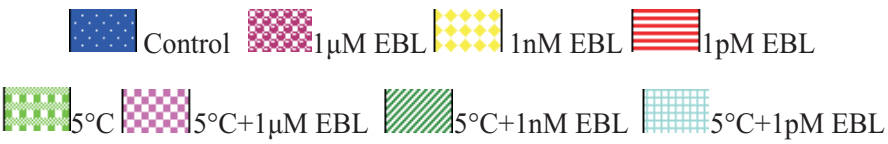


Fig 2. 24-Epibrassinolide affect antioxidant levels in *B. juncea* seedlings under both control and cold stress conditions. Five hundred mg fresh sample tissue was collected on 10th day, homogenized and then centrifuged. Resulting supernatant was used for quantifying the content of non-enzymatic antioxidants as (A) Carotenoids, (B) Vitamin A, (C) Vitamin C and (D) Vitamin E. Error bars represent standard deviation (SD) and different letters above the bars represent a significant difference as determined by one-way ANOVA.



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