

28-Homobrassinolide-Induced Exaggerated Growth, Biochemical Molecular Aspects of *Brassica Juncea* L. RLM-619 Seedlings under High Temperature Stress

Sandeep Kumar^{1*}, Geetika Sirhindi¹ and Renu Bhardwaj²

¹Department of Botany, Punjabi University, Patiala -147 002, India

²Department of Botanical & Environmental Sciences, GNDU, Amritsar, India

Abstract

The present study was pertaining to study the mechanism and signaling of BRs under high temperature stress in *Brassica juncea* L. plants. Results advised that high temperature stress reduced the shoot length, root length, fresh weight of the plants but 28-homobrassinosteroids (28-homoBL) treatment reduced the toxic effect of high temperature stress by improving the same of *B. juncea* L. plants. To ascertain these free radical scavenging enzymes (superoxide dismutase (SOD), Catalase (CAT), Peroxidase (POD), Ascorbate peroxidase (APOX), Polyphenol oxidase (PPO) and Indole acetic acid oxidase (IAAO) and lipid oxidation (MDA) were assayed along with photosynthetic pigments, total carbohydrates, reducing sugars and non-reducing sugars. All studied scavenging enzymes activities were ameliorated by the homoBL treatments. The pigments decreased under temperature stress were also protected by homoBL treatments. Proteins profiling by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were also studied to know the behavior of proteins under temperature stress as well as with the treatment of homoBL. Protein bands disappeared in temperature stress while in the presence of homoBL treatment existing proteins displayed more accumulation leading to the appearance of new bands. Native-PAGE of SOD, CAT and POD suggested the presence of in isoenzymic forms in cells. Application of different concentration of homoBL increased intensities of these isoenzymes under temperature and normal condition as compared to control untreated seedlings. The gene expression of SOD and CAT also suggested that synthesis these enzymes were decreased under high temperature stress but ameliorated with the treatment of homoBL. Present study demonstrated that 28-homoBL have stress-ameliorative properties in *B. juncea* seedlings exposed to high temperature stress by improving seedling growth and enhancing protein content as well as activities of SOD, CAT, APOX, PPO and IAA Oxidase. Further, it was suggested by decreasing the MDA content after the treatments of 28-homoBL. This study demonstrate the culmination of BR's as an anti-stressor for protection of plant exposed to high temperature stress but extensive studies are still needed to know the various aspects related to stress and role of brassinosteroids in regulating them at molecular and signaling level.

Keywords: 28-homobrassinolide; *Brassica juncea* L; Reactive oxygen species; High temperature stress

Introduction

It is predicted that increases in “greenhouse gases” concentration will result in the rise in mean temperatures of about 2°C by the middle of the next century. Although yields of temperate crops increase with enhanced CO₂ concentrations, this may be offset by the negative effects of warmer temperatures in determinate crops. The duration of grain filling in crop plants are determined principally by temperature. In winter crop, high temperatures (~31°C) after anthesis can decrease the rate of grain-filling, whilst high temperatures imposed before anthesis can also decrease yield of crop plants (In winter crop, high temperature episodes occurring near to anthesis can reduce the number of grains per ear and the subsequent rate of increase in harvest index, resulting in smaller grain yields. High temperature stress also leads to over production of reactive oxygen species (ROS) in plants which are highly reactive and can cause damage to proteins, lipids, carbohydrates, RNA and DNA ultimately results in death of cell [1]. Plants have evolved mechanisms on morphological, physiological and molecular level to survive with modulated environmental conditions [2].

Various strategies have been employed to enhance plant growth and productivity under environmental stress. Use of phytohormones is one of the simpler approaches to make crops resistible to negative environmental conditions and perform well by acclimatizing at morphological, physiological, biochemical and molecular level.

The use of plant growth regulators (PGR_s) in agriculture to promote plant growth production and quality is becoming more common [3-6]. Brassinosteroids is a sixth class of phyto-hormone which plays an advantageous role in enhancing crop productivity under adverse conditions [7,8]. BRs are very peculiar in regulating the activities of antioxidant defense system at biochemical and also regulate the compatible solutes under stress condition in plants. Although studies related to the role of brassinosteroids in controlling the metabolic activities of antioxidant defense system enzymes at biochemical and molecular level are available, a comprehensive study is required to gain a deeper insight of the above mechanism. Therefore present study was pertaining to study the mechanism and signaling of BRs under high temperature stress in *B. juncea* plants.

***Corresponding author:** Sandeep Kumar, Department of Botany, Punjabi University, Patiala -147 002, India, Tel: +91 9810685352, E-mail: sandeep20684@gmail.com

Received April 10, 2014; **Accepted** May 24, 2014; **Published** May 30, 2014

Citation: Kumar S, Sirhindi G, Bhardwaj R (2014) 28-Homobrassinolide-Induced Exaggerated Growth, Biochemical Molecular Aspects of *Brassica Juncea* L. RLM-619 Seedlings under High Temperature Stress. J Plant Biochem Physiol 2: 127. doi:10.4172/2329-9029.1000127

Copyright: © 2014 Kumar S, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Materials and Methods

28-Homobrassinolide (28-homoBL) was purchased from Sigma-Aldrich Chemicals Pvt. Ltd, India. The stock solution (10^{-4} M) of 28-homoBL was prepared by dissolving required quantity of 28-homoBL in 5 ml ethanol, in a 100 ml volumetric flask. The desired concentrations (10^{-6} , 10^{-8} and 10^{-10} M) of 28-homoBL were prepared by the dilution of stock solution. Seeds of *B. juncea* L. cv. RLM-619 were procured from the Department of Plant Breeding, Punjab Agriculture University, Ludhiana, India. Healthy seeds of uniform size were surface-sterilized with 0.05% (w/v) Sodium hypochlorite solution followed by repeated washing with deionised water. The surface sterilized seeds were sown in Petri-dish grown in seed germinator with control conditions (16 h Dark, $200 \mu\text{mol PAR m}^{-2} \text{s}^{-1}$, 25°C and 80% relative humidity) for 7 days. 5 h temperature treatment (35 , 40 and 45°C) was given to 7 days old seedlings for 3 consecutive days and on 10th day seedlings were harvested for various physiological, biochemical and molecular analysis. Morphological data in terms of shoot, root length and fresh weight was measured on 10th days after harvesting.

Determination of carbohydrate, sugar, reducing and reducing sugar content

Carbohydrate content was estimated by the method of Dubois et al. [9]. Reaction mixture contains phenol reagents, H_2SO_4 and samples were incubated at room temperature for 30 minutes. Absorbance was taken at 485 nm and amount of carbohydrate was expressed as mg g^{-1} dry weight. Total sugar was estimated following the method of Loewus 1952. Reaction mixture contains 0.05 ml of ethanol extract added 0.95 ml of distilled water and then added 3ml cold anthrone reagent with constant shaking. Reaction mixture was heated for 10 min in boiling water bath and cooled rapidly at room temperature. Absorbance was measured at 630 nm. Amount of total sugars content was calculated and expressed as mg g^{-1} dry weight. Reducing sugar content was estimated by the method of Nelson [10]. Absorbance was measured at 620 nm expressed as mg g^{-1} fresh weight. Non reducing sugar was calculated by subtracting the reducing sugars content from the total sugar and expressed as mg g^{-1} dry weight.

Determination of pigments (Chl a, b and carotenoids)

Total chlorophyll (Chl), chlorophyll a, b and carotenoid (Car) content were quantified by following the method Lichtenthaler et al. Fresh leaves were homogenized in 3.0 ml of 70% acetone followed by its centrifugation at 12000X g for 15 min. Absorbance of supernatant was observed at 645, 663 and 470 nm and 70% acetone was taken as blank. The contents of total chlorophyll, chlorophyll a, chlorophyll b and carotenoid were calculated and presented in terms of mg.g^{-1} fresh weight (Table 1).

Determination of vitamin C and proline content

Known weight of fresh seedlings was homogenized in metaphosphoric citrate buffer for quantifying vitamin C using the method of Chinoy et al. [11]. Homogenized extract was centrifuged at 3000X g for 5 min using cooling centrifuge and to the 6 ml supernatant 2 ml of 2, 6-dichlorophenol indophenols was added as dyeing agent. Absorbance was taken at 530 nm immediately after adding dye and amount of vitamin C was calculated using standard growth values in mg g^{-1} fresh weight. Proline content was estimated spectrometrically following the method of Bates et al. [12]. 1g fresh leaf tissue was homogenized in 3% sulphosalicylic acid, centrifuged at 12000X g for 10 min. 2 ml supernatant, 2 ml acid ninhydrin and 2 ml glacial acetic acid was added in test tube and reaction was made at 100°C for 1 h. Absorbance of red colored praline-ninhydrin product was measured at 520 nm.

Determination of lipid peroxidation

Lipid Peroxidation was determined by measuring the content of malondialdehyde (MDA) using the method of Heath and Peacker [13]. The sample were homogenized in 10% (w/v) tri-chloroacetic acid and centrifuged at 1000X g for 10 min. supernatants were mixed with equal volume of 10% TCA containing 0.6% (w/v) TBA. Absorbance was taken at 450, 532 and 600 nm after centrifuged at 1000X g for 10 min again.

Determination of oxidative enzymes

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by measuring the ability of the enzymes extract to inhibit the photochemical reduction of nitrobluetetrazolium (NBT) (Kono 1978). For total SOD assay, 3 ml reaction mixture was prepared containing 50 mM sodium carbonate (pH 10.2), 24.1 M NBT, 0.1 mM EDTA, 1 mM hydroxylamine, 0.03% (v/v) Triton X-100 and 70 μl enzymes extract. Absorbance was recorded at 560 nm for 2 min. One unit of SOD activity was defined as the amount of enzyme required that caused 50% of NBT reduction. Activity of ascorbate peroxidase (APOX, EC 1.11.1.11) was determined spectrophotometrically as described by Nakano and Asada [14]. Catalase (CAT, EC 1.11.1.6) activity was assayed by measuring the initial rate of H_2O_2 disappearance using the method of Aebi [15]. Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was assayed using the method of Hossain et al. [16]. 3 ml reaction mixture was prepared by using 50 mM Tris-HCl (pH 7.6) contain 2.5 mM Ascorbic acid, 0.1 mM reduced nicotinamide adenine dinucleotide (NADH), 0.14 units of ascorbic acid oxidase and 100 μl of enzyme extract. The enzyme activity was measured by following the decrease in absorbance at 340 nm using extinction coefficient of $6.2 \text{ mM}^{-1} \text{cm}^{-1}$. Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was measured following the method given by Dalton et al. [17]. Guaiacol peroxidase (POD, EC 1.11.1.7) activity was assayed using the method of Putter J

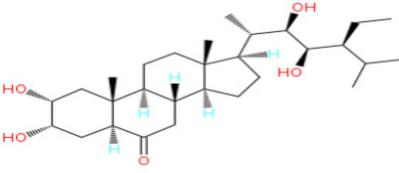
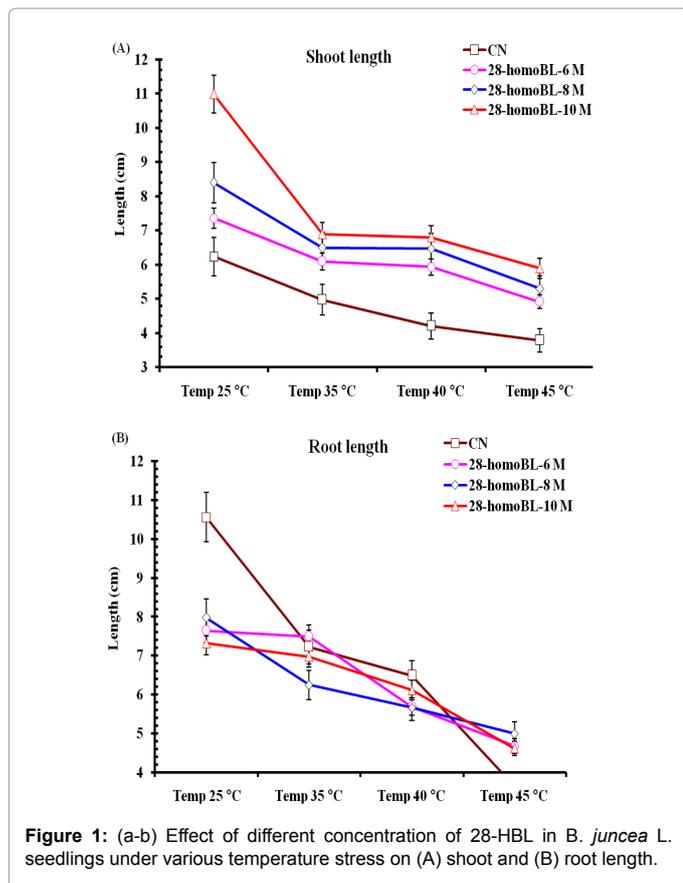
1.	28-homoBL		28-homobrassinolide	478.704	Sigma Aldrich, USA
----	-----------	---	---------------------	---------	--------------------

Table 1: Structure, molecular weight of plant steroids.



1971 with some modifications. 3 ml reaction mixture was made by 50 mM Potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 12.3 mM H_2O_2 and 100 μ l enzyme extract. POD activity was determined by measuring the absorbance at 436 nm and using an extinction coefficient of $26.6 \text{ mM}^{-1}\text{cm}^{-1}$. One unit of POD activity represents the amount of enzyme catalyzing the oxidation of 1 mol of guaiacol in 1 min. PPO (E.C. 1.10.3.1) activity was measured using the method of Bastin and Unleir [18]. The reaction mixture contained 60 mM Phosphate buffer (pH 7.0) and 0.01 M chlorogenic acid and incubated at $30 \pm 2^\circ\text{C}$ for one hour. Absorbance was taken at 430 nm and enzyme activity was expressed as $\text{mol}^{-1}\text{min}^{-1}\text{mg}^{-1}$ protein. IAAO (E.C. 1.11.1.7) activity was measured according the method of Gordon and Weber [19].

Determination of protein content

Protein estimation was done following the method following the method of Lowry et al. [20].

Native PAGE and enzyme activity staining

Native polyacrylamide gel electrophoresis (PAGE) was performed at 4°C , 180 V, following Laemmli [21]. The enzyme solutions were subjected to native PAGE with 10% polyacrylamide gel SOD for POD and CAT. SDS was omitted from the PAGE. Activity stain for each enzyme was carried out as follows. POD, gel was incubated in 25 mM potassium phosphate buffer (pH 7.0) and then the gel was submerged again in the same buffer (fresh amount) containing 18 mM guaiacol and 25 mM H_2O_2 , until the POD activity-containing band was visualized carefully according to Fielding and Hall [22]. For CAT, gel was incubated in 3.27 mM H_2O_2 solution for 25 min and then the gel was washed in distilled water and submerged in a solution composed of

potassium ferricyanide 1% and ferric chloride 1% for 4 min according to Woodbury et al. [23].

Statistical analysis

All analyses were done on a completely randomized design. All data obtained was subjected to one-way analysis of variance (ANOVA) and the mean differences were compared by Turkey's test. Each data was the mean of three replicates ($n=3$) except for shoot lengths of *B. juncea* L. seedlings ($n=10$) and comparisons with P -values ≤ 0.05 were considered significantly different. In all the figures, the spread of values is shown as error bars representing standard errors of the mean.

Results and Discussion

Brassinosteroids stimulate plant growth and development under normal conditions as well as under inadequate conditions of temperature [24]. Present study revealed that temperature stress reduced plant shoot and root growth. Presoaking treatment of BRs made the seedlings more tolerant to abide high temperature shock treatments. Supplementation of BRs ameliorates both shoot and root growth under temperature stress (Figures 1a and 1b). Asthir and Deep [25] also observed similar results in wheat seedlings root and shoot growth. It was found to be inhibited due to heat exposure but applications of homoBL ameliorate growth by mitigating the toxic effect of heat stress. This enhancement was found due to the increased concentrations of Ca^{2+} ions by BRs treatment [26]. It may be possible that there is some cross talk between Ca^{2+} signaling and brassinosteroids at cellular level. Ca^{2+} concentrations in growing parts of the control plants the required signaling cascade under stress as well as with BRs treatments. In present results high temperature stress reduced plant shoot and root growth in *B. juncea* seedlings.

High temperature treatments degraded photosynthetic pigments and BRs treatments provide the protection to the photosynthetic pigments. Among different concentrations of 28-homoBL, 10^{-8} M homoBL ($0.095 \pm 0.00029 \text{ mg.g}^{-1}$ fresh weight) was the best for Chlorophyll a at normal room temperature and also in high temperature stress as compared CN untreated seedlings revealed $0.05 \pm 0.00023 \text{ mg.g}^{-1}$ fresh weight (Figures 2a-d). Detrimental effect of high temperature on Chl a content $0.01 \pm 0.00001 \text{ mg.g}^{-1}$ fresh weights was observed at 45°C . But it was measured $0.053 \pm 0.00004 \text{ mg.g}^{-1}$ fresh weight with treatment of homoBL at same temperature. Similarly chl b content decrease 31.76% at 45°C $0.58 \pm 0.0026 \text{ mg.g}^{-1}$ fresh weight as compared to CN seedlings ($0.85 \pm 0.0056 \text{ mg.g}^{-1}$ fresh weights). Opposing decrease in photosynthetic pigments in rape leaves when exposed to low temperature. Present result of seed priming with 28-homoBL help in alleviating the effect of high temperature by protecting the photosynthetic pigments.

In present study total carbohydrates, total soluble sugar; reducing and non-reducing sugar ameliorated under extreme condition of temperature (Figure 3a-d). Results suggested that with increase in temperature from 35 to 45°C , total carbohydrates accumulated in BRs treated and untreated CN seedlings. It was further ameliorated in the seedlings supplemented with different concentration of homoBL. It was 21.64% more in 10^{-8} M homoBL treatment as compared to CN untreated seedlings exposed to 40°C temperatures stress. Present results also revealed that the seeds primed with different concentrations of homoBL and then exposed at to high temperature showed more accumulation of carbon compounds although photosynthetic degradation was still at higher rate. Carbohydrates, total soluble sugar, reducing and non-reducing sugars are primary source of energy and carbohydrates also accumulates under stress full conditions in plants to act as osmo-protectent [27]. It also due to loss of chlorophyll and carotenoids and due

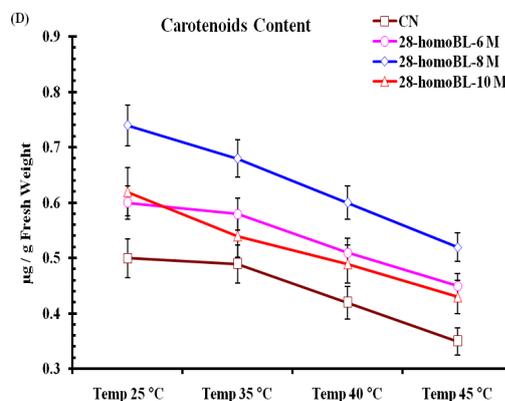
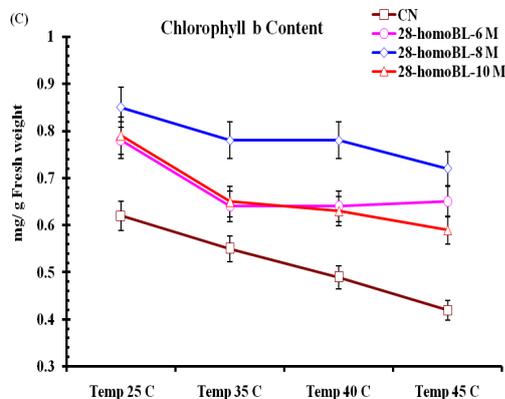
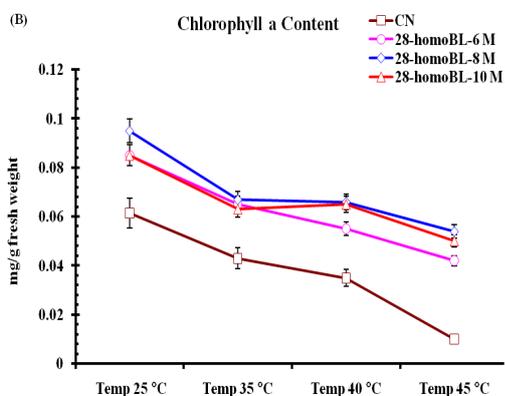
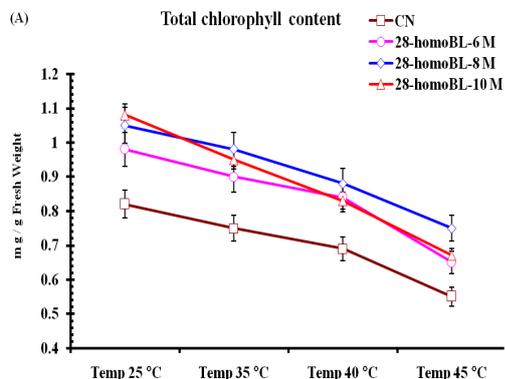


Figure 2: (a-d) Effect of different concentration of 28-HBL in *B. juncea* L. seedlings under various temperature stresses on (A) total chlorophyll content (B) Chl. a (C) Chl. b and (D) Carotenoids content.

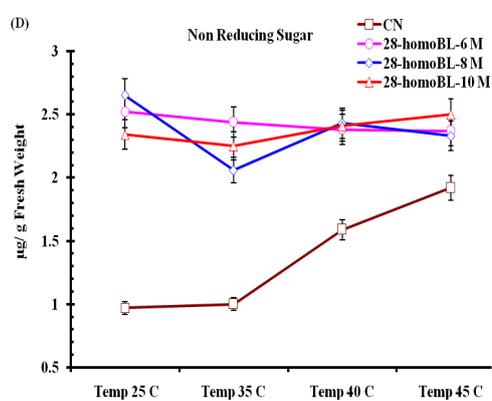
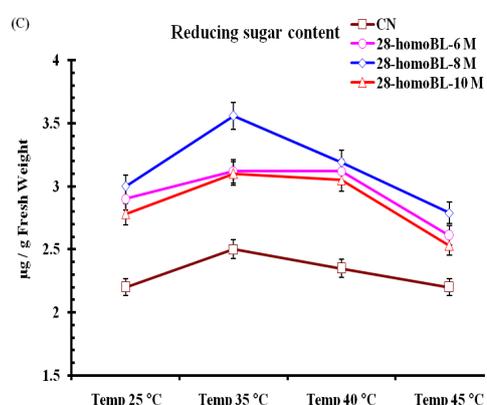
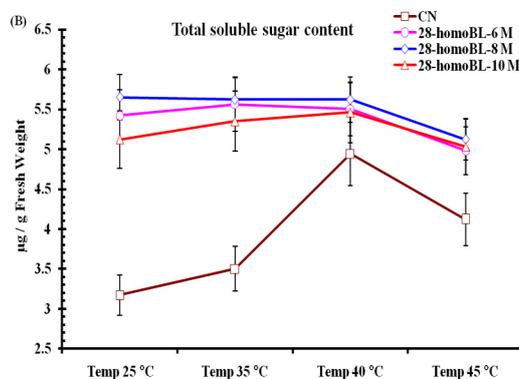
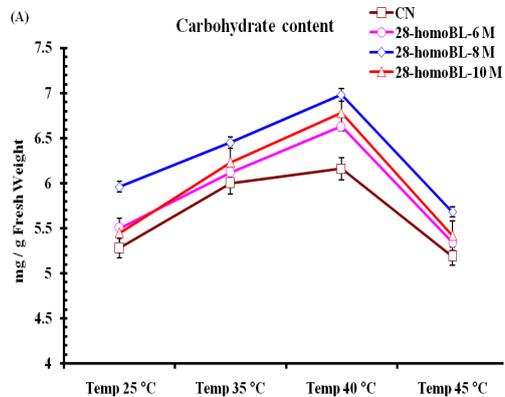


Figure 3: (a-d) Effect of different concentration of 28-HBL in *B. juncea* L. seedlings under various temperature stress on (A) total carbohydrate content (B) total soluble sugar (C) reducing sugar and (D) non-reducing sugar content.

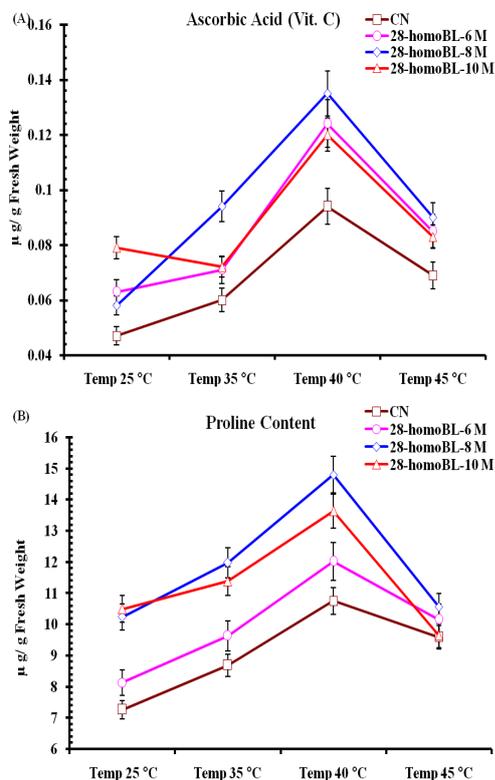


Figure 4: (a-b) Effect of different concentration of 28-HBL in *B. juncea* L. seedlings under various temperature stress on (A) vit. C content (B) proline content.

oxidation under high temperature of these pigments which decreases the metabolic rate leads to reduced carbon metabolism. Reported that BR₂₇ decreased sugar content in winter oilseed rape callus.

Ascorbic acid content also increase significantly in 10⁻¹⁰ M concentration of homoBL treated seedlings as compared to control seedlings and after the high temperature treatment it was further increase with homo BL treatments (Figure 4a). This was 40.50% more than respective control untreated seedlings. 28-homoBL also proved to be beneficial in ameliorating AsA at all temperatures. In homoBL treated seedlings, 10⁻⁸ M concentration was best for AsA (13.51 ± 2.74 µg g⁻¹ FW) content at 40°C. Whereas CN seedlings had 5.5 ± 0.99 µg.g⁻¹ FW AsA content. This amelioration was 59.28% more than the CN untreated seedlings[28]. In their experiment on *Lycopersicon esculentum* L. reputed exogenous application of BR₂₇ alters the ascorbic acid under normal conditions. Significant effect of temperature stress and 28-homoBL was observed on proline accumulation in *B. juncea* L. seedlings. The amelioration of proline content was 61.68% high than CN (Figure 4b). Proline content in higher plants increases under different environmental stress as observes in H₂O₂, drought, high salinity [29] high light and UV radiation [30], heavy metals [31] and oxidative stress [32]. Present studies suggested that under high temperature proline content increased to assertively which further ameliorated when seedlings grown with 28-homoBL primed seeds [33]. Indicated the ameliorative effect of 24-epiBL and 28-homoBL in Cd-stress alleviation which was associated with enhanced levels of proline. Under high temperature stress accumulation of proline strongly protects *B. juncea* seedlings from toxic effect of ROS produces in plant cells. Under high

temperature which may represent a secondary defensive mechanism against oxidative stress of high temperature.

Total protein is the most sensitive component of structure and storage start degradation during in adequate conditions. Total protein under present study significantly decreased under present high temperature stress. Seed primed with 28-homoBL when exposed to high temperature found to be more tolerant in terms of their protein degradation rate. Seedlings having only 28-homoBL pre-sowing treatment grow under normal temperature conditions decisively increased their protein content (Figure 5a). While studying Brassinosteroids functions to protect the translational machinery and heat shock protein synthesis in *B. napus* L. following thermal stress. It found that treatment of *B. napus* L. seedlings with 24-epiBL leads to a significant increase in their basic growth and thermo-tolerance properties. EBR treatment resulted higher accumulation of four major classes of heat shock protein (Hsps) as compared to untreated seedlings.

Thermo-tolerance in plants is mediated via accumulation of osmo-protectant like proline and vitamins along with phenolic compounds and auxins also found to involved in such tolerance mechanisms. However higher temperature stress generates high level of ROS which causes oxidative damage leading to membrane destruction which in terms affects the levels MDA content and antioxidant enzymes [7,8]. Over production of MDA content leads to oxidative degradation of phospholipids of membranes, ensuing in disruption of the permeability of cell membrane. However, it is feasible that treatment of brassinosteroids contribute in inhibition of lipid degradation as well

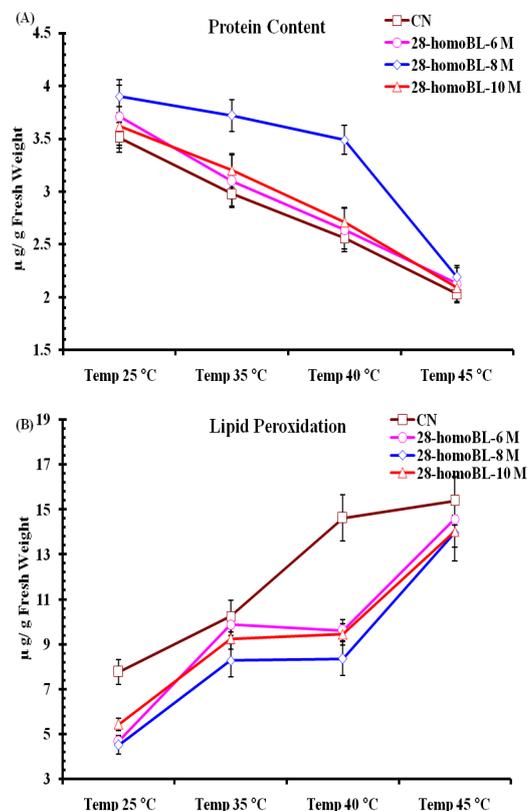
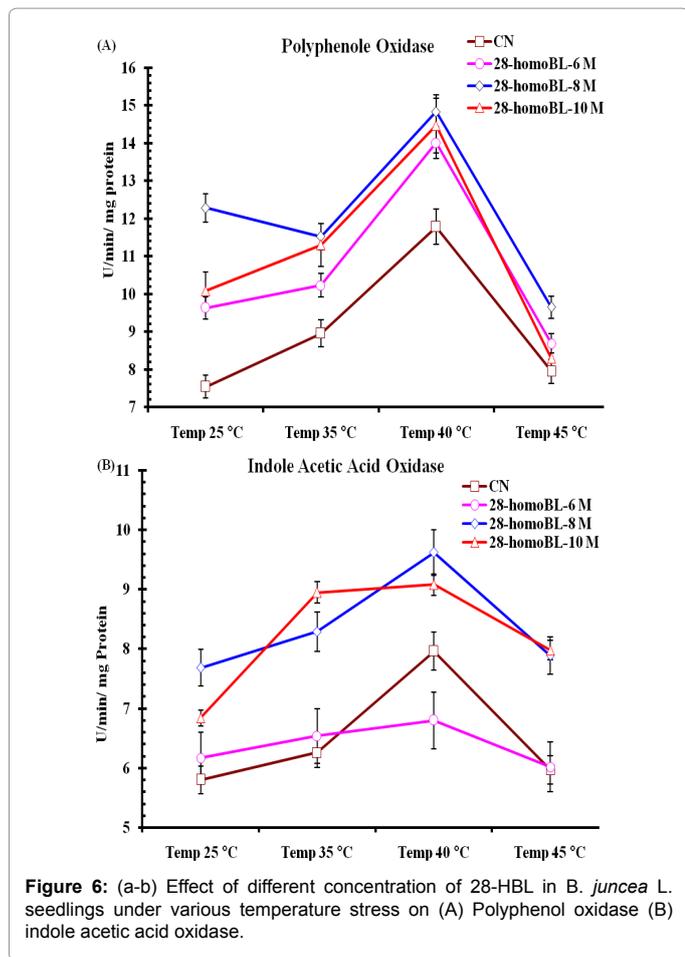


Figure 5: (a-b) Effect of different concentration of 28-HBL in *B. juncea* L. seedlings under various temperature stress on (A) protein content (B) lipid peroxidation (MDA) content.



as stimulation of activities of antioxidant enzymes under stress. In present study 28-HBL, enhances thermo-tolerance generated by high temperature exposure has been observed. This aligns with the prior reports of Sirhindi et al. [34] in *B. juncea* under various temperature stresses. Alteration of MDA content may suggest that 28-HBL treated seeds were less affected by thermal stress than the untreated control (7.8 ± 0.26) in *B. juncea* seedlings. The maximum increase in MDA content was observed at 45°C (15.14 ± 1.53) and this increase in MDA content was reduced to significant low level by 28-HBL seed priming (Figure 5b). The maximum reduction in MDA content was recorded in seedlings treated with 10^{-8} M HBL under 40°C temperature stress (8.29 ± 1.35). It was also observed that seedlings treated with 28-HBL alone could lower MDA level than control untreated seedlings at normal temperature (7.8 ± 0.26).

Thermal stress induces production of phenolic compounds, such as flavonoids and phenyl propanoids [33,35,36]. Synergistic effect of high temperature and 28-HBL was found in *Brassica juncea* seedlings Polyphenol oxidase activities (PPO). It was found that seedlings having pre-sowing soaking treatment of 28-homoBL increase their PPO activity to further enhanced levels than control (7.54 ± 1.62). The maximum increase in PPO activity was observed at (14.83 ± 2.31) and this increased in PPO was further enhanced significantly by 28-homoBL seed priming treatment. The maximum activity of PPO was observed in seedlings raised from seeds primed with 10^{-8} 28-HBL at 40°C. It was also observed that 28-HBL alone under normal temperature help in ameliorating PPO activity in treated seedlings than control untreated

seedlings (7.54 ± 1.62)) (Figure 6a) [37] and [38] reported the activity PPO increases in response to different types of biotic and abiotic stress [39]. Reported accumulation of soluble phenolic compound but lower activities of Peroxidase and PPO activity in tomato and watermelon plants, which indicated that thermal stress induces accumulation of phenolic in plants by activating their biosynthesis as well as inhibiting their oxidation. Related PPO activity to the appearance of physiological injuries caused by thermal stress in *Mahonia repens* plants. The specific activity of IAA oxidase in seedlings of *B. juncea* L. exposed to high temperatures was observed in present study. This increase was further ameliorated in seedlings raised under normal temperature after seeds treated with different concentration of 28-homoBL. 10^{-8} M homoBL was found to be best in present case study.

The enzymatic activity sharply increased during temperature exposure raised from 35 to 40°C, and its activity but detrimental decrease when temperature rose to 45°C (Figure 6b). 28-HBL treatment further ameliorated IAAO activity under normal and thermal stressed seedlings concluded that there must be some cross talk at signaling level between IAAO and HBL and 28-HBL might be regulated IAAO activity for various growth and developmental mechanisms under normal temperature and further enhanced under thermal stress conditions [40] and [7] studied IAAO activity in some forest tree and *B. juncea*, respectively under biotic stress and found that IAAO activity increased under such stress conditions. These results confirmed our previous results of 24-eBL regulation thermal stress in *B. juncea* L. [8].

The studies carried out on antioxidant enzyme activities in *B. juncea* L. seedlings exposed to different high temperature conditions revealed the considerable effect of 28-homoBL treatments. In present study activity of SOD increased significantly under temperature stress and maximum activity was observed at 40°C (34.88 ± 3.56). However, when temperature increased to 45°C, SOD activity decreased to lowest level (19.22 ± 1.23). Seed priming with different concentration of 28-HBL alone significantly alleviate the levels of SOD activity under normal temperature conditions. Our previous reports also showed that exogenous application of BRs modulate antioxidant enzyme activity in normal and stressed *Brassica juncea* plants [36,40]. Maximum activity of SOD was reported in Indian mustard seedlings treated with 10^{-8} M 28-HBL exposed to 40°C temperature shock (34.88 ± 3.56) as compared to only 10^{-8} M 28-HBL or temperature (Figure 7a).

Activity of CAT showed sharp increase in its activity in seedlings exposed to high temperatures and maximum activity was found at 40°C in distilled water seedlings. However, in seedlings raised from seeds primed with different concentrations showed significant high level of CAT enzyme activity under normal and high temperature conditions. Maximum CAT activity was observed at 10^{-8} M 28-HBL treated seedlings at 40°C (50.51 ± 4.56). Very smooth increase in CAT enzyme activity was observed in seedlings treated with 10^{-8} M homoBL with increase in temperatures from 25-45°C which otherwise sharp and dynamic in other treated seedlings at different temperature (Figure 7b). Lipid metabolism in peroxisomes produced H_2O_2 on which CAT acts to scavenge it into O_2 and H_2O . Under stress conditions this ROS (H_2O_2) produced at high rate which has to be detoxified and for this CAT enzyme activity increase. In present study it was observed that under normal conditions of growth and development presence of 28-HBL decisively increase CAT activity. This might be due to the reason that 28-HBL increases lipid metabolism which ultimately leads to more productivity of vegetative oil contents. Activities of APOX and POD were increased in seedlings exposed to high temperature alone. Maximum APOX (value) and POD (value) activity were observed at 40°C when

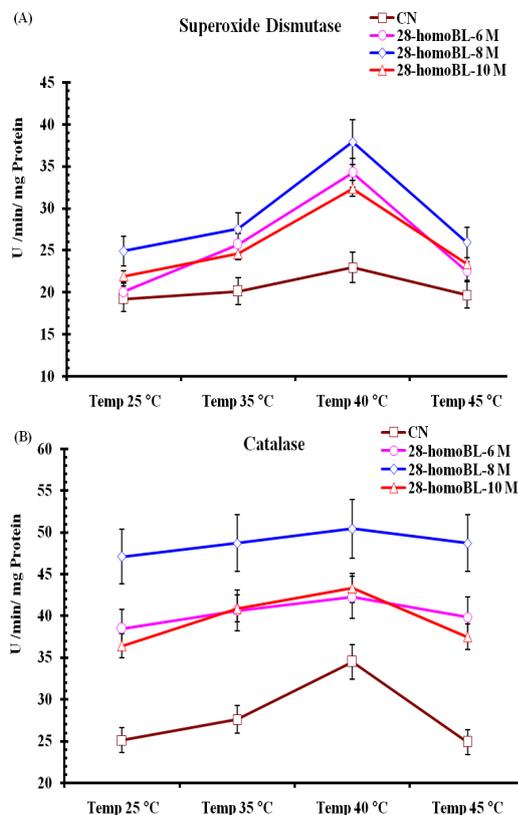


Figure 7: (a-b) Effect of different concentration of 28-HBL in *B. juncea* L. seedlings under various temperature stress on (A) Superoxide dismutase (B) Catalase.

seedlings were raised in distilled water only. Supplementation of 28-HBL at pre-sowing seed level enhanced activities of APOX and POD decisive level. Maximum APOX activity was found in 10⁻⁸ M HBL treated seeds at 40°C (26.12 ± 2.34). Similarly POD activity was also maximum in 10⁻⁸ M HBL treated seeds at 40°C (17.77 ± 1.89) (Figure 8a-b). [7] Reported in radish seedlings increased activity of APOX in 28-HBL treated seeds under normal and metal stress conditions. In some work they reported decrease in POD activity in presence of 28-HBL alone or supplemented with Cr stress. Both APOX and Pod are involved in removal of H₂O₂ produced during metabolism in the cytoplasm using different substrate to detoxify H₂O₂ produced during metabolism in the cytoplasm using different substrates. APOX use ascorbic acid as substrate to detoxify H₂O₂ by reduced itself to dehydroascorbate and then monodehydroascorbate, which ultimately manage and able to maintained the altered redox status of cells under normal conditions [41]. Established that ascorbic acid in its reduced form in essential for ROS scavenging and its cellular pool is maintained by DHAR and MDAR using NADPH as reducing power. From present findings we try to establish that 28-HBL possibly maintained the altered redox status of cells b regulating activities of antioxidant enzymes viz SOD, CAT, APOX, GR, DHAR, MDHAR etc. 28-HBL might be able to re-established redox potential by reducing lipid peroxidation of phospholipids in cell membrane and accumulation of certain osmo-protectants like proline.

Activities of DHAR and MDHAR decisively increased from (DHAR value) and (MDHAR value) respectively under control

untreated seedlings to (DHAR) and value (MDHAR) in 28-HBL treated seedlings. Under temperature treatments DHAR and MDHAR activities eventually increased and maximum DHAR activity was observed in 10⁻⁸ M HBL treated seedlings at 40°C (Figure 9a-b).

Overproduction of ROS during thermal stress is responsible for oxidative degradation of cell membrane which is confirmed from higher values of MDA content in seedlings exposed to high temperature. Seed priming treatments with 28-HBL helped in reducing this lipid peroxidation to significantly low levels as compared to untreated seedlings under various temperature treatments. It might be due to the reason that BRs which are modified sterols can able to modulate the activity of proteins and other antioxidant enzymes within the membrane by affecting either protein confirmation (functionally) or protein activity by direct protein -sterol interactions under thermal stress.

To analyses of rates of SOD, POD, APOX, CAT, DHAR and MDHAR revealed that temperature stress ameliorate internal immune metabolism of plant to produce certain level of high resistance. Seed priming treatment with 28-homoBL enhanced this thermo-tolerance level to further heights. High temperature resulted in high production of ROS, the toxic effect of which was mitigated up to certain level by antioxidant enzyme system which scavenge these free radicals. When seeds prior to the temperature exposure were primed with different concentrations of 28-homoBL, scavenging activity of these antioxidant increased. Under temperature stress the cellular homeostasis disturb due to high rate of ROS production than their mitigation by various antioxidant enzymes. High radicals (O₂^{·-}), hydrogen peroxide (H₂O₂),

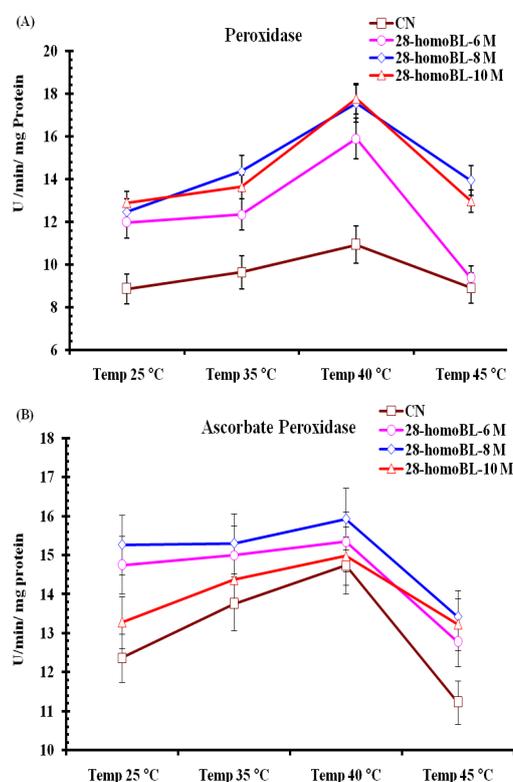


Figure 8: (a-b) Effect of different concentration of 28-HBL in *B. juncea* L. seedlings under various temperature stress on (A) peroxidase (B) ascorbate peroxidase.

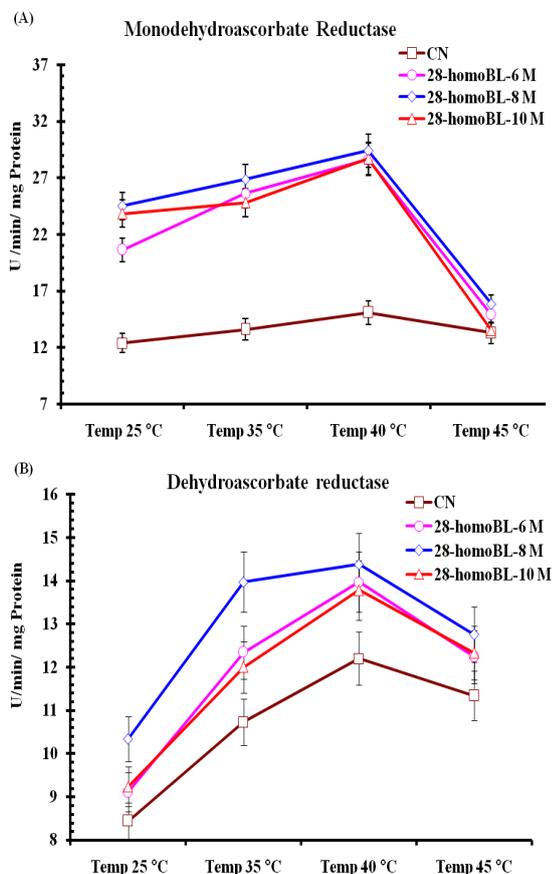


Figure 9: (a-b) Effect of different concentration of 28-HBL in *B. juncea* L. seedlings under various temperature stress on (A) monodehydroascorbate reductase (B) dehydroascorbate reductase.

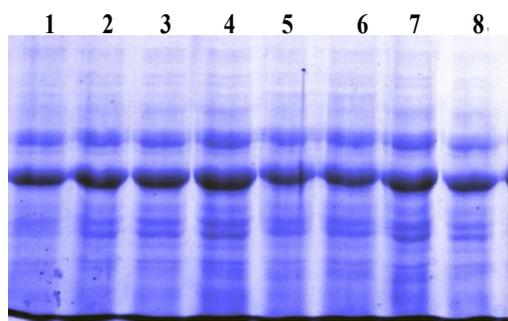


Figure 10: Protein profiling of *Brassica juncea* L. seedlings at 10 days growing with different concentration of 28-homoBL (0, 10⁻⁶ M, 10⁻⁸ M and 10⁻¹⁰ M) under control and 5 h temperature stress condition. Lanes:-1: CN (control); 2: Temp.-40°C; 3: 28-homoBL-10⁻⁶M; 4: 28-homoBL-10⁻⁸M; 5: 28-homoBL-10⁻¹⁰M; 6: 28-homoBL-10⁻⁶M+ Temp.-40°C; 7: 28-homoBL-10⁻⁸M+Temp.40°C; 8: 28-homoBL-10⁻¹⁰M+Temp.-40°C.

hydroxyl radicals (HO[•]), peroxy radical (ROO[•]), etc. [42]. These reactive species are symptoms of cellular injury due to high temperature [43]. High production of ROS causes autocatalytic peroxidation of membrane lipids and pigments thus leading to the loss of membrane semi-permeability and modifying its functions [43]. Thus, under the

influence of enhanced ROS production, plant can maintain its ROS-scavenging antioxidant enzymes such as SOD, CAT, APOX, POD, DHAR and MDHAR etc. to higher levels to detoxify the ROS toxicity [41]. Under thermal stress well regulated more efficient antioxidant system was studied by member of workers [44,45] Recently, studies available showed that different molecular signaling such as BR, abscisic acid (ABA) Ca²⁺ calmodulin (CaM), hydrogen peroxide (H₂O₂) mitogen-activated protein kinase (MAPK) cascade and nitric oxide (NO) are involved in regulation of plant responses to various environmental stresses including heat stress [42,46] (Figure 10).

In part of the native polyacrylamide gel electrophoresis (PAGE) analysis, three isozymes bands of SOD i.e. Mn-SOD, Fe-SOD and Cu/Zn-SOD were detected (Figure 11). But the intensities of these bands were increased under temperature stress as well as with brassinosteroids treatments which is again confirmation of biochemical results of total SOD activities. In addition, three bands were also detected for CAT isozymes, which showed higher intensity under high temperature in brassinosteroids treated seedlings (Figure 12). Native polyacrylamide gel electrophoresis (PAGE) analysis for Peroxidase (POD) enzyme showed three isozymes which also showed increase in their intensities under temperature treatments supplemented with BRs (Figure 13). Similar result was observed by [28] in *Lycopersicon esculentum* under drought stress. SDS-PAGE also revealed that protein was degraded under temperature stress and ameliorated by brassinosteroids treatments under normal as well as under temperature stress condition in *B. juncea* L. seedlings.

Conclusion

High temperature stress indicated detrimental effects on several key physiological processes such as photosynthetic pigments, carbon

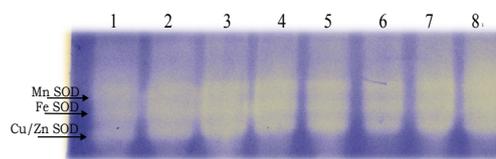


Figure 11: Superoxide dismutase isoenzyme patterns of *Brassica juncea* L. seedlings at 10 days growing with different concentrations of 28-homoBL (0, 10⁻⁶ M, 10⁻⁸ M and 10⁻¹⁰ M) under control and 5h temperature stress condition. Lanes:-1: CN (control); 2: Temp.-40°C; 3: 28-homoBL-10⁻⁶M; 4: 28-homoBL-10⁻⁸M; 5: 28-homoBL-10⁻¹⁰M; 6: 28-homoBL-10⁻⁶M+ Temp.-40°C; 7: 28-homoBL-10⁻⁸M+Temp.40°C; 8: 28-homoBL-10⁻¹⁰M+Temp.-40°C.

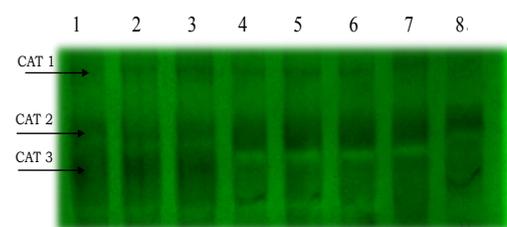


Figure 12: Catalase isoenzyme patterns of *Brassica juncea* L. seedlings at 10 days growing with different concentrations of 28-homoBL (0, 10⁻⁶ M, 10⁻⁸ M and 10⁻¹⁰ M) under control and 5h temperature stress condition. Lanes:-1: CN (control); 2: Temp.-40°C; 3: 28-homoBL-10⁻⁶M; 4: 28-homoBL-10⁻⁸M; 5: 28-homoBL-10⁻¹⁰M; 6: 28-homoBL-10⁻⁶M+ Temp.-40°C; 7: 28-homoBL-10⁻⁸M+Temp.40°C; 8: 28-homoBL-10⁻¹⁰M+Temp.-40°C.

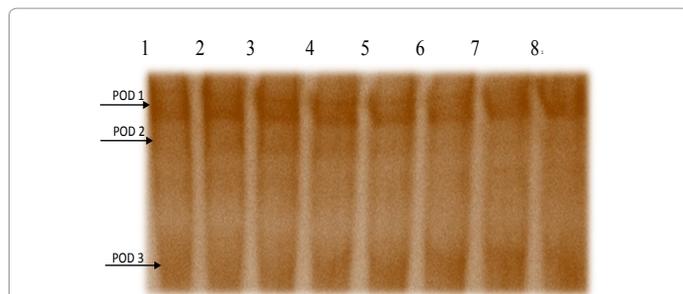


Figure 13: Peroxidase isoenzyme patterns of *Brassica juncea* L. seedlings at 10 days growing with different concentrations of 28-homoBL (0, 10^{-8} M, 10^{-6} M and 10^{-10} M) under control and 5h temperature stress conditions. Lanes:-1: CN (control); 2: Temp.-40°C; 3: 28-homoBL 10^{-8} M; 4: 28-homoBL- 10^{-8} M; 5: 28-homoBL- 10^{-10} M; 6: 28-homoBL- 10^{-6} M+ Temp.-40°C; 7: 28-homoBL- 10^{-8} M+Temp.40°C; 8: 28-homoBL- 10^{-10} M+Temp.-40°C.

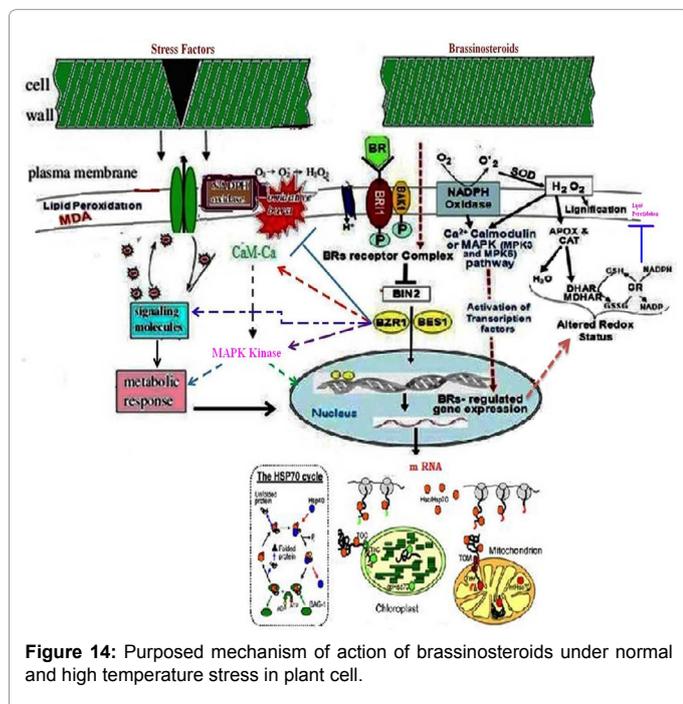


Figure 14: Purposed mechanism of action of brassinosteroids under normal and high temperature stress in plant cell.

metabolism, lipid peroxidation and antioxidant defense system. The main toxic effect of high temperature appeared to be on photosynthesis, degradation of proteins, increased lipid peroxidation. Supplementations of 28-homoBL treatment improve seedling/plant growth by protecting the proteins from thermal degradation by ameliorating the free radical scavenging system. BRs are present in plants accountable for pleiotropic effects in plants under normal and stress environment. A probable mechanism has been proposed based on the information of BRs mechanism available in literature and results obtained in present study. This study explores another role of BRs as an anti-stressor for protection of plant from thermal stress by modulating the metabolic machinery of plants.

Acknowledgement

Financial assistance from University Grants Commission, New Delhi is duly acknowledged.

References

1. Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* 48: 909-930.

2. Zinn KE, Tunc-Ozdemir M, Harper JF (2010) Temperature stress and plant sexual reproduction: uncovering the weakest links. *J Exp Bot* 61: 1959-1968.

3. A Levent Tuna, Cengiz Kaya, Murat Dikilitas, Ibrahim Yokas, Betül Burun, et al. (2007) Comparative effects of various salicylic acid derivatives on key growth parameters and some enzyme activities in salinity stressed maize (*Zea mays* L.) plants. *Pak J Bot* 39:787-798

4. Nitika Arora, Renu Bhardwaj, Priyanka Sharma, Hardesh KA (2008) 28-Homobrassinolide alleviates oxidative stress in salt treated maize (*Zea mays* L.) plants. *Braz J Plant Physiol* 20:153-157.

5. Ashraf M, Akram NA, Arteca RN, Foolad MR (2010) the physiological, biochemical and molecular roles of brassinosteroids and salicylic acid in plant processes and salt tolerance. *Crit Rev in Plant Sci* 29:162-190.

6. Sirhindi GK, Bhardwaj M, Kumar RS, Pradhan SK (2011) Effect of 24-epibrassinolide on activity of antioxidant enzymes in *Brassica juncea* L. under H₂O₂ stress. *Indian J Plant Physiol* 16 :68-71

7. Sharma I, Pati PK, Bhardwaj R (2011) Effect of 28-homobrassinolide on antioxidant defence system in *Raphanus sativus* L. under chromium toxicity. *Ecotoxicology* 20: 862-874.

8. Manish Kumar, Geetika Sirhindi, Renu Bhardwaj, Sandeep Kumar, Gagandeep Jain (2010) Role of 24-epibrassinolide in amelioration of high temperature stress through antioxidant defense system in *Brassica juncea* L. 55-58

9. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* 28: 350-356.

10. Nelson NJ (1957) Colorimetric Analysis of Sugars, In: Colowick S.P. and N.O. Kaplan (eds), *Methods in Enzymology* 3, Academic Press, New York, 85-86

11. Chinoy JJ, Singh YD, Gurumurti K (1976) Colorimetric determination of ascorbic acid turnover in plants. *Ind J Plant Physiol* 19:122-30.

12. Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. *Plant Soil* 39:205-207.

13. Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* 125: 189-198.

14. Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant and Cell Physiol* 22:867-880.

15. Aebi H (1983) Catalase In: Bergmeyer HU (Ed) *Methods of Enzymatic Analysis*. Verlag Chemie Weinhan 673-684.

16. Hossain MA Nakano Y Asada K (1984) Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. *Plant Cell Physiol* 25:385-395

17. Dalton DA, Russell SA, Hanus FJ, Pascoe GA, Evans HJ (1986) Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. *Proc Natl Acad Sci U S A* 83: 3811-3815.

18. Bastin M, Unlüner O (1972) Effect of actinomycin D on the formation of enzymes in Jerusalem artichoke tuber slices. *Planta* 102: 357-361.

19. Gordon SA, Weber RP (1951) the effect of x-radiation on indoleacetic acid and auxin levels in the plant. *Ameri Jour of Botany*. 37: 78-81

20. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275.

21. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

22. Fielding JL, Hall JL (1978) a biochemical and cytological study of peroxidase activity in roots of *Pisum sativum*. *J Exp Bot* 29:969- 981

23. Woodbury W, Spencer AK, Stahman MA (1971) An improved procedure using ferricyanide for detecting catalase isozymes. *Anal Biochem* 44: 301-305.

24. Krishna P (2003) Brassinosteroid-Mediated Stress Responses. *J Plant Growth Regul* 22: 289-297.

25. Asthir B, Deep A (2011) thermotolerance and antioxidant response induce by putrescine and heat acclimation in wheat seedlings. *Seed sci biotech* 5: 42-46.

26. Kleinhenz MD, Palta JP (2002) Root zone calcium modulates the response of potato plants to heat stress. *Physiol Plant* 115: 111-118.

27. Lobato AKS, Neto CFO, Costa RCL, Santos Filho BG, Cruz FJR, et al. (2008) Biochemical and physiological behavior of *Vigna unguiculata* (L.) Walp. Under water stress during the vegetative phase. *Asian J Plant Sci* 7:44-49
28. Behnamnia M, Kalantari KM, Rezanejad F (2009) Exogenous application of brassinosteroid alleviates drought-induced oxidative stress in *Lycopersicon esculentum* L. *General and Applied Plant Physiol* 35:22-34.
29. Yoshida Y, Kiyosue T, Katagiri T, Ueda H, Mizoguchi T, et al. (1995) Correlation between the induction of a gene for delta 1-pyrroline-5-carboxylate synthetase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. *Plant J* 7: 751-760.
30. Saradhi PP, Alia, Arora S, Prasad KV (1995) Proline accumulates in plants exposed to UV radiation and protects them against UV induced peroxidation. *Biochem Biophys Res Commun* 209: 1-5.
31. Yang SL, Lan SS, Gong M (2009) Hydrogen peroxide-induced proline and metabolic pathway of its accumulation in maize seedlings. *J Plant Physiol* 166: 1694-1699.
32. Anuradha S, Rao SR (2007) the effect of brassinosteroids on radish (*Raphanus sativus* L.) seedlings growing under cadmium stress. *Plant Soil Environ* 53:456-472.
33. Nozzolillo C, Isabelle P, Das G (1990) Seasonal changes in phenolics constituents of jack pine seedlings (*Pinus banksiana*) in relation to the purpling phenomenon. *Canad J Botany* 68: 2010-2017
34. Sirhindi G, Kumar S, Bhardwaj R, Kumar M (2009) Effects of 24-epibrassinolide and 28-homobrassinolide on the growth and antioxidant enzyme activities in the seedlings of *Brassica juncea* L. *Physiol Mol Biol Plants* 15: 335-341.
35. Christie PJ, Alfenito MR, Walbot V (1994) Impact of low temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta* 194:541-549.
36. Bharti AK, Khurana JP (1997) Mutants of *Arabidopsis* as tools to understand the regulation of phenylpropanoid pathway and UVB protection mechanisms. *Photochem Photobiol* 65: 765-776.
37. Pandolfini T, Gabrielli R, Comparino C (1992) Nickel toxicity and peroxidase activity in seedlings of *Triticum aestivum* L. *Plant Cell Environ* 15: 719-725.
38. Smith-Becker J, Marois E, Huguet EJ, Midland SL, Sims JJ, et al. (1998) Accumulation of salicylic acid and 4-hydroxybenzoic acid in phloem fluids of cucumber during systemic acquired resistance is preceded by a transient increase in phenylalanine ammonia-lyase activity in petioles and stems. *Plant Physiol* 116: 231-238.
39. Rivero RM, Ruiz JM, García PC, López-Lefebvre LR, Sánchez E, et al. (2001) Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. *Plant Sci* 160: 315-321.
40. Gupta JP (2011) auxin and IAA oxidase activity related to the leaves gall formation in some forest trees. *Sci Res Rep* 1:108-111.
41. Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* 7: 405-410.
42. Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: an overview. *Environmental and Experimental Botany* 61:199-223.
43. Xu S, Zhang Li, J Wei X, Cui H (2006) effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts in two cool-season turfgrass species under heat stress. *Envir Exp Bot* 56:274-285
44. Suzuki N, Mittler R (2006) Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiol Plant* 126:45-51.
45. Kotak S, Larkindale J, Lee U, von Koskull-Döring P, Vierling E, et al. (2007) Complexity of the heat stress response in plants. *Curr Opin Plant Biol* 10: 310-316.
46. Saidi Y, Finka A, Goloubinoff P (2011) Heat perception and signalling in plants: a tortuous path to thermotolerance. *New Phytol* 190: 556-565.