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Physiological and Biochemical Effects of 24-Epibrassinolide on Heat-Stress Adaptation in Maize (*Zea mays* L.)

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Abstract

Brassinosteroids (BRs) are a family of about 70 structurally related polyhydroxy steroidal phytohormones that regulate a number of physiological processes in plants. Among these, brassinolide (BL), 28-homobrassinolide (28-homoBL) and 24-epibrassinolide (24-EpiBL) are more common. The present study aims at studying the usefulness of 24-epiBL in ameliorating the impacts of heat-stress in maize along with its role in regulating cellular antioxidant defense system. Maize hybrid PMH 3 was grown in pots in a green house maintained at 14 hours day (25˚C)/10hours night (17˚C). A solution of 24-epiBL (1 µM) was applied externally at V_4 stage. Leaf tissues were sampled from both treated and control plants. Subsequently, both the groups of pots were placed in plant growth chamber maintained at high temperature (48˚C; RH 50%). Plants were sampled for biochemical analysis after 3, 6, 9, 24 and 48 hours of high temperature exposure. Exogenous application of 24-EpiBL arrested protein degradation and enhanced cell membrane stability, as compared to the control. The biochemical activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and peroxidase (POX) were found to be dynamically and variably modulated post 24-epiBL treatment. Thus, the study supports the role of BRs as anti-stress agents.

Keywords

Brassinosteroid, Maize, Heat-Stress

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1. Introduction

Climate change and associated extreme weather events have made high temperature a major threat to crop production worldwide. High temperatures beyond a critical threshold enhance the production of Reactive Oxygen Species (ROS) in plants, which damages the membranes and degrade proteins, lipids and other bio-molecules bringing about a detrimental change in the metabolism [1]. Tolerance to heat-stress involves several mechanisms like, synthesis of heat shock proteins (HSPs), compatible osmolytes, factors that regulate membrane fluidity, activation of the antioxidative systems to scavenge the ROS, etc. [2]. Plant antioxidant defence system consists of the enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione peroxidise (GPX) along with non-enzymatic components such as compatible solutes, reduced glutathione and ascorbic acid [1]. Phytohormones like auxins, gibberellins, ethylene, jasmonic acid and salicylic acid have been reported to play stress protective role in plants [2]-[4].

Brassinosteroids (BRs) are polyhydroxy steroidal phytohormones that regulate many physiological processes in plants including seed germination, photo-morphogenesis, photosynthesis, flowering, sucrose translocation, assimilate partitioning, senescence and responses to different abiotic and biotic stresses [5] [6]. Nearly 70 BR related compounds have been identified so far that are bioactive [7]. Among these, brassinolide (BL), 28-homobrassinolide (28-HomoBL) and 24-epibrassinolide (24-EpiBL) are commonly used in most of the experimental studies [8].

In recent times, a number of studies have documented the stress-mitigating roles of BRs in plants exposed to various abiotic stresses [8]. BRs play a vital role in protecting the translational machinery through the induction of HSPs under high temperatures [9] [10]. Mazorra *et al.* [11] observed that pre-incubation of tomato leaf discs in cell culture with 24-epiBL controlled cell damage under heat-stress, which was attributed to BR induced expression of HSPs and their enhanced accumulation. The activities of the enzymes of the antioxidant defence system such as CAT, SOD and POX were also enhanced. The stress ameliorative properties of BRs mitigate the harmful impacts of high temperatures through the modulation of the components of the anti-oxidative defence system [11]-[16]. Young rice seedlings exposed to high temperatures, when sprayed with BR had increased POX and SOD enzyme activities, reduced malondialdehyde (MDA) levels and enhanced membrane stability [14]. Treatment of 28-homoBL in *Vigna radiata* c.v. T-44 plants grown under high temperature led to an increase in leaf water potential (ψ) and membrane stability index (MSI) due to enhanced activities of antioxidant enzymes and the level of proline [16]. Similarly, 24-epiBL pre-treatment of high-temperature grown tomato plants, led to an increase in the activities of APX, GPX, SOD and CAT and reduction of H_2O_2 and MDA content. These factors led to the alleviation of heat-induced inhibition of photosynthesis by increasing carboxylation efficiency [15]. Exogenous application of 24-EpiBL was found to induce tolerance to high temperature in BR-deficient tomato mutant, *extreme dwarf* d^x [12]. It was also reported that tomato line overexpressing the dwarf, BR-biosynthesis gene 35SD, showed enhanced heat-stres tolerance. The enhanced tolerance was attributed to higher activities of antioxidant enzymes.

The mechanism of BR action under high temperatures has not been studied well in the non-model crop plants such as maize. Maize (*Zea mays* L.) is the grain crop with highest global production and holds great economic significance for world agriculture. Maize is cultivated in diverse ecologies and heat-stress is one of the major factors limiting its productivity in the tropics and the sub-tropics. We examined the effect of high temperature on the antioxidant defence system and the role of 24-EpiBL in adaptation to high temperature induced oxidative stress in maize. Understanding the BRs mediated stress adaptation in maize might pave the way for developing appropriate spray based technologies for mitigating heat-stress in this crop.

2. Materials and Methods

2.1. Plant Growth and Treatments

Seeds of a single-cross maize hybrid PMH 3 (LM 17 × LM 14) (obtained from Directorate of Maize Research, New Delhi) were grown in thermacol pots (8 cm length × 7 cm diameter) filled with 150 g sand + vermiculite + coco peat (in the ratio 0.5:2:1) in green-house. At V_4 stage, the plants were transferred to plant growth chamber (Sanyo Versatile Environmental Test Chamber, Japan, Model MLR-351H) maintained at 14 hours day (25˚C)/10 hours night (17˚C), relative humidity (RH) of 50% and photosynthetic photon flux density (PPFD) of 170 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for an acclimation period of 1 week. After the acclimation period, the plants were divided into two

groups—the first group of plants were sprayed with 1 µM 24-EpiBL and Tween-20 dissolved in distilled water, while the other group was sprayed with only Tween-20 dissolved in distilled water (mock spray). After 24 hours, both the groups of plants were placed in another plant growth chamber kept ready at 14 hours day (48˚C)/10 hours night (17˚C), RH 50%, and PPFD 170 µmol·m^{-2}·s^{-1} for 2 days (heat-stress treatment). The plants were sampled for biochemical analysis at 0, 3, 6, and 9 hours (h) of the first day of high temperature exposure (48˚C). For survival studies, the plants were transferred to the greenhouse for recovery after 3, 6, 9, 24 and 48 h.

2.2. Measurement of the Lipid Peroxidation

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) content produced by the thiobarbituric acid reactive substance (TBARS) [17]. A pre-weighed (0.1 g) fresh leaf sample was ground homogeneously in 3 mL 0.1% trichloro acetic acid (TCA) and centrifuged at 10,000 rpm for 20 min at 4˚C. Four mL of 0.5% thiobarbituric acid in 20% TCA was added to 1 mL supernatant and incubated in the water bath at 95˚C for 30 min. The reaction was terminated in ice and then centrifuged at 10,000 rpm for 10 min. Absorbance of the supernatant was determined at 532 and 600 nm. After subtracting the non-specific absorbance at 600 nm, the MDA concentration (µmol·g^{-1} FW) was determined using the extinction coefficient of 155 mM^{-1}·cm^{-1}.

2.3. Crude Extract Preparation and Total Protein Estimation

Leaf tissue (0.5 g) was grinded in liquid nitrogen in ice cold mortar and pestle and transferred into oak ridge centrifuge tubes. Five mL of freshly prepared extraction buffer [50 mM sodium phosphate buffer, pH 7.5, 1 mM polyethylene glycol (PEG), 1 mM phenylmethylsulfonyl fluoride (PMSF), 8% (w/v) polyvinylpyrrolidone (PVP), 0.01% (v/v) Triton X-100 added in 100 mL distilled water] was added and the centrifuge tube was vortexed. The tubes were centrifuged at 12,000 rpm for 20 min at 4˚C and the supernatant was transferred to the eppendorf tubes and stored at −20˚C. The supernatant collected was used for estimation of different antioxidant enzyme assays after measuring the total protein content [18].

2.4. Antioxidant Enzymes Assays

CAT activity was determined spectrophotometrically by following the decrease in absorbance at 240 nm [19]. The reaction mixture contained 0.1 M sodium phosphate buffer (pH 6.8), 10 mM H_2O_2 and the reaction was started by adding 0.02 mL of enzyme extract. The decrease in absorbance was recorded at every 30 s up to 2 min. One unit of CAT activity was defined as the amount that caused a change of 0.01 in absorbance. The specific activity of CAT was expressed as U·mg^{-1} protein. SOD activity was assayed by monitoring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) [20]. The reaction mixture of 3 mL contained 50 mM potassium phosphate buffer, 200 mM L-methionine, 3 mM ethylene diamine tetra acetic acid (EDTA), 1.5 mM sodium carbonate (Na_2CO_3), 2.25 mM NBT and 2 µM riboflavin. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT at 560 nm. The specific activity of SOD was expressed as U·mg^{-1} protein. Non-specific POX activity was determined by measuring peroxidation of hydrogen peroxide with guaiacol as an electron donor [21]. The reaction mixture contained 50 mM sodium acetate buffer, 25 mM guaiacol, 25 mM H_2O_2 and 0.05 mL of enzyme extract. The increase in absorbance at 470 nm due to the guaiacol oxidation was recorded for 3 minutes. One unit of POX activity was defined as the amount that caused a change of 0.01 in absorbance. The specific activity of POX was expressed as U·mg^{-1} protein.

3. Results and Discussion

3.1. Plant Survival Studies

After the foliar application of 24-EpiBL, the maize seedlings were subjected to heat-stress (48˚C, 14 h day/17˚C, 10 h night cycle, with RH of 50% throughout) for 48 h. After 3 and 6 h of heat-stress exposure, damaged leaf tips and damaged leaf margins were observed, respectively. A 9 h exposure to heat-stress resulted in drooping of leaves and after 24 h, leaf rolling was observed. Extended heat-stress exposure of 48 h killed the plants. After heat-stress, the plants were transferred to the green house for recovery and watered daily. The plants exposed to high temperature for 3, 6, and 9 h recovered well and exhibited good growth. On the other hand, the plants subjected to 24 h of heat-stress recovered slowly. While the plants subjected to 48 h of heat-stress did not recover at all. No significant visual differences were observed between 24-EpiBL treated and untreated plants. It is well

known that heat-stress induces changes in respiration and photosynthesis and thus leads to a shortened life cycle and diminished plant productivity [22]. Also, by causing injuries to the cell membrane, microtubular organization and ultimately to the cytoskeleton, heat-stress changes membrane permeability and alters cell differentiation, elongation and expansion leading to the physical plant damage [23]-[25]. However, external BR application protects the translational machinery and aids higher expression of heat shock proteins [9] thereby protecting the treated plants from heat-stress to a higher extent as compared to the untreated plants.

3.2. Lipid Peroxidation

Maize seedlings were exposed to heat-stress, with or without 24-EpiBL pre-treatment and the extent of heat induced oxidative damage was evaluated by measuring the MDA content (**Figure 1**). Surprisingly, the foliar application of 24-EpiBL itself at the beginning of the experiment (0 h) led to a significant increase in the MDA content. Thus, 24-EpiBL pre-treatment itself could have caused production of ROS, leading to membrane damage. This is consistent with the prevailing theory that BRs stimulates ROS production and this BR-induced ROS is important for subsequent establishment of stress tolerance [26]. Xia *et al.* [27] observed that cucumber leaves treated with BR inhibitor have reduced ROS level, whereas exogenous BRs induced moderate ROS accumulation mostly in the apoplast. A narrow range of BR concentration was found to cause a moderate increase in the ROS concentration, which in turn triggered a more reduced cellular redox state essential for BR-induced CO_2 assimilation. It is, therefore, likely that BR-induced ROS may also play a critical role in BRs-regulated plant growth and development [26].

On the other hand, under heat-stress, significant differences were observed between the 24-EpiBL pre-treated and the control groups at different time points. MDA content in the 24-EpiBL pre-treated and as well as the control groups reached a peak at 6 h after heat-stress treatment, and then decreased at 9 h. MDA content in the 24-EpiBL pre-treated group was significantly lower than the control group under different duration of exposure to heat-stress, indicating the ameliorative effects of 24-EpiBL on heat induced membrane damage. As compared to the mock sprayed plants, the MDA content was lower by 62.93, 19.35, and 30.25% in the 24-EpiBL sprayed plants at 3, 6 and 9 h of heat-stress exposure respectively (**Figure 1**). These results are similar to those obtained by Cao *et al.* [14] and Ogweno *et al.* [15] who also reported a decrease in the MDA content upon BR treatment under heat-stress. The 24-EpiBL-mediated strengthening of antioxidant defence system and eventually decreased membrane lipid peroxidation could have resulted in lower MDA content [6].

3.3. Total Protein Content

In the control group, the amount of total soluble proteins showed moderate decline upon heat-stress treatment (**Figure 2**). This is consistent with the earlier reports that showed a decline in total soluble protein under condi-

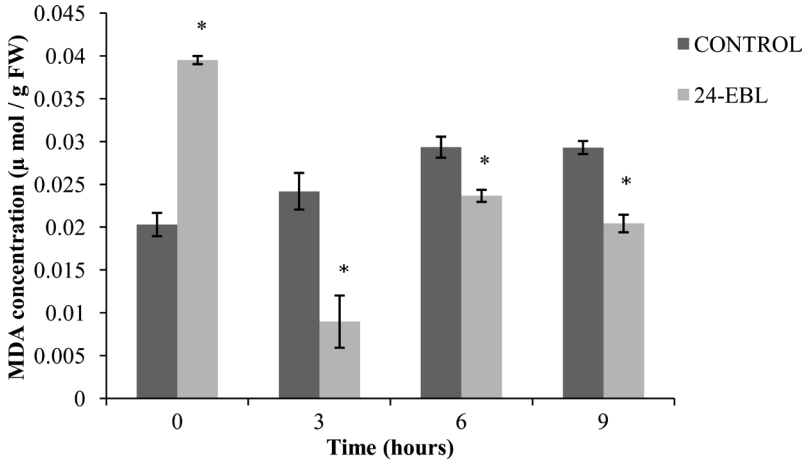

Figure 1. Effect of 24-epibrassinolide on lipid peroxidation under heat-stress. The maize plants (PMH-3) were sprayed with 1 µM 24-epibrassinolide and Tween-20 dissolved in distilled water at the V_4 stage. Leaf samples were collected after 0, 3, 6 and 9 hours of heat-stress treatments for biochemical analysis of MDA concentration (µmol·g^{-1} FW). Data represents mean ± S.E. of three replicates, asterisk indicates significant difference compared to the control at $P < 0.05$.

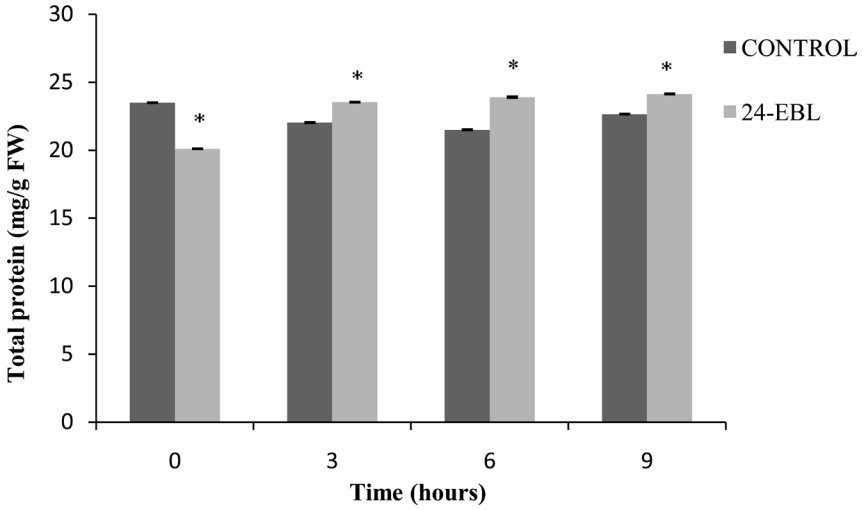

Figure 2. Effect of 24-epibrassinolide on total soluble protein accumulation under heat-stress. The maize plants (PMH-3) were sprayed with 1 μM 24-epibrassinolide and Tween-20 dissolved in distilled water at the V_4 stage. Leaf samples were collected after 0, 3, 6 and 9 hours of heat-stress treatments for determination of total protein content ($mg \cdot g^{-1}$ FW). Data represents mean ± S.E. of three replicates, asterisk indicates significant difference compared to the control at $P < 0.05$.

tions of heat stress in other plant species [28] [29]. In unstressed condition (t = 0 h), 24-EpiBL pre-treatment caused marginal decrease in protein accumulation, probably due to ROS induced protein degradation. However, under heat-stress conditions, 24-EpiBL pre-treatment led to marginal but consistent enhancement of total soluble proteins at all the three time points of heat-stress exposure (3 h, 6 h and 9 h). The soluble proteins increased by 6.79, 11.16 and 6.55% in 24-EpiBL pre-treated group as compared to the untreated group under heat stress at 3, 6 and 9 h respectively. Application of 24-EpiBL could have arrested the protein degradation, probably due to the alleviation of heat-induced inhibition of photosynthesis [15], or by inducing the activities of antioxidant enzymes [14]-[16]. In *Brassica napus* seedlings, 24-EpiBL application led to greater accumulation of 47 kDa rubisco holoenzyme and HSPs [30]. The increased accumulation of HSPs in BR treated seedlings resulted from higher HSP synthesis, as several translation initiation and elongation factors were found to be present at significantly higher levels in BR-treated seedlings as compared to untreated seedlings [9]. In tomato plants exposed to heat-stress, more accumulation of mitochondrial small HSPs was reported in 24-EpiBL treated plants as compared to the untreated [31]. Thus, HSPs might constitute the major group of proteins in the overall proteins that were found to be enhanced upon 24-EpiBL treatment under heat-stress.

3.4. Antioxidant Enzymes

The 24-EpiBL mediated modulation of specific activities of the three key antioxidant enzymes (CAT, SOD and POX), exhibited dynamic and variable responses under heat stress (**Figure 3**). On the other hand, the effect of heat alone on the antioxidant activities depicted a more consistent and expected response. In general, heat-stress led to enhancement of activities of antioxidant enzymes. For example, the CAT activity under untreated conditions increased by 3.44, 29.01, and 109.37% at 3, 6 and 9 h of heat exposure respectively as compared to the plants not exposed to heat stress (t = 0). Application of 24-EpiBL did not had any significant effect on the CAT activity at all the time points of heat-stress exposure, except at 9 h of heat-stress exposure, when the CAT activity reduced by 13.88%. Like CAT, SOD activities under heat-stress alone showed consistent enhancement for various time points of heat-stress exposure duration. The SOD activity was found to be increased by 5.58, 51.61 and 56.46% at 3, 6, and 9 h of heat-stress exposure as compared to the plants not exposed to heat-stress. In contrast, in the 24-EpiBL treated plants the SOD activities were found to be decreased by 13.54, 17.34, and 26.27% at 0, 3 and 6 h of heat-stress exposure, respectively as compared to the mock treated plants. However, at 9h of heat-stress exposure there was 5.24% enhancement in SOD activity in 24-EpiBL treated plants as compared to the mock treated plants. Heat mediated enhancement of antioxidant enzyme activity was also observed in case of POX, at least for two out of three time points of heat-stress exposure (t = 3 and 6 h). A consistent decline of 41.56, 28.15 and 49.10% was observed at 3, 6 and 9 h of high temperature stress in the 24-EpiBL treated plants

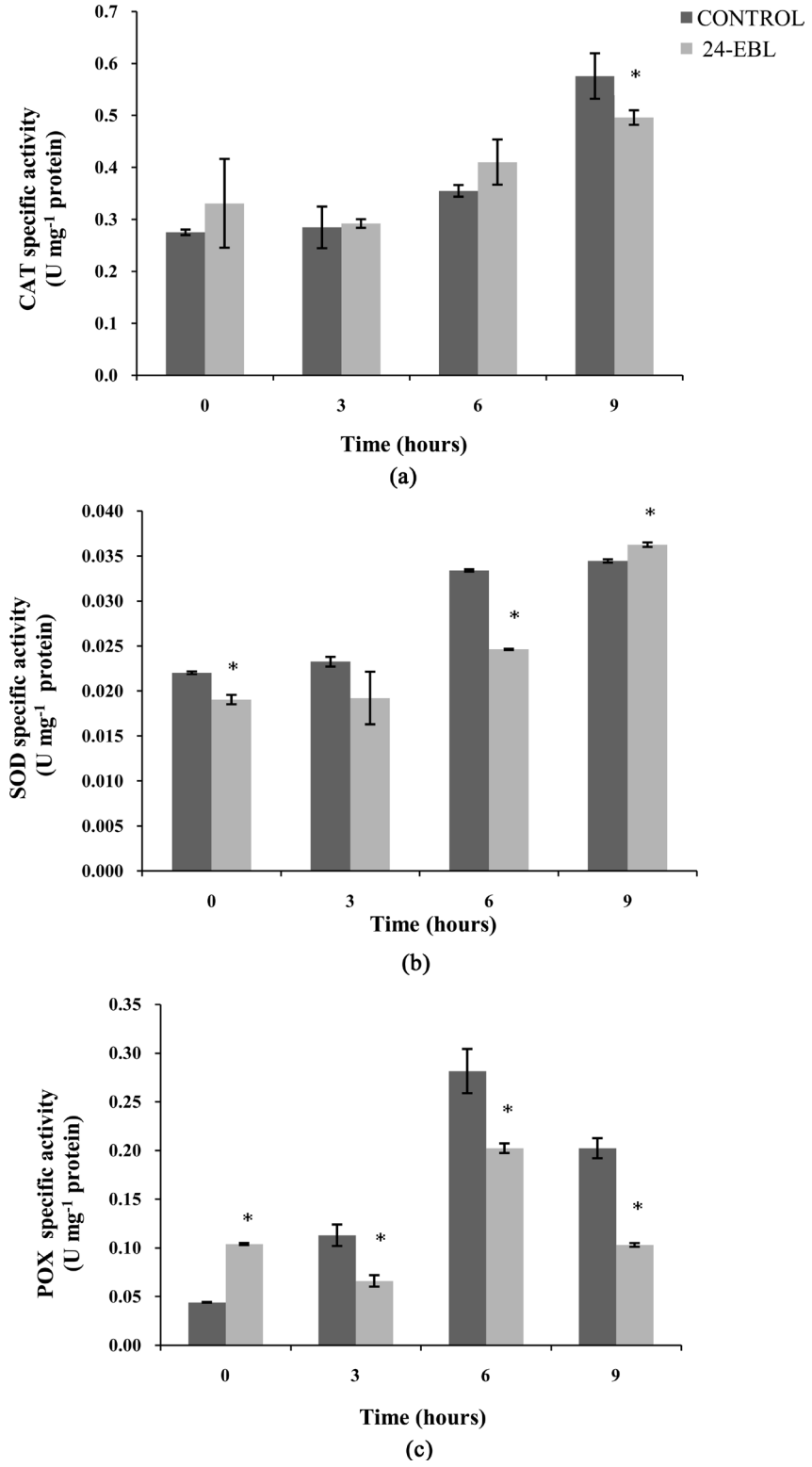

Figure 3. Effect of 24-epibrassinolide on maize plants (PMH-3) under heat-stress. The plants were sprayed with 1 μM 24-epibrassinolide and Tween-20 dissolved in distilled water at the V4 stage. Leaf samples were collected after 0, 3, 6 and 9 hours of heat-stress treatments for biochemical analysis. Specific activities of (a) catalase (CAT); (b) superoxide dismutase (SOD) and (c) peroxidase (POX) were determined ($U \cdot mg^{-1}$). Data represents mean ± S.E. of three replicates, asterisk indicates significant difference compared to the control at $P < 0.05$.

as compared to control plants. When there was no exposure to heat-stress, just 24-EpiBL treatment led to 136.04% enhancement in POX activity as compared to control. Thus, 24-EpiBL mediated modulation of various antioxidant enzyme activities in maize plants exposed to heat-stress was found to be dynamic and variable.

With respect to 24-EpiBL mediated modulation of antioxidant enzyme activities under heat-stress, our findings are in agreement with findings of Mazorra *et al.* [12]. In wild type tomato plants, exogenous application of 1 μM epi-brassinolide (EBL) did not significantly altered the activities of SOD, CAT and APX. It is possible that the induction of thermotolerance by external application of 24-EpiBL may not be directly related to the role of 24-EpiBL in modulating the antioxidant enzyme activities. BRs are known to influence other phytohormones levels such as ethylene, salycilic acid and abscisic acid [32] [33] that also increase thermotolerance and regulate oxidative stress under high temperature [34]-[36]. It is also known that sensitivity to exogenously applied BR is greater in tissues that lack it [37]. Thus, endogenous BR levels and their response to changing thermal regime can have noisy effect on externally applied BR. For such studies, it is important to discriminate the biological effect of endogenous BR signalling and modulating effects of exogenous synthetic BR. However, there is a lack of appropriately characterized and phenotypically inconsequential BR biosynthesis mutant lines in maize suitable for such studies.

4. Conclusion

The present study reveals the usefulness of 24-EpiBL in ameliorating the negative impacts of heat-stress in maize. High temperature beyond a critical threshold enhances the ROS generation which damages the membranes and alters the cellular metabolism. Exogenous application of 24-EpiBL was found to increase the total soluble protein content and protect the plasma membrane from oxidative damage. Thus, the study supports the role of BRs as anti-stress agents. However, further investigations are required to gain insights into the molecular-genetic mechanisms of BRs action, especially their role in modulating the antioxidant defence system under stress.

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