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ORIGINAL ARTICLE

28-homobrassinolide Protects Photosynthetic Machinery in Indian mustard Under High Temperature Stress

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High temperature is a serious threat to crop production. Brassinosteroids (BRs), a group of plant steroidal hormones, can reduce effects of abiotic stresses. The present study was aimed to study the potency of brassinosteroids on high temperature induced changes in Indian mustard (*Brassica juncea* L.) for effects on growth, chlorophyll, photosynthesis, photosystem II, antioxidant system and proline. Surface sterilized seeds of Indian mustard were sown in pots, grown for 21 days and treated with double distilled water or 0.01 µM of 28-homobrassinolide. Treated plants, after 24 h, were exposed to 30°C or 40°C for 48 h. One set of plants were kept at ambient temperature, 25°C, as the control. Plants were harvested at 30 days stage of growth to assess the various parameters. Plants exposed to 40°C had a decline in growth, leaf water potential, chlorophyll, photosynthetic rate, and activities of carbonic anhydrase (E.C.4.2.1.1) and nitrate reductase (E.C.1.6.1.1). The 28-homobrassinolide alone improved growth and photosynthesis responses along with various enzymes activities. Treatment of plants with HBL prior to exposure to 40°C, partially reduced damage and completely controlled damage when exposure was to 30°C. Levels of the antioxidative enzymes catalase (E.C.1.11.1.6), peroxidase (E.C.1.11.1.7), and superoxide dismutase (E.C.1.15.1.1), and the level of proline increased in response to 30 or 40°C and were further enhanced in the presence of 28-homobrassinolide. Plants grown under high temperature had increased levels of H_2O_2; application of HBL before temperature treatment decreased H_2O_2 content compared to the control. Elevated levels of antioxidative enzymes and proline might be responsible for conferring tolerance to high temperature stress in Indian mustard and overcome the loss of productivity of the crop.

Key words: Antioxidative enzymes, Brassinosteroids, Brassica juncea, Photosynthesis, Proline

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Key words: Antioxidative enzymes, Brassinosteroids, Brassica juncea, Photosynthesis, Proline

Environmental stresses affect plant growth and productivity. It is believed that two-thirds of yield potential of major crops is lost due to unfavorable environmental conditions. High temperature is a serious threat to crop productivity. Each degree centigrade rise in average temperature may reduce crop yield up to 17% (Lobell and Asner, 2003). High temperature severely damages mesophyll cells, increases permeability of the plasma membrane (Zhang *et al.,* 2005) and results in reduced water

availability (Simoes Araujao *et al.,* 2003). Photosynthesis is completely inhibited by high temperature before other symptoms are detected (Berry and Borjkman, 1980). Injury due to extended periods of high temperature could inactivate enzymes in chloroplast and mitochondria, protein synthesis, protein degradation and loss of membrane integrity (Howarth, 2005). High temperature alters cellular metabolite homeostasis and promotes production of ROS (Mittler, 2002). However, there is a well-developed ROS scavenging system in plants, i.e., superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and guaicol peroxidase (GPOD). High temperature induces increases in activities of SOD, APX, GPOD and levels of hydrogen peroxide and MDA in tomato (*Lycopersicon esculentum* Mill.) (Ogweno *et al.,* 2008).

Brassinosteroids (BRs) are a class of plant steroidal hormones with biological activity that includes stem elongation, pollen tube growth, leaf bending and epinasty, xylem differentiation, and syntheses of nucleic acids and proteins (Clouse and Sasse, 1998; Khripach *et al.,* 2003; Hayat and Ahmad, 2003; Yu *et al.,* 2004). Moreover, BRs play a protective role against abiotic and biotic stress from heavy metal, salinity, drought, chilling and pathogenic infections (Rao *et al.,* 2002; Sasse, 2003; Bajguz and Hayat, 2009). The BR analogue 28-homobrassinolide (HBL) is relatively stable under field conditions (Khripach *et al.,* 2003). However, very little information is available regarding prior application of BRs to confer tolerance on the onset of high temperature stress in plants, particularly in Indian mustard (*Brassica juncea* L.). Indian mustard is important for its content, 30 to 48%, of edible oil. In addition leaves, seed, and the stem are edible. Mustard is cultivated in tropical and temperate zones and requires relatively cool temperatures for satisfactory growth. However, high temperature leads to loss in productivity. The present work was designed to examine the potency of HBL to protect photosynthetic machinery in Indian Mustard (*Brassica juncea*) under high temperature stress by modulating the antioxidant enzymes and osmoprotectant.

MATERIALS AND METHODS

Seeds of *Brassica juncea* were surface sterilized with 0.01% (w/v) $HgCl_2$ solution for 1 min and rinsed with double distilled water (DDW). Surface sterilized seeds were sown in 15.24 cm diameter earthen pots filled with sandy loam soil and cow manure (6:1 v:v) and allowed to grow under ambient environmental conditions i.e. temperature 25±2°C on a 11 h photoperiod, humidity 50±2% and precipitation 31±5 mm in the net house of the Department of Botany, Aligarh Muslim University, Aligarh, India. At 21 days stage of growth, 30 pots were divided into 6 sets and each sets representing one treatment consist of 5 pots each (replicates). Set 1; treated with DDW and grown at 25°C (control); Set 2; treated three times with about 1 mL of 0.01 µM of HBL at 25°C; concentration selected on the basis of an earlier study (Fariduddin *et al.,* 2004); Set 3; treated with DDW and exposed to 30°C for 48 h at 22 days stage of growth; Set 4; treated with DDW and exposed to 40°C for 48 h at 22 days stage of growth; Set 5; treated three times with about 1 mL of 0.01 µM of HBL at 21 days stage of growth and exposed to 30°C for 48 h at 22 days stage of growth; and Set 6; treated three times with about 1 mL of 0.01 µM of HBL at 21 days stage of growth and exposed to 40°C for 48 h at 22 days stage of growth. However, the seedlings were exposed to higher temperature (30/40°C) by placing them in growth chamber (MAC Plant Growth

Chamber, New Delhi, India). At 30 days stage of growth plants from each set were harvested to assess growth, photosynthetic and biochemical parameters. The experiment was completely randomized design.

Plants were removed from pots along with the soil and dipped in a bucket filled with water to remove adhering soil particles. Lengths of roots and shoots were measured. Plants were weighed to determine their fresh mass and then placed in an oven run at 60°C for 72 h to asses the dry mass of the same seedlings.

Leaf water potential in fresh leaf samples was measured with an water potential system (PSYPRO, WESCOR Inc., Logan, USA).

Chlorophyll in leaves was measured with a chlorophyll meter (model 502, SPAD, Konica Minolta Sensing, Osaka, Japan).

Net photosynthesis rate (A), stomatal conductance (gs), water use efficiency (WUE), internal CO_2 concentration (Ci), and transpiration rate (E) were determined on the third fully expanded leaves between 1100 and 1200 hrs using a portable infra-red gas analyzer portable photosynthetic system (model 6400, LI-COR, Lincoln, NE, USA).

Chlorophyll fluorescence as maximum quantum yield of Photosystem II (Fv/Fm) was monitored with a Leaf Chamber Fluorometer (model 6400-40, LI-COR). All measurements were at a photon flux density (PFD) of 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a constant air flow rate 500 $\mu mol \cdot s^{-1}$. The minimal fluorescence level (Fo) was determined by modulated light, which was sufficiently low (<1 $\mu mol \cdot m^{-2} \cdot s^{-1}$) not to induce significant variable fluorescence. The maximum fluorescence (Fm) was determined by a 0.8 s saturation pulse at 4,200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on leaves that had been dark adapted for 30 min.

Activity of nitrate reductase (NR) was measured following the method of Jaworski (1971). Fresh leaf samples were cut into small pieces and transferred to plastic vials containing phosphate buffer (pH 7.5) followed by addition of potassium nitrate and isopropanol solutions. The reaction mixture was incubated at 30°C, for 2 h followed by addition of N-1-naphthyletylenediamine dihydrochloride and sulphanilamide. Color absorbance was read at 540 nm and compared with the calibration curve. Activity of NR was computed on a fresh weight basis. Activity of carbonic anhydrase (CA) was determined following the procedure of Dwivedi and Randhawa (1974). Leaf samples were cut into small pieces and suspended in cystein hydrochloride solution. Samples were incubated at 4°C for 20 min. The pieces were blotted and transferred to test tubes containing phosphate buffer (pH 6.8) followed by addition of alkaline bicarbonate solution and bromothymol blue indicator. Test tubes were incubated at 5°C for 20 min. The reaction mixture was titrated against 0.05N HCl after addition of 0.2 mL of methyl red indicator.

For assaying activity of peroxidase (POX), catalase (CAT), and superoxide dismutase (SOD), 0.5 g of leaf tissue was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% soluble polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 g for 10 min at 4°C and the supernatant obtained used as crude enzyme extract.

The CAT activity was assayed following the method of Chance and Maehly (1956). The reaction mixture for CAT consisted of phosphate buffer (pH 6.8), 0.1 M H_2O_2 and 1.0 mL enzyme extract as a substrate. To stop the reaction H_2SO_4 was added after incubation for 1 min at 25°C. Disappearance of

H_2O_2 was detected by titrating the reaction mixture against a potassium permanganate solution. The reaction mixture without enzyme was the blank.

For estimation of POX activity (Chance and Maehly, 1956), the enzyme extract (0.1 mL) was added to the reaction mixture consisting of pyrogallol phosphate buffer (pH 6.8) and 1% H_2O_2. Due to formation of tetraguaiacol, the change in absorbance was read for 2 min, at an interval of 20 s, at 420 nm on a spectrophotometer. The control was prepared by adding double distilled water instead of enzyme extract.

The activity of superoxide dismutase was assayed by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The reaction mixture containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 74 mM NBT, 2.0 mM riboflavin, 0.1 mM EDTA and 0.5 mL enzyme extract and was placed under a 15W fluorescent lamp. The reaction was started by switching on the light and was allowed to run for 10 min. The reaction was stopped by switching off the light. Inhibition by 50% due to light was considered as one enzyme unit.

Proline content in fresh leaf samples was determined with the method of Bates et al. (1973). Samples were extracted in sulphosalicylic acid. To the extract equal volumes of glacial acetic acid and ninhydrin solutions were added. Samples were heated at 100°C, to which 5 mL of toluene was added. The absorbance of toluene layer was read at 528 nm on a spectrophotometer.

The hydrogen peroxide content was determined by Jana and Choudhari (1981). The H_2O_2 was extracted by homogenizing a 500 mg plant sample in 3.0 mL of phosphate buffer (50 mM, pH 6.8). The homogenate was centrifuged at 6000 g for 25 min. Three-mL of extract was mixed with 0.1% titanium chloride in 20% (v/v) sulphuric acid and the mixture again centrifuged at 6000 g for 15 min. Absorbance of the color was read at 410 nm, on a spectrophotometer and compared with the calibration curve. The H_2O_2 content was computed on a fresh weight basis.

Data were analyzed using SPSS for windows (ver. 17, SPSS, Chicago, IL). Standard error was calculated and analysis of variance performed. Least significance difference was used to separate treatment means.

RESULTS

Treatment affected all growth parameters (Fig. 1a, b). The HBL alone increased plant shoot and root lengths, and fresh and dry mass. Plants exposed to 40°C had reductions in all growth parameters. However, application of HBL alone before exposing plants to 30°C produced higher values compared with the control, but less than when treated with HBL alone.

Application of HBL alone increased leaf water potential over controls. However, exposure to 30°C or 40°C lowered leaf water potential compared to controls. The combination of HBL and 30°C increased leaf water potential over the control and that with 30°C (Fig. 1d).

Plants exposed to 30°C or 40°C had reduced SPAD values of chlorophyll, net photosynthetic rate (A) and related attributes along with maximum quantum yield of PSII (Fig. 1c, 2a-e, 3e). Moreover, 40°C was more deleterious than 30°C and reduced SPAD values for chlorophyll, A, gs, Ci, WUE, transpiration rate (E) and FV/Fm by 32.52, 45.05, 42.12, 38.88, 6.70, 41.07 and 39.88% over controls (Fig. 1c, 2a-e). Application of HBL alone improved values of all parameters over controls, and prior treatment of plants also reduced impact of high

temperatures which was more prominent in plants exposed to 30°C where values exceeded controls.

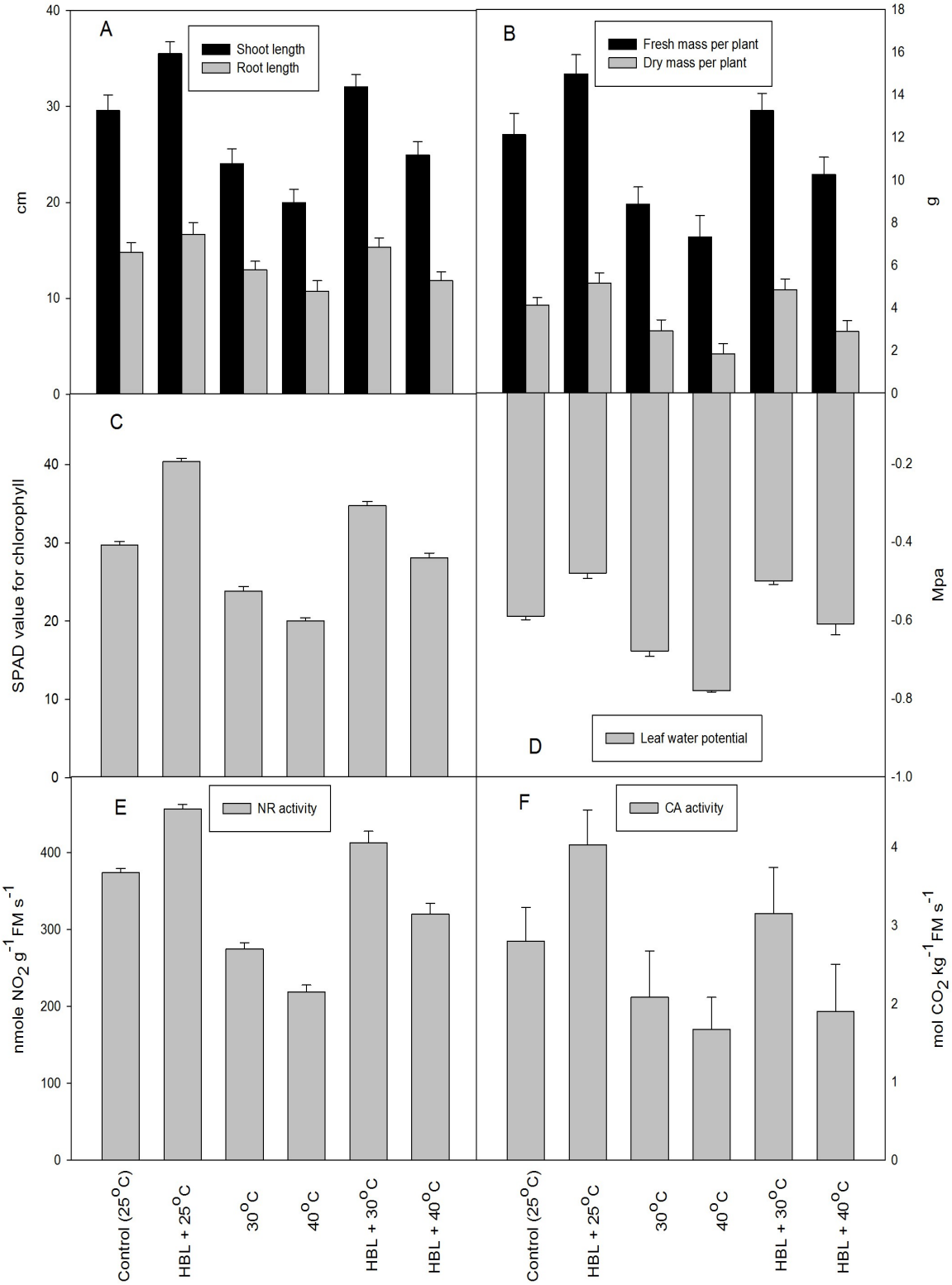

Figure 1 : Effect of 28-homobrassinolide [HBL; 0.01 µM] on (a) shoot and root lengths, (b) fresh and dry mass per plant, (c) SPAD value of chlorophyll, (d) leaf water potential, (e) NR activity and (f) CA activity in *Brassica juncea* exposed to high temperature stress (30°C or 40°C) at 30 days after sowing.

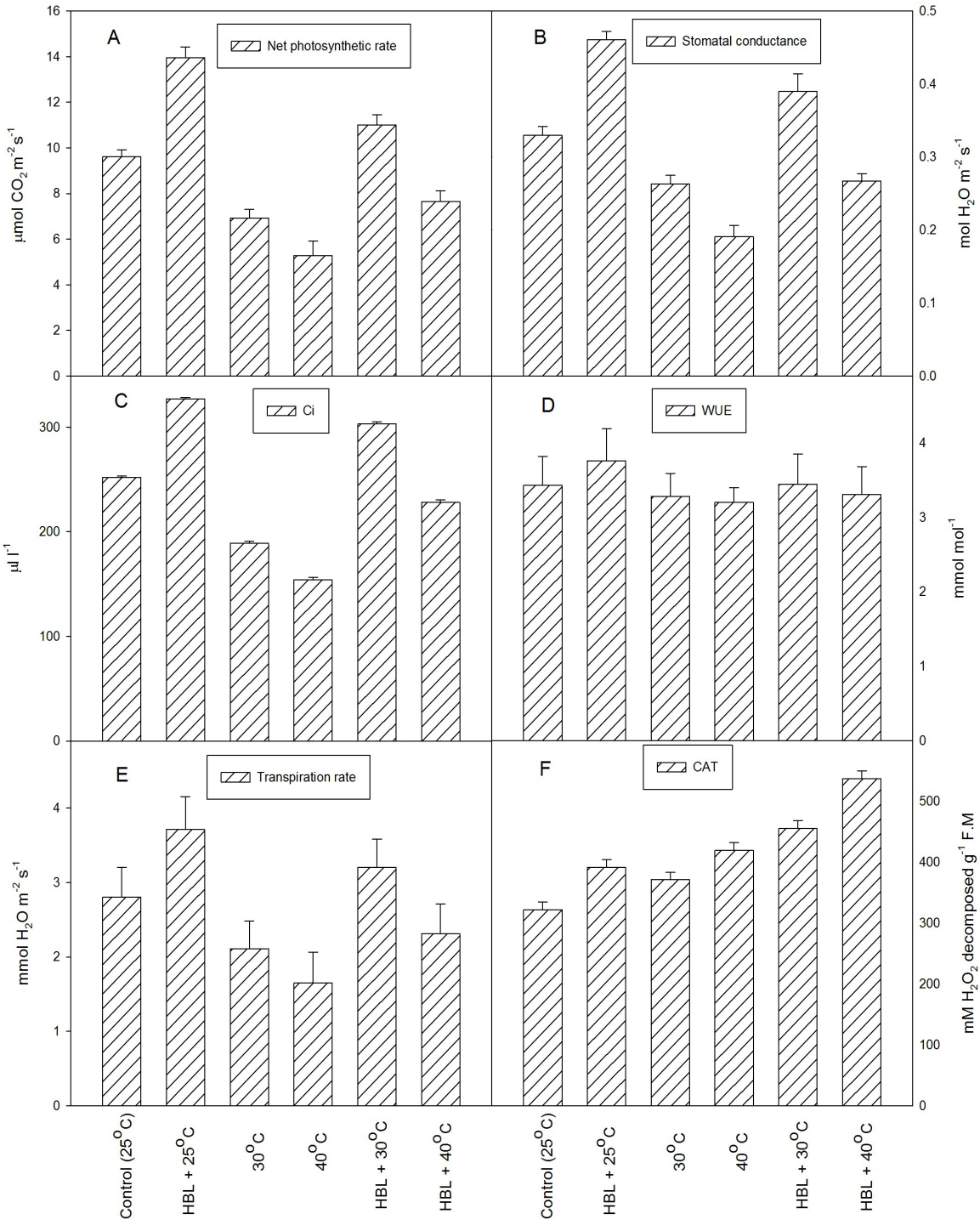

Figure 2 : Effect of 28-homobrassinolide [HBL; 0.01 µM] on (a) net photosynthetic rate, (b) stomatal conductance, (c) internal CO_2 concentration, (d) water use efficiency, (e) transpiration rate and (f) catalase (CAT) activity in *Brassica juncea* exposed to high temperature stress (30° C or 40° C) at 30 days after sowing.

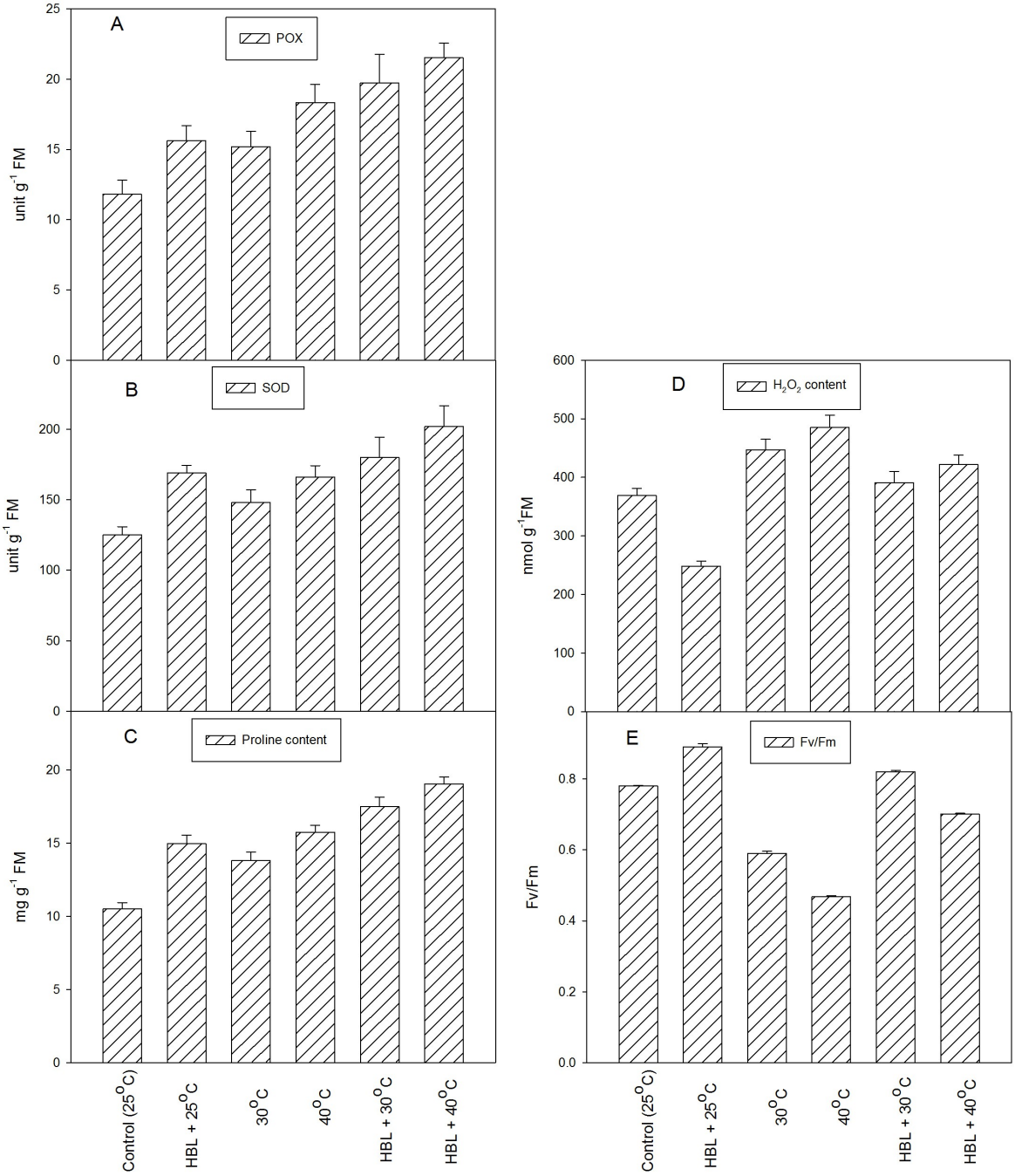

Figure 3 : Effect of 28-homobrassinolide [HBL; 0.01 µM] on (a) peroxidase activity, (b) superoxide dismutase activity, (c) proline content (d) H_2O_2 content and (e) maximum quantum yield of PSII in *Brassica juncea* exposed to high temperature stress (30° C or 40° C) at 30 days after sowing.

Activity of NR and CA were improved by application of HBL alone over controls (Fig. 1e, f). Exposure to 40°C was more detrimental than 30°C with values decreased by 41.44 and 40.35% below controls, respectively. The effect generated by 40°C was partly neutralized by prior application of HBL.

Plants exposed to 30 or 40°C increased the activity of antioxidative enzymes i.e. CAT, POX, and SOD. In addition to this, application of HBL prior to the onset of 30 or 40°C further stimulated the activity of antioxidative enzymes and the maximum values for CAT, POX and SOD were noted in plants

exposed to the combination of HBL and 40°C that were respectively 66.77, 82.45, and 61.60% greater than their controls. Proline, an osmoprotectant, increased with increase of temperature. The maximum proline level was in plants exposed to both HBL and 40°C in combination.

The H_2O_2 content was lower than the control, in plants exposed to HBL (Fig. 3d). However, 30 and 40°C increased H_2O_2 contents in plants, but effect of temperature was reduced by treating plants with HBL before exposure to temperatures.

DISCUSSION

High temperature reduced plant growth but these effects were partly overcome by HBL. This might have been induced by involvement of specific signals generated by BRs to enhance rate of cell expansion (Clouse and Sasse, 1998) and/or cell division (Nakaya *et al.,* 2000). Similar growth promoting effects of BRs have been reported in other plants (Sasse, 2003). The slower growth rate was associated with loss of leaf water potential under high temperature which agrees with Wahid and Clause (2007) in sugarcane (*Saccharum officinarum* L.), tomato (*Lycopersicon esculentum* Mill) (Morales *et al.,* 2003) and drought stressed *Lotus creticus* L. (Anon *et al.,* 2004).

Photosynthesis and its attributes, along with maximum quantum yield of PSII (Fv/Fm), are important markers to assess the impact of high temperature (Wahid *et al.,* 2007). Photochemical reactions in thylakoid lamellae and carbon metabolism in stroma of chloroplasts are primary sites of heat injury (Wise *et al.,* 2004). High temperature reduced maximum quantum yield of photosystem II (Fv/Fm) which was presumed to be due to perturbations of photochemical reactions in thylakoid lamellae. Temperature alters energy distribution and effects activities of enzymes of carbon metabolism, particularly Rubisco. This occurs by altering the rate of RUBP regeneration by disruption of electron transport and inactivation of oxygen evolving enzymes of PS II (Salvucci and Crafts-Brandner, 2004). These changes in plants led to reduced net photosynthesis, but treatment with HBL prior to exposure to high temperature increased photosynthesis rate (Morales *et al.,* 2003). The slower rate of photosynthesis in heat treated plants is likely due to low internal carbon dioxide concentration due to impact on stomatal conductance and leaf chlorophyll content (Karim *et al.,* 1997; Cicek and Cakirlar, 2008). Temperature stress generates ROS that have an adverse impact on the photosynthetic processes (Camejo *et al.,* 2006; Guo *et al.,* 2006). The decrease in internal carbon dioxide level may be the expression of the loss in the activity of Rubisco (Morales *et al.,* 2003; Salvucci and Crafts-Brander, 2004). Since HBL treatment prior to temperature treatment significantly improved values for all parameters, the rate of photosynthesis was also improved. Activity of HBL probably involves expression of specific genes responsible for synthesis of enzymes of chlorophyll synthesis and others involved in photosynthesis (Yu *et al.,* 2004).

A decline in CA activity could have been due to high temperature induced inactivation of Rubisco (Morales *et al.,* 2003; Salvucci and Crafts-Brander, 2004). The decrease in NR activity could be due to enzyme inhibition and/or metabolism dysfunction (Hopkins, 1995), as well as biochemical adaptation to conserve energy by stopping nitrate assimilation at the initial stage (Tikhomirova, 1985). The ability of the seedlings to generate higher NR and CA activities was attained by treatment with HBL. The destructive action of high temperature was

overcome to a greater extent by prior application of plants to HBL. A higher substrate NO_3 level in presence of BRs could be the reason for increased NR activity (Mai *et al.,* 1989; Solomanson and Barber, 1990). Hormones are involved in transcription and/or translation of enzyme proteins (Kalinich *et al*., 1985; Bujguz, 2000; Fariduddin *et al*., 2004; Hayat *et al*., 2007; Hasan *et al*., 2008; Fariduddin, 2009).

Plants have an ability to protect themselves from adverse conditions and in doing accumulate osmolytes including proline (Sairam and Tyagi, 2004). Proline synthesis buffers cellular redox potential under high temperature (Wahid and Clouse, 2007). Application of HBL further enhances proline content, making plants more resistant to high temperature (Ali *et al.,* 2007; Hayat *et al.,* 2007; Hasan *et al.,* 2008; Fariduddin *et al.,* 2009).

In addition to causing tissue dehydration, high temperature induces oxidative stress (Liu and Huang, 2000) which could oxidize proteins, lipids and nucleic acids leading to mutation at the cellular level (Halliwell and Gutteridge, 1985; Sairam and Tyagi, 2004). To protect themselves from oxidative stress plants have endogenous enzymes, CAT, POX, SOD, and glutathione reductase. High temperature stress increased activity of these enzymes to boost resistance of plants (Rivero *et al.,* 2004). The application of HBL alone, or in association with high temperature, increases activities of peroxidase, catalase and superoxide dismutase (Mazzora *et al.,* 2002; Nunez *et al.,* 2003; Cao *et al.,* 2005). The impression is that BRs generate a system (transcription and/or translation) that improves the degree of tolerance in plants to overcome abnormal metabolism. It has been shown that the *det2* Arabidopsis mutant, which is blocked in the biosynthetic pathway of BRs (Cao *et al*., 2005) exhibited abnormal growth features that could be significantly reverted by the exogenous application of 24-epibrassinolide. These plants were also resistant to oxidative stress. The enhanced resistance to oxidative stress was correlated with a constitutive increase in SOD activity and increased transcript levels of the defense gene CAT (Cao *et al.,* 2005). A possible explanation for the fact that *det2* mutant exhibited an enhanced oxidative stress resistance is that the long-term BR deficiency in the *det2* mutant results in a constant *in vivo* physiological stress that, in turn, activates the constitutive expression of some defense genes and activities of related enzymes. It has been demonstrated that ATPA2 and ATP24a genes coding peroxidases were constitutively upregulated in the *det2* Arabidopsis mutant (Goda *et al.,* 2002). Bajguz and Hayat (2009) and Fariduddin *et al*., (2014) reported activation of antioxidative enzymes and proline by brassinosteroids. Drought (Li *et al*., 2012) and cadmium (Hayat *et al*. 2007) and aluminium (Ali *et al*. 2008) also have been reported

This study concluded that the exposure of plant to high temperature restrict plant growth and lowered the photosynthetic efficiency. However, plant gained more tolerance to high temperature stress by modulating antioxidant system if they were given prior treatment of HBL before onset of stress and also protect the photosynthetic machinery.

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