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28-homobrassinolide improves growth and photosynthesis in *Cucumis sativus* L. through an enhanced antioxidant system in the presence of chilling stress

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Abstract

The ameliorative role of 28-homobrassinolide under chilling stress in various growth, photosynthesis, enzymes and biochemical parameters of cucumber (*Cucumis sativus* L.) were investigated. Cucumber seedlings were sprayed with 0 (control), 10^{-8}, or 10^{-6} M of 28-homobrassinolide at the 30-day stage. 48 h after treatment plants were exposed for 18 h to chilling temperature (10/8°C, 5/3°C). The most evident effect of chilling stress was the marked reduction in plant growth, chlorophyll (Chl) content, and net photosynthetic rate, efficiency of photosystem II and activities of nitrate reductase and carbonic anhydrase. Moreover, the activities of antioxidant enzymes; catalase (E.C. 1.11.1.6), peroxidase (E.C.1.11.1.7), superoxide dismutase (E.C. 1.15.1.1) along with the proline content in leaves of the cucumber seedlings increased in proportion to chilling temperature. The stressed seedlings of cucumber pretreated with 28-homobrassinolide maintained a higher value of antioxidant enzymes and proline content over the control suggesting the protective mechanism against the ill-effect caused by chilling stress might be operative through an improved antioxidant system. Furthermore, the protective role of 28-homobrassinolide was reflected in improved growth, water relations, photosynthesis and maximum quantum yield of photosystem II both in the presence and absence of chilling stress.

Additional key words: antioxidant enzymes; brassinosteroids; chilling stress; chlorophyll fluorescence; *Cucumis sativus*; photosynthesis.

Introduction

Low temperature (chilling and frost stress) is a major limiting factor for the productivity of many thermophilic plants such as cucumber and tomato. Plant indigenous to tropical and subtropical climates experience a physiological dysfunction called chilling injury when exposed to nonfreezing temperatures below ~ 12°C for periods in excess of a critical period of time (Saltveit and Morris 1990, Saltveit 2001). However, symptoms of chilling stress/injury include reduced growth and photosynthetic capacity, necrosis and discoloration and abnormal ripening and increased susceptibility to disease. Chilling stress has a direct impact on the photosynthetic apparatus, essentially by disrupting all major components of photosynthesis, including thylakoid electron transport, the carbon reduction cycle and the stomatal control of CO_2 supply, together with an increased accumulation of carbohydrates, peroxidative destruction of lipids and disturbance of the water balance (Allen and Ort 2001). The development of chilling injury symptoms may, in part, be due to the production of activated oxygen species (AOS) (superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radical) causing damage to proteins, nucleic acids and cell membranes (Wise and Naylor 1987). Although the generation of AOS is a common event during growth and developmental processes, it increases substantially under abiotic stress, such as chilling, heat, drought, pollutants, and UV radiation. The development of the symptoms of chilling

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Abbreviations: AOS – active oxygen species; BRs – brassinosteroids; C_i – internal carbon dioxide concentration; CA – carbonic anhydrase; CAT – catalase; CO_2 – carbon dioxide; CS1 – chilling stress 1; CS2 – chilling stress 2; DAS – days after sowing; DDW – double distilled water; DM – dry mass; E – transpiration rate; EBR – epi-brassinolide; FM – fresh mass; g_s – stomatal conductance; HBL – 28-homobrassinolide; LSD – least significant difference; NR – nitrate reductase; P_N – net photosynthetic rate; POX – peroxidase; PPFD – photosynthetic photon flux density; PSII – photosystem II; RWC – relative water content; SOD – superoxide dismutase; TM – turgor mass; UV – ultraviolet; WUE – water-use efficiency; Ψ_w – leaf water potential.
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injury is frequently coincident with peroxidation of fatty acids. The synthesis of lipids, antioxidants (catalase, peroxidase, superoxide dismutase and glutathione reductase) and nonenzymatic low molecular mass antioxidants (proline, carotenoids, flavonoids) as well as other phenolic compounds are part of a complex mechanism of chilling tolerance that involves both restricting the production of AOS and protection from the AOS produced (Walker and McKersie 1993). Therefore, it is clear that the capacity of plants to enhance free radical scavenging could be improved by increasing the endogenous level of both enzymatic and nonenzymatic antioxidant systems.

Brassinosteroids (BRs) are a new group of steroidal plant hormones which occur ubiquitously in the plant kingdom. They have diverse physiological roles on various plant activities in plants such as cell division, cell elongation, vascular differentiation, pollen tube growth, ethylene production, senescence, enzyme activation, gene regulation, protein and nucleic acid biosynthesis, photosynthesis (Clouse and Sasse 1998, Sasse 2003). Moreover, Yu *et al*. (2004) reported that one of the analogues of BRs, epibrassinolide, increased the activity of Rubisco, the maximum quantum yield of photosystem II, and photosynthetic rate in *C. sativus*. In addition to this, BRs have also been reported to play a vital role in plants to counter various biotic and abiotic stresses in diverse plant species. They have the ability to protect plants against moisture/water stress (Sairam 1994, Fariduddin *et al*. 2009b), NaCl stress (Ozdemir *et al*. 2004, Ali *et al*. 2007), heavy metal stresses such as cadmium (Hasan *et al*. 2008), nickel (Alam *et al*. 2007), and copper (Fariduddin *et al*. 2009a), also cold stress (Kagale *et al*. 2007), high- and low-temperature stress (Wilen *et al*. 1995) and various biotic stresses (Clouse and Sasse 1998). However, the ameliorative role of BRs in relation to chilling stress is yet to be established in detail so that the present study was hypothesized that 28-homobrassinolide will equip the *C. sativus* L. plants against the chilling stress through improved photosynthetic activities and changes in the antioxidant system.

Materials and methods

28-homobrassinolide (HBL) was obtained from *Sigma-Aldrich Chemicals Pvt. Ltd*, India. The stock solution (10^{-4} M) of HBL was prepared by dissolving required quantity of HBL in 5 ml of ethanol, in a 100-ml volumetric flask. The desired concentrations (10^{-8} M and 10^{-6} M) of HBL were prepared by the dilution of stock solution. 0.05 % of surfactant Tween-20 was added to the HBL solutions before application.

Seeds of cucumber (*Cucumis sativus* L. cv. summer best were purchased from *National Seed Corporation Ltd*., Pusa, New Delhi, India. Healthy seeds of uniform size were surface-sterilized with 0.05% (w/v) sodium hypochlorite solution followed by repeated washings with deionised water. The surface-sterilized seeds were sown in acid-washed sand, moistened with deionised water in plastic pots (25 cm^3). These seeds were allowed to germinate and the resulting seedlings were grown in a climate chamber (14 h light, 200 μmol PAR m^{-2} s^{-1}, 25°C, 60% relative air humidity) for 30 days. The pots were supplied with full nutrient solution (Hewitt 1966) from 7 d after sowing (DAS) onwards. To maintain the sufficiency of nutrients, the supply of nutrient solution was maintained throughout the duration of the experiment. At the 30-day stage plants were sprayed with 0 (control), 10^{-8} or 10^{-6} M of 28-homobrassinolide (HBL). Each seedling was sprinkled three times. The nozzle of sprayer was adjusted in such a way that it pumped out 1 ml of solution in one sprinkle. Therefore, each plant received 3 ml of deionised water or HBL solution. Forty eight hours after treatment plants were exposed to chilling stress 1 (CS1), *i.e*. 10/8°C or chilling stress 2 (CS2), *i.e*. 5/3°C for 18 h. The other conditions were similar to those before chilling stress. After chilling stress, the plants were allowed to grow under normal conditions for 10 days. Each treatment involved 5 pots (replicate) with three plants per pot which were maintained under completely randomized block design. The plants were harvested 10 d after hormone treatment to assess the growth characteristics, photosynthesis, and biochemical attributes.

One plant from each pot was taken along with sand, dipped in bucket, and filled with water. Then plants were moved smoothly to remove the adhering sand particles and the length of shoots and roots was measured by using a cm scale. The plants were then blotted and weighed to record the fresh mass and then placed in an oven, run at 60°C for 72 h. The samples were weighed again after allowing them to cool at room temperature, to record their dry mass. The leaf area was measured manually by using a graph sheet, where the squares covered by the leaf were counted to note the leaf area.

The relative water content (RWC) was determined in fresh leaf discs of 2-cm diameter, excluding midrib. Discs were weighed quickly and immediately floated on double distilled water (DDW) in Petri dishes to saturate them with water for the next 24 h, in the dark. The adhering water of the discs was blotted and the turgor mass was noted. Dry mass of the discs was taken after dehydrating them at 60°C for 72 h. RWC was calculated by using the values in the following formula:

$$\text{RWC} = \frac{\text{FM} - \text{DM}}{\text{TM} - \text{DM}} \times 100$$

The leaf water potential (Ψ_w) was measured by the use of *Psypro Water Potential System* (*Wescor Inc*., Logan, UT, USA).

The SPAD Chl value in fresh leaf was measured by using a SPAD Chl meter (*SPAD-502*, *Konica, Minolta Sensing, Inc*., Japan). Maximum quantum yield of photosystem II (F_v/F_m) was measured by using a leaf chamber fluorometer (*LI-COR 6400-40*, *LI-COR*, Lincoln, NE, USA). All the measurements carried out at a photosynthetic photon flux density (PPFD) of 1,500 μmol m^{-2} s^{-1} with a constant airflow rate of 500 μmol s^{-1}. The minimal fluorescence level (F_o) was determined by modulated light, which was sufficiently low (< 1 μmol m^{-2} s^{-1}) not to induce any significant variable fluorescence. The maximal fluorescence (F_m) was determined by a 0.8-s saturation pulse at 4,200 μmol m^{-2} s^{-1} on dark-adapted leaves (30 min). The sampled leaf was dark-adapted for 30 min prior to measurement of F_v/F_m.

Gas-exchange parameters were determined on the third fully expanded leaves between 11:00–12:00 h by using an infrared gas analyzer (IRGA) portable photosynthetic system (*LI-COR 6400*, *LI-COR*, Lincoln, NE, USA). To measure net photosynthetic rate (P_N) and its related parameters [stomatal conductance (g_s), transpiration rate (E), water-use efficiency (WUE), *i.e*. P_N/E, and internal CO_2 concentration (C_i)], the air temperature, relative humidity, CO_2 concentration and PPFD were maintained at 25°C, 85%, 600 μmol mol^{-1} and 800 μmol mol^{-2} s^{-1}, respectively.

The activity of nitrate reductase (NR) and carbonic anhydrase (CA) was measured following the method described by Jaworski (1971) and Dwivedi and Randhawa (1974), respectively. The activity of NR [nmol (NO_2) g^{-1}(FM) s^{-1}] was computed on a fresh mass (FM) basis and the results of CA activity were expressed as mol(CO_2) kg^{-1}(leaf FM).

The proline content in fresh leaves was determined by adopting the method of Bates *et al*. (1973). Proline was extracted in sulphosalicylic acid. To the extract, an equal volume of glacial acetic acid and ninhydrin solutions were added. The sample was heated at 100°C, to which 5 ml of toluene was added after cooling. The absorbance of the toluene layer was read at 528 nm, on a spectrophotometer.

For the estimation of peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD), leaf tissue (0.5 g) was homogenized in 5 ml of 50 mM phosphate buffer (pH 7.0) containing 1% insoluble polyvinylpyrrolidone. The homogenate was centrifuged at 27,600 × *g* for 10 min and the supernatant was used as the source of enzymes for estimation. The extraction was carried out at 4°C.

For the 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. The change in the absorbance was read at every 20 s for 2 min at 420 nm on a spectrophotometer. A control set was prepared by adding DDW instead of enzyme extract.

The reaction mixture for catalase consisted of phosphate buffer (pH 6.8), 0.1 M H_2O_2 and enzyme extract (1.0 ml). H_2SO_4 was added to the reaction mixture, after incubating it for 1 min at 25°C, and was titrated against potassium permanganate solution (Chance and Maehly 1956).

The activity of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium using the method of Beauchamp and Fridovich (1971). The reaction mixture containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium, 2 mM riboflavin, 0.1 mM EDTA and 0–50 μl enzyme extract and was placed under 15-W fluorescent lamp. The reaction was started by switching on the light and was allowed to run for 10 min. Fifty percent inhibition by light was considered as one enzyme unit.

The experiment was conducted according to completely randomized block design. A total of 5 replicates for each treatment were taken. Data were statistically analyzed using *SPSS 17.0 for Windows* (*SPSS*, Chicago, IL, USA). Standard error was calculated and analysis of variance (*ANOVA*) was performed on the data to determine the least significance difference (LSD) between treatment means with the level of significance at $P<0.05$.

Results

Growth characteristics: All the growth traits (shoot and root length, fresh and dry mass of whole plant and leaf area) showed significant reduction in proportion to chilling stresses (Fig. 1*A-C*). CS2 (5/3°C) proved to be more injurious to plants and decreased the above said parameters by 30%, 23%, 28%, and 30%, over their respective controls. However, the plants treated with HBL (10^{-8} M) showed a remarked increase in the growth traits. Moreover, the deleterious effect caused by CS1 is more than overcome by the lower concentration of HBL and substantially reversed by the higher one.

RWC and Ψ_w: At the 40-day stage, chilling stresses (CS1 and CS2) significantly reduced both the Ψ_w and the leaf RWC but CS2 proved to be more deleterious as compared to CS1. In the absence of chilling stresses, the pretreatment of HBL (10^{-8} M and 10^{-6} M) was found to improve the level of both RWC as well as Ψ_w as compared to control. The lower concentration of HBL (10^{-8} M) proved to be best in the presence or absence of chilling stress (CS1 and CS2) (Fig. 1*D,E*).

Activities of nitrate reductase (NR) and carbonic anhydrase (CA): Chilling stress (CS2) significantly reduced the activities of NR and CA over the nonstressed plants (control) by 27% and 24%, respectively (Figs. 1*F,* 2*A*).

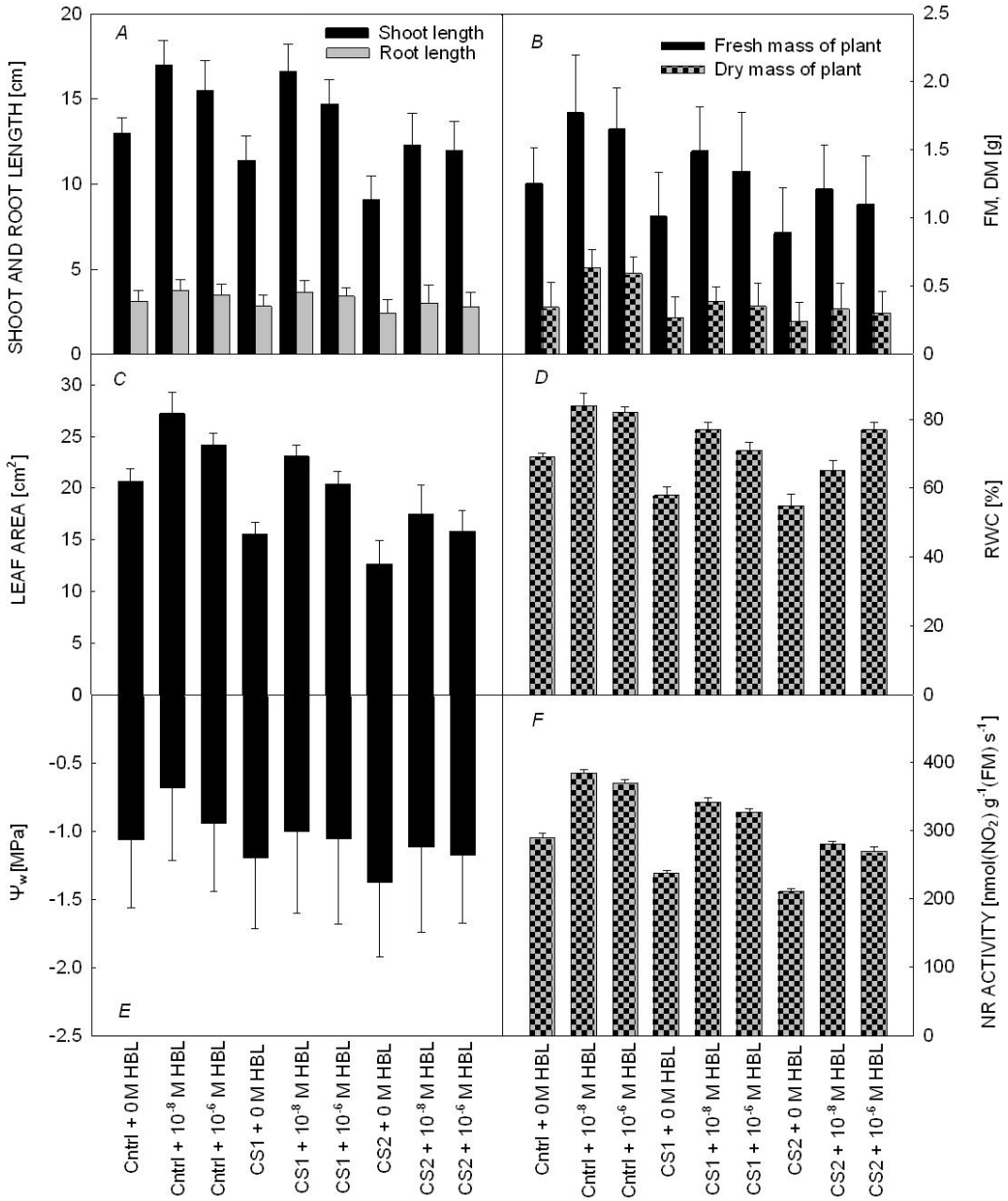

Fig. 1. Effect of 28-homobrassinolide (HBL) (10^{-8} and 10^{-6} M) on (*A*) shoot and root length, (*B*) fresh (FM) and dry mass (DM) (*C*) leaf area, (*D*) leaf relative water content (RWC), (*E*) leaf water potential (Ψ_w), and (*F*) nitrate reductase (NR) activity in *Cucumis sativus* L. exposed to chilling stress [10/8°C (CS1) or 5/3°C (CS2)] at 40 DAS; *vertical bars* show standard errors; all the data are means of five replicates. Cntrl – control.

Furthermore, this loss in the activities of both NR and CA were partially neutralized by pretreatment of HBL (10^{-8} M).

SPAD Chl and Chl fluorescence: The chilling stress (CS2) significantly lowered the Chl content (SPAD level) (Fig. 2*B*) and the maximum quantum yield of PSII (F_v/F_m) (Fig. 2*C*). However, the plant exposed to HBL (10^{-8} M) alone possessed more values in comparison to water sprayed plant (control). Moreover, the damaging effect generated by CS2 was partially neutralized by 10^{-8} M of HBL (Fig. 2*B,C*).

Photosynthesis and related attributes: The plants pretreated with HBL (10^{-8} M) significantly increased the values against untreated plants (control) for P_N, g_s, C_i, WUE and *E* (Fig. 2*D–F,* 3*A–B*). However, the chilling stress (CS2) decreased all the above parameters by 37%, 28%, 33%, 9% and 22% over their respective control. Furthermore, stressed seedlings (CS2) pretreated with

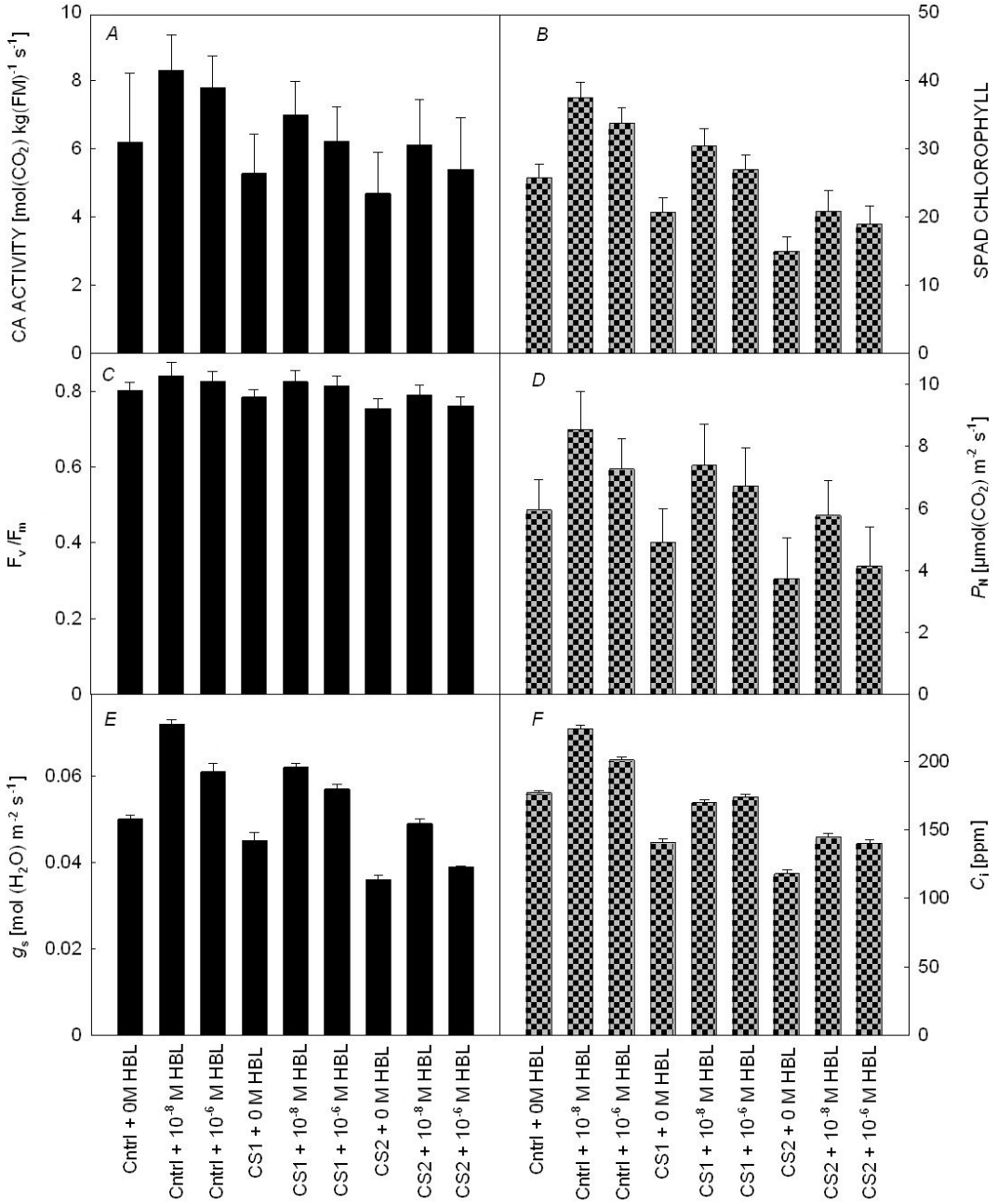

Fig. 2. Effect of 28-homobrassinolide (HBL) (10^{-8} and 10^{-6} M) on (*A*) carbonic anhydrase (CA), (*B*) SPAD chlorophyll, (*C*) maxium quantum yield of PSII (F_v/F_m), (*D*) net photosynthetic rate (P_N), (*E*) stomatal conductance (g_s), and (*F*) internal carbon dioxide concentration (C_i) in *Cucumis sativus* L. exposed to chilling stress [10/8°C (CS1) or 5/3°C (CS2)] at 40 DAS; *vertical bars* show standard errors; all the data are means of five replicates. Cntrl – control.

HBL (10^{-8} M) had higher values as compared to stressed plants (CS2) alone.

Activity of antioxidant enzymes: The activities of antioxidant enzymes (CAT, POX, and SOD) exhibited a significant increase in response to chilling stresses and/or pretreatment with HBL (Fig. 3*C–E*). Interestingly, the plants exposed to CS2 (5/3°C) in association with either of the pretreatment of HBL (10^{-8} and 10^{-6} M) generated the most favorable response. The combination of pretreatment of HBL (10^{-8} M) and chilling stress (CS2), *i.e.* (5/3°C) was most effective and increased the activity of CAT, POX, and SOD by 32%, 150%, and 80%, respectively, over their controls.

Proline content increased in the plants subjected to chilling stresses and/or HBL (Fig. 3*F*). In addition, the maximum content of proline was recorded in plants having pretreatment of HBL (10^{-8}M) as well as chilling stress (CS2).

Discussion

The present study revealed that P_N and their related parameters (g_s, C_i, WUE, and E) as well as Chl content, CA and NR activity decreased in proportion to chilling stress *i.e.* CS1 and CS2 (Fig. 3*A,B*). Photosynthesis is the first most vulnerable physiological process inhibited by chilling stress (Berry and Björkman 1980). Chilling directly affect the functioning of photosynthetic apparatus, essentially by disrupting all major components of photosynthesis, including the thylakoid electron transport, the carbon reduction cycle and the stomatal control of CO_2 supply (Allen and Ort 2001).

The Chl level (SPAD value) and maximum quantum yield of PSII (F_v/F_m) decreased with the increasing level of chilling stress (Figs. 1*F,* 2*B*). Chilling stress has been shown to induce changes in the ultrastructure of chloroplast (Kratsch and Wise 2000) and the chloroplast may lose its capacity to capture light energy after a long chilling period (Yang *et al*. 2005). Many reports have shown that the photosynthetic efficiency of chilling-sensitive plants is greatly reduced by cold treatment (Wang 1982, Aro *et al*. 1993, Sonoike 1999, Yang *et al*. 2005). In chilled plants, light absorbed by PSII is in excess of that can be utilized by the Calvin cycle, therefore heat is dissipated within the PSII antennae, decreasing the F_v/F_m ratio (Björkman and Demmig 1987). Furthermore, pretreatment with HBL enhances the Chl content and the F_v/F_m ratio in stressed plants as well as in the nonstressed ones (Figs. 1*F,* 2*B*). This might be the possible reason for the improved photosynthetic parameters. Yu *et al*. (2002, 2004) found that treatment of *C. sativus* by epibrassinolide improved the photosynthetic capacity through increased PSII quantum yield and activity of Rubisco (Fig. 2*B*). Moreover, application of HBL also improved net photosynthetic rate in *Vigna radiata* (Fariduddin *et al.* 2004) and also under various stresses *viz*. moisture stress in wheat (Sairam 1994), thermotolerance of brome grass (Wilen *et al*. 1995), cold treated rape leaves (Janeczko *et al*. 2007), and various heavy-metal stresses (Ali *et al*. 2008, Hasan *et al*. 2008, Fariduddin *et al*. 2009a). In the present study, the activities of the CA and NR were decreased under chilling stress (Fig. 3*A, B*). Zhou *et al*. (2007) reported that chilling stress of 14°C and 7°C significantly decreased the maximum velocity of RuBP carboxylation by Rubisco (V_{cmax}) and the maximum potential rate of electron transport (J_{max}) and finally led to inactivation of Rubisco (Crafts-Brander and Salvucci 2002, Morales *et al*. 2003, Salvucci and Crafts-Brander 2004). This may be the cumulative reason for the decline of CA activity whereas, the decrease in the activity of NR under chilling stress could be due to an expression of stress induced enzymes 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). However, application of HBL to these stressed plants improved the level of NR and CA (Fig. 3*A, B*). The possible reason for elevated level of NR is that BRs increased the level of nitrate (NO_3^-), the substrate of NR activity (Mai *et al*. 1989, Solomanson and Barber 1990) and also because of the involvement of hormone in transcription and/or translation (Kalinich *et al*. 1985, Bajguz 2000). Similar effects of BRs on the activities of NR and CA have also been reported in *V. radiata* (Fariduddin *et al*. 2004), *Brassica juncea* (Hayat *et al*. 2007) and chickpea (Hasan *et al*. 2008). Growth and development of plants are directly linked with P_N. Chl content (SPAD level) as well as P_N and their related attributes (Figs. 2*B,D–F,* 3*A–B*) of the chilling stressed plants were significantly reduced over the nonstressed plants. All these factors might have led in reduction of growth parameters (FM and DM of plant, length of roots and shoots and leaf area of plant) of chilling-stressed plants (Fig. 1*A–C*). Saltveit and Morris (1990) reported that chilling injury reduced growth, caused tissue discoloration and increased water loss, which was the result of suppressed expression of genes that are active at warmer temperatures. If the suppressed genes are essential, the plant's normal growth would be severely hampered and might lead to the death of the plants (Yang *et al*. 2005). Therefore, suppression of normally expressed essential genes by chilling stress should be one of the most important factors rendering plants to become chilling sensitive (Yang *et al*. 2005) and inhibition of plant growth (Fig. 1*A–C*). Application of HBL to the nonstressed plants increased FM and DM of whole plants as well as leaf area (Fig. 1*B*). Cucumber plants treated with BRs grew better under chilling stress (5°C) as compared to controls and BRs also had a growth-promoting effects in rice growing under low temperatures (Krishna 2003). Kagale *et al*. (2007) reported that BRs treatment enhanced tolerance to drought and cold stress in both *Arabidopsis thaliana* and *Brassica napus* seedlings. Moreover, in response to low temperatures, with the exception of hsp90, transcripts of all genes accumulated to higher levels in EBR-treated *A. thaliana* seedlings and particularly the expression of CBF1 mRNA was higher in BRs-treated *A. thaliana* seedlings (Kagale *et al*. 2007). Higher expression of transcription factors involved in activating the CBF regulator, which in turn protect plants from drought and cold stresses, supports the idea that BRs-treated seedlings are better protected than untreated seedlings in response to stress. Moreover, BRs enhanced tolerance of seedlings to a variety of abiotic stresses and this effect involves changes in expression of gene encoding for both structural and regulatory proteins (Kagale *et al*. 2007). However, a stimulatory effect of BRs on the growth of maize and cucumber seedlings was also seen under chilling stress (Katsumi 1991).

In the present study, chilling stress was found to

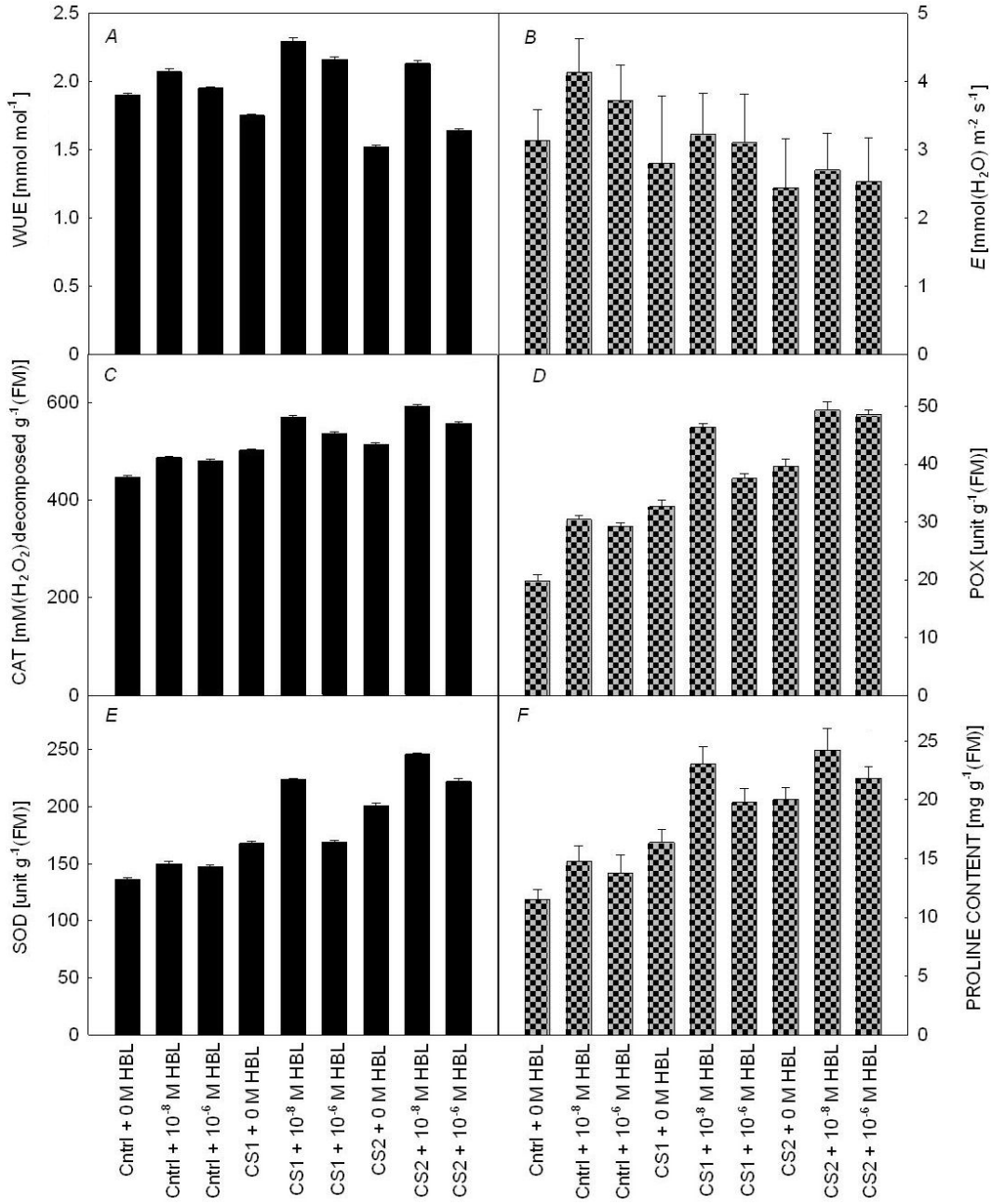

Fig. 3. Effect of 28-homobrassinolide (HBL) (10^{-6} and 10^{-8} M) on (*A*) water-use efficiency (WUE), (*B*) transpiration rate (*E*), (*C*) catalase (CAT) activity, (*D*) peroxidase (POX) activity, (*E*) superoxide dismutase (SOD) activity, and (*F*) proline content in *Cucumis sativus* L. exposed to chilling stress [10/8°C (CS1) or 5/3°C (CS2)] at 40 DAS; *vertical bars* show standard errors; all the data are means of five replicates. Cntrl – control.

adversely affect all water relation parameters (*i.e.* RWP and Ψ_w) of cucumber plants (Fig. 1*D,E*). Increased loss of water is a symptom of chilling injury (Saltveit and Morris 1990). A sharp increase in ABA content in the xylem sap, accompanied by decreased g_s and Ψ_w, were observed in cucumber after a chilling stress at 7°C (Zhou *et al*. 2007).

Consequently, there is movement of unfrozen water down the water potential gradient from inside the cell to the intercellular spaces (Sutka and Galiba 2003). As well chilling stress and hypoxia can indirectly cause osmotic stress *via* effects on water uptake and loss (Bajguz and Hayat 2009). However, exogenous application of HBL on these chilling stressed plants increased the above parameters in the present study. BRs are known to increase water relations such as increased RWC and water uptake (Ali *et al*. 2005). Osmoregulation and maintenance of cell turgor potential by the accumulation of organic or inorganic solutes or by both of these is

considered to be an important process for normal cellular metabolism (Taiz and Zeiger 2006). In the present study, there was a significant increase in the proline content under chilling stress. Proline accumulation has often been shown to occur in plants as a consequence of environmental stress (Sutka and Galiba 2003), this is because plants have an ability to protect themselves from adverse conditions and to do so they accumulate osmolytes, *i.e*. proline (Sairam and Tyagi 2004, Kavi-Kishore *et al*. 2005). However, the application HBL further increased the proline content in chilling stressed plants as well as nonstressed plants. Therefore, the maximum value for proline content was recorded in plants subjected to chilling stress along with application of HBL (Fig. 3*F*). The physiological significance of this increased accumulation is assumed to be associated with the ability of proline to act as osmoregulator, as a protective agent for cytoplasmic enzymes and the membrane, or as a storage compound during post-stress growth. There is also a report of significant positive correlations between proline level and frost tolerance in a broad spectrum of plants (Sutka and Galiba 2003). Similar observations were also reported earlier by other workers under different stress conditions in *B. juncea* (Hayat *et al*. 2007), and *Cicer arietinum* (Ali *et al*. 2007, Hasan *et al*. 2008).

AOS/ROS act both as cytotoxic compounds and as mediators in the induction of stress tolerance (Lee and Lee 2000). In order to protect cellular membranes and organelles from the damaging effects of AOS/ROS, a complex endogenous antioxidant system (CAT, POX, SOD, glutathione reductase) is very important in plant. Chilling stress elevates the level of these antioxidant enzymes to protect against chilling injury (Oidaira 2000, Lee and Lee 2000). In the present investigation it was observed that pretreatment with HBL further increased the activities of CAT, POX, and SOD in chilling-stressed plants (Fig. 3*C–E*). Furthermore, water-stressed maize seedlings treated with BR had increased activities of SOD, CAT, APX, as well as ascorbic acid and carotenoid content (Van Staden *et al*. 1998). On the other hand, BRs elevated the activity of CAT and reduced the activities of POX and ascorbic acid oxidase under osmotic stress conditions in sorghum (Vardhini and Rao 2003). It has been also reported that oxidative stress related gene encoding monodehydroascorbate reductase thioredoxin, the cold and drought stress response genes COR47 and COR78 have been identified by microarray analysis of either BRs-deficient or BRs-treated plants (Mussig *et al*. 2002). Therefore, Cao *et al*. (2005) demonstrated on the basis of molecular, physiological, and genetic approaches that the enhanced oxidation stress resistance in *det2* plants was correlated with a constitutive increase in SOD activity and increased transcript levels of the defense gene CAT. Therefore, a possible explanation for the fact that the *det2* mutant exhibited an enhanced oxidative stress resistance is that the long-term BR deficiency in *det2* mutant results in a constant *in vivo* physiological stress that, in turn, activates the constitutive expression of some defense genes and, consequently, the activities of related enzymes. If this is the case, it may suggest that endogenous BRs in wild-type plants somehow act to repress the transcription or posttranscription activities of the defense genes to ensure normal growth and development of plants. However, it is still not clear whether BRs directly or indirectly modulate the responses of plants to oxidative stress (Cao *et al*. 2005). Moreover, similar action of BRs had also been reported in *B. juncea* and *C. arietinum* subjected to heavy metal stress (Hayat *et al*. 2007, Hasan *et al*. 2008, Fariduddin *et al*. 2009a) and Ali *et al*. (2008) also reported the protective role of BRs in *V. radiata* when exposed to saline and/or nickel stress.

It may be concluded that the exposure of cucumber plants to either of the tested chilling stresses resulted in retardation of growth and development of plants. However, this chilling injury was overcome by application of HBL to the plants, before the chilling stress was applied through elevation of osmolyte levels, *i.e*. proline and also of the level of antioxidant enzymes and this was reflected in improved growth and photosynthetic parameters.

References

Alam, M.M., Hayat, S., Ali, B., Ahmad, A.: Effect of 28 homobrassinolide on nickel induced changes in *Brassica juncea.* – Photosynthetica **45**: 139-142, 2007.

Ali, B., Hassan, S.A., Hayat, S., Hayat, Q., Yadav, S., Fariduddin, Q., Ahmad, A.: A role for brassinosteroids in the amelioration of aluminium stress through antioxidant system in mung bean (*Vigna radiata* L. Wilczek). – Environ. Exp. Bot. **62**: 153-159, 2008.

Ali, B., Hayat, S., Fariduddin, Q., Ahmad. A.: 24-Epibrassinolide protects against the stress generated by salinity and nickel in *Brassica juncea*. – Chemosphere **72**: 1387-1392, 2008.

Ali, B., Hayat, S., Ahmad, A.: 28-Homobrassinolide ameliorates the salt stress in chickpea (*Cicer arietinum* L.). – Environ. Exp. Bot. **59**: 217-223, 2007.

Ali, M.B., Hahn, E.J., Paek, K.Y.: Effects of temperature on oxidative stress defense systems, lipid peroxidation and lipoxygenase activity in *Phalaenopsis*. – Plant Physiol. Biochem. **43**: 213-223, 2005.

Allen, D.J., Ort, D.R.: Impact of chilling temperatures on photosynthesis in warm-climate plants. – Trends Plant Sci. **6**: 36-42, 2001.

Aro, E.-M., Virgin, I., Andersson, B.: Photoinhibition of photosystem II. Inactivation, protein damage and turnover. – Biochim. Biophys. Acta **1143**: 113-134, 1993.

Bajguz, A.: Effect of brassinosteroids on nucleic acid and protein content in cultured cells of *Chlorella vulgaris*. – Plant Physiol. Biochem. **38**: 209-215, 2000.

Bajguz, A., Hayat, S.: Effects of brassinosteroids on the plant responses to environmental stresses. – Plant Physiol. Biochem. **47**: 1-8, 2009.

Bates, L.S., Waldren, R.P., Teare, I.D.: Rapid determination of free proline for water stress studies.- Plant Soil **39**: 205-207, 1973.

Beauchamp, L.D., Fridovich, I.: Superoxide dismutase improved assays and assay applicable to acrylamide gels. – Ann. Biochem. **44**: 216-287, 1971.

Berry, J., Björkman, O.: Photosynthetic response and adaptation to temperature in higher plants. – Annu. Rev. Plant. Physiol. **31**: 491-543, 1980.

Björkmann, O., Demmig, B.: Photon yield of O_2 evolution and chlorophyll fluorescence characterstics at 77 K among vascular plants of different origins. – Planta **170**: 489-504, 1987.

Cao, S., Xu, Q., Cao, Y., Qian, K., An, K., Zhu, Y., Hu, B.Z., Zhao, H.F., Kuai, B.K.: Loss-of-function mutations in *DET2* gene lead to an enhanced resistance to oxidative stress in Arabidopsis. – Physiol. Plant. **123**: 57-66, 2005.

Chance, B., Maehly, A.C.: Assay of catalase and peroxidases. – Meth. Enzymol. **2**: 764-775, 1955.

Clouse, S.D., Sasse, J.M.: Brassinosteroids: Essential regulators of plant growth and development. – Annu. Rev. Plant Physiol. Plant Molecular Biol. **49**: 427-451, 1998.

Crafts-Brandner, S.J., Salvucci, M.E.: Sensitivity of photosynthesis in a C_4 plant maize to heat stress. – Plant Physiol. **129**: 1773-1780, 2002.

Dwivedi, R.S., Randhawa, N.S.: Evolution of a rapid test for the hidden hunger of zinc in plants – Plant Soil **40**: 445-451, 1974.

Fariduddin, Q., Ahmad, A., Hayat, S.: Responses of *Vigna radiata* to foliar application of 28-homobrassinolide and kinetin. – Biol. Plant. **48**: 465-468, 2004.

Fariduddin, Q., Yusuf, M., Hayat, S., Ahmad A.: Effects of 28-homobrassinolide on antioxidant capacity and photosynthesis in *Brassica juncea* plants exposed to different levels of copper. – Environ. Exp. Bot. **66**: 418-424, 2009a.

Fariduddin, Q.**,** Khanam, S., Hasan, S.A., Ali, B., Hayat, S.A., Ahmad, A.: Effect of 28-homobrassinolide on the drought stress-induced changes in photosynthesis and antioxidant system of *Brassica juncea* L. – Acta. Physiol. Plant. **31**: 889-897, 2009b.

Gomez, K.A., Gomez, A.A.: Statistical Procedures for Agricultural Research. – John Wileys & Sons, New York 1984.

Grove, M.D., Spencer, G.F., Rohwedder, W.K., Mandava, N.B., Worley, J.F., Warthen, J.D., Jr., Steffens, G.L., Flippen-Anderson, J.L., Cook, J.C., Jr.: Brassinolide a plant growth promoting steroid isolated from *Brassica napus* pollen. – Nature **281**: 216-217, 1979.

Hasan, S.A., Hayat, S., Ali, B., Ahmad, A.: 28-homobrassinolide protects chickpea (Cicer arietinum) from cadmium toxicity by stimulating antioxidants. – Environ. Pollut. **151**: 60-66, 2008.

Hayat, S., Ali, B., Hasan, S.A., Ahmad, A.: Brassinosteroid enhanced the level of antioxidants under cadmium stress in *Brassica juncea*. – Environ. Exp. Bot. **60**: 33-41, 2007.

Hewitt, E.J.: Sand and Water Culture Methods used in the Study of Plant Nutrition. – Commonwealth Agricultural Bureaux, Farnham Royal, Kent 1966.

Hopkins, W.J.: Introduction to Plant Physiology. – John Wiley & Sons, New York 1995.

Janeckzo, A., Gullner, G., Skoczowski, A., Dubert, F., Barna, B.: Effects of brassinosteroid infilteration prior to cold treatment on ion leakage and pigment contents in rape leaves. – Biol. Plant. **51**: 355-358, 2007.

Jaworski, E.G.: Nitrate reductase assay in intact plant tissues. – Biochem.Biophy. Res. Commun. **43**: 1274-1279, 1971.

Kagale, S., Divi, U.K., Kronchko, J.E., Keller, W.A., Krishna, P.: Brassinosteroid conifers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. – Planta **225**: 353-364, 2007.

Kalinich, J.F., Mandava, N.B., Todhunter, and J.A.: Relationship of nucleic acid metabolism on brassinolide-induced responses in beans. – J. Plant Physiol. **120**: 207-214, 1985.

Katsumi, M.: Physiological modes of brassinolide action in ccumber hypocotyls growth. – In: Cutler, H.G., Yokota, T, Adam, G. (ed.): Brassinosteroids: Chemistry, Bioactivity and Applications. American Chemical Society Symposium Series 474. Pp. 246-254, American Chemical Society, Washington, 1991.

Kishore, P.B.K., Sangam, S., Amrutha, R.N., Laxmi, P.S., Naidu, K.R., Rao, K.R.S.S., Rao, S., Reddy, K.J., Theriappan, P., Sreenivasulu, N.: Regulation of proline biosynthesis degradation uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. – Current Science **88**: 424-438, 2005.

Kratsch, H.A., Wise, R.R.: The ultrastructure of chilling stress. – Plant Cell Environ. **23**: 337-350, 2000.

Krishna, P.: Brassinosteroid-mediated stress responses. – J. Plant Growth Regul. **22**: 289-297, 2003.

Lee, H.D., Lee, B.C.: Chilling stress induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. – Plant Science **159**: 75-85, 2000.

Mai, Y.Y., Lin, J.M., Zeng, X.L., Pan, R.J.: Effect of homobrassinolide on the activity of nitrate reductase in rice seedling. – Plant Physiol. Commun. **2**: 50-52, 1989.

Morales, D., Rodríguez, D., Dell'Amico, J., Nicolás, E., Torrecillas, A., Sánchez Blanco, M.J.: High temperature pre conditioning and thermal shock imposition affect water relations gas exchange and root hydraulic conductivity in tomato. – Biol. Plant. **47**: 203-208, 2003.

Mussig, C., Fischer, S., Altmann, T.: Brassinosteroid regulated gene expression. – Plant Physiol. **129**: 1241-1251, 2002.

Oidaira, H., Sano, S.; Koshiba, T.; Ushimaru, T.: Enhancement of antioxidative enzyme activities in chilled rice seedlings. – J. Plant Physiol. **156**: 811-813, 2000.

Ozdemir, F., Bor, M., Demiral, T., Turkan, I.: Effects of 24-epibrassinolide on seed germination, seedling growth, lipid peroxidation, proline content and antioxidant system of rice (*Oryza sativa* L.) under salinity stress. – Plant Growth Regul. **41**: 1-9, 2004.

Sairam, R.K.: Effects of homobrassinolide application on plant metabolism and grain yield under irrigated and moisture stress conditions of two wheat varieties. – Plant Growth Regul. **14**: 173-181, 1994.

Sairam, R.K., Tyagi, A.: Physiology and molecular biology of salinity stress tolerance in plants. – Current Sci. **86**: 407-421, 2004.

Saltveit, M.E., Morris, L.L.: Overview on chilling injury of horticultural crops. – In: Wang, C.Y. (ed.): Chilling Injury of Horticultural Crops. Pp 3-15. CRC Press, Boca Raton 1990.

Salveit, M.E.: Chilling injury is reduced in cucumber and rice seedlings in tomato pericarp discs by heat-shocks applied after chilling. – Postharvest Biol. Tech. **21**: 169-177, 2001.

Salvucci, M.E., Crafts-Brander, S.J.: Relationship between the heat tolerance of photosynthesis and the thermal stability of Rubisco activase in plants from contrasting thermal environments. – Plant Physiol. **134**: 1460-1470, 2004.

Sasse, J.M.: Physiological actions of brassinosteroids: An

update. – J. Plant Growth Regul. **22**: 276-288, 2003.
Solomonson, L.P., Barber, M.J.: Assimilatory nitrate reductase functional properties and regulation. – Annu. Rev. Plant Physiol. Mol. Biol. **41**: 225-25, 1990.
Sonoike, K.: The different roles of chilling temperature in the photoinhibition of photosystem I and photosystem II. – J. Photochem. Photobiol. **48**: 136-141, 1999.
Sutka, J., Galiba, G.: Abiotic Stresses: Cold Stress. – Agr. Res. Inst. Hung. Acad. Sci., Martonvasar 2003.
Taiz, L., Zeiger.: Plant Physiology. 4th Ed. – Sinauer Assoc. Publ., Sunderland 2006.
Tikhomirova, E.V.: Changes in nitrogen metabolism in millet and elevated temperatures. – Field Crops Res. **11**: 259-264, 1995.
van Staden, L., Jagr, A.K.: Effects of plant growth regulators on the antioxidant system in seedlings of two maize cultivars subjected to water stress. – Plant Growth Regul. **25**: 81-87, 1998.
Vardhini, B.V., Rao, S.S.R.: Amelioration of osmotic stress by brassinosteroids on seed germination and seedling growth of three varieties of sorghum. – Plant Growth Regul. **41**: 25-31, 2003.
Walker, M.A., McKersie, B.D.: Role of the ascorbate-glutathione antioxidant system in chilling resistance of tomato. – J. Plant Physiol. **141**: 234-239, 1993.
Wang, C.Y.: Physiological and biochemical responses of plants to chilling stress. – Hortsci. **17**: 173-186, 1982.
Wilen, R.W., Sacco, M., Gusta, L.V., Krishna, P.: Effects of 24-epibrassinolide on freezing and thermotolerance of bomegrass (*Bromus inermis*) cell cultures. – Physiol. Plant. **95**: 195-202, 1995.
Wise, R.R., Naylor, A.W.: Chilling-enhanced photo-oxidation. The peroxidative destruction of lipids during chilling injury to photosynthesis and ultrastructure. – Plant Physiol. **83**: 272-277, 1987.
Yang, M.T., Chen, S.L., Lin, C.Y., Chen, Y.M.: Chilling stress suppresses chloroplast development and nuclear gene expression in leaves of mung bean seedlings. – Planta **221**: 374-385, 2005.
Yu, J.Q., Huang, L.F., Hu, W.H., Zhou, Y.H., Mao, W.H., Ye, S.F., Noques, S.: A role of brassinosteroids in the regulation of photosynthesis in *Cucumis sativus*. – J. Exp. Bot. **55**: 1135-1143, 2004.
Yu, J.Q., Zhou, Y.H., Huang, L.F., Allen D.J.: Chill induced inhibition of photosynthesis: Genotype variation within *Cucumis sativus*. – Plant Cell Physiol. **43**: 1182-1188, 2002.
Zhou, Y.H., Huang, L.F., Zhang, Y.L., Shi, K., Yu, J.Q., Nogués, S.: Chill induced decrease in capacity of RuBP carboxylation and associated H_2O_2 accumulation in cucumber leaves are alleviated by grafting onto fig leaf gourd. – Ann. Bot. **100**: 839-848, 2007.