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Comparative effect of 28-homobrassinolide and 24-epibrassinolide on the growth, carbonic anhydrase activity and photosynthetic efficiency of *Lycopersicon esculentum*

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Abstract

The present piece of work highlights the comparative effects of two active forms of brassinosteroids (BRs), 28-homobrassinolide (HBL) and 24-epibrassinolide (EBL), on growth parameters, carbonic anhydrase activity and photosynthetic parameters in *Lycopersicon esculentum* (cv. K-21) sampled at 45 (24 h after spray) and 60 days after sowing, under natural conditions. Out of the two active forms of BR, EBL proved better than HBL in improving the above parameters, when applied as foliar spray. Of the three concentrations (10^{-6} M, 10^{-8} M or 10^{-10} M) of HBL and EBL, 10^{-8}M proved best in both cases.

Additional key words: brassinosteroids; carbonic anhydrase; chlorophyll; net photosynthetic rate.

Introduction

Brassinosteroids (BRs) are a relatively new class of plant steroidal hormones which have a diverse role in plant development. They are found in low concentrations throughout the plant kingdom and are widely distributed in lower and higher plants. They have been detected in almost all plant organs such as pollen, anthers, seeds, leaves, stems, roots, and flowers. They are also present in insect and crown galls, for example those formed by *Castanea crenata*, *Distylium racemosum* or *Catharanthus roseus*. These infected plants have higher levels of BRs than the normal tissues. Younger tissues are a richer source of BRs than mature tissues. Studies conducted so far have revealed that BRs elicit a wide range of morphological and physiological responses in plants (reviewed by Hayat *et al.* 2010a, Bajguz and Hayat 2009). Roles of BRs include their ability to cause cell elongation and cell division in stems, inhibit root growth, promote xylem differentiation, and abscission (Mandava 1988, Nemhauser *et al*. 2004), induce synthesis of nucleic acid and protein (Khripach *et al*. 2003), activate several enzymes (Hasan *et al*. 2008), and increase fruit set (Kamuro and Takatsuto 1991, Ali *et al*. 2006). Increasing stress tolerance in the plants is another role assigned to BRs (Clouse and Sasse 1998). Among abiotic stresses, BR has been reported to counter high and low-temperature stress (Kulaeva *et al*. 1991, Wilen *et al*. 1995), moisture stress (Sairam 1994, Hayat *et al.* 2008), drought stress (Schilling *et al*. 1991, Fariduddin *et al.* 2009a), heavy-metal stress (Alam *et al.* 2007, Hayat *et al.* 2007b, Ali *et al.* 2008a, Hasan *et al*. 2008, Fariduddin *et al.* 2009b, Sharma *et al*. 2007, Bhardwaj *et al*. 2007), salinity stress (Ali *et al*. 2007, 2008b) and nitrosative stress (Hayat *et al.* 2010b).

One of such roles of BRs in higher plants is their involvement in the regulation of photosynthesis. The exogenous application of BRs enhanced the net photosynthetic rate in several plant species, *e.g., Brassica juncea* (Hayat et al. 2000, 2001a, 2007a, Ali *et al.* 2008b, Fariduddin *et al.* 2009a,b), *Cucumis sativus* (Yu *et al.* 2004, Xia *et al.* 2006), *Glycine max* (Zhang *et al.* 2008), *Lycopersicon esculentum* (Singh and Shono 2005), *Oryza sativa* (Farooq *et al.* 2009), *Vigna radiata* (Fariduddin *et al.* 2003, 2004, Ali *et al.* 2008a) or *Triticum aestivum* (Sairam 1994, Ali *et al.* 2008c). The foliar application of 24-epibrassinolide (EBL) enhanced the light-saturated net CO_2 assimilation rate and carboxylation rate of Rubisco, thereby increasing the capacity of CO_2 assimilation in the Calvin-Benson cycle (Yu *et al.* 2004, Xia *et al.* 2009).

The chlorophyll (Chl) content was increased in the leaves of *Vigna radiata* (Bhatia and Kaur 1997) and

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Abbreviations: BRs – brassinosteroids; C_i – intercellular carbon dioxide concentration; CA – carbonic anhydrase activity; Chl – chlorophyll; DAS – days after sowing; DDW – double distilled water; E – transpiration rate; EBL – 24-epibrassinolide; FM – fresh mass; g_s – stomatal conductance; HBL – 28-homobrassinolide; LSD – least significant difference; PAR – photosynthetically active radiation; P_N – net photosynthetic rate; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase.

in *Brassica juncea* (Hayat *et al*. 2001a) due to 28-homobrassinolide (HBL) and in *Cucumis sativus* (Yu *et al*. 2004) and *Vicia faba* (Pinol and Simon 2009) due to EBL, when the hormones were applied directly to the foliage. Similarly, the values for the above parameters increased in the leaves of rice (Wang 1997), *Cicer arietinum* (Fariduddin *et al*. 2000), *Brassica juncea* (Hayat and Ahmad 2003). *Vigna radiata* (Fariduddin *et al*. 2003) and *Pelargonium graveolens* (Swamy and Rao 2009) raised from seeds given a presowing treatment with BRs.

Carbonic anhydrase (CA) is the second most abundant soluble protein, other than Rubisco, in C_3-chloroplasts (Reed and Graham 1981, Okabe *et al*. 1984). It is a zinc containing protein with a molecular mass of 180 kDa (Lawlor 1987) and it is a ubiquitous enzyme among living organisms. It catalyzes the reversible interconversion of bicarbonates (HCO_3^-) and CO_2 (Sultemeyer *et al*. 1993). The rate of conversion of HCO_3^- to CO_2 is normally slow in alkaline conditions. However, CA activates the formation of CO_2 (Lawlor 1987). In C_3 plants, CA has a close association with Rubisco where it elevates the level of CO_2 at its active site (Badger and Price 1994). An increase in the activity of CA in the leaves was attained by the application of HBL to the shoots of the *Brassica juncea* (Hayat *et al*. 2000, 2001a). Moreover, the seedlings of wheat and mung bean raised from the seeds treated with HBL possessed high CA activity in their leaves (Hayat *et al*. 2001b, Fariduddin *et al*. 2003). Seed treatment with EBL also reduced the toxic effects of cadmium on carbonic anhydrase activity (Anuradha and Rao 2009).

The present investigation was proposed with an aim to compare the effects of two analogues of BRs (EBL/HBL) on tomato plants by studying various growth and photosynthetic parameters.

Materials and methods

Hormone preparation: HBL and EBL were obtained from *Godrej Agrovet Ltd.,* India, and *Sigma Chemicals, St. Louis,* USA, respectively. Stock solution (10^{-4} M) was prepared by dissolving required quantity of hormone in 5 ml of ethanol. Five ml of surfactant "Tween-20" was added and it was made up to 100 ml using double distilled water (DDW). The desired concentrations of HBL or EBL were prepared by the dilution of stock solution.

Plant material and experimental setup: Authentic seeds of *Lycopersicon esculentum* cv. K-21 were procured from *National Seed Corporation Ltd.,* Pusa, New Delhi. Healthy uniform seeds were surface-sterilised with 0.01% (w/v) mercuric chloride solution and repeated washings with DDW to remove the adhering particles of mercuric chloride. These sterilised seeds were sown in earthen pots filled with sandy loam soil mixed with farmyard manure in the ratio of 9:1 to create the nursery. The experiment was carried out in October–December, 2008 in a net-house under natural environmental conditions with an optimum temperature of 21 to 24^{o}C. The mean irradiance was 350 µmol m^{-2} s^{-1} and the air humidity was 75%. The pots were treated with the recommended dose of fertilizer. The seedlings at 20 days after sowing (DAS) were subsequently transplanted to pots (25 × 25 cm), under conditions similar to those of the nursery pots. The foliage of the plants was sprayed with the required concentrations of HBL/EBL (10^{-6} M, 10^{-8} M or 10^{-10} M) at 44 DAS. Plants sprayed with DDW were used as controls. Each plant was sprinkled three times in the morning. The nozzle of the sprayer was adjusted in such a way that it pumped out 1 ml of DDW, HBL or EBL solutions in one sprinkle. The plants were then sampled at 45 DAS (24 h after spray) and 60 DAS to assess various parameters. Only the data from the final sampling (*i.e*. 60 DAS) are presented here since the pattern of responses in photosynthetic parameters was similar at both the sampling stages (45 and 60 DAS).

Growth analysis: The plants were removed from the pots along with soil and were dipped in a bucket filled with tap water. The plants were gently moved to remove adhering soil particles. The length and fresh mass of roots and shoots were measured using a meter scale and an electronic balance, respectively. The leaf area was measured manually using graph sheet, where the squares covered by the leaf were counted. The plants were then placed in an oven at 80°C for 72 h. The dried plants were then weighed to record plant dry mass.

SPAD Chl and gas-exchange parameters: The SPAD value of Chl was measured with a SPAD Chl meter (*SPAD-502, Konica, Minolta Sensing, Inc.,* Japan). Gas-exchange parameters [net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and transpiration rate (E)] in a well expanded upper third leaf attached to the mother body were measured using an infrared gas analyser portable photosynthetic system (*LI-COR 6400, LI-COR,* Lincoln, NE, USA), between 11:00–12:00 h under clear sunlight. The atmospheric conditions during measurement were photosynthetically active radiation (PAR), 1016 ± 6 µmol m^{-2} s^{-1}, relative humidity 60 ± 3%, atmospheric temperature 22 ± 1^{o}C and atmospheric CO_2 360 µmol mol^{-1}. The duration of the measurement of each sample was 10 min after the establishment of steady-state conditions inside the measurement chamber.

Carbonic anhydrase (CA) activity was determined by the procedure described earlier by Dwivedi and Randhawa (1974). 0.2 g of fresh leaf samples were cut into small pieces and suspended in 10 ml of 0.2 M cysteine hydrochloride solution. The samples were incubated at 4°C for 20 min. The pieces were blotted dry and transferred to test tubes containing 4 ml of phosphate buffer (pH 6.8) followed by the addition of 4 ml of 0.2 M alkaline bicarbonate solution and 0.2 ml of 0.002% bromothymol blue indicator. The test tubes were incubated at 4°C for 20 min. The reaction mixture was titrated against 0.05 N HCl, after the addition of 0.2 ml of methyl red indicator. The results are expressed as mol (CO_2) kg^{-1}(leaf FM) s^{-1}.

Statistical analysis: The experiment was conducted according to simple randomized block design. Each treatment was replicated three times with three plants per each replicate. Treatment means were compared by the analysis of variance using *SPSS 17.0 for Windows* (*SPSS*, Chicago, IL, USA). Least Significant Difference (LSD) between treatment means was calculated at the 5% level of probability. The *Duncan*'s multiple range test (DMRT) has been applied to the data to separate the means.

Results

Growth characteristics: Figs. 1 and 2 show that treatment with BRs significantly increased the rate of growth of tomato plants. Plants exposed to HBL (10^{-8} M) or EBL (10^{-8} M) showed maximum increase in their root and shoot length over their respective controls, at 60 DAS. Foliar application of EBL proved more effective than HBL (although the differences between the effects of HBL and EBL were usually not statistically significant). The percent increase in the length, fresh and dry mass of root and shoot by HBL (10^{-8} M) was 46.9%, 42.4%, 47.6%, 50.4%, 50.5%, 56.4% and by EBL (10^{-8} M) 59.7%, 60.2%, 65.6%,65.4%, 64.6%, and 71.5% at 60 DAS with respect to their controls. The leaf area also increased 32.8% by HBL (10^{-8} M) and 46.5% by EBL (10^{-8} M) at 60 DAS over their respective controls (Fig. 2). It is clear that EBL therefore generated a greater growth response than HBL.

SPAD Chl and gas-exchange parameters: As depicted in Fig. 3*A*, application of HBL (10^{-6}, 10^{-8}, or 10^{-10} M) increased the SPAD level by 25.2%, 30.5%, and 21.2% at 60 DAS over the control. EBL (10^{-8} M) increased the value of SPAD Chl by 37.4% at 60 DAS over that of the control. Out of the two hormone analogues EBL again was more effective than HBL in its response.

The parameters of leaf gas exchange in 60-day-old plants were high on receiving brassinosteroid analogues (HBL/EBL) (Fig. 3*B,C,D,E*). The maximum increase in P_N of about 29.5% was recorded in the leaves of plants sprayed with 10^{-8} M of EBL, whereas, with 10^{-8} M of HBL the increase was about 23.8% at 60 DAS. The other gas-exchange parameters (g_s, C_i, and E) exhibited a trend similar to that of P_N. The per cent increase in g_s, C_i, and E was 53.7%, 12.6%, and 31.2% or 70.0%, 18.9%, and 43.6% by 10^{-8} M of HBL or EBL respectively, at 60 DAS.

Carbonic anhydrase (CA) activity: Brassinosteroid analogues (HBL/EBL), at three concentrations (10^{-6}, 10^{-8} or 10^{-10} M) when applied to the plant foliage improved CA activity to a significant level (Fig. 3*F*). HBL (10^{-8} M) increased the value of CA activity by 24.6% at 60 DAS over the control plants (Fig. 3*F*). Maximum enzyme activity was recorded at 60 DAS in the leaves sprayed with EBL (10^{-8} M) which was 35.2% higher than that of the control.

Discussion

Brassinosteroids, steroidal plant hormones, are reported to control various developmental processes such as promotion of cell division and elongation, photomorphogenesis, seed germination and ethylene biosynthesis (Müssig *et al.* 2002). In agriculture, the potential of brassinosteroids application is based on their ability to increase yield of crops (Khripach *et al.* 2000). They have also the capability to protect plants from various environmental stresses such as drought, extreme temperatures, heavy metals, herbicidal injury, and salinity (Bhardwaj *et al.* 2006, Hayat *et al.* 2010c, Hasan *et al.* 2008, Janeczko *et al.* 2008). The present study showed that both the brassinolides tested stimulated plant growth. Moreover, the medium concentration of EBL tested (10^{-8} M) had the most significant impact on the growth characteristics (in terms of length, fresh and dry mass of roots and shoots, and leaf area) of the plants (Figs. 1, 2). BRs are recognised to mediate growth through the regulation of gene expression (Felner 2003). BRs activate the *BRU1* and *TCH4* genes encoding xyloglucan endotransglycoxylase (XET) and expansins (Cosgrove 1997). These enzymes are responsible for cell wall loosening. Simultaneously, BRs also maintain a healthy metabolic state in a cell and organs (Ali *et al.* 2006), which fulfils the demand for additional structural material and the energy needed for the growth of the cell wall as well as that of the plant as a whole.

The activity of CA enzyme that catalyses interconversion of CO_2 and HCO_3^- is regulated by photon flux density, CO_2 concentration, availability of zinc (Tiwari *et al.* 2005), and the expression of genes encoding CA protein (Kim *et al.* 1994). Higher CA activity can also be achieved by the application of BRs to plants as shown in *Brassica juncea*, under nickel stress (Alam *et al.* 2007) and *Vigna radiata* under aluminium stress (Ali *et al.* 2008a). Similarly, our tomato plants grown under

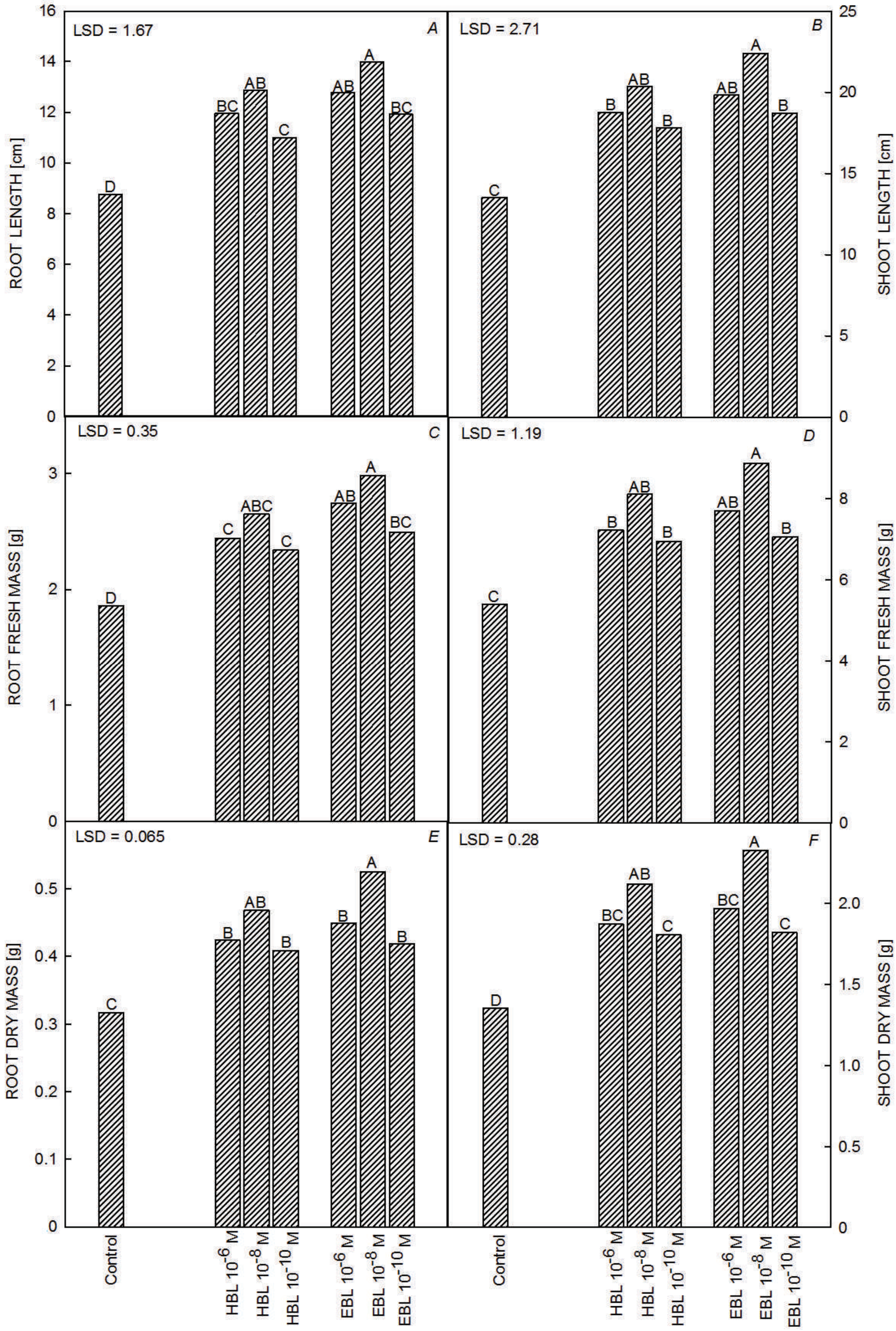

Fig. 1. Effect of 28-homobrassinolide (HBL) or 24-epibrassinolide (EBL) (0, *i.e.* control, 10^{-6}, 10^{-8} or 10^{-10} M) applied foliarly at 44 DAS on the length of (*A*) root and (*B*) shoot, fresh mass of (*C*) root and (*D*) shoot, dry mass of (*E*) root and (*F*) shoot of tomato (*Lycopersicon esculentum* L. cv. K-21) measured in plants at 60 DAS. The *letters* A–D above the bars denote the statistical significance (*Duncan's* multiple range test) of the differences between individual treatments (only those marked with *different letters* differ significantly at $P<0.05$).

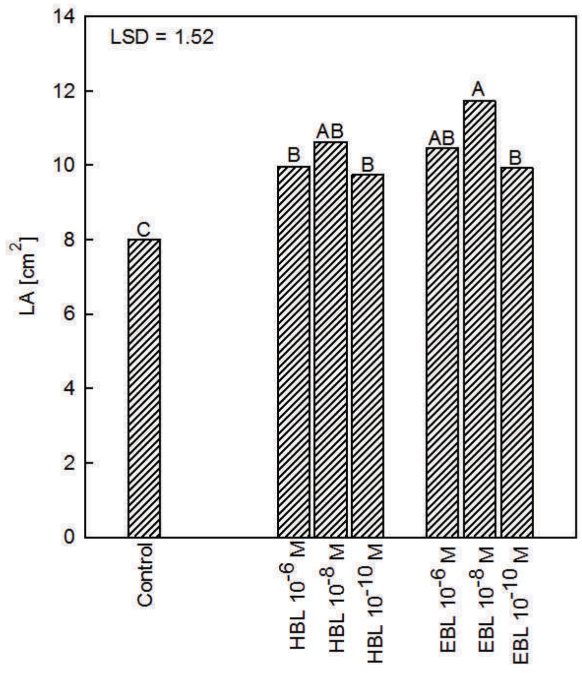

Fig. 2. Effect of 28-homobrassinolide (HBL) or 24-epibrassinolide (EBL) (0, *i.e.* control, 10^{-6}, 10^{-8} or 10^{-10} M) applied foliarly at 44 DAS on the leaf area (LA) of tomato (*Lycopersicon esculentum* L. cv. K-21) measured in plants at 60 DAS. The *letters* A–D above the bars denote the statistical significance (*Duncan's* multiple range test) of the differences between individual treatments (only those marked with *different letters* differ significantly at $P<0.05$).

nonstress conditions exhibited higher CA activity on the application of either of the BRs (Fig. 3*F*) which could be an expression of the impact of BRs on translation and/or transcription (Khripach *et al.* 2003). Moreover, BRs speed up CO_2 assimilation in photosynthesis (Yu *et al.* 2004), therefore, both HBL and EBL improved all the photosynthetic parameters (P_N, g_s, and C_i) (Fig. 3*B,C,D*). BRs also have a positive impact on Rubisco activity (Braun and Wild 1984), a key enzyme in photosynthetic carbon fixation. Improved water relations (Ali *et al.* 2005), higher activities of CA (Fig. 3*F* and Ali *et al.* 2006) and Rubisco (Braun and Wild 1984) in plants treated with BRs are naturally expected to improve the g_s and culminate in higher P_N (Fig. 3). High P_N and related improved parameters in response to BRs under differential abiotic stresses have also been reported by others (Ali *et al.* 2008a,b, Hayat *et al.* 2000, 2001a, 2007a,b, Fariduddin *et al.* 2009a,b).

EBL was more effective than HBL in increasing the photosynthetic parameters (Fig. 3 and Ali *et al.* 2008a). Our results are consistent with those of Singh and Shono (2005) in tomato and Swamy and Rao (2009) in geranium plants. Application of BRs also enhanced the other parameters (g_s, C_i, and E) tested in the present study and EBL again was more effective than HBL (Fig. 3*C*, *D*,*E*). These results are in conformity with those of Ali *et al.* (2008a,b) and Fariduddin *et al.* (2009a). It can be inferred that BR increased g_s, which is correlated with P_N, and this can be brought about because there is a greater amount of CO_2 available for its fixation by photosynthetic enzymes (Hola 2011).

BRs fed to the nonstressed plants also significantly increased the Chl content (Fig. 3*A*) which supports the findings of Braun and Wild (1984), Bhatia and Kaur (1997), Hayat *et al.* (2000, 2001a), Fariduddin *et al.* (2003), Yu *et al.* (2004) and Ali *et al.* (2006, 2007). The most likely reason supporting the increase in Chl content is possibly the BL-induced impact on transcription and/or translation (Khripach *et al.* 2003) by involving the expression of specific genes responsible for the synthesis of the enzymes that determine Chl biosynthesis. Application of BRs on the other hand also results in retarding the rate of Chl degradation and also of the proteins associated with these pigments, particularly proteins of light-harvesting complexes in chloroplast thylakoid membranes (Hola 2011). This increase in the Chl content, under the impact of BRs could possibly lead to the improvement of the light-capturing efficiency which would result in higher photosynthetic rate (Fig. 3*B* and Hola 2011). Similar type of observations have also been reported by Ali *et al.* (2008a,c) in mung bean and mustard plants when sprayed by BRs.

The present study revealed that both analogues of BRs were significantly effective in enhancing the growth and photosynthetic efficiency of tomato plants. Of the two forms, EBL was found to be more effective than HBL. The mechanism by which EBL excels over HBL has not yet been elucidated. The most likely reason may be the difference in structure and stability of these two analogues of BRs (Khripach *et al*. 2003). Generally BRs have an S-oriented alkyl group at C-24 of side chain but EBL exceptionally has an R-oriented alkyl group at the side chain of steroid nucleus. It can be concluded that binding of EBL to the receptor leads to more distorted three dimensional conformational states as compared to HBL. This thermodynamically new active stable state seems to be more actively involved in triggering a wide array of signalling cascades than HBL.

It is concluded from the results of the present investigation that foliar spraying of tomato plants with BRs enhances plant growth and the rate of photosynthesis and also the EBL form is significantly more effective than the HBL form.

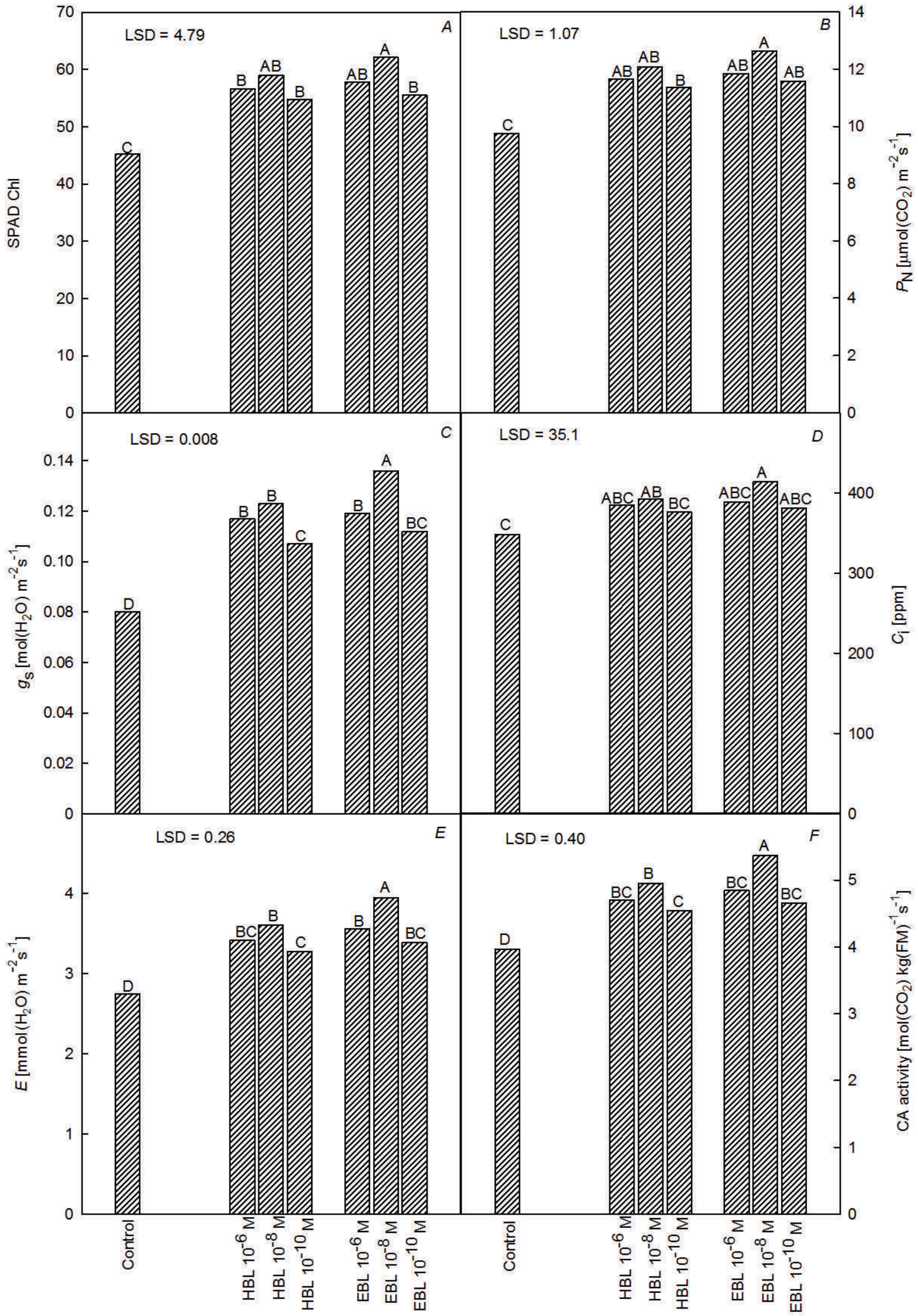

Fig. 3. Effect of 28-homobrassinolide (HBL) or 24-epibrassinolide (EBL) (0, *i.e.* control, 10^{-6}, 10^{-8} or 10^{-10} M) applied foliarly at 44 DAS on the (*A*) SPAD Chl, (*B*) net photosynthetic rate (P_N), (*C*) stomatal conductance (g_s), (*D*) intercellular CO_2 concentration (C_i), (*E*) transpiration rate (*E*) and (*F*) carbonic anhydrase (CA) activity of tomato (*Lycopersicon esculentum* L. cv. K-21) measured in plants at 60 DAS. The *letters* A–D above the bars denote the statistical significance (*Duncan´*s multiple range test) of the differences between individual treatments (only those marked with *different letters* differ significantly at $P<0.05$).

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