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BRIEF COMMUNICATION

Effect of 24-epibrassinolide on drought stress-induced changes in *Chorispora bungeana*

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Abstract

Brassinosteroids (BRs) have been proposed to increase the resistance of plants to drought stress. The effect of foliar application of 0.1 μM 24-epibrassinolide (EBR) on chlorophyll (Chl) content, photosystem 2 (PS 2) photochemistry, membrane permeability, lipid peroxidation, relative water content (RWC), proline content, and the antioxidant system in drought-stressed *Chorispora bungeana* plants was investigated. The results showed that polyethylene glycol (PEG) induced water stress decreased RWC, Chl content and variable to maximum Chl fluorescence ratio (F_v/F_m) less in plants pretreated with EBR than in non-pretreated plants. In addition, lipid peroxidation, measured in terms of malondialdehyde content, membrane permeability and proline content in drought-stressed plants were less increased in EBR pretreated plants, while antioxidative enzyme activities and reduced ascorbate and glutathione contents were more increased in EBR pretreated than in non-pretreated plants. These results suggested that EBR could improve plant growth under drought stress

Additional key words: antioxidative enzymes, brassinosteroids, chlorophyll, lipid peroxidation, membrane permeability, photosystem, proline, reactive oxygen species.

Drought is a major environmental factor restricting plant growth and productivity. Thus, development of methods to induce drought stress tolerance in plants is of vital importance. Water stress causes significant declines in leaf water potential and relative water content (RWC) and directly affects many aspects of plant physiology such as membrane stability, nitrate reductase activity, chlorophyll (Chl) content and photosynthetic parameters (Correia *et al.* 2006, Santos *et al.* 2009). Drought can also cause oxidative damage due to excessive generation of reactive oxygen species (ROS), which can cause deterioration of membrane lipids and proteins, leading to increased membrane leakage of solutes (Moran *et al.* 1994). However, plants evolved the defense system, which includes antioxidative enzymes [catalase (CAT, EC 1.11.1.6), peroxidases (POD, EC 1.11.1.7), superoxide dismutases (SOD, EC 1.15.1.1), glutathione reductase (GR, EC 1.6.4.2) and ascorbate peroxidase (APX, EC 1.11.1.11)], low molecular mass antioxidants ascorbate (AsA), glutathione (GSH), and some compatible solutes such as betaines and proline (Foyer and Noctor 2005, Veljovic-Jovanovic *et al.* 2006).

Brassinosteroids (BRs) play an essential role in plant growth and development (Clouse and Sasse 1998). Moreover, BRs are recognized to have an ameliorative role in plants subjected to various biotic and abiotic stresses, such as high temperature (Ogweno *et al.* 2008), excess of heavy metals (Fariduddin *et al.* 2009), and

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Abbreviations: APX - ascorbate peroxidase; AsA - reduced ascorbate; BRs - brassinosteroids; CAT - catalase; Chl - chlorophyll; EBR - 24-epibrassinolide; EDTA - ethylenediaminetetraacetic acid; EL - electrolyte leakage; F_0, F_m - minimum and maximum fluorescence in dark-adapted leaves; F_v/F_m - variable to maximum fluorescence ratio; GR - glutathione reductase; GSH - reduced glutathione; MDA - malondialdehyde; PEG - polyethylene glycol; PS 2 - photosystem 2; ROS - reactive oxygen species; RWC - relative water content; SOD - superoxide dismutase; Φ_{PS2} - the quantum yield of PS 2.
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salinity (Ali *et al.* 2007). The aim of this study was to investigate effects of 24-epibrassinolide (EBR; one of the most active and stable forms of BRs) pretreatment for improving plants tolerance against drought stress and the role of the antioxidant defense system in this process.

Seeds of *Chorispora bungeana* Fisch. & C.A. Mey, a herb plant of the family *Brassicaceae*, were surface sterilized with 1 % sodium hypochlorite solution plus 0.1 % *Tween-20* and washed several times with deionized water. Cotyledons were cut off and planted on Murashige and Skoog (1962; MS) solid medium containing 0.2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid and 0.2 mg dm^{-3} 6-benzyladenine. The cultures were grown under a 16-h photoperiod, irradiance of 120 μmol m^{-2} s^{-1} (supplied by cool white fluorescent tubes), relative humidity of 55 ± 5 %, and temperature of 23 ± 2 °C. After 20 d, growing tissues were transferred to MS solid medium containing 0.4 mg dm^{-3} gibberellin and 0.6 mg dm^{-3} kinetin. The regenerants were then sub-cultured every two weeks, and after several cycles, the plantlets were transferred to earthenware pots filled with soil and farmyard manure (6:1). Plants were watered with full strength Hoagland solution and when seedlings were approximately 5 cm tall, a 0.1 μM aqueous EBR (*Sigma Aldrich*, St. Louis, USA) solution containing 0.1 % *Tween 20* was sprayed thrice on the leaves every 2 h, with control plants similarly sprayed with water containing only *Tween 20*. The selected EBR concentration was based on earlier work performed by Xia *et al.* (2009). One day after spraying, drought stress was induced by watering the plants with the Hoagland solution containing 20 % polyethylene glycol 6000 (PEG). The plants were divided into four groups: well-watered and non-EBR treated (WW), drought stressed and non-EBR treated (DS), EBR treated and well watered (EBR+WW) and EBR treated and drought stressed (EBR+DS). Leaves were collected 72 h after PEG treatment, immediately frozen in liquid nitrogen, and stored at -80 °C for the subsequent analyses.

Chl *a* and *b* were extracted using 80 % acetone on ice and determined according to Lichtenthaler (1987). The Chl fluorescence parameters were measured on attached leaves using a portable pulse modulation fluorometer (*PAM-2100*, *Heinz Walz*, Effetrich, Germany). The minimum fluorescence (F_0) was read under a weak modulated light after 30 min of dark adaptation, and the maximum fluorescence (F_m) was obtained by applying a 0.8 s saturating pulse. Maximal photochemical efficiency of photosystem 2 (PS 2) was calculated as $F_v/F_m = (F - F_0)/F_m$, and the quantum yield of PS 2 [$\Phi_{PS2} = (F_m' - F_s)/F_m'$], where F_m' is maximum fluorescence after light-adaptation, and F_s steady-state fluorescence (Genty *et al.* 1989).

Membrane permeability was estimated by measuring electrolyte leakage (EL) according to Blum and Ebercon (1981) using a conductivity meter (*DDSJ-308A*, Shanghai, China). Malondialdehyde (MDA) was extracted with 10 % (m/v) trichloroacetic acid and after reaction with 0.6 % thiobarbituric acid the absorbance was determined at 450, 532, and 600 nm according to Hodges *et al.* (1999). RWC was estimated gravimetrically according to Bajji *et al.* (2001) and the proline content was determined by the method of Bates *et al.* (1973).

Leaf samples (0.2 g) were homogenized in 3 cm^3 of 50 mM potassium phosphate buffer (pH 7.8) containing 0.2 mM EDTA and 1 % (m/v) polyvinylpyrrolidone, and then centrifuged at 15 000 *g* for 20 min at 4 °C. The supernatant was then used for enzymatic activity determinations. Protein content was determined according to the method of Bradford (1976) with bovine serum albumin as a standard. SOD activity was assayed by measuring the ability to inhibit the photochemical reduction of nitroblue tetrazolium using the method of Stewart and Bewley (1980). CAT activity was measured as a decline in absorbance A_{240} in 50 mM potassium phosphate buffer (pH 7.0) according to Abassi *et al.* (1998). APX activity was measured by a decrease in A_{290} due to AsA oxidation according to Nakano and Asada (1981). GR activity was measured as a decrease in A_{340} due to oxidation of NADPH according to Foyer and Halliwell (1976). AsA content was measured in extracts according to the method of Law *et al.* (1983), and GSH content was determined by the method of Griffith (1980).

The experiment was repeated twice with three replicates each time. The values for the parameters were analyzed by analysis of variance (*ANOVA*) using *SPSS* (version *15.0*). Means were compared using the Duncan multiple range test at the 5 % probability level.

When subjected to drought stress, leaves of *C. bungeana* showed curled and severely wilted appearance and subsequent chlorosis for up to 72 h of treatment. Concurrently, both Chl *a* and Chl *b* (and hence total Chl) contents decreased significantly in these plants, and this loss of Chl could be related to photoinhibition or ROS formation, as demonstrated by the observed increased lipid peroxidation (Table 1). Moreover, the decrease in Chl content may be attributed to decreased activity of protochlorophyllide reductase (Stobart *et al.* 1985), the key enzyme that determines the biosynthesis of Chl, or increased activity of Chl-degrading enzyme chlorophyllase (Reddy and Vora 1986). Actually, most of enzymes responsible for the synthesis of Chl and photosynthesis are affected by drought stress. However, the application of EBR exhibited significant improvement in *C. bungeana* phenotypic appearance, and increased Chl content in the plants grown with or without drought stress, which suggested that EBR may affected the Chl synthesis as well as photosynthesis (Yu *et al.* 2004). In addition, it was probable that EBR had a protective effect on pigment-protein complexes resulting in decreased degradation of Chl.

The F_v/F_m value, which serves as a measure of the functional status of the oxygen-evolving complex, is usually used as a sensitive indicator for assessing plant photosynthetic performance, and a decrease in F_v/F_m indicates the extent of photoinhibition caused by environmental stresses (Maxwell and Johnson 2000).

Table 1. The effect of EBR on Chl content, chlorophyll fluorescence, electrolyte leakage, MDA content, RWC, proline content, activities of antioxidative enzymes and contents of low molecular antioxidants in leaves of *Chorispora bungeana* plants grown in full strength Hoagland solution without (WW) and with (DS) 20 % PEG for 3 d. Means ± SD of two independent experiments (n = 6). Means within a row followed by different letters are significantly different at $P < 0.05$ based on Duncan test.

Parameters	WW	EBR+WW	DS	EBR+DS
Chl *a* [mg g^{-1}(f.m.)]	0.80 ± 0.01b	0.83 ± 0.02a	0.55 ± 0.02d	0.58 ± 0.01c
Chl *b* [mg g^{-1}(f.m.)]	0.21 ± 0.01a	0.21 ± 0.01a	0.14 ± 0.01b	0.16 ± 0.01b
Chl (*a*+*b*) [mg g^{-1}(f.m.)]	1.01 ± 0.01a	1.04 ± 0.02a	0.69 ± 0.03c	0.74 ± 0.01b
F_v/F_m	0.80 ± 0.02b	0.83 ± 0.01a	0.51 ± 0.02d	0.57 ± 0.02c
Φ_{PS2}	0.80 ± 0.02a	0.82 ± 0.01a	0.52 ± 0.02c	0.57 ± 0.03b
F_m	1.21 ± 0.10a	1.28 ± 0.09a	0.69 ± 0.03b	0.74 ± 0.05b
F_0	0.25 ± 0.04b	0.21 ± 0.01b	0.34 ± 0.03a	0.32 ± 0.01a
electrolyte leakage [%]	13.27 ± 1.32c	12.10 ± 1.29c	33.84 ± 1.16a	31.07 ± 1.21b
MDA [nmol g^{-1}(f.m.)]	14.01 ± 0.74c	12.47 ± 0.71c	28.64 ± 0.77a	26.56 ± 1.03b
RWC [%]	81.10 ± 2.52a	84.10 ± 1.41a	55.57 ± 3.19b	59.33 ± 1.91b
Proline [mg g^{-1}(f.m.)]	0.24 ± 0.01c	0.22 ± 0.01c	0.41 ± 0.01a	0.38 ± 0.01b
SOD activity [U mg^{-1}(protein)]	4.93 ± 0.24c	5.42 ± 0.37c	8.32 ± 0.22b	8.87 ± 0.26a
CAT activity [μmol(H_2O_2) mg^{-1}(protein) min^{-1}]	0.44 ± 0.03d	0.51 ± 0.03c	0.86 ± 0.02b	0.91 ± 0.02a
APX activity [μmol(AsA) mg^{-1}(protein) min^{-1}]	1.72 ± 0.08d	1.98 ± 0.19c	4.33 ± 0.09b	4.61 ± 0.14a
GR activity [μmol(NADPH) mg^{-1}(protein) min^{-1}]	0.15 ± 0.01c	0.16 ± 0.01c	0.29 ± 0.01b	0.31 ± 0.01a
AsA [μmol g^{-1}(f.m.)]	5.16 ± 0.09d	5.36 ± 0.07c	7.20 ± 0.14b	7.58 ± 0.10a
GSH [μmol g^{-1}(f.m.)]	8.68 ± 0.49c	8.36 ± 0.23c	10.91 ± 0.20b	11.62 ± 0.36a

Compared to well-watered seedlings, F_v/F_m and Φ_{PS2} decreased remarkably when *C. bungeana* were exposed to drought stress for 3 d (Table 1), showing clearly that drought stress caused a considerable injury of photosynthetic apparatus. Similar results were reported by Cai *et al.* (2010) in *Incarvillea delavayi*. However, EBR pretreatment reduced the decreases in F_v/F_m and Φ_{PS2} in the drought-treated plants, which indicated that exogenous EBR was able to alleviate photoinhibition caused by drought stress and had a strong protective effect on the structure and function of the oxygen-evolving complex of PS 2 in these leaves. Similarly, Xia *et al.* (2009) found that exogenous EBR prevents chilling stress caused photoinhibition in cucumber. The drought-induced decrease in F_v/F_m resulted from concurrent decrease in F_m and increase in F_o compared to the well watered values. Nevertheless under drought, no significant differences between EBR-pretreated and non-EBR-pretreated F_m and F_o were observed (Table 1).

Drought stress increased membrane permeability of *C. bungeana* leaves, but EBR pretreatment significantly ameliorated membrane deterioration (Table 1). In drought-stressed plants, the MDA content, was significantly higher than in the non-drought controls, indicating that pronounced lipid peroxidation occurred under PEG-induced stress. Under drought stress, EBR pretreatment led to greatly reduced MDA content compared with drought stress alone. EBR pretreatment enhanced the RWC of drought-stressed plants, compared with non-pretreated drought-stressed plants whose RWC drastically decreased (Table 1). However, under stress-free conditions, there were no significant differences in membrane permeability, MDA content, and RWC in EBR-pretreated and control seedlings (Table 1).

Proline performs an important function not only as compatible osmolyte but also in scavenging free radicals and in facilitating corrections of altered redox potentials through replenishment of the $NADP^+$ supply (Jain *et al.* 2001). In stress-free *C. bungeana* plants, the proline content was not significantly changed by EBR pretreatment. There was remarkable increase in proline content in drought-stressed seedlings compared to well watered seedlings, but EBR pretreatment resulted in less increase in proline content in stressed plants (Table 1). One reason might be that the expression of Δ^1-pyrroline-5-carboxylate synthase, which catalyzes the rate-limiting step of proline biosynthesis, was inhibited by EBR (Ábrahám *et al.* 2003).

The antioxidative enzyme (SOD, CAT, APX, and GR) activities exhibited increasing trends in response to both drought stress alone and drought with EBR pretreatment (Table 1). However, as shown here and by others (Lee *et al.* 2009), these enzymes usually did not match the increasing ROS production under severe drought stress. Here, EBR pretreatment significantly enhanced the activities of all these enzymes in drought-stressed plants compared with those of drought-stressed alone (Table 1). Thus, it was clear that EBR increased antioxidative enzyme activities and caused enhancements in the ROS scavenging capacities of these seedlings, resulting in alleviating oxidative injury and concurrently improving the plants resistance to drought stress. Extensive reports have also shown that the application of BRs modified antioxidative enzyme activities under salinity (Ali *et al.* 2007), and heavy metal stress (Fariduddin *et al.* 2009). Previous studies have

demonstrated that elevation of antioxidative enzyme activities by BRs is a gene regulated phenomenon (Xia *et al.* 2009). Therefore, enhanced antioxidative enzyme activities observed here may have been the result of *de novo* synthesis and/or activation of the enzymes, mediated through transcription and/or translation of specific genes (Fariduddin *et al.* 2009).

AsA and GSH are substrates in the AsA - GSH cycle and also act as nonezymatic antioxidants involved in defense against oxidative stress. It is well known that APX and GR are the key enzymes participating in scavenging H_2O_2 within a cell *via* the Asada-Halliwell pathway (Foyer *et al.* 1994). Lin and Pu (2010) found that APX activity increased to higher values in salt-stress tolerant sweet potato than in salt-sensitive ones, and the expression of APX isoforms in response to salinity was tissue specific and also dependent on stress duration. Here, drought stress caused increases in APX and GR activities, resulting in AsA and GSH accumulation (Table 1). Furthermore, EBR pretreatment enhanced APX and GR activities, and AsA and GSH contents. It was also demonstrated that higher AsA and GSH contents assisted alleviation of drought-induced oxidative damage and partially increased the drought stress tolerance of these seedlings.

In conclusion, the present results could be used to explain the protective role of EBR on Chl content, the photochemical activity of PS 2, membrane lipids and proteins. The ameliorative effects by EBR were closely associated with EBR-induced changes in antioxidative enzyme activities and antioxidant contents. The results here suggested that EBR could improve plant growth under drought stress.

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