

Effect of Exogenous 24-Epibrassinolide on Salt Resistance of watermelon (*Citrullus lanatus* L.) under Salinity Stress

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Abstract. The present study investigates the role of 24-epibrassinolide (EBL) in inducing plant tolerance to salinity. Seedlings of watermelon (*Citrullus lanatus* L.) were grown in the presence of 70 mM NaCl and were sprayed with 10⁻⁶ M EBL at 7 days after transplantation and were sampled at 28 day. The plants exposed to NaCl exhibited a significant decline in relative growth rate, net CO₂ assimilation, stomatal conductance, transpiration and water use efficiency. However, the follow up treatment with EBL significantly improved the above parameters. EBL treated plants had greater relative growth rate compared to untreated plants when exposed to salt stress. Application of EBL increased photosynthesis by increasing stomatal conductance in both control and salt stressed plants and may have contributed to the enhanced growth. The water use efficiency was improved because CO₂ assimilation is more important than the transpiration.

Introduction

Salinity has been considered as one of the serious environmental stress on plant growth and development as well as crop production. About 20% of irrigated agricultural land is adversely affected by salinity^[1]. Moreover about one-third of the irrigated land on the earth is affected by salinity^[2]. Through its osmotic effect, salinity perturbs a multitude of physiological processes including photosynthesis, a process which is the main determinant of the dry matter accumulation and productivity of the crops. The decline in photosynthesis observed under salt stress is not only attributed to stomata closure leading to a reduction of intercellular CO₂ concentration, but also to non-stomata factors^[3]. Nonstomatal restriction of net assimilation may originate from a reduced efficiency or regeneration capacity of ribulose-1,5-biphosphate (RuBP) carboxylase, a sensitivity of photosystem II to NaCl, or a reduced leaf chlorophyll concentration^[4]. Salinity reduces the ability of plants to utilize water and causes a reduction in growth rate, as well as changes in plant metabolic processes^[5]. All these events lead to poor plant growth and productivity.

Various agronomic and physiological practices are applied to minimize the adverse effects of salinity on the normal functioning of plants. Moreover, plant growth regulators, both natural and synthetic, are widely applied to agricultural crops as a means of crop improvement^[6].

Brassinosteroids (BRs) represent a group of naturally occurring steroidal lactones widely distributed in plant kingdom. Studies with BR biosynthesis mutants and BR insensitive mutants of *Arabidopsis thaliana* have also provided evidence that BRs are essential for plant growth^[7]. Since the identification of the first BRs, almost one hundred structurally related compounds were isolated from various parts of many plant species^[8]. BRs promote plant growth through multiple mechanisms like cell division, cell elongation, vascular differentiation, germination, rhizogenesis, flowering, and modulation of gene expression^[9]. Exogenous application of BRs promote leaf photosynthesis by positively regulating synthesis and activation of a variety of photosynthetic enzymes including Rubisco^[10], and this activity of BRs may contribute to increased crop yield after BRs application^[11,12]. In addition to stimulating growth, BRs have an anti-stress effect on plants. Previous studies showed that salt tolerance of rice^[13], chickpea^[14], Indian mustard^[15] and mung bean^[6] could be markedly enhanced by the application of brassinosteroids.

Watermelon (*Citrullus lanatus*) is considered sensitive^[4] or moderately sensitive to salt stress^[16]. Salinity hampers watermelon growth more during vegetative phase^[17]. Therefore, the present study was conducted with an objective to ameliorate watermelon salt tolerance by using 24-epibrassinolide, a highly active and stable steroidal hormone^[18]. For this purpose, watermelon plants were sprayed with 24-epibrassinolide solution and the effects of salt exposure for 28 days on growth and photosynthesis parameters were studied.

Materials and methods

Watermelon plant growth

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai var. *lanatus*) cultivar Jing Xin was used in the present study. Jing Xin is a twenty year old cultivar but it is still one of the most important commercial selections worldwide and produces elongated fruits characterized by green skin with dark green stripes and sweet, firm flesh containing seeds. Watermelon seeds were purchased from local dealers and grown in green house at Wuhan Agricultural and Sciences Institute, at controlled conditions.

Salt treatment

The seedlings were irrigated with salt water (70 mM NaCl) and were sprayed with distilled water (control) or 10^{-6} M 24-epibrassinolide (EBL was dissolved in a minimal volume of ethanol and then made up to volume with distilled water as described in our previous study^[19]). The plants were sprayed once on the leaves early in the morning. A Hoagland's solution was added weekly to the plants.

Growth analysis

Plant growth was determined destructively over two harvests, the first at the time of EBL application (t_1) and the second occurred 21 days after EBL application (t_2), to examine plant responses to EBL treatment. The leaf area was measured using a LI-3100 leaf meter (LI-COR. Inc., Lincoln, NE, USA). Plants materials (leaves, stem and roots) were dried in a 70 °C oven for 3 days to determine dry weights. Six individual plants were randomly selected per treatment at each harvest. The relative growth rate (RGR, in $\text{mg}\cdot\text{g}^{-1}\cdot\text{j}^{-1}$), the net assimilation rate (NAR, in $\text{mg}\cdot\text{cm}^{-2}\cdot\text{j}^{-1}$) and the leaf area ratio (LAR, in $\text{cm}^2\cdot\text{g}^{-1}$) of each plant was estimated using the equations:

$$\text{RGR} = (\ln W_2 - \ln W_1) / (t_2 - t_1)$$

$$\text{NAR} = (W_2 - W_1) [(\ln A_2 - \ln A_1) / (A_2 - A_1)(t_2 - t_1)]$$

$$\text{LAR} = 0.5[(LA_1/W_1) + (LA_2/W_2)]$$

Where W_1 and A_1 are the plant dry weight and total leaf area, respectively, at the initial time (t_1), and W_2 and A_2 are the plant dry weight and total leaf area at the final harvest (t_2)^[20].

Chlorophyll fluorescence

Chlorophyll fluorescence measurements were made in attached leaves with a portable fluorometer F.I.M,1500,ADC (Fluorescence Induction Monitor 1550, Analytical Development Company Limited). Leaves were dark-adapted for 30 min, then dark fluorescence F_o , maximal fluorescence F_m , and photochemical yield F_v/F_m ($F_v = F_m - F_o$) were recorded.

F_o is the initial fluorescence emission by antenna Chl a molecules. F_m is the maximum total fluorescence value; $F_v = F_m - F_o$ is the variable fluorescence. The F_v/F_m ratio measures the efficiency of excitation energy captured by open PSII reaction centres representing the maximum capacity of light-dependent charge separation^[21].

Gas exchange

Photosynthetic rate (P_n), stomatal conductance (G_s) and transpiration (E) measurements were made on the upper fourth or fifth fully expanded leaf, between 9:00 h and 11:00 h using a portable gas exchange system (CI-301 CO₂ GAS ANALYSER, CID, Inc.). The atmospheric conditions during the experiments were: PAR, $1100 \pm 50 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$; C_i , $280 \pm 15 \mu\text{mol} \cdot \text{mol}^{-1}$; atmospheric CO₂, $355 \pm 5 \mu\text{mol} \cdot \text{mol}^{-1}$; relative humidity, $65 \pm 5\%$; atmospheric temperature, $25 \pm 2^\circ\text{C}$.

Statistical analysis

Data was subjected to one-way analysis of variance (ANOVA) and the mean differences were compared by Duncan Test. Each value was the mean of six replicates ($n=6$) and comparisons with P-values <0.05 were considered significantly different.

Table 1. Evaluation of relative growth rate (RGR), net assimilation rate (NAR) and leaf area ratio (LAR) of watermelon plants (var. Beldi) were subjected to foliar application of 24-epibrassinolide (EBL) under control or saline condition.

Treatments	RGR($\text{mg} \cdot \text{g}^{-1} \cdot \text{j}^{-1}$)	NAR($\text{mg} \cdot \text{cm}^{-2} \cdot \text{j}^{-1}$)	LAR($\text{cm}^2 \cdot \text{g}^{-1}$)
Control	55.8b	191.1b	21.12b
EBL	69.1a	215.3a	23.34ab
NaCl	47.4c	174.4c	20.05b
NaCl +EBL	60.7b	196.3b	23.14ab

Data were reported as means ($n=6$). Means were separated by Duncan's test, different letters in a single column show statistically significant differences for $P < 0.05$.

Table 2. Chlorophyll fluorescence parameters (F0, Fm and Fv/Fm) of watermelon plants (var. Beldi) were subjected to foliar application of 24-epibrassinolide (EBL) under control or saline condition.

Treatments	Fo	Fm	Fv/Fm
Control	286a	1593a	0.818a
EBL	285a	1538a	0.812a
NaCl	289a	1579a	0.815a
NaCl +EBL	281a	1508a	0.813a

Data were reported as means (n=6). Means were separated by Duncan's test, different letters in a single column show statistically significant differences for P<0.05.

Table 3. Effects of 24-epibrassinolide (10^{-6} M) and NaCl-stress (70mM) on the photosynthetic rate (Pn, $\mu\text{mol CO}_2 \text{ m}^{-2}\cdot\text{s}^{-1}$), stomatal conductance (Gs, $\text{mmol m}^{-2}\cdot\text{s}^{-1}$), transpiration (E, $\text{mmol H}_2\text{O m}^{-1}\cdot\text{s}^{-1}$) and water use efficiency (WUE, $\mu\text{mol CO}_2/\text{mmol H}_2\text{O}$) in watermelon leaves.

Treatments	Pn	E	Gs	WUE
Control	14.1b	3.4a	209b	3.9ab
EBL	16.2a	3.6a	328a	4.2a
NaCl	9.3d	2.7b	216d	3.1bc
NaCl +EBL	11.9c	3.1ab	268c	3.4b

Data were reported as means (n=6). Means were separated by Duncan's test, different letters in a single column show statistically significant differences for P<0.05.

Results

Growth analysis

Application of 70 mM NaCl to watermelon plants adversely influenced relative growth rate (RGR) as compared with control plants (Table 1). Analysis of RGR components shows that this inhibition is due to a significant reduction of net assimilation rate (NAR) and not to the leaf expansion (LAR). When plants grown in the presence or absence of sodium chloride are subjected to EBL treatment, RGR was significantly improved compared with control plants (Table 1). This increase is accompanied by significant increases in the NAR and unchanged for the LAR.

Chlorophyll fluorescence

The changes in PSII photochemistry were investigated in the dark-adapted leaves. Table 2 shows that neither NaCl nor EBL application affected the minimal Chl *a* fluorescence, F_0 , the maximal Chl *a* fluorescence, F_m and therefore the maximum quantum efficiency of PSII photochemistry (F_v/F_m).

Gas exchange

The net assimilation (P_n), transpiration (E) and stomatal conductance (G_s) were significantly ($p < 0.05$) declined in the plants, exposed to NaCl stress (Table 3). It appears from our results that the decline in net photosynthesis (P_n) is mainly attributed to the decrease in stomatal conductance, but not to the damage of the photosynthetic apparatus, since the activity of PSII (estimated by the ratio of F_v/F_m) was preserved in the dark adapted leaves under NaCl-stress (Table 2).

Treatment with EBR has a positive effect on the photosynthetic activity of control and NaCl-stressed plants, which is associated with a parallel positive effect on stomatal conductance. In relative terms, the impact of EBR on carbon fixation is greater than the impact on stomatal conductance (and thus the leaf transpiration); the result is a positive effect of EBR on plant water use efficiency (Table 3).

Discussion

The growth analysis showed that 70 mM NaCl treatment induced a significant reduction in relative growth rate (RGR) of watermelon plants as compared to the control (Table 1). This is consistent with previous reports that watermelon plants are relatively sensitive to salt stress^[4,16,22]. This reduction may be the result of slower leaf expansion (LAR)^[23] or the reduction of photosynthetic activity (NAR)^[24]. Our results show that inhibition of biosynthetic activity (RGR) was estimated by a decline in photosynthesis (NAR) rather than a reduction in leaf area (LAR). This suggests that photosynthesis is the limiting factor for growth. Changes in CO₂ assimilation may be attributable to either stomatal or non-stomatal factors or both^[25]. Our data show that the presence of salt in the root medium caused a reduction in stomatal conductance (G_s) of watermelon plants (Table 2). Similar results were obtained by Martinez-Ballesta^[26] and Lycoskoufis^[4], where G_s was reduced by salt treatments. A decrease in G_s was observed for the NaCl treatments, probably caused by closure of the stomata or a decrease in water uptake through the roots^[26]. The decrease in water flow by salinity may cause lowering in leaf water content that would result in stomatal closure in order to maintain their water status^[27]. The stomatal closure is likely the first plant defense against desiccation and an important factor to control carbon fixation^[28]. Measurements of Chl *a* fluorescence parameters were attempted to evaluate the direct effects of salt stress on PSII photochemistry. Results revealed that NaCl treatment have no effect on leaf photochemistry, and photosystem II (PSII) remained resistant to salt stress as reported by Belkhodja^[29] and Zribi^[30], they showed no significant change in the photosynthetic quantum yield (F_v/F_m) in response to NaCl treatments. It seems from the results that the decline in net photosynthesis (P_n) may result from a reduced stomatal conductance, but not to injuries to the photosynthetic apparatus, since PSII activity (estimated by the ratio F_v/F_m) of dark adapted leaves were preserved under salt stress (Table 2). Parallel decreases in stomatal conductance and net photosynthesis due to NaCl salinity have been reported for watermelon plants^[16,26,31].

Exogenous application of EBL improved the RGR of watermelon plants. These results are not surprising since brassinosteroids were implicated in cell elongation and differentiation^[32]. However, the increase in RGR was attributed to the increase in the physiological growth parameter (NAR) rather

than the morphological growth parameter (LAR). This suggests that EBL-induced salt tolerance in watermelon plant. Since salinity inhibits plant growth by adversely affecting various physiological and biochemical processes including photosynthesis, antioxidant capacity, and ion homeostasis, it is suggested that BRs enhanced growth of salt stressed plants might have been due to BRs-induced changes in these physiological or biochemical processes^[6,13,14,15]. For example, Ali^[14] reported that EBL increased growth in Indian mustard plants could be related to enhanced activity of antioxidative enzymes and proline level that protect the plants from oxidative damage. Therefore, photosynthesis which is a major controlling factor for plant growth and yield might have been increased due to EBL application^[12]. In this study, foliar application of EBL enhanced CO₂ assimilation rate in watermelon plants. Therefore, the promotion of growth in watermelon plants by EBL under NaCl-stress appears at least correlatively linked with enhanced photosynthesis. Increased growth and CO₂ assimilation following the application of BRs under salt stress have been reported in case the mustard^[13,14], and mung bean plant^[6]. EBL activated the rate of photosynthesis may be by increasing stomatal conductance (Table 3), facilitating the diffusion of carbon dioxide into the stomatal cavity^[33]. However, EBL has no effect on quantum efficiency (Table 2). Similarly, no significant changes of Fv/Fm value in wheat, grown hydroponically in EBL-containing medium^[14] or foliar sprayed^[34] (Shahbaz *et al.*, 2008). Yu^[12] noted that EBL significantly increased the light saturated net CO₂ assimilation rate from 3 h to 7 d after spraying with 0.1 mg l⁻¹ EBL in watermelon. Increased CO₂ assimilation rate in EBL treated leaves was accompanied by a higher quantum yield of PSII electron transport, mainly due to a significant increase in the photochemical quenching and unchanged efficiency of energy capture by open PSII reaction centers^[12]. Moreover, BRs had a positive effect on the activation of Rubisco based on increased maximum Rubisco carboxylation rates ($V_{c,max}$), total Rubisco activity and, to a greater extent, initial Rubisco activity induced by an enhanced expression of genes encoding other Calvin cycle genes after BRs treatment might also play a positive role in RuBP regeneration/ J_{max} , thereby increasing maximum carboxylation rate of Rubisco ($V_{c,max}$). Thus, BRs promote photosynthesis by positively regulating synthesis and activation of a variety of photosynthetic enzymes including Rubisco^[10].

In conclusion, the foliar spray of 24-epibrassinolide has a positive effect on growth and plant metabolism. Indeed, it reduces some disruption caused by NaCl-stress: attenuation of growth inhibition and stimulation of photosynthetic characteristics of watermelon under control or saline conditions. These results suggest a potential use of 24-epibrassinolide which to be confirmed by other experiments and field trials.

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