



Exogenous 24-Epibrassinolide Alleviates Effects of Salt Stress on Chloroplasts and Photosynthesis in *Robinia pseudoacacia* L. Seedlings

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Abstract

The brassinosteroids (BRs) constitute a recently defined class of plant hormone that can enhance the resistance of plants to multiple stresses. However, the effects of BRs on salt-stressed woody plants, notably on photosynthesis and chloroplast ultrastructure, have received little attention. Black locust (*Robinia pseudoacacia* L.) seeds and seedlings were pretreated with $1.04 \mu\text{mol L}^{-1}$ 24-epibrassinolide (24-*epiBL*) by soaking and root dipping, respectively, and grown under non-saline or saline conditions (0, 100, 200 mmol L^{-1} NaCl). Salinity stress decreased photosynthesis, chlorophyll concentration, transpiration, and stomatal conductance but also decreased the water-use efficiency, while chlorophyll fluorescence indicated a decrease in photochemical quenching and in maximum potential quantum efficiency. Indicators of oxidative stress (for example, H_2O_2 and antioxidant enzymes), membrane leakage, and amounts of Na^+ ions in leaves and chloroplasts were increased and, at the highest stress, chloroplast ultrastructure was severely disrupted. Exogenous 24-*epiBL* improved membrane stability and reduced foliar Na^+ levels, while substantially alleviating stress-induced changes in photosynthetic gas exchange. Improvements in chlorophyll content and indicators of oxidative stress were not as large but were still highly significant. Thylakoid membrane structure was protected. Both methods of applying 24-*epiBL* were effective, but root-dipped seedlings performed marginally better. The results suggest that treatment of black locust seedlings with 24-*epiBL* prior to planting may improve performance and aid establishment on salt-affected soils.

Keywords 24-*epiBL* · Salt stress · Chloroplast · Photosynthesis · Ultrastructure

Introduction

Abiotic stresses such as soil salinity, drought, flooding, or extreme temperatures reduce crop productivity and impact global food security (Thakur et al. 2010). Worldwide, over 1 billion hectares of land have been damaged by salt (Shahid

2013) causing huge losses in agricultural production (Bartels and Sunkar 2005; Wang et al. 2003). Vegetative bioremediation is a crucially important reclamation approach on saline-alkali soils (Qadir and Oster 2004); for example, trees can be used in agroforestry applications to reduce dryland salinity and related soil degradation problems (Ellis and van Dijk 2009).

China, the world's most populous country (1.38 billion people in 2015) has the third largest area of saline soils (366,500 km^2), accounting for 4.88% of the country's arable land (Li et al. 2014). Black locust (*Robinia pseudoacacia* L.), an arboreal member of the *Fabaceae* indigenous to the eastern United States, has been widely cultivated in China since the early twentieth century (Tateno et al. 2007; Akatov et al. 2016) where it is now the third most planted tree after hybrid poplar and eucalyptus (DeGomez and Wagner 2001). The species has also been introduced to Africa, Europe, Australia, New Zealand, and other parts of Asia and North America. The popularity of black locust is connected

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with rapid growth, symbiotic nitrogen-fixation, adaptability to a wide range of environmental conditions, abundant seed crops, and high timber and honey yields. It is a major tree species for planting into degraded areas such as exposed sand (Rahmonov 2009), mine wastes (DeGomez and Wagner 2001), and saline–alkali soils (Wang et al. 2013) for purposes of soil amelioration, erosion control, and reforestation (Sitzia et al. 2012). Studies have shown, however, that black locust is only weakly salt-tolerant, withstanding salinity contents of up to only 0.3% under laboratory conditions, or to just over 0.5% under field conditions (Liu et al. 2010). As of 2001 (CSDI of Ministry of Construction, China), saline–alkali soils in China have been classified into four degree levels according to the content of NaCl per 100 g dry weight of soil: weak (0.3–1%), moderate (1–5%), heavy (5–8%), and super heavy (> 8%). Therefore, the salt tolerance of black locust needs improvement if it is to be successful on even weakly affected soils.

Plant hormones have long been implicated in regulating plant development and tolerance or susceptibility to diverse stresses, including salinity stress. Brassinosteroids (BRs) are a group of plant steroid hormones originally indicated by the work of Mitchell et al. (1970), and brassinolide was the first BR to be isolated and identified (Grove et al. 1979). Brassinosteroids are perceived by a family of plasma membrane-localized leucine-rich-repeat-receptor kinases in interaction with a co-receptor. The ensuing signal cascade releases several transcription factors that regulate the expression of over a 1000 genes involved in a broad range of physiological functions. BRs participate in the regulation of cell division and expansion, germination, vascular differentiation, root growth, vegetative and reproductive development, homeostasis, and programmed cell death (Kemmerling et al. 2007; Divi and Krishna 2009; Park et al. 2010).

Protective effects of BRs against biotic and abiotic stresses have been frequently reported; e.g., in wheat (Talaat and Shawky 2012), rice (Thussaganpanit et al. 2015), zucchini (Tao et al. 2015), tea (Li et al. 2016), radish (Choudhary et al. 2011), *Arabidopsis* (Park et al. 2010), grape (Wang et al. 2015), and eggplant (Wu et al. 2014). BR-mediated responses to stress occur across different levels of organization and are not fully understood (Sharma et al. 2017). A general observation, however, is that application of BRs lowers oxidative stress. Cui et al. (2016) showed that exogenous application of 0.1 μM 24-epibrassinolide (24-*epiBL*) promoted antioxidant enzyme systems in tomato. Similarly, a 24-*epiBL* pretreatment (0.2–0.3 mM) could restrain reactive oxygen species (ROS) and lipid peroxidation of young grapevine seedlings under chilling stress (Xi et al. 2013). Brassinosteroids may reduce oxidative stress in part through interactions with signal transduction pathways for salicylic acid (Sharma et al. 2017), ethylene (Wei et al. 2015), and nitric oxide (Zhu et al. 2015).

The growth and productivity of plants has a close relationship with photosynthesis, which is one of the most severely affected processes during salinity stress (Sudhir and Murthy 2004). Under salt stress, the root application of 24-*epiBL* was able to enhance the photosynthetic capacity of wheat plants (Ali et al. 2008b). Similarly, foliar application of 24-*epiBL* enhanced photosynthesis in *Brassica juncea* (L.) Czern. (Ali et al. 2008a). Hayat et al. (2010) reported that BRs could maintain photosynthesis by protecting the quantum yield of PSII under salt stress. Existing reports of the effects of BRs on salt-resistance mainly deal with herbaceous crops, but there is little data pertaining to woody plants, particularly at the organelle (that is, chloroplast) level.

To devise new strategies to enhance salt-affected soils, we sought to improve the early tolerance of black locust to salinity stress. To that end, we compared growth and photosynthesis in control and 24-*epiBL*-treated plants exposed to two different salt levels and conducted measurements of oxidative stress, membrane integrity, chlorophyll content, Na^+ content, fluorescence parameters, and chloroplast ultrastructure. Our objective was to determine whether exogenous 24-*epiBL* could improve the performance of black locust seedlings exposed to higher levels of salt than they would normally tolerate, to enhance the availability of alkaline land for food production.

Materials and Methods

Plant Material and 24-*epiBL* Treatments

The seeds of black locust (*Robinia pseudoacacia* L.) used in this study were obtained from South Forest Tree and Seed Testing Center of the State Forestry Administration in China and experiments were conducted from September 2014 to July 2015. Seeds were surface sterilized in hot deionized water (85–95 °C) for a few seconds to remove the waxy cuticle. Seeds were then soaked in either distilled water or 1.04 μM 24-*epiBL* (Sigma-Aldrich, St. Louis, MO, USA) for 24 h before sowing into trays containing quartz sand moistened with either distilled water or 1.04 μM 24-*epiBL*, respectively. The 24-*epiBL* was prepared by first dissolving 0.1 mg 24-*epiBL* in 10 mL absolute ethanol as a stock solution; this was diluted just before use to yield the 1.04 μM treatment solution in 0.1% Teepol. Seeds were germinated at 25 °C under constant light at a photosynthetic photon flux density (PPFD) of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After 10 days, when cotyledons were fully expanded, seedlings were transplanted into seedling bags (10 cm diameter \times 15 cm depth) filled with a mixture of vermiculite, peat, and perlite (1:2:2, v/v). Seedlings were cultivated under controlled conditions in a greenhouse at the College of Forestry, Nanjing Forestry

University (32°7'N, 119°12'E), Jiangsu Province, China. Supplemental lighting provided a minimum 12-h photoperiod and PPFD of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and the day/night temperature regime was 26 °C/16 °C.

Seedlings grew without stress for 6 months until the spring of 2015, by which time they were ~45 cm tall. At this point, all seedlings were washed of rooting medium. Seedlings from seeds pretreated with 24-*epiBL* were replanted directly into plastic pots (40 cm × 25 cm × 15 cm; 1 plant per pot) containing coarse sand and vermiculite 2:1 (v:v). Seedlings from untreated seeds were split into two groups and root-dipped for 48 h in either distilled water or 1.04 μM 24-*epiBL* (refreshed every 12 h) prior to being replanted in the same way. Afterwards, seedlings were watered to the drip-point every 7 days with a modified Hoagland's nutrient solution (Hoagland and Arnon 1950) containing 0.2 mM KH_2PO_4 , 1.0 mM K_2SO_4 , 2.0 mM $\text{Ca}(\text{NO}_3)_2$, 0.5 mM MgSO_4 , 200 μM Fe-EDTA, 5 μM H_3BO_3 , 2 μM MnSO_4 , 0.5 μM ZnSO_4 , 0.3 μM CuSO_4 , and 0.01 μM $(\text{NH}_4)_2\text{Mo}_7\text{O}_{24}$, with pH adjusted to 7.0.

Groups and Salt Stress Treatments

After a further 2 weeks, seedlings of uniform height (~50 cm) were selected for the salt stress treatments. There were three groups: seedlings never exposed to 24-*epiBL* (CK); seedlings from seeds treated with 24-*epiBL* (Sew); and seedlings root-dipped in 24-*epiBL* (Diw). Fifteen seedlings of each group were subjected to each of three levels of salt stress: 0, 100, and 200 mM NaCl, all in Hoagland's solution. The overall experiment, therefore, consisted of nine treatment combinations in a 2-way factorial: CK-0, CK-100, CK-200, Sew-0, Sew-100, Sew-200, Diw-0, Diw-100, and Diw-200. Pots were flushed with their respective nutrient solutions and then, thereafter, the water content was kept near saturation (40%) by automatic drip-feeding with fresh solution delivered by an Intelligent Automatic Watering System (patent CN 201398356 Y). Data for gas exchange, chlorophyll fluorescence, antioxidant systems, and chloroplast ultrastructure were collected as described below, beginning 45 days after treatments commenced. Over the measurement period, the day/night temperature regime was 29 °C/18 °C, relative humidity varied from 45 to 80%, and natural lighting provided a photoperiod of about 14 h and a mid-day PPFD of ~1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Determination of Gas Exchange Variables

Leaf gas exchange was measured using an infrared gas exchange analyzer (LI-6400, LI-COR Inc., Lincoln, NE, USA) from 09:30 to 11:30 h local time, on clear days in May. The net photosynthetic rate (A_n), stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration

(C_i) were measured on 0.7 cm × 3.0 cm (2.1 cm²) of leaf area using an ambient CO_2 concentration (C_a) of 380 $\mu\text{mol mol}^{-1}$. The relative air humidity and leaf temperature in the chamber were maintained at ~35% and 25 °C, respectively, while PPFD ranged from 1200 to 1400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For each treatment, four fully expanded leaves from each of three seedlings were assayed. The stomatal limitation (L_s) and the water-use efficiency (WUE) were calculated as $L_s = 1 - C_i/C_a$ and $WUE = A_n/E$, respectively, according to Li et al. (2013).

Chlorophyll Fluorescence

Chlorophyll fluorescence was measured using a portable fluorometer (PAM-2500; Walz, Effeltrich, Germany) as described by Roháček (2002). The data were taken on the fourth fully expanded leaf of each seedling, numbered basipetally. The minimal (F_o) and maximal (F_m) chlorophyll fluorescence emission was determined after 30 min of dark adaptation, and minimal (F'_o) and maximal (F'_m) fluorescence level in the light-adapted state, and the steady-state value of fluorescence (F_s), were measured after light adaption at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. We report the maximum quantum yield of PSII as F_v/F_m , the size and number of open reaction centers as F_v/F_o , photochemical quenching (Q_p) as $(F'_m - F_s)/(F'_m - F'_o)$, and non-photochemical quenching (NPQ) as $(F_m - F'_m)/F'_m$.

Estimation of Membrane Stability Index

The membrane stability index (MSI) was estimated by taking 200 mg leaf material in 10 mL of double-distilled water (DDW) in two sets. One set was heated at 40 °C for 30 min in a water bath and the electrical conductivity (C_1) was measured on a conductivity meter. The second set was boiled at 100 °C in a boiling water bath for 10 min and its conductivity was also measured (C_2). MSI was calculated using the formula given by Sairam (1994):

$$MSI = \left[1 - \frac{C_1}{C_2} \times 100\% \right].$$

Determination of Relative Water Content

Relative water content (RWC) was determined on fresh leaf discs of 2 cm diameter. Discs were weighed quickly (FW) and immediately floated on DDW for 24 h in the dark. Discs were blotted dry and reweighed to obtain mass at full turgor (TW), then dried at 70 °C for 48 h to obtain the dry mass (DW). RWC was calculated according to Hayat et al. (2007):

$$RWC = \frac{FW - DW}{TW - DW} \times 100\%.$$

Element Analysis by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

At the final harvest time (day 50), leaves (numbers 6–8, counting from the base of the shoot) were sampled from control and salt-stressed plants. Following the procedures of Hajibagheri and Flowers (1984), 30–50 mg of oven-dried leaf tissue was added to 2 mL 65% HNO₃ and pressure-extracted for 12 h at 140 °C on a SpeedDigerter K-436 (BUCHI, Switzerland) until the liquid was almost clear. It was then filtered, diluted as necessary, and used for elemental analysis on a ICP-OES spectrometer (Spectro Ciros CCD, GmbH & Co KG, Kleve, Germany) at wavelengths of 226–502 nm (Abo-Ogiala et al. 2014).

Observation of Chloroplast Ultrastructure

The ultrastructure of the chloroplasts was observed as described by Shu et al. (2013). Leaf segments were cut into pieces of approximately 1 mm². The leaf pieces were immersed in a mixture of 3% glutaraldehyde and 1% formaldehyde in a 0.1 M phosphate buffer (pH 7.4) for 2 h (primary fixation) and then 2% osmic acid in the same buffer for 2 h (secondary fixation). After dehydration in acetone and embedding in Durcupan ACM (Fluka), ultrathin sections were cut, stained with uranium acetate and lead citrate in series, and examined under a Hitachi transmission electron microscope (Carl Zeiss, Göttingen, Germany) using an 80-kV acceleration voltage.

Isolation of Chloroplasts

Chloroplasts were isolated from fully expanded leaves by differential and density gradient centrifugation as described by Cerović and Plesnicar (1984) and Song et al. (2006) with some modifications. Briefly, 5 g of leaflets were chopped using a blender (HR-2826; Philips, Zhuhai, China) with five volumes of a medium containing 0.4 M sucrose, 2 mM EDTA, 5 mM MgCl₂·6H₂O, and 0.1% BSA in 50 mM Tris-HCl buffer (pH 7.6). The homogenate was squeezed through six layers of gauze and the filtrate was centrifuged at 2000×g for 2 min. The resulting pellet was resuspended in 2 mL of medium containing 0.3 M sorbitol, 2 mM EDTA, and 1 mM MgCl₂·6H₂O in 50 mM HEPES-KOH buffer (pH 7.8), which was put into a tube containing 8 mL of resuspension medium plus 40–80% (v/v) Percoll and centrifuged for 3 min at 2000×g. The interlayer (7 mL) between 40% and 80% Percoll contained intact, isolated chloroplasts. All procedures were carried out at 4 °C.

A 2 mL aliquot of the interlayer was brought up to 10 mL with 0.05 M sodium phosphate buffer (pH 7.8) containing 5 mM EDTA, for analysis of antioxidant enzymes, H₂O₂, and malondialdehyde (MDA). For measurement of chlorophyll a

(Chl *a*), chlorophyll b (Chl *b*) and total chlorophyll, another 2 mL aliquot was spun down (1000×g for 10 min at 4 °C) and the pelleted chloroplasts were resuspended in 8 mL of 80% acetone and assayed spectrophotometrically according to Arnon (1949) by reading the absorbance at 645 and 663 nm. For chloroplast Na⁺ content, pelleted chloroplasts from a final 1 mL aliquot were extracted with 5 mL of 0.5 M HCl by shaking on a water bath at 50 °C for 45 min. The volume was brought up to 10 mL with 0.5 M HCl before filtering and diluting for ICP-OES as described above.

Assays for H₂O₂, MDA, and Antioxidant Enzyme Activities

The H₂O₂ assay was based on the formation and spectrophotometric measurement of a titanium peroxide complex according to Patterson et al. (1984) as modified by Diao et al. (2014). MDA was measured in terms of thiobarbituric acid reactive substances (TBARS) following Heath and Packer (1968). Superoxide dismutase (SOD) was assayed by monitoring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm (Beyer and Fridovich 1987). One unit of SOD activity is the amount required to reduce the rate of reduction by 50% at 560 nm. Ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981) by following the decrease in absorbance at 290 nm as ascorbate was oxidized by added H₂O₂. One unit of APX is the amount of enzyme required to oxidize ascorbate at a rate of 1 mM min⁻¹.

Statistical Analysis

All biochemical analyses had at least three biological replicates. Data were statistically analyzed with R 3.3.0 (SPSS Inc., Chicago, IL, USA) using a two-factorial completely randomized design. Two-way analyses of variance (ANOVA) were employed to test the effects of salt stress, 24-*epiBL* and their interaction, and means were separated using Duncan's multiple range tests. Where necessary (APX, MDA, *MSI*, and Na⁺ concentrations in leaves and chloroplasts), data were square root and/or log transformed to meet assumptions of normality and homogeneity of variance. Standard error bars in figures are based on untransformed data. Differences were considered significant at *P* < 0.05.

Results

Gas Exchange

In the absence of salt stress, neither of the 24-*epiBL* treatments had a significant effect on photosynthesis (*A_n*), transpiration (*E*), stomatal conductance (*g_s*), water-use efficiency

(WUE), or the degree of stomatal limitation (L_s), but the Diw treatment did result in a lower intercellular CO_2 concentration (C_i). Salt stress, in the absence of any $24-epiBL$ treatment, reduced A_n to about a quarter the control rate (Fig. 1a). This dramatic inhibition of photosynthesis was partially prevented by treatment with $24-epiBL$, particularly with Diw. Indeed, $24-epiBL$ -treated plants had photosynthetic rates that were 3.0–3.8 times higher than CK-100 or CK-200 plants. Transpiration rates showed very similar though less dramatic differences. Concurrent with the effects of salt stress on A_n and E was a general reduction in g_s . Stomatal conductance was unaffected by $24-epiBL$ treatment at 100 mM NaCl but both Sew and Diw prevented any further decrease in g_s at 200 mM NaCl. Overall, the net effect of the changes in A_n and E under salt stress was a large reduction in WUE (Fig. 1e), but this effect was entirely reversible by pretreatment with $24-epiBL$. These changes in WUE occurred in the absence of any consistent change in C_i or L_s ; raising the possibility that the effects of salt stress, and the protective effects of $24-epiBL$, were at the level of the mesophyll.

Chlorophyll Fluorescence

The $24-epiBL$ pretreatments had no effect on the maximum quantum yield of PSII (F_v/F_m) or the potential photochemical efficiency of PSII (F_v/F_o) in unstressed black locust seedlings, but did increase both the photochemical (Q_p) and non-photochemical (NPQ) quenching of fluorescence in these seedlings (Fig. 2), suggesting an increase in open PSII reaction centers and enhanced heat dissipation of excitation energy. Salt stress led to a small decrease in F_v/F_m ($P=0.002$), which was prevented by $24-epiBL$ pretreatment of roots (Diw) but not seeds (Sew). There were large decreases in F_v/F_o at 100 mM NaCl ($P<0.001$) but no further decline in this variable at 200 mM NaCl (Fig. 2b). As in the zero salt controls, the $24-epiBL$ pretreatments had no effect on F_v/F_o . Non-photochemical quenching was about 1.2–1.3 times higher at 100 mM NaCl, and perhaps slightly more at 200 mM NaCl (Fig. 2c). Although $24-epiBL$ increased NPQ under control conditions, it had no further effect under salt stress ($P=0.011$ for the interaction effect). Photochemical quenching was substantially reduced by salt stress ($P<0.001$) and more so at 200 than at 100 mM NaCl (Fig. 2d). Pretreatment of seeds with $24-epiBL$ enhanced Q_p by 31% at 100 mM NaCl but was not significantly effective at 200 mM; in contrast, unlike under control conditions, the root-dipping pretreatment was without effect under either level of salinity stress.

Chloroplast Ultrastructure and Chlorophyll Levels

Alterations in the structure of chloroplasts and thylakoids induced by salt stress are illustrated in Fig. 3. Relative to

controls (Fig. 3a, b), the chloroplasts of salt-stressed plants were swollen and partly separated from the plasma membrane (Fig. 3c, e). The shape of chloroplasts changed from elliptical to nearly round, and the lamellar structure of thylakoids was less regular, particularly at 200 mM NaCl (Fig. 3e, f). Plastoglobuli were swollen but fewer in number compared to the control. Under the higher salt stress (200 mM NaCl), the cell wall was bent and starch granules were smaller and less well defined (Fig. 3e, f). These severe impacts on chloroplast structure were partly alleviated by the $24-epiBL$ pretreatments. Application of $24-epiBL$ increased the number of plastoglobuli and maintained the fabric of thylakoid lamellae relative to stressed controls (Fig. 3g–n). At 100 mM NaCl, both $24-epiBL$ pretreatments decreased the swelling of chloroplasts (compare panels g and i with c) and preserved the structure of thylakoid lamellae (compare panels h and j with d). Swelling of chloroplasts at 200 mM NaCl was not avoided by the $24-epiBL$ pretreatments, but lamellae appeared relatively normal, particularly with the Diw pretreatment (Fig. 3k–n).

Pretreatment with $24-epiBL$ had a highly significant positive effect on Chl *a*, Chl *b*, and total chlorophyll concentrations of chloroplasts (Table 1). Concentrations reached their highest levels in Diw, but these were only significantly greater than Sew in the case of Chl *a*. Salt-stressed plants had substantially lower chlorophyll concentrations than the unstressed controls. The $24-epiBL$ pretreatments partially ameliorated this decline, but not statistically so in all cases (the interaction effect was significant for Chl *a* and total chlorophyll, but not for Chl *b*). The root-dipping pretreatment was the most effective, increasing total chlorophyll by 81% at 200 mM NaCl (Table 1).

Tissue and Chloroplast Na^+ Concentrations

Sodium concentrations in leaves (per unit fresh mass) and chloroplasts (per unit protein) followed very similar trends (Fig. 4). The 100 and 200 mM NaCl treatments increased foliar Na^+ by 2.8-fold and 11.7-fold, and chloroplast Na^+ by 5.5-fold and 16.8-fold, respectively. The $24-epiBL$ pretreatments reduced Na^+ levels in leaves of controls and salt-stressed plants, with root dipping being most effective in the latter (Fig. 4a). In contrast, the $24-epiBL$ pretreatments did not result in lower Na^+ in chloroplasts at 200 mM NaCl, but did result in a 24–28% reduction at 100 mM NaCl (interaction effect significant at $P=0.003$).

Membrane Stability Index and Relative Water Content

The effects of salt stress, pretreatment with $24-epiBL$, and their interaction on membrane stability were all highly significant (Fig. 5a). The $24-epiBL$ pretreatments had no effect

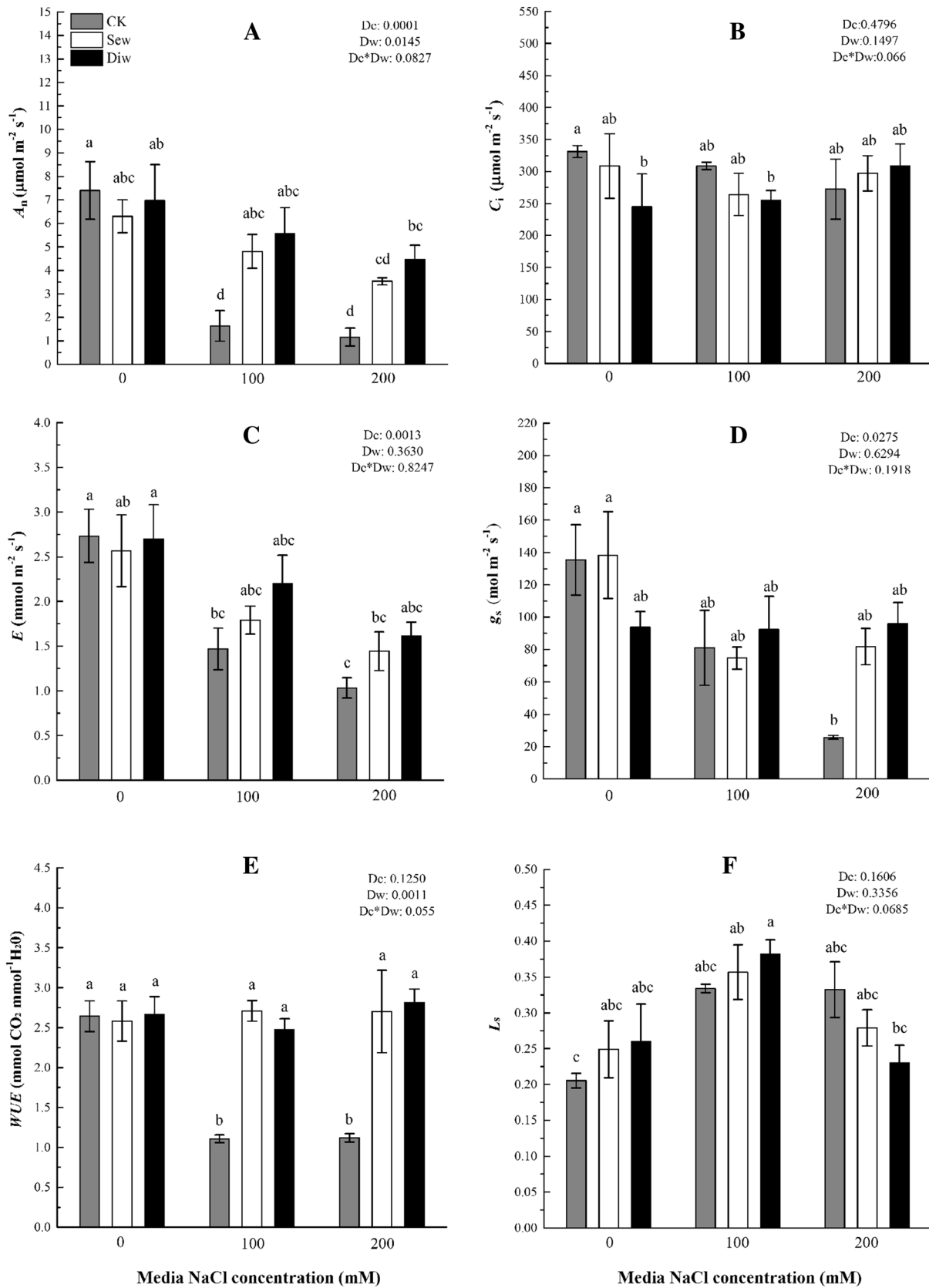


Fig. 1 Changes in **a** net photosynthetic rate (A_n), **b** intercellular CO_2 concentration (C_i), **c** transpiration rate (E), **d** stomatal conductance (g_s), **e** water-use efficiency (WUE), and **f** stomatal limitation (L_s) in leaves of black locust seedlings at three levels of salt stress, with or without prior treatment with 24-*epiBL* (CK: no 24-*epiBL*; Sew: seeds soaked in 1.04 μM 24-*epiBL*; Diw: roots dipped in 1.04 μM 24-*epiBL*). Each bar represents the mean (\pm SE) of three replicates. D_c , D_w , and $D_c \cdot D_w$ are the P values for the salt concentration effect, the 24-*epiBL* application effect, and the interaction effect between salt concentration and 24-*epiBL* application, respectively. Different letters above bars show significant differences between means ($P < 0.05$)

on the MSI in the absence of stress. However, under the NaCl treatments, both Sew and Diw were dramatically effective in maintaining the MSI near the control CK-0 level. In sharp contrast, the MSI fell to 67.8% and 46.1% of the control level at 100 and 200 mM NaCl, respectively, in seedlings that received no 24-*epiBL* pretreatment. Changes in relative water content (RWC) were similar in direction to changes in MSI , but not as pronounced (Fig. 5b). Only the Diw pretreatment was significantly different from the seedlings that were not treated with 24-*epiBL*, and only under salt stress.

H_2O_2 , MDA, and Antioxidant Systems

Chloroplasts of salt-stressed plants (CK-100, CK-200) had increased levels of H_2O_2 (Table 2) and, especially, MDA when compared to the unstressed control (CK-0). Pretreatment with 24-*epiBL* had no effect in the absence of salt stress but substantially reduced accumulations of H_2O_2 and MDA in the presence of stress. Again, Diw was generally more effective than Sew, but not statistically so in every case (interaction effect significant at $P < 0.0001$). Root dipping decreased the amounts of H_2O_2 and MDA by 68% and 92% at 100 mM NaCl, and by 59% and 74% at 200 mM NaCl.

Activities of APX and SOD were increased by 2.37 and 2.65 times, respectively, in chloroplasts under 100 mM NaCl stress, but under 200 mM NaCl the APX activity decreased dramatically (by 85% relative to CK-0) and SOD activity was also reduced but still exceeded the unstressed controls by 1.58 times (Table 2). Treatment of no salt controls with 24-*epiBL* increased the APX activity by 73–79% but had no effect on SOD activity in CK-0 plants. Both 24-*epiBL* pretreatments increased the chloroplast APX activity by 2.4–3.2 times in stressed plants, but only Diw stimulated the SOD activity and only at 200 mM NaCl (by 34%).

Discussion

Numerous workers have reported the benefits of 24-*epiBL* applications on plants and the efficacy of those treatments in alleviating the symptoms of abiotic stress, including high- and low-temperature stress, freezing, drought, heavy

metals, and salt stress. The dose and method of application of 24-*epiBL* varies from study to study and species to species. For example, protective effects of exogenous 24-*epiBL* have been reported for heat stress in rice (Thussaganpanit et al. 2015), melon (Zhang et al. 2013), and eggplant (Wu et al. 2014), chilling in cucumber (Jiang et al. 2013), zinc-induced oxidative stress in radish (Ramakrishna and Rao 2012), and salt stress in wheat (Talaat and Shawky 2012). There are few reports, however, that involve woody plants. In experiments with grapevine, Xi et al. (2013) found that exogenous 24-*epiBL* enhanced the activity of antioxidants systems during chilling stress, whereas Wang et al. (2015) reported that 24-*epiBL* alleviated stomatal and non-stomatal limitations on photosynthesis and associated effects on chlorophyll fluorescence and chloroplast ultrastructure during water stress. Below, we evaluate the impact of salt stress on photosynthesis and related variables in black locust, and follow this with a discussion of how these impacts were ameliorated by exogenous 24-*epiBL*.

Impacts of Salinity Stress on Black Locust Seedlings

As shown in Fig. 1, even the 100 mM NaCl treatment caused a severe decline in A_n in black locust seedlings. Stomatal conductance and transpiration were also reduced, suggesting a diffusion limitation on A_n associated with osmotic stress. The possibility that there was some osmotic stress is supported by a reduction in RWC , albeit a non-significant one (Fig. 5b). The C_i , however, was relatively constant, consistent with a concurrent biochemical limitation on A_n (Flexas and Medrano 2002). Consequently, although the calculated stomatal limitation (L_s) in salt-stressed plants was higher than in controls, the difference was not significant (Fig. 1f). Indeed, salinity had less severe impacts on g_s and E than on A_n , resulting in a large drop in WUE . The drop in WUE independent from any significant change in C_i or L_s suggests a negative effect of salt stress on the mesophyll conductance (g_m). We did not explore this possibility, but the change in shape of the chloroplasts to a more rounded form, less appressed to the cell wall (Fig. 3c, e), would lengthen the aqueous phase of the CO_2 diffusion pathway (Milla-Moreno et al. 2016). Xu et al. (2013) found that g_m was much more seriously affected than g_s in poplar (*Populus cathayana* Rehder) under alkaline (Na_2CO_3) stress. Swelling of chloroplasts and their positioning towards a more central location less closely associated with the cell wall are common phenomena in salt-stressed crop plants; for example, rice (Yamane et al. 2008) and potato (Gao et al. 2014).

Chlorophyll fluorescence monitoring has been widely used to assay the health of the photosynthetic mechanism under environmental stress (Sayed 2003). The incremental decrease in F_v/F_m we observed in salt-stressed plants indicates a progressive reduction in the maximum quantum

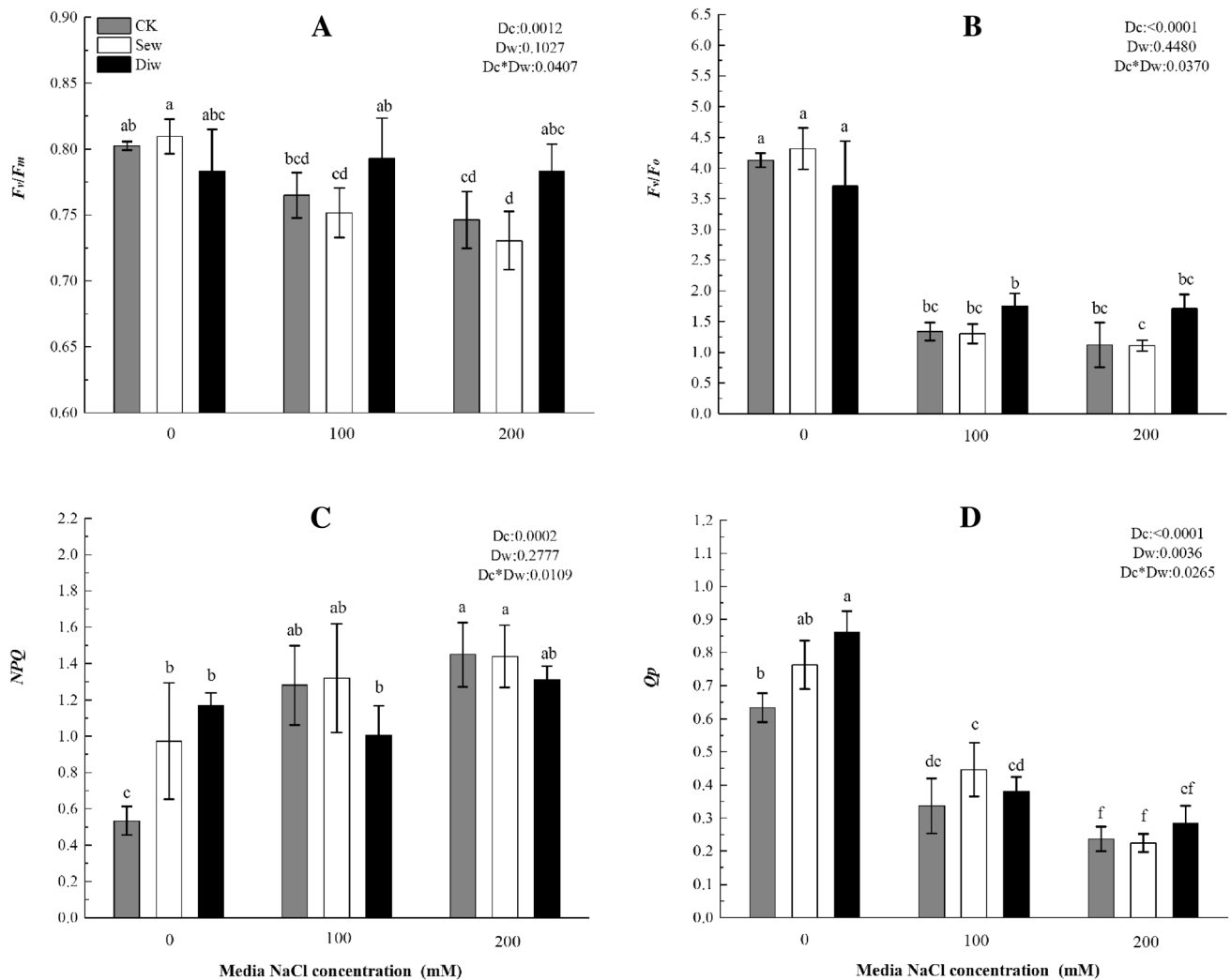


Fig. 2 Effects of exogenous 24-*epiBL* on chlorophyll fluorescence parameters in leaves of black locust seedlings at three levels of salt stress, with or without prior treatment with 24-*epiBL* (CK:

no 24-*epiBL*; Sew: seeds soaked in 1.04 μM 24-*epiBL*; Diw: roots dipped in 1.04 μM 24-*epiBL*). Other details as in Fig. 1

yield of photosynthesis (Fig. 2), whereas the sharp decrease in F_v/F_o suggests a more immediate impact on the water-splitting complex (Kalaji et al. 2011). Photochemical quenching (Q_p) decreased while NPQ increased, consistent with reduced rates of carbon fixation and greater energy dissipation as heat (Oxborough 2004). Our findings are in agreement with the salt stress studies of Wu et al. (2012) on eggplant, Wang et al. (2007) on poplar, and Shu et al. (2013) on cucumber; the latter authors found that once the photosynthetic capacity was too low to use all incident radiation absorbed, excess excitation energy was dissipated in the thylakoids to reduce photoinhibition. Shu et al. (2013) also reported reductions in Chl *a*, Chl *b*, and total chlorophyll in leaves of salt-stressed cucumber that were similar to, but not as dramatic as the reductions in black locust chloroplast chlorophyll levels that we observed (Table 1).

Another indication that specific ion stress, rather than osmotic stress, was primarily responsible for the negative impacts on black locust seedlings is seen in the cell membrane stability index (*MSI*), which was sharply reduced by salt stress (Fig. 5a). Talaat and Shawky (2012) observed a similar reduction in *MSI* in salt-stressed wheat, but in contrast to our results there was also a substantial decrease in *RWC*. We found that Na^+ concentrations of intact leaf tissue and isolated chloroplasts roughly paralleled each other and were increased several fold (more in chloroplasts) when black locust seedlings were grown with NaCl (Fig. 4). Similarly, Wang et al. (2007) reported large accumulations of Na^+ and Cl^- in chloroplasts of salt-stressed poplar. In the present work, there was a simultaneous increase in chloroplast H_2O_2 and MDA (Table 2), indicative of oxidative stress. Diao et al. (2014) observed similar increases in chloroplast H_2O_2 and

Fig. 3 The effects of salt stress on the ultrastructure of chloroplasts and thylakoid membranes in leaves of black locust seedlings at three levels of salt stress, with or without prior treatment with *24-epiBL*. **a, b** CK-0; **c, d** CK-100; **e, f** CK-200; **g, h** Sew-100; **i, j** Diw-100; **k, l** Sew-200; **m, n** Diw-200. The second fully expanded leaves, numbered basipetally, were sampled for ultramicroscopic observation after 50 days of salt stress. *CW* cell wall, *PM* plasma membrane, *ChM* chloroplast membrane, *SG* starch granule, *OS* osmiophilic plastoglobuli, *Thl* thylakoid, *G* grana, *Thl* thylakoid lamella. Scale bars for chloroplasts and thylakoids are 5 μ m and 1 μ m, respectively

MDA levels in salt-stressed tomato. We also measured APX and SOD activity, but changes in these activities were less consistent. The efficiency of the ROS scavenging system is believed to play an important role in protecting cells from oxidative damage (Mittler 2002). It is unclear whether the oxidative stress was a symptom or cause of the increase in *MSI* and/or photoinhibition, but the three symptoms are likely to be related. Agrawal et al. (2013) observed a similar overall syndrome in response to salinity in leaves of the Indian jujube (*Ziziphus mauritiana* L.) tree. In black locust, this syndrome is accompanied by changes in chloroplast ultrastructure (Fig. 3), including chloroplast swelling, disruption of thylakoid membranes, larger but fewer plastoglobuli, and smaller, less distinct starch granules.

Similar effects on thylakoid membranes were apparent in potato (Gao et al. 2014) and rice plants (Yamane et al. 2008) exposed to salinity, in the latter case in association with decreases in F_v/F_m and increases in F_o . Likewise, drought stress caused chloroplast swelling and disrupted the lamellar arrangement of thylakoids in poplar (Zhang et al. 2012) but, in contrast to the effects of salt stress on black locust, increased not only the size but also the number of plastoglobuli.

Protective Effects of *24-epiBL*

Beyond some increases in NPQ and Q_p , and higher activities of APX and SOD, there were no observable effects of our *24-epiBL* pretreatments on unstressed black locust seedlings. There were, however, very strong positive effects of *24-epiBL* on the salt-stressed seedlings. The most dramatic results were a complete or almost complete reversal of the effects of salinity stress on A_n , *MSI*, and *WUE*. The positive effect on *WUE* in combination with little change in g_s suggested that *24-epiBL* promotes photosynthesis at the level of the mesophyll, by protecting the photosynthetic apparatus and by helping to maintain g_m . This is consistent with the maintenance of *MSI*, *RWC*, and the positive effects of *24-epiBL* on chloroplasts (improved shape and positioning, lower Na^+ content, increased numbers of plastoglobuli and enhanced condition of thylakoid membranes), less severe reductions in chlorophylls, and improvements in F_v/F_m . Pretreatment with *24-epiBL* also reduced levels of oxidative

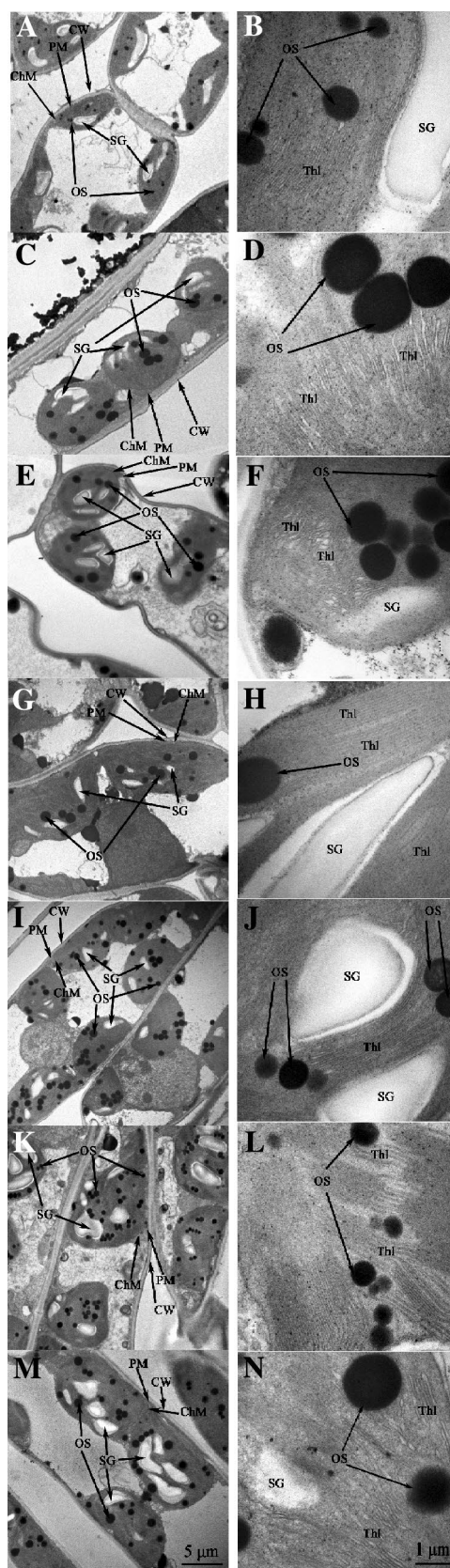


Table 1 Effect of salt stress on chlorophyll a (Chl *a*), chlorophyll b (Chl *b*), and total chlorophyll in black locust seedlings grown with or without 24-*epiBL* pretreatment (CK: no 24-*epiBL*; Sew: seeds soaked in 1.04 μM 24-*epiBL*; Diw: roots dipped in 1.04 μM 24-*epiBL*)

	NaCl treatment (mM)	24- <i>epiBL</i>			Dc	Dw	Dc*Dw
		CK	Sew	Diw			
Chl <i>a</i> ($\mu\text{g mg}^{-1}$ protein)	0	21.99 \pm 1.82c	26.72 \pm 1.23b	29.31 \pm 2.35a	<0.0001	<0.0001	0.0002
	100	13.20 \pm 0.64e	16.64 \pm 0.79d	16.53 \pm 1.09d			
	200	6.04 \pm 0.79f	7.32 \pm 0.5f	11.39 \pm 0.93e			
Chl <i>b</i> ($\mu\text{g mg}^{-1}$ protein)	0	11.08 \pm 2.38a	11.16 \pm 3.67a	11.76 \pm 0.95a	<0.0001	0.0252	0.1933
	100	6.72 \pm 1.87b	10.74 \pm 0.74a	11.97 \pm 0.88a			
	200	2.41 \pm 0.4c	3.06 \pm 0.42c	3.94 \pm 0.11bc			
Total chlorophyll ($\mu\text{g mg}^{-1}$ protein)	0	33.14 \pm 5.33b	37.80 \pm 3.57a	41.07 \pm 3.24a	<0.0001	0.0003	0.0185
	100	19.92 \pm 2.12d	27.38 \pm 0.19c	28.50 \pm 0.86c			
	200	8.45 \pm 0.48f	10.38 \pm 0.85f	15.33 \pm 1.02e			

Dc, Dw, and Dc*Dw are the *P* values for the salt concentration effect, the 24-*epiBL* application effect, and the interaction effect between salt concentration and 24-*epiBL* application, respectively. Different letters following means (\pm SE) indicate significant differences (*P* < 0.05)

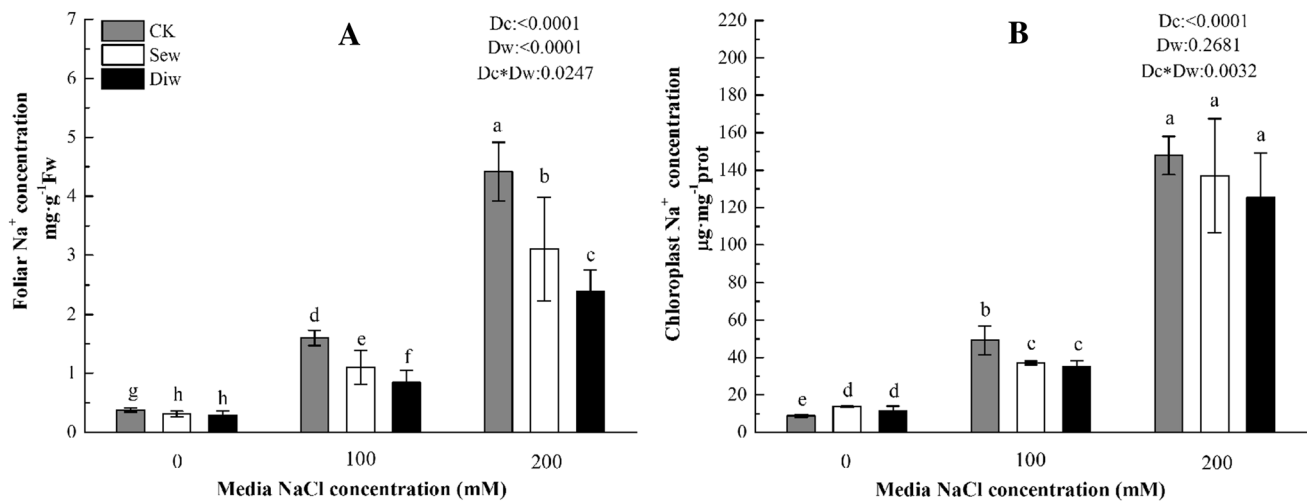


Fig. 4 Changes in Na⁺ concentrations in **a** leaves and **b** chloroplasts of black locust seedlings at three levels of salt stress, with or without prior treatment with 24-*epiBL* (CK: no 24-*epiBL*; Sew: seeds soaked

in 1.04 μM 24-*epiBL*; Diw: roots dipped in 1.04 μM 24-*epiBL*). Other details as in Fig. 1

stress, as evidenced by reduced levels of H₂O₂ and MDA in the salt-stressed plants.

Although both 24-*epiBL* pretreatment protocols provided substantial protection, seed soaking (Sew) was somewhat less effective than root dipping (Diw), particularly at the highest stress level (200 mM NaCl). Root dipping resulted in better maintenance of total chlorophyll concentrations, higher F_v/F_m , higher F_v/F_0 , lower foliar Na⁺, lower MDA levels, lower H₂O₂ levels, and greater SOD activity. The lower effectiveness of seed soaking was likely caused by a greater dilution of applied 24-*epiBL* by biomass accretion and the greater time since treatment, an intervening period of over 6 months.

Many authors report similar effects of exogenous 24-*epiBL* in alleviating the symptoms of salt and other

stresses. Wu et al. (2017) reported that spraying the foliage of perennial ryegrass seedlings with 24-*epiBL* could increase their chlorophyll content, A_n , g_s , and antioxidant enzyme activities to protect them from salinity. Treatment of salt-stressed barley seedlings with 24-*epiBL* significantly increased growth and RWC, and improved salinity tolerance by reducing K⁺ efflux from roots via depolarization-activated K⁺ channels (Azhar et al. 2017). Similar to the present paper, but working with a moderately salt-sensitive cultivar of wheat, Ali et al. (2008b) concluded that improvements in photosynthesis induced by pretreatment with 24-*epiBL* were related to non-stomatal factors and the removal of biochemical limitations in the mesophyll.

Under drought conditions induced by polyethylene glycol (PEG), Wang et al. (2015) reported that application of

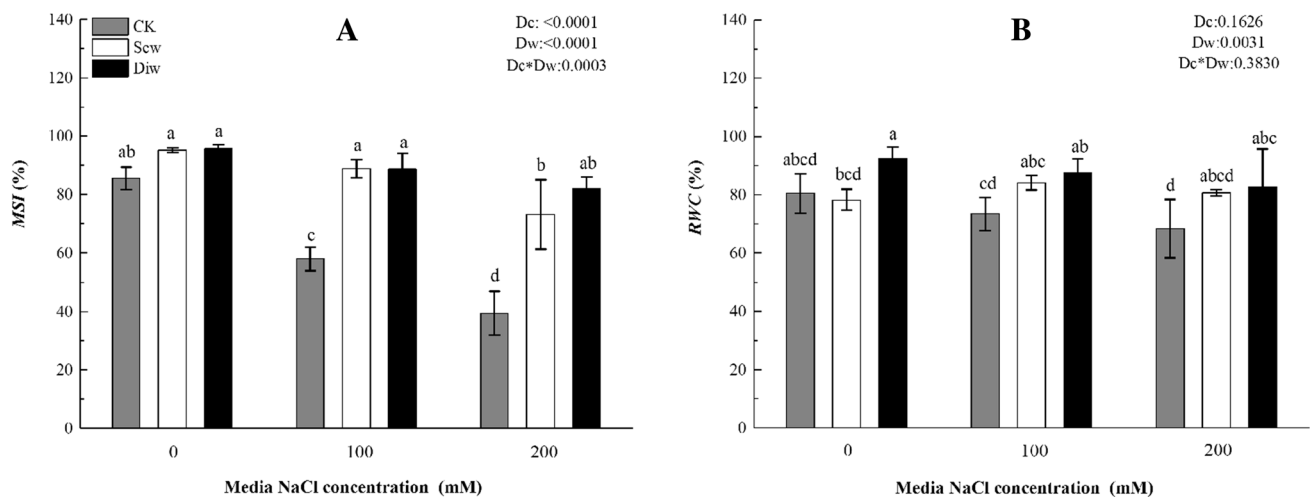


Fig. 5 Changes in **a** membrane stability index (*MSI*) and **b** relative water content (*RWC*) of leaves of black locust seedlings at three levels of salt stress, with or without prior treatment with *24-epiBL* (CK:

no *24-epiBL*; Sew: seeds soaked in $1.04 \mu\text{M}$ *24-epiBL*; Diw: roots dipped in $1.04 \mu\text{M}$ *24-epiBL*). Each bar represents the mean (\pm SE) of three replicates. Other details as in Fig. 1

Table 2 Effect of salt stress and *24-epiBL* addition on H_2O_2 and MDA levels, and APX and SOD activities of chloroplasts of black locust seedlings grown with or without *24-epiBL* pretreatment (CK:

no *24-epiBL*; Sew: seeds soaked in $1.04 \mu\text{M}$ *24-epiBL*; Diw: roots dipped in $1.04 \mu\text{M}$ *24-epiBL*)

NaCl treatment (mM)	<i>24-epiBL</i>	H_2O_2 (mmol mg^{-1} prot)	MDA (nmol mg^{-1} prot)	APX (units mg^{-1} prot)	SOD (units mg^{-1} prot)
0	CK	$0.25 \pm 0.043\text{d}$	$7.0 \pm 0.022 \text{ g}$	$1.06 \pm 0.28\text{c}$	$11.73 \pm 0.75\text{e}$
	Sew	$0.28 \pm 0.037\text{d}$	$6.3 \pm 0.23 \text{ g}$	$2.00 \pm 0.24\text{b}$	$13.05 \pm 2.33\text{e}$
	Diw	$0.33 \pm 0.085\text{d}$	$5.8 \pm 0.21 \text{ g}$	$2.03 \pm 0.56\text{b}$	$13.25 \pm 1.65\text{e}$
100	CK	$1.07 \pm 0.081\text{b}$	$200.0 \pm 9.5\text{b}$	$2.51 \pm 0.70\text{b}$	$31.09 \pm 0.94\text{ab}$
	Sew	$0.64 \pm 0.032\text{c}$	$22.0 \pm 3.7\text{e}$	$4.5 \pm 0.097\text{a}$	$33.84 \pm 1.11\text{bc}$
	Diw	$0.34 \pm 0.035\text{d}$	$16.0 \pm 1.0\text{f}$	$4.33 \pm 0.23\text{a}$	$41.57 \pm 1.81\text{a}$
200	CK	$2.54 \pm 0.28\text{a}$	$310.0 \pm 70.0\text{a}$	$0.16 \pm 0.040\text{e}$	$18.49 \pm 0.95\text{d}$
	Sew	$1.19 \pm 0.12\text{b}$	$130.0 \pm 14.0\text{c}$	$0.36 \pm 0.049\text{de}$	$18.86 \pm 1.84\text{d}$
	Diw	$1.05 \pm 0.19\text{b}$	$82.0 \pm 4.4\text{d}$	$0.51 \pm 0.027\text{d}$	$24.83 \pm 0.66\text{c}$
	Dc	<0.0001	<0.0001	<0.0001	<0.0001
	Dw	<0.0001	<0.0001	<0.0001	0.0103
	Dc*Dw	<0.0001	<0.0001	0.224	0.4077

Dc, *Dw*, and *Dc*Dw* are the *P* values for the salt concentration effect, the *24-epiBL* application effect, and the interaction effect between salt concentration and *24-epiBL* application, respectively. Different letters following means (\pm SE) indicate significant differences ($P < 0.05$)

24-epiBL to grape seedlings increased their chlorophyll content, maximal fluorescence, F_v/F_m , and *NPQ*. At the same time, chloroplasts were less swollen and the lamellae maintained a relatively normal appearance. Treatment with *24-epiBL* similarly reduced the effects of PEG-induced water stress in blue mustard (*Chorispora bungeana* Fisch. & C.A. Mey.) plants as evidenced by higher F_v/F_m and Chl *a* content, accompanied by up-regulation of the ROS scavenging system and reduced lipid peroxidation (Li et al. 2012). The latter function might reflect the involvement of BRs in modifying plasma membrane stability during stress (Hamada 1986). Ahammed et al. (2013) showed that pretreatment

of tomato seedlings with *24-epiBL* improved the ability of the ROS scavenging system to reduce the harm caused by H_2O_2 accumulation and membrane lipid peroxidation upon exposure to polychlorinated biphenyls.

Although BRs are widely considered to regulate tolerance to various stresses in plants, little is known regarding the underlying mechanisms. Proximally, they may act in a manner similar to other agents where improved salt tolerance has been associated with many of the same physiological and structural changes reported here. For example, in studying the effects of silicon and salt stress on Japanese honeysuckle (*Lonicera japonica* L.), Gengmao

et al. (2015) observed that exogenous K_2SiO_3 helped to maintain chloroplast lamellae and reduce foliar Na^+ levels, leading to increased A_n . Hu et al. (2014) reported similar effects of Si in maintaining chloroplast ultrastructure (including plastoglobuli and starch grains) in salt-stressed tomato accompanied by an improvement in A_n and Q_p , and reduced NPQ , in parallel with increased g_s and decreased L_s , but without any change in C_i . Similarly, Shu et al. (2013) reported that application of spermine to salt-stressed cucumber improved chlorophyll fluorescence parameters and increased the activities of antioxidant enzymes and levels of antioxidants in chloroplasts. Mycorrhization of poplars by *Paxillus involutus* (Batsch) Fr. improved their salt tolerance by reducing the amounts of Na^+ in leaves (Luo et al. 2011). Higher salinity tolerance in Euphrates poplar (*Populus euphratica* Oliv.) relative to a hybrid cultivar (*P. × popularis*) was associated with a lower Na^+ content in the chloroplasts of the former (Wang et al. 2007).

Conclusion

In conclusion, our results show that salt stress induces photoinhibition in black locust seedlings. Exogenous 24-*epiBL* pretreatments alleviated the stress-induced inhibition of photosynthesis by reducing Na^+ -ion toxicity and ROS in chloroplasts, and by stabilizing chloroplast and thylakoid membrane structure. Applications of 24-*epiBL* by seed soaking or by root dipping were both effective, but plants produced using the latter method performed somewhat better under salt stress. The beneficial influence of 24-*epiBL* was relatively long-lasting (several months from time of application), and potentially long enough to promote establishment in practice, on salt-affected soils in agroforestry applications in the field.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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