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Pretreatment with 24-Epibrassinolide Synergistically Protects Root Structures and Chloroplastic Pigments and Upregulates Antioxidant Enzymes and Biomass in Na^+-Stressed Tomato Plants

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Abstract

Salt stress reduces plant growth by negatively interfering with the division rate and cellular expansion, limiting the growth and development of the roots, stems, and leaves. 24-Epibrassinolide (EBR) is a molecule extracted from plant tissues and is a plant growth regulator with a high capacity to modulate tolerance to abiotic stresses. The objective of this study was to verify the possible improvements promoted by pretreatment with EBR in salt-stressed tomato plants, evaluating the variables related to root anatomy, photosynthetic pigments, antioxidant system, and biomass accumulation. The experiment comprised four treatments: two salt conditions (0 and 150 mM NaCl, described as Na^+ (−) and Na^+ (+), respectively) and two concentrations of 24-epibrassinolide (0 and 100 nM EBR, described as EBR (−) and EBR (+), respectively). EBR modulated the protection and vascularization of root structures, as demonstrated by the increases in epidermis thickness (12%) and metaxilem diameter (119%), respectively. This steroid relieved oxidative damage, which was clearly linked to elevated activities of superoxide ascorbate peroxidase (24%) and guaiacol peroxidase (31%). EBR also benefited photosynthetic pigments, reducing the degradation of chlorophylls. In addition, pretreatment with EBR favoured a higher biomass, which was due to positive effects on leaf and root tissues, including better performance of photosynthetic machinery.

Keyword Brassinosteroids · Chlorophylls · Growth · Peroxidase · Root metaxylem

Abbreviations

APX	Ascorbate peroxidase
BRs	Brassinosteroids
CAR	Carotenoids
CAT	Catalase
Chl *a*	Chlorophyll a
Chl *b*	Chlorophyll b
C_i	Intercellular CO_2 concentration
CO_2	Carbon dioxide
E	Transpiration rate
EBR	24-Epibrassinolide
EDS	Equatorial diameter of the stomata
EL	Electrolyte leakage
ETAb	Epidermis thickness from abaxial leaf side
ETAd	Epidermis thickness from adaxial leaf side
ETR	Electron transport rate
ETR/P_N	Ratio between the apparent electron transport rate and net photosynthetic rate
EXC	Relative energy excess at the PSII level
F_0	Minimal fluorescence yield of the dark-adapted state
F_m	Maximal fluorescence yield of the dark-adapted state
F_v	Variable fluorescence
F_v/F_m	Maximal quantum yield of PSII photochemistry
g_s	Stomatal conductance
H_2O_2	Hydrogen peroxide

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K	Potassium
LDM	Leaf dry matter
MDA	Malondialdehyde
Mg	Magnesium
Na^+	Sodium
NPQ	Nonphotochemical quenching
O_2^-	Superoxide
PDS	Polar diameter of the stomata
P_N	Net photosynthetic rate
P_N/C_i	Instantaneous carboxylation efficiency
POX	Peroxidase
PPT	Palisade parenchyma thickness
PSII	Photosystem II
q_P	Photochemical quenching
RCD	Root cortex diameter
RDM	Root dry matter
RDT	Root endodermis thickness
RET	Root epidermis thickness
ROS	Reactive oxygen species
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SD	Stomatal density
SDM	Stem dry matter
SF	Stomatal functionality
SI	Stomatal index
SOD	Superoxide dismutase
SPT	Spongy parenchyma thickness
TDM	Total dry matter
Total Chl	Total chlorophyll
VCD	Vascular cylinder diameter
WUE	Water-use efficiency
Φ_{PSII}	Effective quantum yield of PSII photochemistry.

Introduction

Tomato is considered a model plant in research conducted in the agricultural sector (Suresh et al. 2014) because it has interesting characteristics, such as a short cycle, rapid growth, easy pollination, wide adaptability to climatic conditions, and high rates of seed production (Bai and Lindhout 2007; Gerszberg et al. 2015; Gerszberg and Hnatuszko-Konka 2017). In this context, this species plays an important role in the generation of knowledge, more specifically on the deleterious effects caused by salt stress (Zheng et al. 2016) and ways to mitigate these interferences on metabolism (Shu et al. 2016) and growth in higher plants (Maia et al. 2018).

Soil salinity is a problem in several regions of the world, including agricultural areas (Qureshi et al. 2008; Qadir et al. 2014), and the incorrect use of irrigation and inadequate soil management are the main causes of soil salinization due to anthropogenic action (Bouksila et al. 2013). In plants, salt stress generated by sodium (Na^+) causes osmotic and ionic imbalances (Dalio et al. 2011), reducing the plant water potential and impairing plant metabolism (Rengasamy 2010). Na^+ ions also negatively impact nutrient uptake, often causing nutritional deficiency (Hafsi et al. 2017; Cao et al. 2019). Additionally, Na^+ accumulation in the cytoplasm and/or vacuoles causes oxidative stress in the cells (Chaves et al. 2009), resulting in anatomical disturbances and interferences with the photosynthetic machinery, growth rate, and biomass (Ashraf and Harris 2004; Munns and Tester 2008b).

Salt stress reduces plant growth, negatively impacting the division rate and cellular expansion and limiting the growth and development of roots, stems, and leaves (Yu et al. 2015; Forieri et al. 2016a; Khoshbakht et al. 2018). Specifically, in root tissue, this stress induces a decrease in the metaxylem as a response to minimize cavitation and loss of function of vessel elements, which are essential structures for the conduction of water and assimilation of nutrients (Risopatron et al. 2010; Oliveira et al. 2018). Additionally, mild or moderate Na^+ stress can provoke an increase in the thickness of the cell wall in an attempt to reduce the accumulation of this potentially toxic ion; however, at high concentrations, cell plasmolysis often ensues, triggering damage to protective tissues, including the epidermis and endoderm (Silva et al. 2020).

Chlorophyll (Chl) is an essential component of the photosynthetic machinery (Johnson 2016) and is responsible for the absorption of light in specific bands of the spectrum (Chen 2014) and the biosynthesis of carbohydrates indispensable for plant metabolism (Kalaji et al. 2018). Structurally, Chl has a hydrophobic tail that inserts into the thylakoid membrane, a head formed by a porphyrin ring, and a circular group of Mg^{2+} atoms (Hohmann-Marriott and Blankenship 2011). Typically, exposure to Na^+ ions induces intense formation of O_2^- ions, which are generated by the fusion of excess electrons with O_2 molecules in the chloroplasts (Shelke et al. 2017). They cause damage to membranes and disturbances in the centres of PSII reactions (Nishiyama et al. 2004; Kalaji et al. 2011), resulting in photoinhibition (Tavakkoli et al. 2011; Ruban 2015).

Plants under salt stress often overproduce reactive oxygen species (ROS), which superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are the major components (Gill and Tuteja 2010). ROS are highly toxic substances that cause oxidative damage to membranes, proteins and nucleic acids in plant cells (Shahzad et al. 2018). Antioxidant metabolism plays a promising role in mitigating the impacts of ROS accumulation (Kang and Nam 2016). Under oxidative stress, the enzymes superoxide dismutase (SOD), guaiacol peroxidase (POX), catalase (CAT) and ascorbate peroxidase (APX) (He et al. 2016) are responsible for ROS elimination, reducing damage to the photosynthetic apparatus and cell membranes (Guerrero et al. 2015).

24-Epibrassinolide (EBR) is a molecule extracted from plant tissues and is natural and biodegradable (Tanveer et al. 2018). Chemically, it is one of the brassinosteroids (BRs) with plant growth regulatory activities (Müssig 2005). EBR is involved in multiple metabolic processes, including cell division (Tong et al. 2014), cell elongation (Tang et al. 2011), vascular differentiation (Ibanes et al. 2009), reproductive development (Huang et al. 2013; Vogler et al. 2014; Maita and Sotomayor 2015), germination (Wang et al. 2011), formation of the root system (González-García et al. 2011; Cai et al. 2018), senescence, abscission and maturation (Hansen et al. 2009; Mazorra et al. 2013), gene regulation (Mao et al. 2017), and modulation of tolerance to biotic and abiotic stresses (Sasse 2003; Choudhary et al. 2012; Pereira et al. 2019).

The hypothesis of this research was based on the negative effects induced by Na^+ stress in plants, such as disturbances in the anatomy of stem and root tissues (Akhtar et al., 2017; Silva et al., 2020), oxidative damage (Sheikh-Mohamadi et al. 2017), and interference with photosynthetic performance (Hasanuzzaman et al. 2018). On the other hand, studies available in the literature demonstrate that EBR can mitigate the problems associated with this stress (Ahammed et al. 2020), increasing ROS scavenging (El-Mashad and Mohamed 2012; Rattan et al. 2020), and modulating anatomical adaptations (Oliveira et al. 2018). Therefore, the objective of this study is to verify the possible improvements by pretreatment with EBR in salt-stressed tomato plants, evaluating the variables linked to root anatomy, photosynthetic pigments, antioxidant system, and biomass accumulation.

Materials and Methods

Location and Growth Conditions

The experiment was performed at the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55′ S, 47°34′ W). The study was conducted in a greenhouse with controlled temperature and humidity. The minimum, maximum, and median temperatures were 27, 33 and 26.2 °C, respectively. The relative humidity during the experimental period varied between 60 and 80%.

Plants, Containers and Acclimation

Seeds of *Solanum lycopersicum* L. cv. Caline IPA-7 Hortivale™ were germinated using Plantmax™ substrate. Fifteen-day-old seedlings with similar features and sizes were selected and placed in 1.2 l containers (0.15 m in height and 0.10 m in diameter) filled with a mixed substrate of sand and vermiculite in a 3:1 ratio. A solution was used for nutrients (Lima and Lobato 2017), and the ionic force was started at 50% and was modified to 100% after two days. After two days, the nutritive solution remained at total ionic force.

Experimental Design

The experiment was randomized with four treatments, including two salt conditions (0 and 150 mM NaCl, described as Na^+ (−) and Na^+ (+), respectively) and two concentrations of brassinosteroids (0 and 100 nM EBR, described as EBR (-) and EBR (+), respectively). Six replicates for each of the four treatments were conducted, yielding a total of 24 experimental units used in the experiment, with one plant in each unit. Na^+ concentration was defined based in study conducted by Stevens et al. (2006) with tomato plants, while EBR treatment was chosen in agreement with Maia et al. (2018).

24-Epibrassinolide (EBR) Preparation and Application

Twenty-day-old young plants were sprayed with 24-epibrassinolide (EBR) or Milli-Q water (containing a proportion of ethanol that was equal to that used to prepare the EBR solution) at 5-d intervals until day 35. The 0 and 100 nM EBR (Sigma-Aldrich, USA) solutions were prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [ethanol:water (v/v) = 1:10,000] (Ahammed et al. 2013).

Plant Conduction and Water Deficit Treatment

One plant per pot was used to examine the plant parameters. The plants received the following macro- and micronutrients contained in the nutrient solution in agreement with Lima and Lobato (2017). To simulate Na^+ exposure, NaCl was used at concentrations of 0 and 150 mM Na^+, which was applied over 15 days (days 25–40 after the start of the experiment). During the study, the nutrient solutions were changed at 07:00 h at 3-day intervals, with the pH adjusted to 5.5 using HCl or NaOH. On day 40 of the experiment, physiological and morphological parameters were measured for all plants, and leaf tissues were harvested for anatomical and biochemical analyses.

Measurement of Chlorophyll Fluorescence and Gas Exchange

Chlorophyll fluorescence was measured in fully expanded leaves under light using a modulated chlorophyll fluorometer (model OS5p; Opti-Sciences). Preliminary tests determined the location of the leaf, the part of the leaf, and the time required to obtain the greatest F_v/F_m ratio; therefore, the

acropetal third of the leaves, which was the middle third of the plant and adapted to the dark for 30 min, was used in the evaluation. The intensity and duration of the saturation light pulse were 7500 µmol m^{-2} s^{-1} and 0.7 s, respectively. Gas exchange was evaluated in all plants and measured in the expanded leaves in the middle region of the plant using an infrared gas analyser (model LCPro$^+$; ADC BioScientific) in a chamber under constant CO_2, photosynthetically active radiation, air-flow rate, and temperature conditions at 360 µmol mol^{-1} CO_2, 800 µmol photons m^{-2} s^{-1}, 300 µmol s^{-1}, and 28 °C, respectively, between 10:00 and 12:00 h. The water-use efficiency (WUE) was estimated according to Ma et al. (2004), and the instantaneous carboxylation efficiency (P_N/C_i) was calculated using the formula that was described by Aragão et al. (2012).

Measurements of Anatomical Parameters

Samples were collected from the middle region of the leaf limb of fully expanded leaves, and roots were collected 5 cm from the root apex. Botanical material was fixed in FAA 70 for 24 h and then dehydrated in a series of ethanol and butanol before being embedded in histological paraffin (Johansen 1940). Transverse sections were prepared according to the procedures described by Maia et al. (2018). For stomatal characterization, the epidermal impression method was used as described by Segatto et al. (2004). The slides were observed and photographed under an optical microscope (Motic BA 310; Motic Group Co. LTD.) equipped with a digital camera (Motic 2500; Motic Group Co., LTD.). The images were analysed using Moticplus 2.0 software. In both leaf faces, the stomatal density (SD) was calculated as the number of stomata per unit area, and the stomatal functionality (SF) was calculated as the ratio PDS/EDS according to Castro et al. (2009). The stomatal index (SI %) was calculated as the percentage of stomata in relation to the total number of epidermal cells in a given area.

Determination of the Antioxidant Enzymes, Superoxide and Soluble Proteins

Antioxidant enzymes (SOD, CAT, APX, and POX), superoxide, and soluble proteins were extracted from leaf tissues according to the method of Badawi et al. (2004). The total soluble proteins were quantified using the methodology described by Bradford (1976). The SOD assay was measured at 560 nm (Giannopolitis and Ries 1977), and the SOD activity was expressed in mg^{-1} protein. The CAT assay was detected at 240 nm (Havir and McHale 1987), and the CAT activity was expressed in µmol H_2O_2 mg^{-1} protein min^{-1}. The APX assay was measured at 290 nm (Nakano and Asada 1981), and the APX activity was expressed in µmol AsA mg^{-1} protein min^{-1}. The POX assay was detected at 470 nm (Cakmak and Marschner 1992), and the activity was expressed in µmol tetraguaiacol mg^{-1} protein min^{-1}. O_2^- was measured at 530 nm (Elstner and Heupel 1976).

Quantification of Hydrogen Peroxide, Malondialdehyde and Electrolyte Leakage

Stress indicators (H_2O_2 and MDA) were extracted using the methodology described by Wu et al. (2006). H_2O_2 was measured using the procedures described by Velikova et al. (2000). MDA was determined by the method of Cakmak and Horst (1991) using an extinction coefficient of 155 mM^{-1} cm^{-1}. EL was measured according to Gong et al. (1998) and calculated by the formula EL (%) = (EC_1/EC_2) × 100.

Determination of Na^+ Content, Photosynthetic Pigments and Biomass

Na^+ contents were determined using flame photometry (model 910; Analyser) based on procedures described by Carmo et al. (2000). Chlorophyll and carotenoid determinations were performed using a spectrophotometer (model UV-M51; Bel Photonics) according to the methodology of Lichtenthaler and Buschmann (2001). The biomass of roots, stems, and leaves was measured based on constant dry weights (g) after drying in a forced-air ventilation oven at 65 °C.

Data Analysis

Data were subjected to normality of residuals using Shapiro–Wilk test and data applied to one-way ANOVA. Significant differences between the means were determined using the Scott-Knott test at a probability level of 5% (Steel et al. 2006). Standard deviations were calculated for each treatment.

Results

EBR Reduced Na^+ Contents and Protected Tissue Structures in Salt-Stressed Plants

Tomato plants under salinity (150 mM NaCl) presented significant increases in Na^+ contents (Table 1). However, EBR application induced reductions in roots (39%) and leaves (60%) compared to the treatment without EBR. For root structures, plants subjected to NaCl toxicity suffered reductions (Table 2 and Fig. 1). However, the application of 100 nM EBR in plants submitted to 150 mM NaCl promoted increases of 12%, 10%, 9%, 66%, and 119% in RET, RDT, RCD, VCD, and RMD, respectively, compared

Table 1 Na^+ contents in tomato plants sprayed with EBR and exposed to Na^+ stress

Na^+	EBR	Na^+ in root (mg g DM^{-1})	Na^+ in leaf (mg g DM^{-1})
−	−	0.09 ± 0.01^c	0.04 ± 0.01^c
−	+	0.03 ± 0.01^d	0.01 ± 0.01^d
+	−	16.83 ± 0.49^a	7.75 ± 0.04^a
+	+	10.31 ± 0.39^b	3.12 ± 0.02^b

Na^+ = Sodium. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Values described corresponding to means from three repetitions and standard deviations

to comparable treatment without EBR. On leaf structures, salinity provoked negative interferences (Table 2 and Fig. 2). However, plants under the combined actions of NaCl and EBR had ETAd, ETAb, PPT, and SPT values that increased by 6%, 11%, 10%, and 14%, respectively, and the PPT/SPT ratio was reduced by 3% compared to similar treatments without EBR. Plants without NaCl and sprayed with EBR also exhibited increases in ETAd, ETAb, PPT, and SPT of 5%, 10%, 6%, and 13% and a reduction of 6% in PPT/SPT compared to treatment without NaCl and without EBR.

Steroid Alleviated the Damage Provoked by Salinity on the Photosynthetic Machinery

Na^+ toxicity induced decreases in chloroplastic pigments (Table 3). Samples exposed to Na^+ and EBR exhibited increases of 39%, 45%, 65%, and 40% in the variables Chl *a*, Chl *b*, Car, and Total Chl, respectively, but reductions of 3% and 12% in the ratios Chl *a*/Chl *b* and Chl total/Car, respectively, when compared to equal treatment (150 mM NaCl) without EBR. Regarding chlorophyll fluorescence, plants exposed to NaCl toxicity were negatively affected (Table 3 and Fig. 3). Plants under NaCl and EBR exhibited decreases of 10% and 1% in F_0 and F_m, respectively. However, these plants presented increases of 2% and 4% in F_v and F_v/F_m, respectively, compared to the same parameters in plants exposed to a similar treatment without EBR. Plants under the combined effects of Na^+ and EBR exhibited increases in Φ_{PSII} (17%) and ETR (16%) and q_p (2%) and reductions in NPQ (19%), EXC (8%), and ETR/P_N (6%) compared with the same parameters in plants that received an equivalent treatment without EBR. Regarding gas exchange, Na^+-induced toxicity provoked negative repercussions (Table 3). The spray with EBR in plants under salt stress promoted increases in P_N, E, g_s, WUE, and P_N/C_i values by 15%, 6%, 22%, 11%, and 28%, respectively. However, a decrease of 1% in C_i occurred compared to plants exposed to Na^+ without EBR.

EBR Spray-induced Beneficial Effects on Stomatal Characteristics in Plants Under Na^+ Stress

Salinity promoted negative effects on stomatal characteristics (Table 4). On the adaxial surface, plants exposed to NaCl and EBR had increases of 7%, 4%, and 14% in SD, SF, and SI, respectively, showing decreases of 3% in PDS and 7% for EDS. On the abaxial face, EBR promoted 7%, 6%, and 12% increases in SD, SF, and SI variables, as well as reductions of 2% and 7% in PDS and EDS variables, respectively, when compared to the respective parameters in plants that received similar treatments without EBR.

Table 2 Root and leaf structures in tomato plants sprayed with EBR and exposed to Na^+ stress

Na^+	EBR	RET (µm)	RDT (µm)	RCD (µm)	VCD (µm)	RMD (µm)
Root structures						
−	−	28.09 ± 0.38^a	23.06 ± 1.41^a	180.29 ± 17.52^a	287.49 ± 7.42^b	78.20 ± 5.54^a
−	+	29.38 ± 0.98^a	24.05 ± 0.87^a	192.20 ± 5.37^a	308.34 ± 25.82^a	84.28 ± 7.30^a
+	−	22.56 ± 0.45^c	20.50 ± 0.61^b	157.98 ± 14.90^b	156.19 ± 13.81^d	29.38 ± 0.96^c
+	+	25.20 ± 1.84^b	22.60 ± 0.36^a	172.13 ± 13.05^b	259.96 ± 2.39^c	64.34 ± 3.18^b
Na^+	EBR	ETAd (µm)	ETAb (µm)	PPT (µm)	SPT (µm)	Ratio PPT/SPT
Leaf structures						
−	−	31.93 ± 1.74^a	20.82 ± 1.89^a	134.89 ± 4.64^a	125.52 ± 3.47^c	1.08 ± 0.04^a
−	+	33.38 ± 3.08^a	22.82 ± 1.70^a	142.84 ± 11.77^a	141.28 ± 5.69^a	1.01 ± 0.07^a
+	−	30.51 ± 2.75^a	19.41 ± 1.67^a	124.46 ± 6.08^b	117.16 ± 1.72^d	1.06 ± 0.06^a
+	+	32.31 ± 2.82^a	21.51 ± 1.69^a	136.56 ± 6.91^a	133.05 ± 5.75^b	1.03 ± 0.03^a

RET root epidermis thickness, *RDT* root endodermis thickness, *RCD* root cortex diameter, *VCD* vascular cylinder diameter, *RMD* root metaxylem diameter, *ETAd* epidermis thickness from adaxial leaf side, *ETAb* epidermis thickness from abaxial leaf side, *PPT* palisade parenchyma thickness, *SPT* spongy parenchyma thickness. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Values described corresponding to means from six repetitions and standard deviations

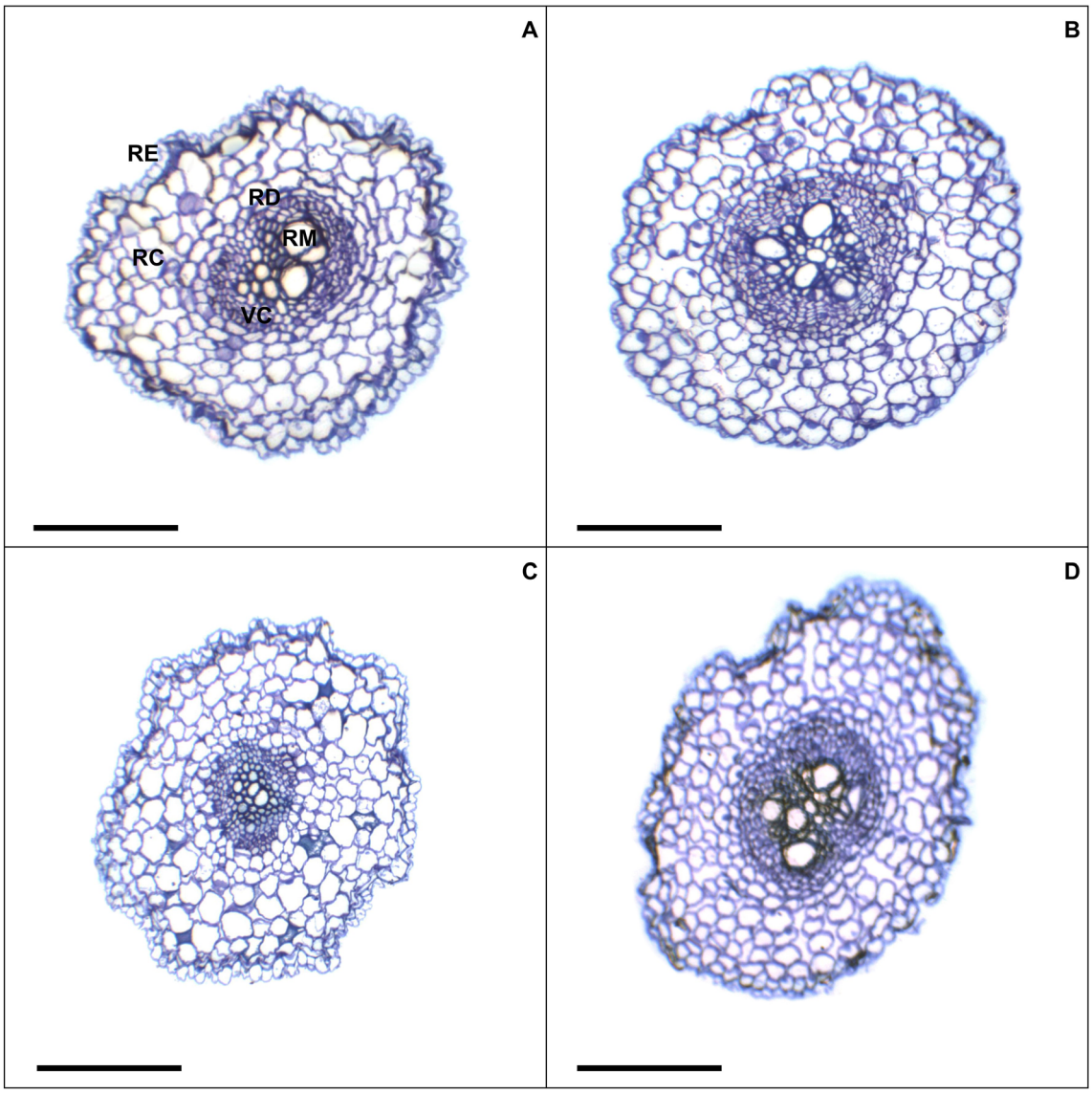

Fig. 1 Root cross sections in tomato plants sprayed with EBR and exposed to salt stress. $-Na^{\pm}$ EBR (**a**), $-Na^{+}/+$EBR (**b**), $+Na^{\pm}$ EBR (**c**) and $+Na^{+}/+$EBR (**d**). *RE* root epidermis, *RC* root cortex, *RD* root endodermis, *VC* vascular cylinder, *RM* root metaxylem. Bars: 200 µm

Redox Metabolism was Upregulated in Plants Sprayed with EBR and Subjected to Salt Toxicity

The high Na^{+} in the solution applied to plants caused increases in antioxidant enzyme activities (Fig. 4). Plants under the combined effects of Na^{+} and EBR exhibited increases of 5%, 22%, 24%, and 31% in SOD, CAT, APX, and POX, respectively, compared to the same parameters in plants that received the same treatment without EBR. Regarding stress indicators, salinity promoted increases (Fig. 5) but plants treated with Na^{+} and EBR presented reductions in O_2^{-}, H_2O_2, EL, and MDA values by 16%, 26%, 19%, and 24%, respectively, compared to the same parameters in plants that received similar treatments without EBR.

Pretreatment with EBR Mitigated the Negative Impacts Associated with Na^{+} on Biomass

Plants treated with Na^{+} exhibited significant decreases in biomass (Figs. 6, 7). EBR spray on plants exposed to NaCl induced increases in LDM, RDM, SDM, and TDM of 24%, 159%, 20%, and 61%, respectively, compared with the same parameters in plants that received equal treatment without EBR.

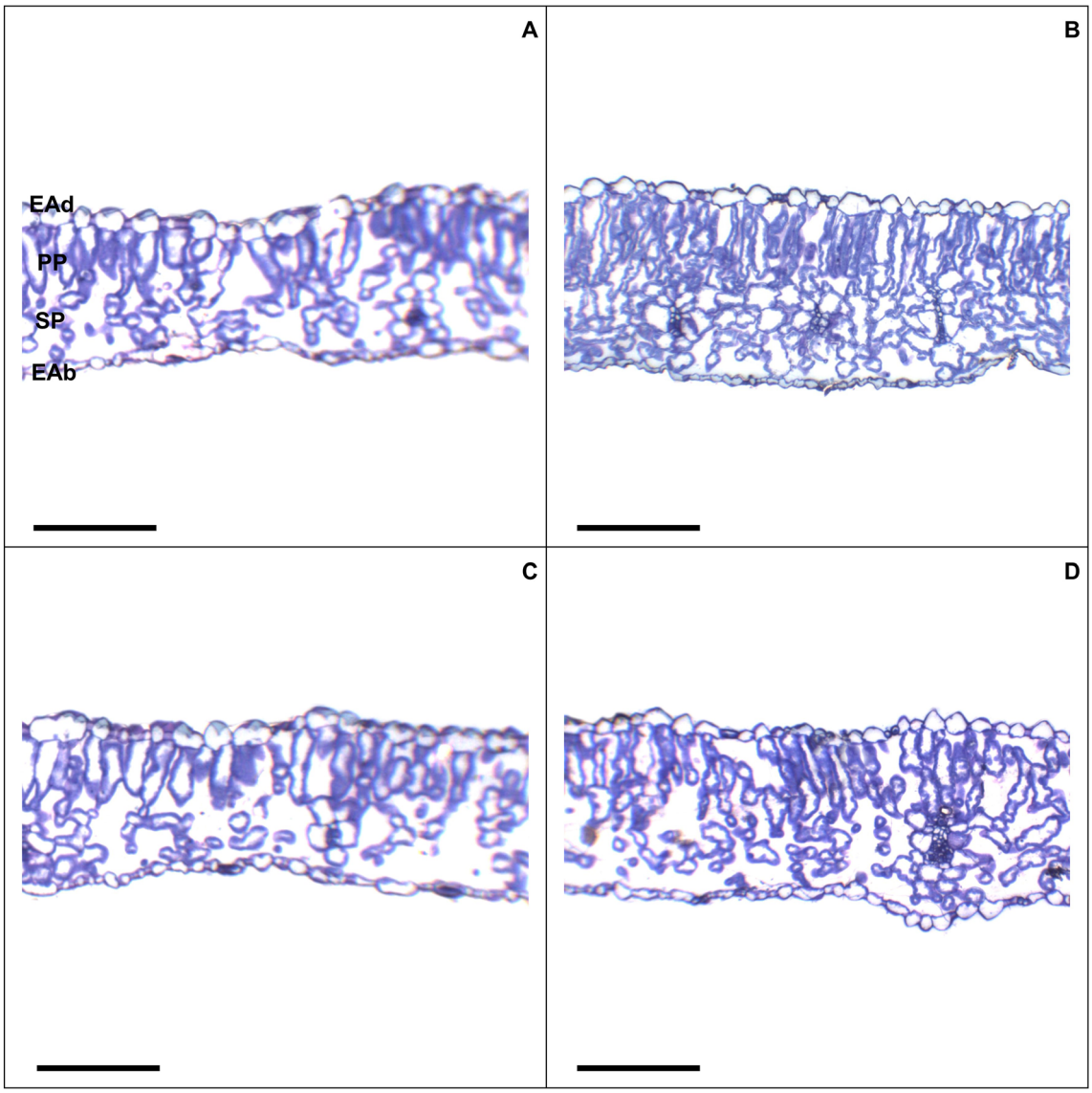

Fig. 2 Leaf cross sections in tomato plants sprayed with EBR and exposed to salt stress. $-Na^{\pm}$ EBR (**a**), $-Na^{+}/+$EBR (**b**), $+Na^{\pm}$ EBR (**c**) and $+Na^{+}/+$EBR (**d**). *EAd* adaxial epidermis, *EAb* adaxial epidermis, *PP* palisade parenchyma, *SP* spongy parenchyma. Bars: 200 µm

Discussion

Treatment with 100 nM EBR reduced the Na^{+} contents in leaf and root tissues of tomato plants exposed to 150 mM NaCl for 15 days. EBR minimized Na^{+} contents, probably due to its beneficial action on cation transporters because under salt stress, K^{+} transporters are often blocked, resulting in Na^{+} uptake and causing reductions in K^{+}/Na^{+} ratios in plants (Sarabi et al. 2017; Alam et al. 2019; Debnath et al. 2019). Na^{+} can accumulate in all plant tissues (leaves, stems, and roots); in other words, these ions can accumulate in the cytoplasm and/or vacuole (Hasegawa 2013) and consequently cause damage to root anatomical structures and photosynthetic machinery (Farooq et al. 2015). Dong et al. (2017) studied the effect of EBR spray on *Triticum aestivum* plants under salinity (120 mM NaCl) and reported that EBR (10 nM) significantly reduced Na^{+} contents in the roots.

EBR promoted increases in structures related to root anatomy (RET, RDT, RCD, VCD, and RMD). This steroid acts on several genes, such as the *ERF2* and *ERF5* genes, which are involved in ethylene biosynthesis and often have effects on the growth and division of meristematic tissues of the root, such as RET, RDT, and RCD (Mussig et al. 2003; Hacham et al. 2011). On the other hand, the plants subjected to 150 mM NaCl presented a reduction in all the anatomical parameters analysed, demonstrating the susceptibility of *Lycopersicon esculentum* roots to salt stress (Hameed et al. 2009; Céccoli et al. 2011). EBR spray induced increases

Table 3 Chloroplastic pigments, chlorophyll fluorescence, and gas exchange in tomato plants sprayed with EBR and exposed to Na^+ stress

Na^+	EBR	Chl *a* (mg g^{-1} FM)	Chl *b* (mg g^{-1} FM)	Total Chl (mg g^{-1} FM)	Car (mg g^{-1} FM)	Ratio Chl *a*/Chl *b*	Ratio Total Chl/Car
Chloroplastic pigments							
−	−	4.11 ± 0.27^b	1.37 ± 0.08^a	5.48 ± 0.28^b	0.63 ± 0.03^b	3.02 ± 0.26^a	8.77 ± 0.77^a
−	+	4.62 ± 0.26^a	1.42 ± 0.13^a	6.04 ± 0.22^a	0.81 ± 0.05^a	3.27 ± 0.32^a	7.46 ± 0.64^b
+	−	2.82 ± 0.17^c	0.78 ± 0.03^c	3.60 ± 0.17^d	0.37 ± 0.01^d	3.63 ± 0.25^a	9.47 ± 0.73^a
+	+	3.92 ± 0.25^b	1.13 ± 0.13^b	5.04 ± 0.22^c	0.61 ± 0.06^c	3.53 ± 0.31^a	8.36 ± 0.26^a

Na^+	EBR	Φ_{PSII}	q_P	NPQ	ETR (µmol m^{-2} s^{-1})	EXC (µmol m^{-2} s^{-1})	ETR/P_N
Chlorophyll fluorescence							
−	−	0.27 ± 0.03^b	0.75 ± 0.05^a	0.22 ± 0.02^b	39.16 ± 3.84^b	0.65 ± 0.43^a	3.28 ± 1.16^b
−	+	0.31 ± 0.01^a	0.78 ± 0.07^a	0.19 ± 0.01^b	44.96 ± 1.62^a	0.61 ± 0.01^b	2.68 ± 0.23^c
+	−	0.24 ± 0.02^b	0.65 ± 0.06^b	0.37 ± 0.03^a	35.85 ± 3.13^b	0.66 ± 0.03^a	5.31 ± 0.49^a
+	+	0.28 ± 0.01^a	0.66 ± 0.06^b	0.30 ± 0.02^a	41.50 ± 1.65^a	0.61 ± 0.04^b	5.01 ± 0.21^a

Na^+	EBR	P_N (µmol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	C_i (µmol mol^{-1})	WUE (µmol $mmol^{-1}$)	P_N/C_i (µmol m^{-2} s^{-1} Pa^{-1})
Gas exchange							
−	−	12.74 ± 1.47^b	2.59 ± 0.10^b	0.24 ± 0.02^b	252.00 ± 7.67^b	6.60 ± 0.19^a	0.049 ± 0.003^b
−	+	16.64 ± 1.12^a	2.96 ± 0.16^a	0.35 ± 0.02^a	243.33 ± 7.55^b	6.92 ± 0.43^a	0.068 ± 0.003^a
+	−	6.83 ± 0.64^c	1.52 ± 0.12^c	0.09 ± 0.01^c	265.17 ± 10.17^a	2.88 ± 0.24^b	0.025 ± 0.001^d
+	+	7.87 ± 1.01^c	1.61 ± 0.13^c	0.11 ± 0.01^c	261.50 ± 18.38^a	3.20 ± 0.24^b	0.032 ± 0.002^c

Chl a chlorophyll *a*; *Chl b* chlorophyll *b*; *Total chl* total chlorophyll, *Car* carotenoids; Φ_{PSII} effective quantum yield of PSII photochemistry, q_P photochemical quenching coefficient, *NPQ* nonphotochemical quenching, *ETR* electron transport rate, *EXC* relative energy excess at the PSII level, *ETR/*P_N ratio between the electron transport rate and net photosynthetic rate, P_N net photosynthetic rate, *E* transpiration rate, g_s stomatal conductance, C_i intercellular CO_2 concentration, *WUE* water-use efficiency, P_N/C_i carboxylation instantaneous efficiency. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Values described corresponding to means from six repetitions and standard deviations

in the RET, RDT, and RCD tissues, mitigating the adverse effects of NaCl because these structures are intrinsically involved in protection under abiotic stress conditions, such as saline stress (Cui 2016). Ribeiro et al. (2019) analysed *Glycine max* seedlings under conditions of water deficiency and reported that EBR had positive effects on RMD, which is an intensely vascularized tissue that contributes to the absorption of water and nutrients; they also verified the positive effects of EBR on RET, RDT, and VCD, indicating that EBR stimulated cell division and root growth.

Plants treated with NaCl and sprayed with EBR had positive outcomes in regard to leaf anatomy variables, such as ETAd, ETAb, PPT, SPT, and PPT/SPT ratio. Increases in PPT and SPT clearly contributed to increases verified in P_N/C_i and P_N, reflecting ETAd and ETAb (Abbruzzese et al. 2009). PPT and SPT are tissues with large amounts of chloroplasts and intracellular spaces and are both efficient channels for CO_2 capture (Pereira et al. 2016; Maia et al. 2018). Salinity promotes negative interferences on gas exchange, as well as it lowers rates of expansion and cell division (Hu and Schmidhalter 2005). However, this steroid probably improved Ca^{+2} uptake, affecting cell expansion and division (Hayat et al. 2012), with a subsequent increase in the ETAd and ETAb values observed in our study. Oliveira et al. (2018) studied the deleterious effects of saline stress (0 and 250 mM NaCl) and the consequences of EBR administration (0 and 50 nM EBR) on young *Eucalyptus urophylla* plants and found increases in ETAd (7%), ETAb (22%), PPT (14%), and SPT (25%) after the application of EBR.

Plants subjected to Na^+ and EBR presented increases in photosynthetic pigments, and this result was related to the role of EBR in mitigating the degradation of chlorophylls occasioned by photoinhibition. Photoinhibition is a phenomenon that occurs when photosynthetic pigments absorb light excessively, which leads to damage at the reaction centres of PSII, suggesting that EBR improves heat dissipation and electron transfer (Munns and Tester 2008b; Pereira et al. 2019). Alzahrani et al. (2019) evaluated the physiological, biochemical, and antioxidant responses of two *Vicia faba* genotypes subjected to three concentrations of NaCl (50, 100, and 150 mM) and found significant reductions of 54%,

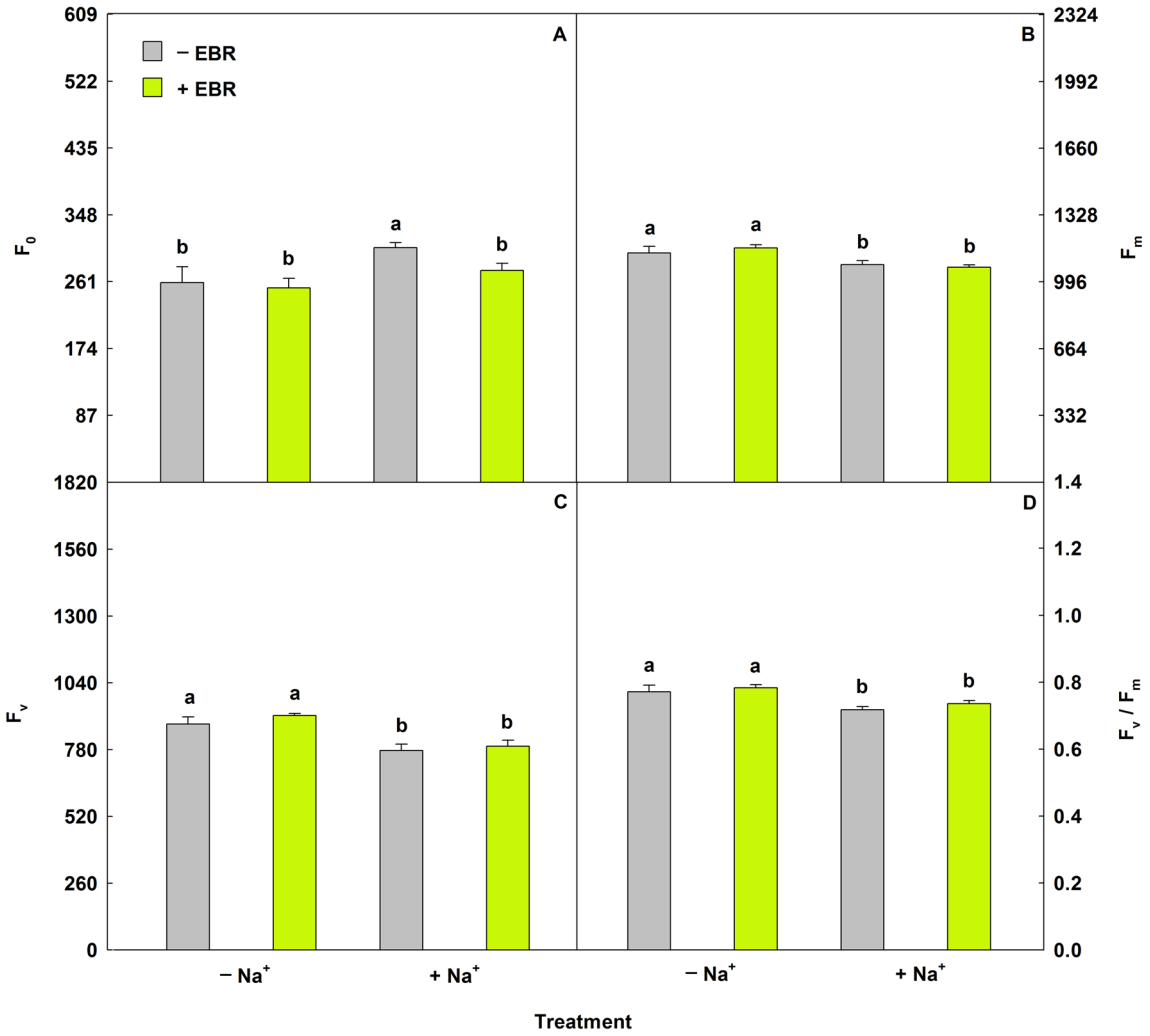

Fig. 3 Minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted state (F_m), variable fluorescence (F_v), and maximal quantum yield of PSII photochemistry (F_v/F_m) in tomato plants sprayed with EBR and exposed to salt stress. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$). Columns corresponding to means from six repetitions and standard deviations

Table 4 Stomatal characteristics in tomato plants sprayed with EBR and exposed to Na^+ stress

Na^+	EBR	SD (stomata per mm^2)	PDS (µm)	EDS (µm)	SF	SI (%)
Adaxial face						
−	−	137.14 ± 12.05^b	18.38 ± 1.03^a	25.52 ± 1.51^c	0.72 ± 0.06^a	12.11 ± 1.03^b
−	+	142.86 ± 0.00^a	16.24 ± 0.98^b	22.62 ± 2.24^d	0.72 ± 0.07^a	13.69 ± 1.01^a
+	−	107.14 ± 2.37^d	19.58 ± 1.34^a	29.26 ± 1.67^a	0.67 ± 0.05^a	9.44 ± 0.65^d
+	+	114.19 ± 1.81^c	18.95 ± 1.62^a	27.34 ± 1.72^b	0.70 ± 0.07^a	10.76 ± 0.75^c
Abaxial face						
−	−	175.57 ± 11.78^a	18.81 ± 0.67^b	25.91 ± 2.39^b	0.73 ± 0.08^a	13.40 ± 0.76^a
−	+	181.43 ± 6.32^a	17.62 ± 1.47^b	24.91 ± 2.31^b	0.71 ± 0.09^a	14.02 ± 0.95^a
+	−	154.14 ± 10.11^c	20.11 ± 1.83^a	28.83 ± 0.91^a	0.70 ± 0.06^a	11.09 ± 0.87^c
+	+	164.29 ± 6.51^b	19.74 ± 1.59^a	26.94 ± 2.17^b	0.74 ± 0.07^a	12.47 ± 0.78^b

SD stomatal density, *PDS* polar diameter of the stomata, *EDS* equatorial diameter of the stomata, *SF* stomatal functionality, *SI* stomatal index. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$). Values described corresponding to means from six repetitions and standard deviations

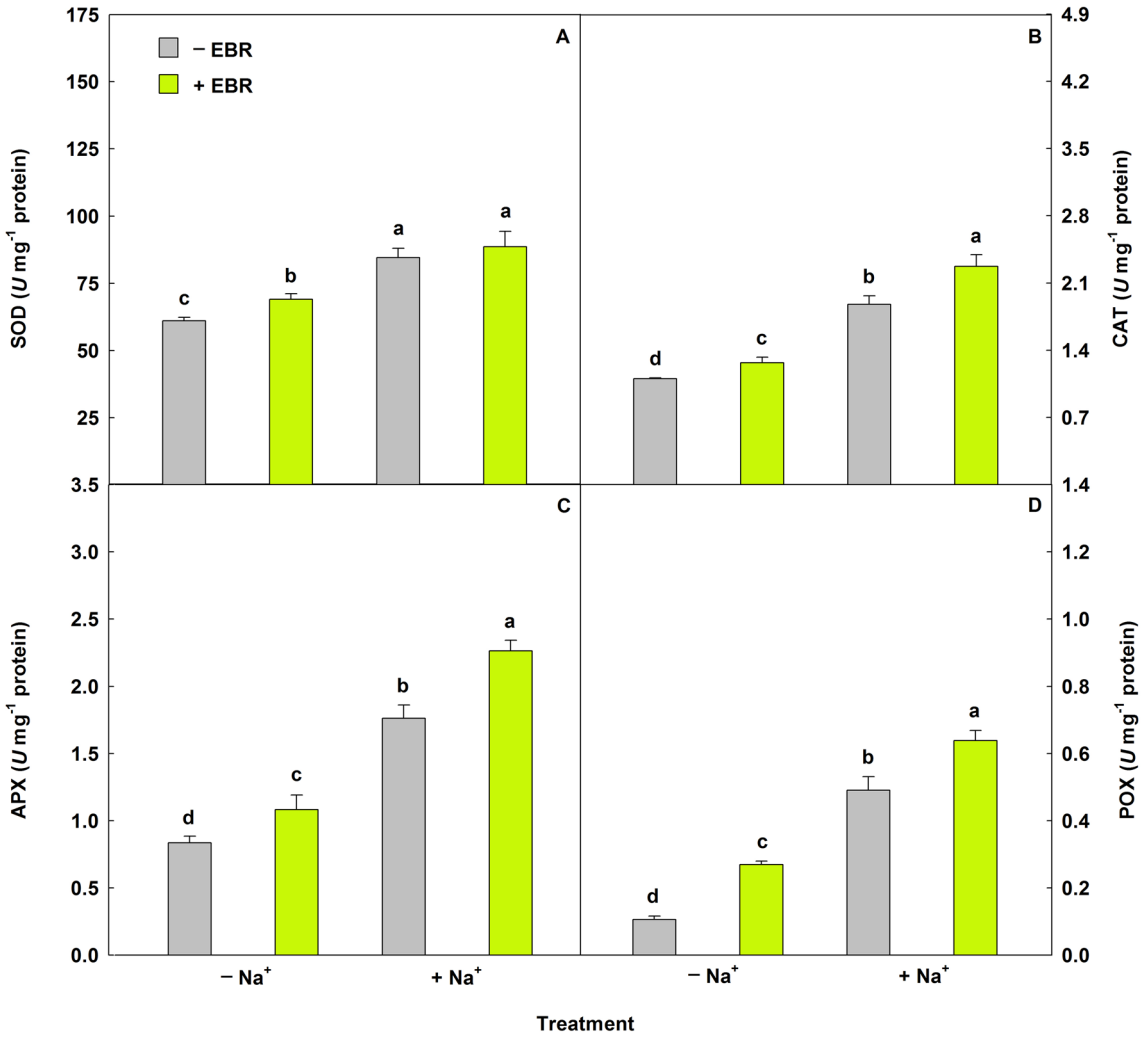

Fig. 4 Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POX) in tomato plants sprayed with EBR and exposed to salt stress. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Columns corresponding to means from six repetitions and standard deviations

51%, and 51% for Chl *a*, Chl *b*, and Car, respectively, when compared to the control in the Hassawi-3 genotype.

Steroids increased F_m, F_v, and F_v/F_m values and decreased F_0, alleviating the damage provoked by salinity on PSII reaction centres. The low F_v/F_m values in plants under salinity are clear indicators of injury to thylakoid membranes due to negative interferences promoted by O_2^- in PSII, with subsequent inhibition of the photosynthesis process (Hussain and Reigosa 2011). In contrast, steroid protects the photosynthetic apparatus from ROS and attenuates the photoinhibition induced by excess NaCl in PSII reaction centres (Ahammed et al. 2012). In other words, steroid used probably interfered on translation of the *psbA* gene, which is required for transcription of pre-D1, a precursor of D1 protein in PSII. Salinity often inhibits the degradation of D1 protein during photoinhibition by blocking the interaction between FtsH, DegP2 proteases, and D1 protein (Ohnishi and Murata 2006). In addition, this study described that EBR caused increases in Φ_{PSII}, ETR, and q_p, while NPQ, EXC, and ETR/P_N declined in plants pretreated with EBR and exposed to Na^+. There results demonstrate the positive effects of this steroid on ETR and better utilization of light energy via q_p, improving Φ_{PSII} (Qin et al. 2011; Qiu et al. 2013; Kahlaoui et al. 2014). Wang et al. (2015) investigated chlorophyll fluorescence, leaf surface morphology, and cell ultrastructure in *Vitis vinifera* plants treated with EBR and exposed to water deficit and detected beneficial outcomes on F_v/F_m and Φ_{PSII}, similar to the results found in this research.

Gas exchange was maximized, with increases in P_N, E, g_s, WUE, and P_N/C_i and a decrease in C_i; these results indicate positive effects associated with exogenous application of EBR. Plants treated with EBR and Na^+ had increases

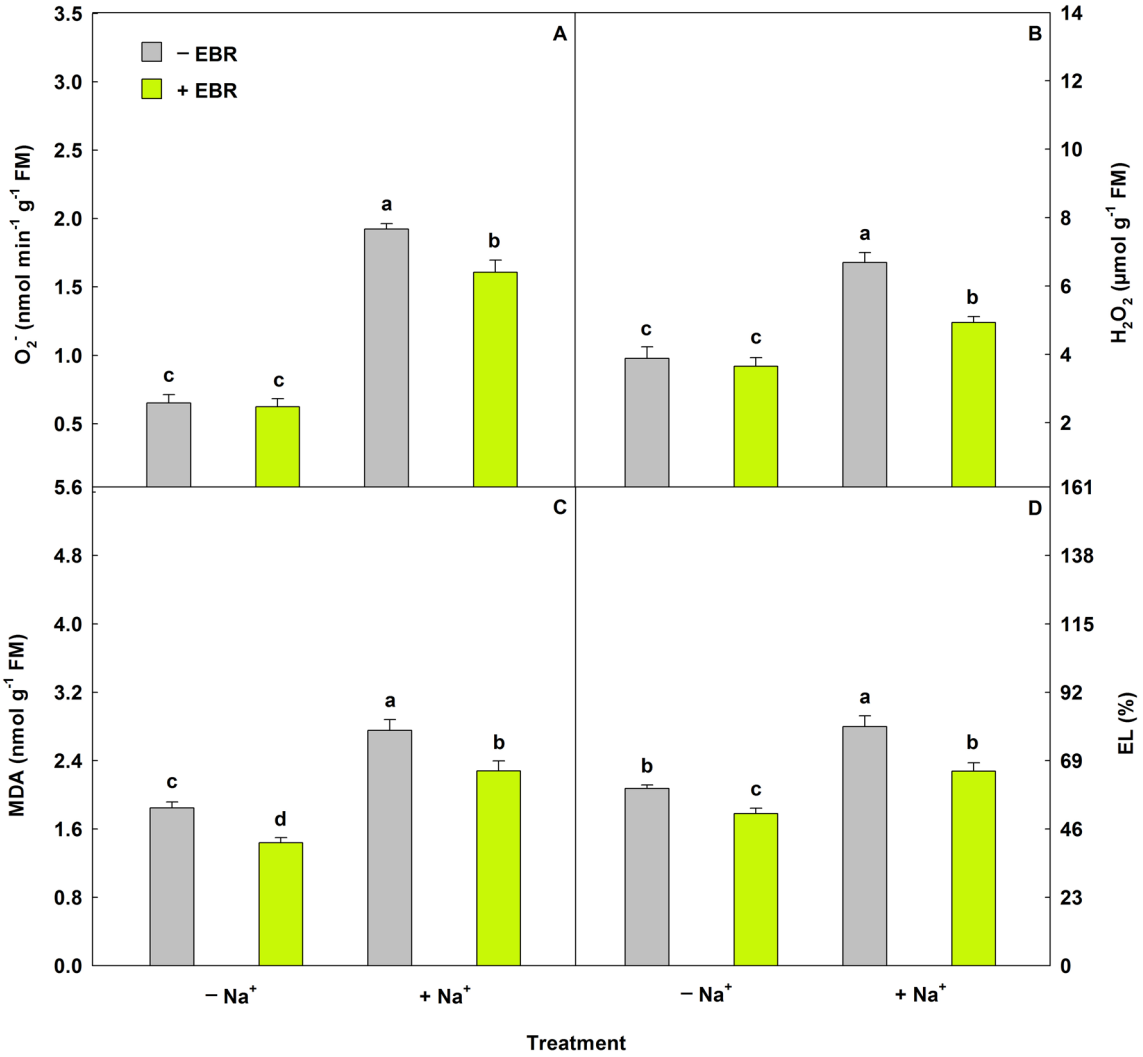

Fig. 5 Superoxide (O_2^-), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and electrolyte leakage (EL) in tomato plants sprayed with EBR and exposed to salt stress. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Columns corresponding to means from six repetitions and standard deviations

in SD and SI as previously reported, suggesting beneficial effects of EBR on the stomatal mechanism (Chaves et al. 2009). This led to an increase in the g_s values, verified in this study in plants under the effect of 100 nM EBR and 150 mM NaCl, which led to higher CO_2 absorption (P_N/C_i) and a probable increase in the activity of RuBisCo, an enzyme responsible for CO_2 fixation in the Calvin Cycle, and an ensuing increase in P_N (Karlidag et al. 2011; Allel et al. 2018). The increase observed in WUE indicates a better utilization of the water resource, a part of the adaptive mechanisms against the deleterious effects caused by the high concentration of Na^+ in the cells of the roots and leaves. In addition, the increase observed in *E* is intrinsically linked to g_s, suggesting that EBR improves water inflow through stomatal adaptations, modulating tolerance to the osmotic and ionic imbalance induced by NaCl (Gupta et al. 2016; Hasanuzzaman et al. 2018). Our research corroborates the results of Shahbaz et al. (2008), who found increases in P_N, *E*, and g_s of two *Triticum aestivum* genotypes treated with EBR (0, 0.0125, 0.025, and 0.0375 mg L^{-1}) and exposed to NaCl (0 and 150 mM).

Functional and anatomical characteristics of the stomata were improved upon treatment with EBR, resulting in increases in SD, SF, and SI values and decreases in PDS and EDS. It is conceivable that EBR stimulated transporters with a high affinity to K^+, therefore, enhancing the absorption of this ion and attenuating the imbalance in the K^+/Na^+ ratio, which is important for the osmoregulation of cells and stomatal functioning (Chen et al. 2005). A consequent increase in SF may have improved the efficiency of the gas exchange process, as previously verified in this study. The absorption and assimilation of Na^+ ions directly interfere

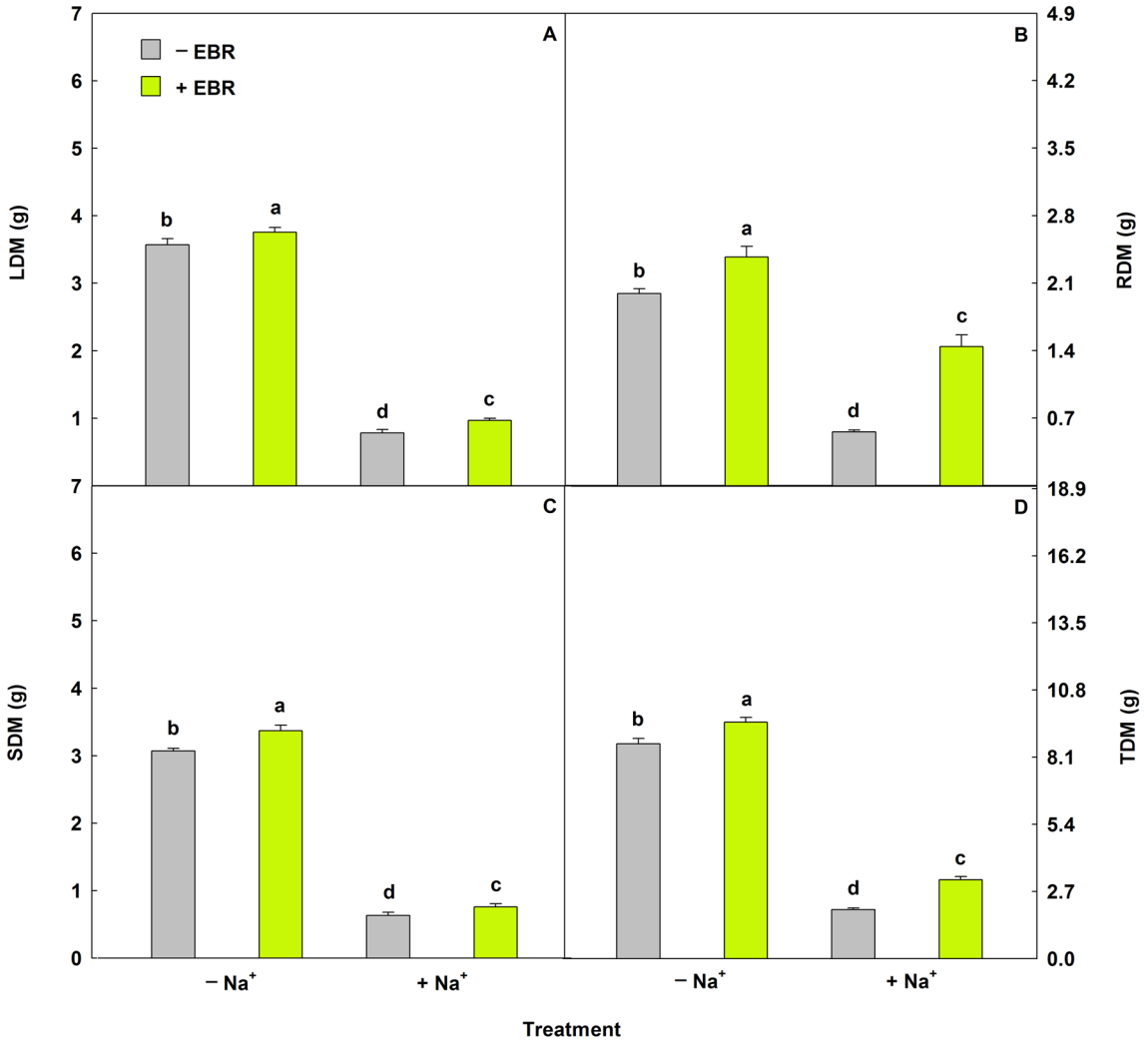

Fig. 6 Leaf dry matter (LDM), root dry matter (RDM), stem dry matter (SDM), and total dry matter (TDM) in tomato plants sprayed with EBR and exposed to salt stress. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Columns corresponding to means from six repetitions and standard deviations

with the functionality and anatomy of the stomata, resulting in reduction of SD and SF. However, plants sprayed with EBR had decreases in PDS and EDS, resulting in higher SD and SI. Khan et al. (2003) reported that smaller and elliptical stomata present higher density and greater functionality compared to large and cylindrical stomata, which has positive effects on WUE, CO_2 absorption, and gas exchange (Souza et al. 2018). Our results confirm the study conducted by Maia et al. (2018) working with two *Lycopersicon esculentum* genotypes (BR-efficient and BR-deficient) sprayed with two EBR concentrations (0 and 100 nM) that showed that EBR increased SD, SF, and SI and decreased PDS and EDS values.

Plants treated with EBR and exposed to Na^+ increased the activities of the SOD, CAT, APX, and POX enzymes, demonstrating the action of EBR in the cellular homeostatic processes linked to the reduced production of oxygen reactive species (ROS). Salinity frequently induces cellular redox imbalance, resulting in the accelerated accumulation of ROS (Parihar et al. 2015); however, our results revealed that EBR positively modulated the activity of enzymes responsible for the elimination of ROS through the antioxidant system (Fita et al. 2015). This system is composed of reactions that promote the detoxification of toxic radicals, such as O_2^- and H_2O_2, with the enzyme SOD being the first defence line by dismutation of O_2^- to H_2O_2 (Apel and Hirt 2004). Dong et al. (2017) studied EBR functions in relation to salt tolerance in *Triticum aestivum* seedlings subjected to three NaCl concentrations (0, 100, and 120 nM) and sprayed with 1, 10, and 100 nM EBR and found increases in the enzymatic activities of SOD and POX after spraying with 10 nM EBR when compared to the same treatment without EBR.

Fig. 7 Upper and side views of tomato plants sprayed with EBR and exposed to salt stress

Steroid induced reductions in O_2^-, H_2O_2, MDA, and EL levels in plants under 150 mM NaCl and maximized the activities of the antioxidant enzymes intrinsically related to ROS removal previously described in this study. Paralelly, Oliveira et al. (2018) found that EBR application improves K^+ uptake, which implies a reduction in the K^+/Na^+ ratio. K^+ ions are responsible for cell osmoregulation, contributing among others to mitigate the production of ROS in chloroplasts in adverse conditions (Pang and Wang 2008; Khoshbakht et al. 2018). Our research corroborates the results described by Sun et al. (2015) investigating the application of EBR in *Lolium perene* plants under salt stress, observing reductions of 29% and 21% for H_2O_2 and MDA, respectively, when sprayed with 10 nM EBR.

Treatment with exogenous EBR attenuated the impacts on biomass in plants exposed to 150 mM NaCl, increasing LDM, RDM, SDM, and TDM. This may be directly related to the improvement promoted by EBR on photosynthetic pigments, PSII efficiency, stomatal characteristics, and antioxidant enzymes. Under salt stress, reduced rates of light absorption (Latrach et al. 2014), insufficient function of the photosynthetic machinery (Forieri et al. 2016a), cellular damage (Hu et al. 2016), and negative reflexes on leaf anatomy (Oliveira et al. 2018) are normally detected, and they result in lower biomass (Sharma et al. 2013). Interestingly, our study discovered multiple positive effects of this steroid; it mitigated the degradation of photosynthetic pigments via cell osmoregulation due to the reduced production of ROS and photochemical dissipation as demonstrated by reductions in NPQ, EXC, and ETR/P_N. In addition, it favoured cell division and expansion, as verified in the anatomical variables PPT and SPT, resulting in increase in growth. Fariduddin et al. (2014) studied the physiological, biochemical, and morphological responses in *Cucumis sativus* plants under salt stress and observed an increase of 47% in TDM after spraying with 10^{-8} M EBR. Lima and Lobato (2017) studied *Vigna unguiculata* plants under water deficit combined with the application of EBR (0, 50, and 100 nM) and reported benefits on biomass, obtaining increases of 11%, 7%, 10%, and 10% for LDM, SDM, RDM, and TDM, respectively.

Conclusions

This research confirmed that pretreatment with EBR in tomato plants attenuated the deleterious effects associated with Na^+ stress. EBR modulated protection and vascularization to root structures, demonstrated by the increases in epidermis thickness and metaxilem diameter, respectively. Concomitantly, this steroid relieved oxidative damage, which was clearly associated with elevation of the activities of antioxidant enzymes, such as superoxide ascorbate peroxidase and guaiacol peroxidase. EBR also had beneficial effects on photosynthetic pigments, alleviating the degradation of chlorophylls, concomitant with reductions in malondialdehyde and electrolyte leakage. In addition, pretreatment with EBR favoured higher biomass accumulation due to positive effects on leaf and root tissues, including improved performance of the photosynthetic apparatus. Therefore, these results suggest that pretreatment with 100 nM EBR clearly provided protection in tomato plants under salt stress.

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Author Contribution AKSL and PA were the advisors of this project, planning all phases of the research and critically revised the manuscript. VQS, WFSM, YCP, BRSS, and EMSGL conducted the experiment and performed physiological, biochemical, anatomical, and morphological determinations, as well as wrote and edited the manuscript. MNA critically revised the manuscript. All authors read and approved final version of manuscript.

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Data Availability Data are available upon request to the corresponding author.

Declarations

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

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