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Research article

Salinity-induced modifications on growth, physiology and 20-hydroxyecdysone levels in Brazilian-ginseng [*Pfaffia glomerata* (Spreng.) Pedersen]

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ABSTRACT

– Salinity is a major threat to agriculture. However, depending on the concentration of soluble salts in soil, increased secondary metabolite levels can occur with no major damages to plant growth and development. The phytoecdysteroid (PE) 20-hydroxyecdysone (20E) is a secondary metabolite with biotechnological, medicinal, pharmaceutical and agrochemical applicability. Here, we characterize the responses (growth and physiology) of *Pfaffia glomerata* under different NaCl concentrations and examine the production of 20E as affected by salinity. Forty-day-old plants grown in greenhouse were exposed to 0, 120, 240, 360 or 480 mM of NaCl for 11 days. Moderate salinity (i.e., 120 mM of NaCl) led to increased 20E concentrations in leaves (47%) relative to the control with no significant effect on photosynthesis and biomass accumulation, thus allowing improved 20E contents on a per whole-plant basis. In contrast, plants under high salinity (i.e., 240–480 mM of NaCl) displayed similar 20E concentrations in leaves compared to the control, but with marked impairments to biomass accumulation and photosynthetic performance (coupled with decreased sucrose and starch levels) in parallel to nutritional imbalance. High salinity also strongly increased salicylic acid levels, antioxidant enzyme activities, and osmoregulatory status. Regardless of stress severity, 20E production was accompanied by the upregulation of *Spook* and *Phantom* genes. Our findings suggest that *P. glomerata* cultivation in moderate salinity soils can be considered as a suitable agricultural option to increase 20E levels, since metabolic and structural complexity that makes its artificial synthesis very difficult.

1. Introduction

Plants are often exposed to several environmental stresses such as drought, waterlogging, temperature extremes and salinity. Salinity affects the soils of more than 100 countries (≅ 1 billion ha of land area) and contributes to the degradation of land and water resources (FAO and ITPS, 2015). Salt stress affects plants in two distinct ways, by inducing osmotic stress causing ion toxicity due to the accumulation of sodium (Na^+) and/or chloride (Cl^-) at toxic concentrations, thus limiting nutrient absorption (e.g., K^+ and Ca^{2+}) and disturbing the ionic balance (Almeida et al., 2017a; Assaha et al., 2017; Wangsawang et al., 2018). As general consequences of these imbalances plant growth and productivity can be severely impaired (Hanin et al., 2016; Acosta-Motos et al., 2017; Negrão et al., 2017).

Overall, plants cope with reductions in soil osmotic potential (osmotic or water-deficit stress) caused by salinity via acclimation mechanisms such as osmotic adjustment and maintenance of cell turgor, which are associated with increases in the levels of solutes such as

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amino acids (e.g., proline), quaternary ammonium compounds (e.g., glycine betaine), tertiary sulfonium compounds (e.g., dimethylsulfoniopropionate), polyols (e.g., mannitol), and soluble sugars (e.g., fructose, trehalose, and raffinose) (Reis et al., 2012; Richter et al., 2015; Farhangi-Abriz and Torabian, 2018). The ionic imbalance caused by salinity can disrupt the metabolic homeostasis, which in turn can promote the reactive oxygen species (ROS) formation at levels detrimental to cells (e.g., superoxide anion (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2)) (Azevedo-Neto et al., 2006; Miller et al., 2010; Hossain et al., 2017), provoking serious damages to the metabolism through the oxidation of lipids, nucleic acids and proteins (Gill and Tuteja, 2010). To cope with salt-induced oxidative stress, plants evolved a sophisticate mechanism encompassing both enzymatic (e.g., superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX)) (Koleška et al., 2017; Vighi et al., 2017; Farhangi-Abriz and Torabian, 2018) and non-enzymatic (e.g., ascorbic acid (ASC), reduced glutathione (GSH), and α-tocopherol) systems (Chakraborty et al., 2016; Arora and Bhatla, 2017; Kaur et al., 2017).

The deleterious effects of salinity caused by osmotic stress, nutritional imbalance, and oxidative stress ultimately promote decreases in leaf area, chlorophyll content, stomatal conductance and photosystem II (PS II) efficiency, which collectively lead to decreased photosynthetic performance on a leaf- and whole-plant scales (Meloni et al., 2003; Chaves et al., 2009; Kan et al., 2017; Çiçek et al., 2017). Nevertheless, salt stress can induce the biosynthesis of molecules from secondary metabolism with high biotechnological interest in plants (Wahid and Ghazanfar, 2006; Akula and Ravishankar, 2011). For example, salt stress increased the contents of gossypol in cotton (Wang et al., 2015), changed the production levels of artemisinin and essential oils in *Artemisia annua* L. (Yadav et al., 2017) and affected the biosynthesis pathways of carotenoids and flavonoids in *Solanum nigrum* (Abdallah et al., 2016). Consequently, the understanding on how salinity can lead to increased levels of metabolites with biotechnological, medicinal, pharmaceutical and agrochemical applicability is of utmost importance and may contribute to the development of agriculture under unfavorable conditions.

Plants have developed several genetic mechanisms that produce secondary metabolites, which play important roles in defense and acclimatation to face biotic and/or abiotic stresses (Barton and Boege, 2017). Here, we stand out the 20-hydroxyecdysone (20E), the main hormone related to ecdysis process in insects (zooecdysteroid), but acting as a secondary metabolite (phytoecdysteroid) in the plant kingdom. The 20E displays potential therapeutic applications (John et al., 2018), and it is produced in relatively large amounts in some species such as *Pfaffia glomerata* (Spreng.) Pedersen (Amaranthaceae).

P. glomerata is a medically and economically important species with proven phytotherapeutic properties (Almeida et al., 2017b; Silva et al., 2017; Dias et al., 2019; Vardanega et al., 2019). It has been demonstrated that application of 20E protects *Triticum aestivum* plants from oxidative imbalance, enhances the anti-oxidative systems and improves resistance to lead stress (Lamhamdi et al., 2016), in addition to enhancing tolerance to salt stress (Li et al., 2018); however, the impacts that abiotic stresses may cause on endogenous levels of 20E are unknown. In phytosterol (plant sterols) pathways, salt stress enhanced the levels of campesterol, stigmasterol, and β-sitosterol in *Avicennia marina* and *Rhizophora stylosa*, a salt secretor and a non-salt secretor plant, respectively (Basyuni et al., 2012), which suggests that salinity may also promote the increase of PEs (i.e., 20E), since this pathway is derived from phytosterols (Tarkowská et al., 2016; Moreau et al., 2018).

In *P. glomerata*, recent information has revealed two Halloween genes – *Spook* (*Spo*) and *Phantom* (*Phm*) – associated with 20E biosynthesis (Batista et al., 2019a), although the 20E biosynthetic pathway is not fully elucidated (Festucci-Buselli et al., 2008a; Ohyama et al., 2009; Tsukagoshi et al., 2016); in addition, the role of 20E is also poorly understood (Festucci-Buselli et al., 2008a; Boo et al., 2013; Thussagunpanit et al., 2017). Thus, the use of experimental strategies to analyze genes involved in 20E biosynthesis may provide a better understanding of the ecological, physiological and biochemical role in plants (Festucci-Buselli et al., 2008b).

Given the facts described above, we hypothesized that salinity can lead to increased 20E contents (absolute amounts) in *P. glomerata*. Here, we firstly characterize the responses of *P. glomerata* to salt stress and, secondly, examine the production of 20E as affected by salinity. We demonstrate that moderate salinity (i.e., 120 mM) does not affect plant growth and photosynthesis but is able to increase the total 20E production on a per plant basis. Our study offers, therefore, novel insights on how *P. glomerata* deals with salt stress and explore underlying mechanisms by which 20E production is affected by salinity.

2. Materials and methods

2.1. Plant growth and experimental design

Plants of *Pfaffia glomerata* (accession 43) used in this study were taken from the Germplasm bank of the Plant Tissue Culture Laboratory (Federal University of Viçosa, Brazil), where 71 accessions are maintained *in vitro*. Voucher material was deposited at the Leopoldo Krieger Herbarium (UFJF, Juiz de Fora, MG, Brazil) under the code number CESJ 63,317.

Explants with a nodal segment (1.5 cm) were cultured in test tubes (22 mm diameter x 150 mm height) containing 10 mL Murashige and Skoog (1962) medium supplemented with MS vitamins, 100 mg L^{-1} myo-inositol and 3% sucrose (w/v), pH was adjusted to 5.7 ± 0.1, solidified with 5.5 g L^{-1} agar (Phytotechnology Laboratories, LLC, Shawnee Mission, KS, USA) and sterilized by autoclaving at 121 °C and 1.5 atm for 20 min. The plants were cultivated for 40 days under 25 ± 2 °C, with 40 μmol m^{-2} s^{-1} of irradiance and 16-h photoperiod.

After the *in vitro* culture, plants were acclimatized for 10 days in 300-mL plastic cups (COPOBRAS, São Ludgero, SC, Brazil). Then, they were transplanted to plastic vessels (24 × 21 × 15 cm; EME-A-EME Ind. Com. Ltda., Petrópolis, RJ, Brazil) filled with 2 kg of commercial substrate Tropstrato HT® (Vida Verde, Mogi Mirim, SP, Brazil) and then the plants were grown in a greenhouse (average temperature 27.5 ± 2 °C; natural light conditions with intensity of ~750 μmol photons m^{-2} s^{-1}). During the greenhouse cultivation (51 days total, being 40 days of growth without salt and 11 days with salt), the plants were fertilized at 15 and 30 days with 5.5 g NPK - 26:06:12 (Polyblen Montanha®, São Paulo, SP, Brazil).

Salt treatments 0 (control), 120, 240, 360 and 480 mM of sodium chloride (NaCl; ACS reagent, ≥ 99%, Sigma-Aldrich, St. Louis, MO, USA) were applied to 40-day-old plants during 11 days. NaCl was dissolved in deionized water at the established concentrations and the plants where was watered to soil saturation within a single step as reported in Cao et al. (2018), except the control that did not receive the salt. Plants were irrigated during the experiment using deionized water (conductivity equal to 0.04 mS). After this period, samplings and measurements were performed. The volume of irrigation water was applied to maintain the soil near field capacity. The experimental design was completely randomized with 7 replicates; the experimental unit was one plant per vessel.

2.2. Growth parameters

Plant tissues were collected at the end of the experiment and separated into root, stem, and leaves, after which they were oven-dried at 50 °C for 72 h to obtain the dry weight (DW). The abscised leaves were collected and their masses included in total plant DW. Plant height was also assessed.

2.3. Tissue nutrient analysis

Leaves were sliced and dried at 50 °C with forced and continuous

Fig. 1. 51-day-old *Pfaffia glomerata* plants exposed to different concentrations of NaCl (0 – control, 120, 240, and 480 mM) for 11 consecutive days. Bar = 10 cm.

Table 1
Growth parameters in 51-day-old *Pfaffia glomerata* plants exposed to different concentrations of NaCl (0 – control, 120, 240, and 480 mM) for 11 consecutive days.

NaCl (mM)	Plant height (cm)	Leaf dry weight (g)	Leaf abscission dry weight (g)	Stem dry weight (g)	Root dry weight (g)	Total dry weight (g)
0	66.0 ± 4.9 a	10.3 ± 0.4 a	0.0 b	8.6 ± 0.4 a	4.4 ± 0.3 a	23.2 ± 1.0 a
120	65.3 ± 7.2 a	8.9 ± 0.6 a	0.0 b	8.4 ± 0.7 a	4.1 ± 0.4 a	21.4 ± 1.3 a
240	55.1 ± 8.3 b	6.6 ± 0.6 b	0.49 ± 0.2 b	4.8 ± 0.6 b	2.2 ± 0.4 b	14.0 ± 1.2 b
360	44.9 ± 9.5 c	4.6 ± 0.6 b	1.41 ± 0.3 a	2.4 ± 0.2 c	0.9 ± 0.2 c	9.3 ± 0.8 c
480	44.8 ± 4.1 c	4.4 ± 0.6 b	2.17 ± 0.3 a	3.2 ± 0.1 bc	1.0 ± 0.1 bc	10.7 ± 0.5 bc

Values represent means ± SE (n = 7). Same letters do not differ at 5% level by Tukey's test.

ventilation until the dry mass stabilized and then were milled (using a Knife Mill, Willey®). To quantify the levels of P, K^+, Ca^{2+}, Mg^{2+}, S, Zn, Cu, Mn, Fe and Na^+, the leaf samples were submitted to nitric-perchloric digestion (4:1) (v/v) and the N the digestion was sulfuric by Kjeldahl method (Sarruge and Haag, 1974). Determination of P was by colorimetry through the ascorbic acid with reading in molecular absorption spectrophotometer (Femto Indústria e Comércio de Instrumentos Ltda., São Paulo, SP, Brazil); K and Na by flame photometry (Corning 400, Corning Inc., NY, USA); Ca, Mg, Fe, Cu, Zn and Mn by atomic absorption spectrophotometry (Mulgrave, Victoria, Australia); and S by turbidimetry as reported in Alvarez et al. (2001).

2.4. Leaf gas exchange and chlorophyll a fluorescence

Gas exchange analyzes were performed on the third fully expanded leaf from the apex of plants using the open gas exchange system Li-6400XT (Li-Cor, Lincoln, NE, USA) equipped with an integrated fluorescence chamber heads (Li-6400-40, Li-Cor, USA). The net CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), transpiration rate (E) and internal CO_2 concentration (C_i) were measured from 08:30 to 12:00 h under an external CO_2 concentration of 400 μmol mol^{-1} air and average temperature of 25 °C. All measurements were conducted under an artificial, saturating light of 1000 μmol m^{-2} s^{-1} that was provided by a light-emitting diode with 10% blue light in order to maximize stomata opening.

Previously dark-adapted (8 h) leaf tissues were illuminated with weak modulated measuring beams (0.03 μmol m^{-2} s^{-1}) to obtain the initial fluorescence (F_0). Saturating white light pulses (8000 μmol m^{-2} s^{-1}; 0.8 s) was applied to ensure maximum fluorescence emissions (F_m), from which the variable-to-maximum Chl fluorescence ratio, $F_v/F_m = [(F_m - F_0)/F_m)]$, was calculated. The coefficient for photochemical quenching (qP) was calculated as $qP = (F_m'-F_s)/(F_m'-F_0')$ and that for non-photochemical quenching (NQP) was calculated as $NQP = (F_m/F_m')-1$. The actual quantum yield of PS II electron transport (Φ_{PSII}) was computed as $\Phi_{PSII} = (F_m'–F_s)/F_m'$). The apparent electron transport rate (ETR) was estimated as ETR = Φ_{PSII}*PPFD × f*α where f is a factor that accounts for the partitioning of energy between PS II and PSI and is assumed to be 0.5, which indicates that excitation energy is distributed equally between the two photosystems, and α is the leaf absorptance by photosynthetic tissues and is assumed to be 0.84 (Maxwell and Johnson, 2000).

2.5. Determination of total chlorophyll and quantification carbohydrates, protein and amino acid levels

Leaf samples (third fully expanded leaf from the apex of plants), collected at the end of the experiment at 13:00 h, were flash frozen in liquid nitrogen, and subsequently ground and lyophilized for analysis. Approximately 30 mg lyophilized tissues were used for extraction with ethanol as described by Praxedes et al. (2006). Photosynthetic pigments were determined as described by Arsovski et al. (2018). Carbohydrates (starch, sucrose, glucose, and fructose) were assessed as described by Fernie et al. (2001). Protein and total amino acid levels were analyzed as reported in Cross et al. (2006).

2.6. Proline content

Proline content was determined following Bates et al. (1973), with minor modifications. Approximately 10 mg of lyophilized leaf tissue (third fully expanded leaf from the apex of plants) were suspended in 1 mL of 3% sulfosalicylic acid (w/v), vortexed (2 times for 20 s) and centrifuged at 6300 g for 10 min. Later, 200 μL of the supernatant was collected, 400 μL of acidic ninhydrin solution (1.25 g of ninhydrin, 30 mL of glacial acetic acid and 20 mL of 6M phosphoric acid) were added

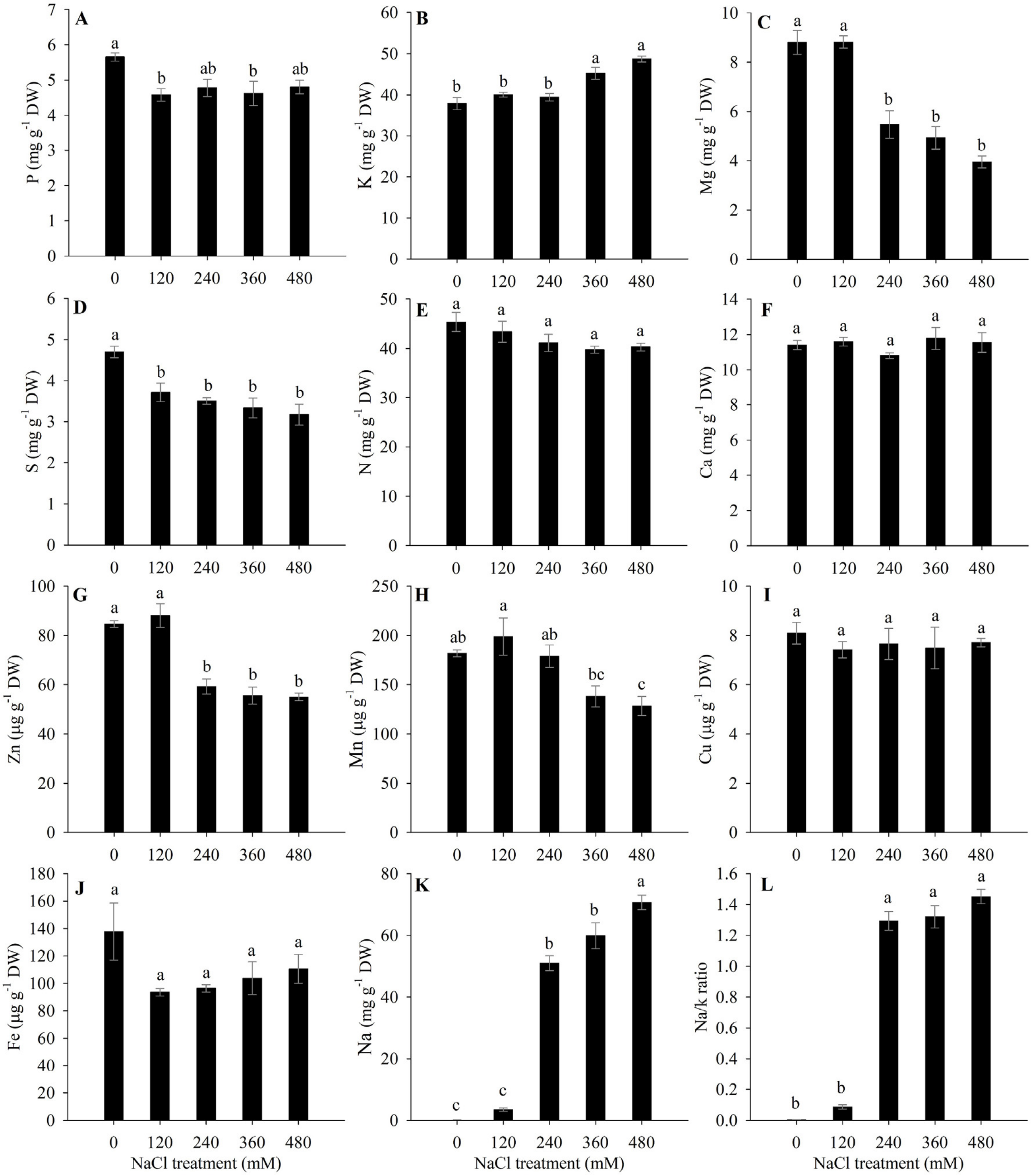

Fig. 2. Levels of macronutrient, micronutrient and sodium in 51-day-old *Pfaffia glomerata* plants exposed to different concentrations of NaCl (0 – control, 120, 240, and 480 mM) for 11 consecutive days. A – Phosphorus (P); B – Potassium (K); C – Magnesium (Mg); D – Sulfur (S); E – Nitrogen (N); F – Calcium (Ca); G – Zinc (Zn); H – Manganese (Mn); I – Copper (Cu); J – Iron (Fe); K – Sodium (Na); and L – Na/K ratio. Values represent means ± SE (n = 5). Same letters do not differ at 5% level by Tukey's test.

and the samples were incubated at 100 °C for 1 h. The reaction was stopped in an ice bath. Then, readings were made at 520 nm and a calibration curve was obtained by preparing proline standard solutions (0–220 µg mL^{-1}; Sigma-Aldrich, St. Louis, MO, USA) and expressed in mmol mg^{-1} dry weight (DW).

2.7. Quantification of polyamines and phytohormones

The quantification was performed according to Napoleão et al. (2017). Approximately 110 mg of fresh leaf tissue (third fully expanded leaf from the apex of plants) were ground in liquid nitrogen followed of extraction by twice with 400 µL of methanol: isopropanol: acetic acid (20:79:1; v/v/v). The samples were vortexed (4 times for 20 s), sonicated (10 min), kept on ice (30 min), centrifuged (20,000 *g*, 10 min at 4 °C), and the supernatant was collected. Then, the supernatant was filtered (Econofltr PVDF 13 mm and 0.2 µm; Agilent Technologies, Santa Clara, CA, USA) and used for liquid chromatography-mass spectrometry (LC-MS) analysis.

Samples of the final supernatant (5 µL) were automatically injected into the LC-QqQ MS (Triple Quadrupole 6430, Agilent Technologies, Waldbronn, Germany) equipped with an Eclipse Plus C18 (2.1 × 50 mm, 1.8 µm) column and guard column Zorbax SB-C18 (1.8 µm, Agilent) at 26 °C. The mobile phase consisted of 0.02% acetic acid in water (solvent A) and 0.02% acetic acid in acetonitrile (solvent B) at a constant flow rate of 300 µL min^{-1}. A linear gradient was applied as follows: 0–11 min and 5%–60% of B, 11–13 min and 60%–95% of B, 13–17 min and 95% of B, 17–19 min and 95%–5% of B and 19–20 min and 5% of B. A mass spectrometer ionisation source ESI

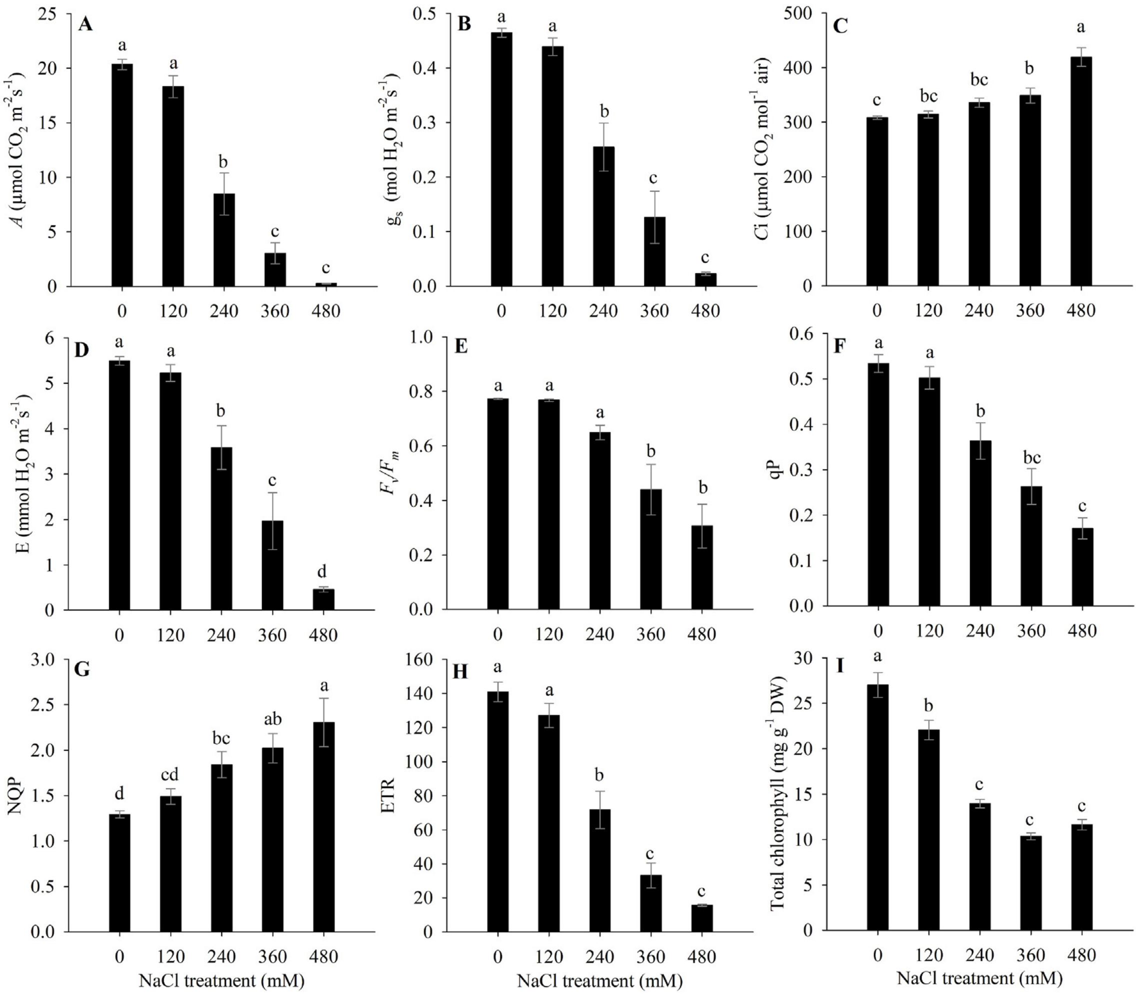

Fig. 3. Gas exchange, chlorophyll fluorescence parameters and photosynthetic pigments in 51-day-old *Pfaffia glomerata* plants exposed to different concentrations of NaCl (0 – control, 120, 240, and 480 mM) for 11 consecutive days. A – Net CO$_2$ assimilation rate (A); B – Stomatal conductance to water vapor (g_s); C – Internal CO$_2$ concentration (C_i); D – Transpiration rate (E); E – PS II maximum quantum yield (F_v/F_m); F – Photochemical quenching (qP); G – Non-photochemical quenching (NQP); H – Electron transport rate (ETR); and I – Total chlorophyll. Values represent means ± SE (n = 7). Same letters do not differ at 5% level by Tukey's test.

(Electrospray Ionisation) was used with the following conditions: gas temperature of 300 °C, nitrogen flow rate of 10 L min^{-1}, nebulizer pressure of 35 psi and capillary voltage of 4000 V. Analysis of polyamines and phytohormones were performed on Multiple Reaction Monitoring (MRM) of ion pairs to use the following mass transitions: putrescine (Put) (89/72), (B) spermidine (Spd) (203/83), (C) spermine (Spm) (146/72), cytokinin (CK) by zeatin (Zea) (220/136), 1-aminocyclopropane-1-carboxylic acid (ACC) (102/56), abscisic acid (ABA) (263/153), salycilic acid (SA) (137/93), and methyl jasmonate (MeJa) (225/151). Put, Spd, Spm, Zea, and ACC were scanned as positive mode whereas ABA, SA, and MeJa were scanned in the negative mode. Polyamines and phytohormones were quantified via calibration curves using authentic standards (1–200 μg and 0.1–500 ng, respectively; all of Sigma-Aldrich, St. Louis, Missouri, USA). The data generated were analyzed in *Mass Hunter Workstation* software and results are expressed on a fresh weight (FW) basis.

2.8. Enzymatic assays

Superoxide dismutase (SOD), catalase (CAT), peroxidases (POD), and ascorbate peroxidase (APX) were extracted by homogenizing 100 mg of frozen fresh material (third fully expanded leaf from the apex of plants) with 1 mL of the extraction buffer (potassium phosphate buffer 0.1 M and pH 6.8; phenylmethylsulfonyl fluoride (PMSF) 1 mM; polyvinylpyrrolidone (PVPP) 1% (w/v); ethylenediaminetetraacetic acid (EDTA) 0.1 mM). The mixture was centrifuged at 10,000 *g* for 15 min, and the supernatant was used as crude enzyme extract. All steps were performed at 4 °C.

SOD activity was determined as described by Del Longo et al. (1993). Samples were exposed to 15-W lamps for 5 min before being quantified by absorbance readings at 560 nm. The blank was obtained under the same conditions but in the absence of light. A SOD unit was defined as the enzyme amount required to inhibit 50% of the nitroblue tetrazolium (NBT) photoreduction (Beauchamp and Fridovich, 1971). CAT activity was determined as described by Havir and McHale (1987). The decrease in the absorbance at 240 nm was monitored and the enzymatic activity was calculated using a molar extinction coefficient equal to 36 M^{-1} cm^{-1}. POD activity was determined as described by Kar and Mishra (1976). The increase in the absorbance at 420 nm was monitored and the enzymatic activity was calculated using a molar extinction coefficient equal to 2.47 mM^{-1} cm^{-1} (Chance and Maehley, 1955). APX activity was determined as described by Nakano and Asada (1981). The decrease in the absorbance at 290 nm was monitored and the enzymatic activity was calculated using a molar extinction coefficient equal to 2.8 M^{-1} cm^{-1}. The result of SOD activity was expressed as U min^{-1} mg^{-1} protein, while CAT, POD, and APX were expressed as μmol min^{-1} mg^{-1} protein.

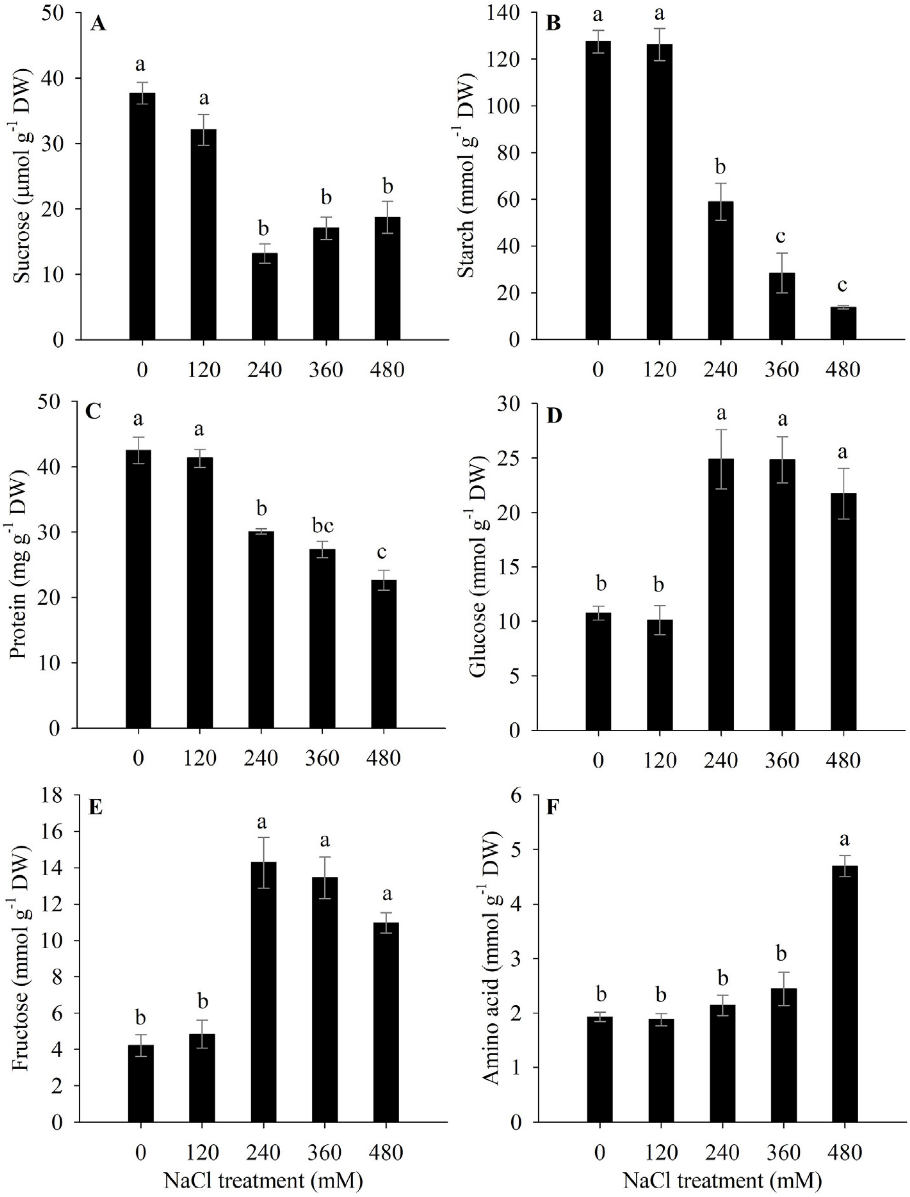

Fig. 4. Metabolite levels and protein and starch content in 51-day-old *Pfaffia glomerata* plants exposed to different concentrations of NaCl (0 – control, 120, 240, and 480 mM) for 11 consecutive days. A – Sucrose; B – Starch; C – Protein; D – Glucose; E – Fructose; and F – Amino acid. Values represent means ± SE (n = 5). Same letters do not differ at 5% level by Tukey's test.

2.9. Determination of 20-hydroxyecdysone levels

The preparation of the methanolic extract was performed as described by Corrêa et al. (2015). The determination of 20E was done using high-performance liquid chromatography (HPLC) on a device Shimadzu SPD-10Avp (Kyoto, Japan) equipped with ultraviolet (UV) detector set at 245 ηm and column Supelco C18 (30 cm × 7.9 mm diameter) with a slow flow of 0.8 mL min^{-1} and column pressure of 97.6 kgf. The mobile phase consisted of isocratic system with a mixed of HPLC grade methanol and deionized water in the ratio (1:1) (v/v). The volume of the injected sample was 20 µL, running for 15 min. The calibration curve was obtained by preparing 20E standard solutions (Sigma-Aldrich, St. Louis, MO, USA) in HPLC grade methanol (0–120 mg L^{-1}).

2.10. RNA extraction, cDNA synthesis, and RT-qPCR analysis

Approximately 100 mg of fresh leaf tissue (third fully expanded leaf from the apex of plants) were ground in liquid nitrogen followed of extraction (total RNA) by TRI Reagent® (Sigma-Aldrich, St. Louis, MO, United States) and treated with DNAse I (Thermo Scientific NanoDrop Technology, Wilmington, DE, USA) to remove genomic DNA contamination. First-strand cDNA was synthesized from 500 ng of the total RNA using the MMLV Reverse Transcriptase (Ludwig Biotec®, Alvorada, RS, Brazil).

Ecdysteroid 25-hydroxylase (Phantom - Cyp306a1) and cytochrome P450 family 307 subfamily A (Spook - Cyp307a1) expression levels were assessed by qRT-PCR with gene-specific primers. qRT-PCR was conducted on a CFX96 Touch™ (BIO-RAD). The *P. glomerata* glycerol-3-phosphate dehydrogenase gene (PgGAPDH) was used as an internal reference gene (Batista et al., 2019b). The primers were obtained from a *P. glomerata* transcriptome (Batista et al., 2019a). PCR programs were as follow: 2 min at 50 °C and 10 min at 90 °C, followed by 40 cycles of 16 s at 95 °C and 1 min at 60 °C, and 15 s at 95 °C, 1 min at 60 °C, 30 s at 95 °C, and 15 s at 60 °C. Transcript levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with three biological

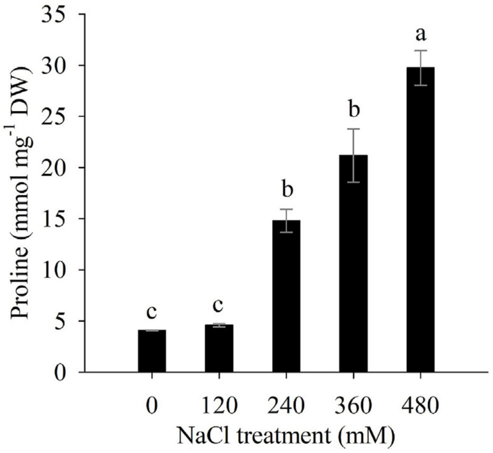

Fig. 5. Proline content in 51-day-old *Pfaffia glomerata* plants exposed to different concentrations of NaCl (0 – control, 120, 240, and 480 mM) for 11 consecutive days. Values represent means ± SE (n = 4). Same letters do not differ at 5% level by Tukey's test.

replicates and at least three technical replicates (reactions).

2.11. Statistical analyses

The experiment was designed in a completely randomized design. The data were statistically analyzed by one-way analysis of variance (ANOVA). Gene expression was tested for significant differences using Dunnett's test ($P < 0.05$), while all other variables by Tukey's test ($P < 0.05$). All of the statistical analyses were performed using the software Genes (Cruz, 2013).

3. Results

3.1. Salt stress inhibits growth

Plants under 120 mM NaCl demonstrated mild symptoms when compared to plants treated with 240, 360 and 480 mM NaCl (Fig. 1). Plants under salt stress showed phenotypic symptoms such as leaf chlorosis and senescence and necrotic spots in the leaf blade (Supplemental Fig. S1), particularly at the highest NaCl levels (≥240 mM).

Compared to control plants, plant height decreased significantly at 240 (17%), 360 (32%) and 480 (32%) mM NaCl. Decreases were also observed in leaf DW (36–57%), stem DW (45–73%), and root DW (50–78%) (Table 1). Leaf abscission was also significant at 240, 360 and

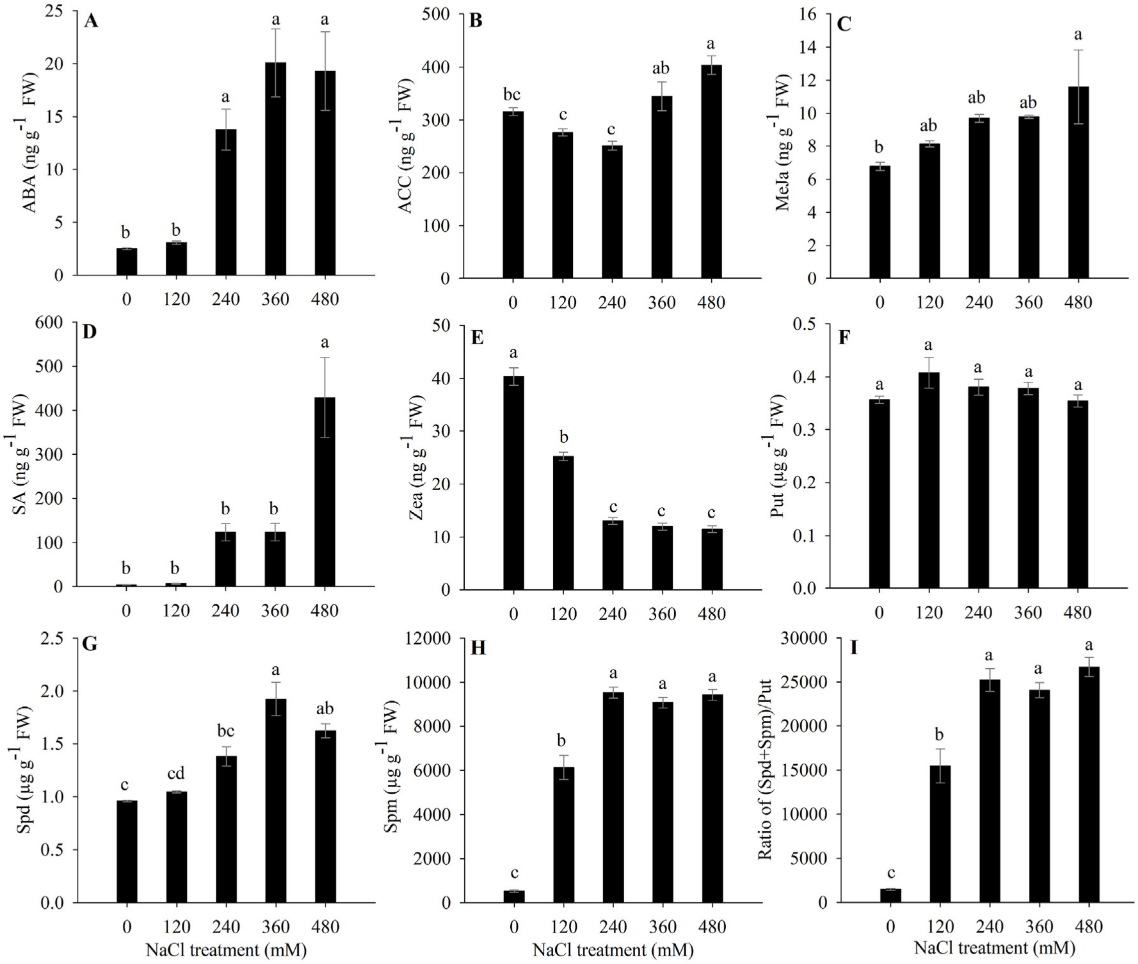

Fig. 6. Phytohormone contents in 51-day-old *Pfaffia glomerata* plants exposed to different concentrations of NaCl (0 – control, 120, 240, and 480 mM) for 11 consecutive days. A – Abscisic acid (ABA); B – 1-carboxylic acid-1-aminocyclopropane (ACC); C – Methyl jasmonate (MeJa); D – Salicylic acid (SA); E – Zeatin (Zea), F – Putrescine (Put); G – Spermidine (Spd); H – Spermine (Spm); and I – Ratio of (Spd + Spm)/Put. Values represent means ± SE (n = 5). Same letters do not differ at 5% level by Tukey's test.

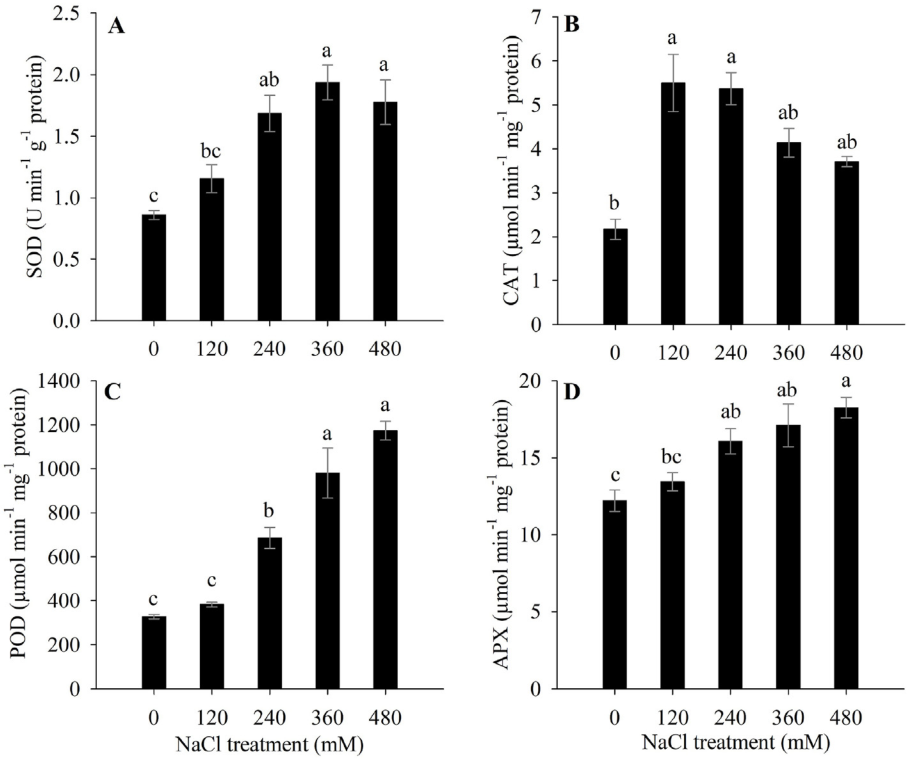

Fig. 7. The activities of antioxidant enzymes in 51-day-old *Pfaffia glomerata* plants exposed to different concentrations of NaCl (0 – control, 120, 240, and 480 mM) for 11 consecutive days. A – Superoxide dismutase (SOD); B – Catalase (CAT); C – Peroxidases (POD); and D – Ascorbate peroxidase (APX). Values represent means ± SE (n = 4). Same letters do not differ at 5% level by Tukey's test.

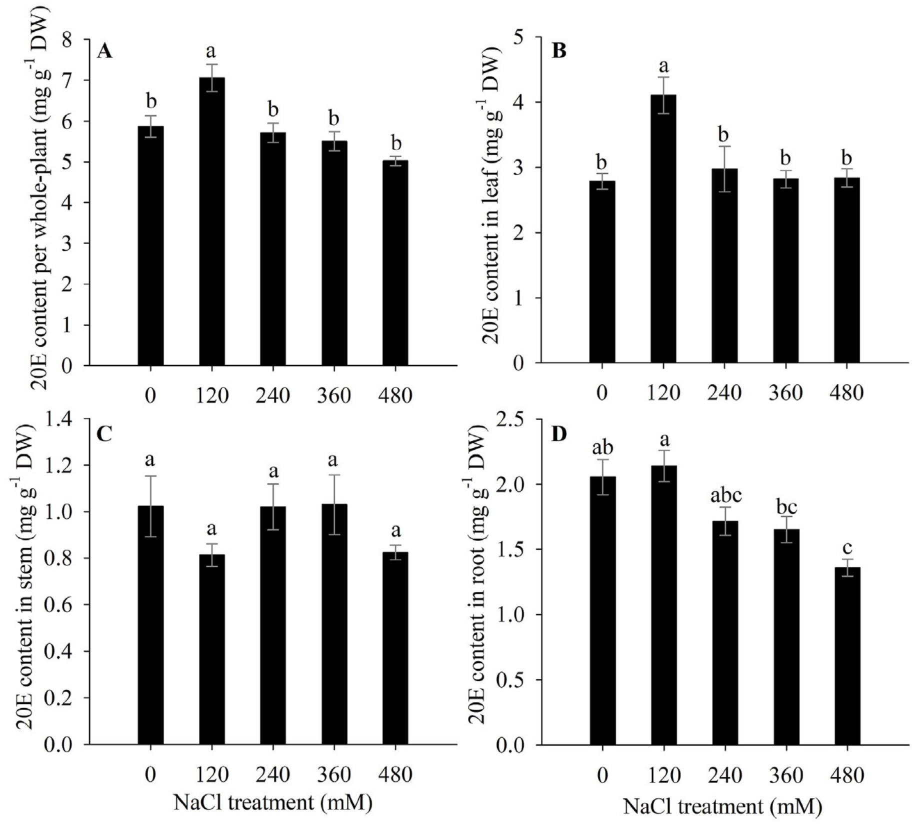

Fig. 8. 20-hydroxyecdysone (20E) levels in 51-day-old *Pfaffia glomerata* plants exposed to different concentrations of NaCl (0 – control, 120, 240, and 480 mM) for 11 consecutive days. The content of 20E per: A – plant, B – leaf, C – stem, and D – root. Values represent means ± SE (n = 7). Same letters do not differ at 5% level by Tukey's test.

480 mM treatments (resulting in leaf losses of 0.5, 1.4 and 2.2 g, respectively) as compared with 0 or 120 mM salt treatments (which did not displayed leaf abscission) (Table 1). Taken together, these differences caused decreases in total DW at 240, 360 and 480 mM treatments (40–60%) (Table 1).

3.2. Salt stress promotes leaf nutritional imbalances

Salt stress induced significant changes in leaf nutritional status. K^+ content increased by 360 and 480 mM (2 times in both), as well as Na^+ increased considerably in 240–480 mM NaCl (406–562 times), which

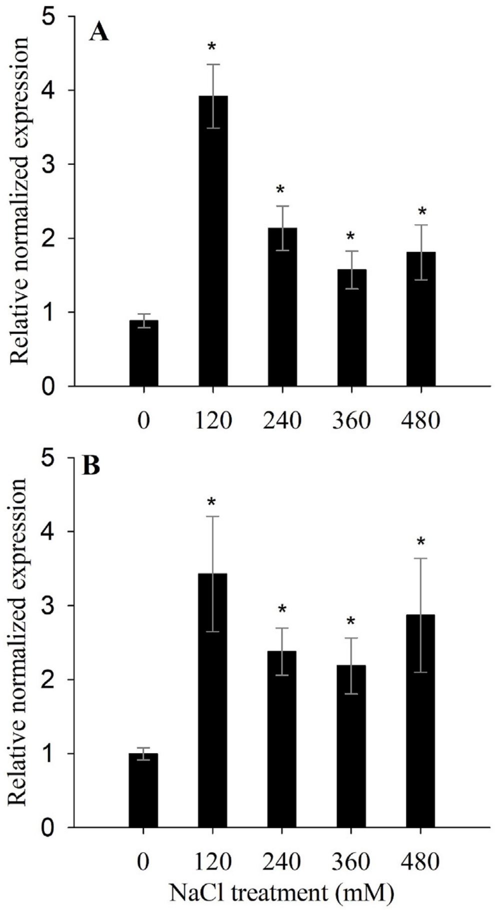

Fig. 9. Normalized relative expression of *Phantom* and *Spook* genes in 51-day-old *Pfaffia glomerata* plants exposed to different concentrations of NaCl (0 – control, 120, 240, and 480 mM) for 11 consecutive days. A – *Spook* gene and B – *Phantom* gene. Gene expression relative to the control gene *glyceraldehyde-3-phosphate dehydrogenase*. Values represent means ± SD (n = 4), $^*p < 0.05$ by Dunnett's test.

led to a high Na^+/K^+ ratio in these treatments (Fig. 2). In parallel, salt stress reduced the levels of P, Mg^{2+}, S, Zn and Mn in 240–480 mM NaCl treatments, while the other nutrients (N, Ca^{2+}, Cu and Fe) were not responsive (Fig. 2).

3.3. *Salt stress negatively affects photosynthetic performance*

The *A* decreased significantly by 58, 85 and 99% at 240, 360 and 480 mM NaCl, respectively, relative to the control plants (Fig. 3A). This was accompanied by decreases in g_s, 45, 73 and 95% at 240, 360 and 480 mM of NaCl, respectively (Fig. 3B). *E* responded to salt treatments in a similar way as that of g_s (Fig. 3D). In contrast, C_i significantly increased in plants under 360 and 480 mM of NaCl (13 and 36%, respectively) when compared to the control plants (Fig. 3C).

Compared with controls, there were significant decreases in F_v/F_m (43 and 60% at 360 and 480 mM NaCl, respectively) (Fig. 3E), in qP (32, 51 and 68% at 240, 360 and 480 mM NaCl, respectively) (Fig. 3F), in ETR (49, 76 and 89% at 240, 360 and 480 mM NaCl, respectively) (Fig. 3H), and total chlorophyll content (18, 48, 62 and 57% at 120, 240, 360 and 480 mM NaCl, respectively) (Fig. 3I). In contrast, NQP increased significantly (42, 56 and 78% at 240, 360 and 480 mM NaCl, respectively) (Fig. 3G).

3.4. *Salt stress strongly affected primary leaf metabolite levels and protein and starch concentrations*

Salt stress reduced metabolite levels related to energy reserves (sucrose and starch) and also the protein content at 240, 360 and 480 mM compared to controls (Fig. 4A–C, respectively). In contrast, salt stress increased the levels of metabolites with osmoregulatory roles, such as glucose and fructose whose contents increased at 240, 360 and 480 mM NaCl (Fig. 4D and E), and amino acids which increased significantly at 480 mM compared to controls (Fig. 4F). Notably, there were remarkable increases in proline content (262, 418 and 626% at 240, 360 and 480 mM NaCl, respectively) relative to controls (Fig. 5).

3.5. *Salt stress induces changes in leaf phytohormone levels*

Phytohormone levels varied significantly compared with controls, with increases in ABA (6–9 times in 240–480 mM NaCl) (Fig. 6A), ACC (2 times in 360 and 480 mM NaCl) (Fig. 6B), MeJa (2–3 times in 240–480 mM NaCl) (Fig. 6C) and SA (33–113 times in 240–480 mM NaCl) (Fig. 6D), while Zea showed reductions (68, 70 and 72% at 240, 360 and 480 mM NaCl, respectively) (Fig. 6E). Increases were also observed in Spd (3 times in 360 and 480 mM NaCl) and strongly in Spm (13–19 times in 240–480 mM NaCl) (Fig. 6G and H, respectively), which led to a high (Spd + Spm)/Put ratio in all NaCl treatments (11–19 times) (Fig. 6I). Put was unresponsive to the treatments (Fig. 6F).

3.6. *Salt stress increase activities of antioxidant enzymes*

Compared to control plants, their salt-stressed counterparts displayed significant increases in SOD (96, 125 and 106% at 240, 360 and 480 mM, respectively) (Fig. 7A), POD (110, 200 and 259% at 240, 360 and 480 mM, respectively) (Fig. 7C) and APX (32, 40 and 50% at 240, 360 and 480 mM, respectively) (Fig. 7D), while the greatest increases (~150%) in CAT were observed at 120 and 240 mM NaCl, against 91 and 71% under 360 and 480 mM NaCl, respectively (Fig. 7B).

3.7. *Moderate salinity increase 20E contents*

Whole-plant 20E content increased significantly under 120 mM NaCl (20%) when compared to control treatment, while the other treatments (e.g., 240, 360 and 480 mM NaCl) displayed similar 20E content when compared to control (~5.5 mg g^{-1} DW) (Fig. 8A). In comparison with controls, 20E concentration increased in leaves (47%) but only at 120 mM NaCl (Fig. 8B), but remained unresponsive to salt treatments in the stems (ranging from 0.8 to 1.0 mg g^{-1} DW) (Fig. 8C), whereas in the roots it decreased significantly at 240 (17%), 360 (20%) and 480 (34%) mM NaCl (Fig. 8D).

3.8. *Salt stress upregulates the Spook and Phantom genes*

The expression of 20E biosynthesis genes was positively influenced by all NaCl concentrations. Compared to controls, *Spook* and *Phantom* were upregulated (4–5 times) under 120 mM NaCl salt stress, but to a lesser extent (3 fold) in the other treatments (240–480 mM NaCl) (Fig. 9A and B).

4. Discussion

To the best of our knowledge, this study is the first to investigate regulation and expression of 20E in response to short-term salt stress. Our data confirmed the hypothesis that salinity enhances 20E production, but this increase occurred only at 120 mM of NaCl, since in larger concentrations the plants display serious disturbances, although increased expression of the 20E biosynthesis pathway genes occurs at all levels of stress. Moderate salinity (120 mM of NaCl) did not affect photosynthesis, carbohydrate and amino acid pools and ultimately biomass accumulation, but was able to induce higher 20E concentrations and thus higher absolute contents (amounts) of 20E on a per whole-plant basis could be obtained. In contrast, growth was severely affected with increasing high salinity (240–480 mM of NaCl) due probably to the impact caused by decreases in photosynthetic performance (and low assimilate availability, e.g., sucrose and starch), which, despite not causing reductions in the concentrations of 20E, led to decreased contents (amounts) of 20E on a per whole-plant basis due to lower plant biomass.

We observed proportionally larger decreases in A than in g_s and ETR, concomitantly with increases in C_i, at concentrations of NaCl ≥ 240 mM. Therefore, impairments to A are unlikely to have been associated with diffusive or photochemical limitations; rather the observed decreases in A were associated with dysfunctions at the level of biochemical reactions to CO_2 fixation. In any case, a range of dysfunctions at the photochemical level were noted under severe salt stress, as inferred from the decreased PSII photochemical efficiency (analyzed as F_v/F_m), which suggests the occurrence of chronic photoinhibition to photosynthesis (Krause and Weis, 1991). Concordant decreases in the fraction of absorbed light that is dissipated photochemically (estimated as q_P) were also noted, suggesting that the stressed plants were likely unable to fully capture and exploit the absorbed energy. In as much as these results were accompanied by lower decreases in ETR than in A, an excess reducing power is expected to be created that can trigger a range of photoinhibitory and photooxidative effects (Maxwell and Johnson, 2000). Here we suggest that the salt-stressed plants cope with such an excess reducing power via heat dissipation, as evidenced by the increased NPQ (Krause and Weis, 1991; Logan et al., 2007). Nevertheless, heat dissipation was proven not to be enough to prevent photoinhibition given the observed decreases in F_v/F_m and other dysfunctions such as leaf chlorosis and abscission. Collectively, imbalances in the photosynthetic apparatus are believed to result in increased ROS production (oxidative stress), which in turn explain the increased activities of some antioxidant enzymes we analyzed.

In addition to altering their antioxidant system, plants dealt with salt stress (240–480 mM of NaCl) by promoting osmotic adjustment (as judged from the increases in sugars such as glucose and fructose, amino acids and proline). These adjustments are consistent with our data of plant water potential, which was unresponsive to the treatments regardless of the osmotic stress that is created by the addition of salts (data not shown). Cytoplasmic osmolytes, besides facilitating water absorption, can also protect and stabilize structures and macromolecules (proteins, membranes, chloroplasts, and lysosomes) (Bohnert et al., 1995; Park et al., 2016). Such a protective function might be also achieved via the enhanced PA (e.g., spermine and spermidine) levels in salt-stressed plants (Sudhakar et al., 2015). Our data additionally suggest an overall hormonal reprogramming due to the stress, e.g., changes in SA, ACC (precursor of ethylene) and CKs which may be involved in signaling to improve salt resistance through osmotic adjustment (Gharbi et al., 2017). Increased ACC and decreased Zea can help to explain leaf senescence and abscission (Ghanem et al., 2008). Increases in ABA levels can trigger stomata closure, thus helping to decrease water loss (Fahad et al., 2015). Finally, the levels of SA, which increased sharply under high NaCl concentrations, can be a strategy for mitigating the negative effects of salt stress (Zheng et al., 2018) via counteracting the tradeoffs between growth and defense (Shakirova, 2007; Rivas-San Vicente and Plasencia, 2011). Taken all of the above information together, our data suggest a range of strategies of *P. glomerata* plants to cope with salt stress. Nonetheless, these acclimation responses were proven unsuccessful given that plant growth (coupled with photosynthetic performance and nutritional imbalances) was severely impaired at concentrations equal or above 240 mM NaCl.

All these changes caused by salinity in photosynthetic performance, ion homeostasis and oxidative balance may activate secondary metabolic pathways through ROS production (here, indirectly suggested by the increase in antioxidant enzymes) (Gengmao et al., 2015). The current finding of our research is that salt stress upregulated the expression of genes related to 20E biosynthesis pathway, suggesting that abiotic factors may shift the endogenous levels of 20E in plants. Here, possibly, the incipient stress (osmotic stress) and ion toxicity caused by excess salts promoted ROS accumulation which, in turn, may have induced the increase of 20E in *P. glomerata* given that ROS induce the expression of several genes related to the biosynthesis of secondary metabolites (Zhao et al., 2005). However, 20E concentration was not further increased with salinity in a concentration-dependent manner, despite the marked overexpression of *Spook* and *Phantom* genes. Likely, severe salt stress was able to dramatically disrupt carbon metabolism (with concomitantly decreased levels of sucrose and starch) and, as a consequence, 20E concentrations were held in check given that the investment in secondary metabolites is linked to carbon availability at the whole-plant scale (Huang et al., 2017).

In conclusion, our results show that *P. glomerata* counteracts salt stress via a range of acclimatory strategies (e.g., osmotic adjustment and increased activities of antioxidant enzymes), which might involve an orchestrated reprogramming of hormone levels. However, these strategies were proven unsuccessful given that ion imbalances and depressed photosynthetic performance led to marked decreases in plant growth at NaCl concentrations ≥ 240 mM. Most importantly, moderate salinity (120 mM of NaCl) did not affect photosynthesis and biomass accumulation, and was able to upregulate 20E levels, and thus allowing improved 20E contents in a whole-plant basis. Also, moderate salinity could be used as a strategy to improve 20E production in *P. glomerata*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.05.002.

Author contributions

SHSF, DSB and WCO designed the experiment. SHSF, DSB, CEV, KC, POS, TDS, EAF, LNFC and RTA performed analyses. SHSF, DSB, JM, RAFB, FM DaMatta and WCO contributed to the data interpretation and to the writing of the paper with contributions of all the authors. All authors read and approved the final paper.

Conflict of interest

All authors declare no conflict of interest.

Contribution

In the present study we hypothesized that salt stress, depending on its severity, can lead to increased 20-hydroxyecdysone (20E) contents (absolute amounts) in *Pfaffia glomerata*. Here, we firstly characterize the responses of *P. glomerata* to salt stress and, secondly, examine the production of 20E as affected by salinity. We highlight the interesting aspect of the work is the expression of two Halloween genes (*Phantom* and *Spook*) in 51-day-old *Pfaffia glomerata* plants exposed to different concentrations of NaCl. Our findings suggest that *P. glomerata* cultivation in moderate salinity soils can be considered as a suitable agricultural option to increase 20E levels, since metabolic and structural complexity that makes its artificial synthesis very difficult.

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