Physiol Mol Biol Plants (March 2021) 27(3):457–468
https://doi.org/10.1007/s12298-021-00957-9

RESEARCH ARTICLE

Salt tolerance of *Calotropis procera* begins with immediate regulation of aquaporin activity in the root system

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Received: 2 January 2021 / Revised: 10 February 2021 / Accepted: 12 February 2021 / Published online: 20 February 2021

Abstract The ability to respond quickly to salt stress can determine the tolerance level of a species. Here, we test how rapidly the roots of *Calotropis procera* react to high salinity conditions. In the first 24 h after saline exposure, the plants reduced stomatal conductance, increased CO_2 assimilation, and water use efficiency. Thus, the root tissue showed an immediate increase in soluble sugars, free amino acid, and soluble protein contents. Twelve aquaporins showed differential gene expression in the roots of *C. procera* under salinity. Transcriptional upregulation was observed only after 2 h, with greater induction of *CpTIP1.4* (fourfold). Transcriptional downregulation, in turn, occurred mainly after 8 h, with the largest associated with *CpPIP1.2* (fourfold). *C. procera* plants responded quickly to high saline levels. Our results showed a strong stomatal control associated with high free amino acid and soluble sugar contents, regulated aquaporin expression in roots, and supported the high performance of the root system of *C. procera* under salinity. Moreover, this species was able to maintain a lower Na^+/K^+ ratio in the leaves compared to that of the roots of stressed plants. The first response of the root system, after immediate contact with saline solution, present an interesting scenario to discuss.

Keywords Gas exchange · Root physiology · Salinity · Salt stress · Tonoplast intrinsic proteins

Introduction

Calotropis procera is a perennial Asian shrub with a significant adaptation to adverse climatic and poor soil conditions (Hassan et al. 2015). Among peculiar traits, it presents high photosynthetic performance, stomatal control, and water use efficiency under drought conditions (Rivas et al. 2017; 2020; Tezara et al. 2011;), and high metabolic performance under salinity (Mutwakil et al. 2017). Furthermore, although it originated in arid regions, this species is evergreen with a C_3 photosynthetic metabolism. This set of characteristics makes it a relevant model for ecophysiological studies and an important source of gene pools to identify genes involved in tolerance to abiotic stresses. Among them, high salinity in the soil is one of the major abiotic stresses responsible for desertification processes worldwide, decreasing plant biomass production (Munns and Tester 2008).

The severity of the stress depends on the salt concentration to which the plant has been exposed. When plants are under very high salt concentrations ($\geq$ 200 mM NaCl), it experiences more rapid and intense osmotic and ionic stresses, and exhibits greater susceptibility to plant cell plasmolysis (Shavrukov 2013). Plant water supply becomes precarious when roots are exposed to such high saline concentrations. Plant water relations depend on the root system. Therefore, root acclimatization responses to salt are essential to support water and nutrient conductivities

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12298-021-00957-9.

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(Rodríguez-Gamir et al. 2019) and avoid the accumulation of excess Na^+ (Munns and Tester 2008). Thus, performance of roots under salinity by exploring their biochemical and molecular traits is unclear. Among the mechanisms triggered by roots related to salt tolerance, the performance of osmotic adjustment (Blum 2017; Slama et al. 2015;) through primary metabolite content (Sami et al. 2016) and the control of aquaporin (AQP) activity are worth highlighting (Kapilan et al. 2018; Rodríguez-Gamir et al. 2019). In all plant tissues, AQPs are intrinsic membrane proteins that act in the transport of cell water and small solutes, such as H_2O_2, ammonia, urea, glycerol, and others, in addition to gases and ions (Kapilan et al. 2018; Li et al. 2014). The increase in primary metabolite biosynthesis decreases the osmotic pressure and restores the plant water flow in relation to salinized soil (Blum et al. 2017). These compounds are associated with free radical elimination, cell structure protection, and maintenance of enzymatic activity (Sami et al. 2016; Mansour and Ali, 2017).

The AQPs family plays an important role in the water relations of the entire plant (Singh et al. 2020), which are largely regulated by salt concentrations (Liu et al. 2012) and drought (Li et al. 2014; Rodríguez-Gamir et al. 2019). Based on their phylogenetic distribution, plant AQPs are categorized into five main subfamilies (Bezerra-Neto et al. 2019): plasma membrane intrinsic proteins (PIP), nodulin 26-like intrinsic proteins, tonoplast intrinsic proteins (TIPs), small intrinsic proteins, and uncharacterized intrinsic proteins. These proteins support hydraulic regulation and nutrient transport (Li et al. 2014), stomatal conductance (Rodríguez-Gamir et al. 2019), mesophyll conductance, transpiration and photosynthesis (Moshelion et al. 2015), growth and plant development, and influence ionic homeostasis (An et al. 2017). The functional regulation and role of AQPS in plant growth, development, and physiology in response to abiotic stresses have been widely studied (Bezerra-Neto et al. 2019; Singh et al. 2020). Regarding AQPs, we focused on PIPs and TIPs transcriptional regulation. Most PIPs have been identified in plasma membranes, and they are generally located in organs characterized by a large water flux. TIPs, in turn, play a relevant role in intracellular water movement and non-limiting water flow through membranes (Kapilan et al. 2018; Singh et al. 2020).

This study evaluates the performance of the root system under exposure to high salinity. The main goal was to measure the root dynamics of carbon metabolism traits and the activity of aquaporins in the first 24 h under intense salinity stress. Thus, three questions were raised: (1) *C. procera* able to maintain water status and gas exchange during the first hours of salt exposure to roots?" (2) Are the roots of *C. procera* capable of producing rapid responses to adjust primary metabolites soon after exposure to high salinity? (3) How long does it take for *C. procera* roots to change aquaporin activity in response to high salinity? Considering these questions, there are two hypotheses: (1) the root system of *C. procera* presents biochemical responses that allow it to promote osmoregulation through the accumulation of organic solutes, and (2) under a high salt concentration, the upregulation of the gene expression of *PIPs* and *TIPs* occurs to promote the adjustment of plant water status and maintain water uptake by the root system.

Material and methods

Plant material and growth conditions

Calotropis procera (Aiton) W. T. Aiton (Apocynaceae) seeds were collected in the beach of Maria Farinha (municipality of Paulista) on the coast of Pernambuco state, Brazil (7° 50′ 32.9″ S, 34° 50′ 21.2″ W). The seed surface was sterilized by immersion in 0.5% sodium hypochlorite solution (v/v) for 5 min and washing with deionized water. The seeds were germinated in Petri dishes with wet filter paper and kept in a growth chamber (at 25 °C, 12 h photoperiod, and 70% relative humidity). After root emergence, the seedlings were transferred to pots containing 7 kg of washed sand and maintained in a greenhouse for 3 months. Throughout the experiment the plants grew in a greenhouse where the temperature ranged between 26 to 32 °C, full solar radiation within the environment. The plants were irrigated daily to keep the substrate fully hydrated. Every 15 days, the plants received a complete Hoagland solution.

After ninety days of emergence, the plants were exposed to two treatments: young plants watered daily with 300 mL deionized water (control), and young plants watered daily with 300 mL of 200 mM NaCl solution (salinity). The first watering was performed at 8:00 am in the morning and subsequent watering occurred after 24 h at the end of the daily gas exchange analysis.

The treatments (control and salinity) consisted of 28 plants each, with four time-points and seven replicates. To determine the collection time points of the plant material and the physiological measurements, the initial period of salt application (8:00 am) was used as a reference. The first day of the experiment included the time-points at 2 and 8 h of exposure to salt. Day 2 comprised the time points at 21 and 24 h of salt exposure.

Physiological parameters

Foliar relative water content and soil electrical conductivity

Relative water content (RWC) was measured using a leaf disc collected at 5:00 am, 21 h after exposure to salt, according to the methodology of Barrs and Weatherley (1962). The RWC was calculated using the formula: RWC (%) = (FW − DW/TW − DW) × 100, where DW equals the dry weight, FW is the fresh weight, and TW equals the matter turgid weight. The RWC analysis was performed with three biologic repetitions (each treatment).

Sand samples of 25 g were collected and homogenized in 25 mL of deionized water. It was stirred three times for 2 min. The solution was kept immobile for 24 h and the supernatant was sampled to calculate electrical conductivity using a conductivity meter.

Gas exchange and chlorophyll contents

The stomatal conductance (g_s), CO_2 net assimilation (*A*), and transpiration *(E)* were measured in completely expanded and healthy leaves using a portable infrared gas exchange analyzer (ADC, LCi-Pro Model, Hoddesdon, UK). Photosynthetic photon flux density was determined by measuring the environmental incident radiation. The water use efficiency (WUE) was calculated from the *A*/*E* ratio. Gas exchange was determined with four repetitions (each treatment). The evaluations were performed in the morning (08:00–09:00 am), 24 h after the beginning of salt exposure. The vapor pressure deficit (VPD) was determined according to the method of Campbell and Norman (1998). In the morning, a VPD of 1.87 kPa was determined on the second day (24 h exposure to salt) of the experiment.

The chlorophyll contents were determined using leaves of *C. procera* collected 24 h after salt application. All samples were immediately frozen in liquid nitrogen and stored at − 80 °C. Chlorophyll *a* (CHL*a*) and chlorophyll *b* (CHL*b*) were quantified according to the method of Lichtenthaler and Buschmann (2001). The analyses were performed using a dual-beam spectrophotometer adjusted to the specific wavelength for each organic compound with three repetitions (each treatment).

Biochemical parameters

Proteins, amino acids, and sugars analysis

The analysis of the organic solutes was performed using the roots of *C. procera* collected at different time-points (2, 8, and 24 h) after salt application. All samples were immediately frozen in liquid nitrogen and stored at − 80 °C.

The collected roots were used to quantify soluble sugars (SS) (Dubois et al. 1956), free amino acids (FAA) (Moore and Stein 1948), and total soluble proteins (TSP) (Bradford 1976). The analyses were performed using a dual-beam spectrophotometer adjusted to the specific wavelength for each organic compound. The analysis of the organic solutes was carried out with three repetitions (each treatment).

Measurement of $Na^+$and K^+ contents

To quantify the sodium and potassium contents, roots were collected after 24 h of exposure to salt. After drying in an oven (60 °C), 500 mg of plant material was digested in sulfuric acid P.A. (H_2SO_4) solution in a digester block at 350 °C for 84 min to obtain the sample extract (Thomas et al., 1967). Sodium and potassium contents were determined by flame emission photometry (DM-62, Digimed, São Paulo, Brazil). The Na^+/K^+ ratio was calculated using sodium and potassium content data. The analysis was conducted with four repetitions (each treatment).

Statistical analyses

Data were subjected to a test of normality (Shapiro–Wilk) and homogeneity of variance to determine if the prerequisites for parametric statistics used were met. The dataset that did not meet the requirements was subjected to non-parametric tests. Data on the sodium content, potassium content, Na^+/K^+ ratio, and biochemistry were subjected to a two-factor analysis of variance (ANOVA), where salt treatment versus exposure time to salinity or plant organ were analyzed. The differences in the values were submitted to Duncan test ($p < 0.05$). All data were analyzed using Statistica 8.0 (StatSoft. Inc., Tulsa, OK 74,104, USA).

Molecular analyses

Root material for AQP expression analysis

For the AQP gene expression study, root samples were collected at 2, 8, and 24 h (three replicates) after the salt exposure. All collected samples were immediately frozen in liquid nitrogen and stored at − 80 °C until RNA isolation.

Total RNA isolation and cDNA synthesis

Total RNA was isolated with the SV Total RNA Isolation System Kit (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions. The integrity of RNA was

verified by 1.5% (w/v) agarose gel electrophoresis and quantified by fluorometry (Qubit, Oregon, USA) and stored at − 80 °C until analysis. First-strand cDNA was synthesized from total RNA (1 µg) using GoScript™ Reverse Transcription System Oligo dT (Promega, Fitchburg WI, USA) according to the manufacturer's protocol.

Aquaporin selection and primer design

AQP protein seed sequences obtained from the Universal Protein (Uniprot) and National Center for Biotechnology Information (NCBI) databases were used for local BLAST (tBLASTn; e-value = $\leq 10^{-5}$) against *C. procera* leaf transcriptome generated by our group (Coêlho et al. 2019) and deposited in the NCBI database (accession number PRJNA508417). The retrieved sequences were translated by the ORF-Finder tool and submitted to the CD-Search domain recognition tool for full MIP domain selection. To determine the AQP nomenclature (Table A1), an alignment (MEGA 7 program) with *C. procera* and *Arabidopsis thaliana* AQP sequences was performed to construct a phylogenetic tree (Neighbor-Joining method, Bootstrap 1000 replications) (Tamura et al. 2007) (Fig. A1). The nomenclature was based on sequence homology (Johanson et al. 2001). The alignment was also used to confirm the presence and integrity of the NPA functional motifs and ar / R selective filter, both related to water transport (Wallace and Roberts 2004) (Fig. A2). The selected transcripts were coding six intrinsic plasma membrane proteins—PIPs (two PIP1 and four PIP2) and seven intrinsic tonoplast proteins—TIPs (four TIP1, two TIP2, and one TIP4). Primers were designed using the Primer3 tool (Rozen and Skaletsky 2000) with the following parameters: primer length of 18–22 bp, GC content of 45–55% (50% ideal), melting temperature (Tm) in a range of 58–62 °C (ideal 60 °C), and amplicon lengths of 100–200 bp. Peptidyl-prolyl cis-isomerase 23 (*CYP23*), Actin-104 (*ACT104*), and Ubiquitin carboxyl-terminal hydrolase 25 (*UBP25*) were used as reference genes (RGs) for data normalization, as recommended by Coêlho et al. (2019). The real-time quantitative PCR (qPCR) primer sequences and amplicon characteristics are described in Supplementary Table A1.

Real-time quantitative PCR

The qPCR analysis was performed using LineGene 9600 equipment (Bioer, Hangzhou, China). The reactions were prepared with the GoTaq® qPCR Master Mix (Promega, Fitchburg WI, USA) consisting of 5 µL of SYBR Green Super Mix (Applied Biosystems, Foster City CA, USA), 2 µL of diluted cDNA (1:10), and 0.3 µL of each primer pair (5 µM) in a final volume of 10 µL with sterile ddH_2O. Non-template controls were included for each primer pair.

Table 1 Relative water content (RWC, %), stomatal conductance (g_s, mol m^{-2} s^{-1}), CO_2 assimilation (*A*, µmol m^{-2} s^{-1}), water use efficiency (WUE, mmol m^{-2} s^{-1}), chlorophyll *a* (CHL*a*, $g.kg^{-1}$DW), chlorophyll *b* (CHL*b*, $g.kg^{-1}$DW) in leaves of young *C. procera* plants under salinity conditions in a greenhouse (200 mM NaCl)

Parameter	Treatment	
	Control	200 mM NaCl
RWC	65.6 ± 1.06	63.8 ± 1.07
g_s	0.063 ± 0.003*	0.050 ± 0.000
A	7.7 ± 0.1	9.5 ± 0.3*
WUE	3.2 ± 0.1	5.6 ± 0.2*
CHL*a*	3.6 ± 0.19	4.1 ± 0.24
CHL*b*	1.2 ± 0.08	1.2 ± 0.05

Values followed by * are different according to t-test ($p < 0.05$)

qPCR amplification conditions were programmed to 95 °C for 2 min, followed by 35 cycles at 95 °C for 15 s, 62 °C for 1 min, and 72 °C for 15 s. The reactions were performed in triplicate.

The melting curves were generated by varying the amplification temperature from 65–95 °C. Amplification efficiency (E) was determined for each primer from a standard curve, and generated after serial cDNA dilutions (1:10, 1:100, 1:1000, and 1:10,000) in triplicate and calculated using the equation: E = 10 (− 1/slope of the standard curve) (Rasmussen 2001). The inclinations of the standard curve line in the range from − 3.58 to − 3.10 were considered acceptable for the trial (Biassoni 2014). These line slope values correlated with amplification efficiencies between 90 (E = 1.9) and 110% (E = 2.1).

The Rest2009 software package (REST Standard mode) was used to calculate and analyze the relative expression of the target transcripts. Relative expression was calculated using the formula: E (ΔCq aquaporin)/ E (ΔCq RG), where E represents the average efficiency for each gene, ΔCq is the difference between mean Cq-value of a control sample and the mean Cq-value of treated sample. Such analysis was based on paired comparisons (of target transcript and reference genes under salinity conditions and controls) using randomization and bootstrapping—Pair-wise Fixed Reallocation Randomization Test© (Pfaffl et al., 2002). Hypothesis testing ($p < 0.05$) was applied to determine if differences in the expression of target transcripts under control and treated conditions were significant.

Results

Foliar relative water content and soil electrical conductivity

Leaf RWC analysis revealed no significant difference after 24 h of salinity in relation to the control treatment. The average RWC values were 66 and 64% for the control and salinity treatments, respectively (Table 1). After 24 h under saline stress the soil electrical conductivity was 0.36 ± 0.02 and 0.98 ± 0.15 mS cm^{-1}, for the control and saline treatment, respectively.

Sodium and potassium content and Na^+/K^+ ratio

The application of saline solution resulted in an increase of Na^+ ions in the roots, stems, and leaves. In relation to the control, the greatest increase occurred in the roots (Fig. 1A). On the other hand, *C. procera* showed the ability to maintain K^+ ion content in the three measured organs (Fig. 1B). The Na^+/K^+ ratio remained unchanged in the leaves, however lower than root content values (Fig. 1C).

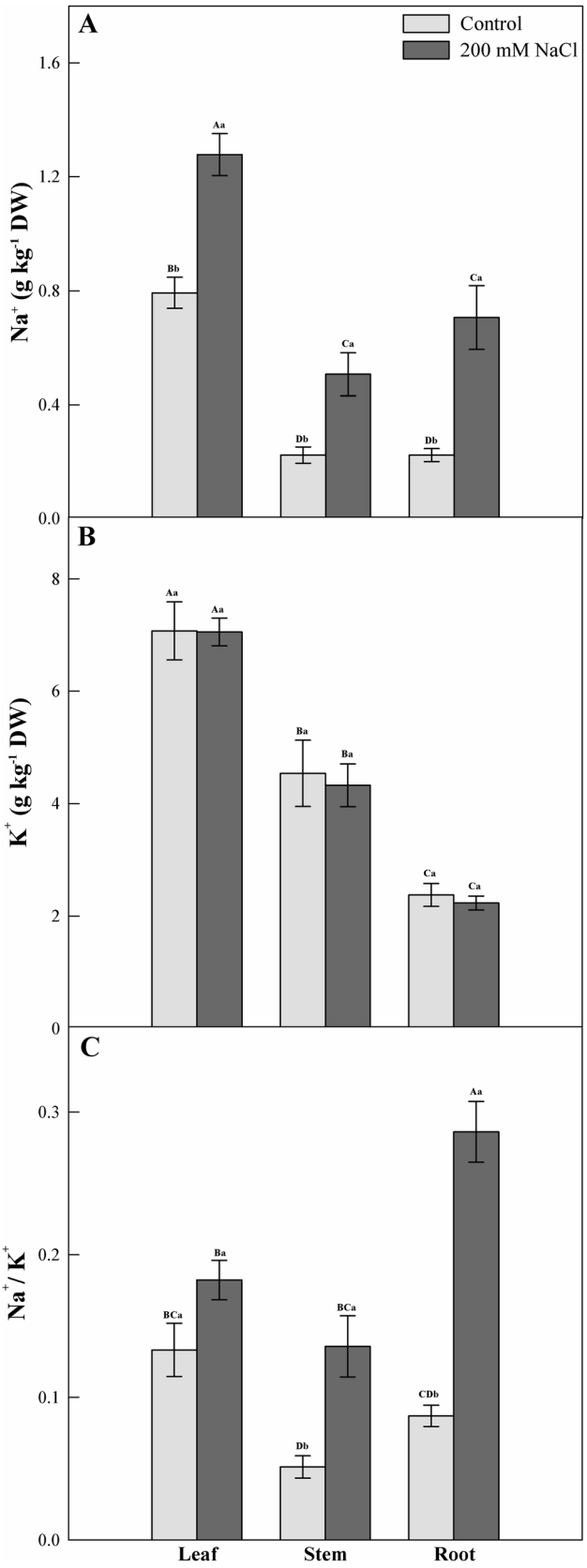
Gas exchange and chlorophyll contents

Stomatal conductance (g_s) was reduced in the salinity group compared to that in the control group after 24 h of exposure to salt (21%) (Table 1). The CO2 assimilation *(A)* increased by 23% (Table 1) in the plants under salinity. Also the water use efficiency (WUE) increased by 74% in salt-exposed plants compared to control plants (Table 1). Furthermore, plants exposed to salt showed no reduction in chlorophyll a (CHL*a*) and chlorophyll b (CHL*b*) after 24 h (Table 1).

Root sugars, amino acid, and protein contents

Salt application promoted rapid changes in the dynamics of root primary metabolism in *C. procera* (Fig. 2). Plants subjected to salinity treatment showed a 1.6-fold increase in soluble sugars (SS) after 2 h of exposure to salt. However, SS decreased (24–24%) after 8 and 24 h of exposure to salt (Fig. 2 A).

Solutes involved in nitrogen metabolism showed fast changes in the dynamics of the root system of plants exposed to salt. Increased FAA (52%) was also observed after 2 h of salt addition (Fig. 2 B). TSP increased by 85% in stressed plants compared to control plants after 8 h of exposure to salt (Fig. 2 C). Compared to the control plants, plants under saline treatment increased FAA content (109%) and decreased TSP (21%) after 24 h of exposure to salt (Fig. 2 B,C).



◀**Fig. 1** Sodium and potassium content and Na^+/K^+ ratio in the leaf, stem and root of young plants of *Calotropis procera* 24 h after exposure to salinity (200 mM NaCl) in a greenhouse assay. Each bar represents (n = 4 ± SE), when followed by same uppercase letters did not differ between treatments among different organs, and same lowercase letters did not differ between treatments at the same organ ($p < 0.05$)

Gene expression of AQPs in the root system

Salt promoted rapid changes in *CpPIP* and *CpTIP* gene expression dynamics in *C. procera* roots. The *CpTIP1.4* isoform was highly upregulated (fourfold), whereas *CpPIP1.2* was the most downregulated (fourfold) (Fig. 3 BJ). Plants subjected to salinity treatment showed upregulation of *CpPIP1.2*, *CpPIP2.4* (Fig. 3 BF), *CpTIP1.1*, *CpTIP1.2*, *CpTIP1.3*, *CpTIP1.4*, and *CpTIP2.1* (Fig. 3 G-K), and downregulation of *CpPIP2.1*, *CpPIP2.2*, *CpPIP2.3* (Fig. 3 E-C), and *CpTIP4.1* (Fig. 3 M) after 2 h of exposure to NaCl. Salinity promoted downregulation of *CpPIP1.2*, *CpPIP2.3*, *CpPIP2.4* (Fig. 3 BEF), *CpTIP1.3*, *CpTIP1.4*, *CpTIP2.1*, *CpTIP2.2* (Fig. 3 I-L), and *CpTIP4.1* isoform (Fig. 3 M) at 8 h. When the plants were exposed for 24 h to NaCl, the *CpPIP1.2* (Fig. 3 B), *CpTIP1.3*, *CpTIP1.4*, and *CpTIP2.2* (Fig. 3 IJL) isoforms were downregulated.

Salinity (200 mM, NaCl) promoted a rapid change in *CpPIP* and *CpTIP* expression levels in *C. procera* roots. Among the seven *CpTIP* isoforms, five (*CpTIPs 1.1, 1.2, 1.3, 1.4*, and *2.1*) were significantly upregulated after 2 h of salinity, three of these (*CpTIPs 1.3, 1.4*, and *2.1*) were repressed during the longest periods of salt exposure (8 and 24 h).

CpPIP 1.2 and *CpPIP 2.4* were also upregulated at 2 h, although the expression was repressed after 8 h. On the other hand, three *CpPIPs* (*2.1, 2.2*, and *2.3*) were downregulated in the first analysis time (2 h).

Discussion

Our results suggest that *C. procera* under salinity stress has fast modulation of root aquaporin activity. Moreover, the increase in CO_2 assimilation, even with reduced stomatal conductance, promotes greater *WUE* in the first 24 h, soluble sugar and protein contents in the first 2 and 8 h of exposure to salt. This species also has an outstanding ability to maintain a higher Na^+/K^+ ratio in the root system and stem, alleviating the ionic imbalance in the leaf tissue.

Under salt exposure, Na^+ accumulation in plant tissues is unavoidable. However, the ability to avoid targeting some tissues may represent a key factor in salinity tolerance. In this regard, several tolerance mechanisms are triggered by the selectivity of Na^+ uptake by roots. Thus, the preferential xylem was loaded over K^+ instead of Na^+, and sodium was removed from the xylem by roots, stems, petiole, and leaf sheaths (Munns and Tester 2008). *C. procera* was able to keep K^+ concentrations significantly unchanged, contributing to metabolism protection. Thus, a balance of K^+ and Na^+ concentrations act to combat ionic toxicity. Ions of K^+ are essential for preventing Na^+ entry, promoting the activation of numerous enzymes, stabilizing protein synthesis, and maintaining homeostasis of cytosolic pH (Munns and Tester 2008; Dreyer and Uozum 2011).

The first question was, "is *C. procera* able to maintain water status and gas exchange during the first hours of salt exposure to roots?" During exposure to salinity, changes in water status are common (Munns and Tester 2008), however small changes in leaf RWC probably occur due to mechanisms of WUE, such as stomatal control (Rodríguez-Gamir et al. 2019), osmotic adjustment (Blum 2017) and transcriptional reprogramming of AQPs (Moshelion et al. 2015).

Indeed, the ability to maintain water status is crucial for maintaining photosynthetic activity under salinity (Munns and Tester 2008). In our study, *C. procera* had robust photosynthetic performance in the face of higher stomatal resistance; thus, WUE increased under salinity. Opposite responses of A and g_s presuppose the influence of non-stomatal factors on photosynthetic rates. The species studied here showed similar behavior in comparison with those in previous studies under water deficit (Rivas et al. 2017). This work argues that the high photosynthesis rate of *C. procera* was supported by an increased mesophilic conductance and electron transport under water deficit. Stomatal control is a plant acclimatization response to low water availability, favoring WUE in *C. procera* (Rivas et al. 2017). The high CO_2 assimilation in *C. procera* at the onset of salinity and increased root soluble sugars could support water uptake.

The second question, was "are roots of *C. procera* capable of producing quick responses to adjust primary metabolites soon after exposure to high salinity?" Increased contents of organic solutes such as soluble sugars, amino acids, and proteins can play a role in osmoregulation contributing to salt tolerance in plants (Blum 2017; Sami et al. 2016). Under salinity conditions, *C. procera* exhibited a quick changes in the dynamics of root organic solutes. After the first two hours under salinity stress, the plants showed the highest SS and FAA contents. The quick responses of these metabolites suggest plasticity and contribute to the clarification of the salinity tolerance of this species (Slama et al. 2015).

Rapid exposure to salinity is not common in the wild, where salinity occurs progressively. However, studies such

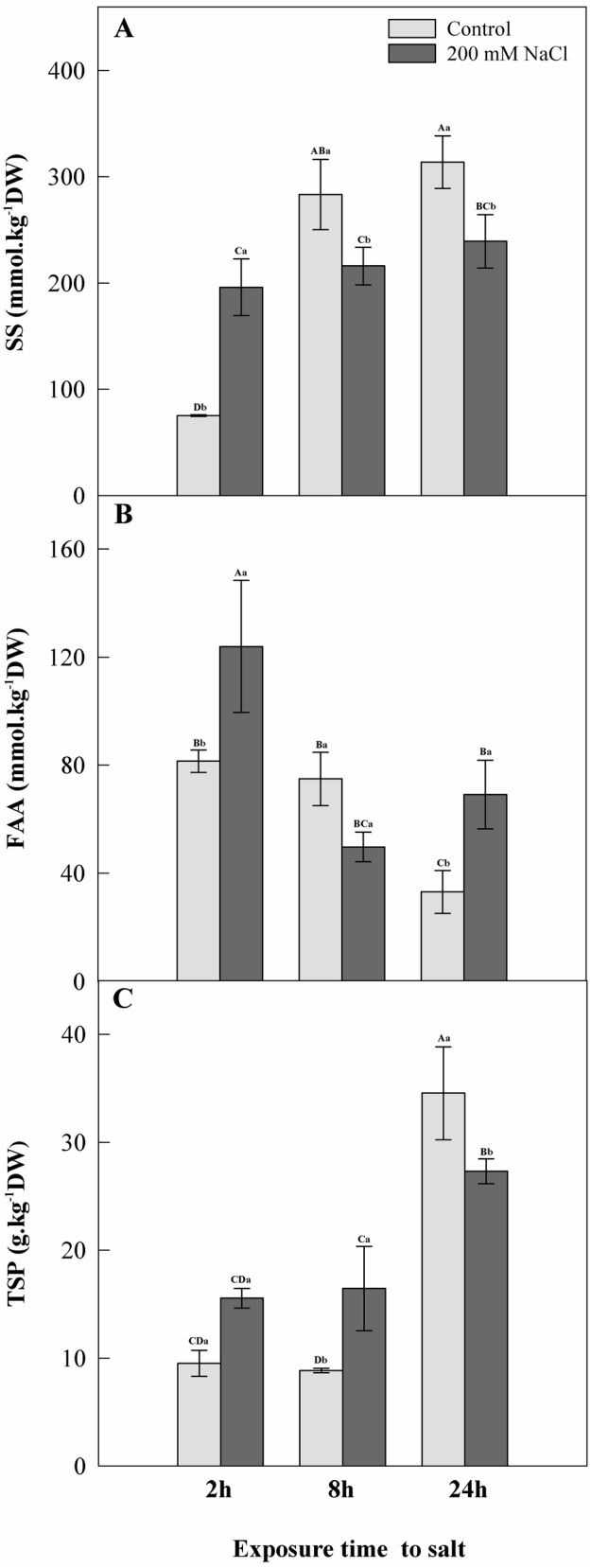


Fig. 2 Root content of **A** soluble sugars (SS), **B** free amino-acids (FAA) and **C** total soluble proteins (TSP) in roots of young plants of *Calotropis procera* under salinity conditions (200 mM NaCl) in a greenhouse assay. Each bar represents (n = 3 ± SE), when followed by same lowercase letters did not differ between treatments within each exposure time-point to salinity and same uppercase letters did not differ between treatments between exposure time-points to salinity ($p < 0.05$)

as ours can demonstrate the ability of a species to use efficient mechanisms very quickly. Amino acids were strongly regulated under exposure to salt. A recent study on *C. procera* metabolic and transcriptomic responses to saline conditions showed evident participation of different amino acids, as an acclimatization response over time (Mutwakil et al. 2017).

Soluble sugars are another important class of metabolites recognized for having numerous functions, mediating vital physiological processes in plants (Sami et al. 2016). The solutes involved in carbon metabolism significantly increased as an initial response to salt exposure. Accumulation of carbohydrates can alleviate the negative effects of excess salt by acting on osmotic homeostasis, eliminating free radicals and stabilizing membranes (Sami et al. 2016). Additionally, sugars are related to stress signaling by modulating gene expression (Martínez-Noël and Tognetti 2018), reducing Na^+ and increasing K^+ uptake under salinity (Sami et al. 2016). Another important aspect of sugar metabolism is its role in sensing embolism in xylem vessels. When there is difficulty in the uptake of water from the soil, for example, under water deficit or high salinity conditions, the risk of embolism in xylem vessels increases and thus further damages the water supply for shoots. The mechanism by which plants can perceive embolism has been investigated. Sugars play an important role in triggering the chain of events that end by refilling xylem vessels (Secchi and Zwieniecki 2011). Indeed, *C. procera* plants were able to maintain RWC even under salinity conditions.

The third question was "how long does it take for *C. procera* roots to change AQP activity in response to salinity?" Once solutes have accumulated and a favorable water potential gradient has been created for hydraulic conductivity in the whole plant, it is necessary to facilitate the water flow through the tissues. Water flow is controlled by the activity of AQPs, and this mechanism is essential for growth in saline environments because these proteins play an important physiological role in water status, controlling hydraulic conductivity in the whole plant, water uptake of roots from the soil solution, and stomatal conductance (Li et al. 2014; Moshelion et al. 2015).

AQPs from *C. procera* roots showed a differential response to salinity. Five *cpTIP* isoforms were up-

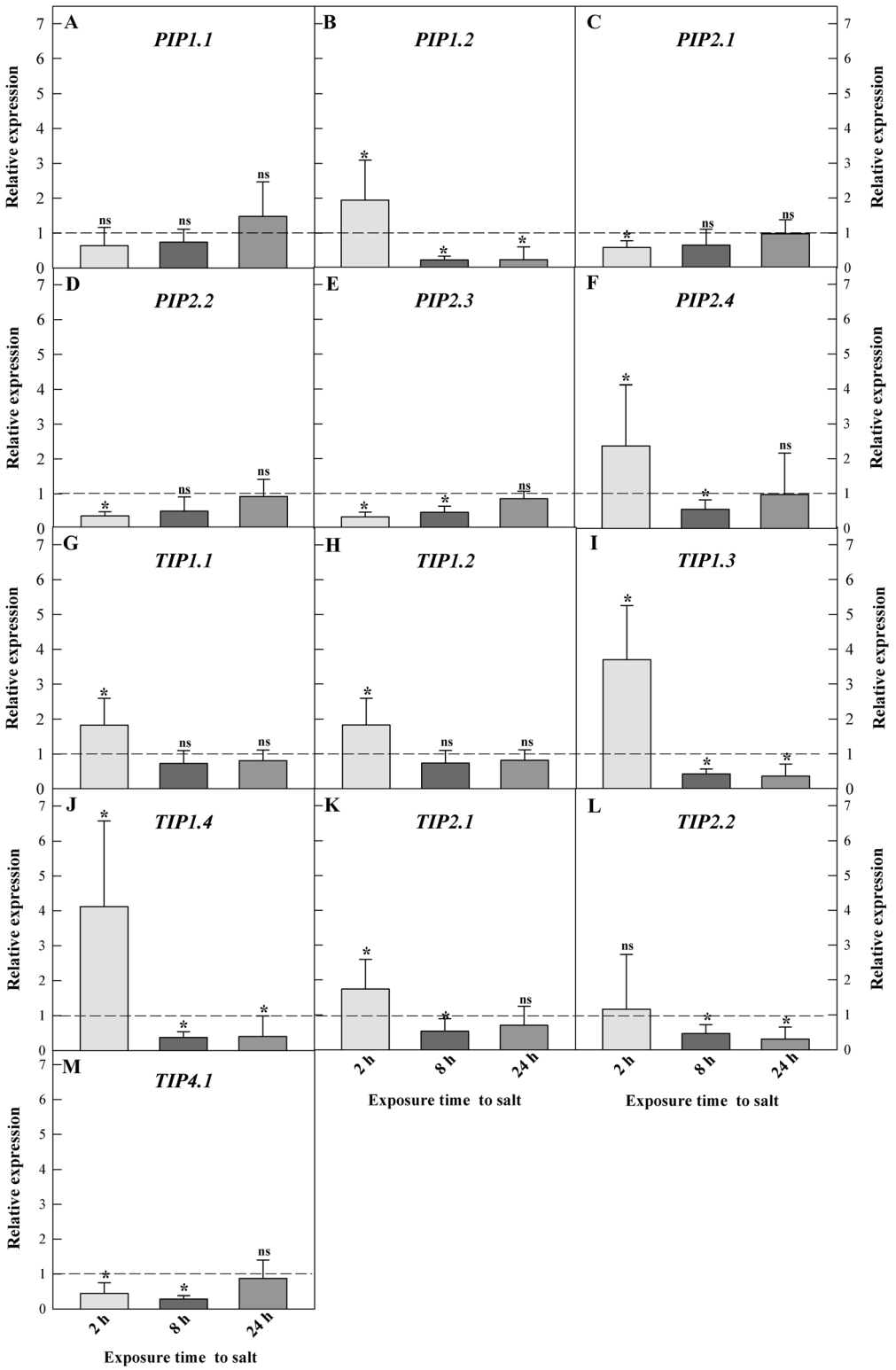


Fig. 3 Relative expression[‡] of aquaporins in roots of young *Calotropis procera* plants under salinity conditions (200 mM NaCl) in a greenhouse assay. **A–F** *Intrinsic plasma membrane proteins—PIPs*. **G–M** *Tonoplast intrinsic proteins—TIPs* (–) Dashed line indicates the limit between the positive (above the line) and negative (below the line) regulation of expression. Values followed by * indicate significant differences between treatments (Control and Salinity) by the Fixed Paired Reallocation Randomization test ($p < 0.05$). [‡] Relative expression was calculated using the formula: E (ΔCq aquaporin)/ E (ΔCq reference genes), where E represents the average efficiency for each transcript (aquaporin and reference genes), ΔCq is the difference between mean Cq-value of each control sample and the mean Cq-value of each treated sample

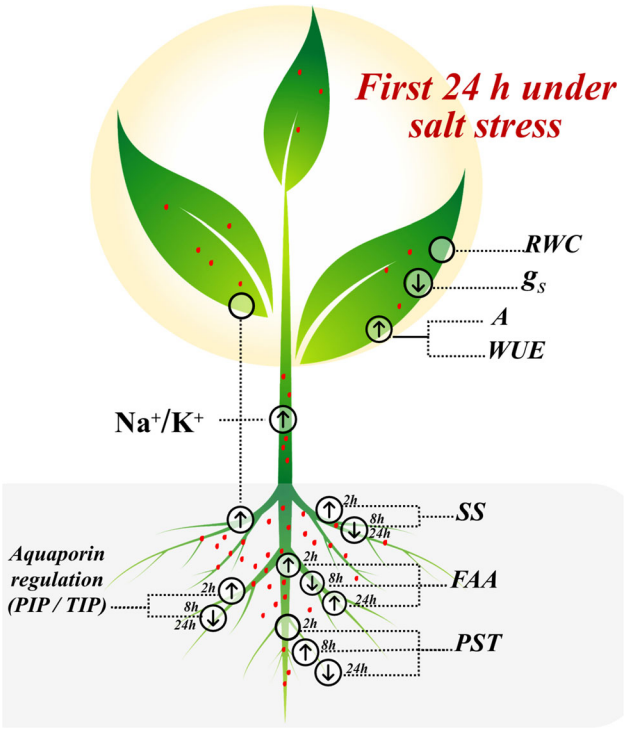


Fig. 4 Major traits that helped the *Calotropis procera* plants tolerate salinity (200 mM) by controlling changes in physiological and biochemical parameters. The arrows indicate the trait behavior during the saline treatment, without change (), increasing (↑) or decreasing (↓) its activity or content in the first 24 h under salt stress. The red dots represent the proportion of the Na^+/K^+ ratio in the three measured tissues. Relative water content (*RWC*), stomatal conductance (g_s), CO_2 assimilation (*A*), water use efficiency (*WUE*), soluble sugars (*SS*), free amino acids (*FAA*), protein soluble total (*PST*), plasma membrane proteins (PIP) and intrinsic tonoplast proteins (TIP)

regulated and five were down-regulated. TIP members are located mainly on the vacuole membrane (tonoplast) and show high activity in water transport (Maurel et al. 2008; Wallace and Roberts 2004). In addition to water, TIPs are capable of transporting glycerol, urea, ammonia, and CO_2. The overexpression of TIPs has been associated with increased salt tolerance in several plant species. Overexpression of *PgTIP1* in soybeans promotes the upregulation of *NHX*, *SOS1*, *CAT1*, and *APX1*, contributing to ion compartmentalization in the vacuole and antioxidant defense under salinity (An et al. 2017). Furthermore, transformed *Arabidopsis* plants overexpressing *SlTIP2.2* show increased activity of antioxidant enzymes (CAT, SOD, POD), K^+/Na^+ ratio, and tolerance to salt stress (Xin et al. 2014).

CpPIP isoforms were also upregulated in *C. procera* roots, but to a lesser extent than *CpTIP1* isoforms. The PIP subfamily acts on water transport with high efficiency, controlling almost the entire membrane water permeability potential by the members PIP1 and PIP2 (Bellati et al. 2010). On the other hand, when the expression of PIPs is inhibited, there is a reduction in water transport in roots, hydraulic conductivity, and leaf water potential, and a delay in plant recovery after water deficit. Additionally, the overexpression of *GmPIP1.6* in roots confers tolerance to salt in soybeans by promoting the maintenance of hydraulic conductivity, increased CO_2 assimilation, and growth (Zhou et al. 2014). Indeed, the overexpression of *PIP2.7* induces a six-fold increase in the hydraulic conductivity of roots in *A. thaliana* conditioned to salt stress (Pou et al. 2016).

The AQP isoforms in *C. procera* had a transient regulation in relation to exposure time to NaCl. Temporal variation in the expression of AQPs was also observed in rice roots under salt stress (Li et al., 2014). The transcriptional and post-transcriptional regulation of AQP isoforms is complex mechanisms (Kapilan et al. 2018), although, among the AQPs studied here, it was possible to trace a pattern of temporal expression, with upregulation at 2 h and downregulation after 8 and 24 h of exposure to salinity.

Previous studies have shown the importance of inducing AQPs to maintain the water status of plant species (Pou et al. 2016; Zhou et al. 2014;) by the coordinated expression of PIPs and TIPs. This is a key point for improving cell water flow and water uptake in roots (Maurel et al. 2015). Indeed, tomato plants that maintain *PIP1* abundance are more tolerant to salt stress than those genotypes with a decreased AQP presence (Han et al. 2020). On the other hand, the downregulation of AQPs negatively influences hydraulic conductivity and stomatal conductance, thus relieving the transpiration rate, which in turn favors the conservation of water in shoots (Moshelion et al. 2015; Skorupa-Kłaput et al. 2015). These proteins also play a role in the hydraulic control of xylem parenchyma cells, highlighting their participation in maintaining xylem transport capacity and restoring vessel functionality after embolism events (Secchi et al. 2017).

At a high salt concentration (200 mM, NaCl), there was upregulation of *CpPIPs* and *CpTIPs* (especially *CpPIP2.4*, *CpTIP1.4*, and *CpTIP1.3*), possibly indicating its involvement in adjusting cell water flow (vacuole-cytosol-apoplast) and increasing water uptake by the root system (Maurel et al. 2015). On the other hand, we propose that the downregulation of some AQPs, especially *CpPIP1.2*, *CpTIP4.1*, and *CpTIP1.4* at 8 h, and *CpPIP1.2*, *CpTIP2.2*, and *CpTIP1.3*, after 24 h of exposure to NaCl, can be a strategy used by *C. procera* to prevent excess salt contact with the root system, which quickly reaches the shoots (Skorupa-Kłaput et al. 2015). Therefore, previous studies have argued that lower aquaporin activity can be offset by an increase in the number of proteins in the plasma membrane. Thus, the intensity of uptake water is increased

without greater activity in each of the channels and the dilution of NaCl within the root cells is maintained (Muries et al. 2011). Most studies do not measure protein synthesis and expression at the same time, but those who did found varied responses, which may be antagonistic or not. This would be due to the stress intensity and duration imposed, as well as the species studied, mainly under saline stress (Yepes-Molina et al. 2020). Under another abiotic stress, such as caused by low temperature in corn plants, the recovery of root hydraulic conductivity is pointed out by the authors due to the greater activity and/or abundance of aquaporins (Aroca et al. 2005).

The plasticity in modulating the primary metabolism and gene expression of AQPs supports the robustness of the *C. procera* root system in response to osmotic and ionic stresses generated by salt stress (Munns and Tester 2008; Shavrukov 2013). Such tolerance mechanisms can act locally to protect cellular processes and maintain the activity of the root system through osmoregulation, detoxification of reactive oxygen species, and storage of cytosolic sodium (An et al. 2017; Han et al. 2020; Mansour and Ali 2017; Sami et al. 2016). It can regulate the water homeostasis of the entire plant (Maurel et al. 2015; Pou et al. 2016; Rodríguez-Gamir et al. 2019).

In summary, root physiological responses of *C. procera* under salinity conditions show that high salt concentrations cause a quick response detectable by analyzing physiological parameters of young plants under greenhouse conditions. Exposure to high NaCl concentrations (200 mM) did not affect the photosynthesis and shoot water status in a short time (24 h exposed to salt). Our first hypothesis was that *C. procera* root system would show biochemical responses that allowed it to promote osmoregulation through the accumulation of organic solutes. Quick changes in the roots of treated plants suggested that *C. procera* metabolites, such as soluble sugars and free amino acids, might be involved in osmoregulation to tolerate osmotic stress. The second hypothesis was that under high salt conditions, the upregulation of the gene expression of PIPs and TIPs would occur to promote the adjustment of plant water status and maintain the water uptake by the root system, maintaining leaf RWC. At the beginning of salinity exposure, there was no difference in RWC between the treated and control plants. The temporal modulation of *CpPIP* and *CpTIP* expressions support the plasticity of the root system in water uptake to mitigate the decreasing water availability in the soil and cellular dehydration generated by the salinized soil. Finally, we concluded that strong stomatal control associated with high amino acid and soluble sugar contents, as well as the regulation of AQP expressions in roots, support the high performance of the root system and alleviates *C. procera* leaf metabolism.

Acknowledgements This work was supported by the National Council for Scientific and Technological Development (CNPq) [CNPq-470247/2013-4; 310871/2014-0; and 433931/2018-3]. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) (Finance Code 001) for scholarship to M.R.C. M.G.S. and A.M.B.I. recognize CNPq for fellowships and financial support. A.M.B.I. recognizes the CAPES BioComputacional Program [88882.160046 / 2013-01] for financial support.

Author contributions MRC and RR conducted the experiment and performed the measurements. MRC analyzed the data and wrote the initial draft first version of the manuscript. MS is the advisor of MRC and participated in the planning of the study, data analysis, and writing of the manuscript. JRCF-N, VP, and AMB-I participated in the molecular data analysis, discussions about results, and helped in the writing of the final manuscript.

Compliance with ethical standards

Conflict of interest No conflict of interest exists in the submission of this manuscript, and the manuscript has been approved by all authors for publication.

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