Effect of salinity on seed germination, growth and metabolic activity of pitaya seedlings [*Stenocereus thurberi* (Engelm.) Buxb.]

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ABSTRACT

The Cactaceae is an important resource in the arid and semi-arid zones of Mexico, this family is naturally distributed in the Americas. Pitaya (*Stenocereus thurberi*) is an important endemic species of the Sonoran Desert that has received little attention despite its diverse uses, distribution and endemism. In the environment where pitayas thrive, salinity is a common condition, and its effects in physiological traits has been poorly recorded in the literature. In this study, NaCl effect was evaluated on germination, and seedlings growth and metabolic changes. For the latter, heat or metabolic activity (q), respiration rate (R_{CO2}), metabolic efficiency (R_q / R_{CO2}) and growth rate ($R_{SG}\Delta H_B$) were determined. These variables were analyzed using isothermal calorimetry at 25°C in pitaya seedlings. Final germination and plantlet survival rate were not affected by salinity; however, mean germination time, aerial biomass production, root growth and the calorimetric variables mentioned above were directly affected according to salt concentration. Although seedling size decreased, contents of chlorophylls a, b, and total chlorophyll (a+b) and total carotenoids showed an increase as a function of NaCl concentration. Variables evaluated showed different salinity tolerance, reaching maximum values between 4,000 and 6,000 ppm of NaCl. As far as pigment synthesis, salinity treatments caused increases in chlorophylls and carotenoids. This increment was more conspicuous at salinity treatments above 8,000 ppm.

Keywords: saline tolerance, calorimetry, salt stress.

INTRODUCTION

Drought and temperature fluctuations are major selection pressure factors, driving the evolution of plant species in arid and semi-arid regions. Cacti are among the more successful species adapting to such conditions, through their crassulacean acid metabolism (CAM). These species close their stomata during the day and open at night, also have tissues capable of storing large amounts of water relative to their volume, and evolved their leaves to thorns to reduce transpiration, therefore optimizing water use. The cacti family distribution is limited to the Americas (Rojas-Aréchiga and Vázquez-Yanes, 2000); while pitaya (*Stenocereus thurberi*) is one of the most emblematic species of the Sonoran Desert, which is

characterized by its cacti abundance and diversity. Pitayas have been used by the Seri and Mayo native peoples as food, medicine, construction, dye source, spirits beverages and as ornaments (Paredes-Aguilar *et al*. 2000). This slow growth species is widely distributed in northwest Mexico within Sonora, Baja California and northern Sinaloa, as well as in Arizona in the USA.

In addition to water scarcity, arid and semi-arid regions of the world, are exposed to a concomitant soil salinization (Schwabe *et al*. 2006), which becomes another important factor restricting plants establishment, growth, and production (Beltrán-Morales *et al*. 2015). Plants growing under saline stress, exhibit an increased energy expenditure to extract water from soils with negative osmotic potential, resulting in reduced cell expansion and tissue growth, decreased flow of assimilates to growing meristems and allowing inorganic ions accumulation (Morales *et al*. 2002).

Although saline stress occurs in all plants, their tolerance level and growth rate reduction vary among species and phenological stages (Parida and Dass, 2005). Because of that, salinity has been extensively analyzed in domesticated plants for crop production; however, the effects of NaCl in cacti have been poorly documented. In spite of *S. thurberi* environmental, traditional and cultural values, its uses, distribution and endemism, the effects of salinity on growth and metabolic variables are rather scarce in the literature. The aim of this study was to assess the effects of salinity on germination, growth and metabolic variables in *S. thurberi* seedlings under controlled laboratory conditions, in order to get a better understanding of its adaptation mechanisms.

MATERIALS AND METHODS

Vegetative material

Mature fruits were collected during the second week of May 2015 from several wild plants in the Carbó area, in the Sonoran Desert of northwest Mexico (29° 32.36' North Latitude, 111° 00.65' West Longitude and 454 m.a.s.l.). Seeds were manually separated and washed with tap water. After rinsing, the seeds were dried and stored in amber vials at 25 ± 2°C until use.

Culture media

MS salts (2.16 $g \cdot L^{-1}$) (Murashige and Skoog, 1962) were used, as well as agar as a base medium (17 $g \cdot L^{-1}$). Salinity treatments were established by adding NaCl at concentrations of 0, 2, 4, 6, 8 and 10 (X 10^3) ppm. After preparation media were sterilized for 15 min (120°C and 1.05 $kg \cdot cm^{-2}$) and poured into Petri dishes. All media were prepared with MilliQ water (Ultrapure Water System. Millipore Co. Molsheim, France) (18.2 MΩ-cm).

Germination

To evaluate the effects on germination, seeds were superficially disinfected with 30% ethanol for one minute and subsequently washed with 2% sodium hypochlorite for 3 min, after that,

100 seeds were allocated to each treatment, divided in four Petri dishes, sealed with paraffin to avoid evaporation.

The experiment was performed at 25 ± 2°C with a 16/8 photoperiod using white light (6.5 $mol \cdot m^{-2} \cdot s^{-1}$). After 20 days in culture, germination percentage and mean germination time (GT_{50}) were determined. The criterion used to evaluate germination was when radicles reached at least 1 mm. GT_{50} is referred to the period when half the seeds germinated on each treatment.

Growth

Measurements were taken with a digital Vernier (model 3415, Traceable Co. Wester, Texas, USA). The length of aerial parts and roots of 40 seedlings at 60 days after germination were recorded. Subsequently, the plants were dried in an oven at 60°C for 48 h (model DX 600, Yamato Scientific Co. Tokyo, Japan). The fresh and dry weights were recorded with an Ohaus, Explorer Pro analytical balance (model EP 214, Ohaus Co. N.J., USA). Once incinerated at 525°C for 4 h, ashes were weighed (AOAC, 1990).

Chlorophylls and carotenoids contents

Chlorophylls and carotenoids were determined on 60 day old plantlets. Fresh seedling tissue weighing one gram was used to determine chlorophylls a, b, and (a+b), as wells as for carotenoid quantification (Lichtenthaler, 1987). Pigments were extracted in 80% cold acetone. Absorbance readings were performed on a Varian UV-VIS spectrophotometer (Model Cary 50, USA). Data were taken at 470, 644 and 662 nm, appropriate conversions were used to yield chlorophylls a, b, a+b and caortenoids values. All reagents were purchased from Sigma-Aldrich.

Calorimetric measurements

Metabolic heat (Rq, $\mu W.mg^{-1}dw$), and CO_2 production (R_{CO2} $nmol\ mg^{-1}dw$) were determined directly from microcalorimetric readings. Based on those two variables, it is possible to get the following relations: Metabolic efficiency = R_q / R_{CO2}, expresses as $kJ \cdot mol^{-1}$, while Growth rate (R_{SG} ΔH_B) = (470)*(R_{CO2})-q, is expressed as $\mu W \cdot mg^{-1}dw$ (Hansen *et al*. 1996; Hansen *et al*. 2004; Weili *et al*. 2008).

Respiratory parameters were determined in a differential scanning calorimeter Multi-Cell DSC 4100 (Calorimetry Science Corporation, Pleasant Grove, Utah, U.S.A.) working in the isothermal mode at 25°C. The instrument is equipped with four sealed hastelalloy cells of 1 cm^3. The fourth cell was used as a reference and remained empty during measurements. To prevent internal humidity condensation, a steady flow of N_2 at 1.75 $mL \cdot min^{-1}$ was used; based on the methodology described by Criddle *et al*. (1990).

Statistical analysis

A completely randomized design was used, and all variables were analyzed by a one-way ANOVA. The germination and growth experiments were performed with four replicates of 25 seeds each, and six repetitions in the calorimetric measurements. A 95% significance level was used; before statistical analysis, germination percentages were normalized using the arcsine square root (Sokal and Rohlf, 1995). Where significant differences were detected, means were compared by Tukey, using the statistical software program SAS (SAS, 1999).

RESULTS AND DISCUSSION

Salinity treatments

Table 1 shows the salinity values reached by the established treatments ranging from 0 to 10,000 ppm NaCl. This imply that a whole salinity gradient was established, as required for the proper evaluation of the above mentioned variables.

Table 1. Effect of NaCl on germination and growth of *S. thurberi*.

NaCl (x 1,000 ppm)	Germination (%)	GT_{50} (days)	Aerial length (mm)*	Root length (mm)*
0	93.0±3.8	3.9±0.1^{a}	8.8±0.5^{a}	6.5±2.0^{a}
2	89.0±5.0	4.1±0.2^{a}	8.7±0.4^{a}	6.4±1.9^{a}
4	97.0±3.8	4.0±0.1^{a}	8.7±1.0^{a}	6.1±2.1^{ab}
6	88.0±3.3	4.5±0.2^{ab}	7.5±0.5^{b}	6.1±2.0^{ab}
8	85.0±5.0	5.7±0.6^{bc}	6.1±0.4^{c}	5.0±2.1^{bc}
10	90.0±5.2	6.1±0.6^{c}	4.8±0.5^{d}	4.4±1.9^{c}

Means with a different letter in the same column are statistically different $p<0.05$.
*n = 40. GT_{50} = mean germination time.

Germination

After 20 days of sowing, salinity treatments did not exert a significant effect on final germination ($F=1.66$, $p=0.19$) as shown in Table 1. Such evidence may suggests a high adaptability of this species to salt stress at this particular phenological stage**,** and the salinity treatments used. However, Goykovic and Saavedra (2007) reported that in many species, salinity tolerance during germination may not be consistent upon further development. Also, these results indicate that pitaya seeds are more tolerant to salt concentration than seeds from other columnar cacti. For instance, *Pachycereus pringlei* seeds are affected by 0.05 M (2922 ppm) NaCl, and only 10% germinate at 0.2M (11680 ppm) NaCl (Nolasco *et al*. 1996). In this regard, Beltran-Morales *et al*. (2015) reported that germination of *Pachycereus pectin-*

aboriginum seeds diminishes, as salinity concentration increases above 2.03 dSm^{-1} (1,300 ppm) NaCl. Thus, other species thriving in the region do not forcefully feature the same adaptation to salinity.

Mean germination time (GT_{50})

The average time required for radicle tips to emerge indicates significant differences within treatments (F=7.27, p=0.0007), with longer germination time at increasing salt concentration (Table 1). Salinity treatments between 0 and 6,000 ppm had the shortest mean germination times, ranging from 3.9 to 4.5 days, while those of 8,000 and 10,000 ppm NaCl took 5.7 and 6.1 days, respectively. A high positive correlation (R^2=0.75) was observed between GT_{50} and salinity treatments. Interestingly, total germination was not affected by salinity; however, the time required to achieve such numbers did increased as salinity increased. De la Barrera and Nobel (2003) reported a delay of four days in seed germination of *S. queretaroensis* when osmotic pressure increased from 0 to -1.0 MPa. Meiado *et al*. (2010), reported that an increase in saline concentration affected germination and promoted a slower unsynchronized germination in seeds of *Cereus jamacaru*. These authors attributed this germination delay to either decreased water availability inside the seed during imbibition, or osmotic stress toxicity. This is in line with our results, as Table 2 shows a decrease in fresh weight and moisture loss with respect to the increase in salinity, which indicates a seedling decreasing ability to extract water from the culture medium.

Growth

In this experiment, final germination reached at least 85% in all treatments, although salinity affected growth of both aerial and root biomasses. Table 1 show values for growth with a clear pattern observed. Shortest lengths were recorded for both aerial and roots biomasses at increasing NaCl concentration values. The aerial part was significantly affected starting from 6,000 ppm NaCl (p=0.0001). In fact, comparing to controls, final size in 6,000, 8,000 and 10,000 ppm NaCl treatments were consistently reduced by 14.8, 30.7 and 45.3%, respectively. On the other hand, root length showed significant reductions (p=0.0001) only at the highest salinity treatments, reaching a 23 and 32% reductions at 8,000 and 10,000 ppm NaCl. Physiologically, such growth reduction results from plant adaptation to survive under salt stress, caused by stomatal closure, which causes poor CO_2 fixation, and therefore an inadequate photosynthetic rate (Zhu, 2001). Major osmotic pressure to maintain turgidity is caused as well (Nobel, 2006). This reduction in growth and yield may be attributed to the production of reactive oxygen species (ROS), nutrient ion and osmotic imbalance. Ion toxicity is caused by accumulation of Na^+ and Cl^- ions under continuous exposure to saline conditions (Singh *et al*. 2016). *Cereus validus* fixed 67% less CO_2 under a salt stress of 400 mM NaCl (Nobel *et al*. 1984).

Biomass and mineral contents

The results in this study showed that fresh and dry biomass weights and water content were affected by saline stress (Table 2).

The fresh weight recorded clearly reflected the effect of salinity (F=45.4, p=0.0001). Reductions along the whole salinity gradient tested were found; while controls yielded a mean of 1,500 mg, plantlets in the extreme treatment reached only 200 mg fw, a conspicuous reduction of 86.7%.

Dry weight was also significantly reduced by increasing salinity (F=5.05, p=0.0001). However, such effects were less notorious, showing values in a range between 28 and 15 mg per plant in controls and the highest salinity treatment, which implies a total reduction of 54%. However, such reduction was significant starting at 6,000 ppm (Table 2). Cha-um *et al*. (2013), reported a similar trend in loss of fresh and dry weights in *Echinopsis calochlora* under exogenous NaCl. Salt stress is inversely related to growth in glycophyte plants, since it causes an inadequate photosynthetic rate and a decrease in cell division and expansion (Zhu, 2001). The lowest growth rate caused by salinity is due to changes in water content, ionic ratios and assimilates distribution (Willadino and Camara 2005; Munns and Tester, 2008).

Table 2. Effect of NaCl on biomass production, water content and ashes of *S. thurberi*.

NaCl (x 1,000 ppm)	Fresh weight (g)	Dry weight (g)	Water content (%)	Dry matter (%)	Ashes (% in fw)
0	1.5±0.3^{a}	0.028±0.1^{a}	98.2±0.3^{a}	1.8±0.9^{a}	0.43±0.04^{a}
2	1.2±0.1^{b}	0.023±0.2^{ab}	98.0±0.5^{ab}	2.0±0.8^{ab}	0.43±0.03^{a}
4	0.9±0.1^{bc}	0.022±0.1^{ab}	97.5±0.3^{ab}	2.5±0.7^{ab}	0.54±0.04^{a}
6	0.6±0.1^{cd}	0.020±0.2^{ab}	96.7±0.7^{bc}	3.3±0.3^{cd}	0.67±0.07^{b}
8	0.4±0.1^{de}	0.017±0.6^{b}	95.7±0.8^{c}	4.3±0.5^{c}	0.94±0.11^{c}
10	0.2±0.1^{e}	0.015±0.6^{b}	93.5±0.9^{d}	6.5±0.3^{d}	1.2±0.12^{d}

Means with different letters in the same column are statistically different p<0.05.
n = 3; fw= fresh weight

In addition, Silveira *et al*. (2005), argued that saline conditions limit plant growth by increasing free radicals derived from electron transfer during photosynthesis and respiration. Tissue water content decreased with increasing salt concentration, as shown in Table 2. A significant difference (p<0.05) was found in treatments beyond 6,000 ppm NaCl, reaching up to a 5% reduction in the 10,000 ppm treatment. Parida and Das (2005) reported that relative water content, leaf water potential, water intake, transpiration, water retention and water use efficiency decrease when plants are under saline stress.

Ashes content increased directly proportional to salt concentration increments (Table 2). Mineral accumulation in plant tissues is probably due to the increase of NaCl concentration in the rooting medium. However, Parida and Das (2005) found that high NaCl content, inhibits

absorption of other minerals, such as Ca, K and Mg, although induces proline accumulation. The same trend in reductions of N, P, K, Ca and Mg in varieties of prickly pear (*Opuntia* spp) under different salinity gradients has been reported (Calderón-Paniagua *et al*. 2001; Franco-Salazar and Véliz 2008). In our study, only total mineral content is reported, however we hypothesize that a similar behavior may occur in *S. thurberi* mineral composition.

Chlorophyll content

Chlorophylls a, b and a+b, and total carotenoids in *S. thurberi* seedlings (Fig. 1) showed a significant increase**,** but only above 8,000 ppm NaCl. Our results indicate that treatments above that threshold, increased chlorophylls and carotenoids synthesis. The latter was probably due to the increasing presence as osmoprotective substances, which leads to water movement from the chlorenchyma to hydro chlorenchyma (Franco-Salazar and Véliz, 2007). Silva-Ortega *et al.* (2008), reported similar results in *O. streptacantha*, concluding that under saline stress, proline helps maintain turgor and photosynthetic activity in species of *Opuntia*. In addition, leaf area reduction due to salinity do not necessarily affect photosynthesis, since plants are capable of performing cellular adjustments and increase chloroplasts density (M**u**nns and Tester, 2008).

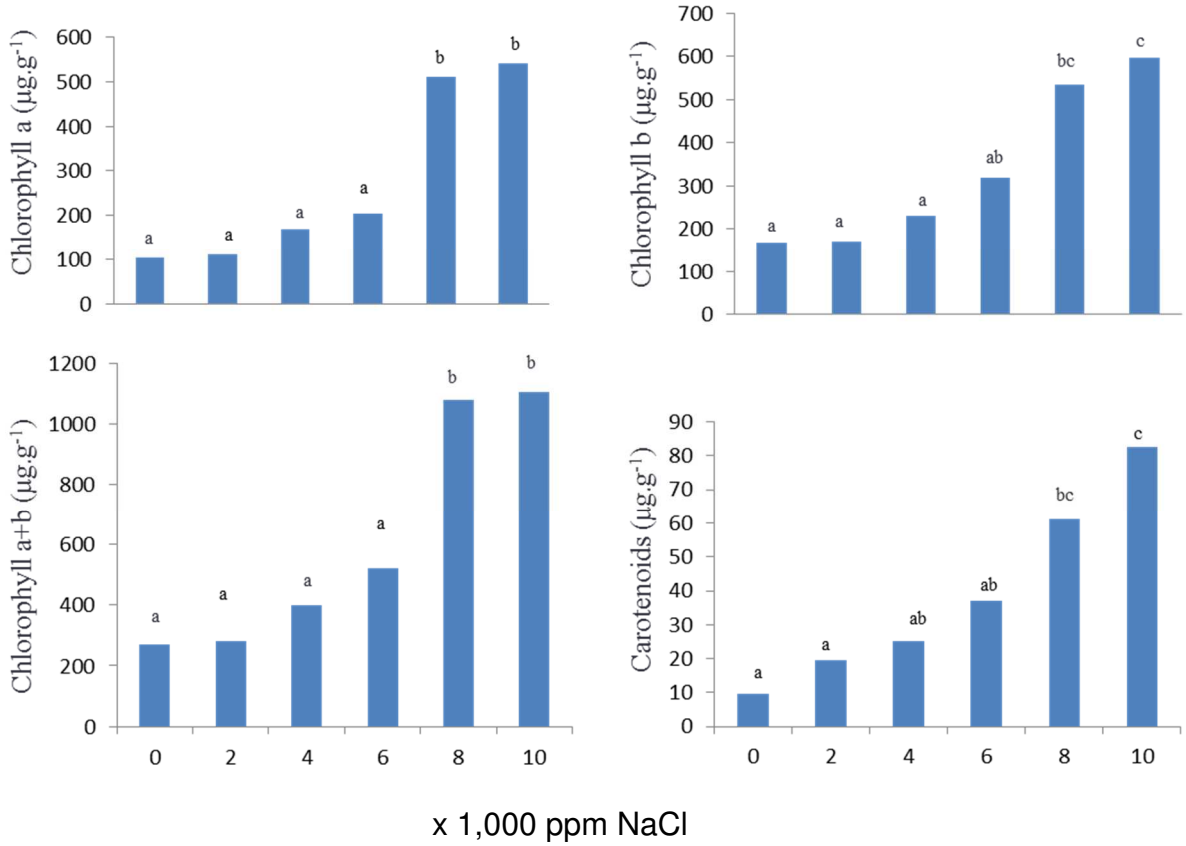

Figure 1. Effects of increasing salinity on 60 days old *S. thurberi* seedlings on contents of chlorophyll a, b, and (a+b), as well as total carotenoids on fresh weight basis.

Aguilar and Peña (2006) reported an increase of chlorophylls a, b and a+b in chlorenchyma of *Opuntia ficus-indica*, when subjected to intermediate water stress. Ruiz *et al.* (2007) reported the same trend in *Agave salmmiana* under water stress. The increase of chlorophylls in *S. thurberi* may be taken as evidence that saline stress do not generate severe damages or modifications in the photosynthetic mechanism, which may represent an indicator of salinity tolerance.

Carotenoid content showed an increase from 2 to 8 times its concentration in comparison to controls. These results suggest that salinity stimulates the production of these pigments in *S. thurberi*. Cela and Munné-Bosch (2012) reported a similar increase in β-carotene production in *Aptenia cordifolia* in response to exposure time to saline conditions. These authors mention that a low demand of photoprotectors is a characteristic in plants resistant to saline stress. Nonetheless, Chávez *et al*. (2015), proposed that the increase of carotenoids is just a mechanism of saline adaptation with the objective of protecting the chlorophyll moiety during photooxidation. In this sense, the increase of carotenoids in *S. thurberi*, could be a strategy of adaptation to saline stress as an osmoprotective compound. Nevertheless, Cha-um *et al.* (2013), reported that carotenoids are very sensitive and decreased significantly in *Echinopsis calochlora* under saline stress.

Calorimetric measurements

Metabolic heat production and respiration rates

Salinity induced significant differences in metabolic heat rate. Pitaya seedlings metabolic heat production showed a particular pattern. It was stimulated up to 4,000 ppm NaCl, reaching 6.4 $\mu W \cdot mgdw^{-1}$; followed by a significant reduction of 58%; however, after this threshold, no significant changes were detected (Table 3). These results indicate that, as far as metabolic heat production, *S. thurberi* seedlings reached a maximum at 4,000 ppm NaCl.

On the contrary, respiration rate did not show significant differences among treatments, although a decreasing response can hardly be ignored. The high variability observed is likely caused by lack of precision when handling the samples, although other physiological causes may play a role as well, such as stomatal closure to prevent moisture loss; therefore, limiting gas exchange. On the other hand, plants under salinity stress probably activate alternative paths in physiological cycles, where they take O_2 to oxidize NADH and produce $FADH_2$ without CO_2 production (Mathews and van Holde, 1996). Although this could explain the reduction of respiration rate, as occurs during seeds germination where glyoxylate cycle is activated (Mathews and van Holde, 1996). Still further research is needed for conclusive evidence to support this hypothesis. Weili *et al.* (2008), cited a similar trend in three species of the genus *Caragana*, where they reported an increase in metabolic activity between 10 and 35°C and a decrease of this variable at higher temperatures.

Metabolic efficiency

Metabolic efficiency of pitaya seedling decreases as the NaCl concentration increases (Table 3), however, the control and 2000 ppm treatments were highly efficient ($p<0.05$), showing 159

and 204 Kj·mol^{-1} respectively, whereas the treatments of 4,000, 6,000 and 8,000 ppm showed little efficiency, indicating stressing conditions for growth. The treatment of 10,000 ppm exceeds the efficiency limits 470 kj·mol^{-1} (Hansen *et al*. 2004) with a value of 563 kj·mol^{-1}. The increase of this variable indicates that much of the energy from substrates dissipates as heat, so it is not used for biomass generation. According to Hansen *et al*. (2004), a value of 470 kj·mol^{-1} or less, indicates that the conditions are optimal for plant growth and development. Millan-Soto *et al*. (2016), reported a decrease in metabolic efficiency (R_q / Rco_2) in four clonal lines of *A. angustifolia* (CAM species) measured at 15°C, they found metabolic efficiency values between 459 and 597 kj·mol^{-1}, while at 45°C ranged from 472 to 745 kj·mol^{-1}.

Specific growth

The results by micro calorimetry indicate a progressive decrement in the specific growth of seedling of pitaya because of the increments in salt concentration (Table 3). However, the maximum growth occurred in control, 2000 and 4000 ppm with 7.83, 7.55 and 5.06 µw·mg^{-1} dw, respectively. Nevertheless, statistically significant changes ($p<0.05$) were recorded from the 6,000 ppm of NaCl and up, where it originates a reduction of 73.7, 88.2 and 91.0% on treatments 6,000, 8,000 and 10,000 ppm NaCl, respectively. These results are consistent with the data on the growth of pitaya seedlings of the present study (Table 1). Millan-Soto *et al.* (2016), reported this same trend in the growth rate of *A. angustifolia* under temperature stress. In this regard Hansen *et al*. (1995), mentioned that energy efficiency is inversely proportional to the loss of metabolic heat energy per mole of CO_2 breathed during dark respiration (R_q / R_{CO2}). If the energy loss is high, the plant is inefficient in conserving the energy to be used in biomass formation and, therefore, growth is limited.

CONCLUSIONS

Based on these results, it is concluded that the salinity treatments used do not affect seed germination, but caused an ion imbalance that negatively affected growth, and therefore biomass production in *S. thurberi* seedlings. However, increasing saline stress caused a concomitant increase in photosynthetic pigments, suggesting the formation of osmolyte compounds that help protect plant cells subjected to the low water potentials caused by saline stress. The salinity stress used, while reducing biomass production, favored chlorophyll and carotenoid content. Although the changes in the variables analyzed were gradual, the significant changes were observed on treatments above 4,000 ppm of NaCl. Calorimetric results indicated that metabolic efficiency decreased to a minimum until 8,000 ppm, while specific growth rate achieved the best response in low salinity treatments.

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