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Antioxidant metabolism of *Chenopodium quinoa* Willd. under salt stress

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ABSTRACT

The aim of this study was to understand the functioning and importance of antioxidant metabolism in the preservation of *Chenopodium quinoa* early seedling tissue against the oxidative damage caused by different concentrations of NaCl (0, 50, 100, 150 and 200 mM). The experimental design was completely randomized, with 5 treatments and 5 replications per treatment. After 7 days of treatments, the shoots and roots were evaluated for biomass allocation, betacyanin and antioxidant metabolism. The activity of superoxide dismutase showed similar behavior in both roots and shoots, and the greater activity of this enzyme was observed in higher concentrations of NaCl. The high SOD activity resulted in an increased production of H₂O₂ only in the shoots. H₂O₂ was probably removed by catalase, which exhibited behavior similar to that of superoxide dismutase. Higher levels of lipid peroxidation as well as lower levels of betacyanin were observed in the shoots at a concentration of 200 mM NaCl. All this metabolic adjustment was made without any impact on seedling dry matter accumulation. In this way, *Chenopodium quinoa* early seedlings are potentially tolerant to salinity, as an adjustment of their antioxidant metabolism was performed without affecting growth.

Key words: antioxidant enzymes, betacyanin, dry matter accumulation, salinity

Metabolismo antioxidante de Chenopodium quinoa Willd submetida à salinidade

RESUMO

Objetivou-se com o presente trabalho entender o funcionamento e a importância do metabolismo antioxidante na preservação dos tecidos de plântulas de *Chenopodium quinoa* submetidas a diferentes concentrações de cloreto de sódio (0, 50, 100, 150 e 200 mM). O delineamento experimental utilizado foi o totalmente casualizado com 5 tratamentos e 5 repetições por tratamento. Após 7 dias de imposição dos tratamentos, parte aérea e raízes foram avaliadas quanto a produção de massa seca, conteúdo de betacianinas e metabolismo antioxidante. A atividade da dismutase do superóxido foi similar tanto na raiz quanto na parte aérea, tendo a sua atividade mais elevada nas maiores concentrações de NaCl. A elevação na atividade da dismutase do superóxido resultou em elevação na produção de H₂O₂ somente na parte aérea. O peróxido de hidrogênio, provavelmente, foi removido pela catalase, a qual apresentou comportamento similar ao da dismutase do superóxido. Maiores níveis de peroxidação lipídica e redução na concentração de betacianinas foram observados na concentração de 200 mM de NaCl na parte aérea. Dessa maneira, plântulas de *Chenopodium quinoa* podem ser consideradas potencialmente tolerantes à salinidade, uma vez que realizam o ajuste do seu metabolismo antioxidante, sem afetar o crescimento vegetal.

Palavras-chave: enzimas antioxidantes, betacianina, acúmulo de massa seca, salinidade

Introduction

Chenopodium quinoa Willd. is a pseudocereal originating from the Andes, popularly known as quinoa, and it belongs to the Chenopodiaceae family, as described by Walter Vent. This species is a facultative halophyte with a wide phenotypic and genetic variability, and it has a high capacity for climate adaptation, having been cultivated from sea level to high altitudes (Jacobsen et al., 2012). The Brazilian cultivation of quinoa has been increasing as this species is tolerant to abiotic stresses such as drought, soil acidity, high temperatures, and salinity (Jacobsen et al., 2003). Additionally, salinization has become a recurrent problem in Brazil, affecting 20-25% of all irrigated areas (FAO, 2006), thereby increasing the interest in crops that are tolerant to such stressful conditions.

Soil salinization caused by saline irrigation water can lead to increasingly unproductive soils (Lima & Bull, 2008). Irrigation water containing high salt concentrations can also lead to salt accumulation and cause damage to plants. As a consequence of excessive absorption through the tissues, mainly of chlorine (Cl^-) and sodium (Na^+) ions, salinity can trigger two types of stress: osmotic and ionic (Tavakkoli et al., 2011).

High levels of sodium chloride in soil can reduce the water absorption by plants by directly affecting the process of stomatal closure and CO_2 absorption (Munns, 2002). The limitation in CO_2 fixation by the Calvin cycle in plants under such conditions decreases the oxidation of NADPH. The electron of the reduced ferredoxin, which would be transferred to NADP^+ , goes to the O_2 , leading to the excessive intracellular production of reactive oxygen species (ROS). ROS are highly cytotoxic by reacting with biomolecules such as lipids, proteins and nucleic acids, causing lipid peroxidation, protein denaturation and DNA mutations, respectively (Gill & Tuteja, 2010).

Therefore, plants have developed different antioxidant defense systems to remove ROS and minimize cellular damage. The enzymatic antioxidant system is one of the protective mechanisms, including superoxide dismutase (SOD), which catalyzes the dismutation of $\text{O}_2^{\cdot -}$ in H_2O_2 and O_2 . The H_2O_2 is converted into O_2 and H_2O by the action of catalase (CAT) and ascorbate peroxidase (APX) (Gill & Tuteja, 2010).

In addition to the enzymatic antioxidant system, betalaines are secondary metabolic compounds found in the stems and leaves of quinoa that also have antioxidant properties (Ribeiro et al., 2014). Betalaines may also act as osmolytes in the protection of physiological processes against abiotic stresses (Wang et al., 2006). Generally, salinity induces the accumulation of amino acids such as phenylalanine, the precursor of the biosynthesis of betalaines, including betacyanins (Tanaka et al., 2008).

Despite this well-known tolerance of quinoa plants to salt stress, few studies have been developed to relate the adaptations of antioxidant metabolism under salt stress (Shabala et al., 2012). Thus, this study aimed to understand the functioning and importance of antioxidant metabolism in the preservation of *Chenopodium quinoa* early seedling tissue against the oxidative damage caused by salt stress.

Material and Methods

Chenopodium quinoa Willd. seeds were put to germinate between two sheets of germination papers, with 10 seeds per line and 10 per column, totaling 100 seeds per paper. Two germination papers were moistened in distilled water, and then the seeds were placed on the first germination paper, and a second paper was placed on top to make a roll. The amount of distilled water placed was determined according to Maia et al. (2012), based on the weight of the paper (2.5 mL g^{-1} paper). The photosynthetic photon flux density in the growth chamber (kind BOD) was approximately $77 \mu\text{mol m}^{-2} \text{ s}^{-1}$ at the level where the plants were grown. The temperature was 25°C during the day and 20°C during the night. The relative humidity was kept at 70% during the day and 90% during the night. Four days after sowing, when the early seedlings were seven centimeters, the following treatments were imposed: Control (distilled water), and 50 mM, 100 mM, 150 mM and 200 mM of NaCl. To submit the early seedlings to stress, the germination papers were exchanged and moistened in the respective saline solutions. After seven days of treatments, the harvesting of roots and shoots was performed.

The experimental design was completely randomized, with 5 treatments (0 mM, 50 mM, 100 mM, 150 mM and 200 mM of NaCl) and 5 replications per treatment. Each experimental plot consisted of 4 rolls with 100 seedlings each. The data were subjected to an analysis of variance using the statistical program SISVAR 4.3 (System Analysis of Variance for Balanced Data) (Ferreira, 2011). The means between the treatments were compared by the Scott & Knott (1974) test at 0.05 probability.

For dry weight, the early seedlings were divided into roots and shoots. The plant material was dried at 70°C until a constant weight, and the dry weight was measured.

The betacyanin content was determined according to Cai et al. (1998). One hundred milligrams of fresh weight were ground in 5 mL of distilled water and were then centrifuged at 13,632 g at 4°C for 25 minutes. The quantification of betacyanin was carried out in the supernatant by absorbance at 536 nm and 650 nm in a spectrophotometer. The concentration of the betacyanin was calculated and expressed by taking into account the coefficient of molar extraction for Amarantina (5.66×10^4), and the result was expressed in Amarantina 100 mg g^{-1} fresh weight. As betacyanin is a pigment found only in stems and leaves, this quantification was performed only in the shoots of quinoa seedlings.

SOD, CAT and APX were extracted according to Biemelt et al. (1998): 0.2 g of fresh leaf were ground in liquid nitrogen and homogenized in 1.5 mL of extraction buffer containing 1.47 mL of potassium phosphate buffer 0.1 M (pH 7.0), 15 μL of EDTA 0.1 M (pH 7.0), 6 μL of DTT 0.5 M, 12 μL of PMSF 0.1 M, ascorbic acid 0.001 M and 22 mg polyvinylpyrrolidone (PVPP). The extract was centrifuged at 12,000 g for 30 minutes at 4°C , and the supernatant was collected and stored at -20°C during the analysis period.

The SOD activity was measured by the ability of the enzyme to inhibit the photoreduction of nitroblue tetrazolium (NBT) (Giannopolitis & Ries, 1977). Aliquots (10 μL) of enzymatic

extract were added to the incubation medium, which contained 100 μL of potassium phosphate 100 mM (pH 7.8), 40 μL of methionine 70 mM, 3 μL EDTA 10 μM , 31 μL of water, 15 μL of NBT 1 mM, and 2 μL of riboflavina 0.2 mM. Tubes containing the reaction medium and 10 μL of sample were illuminated for 7 minutes with a 20 W fluorescent lamp. The same reaction medium without a sample was illuminated as a control. Readings were taken at 560 nm, and the calculation of the enzyme was performed with the following equation: % inhibition = $(A_{560} \text{ sample with enzyme extract} - A_{560} \text{ control without enzyme extract}) / (A_{560} \text{ control without enzyme})$. One unit of SOD is able to inhibit 50% of the photoreduction of NBT under the assay conditions.

CAT was evaluated according Havir & McHale (1987) as follows: aliquots (10 μL) of enzyme extract were added to 170 μL of incubation medium containing 90 μL of potassium phosphate 200 mM (pH 7.0), 71 μL of water and 9 μL of hydrogen peroxide 250 mM, incubated at 28 °C. Enzyme activity was determined by the decrease in absorbance at 240 nm every 15 seconds for 3 minutes, monitored by the consumption of hydrogen peroxide. The molar extinction coefficient used was 36 $\text{mM}^{-1} \text{cm}^{-1}$.

The APX activity was determined by monitoring the rate of oxidation of ascorbate at 290 nm every 15 seconds for 3 minutes. Aliquots (10 μL) of enzyme extract were added to 170 μL of incubation buffer, consisting of 90 μL of potassium phosphate 200 mM (pH 7.0), 9 μL ascorbic acid 10 mM, 62 μL of water and 9 μL of hydrogen peroxide 2 mM (Nakano & Asada, 1981). The molar extinction coefficient was 2.8 $\text{mM}^{-1} \text{cm}^{-1}$.

The ascorbate concentration was determined as described by Arakawa et al. (1981): 50 mg of fresh leaf were macerated in liquid nitrogen, added to 20% PVPP (m/m) and homogenized in 1500 μL of trichloroacetic acid (TCA) 5% (m/v). The homogenate was then centrifuged at 13,000 g for 15 minutes at 4 °C. Aliquots (40 μL) of the supernatant were added to the reaction medium, which was composed of TCA 5% (m/v), ethanol 99.8% (v/v), phosphoric acid (H_3PO_4) 0.4% in ethanol (v/v), bathophenanthroline 0.5% in ethanol (m/v) and FeCl_3 0.03% in ethanol (m/v). The mix was homogenized thoroughly and incubated at 30 °C for 90 minutes. Readings were performed at 534 nm.

H_2O_2 was determined according Velikova et al. (2000): 200 mg of fresh leaf were macerated in liquid nitrogen, added to 20% PVPP (m/m) and homogenized in 1500 μL of trichloroacetic acid (TCA) 0.1% (m/v). The homogenate was centrifuged at 12,000 g for 15 minutes at 4 °C. The H_2O_2 was determined by measuring the absorbance at 390 nm in a reaction medium containing 500 μL of extract, 500 μL of 10 mM (pH 7.0) potassium phosphate buffer and 1000 μL of 1 M potassium iodide.

Lipid peroxidation was determined by the quantification of thiobarbituric acid reactive species, as described by Buege & Aust (1978). Two hundred milligrams of fresh leaf were macerated in liquid nitrogen, added to 20% PVPP (m/m) and homogenized in trichloroacetic acid (TCA) 0.1% (m/v). The homogenate was centrifuged at 10,000 g for 10 minutes at 4°C. Aliquots of the supernatant (250 μL) were added to the reaction

medium [thiobarbituric acid (TBA) 0.5% (m/v) and TCA 10% (m/v)] and were then incubated at 95°C for 30 minutes. The reaction was stopped by rapid cooling on ice, and readings were determined in a spectrophotometer at 535 nm and 600 nm. TBA form complexes of red color with low molecular weight aldehydes, such as malondialdehyde (MDA), a by-product of the peroxidation process. The concentration of the MDA/TBA complex was calculated with the following equation: $[\text{MDA}] = (A_{535} - A_{600}) / (\xi \cdot b)$, where: ξ (extinction coefficient = $1.56 \times 10^5 \text{ cm}^{-1}$); b (optical length = 1).

Results and Discussion

The dry weight of the shoots and roots (Figure 1A and Figure 1B) of early seedlings of *Chenopodium quinoa* were unaffected by different concentrations of sodium chloride (NaCl).

Quinoa early seedlings showed a higher betacyanin content in the treatments from 0 to 150 mM of NaCl as compared to the ones subjected to 200 mM of NaCl (Figure 2).

The activities of the enzymes superoxide dismutase (SOD) and catalase (CAT) were higher in the shoots (Figure 3A;

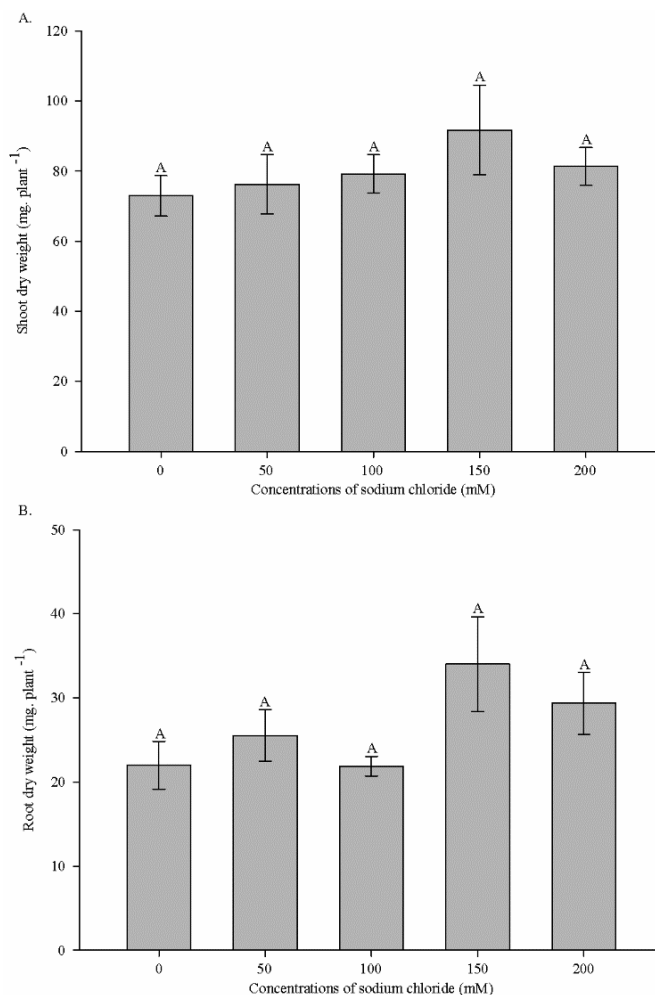


Figure 1. Shoots (A) and roots (B) dry weight of *Chenopodium quinoa* plantlets submitted to increasing concentrations of NaCl (0, 50, 100, 150 and 200 mM) for seven days. Different letters indicate significant differences with 0.05 probability. The bars represent the standard error of the mean

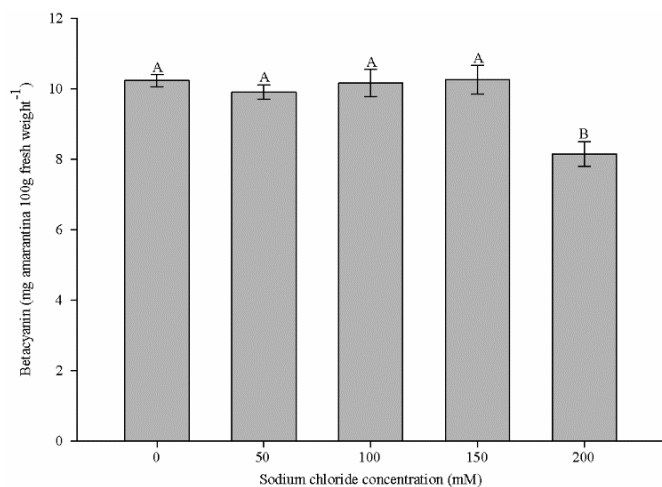


Figure 2. Betacyanin concentrations in shoots of *Chenopodium quinoa* plantlets submitted to increasing concentrations of NaCl (0, 50, 100, 150 and 200 mM) for seven days. Different letters indicate significant differences with 0.05 probability. The bars represent the standard error of the mean.

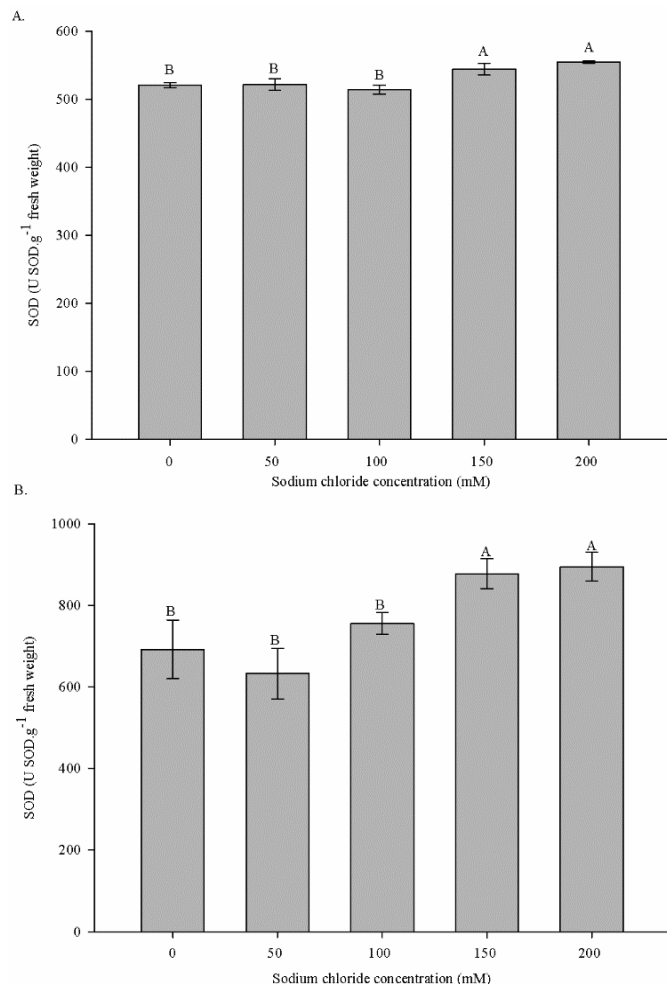


Figure 3. Superoxide dismutase (SOD) activity in shoots (A) and roots (B) of *Chenopodium quinoa* plantlets submitted to increasing concentrations of NaCl (0, 50, 100, 150 and 200 mM) for seven days. Different letters indicate significant differences with 0.05 probability. The bars represent the standard error of the mean

Figure 4A, respectively) and roots (Figure 3B; Figure 4B, respectively) of early seedlings subjected to 150 and 200 mM of NaCl.

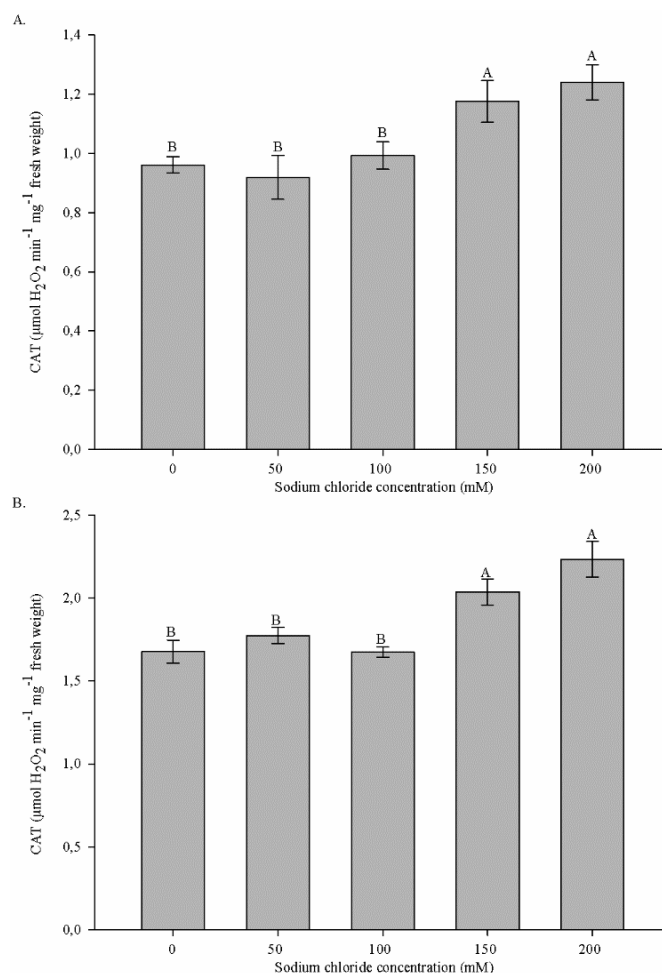


Figure 4. Catalase (CAT) activity in shoots (A) and roots (B) of *Chenopodium quinoa* plantlets submitted to increasing concentrations of NaCl (0, 50, 100, 150 and 200 mM) for seven days. Different letters indicate significant differences with 0.05 probability. The bars represent the standard error of the mean

Higher APX activity was observed in shoots (Figure 5A) of early seedlings subjected to 0 and 50 mM of NaCl, while in roots (Figure 5B), no differences were observed between treatments.

The ascorbate content in shoots (Figure 6A) was higher in early seedlings subjected to 100 to 200 mM of NaCl. In roots (Figure 6B), the highest ascorbate contents were found in control and 50 mM of NaCl.

Higher lipid peroxidation was observed in shoots (Fig. 7A) of *Chenopodium quinoa* early seedlings treated with 200 mM of NaCl. Nevertheless, in the roots (Fig. 7B), no differences were observed between treatments.

Increased production of hydrogen peroxide (H₂O₂) was found in the shoots of early seedlings subjected to 150 and 200 mM of NaCl (Figure 7C). However, in roots (Figure 7D), no differences were observed between treatments.

Regarding the enzymes involved in the removal of ROS, SOD is the first line of defense against oxidative stress (Gill & Tuteja, 2010). The increase in NaCl concentration induced greater SOD activity (Figure 3A), triggering the overproduction of H₂O₂ (Figure 7C). Due to H₂O₂ toxicity, the action of ascorbate peroxidase (APX) and catalase (CAT) is indispensable, aiming for ROS detoxification (Maia

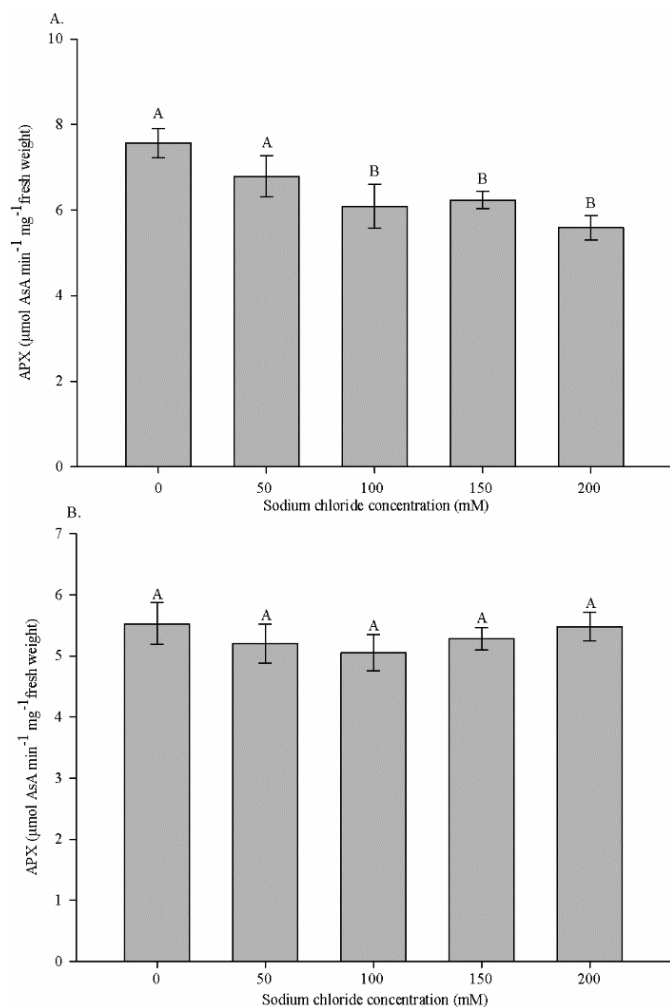


Figure 5. Ascorbate peroxidase (APX) activity in shoots (A) and roots (B) of *Chenopodium quinoa* plantlets submitted to increasing concentrations of NaCl (0, 50, 100, 150 and 200 mM) for seven days. Different letters indicate significant differences with 0.05 probability. The bars represent the standard error of the mean

et al., 2012). The affinity of these enzymes for H₂O₂ is a very important factor to determine the antioxidant action. Considering that CAT has a low affinity to H₂O₂, this enzyme becomes active under higher concentrations of its substrate, whereas APX has a high affinity to H₂O₂ and is capable of eliminating H₂O₂ even at a low concentration (Sharma et al., 2012).

In this work, the higher CAT activity (Figure 4) coincided with higher SOD activity, confirming the assumption that these enzymes act in series. Early seedlings exposed to lower NaCl concentrations showed lower H₂O₂ production (Figure 7C) and higher APX activity (Figure 5A). This result confirms the role of APX acting mainly in lower H₂O₂ concentration (Sharma et al., 2012). The shoots of early seedlings exposed to 150 mM and 200 mM of NaCl required higher CAT activity (Figure 4A) to remove the hydrogen peroxide (Figure 7C) produced, possibly due the higher SOD activity (Figure 3A). This result supports a role of CAT in the antioxidant system, which acts mainly at higher H₂O₂ concentrations (Sharma et al., 2012).

This coordinated activity of antioxidant metabolism was able to keep the lipid peroxidation at similar levels to those of control plants until 150 mM of NaCl. On the other hand, after

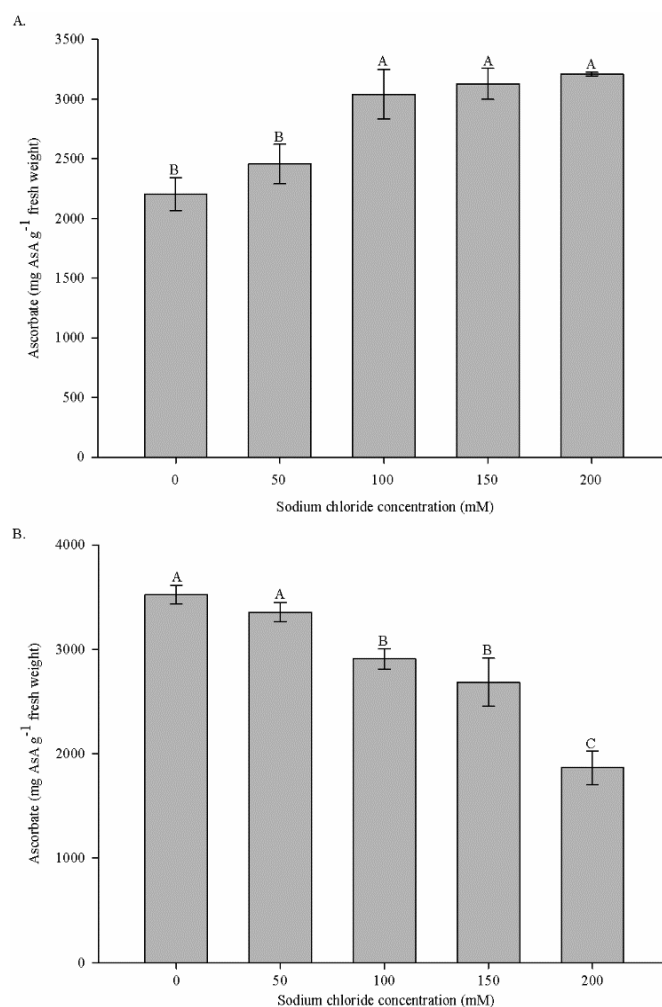


Figure 6. Ascorbate concentration in shoots (A) and roots (B) of *Chenopodium quinoa* plantlets submitted to increasing concentrations of NaCl (0, 50, 100, 150 and 200 mM) for seven days. Different letters indicate significant differences with 0.05 probability. The bars represent the standard error of the mean

200 mM of NaCl, there was higher generation of H₂O₂, and likely the high activity of CAT was not sufficient to avoid lipid peroxidation (Figure 7A).

Increases in CAT activity, similar to those found in this work, have been reported in a wide variety of species, including *Cassia angustifolia* (Agarwal & Pandey, 2004) and *Saccharum officinarum* (Willadino et al., 2011), under salt stress. This increased activity of enzymatic antioxidant metabolism was able to maintain low levels of lipid peroxidation front to salt stress. Thus, the present study confirms for *Chenopodium quinoa* the relationship between tolerance to oxidative stress induced by NaCl and the antioxidant defense system proposed by Agarwal & Pandey (2004).

Higher ascorbate content was found in the shoots (Figure 6A) of early seedlings subjected from 100 to 200 mM of NaCl, which is likely also due to increased H₂O₂ production. Increases in ROS production were followed by increases in SOD (Figure 3A) and CAT activity (Figure 4A). Ascorbate is an antioxidant molecule that acts in the removal of superoxide radicals and hydrogen peroxide in various organelles, and it is produced in higher concentrations under stressful conditions (Gill & Tuteja, 2010).

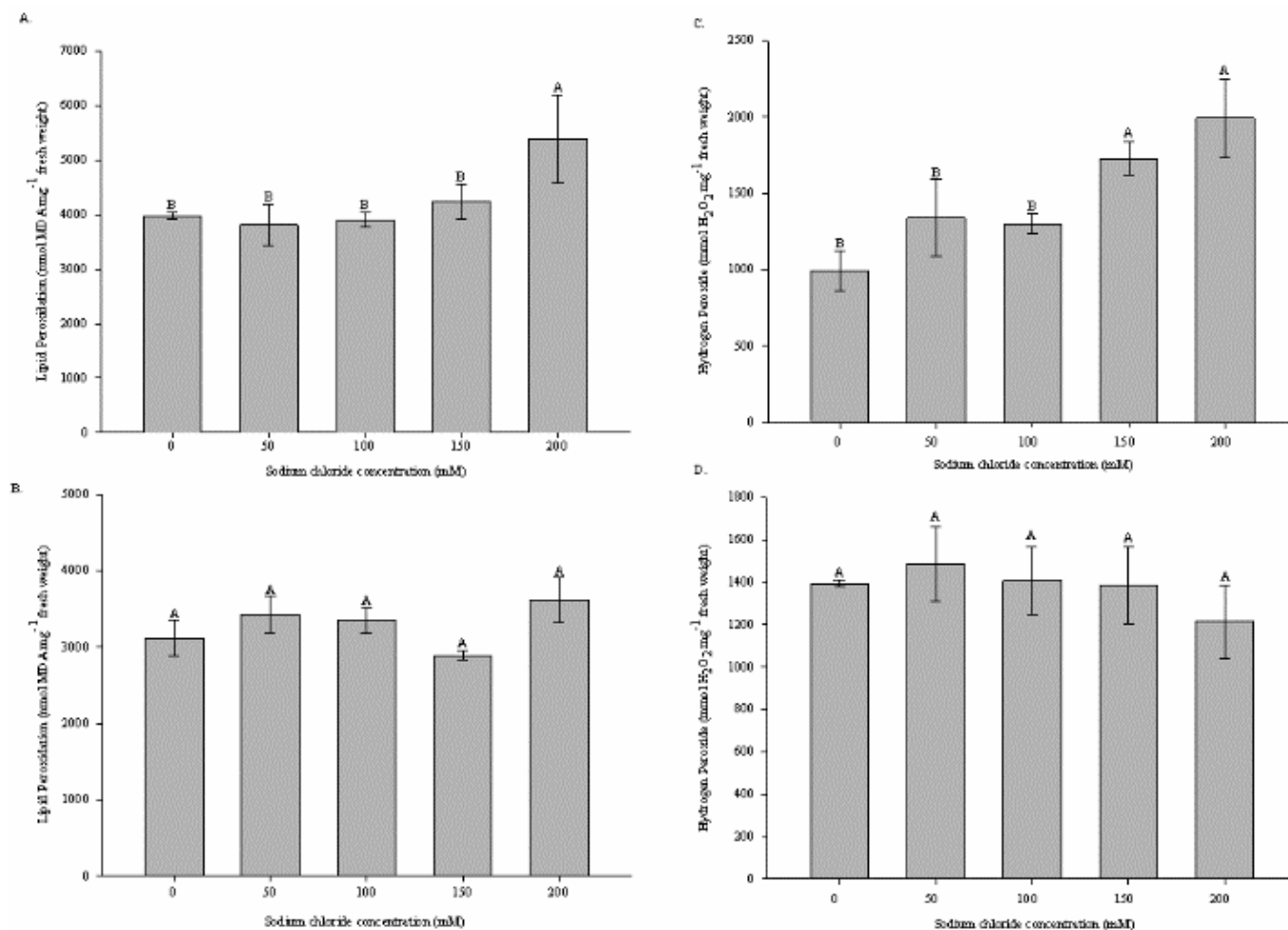


Figure 7. Lipid peroxidation and hydrogen peroxide (H₂O₂) concentration in shoots (A and C, respectively) and roots (B and D, respectively) of *Chenopodium quinoa* plantlets submitted to increasing concentrations of NaCl (0, 50, 100, 150 and 200 mM) for seven days. Different letters indicate significant differences with 0.05 probability. The bars represent the standard error of the mean

Adaptations to salinity can vary among species (Morales et al., 2001; Prisco & Gomes-Filho, 2010), genotypes, stages of development and the individual level of stress imposition (Prisco & Gomes-Filho, 2010). Several studies have shown the negative effects of salinity on the growth of plants such as *Vigna unguiculata* L. (Deuner et al., 2011) and *Helianthus annuus* L. (Carneiro et al., 2011). Nevertheless, this pattern was not found in this study for *Chenopodium quinoa* early seedlings. Therefore, *Chenopodium quinoa* is considered a potentially tolerant species to salt stress (Jacobsen et al., 2012), as even when showing greater lipid peroxidation (Figure 7A), these plantlets were able to maintain the dry weight of their shoots (Figure 1A). Similar results were found in *Glycine max* cv. Pusa 37, a cultivar known to be tolerant to salt stress, maintaining growth despite the elevated lipid peroxidation under high salt concentrations (Hakeem et al., 2012).

Early seedlings subjected to 200 mM of NaCl showed increased lipid peroxidation and decreased betacyanin content. This result suggests a reduction in plant secondary metabolites and a greater investment in plant development and metabolism, maintaining the growth of the seedlings. Nevertheless, at concentrations lower than 200 mM of NaCl, betacyanins can function as osmolytes in the defense of physiological processes against abiotic stress by modulating the pool of amino acids.

Thus, under stressful conditions, plants may need alternative ways of energy dissipation to avoid photoinhibition and photooxidation (Ribeiro et al., 2014).

Moreover, sodium ions are mobile elements transported through xylem from root to shoot by the transpiration stream. For this reason, shoots are more vulnerable than roots under salinity, as the accumulation of toxic ions occurs in shoots (Tester & Davenport, 2003). Thus, in this work, a larger physiological impact was observed in the shoots than in the roots.

Conclusions

Antioxidant enzymes of *Chenopodium quinoa* early seedlings were able to dissipate excess ROS, maintaining low levels of lipid peroxidation until 150 mM of NaCl. Nevertheless, under 200 mM of NaCl, antioxidant enzymes were inefficient at removing excess ROS, triggering greater lipid peroxidation.

Thus, the salinity tolerance shown by *Chenopodium quinoa* early seedlings was due to the activation of the antioxidant system, which allowed for the maintenance of its growth at higher concentrations of NaCl, even showing greater lipid peroxidation.

Acknowledgments

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