

Anatomical and Physiological Responses of Four Quinoa Cultivars to Salinity at Seedling Stage

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Abstract

Objectives: Salinity is one of the most important environmental stress factors that limits both seed germination and seedling develop. Seedling stage is the most susceptible stage of plants to salt stress. The aim of this work is to study the effect of salt stress on anatomical and physiological features of cotyledons and roots of the seedlings of four quinoa cultivars (Witulla, Wariponcho, Pasankalla and CICA). **Methods/ Statistical Analysis:** Seed germination and seedling growth was carried out under 0 and 200 mM NaCl during 10 days. Root length and diameter, and root proton extrusion were measured. Soluble sugars and proline concentrations were determined in both cotyledons and roots. Anatomical attributes of cotyledons (adaxial and abaxial stomatal area and density, palisade and spongy cell layers, palisade cell density) and roots (stele diameter and surface, metaxylem vessel diameter) were also analyzed. **Findings:** Salt stress caused physiological and anatomical changes in cotyledons and roots. Sucrose, glucose and fructose concentrations were more affected in cotyledons than in roots of salt-treated seedlings. Proline accumulation was more affected in roots. Saline stress affected differentially the anatomical attributes of quinoa cultivars. **Application/Improvements:** Quinoa seedlings when subjected to salt stress are able to develop a set of anatomical, physiological and biochemical adaptive mechanisms which seem to occur at different combinations among quinoa cultivars, but giving similar abilities to cope with the excess of salt and survive under extreme conditions.

Keywords: Cotyledon, Proline, Quinoa, Root, Salinity, Sugars

1. Introduction

Salinity constitutes a stress factor that limits the plant growth and development. Saline and salinized soils cover 40-50% of the world's terrestrial surface and a quarter of the cultivated surface worldwide¹, of which 129 million ha

are in South America mainly in the two biggest grain-producing countries: Brazil and Argentina. According to FAO-UNESCO, Argentina is the third country in the world affected by halomorphic processes, covering arid, semiarid and wet sectors used for agricultural practices, with or without irrigation². In the last thirty years, sali-

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nized soils in the world increased at a rate of 3 ha min⁻¹, and considering the current model of climate change, lands under salinization are expected to increase strongly during this century³.

According to the ability to survive in saline environments, plants are grouped into salt-tolerant species (halophytes) and salt-non-tolerant species (glycophytes)⁴. Halophytes only constitute around 1% of the total flora of the Earth, but include some potentially useful alternative crop species such as members of the *Chenopodiaceae* family. *Chenopodium quinoa* Willd, known as “quinoa” or “quinua”, was the main food of American pre-hispanic cultures. Quinoa seed, commonly named “grain”, has a high nutritional quality, since it is rich in essential amino acids, minerals and vitamins⁵. The nutritional value of quinoa and its resistance to face abiotic stresses such as drought, salinity, frost, low temperature and UV-B radiation, as well as the ability to grow on marginal soils, have turned it into studying subject of different research groups in several countries⁶. Despite its high tolerance to salinity *C. quinoa* is not considered as true or obligatory halophyte due to great variability observed in the performance and fitness growth of plants under different salinity levels. In fact, some cultivars can be considered as facultative halophytes (excluding salt excess), meanwhile other ones can be classified as salt tolerant (accumulating salt within the plant)⁷. Variability in salt tolerance observed in quinoa’s cultivars and/or accessions has been related to geographical origin of parenteral lines. Higher salinity tolerance was found in the Bolivian highland (Altiplano) ecotypes compared with Peruvian valley and Chilean coastal ecotypes⁸.

Although halophytes show a wide gradient of salt tolerance, in greater or lesser extent, both glycophytes and halophytes become affected by the excessive concentration of salt in the soil. Beyond toxic effects induced by accumulated Na⁺ and Cl⁻ ions, soil salinity also imposes an osmotic stress on plants by reducing the soil water potential that leads to the restriction of water uptake. Deleterious effects of salinity include, among others, the impairment of photosynthesis, oxidative damage, reduced growth, inhibition of cell division and expansion, dysfunction of cell membranes, and mineral imbalance⁹. Salt tolerance is a multifarious phenomenon involving a variety of changes at molecular, organelle,

cellular, tissular, and whole plant levels. Many studies have shown that plants evoke a variety of protective mechanisms involving metabolic, physiological and morpho-anatomical adaptations, which enable them to cope with the excess of salt. Metabolic adaptations include, among others, the synthesis of several soluble compounds or compatible osmolytes (e.g. sugars, sugar alcohols, polyols, amino acids, sulfonium and quaternary ammonium compounds, soluble polysaccharides, etc.), polyamines, lignin and suberin precursors, waxes, proteins, and antioxidant enzymes¹⁰. Physiological adaptations include, among others, restricted toxic ion uptake, increased succulence, exclusion of toxic Na⁺ and Cl⁻, intracellular sequestration of Na⁺, and activation of root cell membrane H⁺ATPase pumping¹¹. Major morpho-anatomical adaptations include the development of thick epidermis and sclerenchyma, increased density of trichomes and stomata, increased cell size and vacuolar volume, development of excretory structures like vesicular hairs and salt glands, development of bulliform cells, increased root stele diameter, and reinforced exodermis¹². Likewise, the effect of saline stress is also dependent on developmental stage of the plant, being more sensitive in early growth steps. Therefore, it is essential evaluate the effect of salt excess on the seedling phase, considering that is a critical stage in the life cycle of plants in which they grow most rapidly, but also are more vulnerable to abiotic and biotic stresses¹³. In that context, physiological bases of salt tolerance during the early seedling stage are not well understood, and then more studies are need to unravelling the basic mechanisms involved in salt tolerance. Although there is wide knowledge about the physiology and grain yield of several quinoa cultivars under salt stress, there is no information about its anatomy at the seedling stage. The aim of the present work was to assess physiological and anatomical features of different *C. quinoa* cultivars at the seedling stage subjected to saline stress.

2. Materials and Methods

2.1 Plant Material Preparation

Quinoa seeds of four cultivars: Witulla, Wariponcho, Pasankalla and CICA, originating from Perú (Puno region. 15°04’S, 70°07’W, 3800 m asl) were surface sterilized

with 3% NaClO solution during 10 min, washed with distilled water and germinated on trays (15 cm x 20 cm x 7 cm) filled with wet vermiculite (100 seeds per tray, 4 trays per treatment). Vermiculite was wetted either with ¼ strength Hoagland nutrient solution (0.97 mS cm⁻¹ electrical conductivity) or 200 mM NaCl solution prepared using ¼ strength Hoagland solution (17.2 mS cm⁻¹ electrical conductivity). Trays of both control (C), without NaCl, and Treated (T) with 200 mM NaCl were transferred to controlled chamber under 190 µmol m⁻² s⁻¹ PPFD (photosynthetic photon flux density), 25°C day/night temperature, 16 h photoperiod and 70% Relative Humidity (RH) during 10 days. Seedlings with expanded cotyledons were harvested, divided into cotyledons and roots, weighted to obtain the Fresh Weight (FW), and stored at -20°C for chemical analyses. For anatomical studies the samples of cotyledons and roots were immediately fixed in FAA (formaldehyde/acetic acid/ethanol 5:5:90).

2.2 Cotyledon and Root Anatomy

For anatomical studies six cotyledons of different seedlings for each treatment and for each cultivar were used. For abaxial and adaxial epidermis analysis cotyledon samples were clarified with 10% NaClO solution for 12 h at room temperature, washed with distilled water and stained with 1% safranin in ethyl alcohol 50%. To study cells and tissues, freehand cross sections were cut from the middle zone of cotyledons fixed in FAA, stained with safranin, cresyl violet acetate and safranin-fast green, and mounted in water:glycerol (1:1)¹⁴. Microscopic observations were performed using a stereo-microscope (Olympus SZX7, Japan) and micrographs were taken with a digital camera (Canon A620, Power Shot 7.1 MP, Japan). To enhance the visibility of calcium oxalate crystals, transversal sections of cotyledons were also observed by using crossed polarizing filters. Anatomical attributes recorded in both adaxial and abaxial blade epidermis were stomata density and stomata area. In cross sections it was measured the number of cell layers in both palisade and spongy parenchyma, and palisade parenchyma cells per unit area. For anatomical root study, freehand transversal sections were cut 1 cm behind the collar zone from six roots for each treatment and for each cultivar, stained with a triple stain consisting of safranin, astra blue-safranin and

cresyl violet acetate, and mounted in water:glycerol (1:1). Anatomical attributes measured were: total root diameter, stele diameter, percent of stele surface, and metaxylem vessels diameter. Anatomical attributes of cotyledons and roots were measured using an ocular micrometer, which was calibrated with a stage micrometer.

At the end of the experiment the root length of 30 seedlings for each treatment and for each cultivar was measured using a plastic ruler (accuracy ± 0.5 mm).

2.3 Soluble Sugars and Proline Extraction and Quantification

Soluble sugars (sucrose, glucose and fructose) and proline from cotyledons and roots were extracted¹⁵. Fructose was determined by using the resorcinol-thiourea reagent¹⁶. Sucrose was determined by using the resorcinol-thiourea reagent according to Cardini et al. modification¹⁷. Glucose was determined enzymatically by the coupled reaction of glucose oxidase-peroxidase¹⁸. Proline was determined by using the ninhydrin reagent¹⁹. Sugar and proline concentrations were expressed as µmol g⁻¹ FW.

2.4 Plasma Membrane Proton Extrusion

To measure the extrusion of protons from intact seedlings, 15 healthy seedlings for each cultivar and for each treatment were washed with distilled water and transferred to 50 mL of 1 mM Tris-HCl buffer, pH 7.0, containing 0.5 mM CaCl and 50 mM KCl²⁰. The decrease of pH was recorded over 30 min using a glass combination pH-sensitive electrode coupled to a pH meter (Hanna Instrument, USA). High potassium concentration was used to increase the acidification of external medium, and then to obtain more significant differences in pH values (ΔpH). To assess the involvement of plasma membrane H⁺ ATPase activity in proton extrusion, 15 seedlings were transferred to 50 mL of 1.5 mM Na₃VO₄ (specific inhibitor of plasma membrane H⁺ ATPase) for 20 min. After this time, seedling were rinsed with distilled water and the proton extrusion was recorded for 30 min.

2.5 Statistical Analysis

Data were subjected to analysis of variance (ANOVA), and the means were statistically compared by Tukey's test at 5% probability. Values are given as means ± SE. Experiments were repeated at least three times.

3. Results

3.1 Cotyledon Anatomy

Control and salt-treated adaxial cells were isodiametric with straight or curved cell walls (Figure 1 A,B), while abaxial cells were rectangular with cell walls of similar looking, but in some salt-treated cells they were slightly sinuous (Figure 1C,D). Cross sections of salt-treated cotyledons did not show any change in relation to untreated cotyledons in all cultivars (Figure 2). Vascular bundles are collateral with parenchyma sheath (Figure 2A,D). They have a dorsiventral structure, with monostratified and papillose epidermis, smooth cuticle and sunken stomata in both surfaces (Figure 2B,E). Calcium oxalate crystals were observed in control and treated cotyledons mainly in spongy parenchyma, but no differences were observed (Figure 2C,F). Contrarily, anatomical attributes were differentially affected by saline treatment (Table 1). Adaxial and abaxial epidermis of salt-treated cotyledons of Pasankalla and CICA cultivars showed significant increases of stomatal area (50% and 44%, adaxial surface) and (45% and 27%, abaxial surface), when comparing with untreated (control) cotyledons. Stomatal area of Wariponcho cultivars was decreased by saline treatment. For adaxial and abaxial surfaces, decreased values were 14% and 8%, respectively. The Witulla cultivar showed a different trend of stomatal area, being decreased (14%) in adaxial surface and increased (13%) in abaxial one. Under saline treatment the adaxial stomata number increased significantly (55%) in Wariponcho cotyledons whereas in CICA ones decreased significantly (60%) compared with control cultivars. Contrarily, in abaxial surface the stomata number was significantly decreased in all cultivars. Highest decreases were observed in CICA (46%) and Wariponcho (33%). Numbers of palisade parenchyma cell layers in salt-treated Wariponcho and Pasankalla cotyledons were higher than that in control cotyledons. The Witulla cultivar showed the lower number of spongy parenchyma cell layers compared with salt-untreated cultivar. In CICA cultivar there were no changes in cell layers of both palisade and spongy parenchyma. A significant decrease (40.3%) of the palisade cell number per unit area was observed in salt-treated Pasankalla cultivar (Table 1).

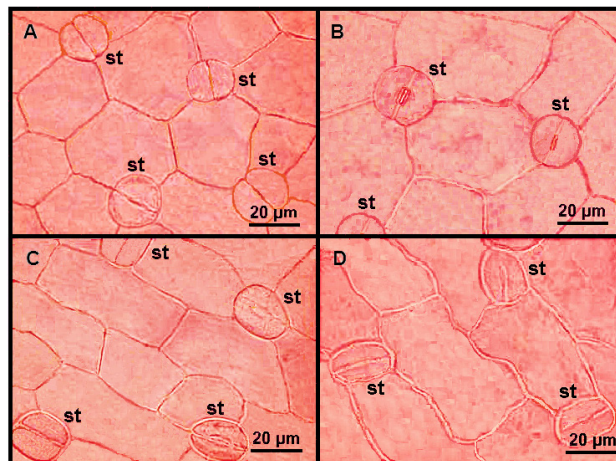


Figure 1. Adaxial and abaxial epidermis of the control cotyledon (A, B) and salt-treated cotyledon (C, D). st: stomata.

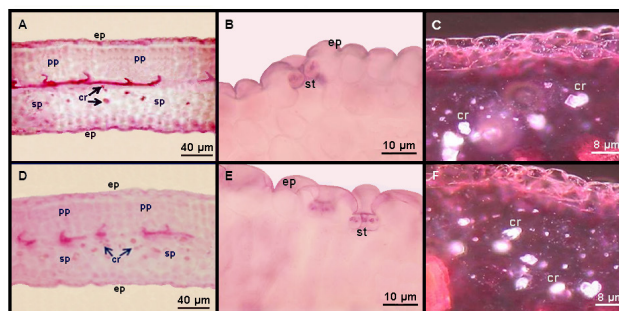


Figure 2. Transversal sections of control (A, B, C) and salt-treated (D, E, F) quinoa cotyledons. Calcium oxalate crystals were observed using polarized light. st: stomata, ep: epidermis, pp: palisade parenchyma, sp: spongy parenchyma, cr: calcium oxalate crystal.

3.2 Root Anatomy

In general, all cultivars showed a well-developed epidermis with a cortex formed by 2-4 layers of parenchyma cells in Witulla and Wariponcho cultivars (Figure 3A-D), and by 4-7 layers in Pasankalla and CICA ones (Figure 3E-H). An endodermis with Casparian strip in radial walls and a diarch stele with monostratified pericycle was observed in all control

Table 1. Anatomical attributes of control and salt-treated quinoa cotyledons. Each value (\pm SE) is the mean of six cotyledons from six individual seedlings. Values labelled with asterisk for each cultivar are significantly different between the control and salt-treated condition at $p < 0.05$

Anatomical attributes	Witulla		Wariponcho		Pasankalla		CICA	
	C	T	C	T	C	T	C	T
Adaxial stomatal area (μm^2)	279.2 \pm 15.7	240.5 \pm 8.6*	401.6 \pm 27.5	378.7 \pm 22.8	239.9 \pm 18.0	358.6 \pm 16.3*	211.9 \pm 10.5	306.1 \pm 7.7*
Abaxial stomatal area (μm^2)	279.2 \pm 6.3	316.2 \pm 9.9	395.4 \pm 16.6	344.3 \pm 8.3*	230.8 \pm 10.8	334.7 \pm 13.5*	172.8 \pm 9.0	219.4 \pm 11.8*
Adaxial stomatal density (stomata mm^{-2})	7.4 \pm 1.0	9.1 \pm 1.7	9.1 \pm 0.6	4.2 \pm 0.7*	5.2 \pm 0.7	6.1 \pm 0.5	5.1 \pm 0.8	8.3 \pm 1.1*
Abaxial stomatal density (stomata mm^{-2})	11.2 \pm 0.9	9.0 \pm 1.0*	9.2 \pm 1.4	6.4 \pm 0.7*	9.2 \pm 0.7	7.0 \pm 0.5*	13 \pm 1.2	7 \pm 1*
Palisade cell layers	2 - 3	2 - 3	2 - 3	3 - 4	2 - 3	3 - 4	2 - 3	2 - 3
Spongy cell layers	4 - 5	3 - 4	5 - 6	5 - 6	5 - 6	5 - 6	5 - 6	5 - 6
Palisade cells per unit area (cell mm^{-2})	349.4 \pm 20.3	392.4 \pm 30.8	339.1 \pm 18.3	335.7 \pm 23.8	433.4 \pm 20.2	308.8 \pm 17.5*	63.9 \pm 36.1	439.7 \pm 30.2

and salt-treated cultivars; however, some anatomical features were differentially affected by saline treatment. Roots of Pasankalla and CICA cultivars of both control and salt-treated seedlings show subepidermal cells with thickened walls that may correspond to the exodermis (Figure 3E-H). However, in Wariponcho cultivar the exodermis only occurs in salt-treated roots (Figure 3D). An increased turgor of the cortex cells in salt-treated roots of Witulla and Wariponcho cultivars was also observed (Figure 3B,D). Root anatomical attributes are shown in Table 2. Root diameter increased in salt-treated seedlings of Witulla (16%), Wariponcho (41%) and Pasankalla (17%) cultivars, whereas there was no significant change in CICA cultivar. Stele diameter and surface percentage in salt-treated CICA cultivar significantly decreased when comparing with control cultivar, while in Witulla, and Wariponcho cultivars significant decreases were only observed in surface percentage. Metaxylem vessel diameters were differentially affected by saline treatment, being significantly increased in Witulla (27%) and Wariponcho (28%) roots and significantly decreased in Pasankalla (35%) and CICA (45%) roots, respectively.

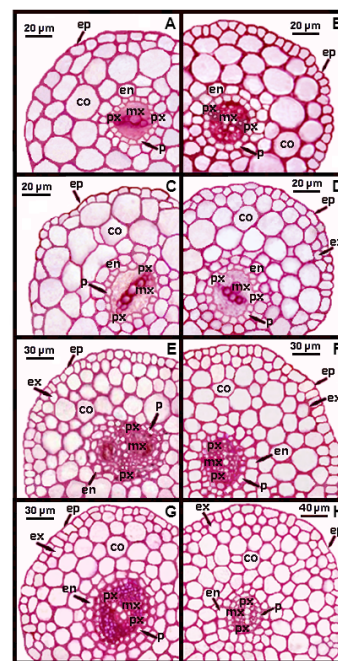


Figure 3. Root cross sections of both control (A, C, E, G) and salt-treated (B, D, F, H) quinoa seedlings. A, B: Witulla, C, D: Wariponcho, E, F: Pasankalla, G, H: CICA. ep: epidermis, ex: exodermis, co: cortex, en: endodermis, mx: metaxylem, px: protoxylem, p: pericycle.

3.3 Root Length

Root length decreased in salt-treated Wariponcho (26%), Pasankalla (35%) and CICA (22%) seedlings, while in salt-treated Witulla cultivar increased (33%) Figure 4.

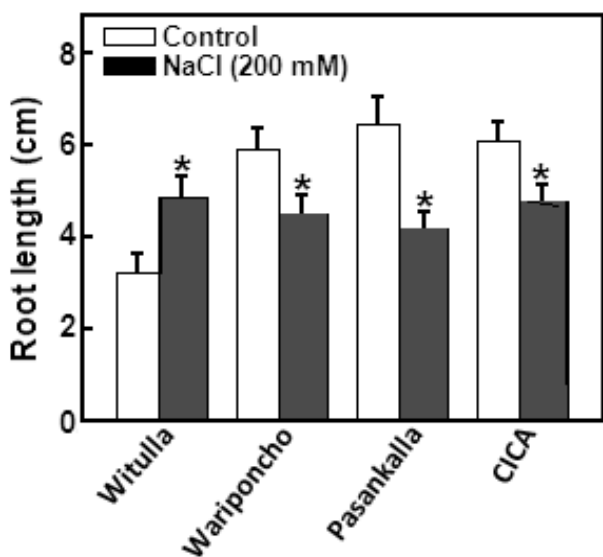


Figure 4. Root length of both control and salt-treated quinoa seedlings. Asterisk on bar for each cultivar indicates significant difference between the control and saline condition at $p < 0.05$ ($n = 30$).

3.4 Soluble Sugars

Sucrose, glucose and fructose in both cotyledons and roots were differentially affected by saline treatment Figure 5. Sucrose content in cotyledons of salt-treated plants was significantly higher in Pasankalla cultivar (135%), whereas in CICA one was significantly lower (54%) compared with control cultivars. In Witulla and Wariponcho cultivars there were no changes in sucrose content. Root sucrose content strongly increased in salt-treated Witulla and Wariponcho roots (417% and 208%), while a less increase occurred in Pasankalla roots (26%) (Figure 5A,B). Glucose contents in both cotyledons and roots of salt-treated cultivars were significantly lower than in controls, excepting in CICA cultivar that showed 54% increase regarding to control (Figure 5C,D). Similarly, cotyledon and root fructose contents were significantly lower in salt-treated cultivars when comparing with salt-untreated controls (Figure 5E,F).

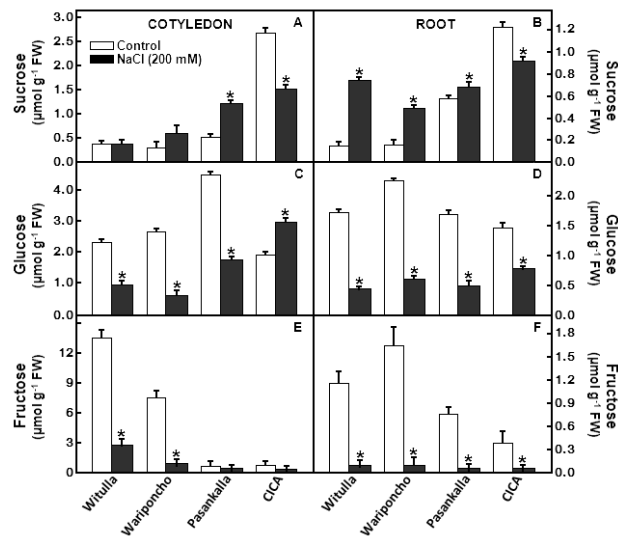


Figure 5. Effect of salinity on sucrose, glucose and fructose contents of different quinoa cultivars grown in presence of 200 mM NaCl. Asterisk on bar for each cultivar indicates significant difference between the control and saline condition at $p < 0.05$ ($n = 6$).

3.5 Proline

Cotyledon and root proline contents were differently affected by saline treatment in all studied cultivars. Proline in both salt-treated and control cotyledons was 10-f higher than that in roots Figure 6. Cotyledon proline content increased strongly in salt-treated CICA cultivar (214%), and also significantly in Pasankalla seedlings (42%), compared with control seedlings (Figure 6A). By contrast, in salt-treated roots proline decreased in Witulla (19%), Wariponcho (31%) and CICA (32%) seedlings, but increased in Pasankalla (89%) ones, regarding to control seedlings (Figure 6B).

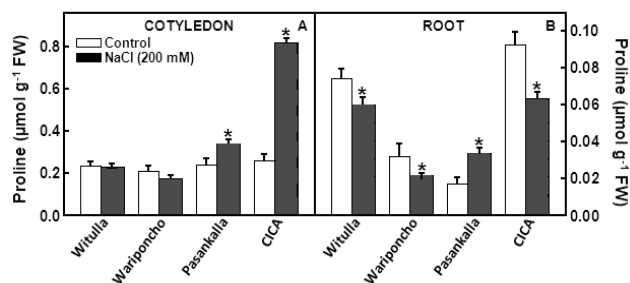


Figure 6. Proline content in cotyledons and roots of both control and salt-treated quinoa seedlings. Asterisk on bar for each cultivar indicates significant difference between the control and saline condition at $p < 0.05$ ($n = 6$).

3.6 Proton Extrusion

A significant increase of H^+ extrusion was observed in salt-treated Pasankalla seedlings, reaching a maximum increase of 346% when comparing with control seedlings. Contrarily, in CICA cultivar a significant decrease (65%) in H^+ extrusion was found. No significant changes were observed in Witulla and Wariponcho H^+ extrusion Figure 7. In presence of Na_3VO_4 a strong inhibition of H^+ extrusion occurred, indicating the involvement of the plasma membrane H^+ -ATPase activity in the efflux of protons (data not shown).

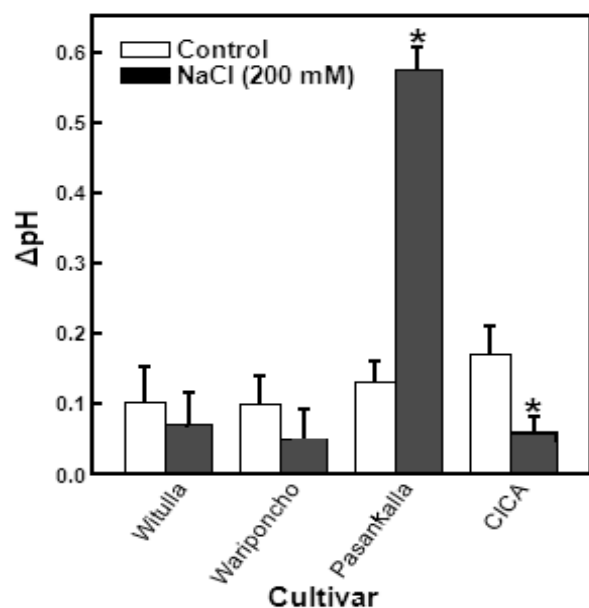


Figure 7. Effect of NaCl on proton extrusion from roots of four quinoa cultivars. Asterisk on bar for each cultivar indicates significant difference between the control and saline condition at $p < 0.05$ ($n = 10$).

4. Discussion

The present work characterizes seedling anatomical structures of four *C. quinoa* cultivars exposed to 200 mM NaCl. Even though salt-treated seedlings did not evidence visible morphological changes associated to salt damage, several modifications at both tissular and cellular organization levels were observed in cotyledons and roots, when comparing with salt-untreated seedlings. Comparison of salt tolerance among cultivars/varieties of crop species at seedling stage is very important in agricultural production. During the seedling stage, salinity shows the most

damaging effects on plant development. Salt tolerance generally increases as the plant matures¹³. It is important to highlight that strategies to face salt-induced stress are diverse and the occurrence of different combinations of mechanisms in different species and/or cultivars can confer similar fitness in front of extreme saline conditions⁹.

Some studies indicate that the reduction of stoma size under saline conditions must be recognized as a strategy to diminish the leaf water loss^{12,21}. In agreement with this assumption, stomata area in adaxial (Witulla) and abaxial (Wariponcho) surfaces was reduced by saline treatment. Salt-treated Wariponcho cotyledons also showed lower stomata density (Table 1). Low stomata density has been recognized as an adaptive trait to cope with saline stress, avoiding excessive transpiration and keeping water availability to maintain the internal medium homeostasis²². Thus, lower stomata density occurring in Wariponcho cultivar can represent an additional trait to survival in salinized soils. Increases of stomatal area and density were also reported in salt-exposed plants²³, just as it was observed in both adaxial and abaxial surfaces of Pasankalla and CICA cultivars Table 1. In this way, the increase of stomatal area has been linked to both enhanced stomatal conductance and higher water use efficiency²⁴, which may indicate the success of a determined species in a particular area.

Increased leaf thickness has been reported as a successful trait for plant species growing under saline conditions. Leaf thickening is considered as a mechanism to increase the water retention by mesophyll tissues in order to counteract salt toxicity²⁵. On the other hand, thick palisade helps in more mesophyll conductance and hence enhances the CO_2 diffusion that may increase the photosynthesis rate²⁶. Furthermore, the process of photosynthesis takes place mainly within palisade cells, and then an increased thickness of the palisade parenchyma allows higher photosynthetic activity and also greater production of carbohydrates²⁷. In agreement with these findings salt-treated Wariponcho and Pasankalla cotyledons exhibit an increased number of palisade parenchyma cell layers and significantly higher content of sucrose compared with salt-untreated cotyledons (Table 1, Figure 5A). Palisade cells of Pasankalla cotyledons also showed increased cell size. We assume that this feature could be related to greater sucrose synthesis occurring in these cotyledons. Our assumption agrees with previous results obtained in *Cucumis melo* that suggest that an increase in the number of large cells promotes the sucrose accumulation²⁸.

By contrast in salt-treated cotyledons of Witulla and CICA cultivars there was no increase of both palisade cell layers and sucrose concentration. Calcium oxalate (CaOx) are widely distributed in plant kingdom and found in over 215 families including the *Chenopodiaceae* family²⁹. Many functions have been attributed to CaOx in plants such as participating in calcium homeostasis and storage, removal of excess oxalate, heavy metal detoxification, tissue support, light gathering and reflection, salt stress and cell homeostasis, and protection against insects and foraging animals, but evidences supporting most of these hypotheses are still insufficient²⁹. Quinoa contains CaOx in glands localized on the surface of both mature and young leaves during the vegetative phase as a drought-tolerant mechanism; however, they are more abundant in the former³⁰. In the present study CaOx crystals were observed in the spongy mesophyll of all analysed cultivars, but there were no significant differences between control and salt-treated cotyledons (Figure 2C,F). Then, it can be assumed that internal CaOx crystals do not play a significant role into NaCl tolerance mechanisms of *C. quinoa* at seedling stage. Further studies are needed to ascertain the role of CaOx crystals in salt-stressed quinoa seedlings.

Growth, architecture and anatomy of roots are often affected by saline stress³¹. Exomorphological attributes of roots such as length, branching and diameter frequently are negatively affected by both ionic and osmotic components of NaCl³². Salt stress can cause morphological and physiological changes in leaves and roots of sensitive species, including root length decrease³². Nevertheless, despite deleterious effects of salt on the root length, several halophytes show increases of this parameter under a moderate saline stress³¹. Data reported here show decreases in root length of salt-treated Wariponcho, Pasankalla and CICA seedlings, but not in Witulla cultivar which shows a significant increase of the root length (Figure 4). Increases and decreases of root length were also communicated for other quinoa cultivars growing under 100-200 mM NaCl^{33,34}. Plants often allocate a greater proportion of their biomass to organs that are involved in acquiring resources that are scarcest³⁵. In this way, Witulla cultivar seems to be more capable to sustain the seedling growth under challenging conditions. In support of this assumption, sucrose level in Witulla roots was significantly higher in salt-treated roots than in salt-untreated ones.

Saline stress also affects the cell division and cell expansion as well as cambial activity of roots which induce

anatomical alterations³⁶. Except for CICA cultivar the root diameter increased significantly in salt-treated seedlings, but the stele diameter did not show significant changes. In CICA seedlings the root diameter was not affected by the salt whereas the stele diameter decreased significantly. Contrarily, the stele area percentage decreased significantly in three of the four studied cultivars Table 2. A noticed trait of salinity is the delay of xylem differentiation in stele tissues, which often promotes the diminution of root xylem vessel diameter, probably caused by a repression in the development of metaxylem vessels and a lower cambial activity³⁷. Agreeing with these findings salt-treated Pasankalla and CICA seedlings showed significant reductions of metaxylem vessel diameters. It is well known that water transport into the vascular cylinder from root to aerial organs is depending on several physicochemical processes such as root pressure, water potential and vessel diameter (capillarity). In this regard the presence of narrower metaxylem vessels even if reduces the axial water conductivity, provides increased capillarity of conductor elements (Jurin's law), giving a more effective transport of water to upper zones of the plant³⁸. In addition, a lower vessel diameter provides better protection against the effect of embolism which may occur under condition of restricted water substitution imposed by salinity. However, reduced area of xylem offered more resistance to water flow, and then more energy is required to transport a determined quantity of water from roots to leaves, which ultimately results in more hampered growth performance of the plant³⁹. In an opposite trend, in Witulla and Wariponcho roots increased metaxylem vessel diameters were observed, as compared with salt-untreated roots. A similar trait has been observed in *Prosopis cineraria* under 200 mM NaCl⁴⁰. Increases in metaxylem vessel diameters could be considered as an adaptive strategy to facilitate water transport. Differences in metaxylem vessel diameters could reflect different strategies of quinoa cultivars to maintain an optimum water balance in seedling tissues and simultaneously cope with NaCl toxicity.

Root cortex area, in general, was higher in salt-treated than in salt-untreated seedlings (Figure 3). Increased root cortex thickness has also been communicated for other halophytes exposed to salt stress⁴¹. Changes in cortex thickness often modify the stele: root cross-section area ratio. For example, it was reported decreases of this ratio in salt-treated *Chloris gayana* seedlings³². In agreement with this finding our results showed significant decreases of the stele: root cross-section area ratio in salt-treated seedlings of all

Table 2. Anatomical attributes of control and salt-treated quinoa roots. Each value (\pm SE) is the mean of six roots from six individual seedlings. Values labelled with asterisk for each cultivar are significantly different between the control and salt-treated condition at $p < 0.05$

Anatomical attributes	Witulla		Wariponcho		Pasankalla		CICA	
	C	T	C	T	C	T	C	T
Root diameter (μm)	320.5 \pm 16.9	370.8 \pm 15.5*	360.0 \pm 15.4	506.3 \pm 62.4*	591.3 \pm 58.3	692.1 \pm 64.3*	526.3 \pm 25.8	516.3 \pm 16.4
Stele diameter (μm)	104.2 \pm 12.5	94.2 \pm 9.9	118.9 \pm 15.6	139.2 \pm 18.2	151.9 \pm 31.5	166.9 \pm 18.3	186.9 \pm 17.1	143.1 \pm 15.8*
Stele surface (%)	32.3	26.1*	31.6	27.5*	24	24.1	35.9	21.7*
Metaxylem vessel diameter (μm)	13.8 \pm 1.4	17.5 \pm 1.2*	17.5 \pm 2.2	22.5 \pm 2.2*	22.9 \pm 1.9	15.0 \pm 2.2*	22.5 \pm 2.2	13.3 \pm 1.3*

studied cultivars (data not shown). By contrast higher stele: root cross-section area ratios were also reported in some halophytic shrubs and in the halophytic legume *Prosopis strombulifera*⁴². Thus, it remains an open question if modified cortex size or stele to root cross-section ratio have regulatory functions for salt retention and/or water flow regulation in quinoa seedlings grown under saline conditions.

Exodermis and endodermis are protective cell layers against water and solute losses occurring in roots of almost all plants including hydrophytic, mesophytic and xerophytic species⁴³. However, they also play a critical role in controlling the radial flow of water. Recently has been proposed that *Chenopodiaceae* halophytes evolutionarily have developed at root level histo-anatomical strategies to controlling the penetration of salt and its retention in this organ. In fact, the occurrence of a well-developed endodermis capable of controlling the penetration and subsequent distribution of the salt has been clearly established in many species⁴⁴. Agreeing with this assumption a well-developed endodermis was observed in both salt-treated and salt-untreated root seedlings of all studied cultivars. Although no differences in exodermis development were found between salt-treated and salt-untreated seedling roots of Pasankalla and CICA cultivars, an earlier salt-induced exodermis development was observed in Wariponcho roots, which could represent an adaptive advantage regarding other cultivars (Figure 3D).

Beyond anatomical strategies developed in seedlings growing under saline conditions to retain the water inside tissues, many plant species have developed several metabolic mechanisms in response to water deficit to maintain both tissular and cellular water content. Under saline stress the accumulation of soluble sugars (e.g. sucrose, trehalose, fructans), sugar alcohols (e.g. mannitol, sorbitol), N-containing compounds (e.g. proline, glycine betaine), and cyclic polyols (e.g., pinitol, ononitol) allows to plants maintain the cellular turgor pressure necessary for both cell expansion and growth, and also to act as osmoprotectant molecules. Overall these compounds are referred as “compatible osmolytes” or “compatible solutes”¹⁰. Data reported in this work reveal different accumulation patterns for sucrose and proline in cotyledons and roots of seedlings grown under 200 mM NaCl (Figures 5A,B and 6A,B) which indicates that quinoa cultivars can display different osmoregulatory efforts to cope the salt stress. Witulla and Wariponcho seem concentrate their osmoregulatory efforts through the sucrose accumulation inside roots⁴⁵, likely to assure better water uptake; while CICA cultivars accumulate proline in cotyledons⁴⁶, likely to benefit both the water transport and photosynthesis. The Pasankalla cultivar accumulates sucrose and proline in both cotyledons and roots, being more efficient to keep an integral osmotic balance against saline stress. According with our results the sucrose accumulation seems to have

more responsiveness as compatible osmolyte than the proline accumulation under moderate saline stress. Low accumulation of proline under moderate salt stress has also been communicated for other quinoa cultivars³³ and durum wheat. Although the accumulation of hexoses has been observed in some halophytes under saline conditions⁴⁷ in the present study significant decreases of both glucose and fructose concentrations in cotyledons and roots were found practically in all cultivars. This fact might reflect for these sugars other metabolic roles such as fuel to sustain the higher demand of metabolic energy necessary to maintain the Na⁺ homeostasis and/or carbon skeleton to support the synthesis of salt-related secondary metabolites rather than their roles as osmoprotectant compounds. Furthermore, it has been proposed that accumulation of organic osmolytes in leaves of quinoa seedlings plays a dual role providing osmotic adjustment and protection of photosynthetic machinery against oxidative stress⁴⁸. On the other hand, soluble sugars also act as molecules signal in abiotic stress responses⁴⁹.

Increases of vanadate-sensitive H⁺ efflux, Na⁺ efflux and K⁺ retention in roots of salt-treated halophytes have been reported⁵⁰. Our results showed a significant increase of the H⁺ extrusion in salt-treated roots of Pasankalla cultivar, while in CICA one a significant decrease occurred (Figure 7). This could indicate that in Pasankalla and CICA roots the H⁺ efflux is mainly controlled by either the plasma membrane H⁺-ATPase activity (in the former) or tonoplast H⁺-pyrophosphatase (H⁺-PPase) activity (in the latter). Agreeing with our assumption, in a previous study it was demonstrated that salt-induced differences between plasma membrane and tonoplast transporter activities greatly contribute to differences in the salinity tolerance of quinoa cultivars^{51–53}. However, further studies are needed to clarify this subject.

5. Conclusions

This work represents, to our knowledge, the first approach to relationships between root and cotyledon anatomy with both physiological and metabolic parameters occurring in quinoa seedlings that grow under moderate saline stress. Most anatomical and physiological results permitted establish that different strategies to face the saline stress occurring at different combinations among quinoa cultivars, but conferring a similar fitness to cope with salt toxicity and also survive under extreme conditions.

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