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REGULAR ARTICLE

The role of cotyledon metabolism in the establishment of quinoa (*Chenopodium quinoa*) seedlings growing under salinity

Ana María C. Ruffino · Mariana Rosa · Mirna Hilal · Juan A. González · Fernando E. Prado

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Abstract A growth chamber experiment was conducted to assess the effect of salinity on emergence, growth, water status, photosynthetic pigments, osmolyte accumulation, and ionic content of quinoa seedlings (*Chenopodium quinoa*). The aim was to test the hypothesis that quinoa seedlings are well adapted to grow under salinity due to their ability to adjust the metabolic functionality of their cotyledons. Seedlings were grown for 21 days at 250 mM NaCl from the start of the germination. Germination percentage and cotyledon area were not affected by salt whereas seedling height decreased 15%. FW increased in both control and salt-treated cotyledons, but the increase was higher under salinity. DW only increased in salt-treated cotyledons. The DW/FW ratio did not show significant differences between treatments. Relative water content, chlorophyll, carotenoids, lipids, and proteins were significantly lower under salinity. Total soluble sugars, sucrose and glucose concentrations were higher in salt-treated than in control cotyledons. Ion concentration showed a different distribution pattern. Na^+ and Cl^- concentrations were higher under salinity, while an inverse result was observed for K^+ concentration. Proline and glycinebetaine concentrations increased under salinity, but the increase was higher in the former than the latter. The osmoprotective role of proline, glycinebetaine, and soluble sugars is discussed.

Keywords Cotyledons · Photosynthetic pigments · Osmoprotection · Glycinebetaine · Proline · Soluble sugars

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A. M. C. Ruffino · M. Rosa · M. Hilal · F. E. Prado
Cátedra de Fisiología Vegetal,
Facultad de Ciencias Naturales e IML,
Miguel Lillo 205,
CP 4000 Tucumán, Argentina

J. A. González
Instituto de Ecología, Area Botánica,
Fundación Miguel Lillo,
Miguel Lillo 251,
CP 4000 Tucumán, Argentina

F. E. Prado (✉)
Facultad de Ciencias Naturales e IML,
Miguel Lillo 205,
CP 4000 San Miguel de Tucumán, Argentina
e-mail: pra@tucbbs.com.ar
e-mail: fepra@csnat.unt.edu.ar

Introduction

Soil salinity affects about 7% of the world's land area; of the 1.5 billion ha that are cultivated, about 5% are affected by salt (Tester and Davenport 2003). Irrigated land produces one-third of the world's food approximately (Munns 2002), so its salinization, often due to poor irrigation practices, is particularly critical. Dryland salinity is also an important, and increasing,

problem in some areas of the world (Tester and Davenport 2003). In growing plants, soil salinity has a complex effect causing disturbance of membrane integrity, nutrient imbalance, and of metabolic activity (Hasegawa et al. 2000). Management practices to reduce soil salt concentrations are expensive and cannot always be applied in the world's underdeveloped and developing countries (Djanaguiraman et al. 2006). A possible alternative is the introduction of species (halophytes) capable of tolerating higher soil salinities with a good adaptability in terms of growth and yield.

Salinity tolerance is a heritable trait with a polygenic character linked to a complex genetic basis that can be used as an efficient criterion for selection of salt resistant populations (Mano and Takeda 1997; Flowers and Colmer 2008). There is also evidence to support the view that salt tolerance is a complex physiological trait affecting entirely the plant's life (Flowers 2004). Germination determines when and how seedling growth begins, and the young seedling is the most vulnerable stage to environmental conditions (Malcolm et al. 2003). The ability of species/cultivars to survive during the germination and early seedling stages is crucial for the growth and yield of the crops as well as for the distribution of species (Ungar 1996; Tobe et al. 2000; Malcolm et al. 2003). In saline environments, seeds and young seedlings are often exposed to salt concentrations higher than older plants because germination usually occurs in the soil surface (Foolad 1999). Detrimental effects of salinity on seed germination and seedling growth have been well characterized (Tobe et al. 2000; Koyro and Sayed 2008; Meloni et al. 2008), but the biochemical mechanisms underlying the establishment of seedlings are still poorly understood.

Several studies showed that even halophytes are particularly salt sensitive during the stages of seed germination and seedling establishment (Ungar 1996; Tobe et al. 2000; Malcolm et al. 2003). However, they have an advantage over plant species that lack strategies to deal with salt in the soil (Khan et al. 2000; Tobe et al. 2000; Rosa et al. 2004; Koyro and Sayed 2008). In many germinating halophytes such as *Cakile maritima*, *Salicornia bigelovii*, *Simmondsia chinensis*, and *Halocnemum strobilaceum* (Glenn et al. 1999), the growth of the embryonic axis is wholly dependent on the transfer of storage materials from the cotyledons (storage-type cotyledon). In others, such as *Chenopodium quinoa*, *Atriplex nummularia*, *Kochia childsii*, *Amaranthus hypochondriacus*, and *Borszczowia aralocaspica* (Shepherd et al. 2005), with scarce storage materials in their cotyledons (non-storage-type cotyledon), the growth of the embryonic axis is largely sustained by cotyledon photosynthesis. These cotyledons contain chlorophyll, possess stomata and are efficient photosynthetic organs (Rosa et al. 2004; Voznesenskaya et al. 2004). Therefore, species with storage-type cotyledons become autotrophic organisms when the first true leaves appear, while those with non-storage-type cotyledons start earlier. This difference has been related to the plasticity of biomass allocation, and used to help understand the plant's growth ability and performance under saline conditions (Ungar 1996; Malcolm et al. 2003; Rosa et al. 2009).

Quinoa (*Chenopodium quinoa*) is an Andean halophytic species emerging as a potential new crop in many regions of the world due to the nutritional composition of their seeds (Repo-Carrasco et al. 2003; Bhargava et al. 2006; Jacobsen 2007). Quinoa possesses non-storage-type cotyledons and exhibits a fast growth rate during the establishment of seedlings (Rosa 2006). Based on present knowledge, the aim of this study was to investigate the effect of salinity on quinoa seedlings establishment. Growth, water status, photosynthetic pigments, soluble sugars, proline, glycinebetaine, protein, and ion content were analysed in cotyledons of growing seedlings to ascertain the hypothesis that during germination and seedling establishment the halophytic species are more tolerant to salinity than glycophytes due to their ability to adjust the metabolic functionality of cotyledons.

Materials and methods

Plant material, germination and seedling growth

Seeds of *Chenopodium quinoa* Willd. cv. Sajama were obtained from Patacamaya Experimental Station (Oruro-Bolivia). Seeds were surface-sterilised with 2% sodium hypochlorite solution for 7 min, washed thoroughly with distilled water, and then sown in 5 cm Petri dishes (50 seeds per dish) on 1 layer of filter paper (Whatman Nº 1) moistened with 2 ml of eighth-strength Hoagland's solution (Hoagland and Arnon 1950) (control), or test solution (eighth-

strength Hoagland's solution containing 250 mM NaCl) (salt-treated). This NaCl concentration corresponds to the salt content present in different salinized areas of the Argentinean Northwest (González, personal communication). In addition, NaCl was used as the sole salinizing agent as it is the main component of the soluble salts present in most saline soils (Yang et al. 2007). Germination was carried out at 20ºC in darkness and the germinated seeds recorded hourly for 14 h. Seeds were considered to have germinated when the emerging radicle was at least 2 mm. Seeds with abnormal germination only represented between 2–4% in both treatments and they were not recorded. Abnormal germination corresponds to shoot growth without radicle extension. Five replicates of five Petri dishes each were used for each treatment.

For seedling growth, sterilised seeds were sown on wet vermiculite in plastic boxes (20 × 15 × 5 cm) in a controlled environmental chamber with a 14 h (25ºC) light/10 h dark (20ºC) cycle, relative humidity 70%, and photosynthetic photon flux density (400–700 nm) of 180 μE m^{-2} s^{-1}, supplied by cool white fluorescent lamps. Boxes were irrigated every two days with both control and test solution until the solution drained through the box: this procedure was sufficient to maintain salinity level in the boxes. Salt concentration was estimated through the electrical conductivity of effluent solutions. Values of electrical conductivity for control and test solution were 0.65 dS m^{-1} and 26.6 dS m^{-1}, respectively. Cotyledons of control and salt-treated seedlings were harvested 8 h after light was turned on at 6, 12 and 21 days after sowing. Samples were frozen in liquid nitrogen and stored at −80ºC for chemical analyses. Final harvest day was coincident with time that first true leaves have a similar size to cotyledons. Five replicates of 200 seedlings each were used for each treatment.

Growth parameters and relative water content (RWC) measurements

Cotyledon growth was measured based on area increase of one pair of cotyledons. The area was defined as length x width. This value, although not the exact area of the cotyledon, is proportional to its area. Cotyledon measurements were performed using a digital vernier caliber (Hitachi, Japan). Twenty pairs of cotyledons from different replicates were selected at random for each measurement. Seedling height was measured with a plastic ruler (accuracy±0.5 mm). Cotyledon RWC was calculated as: RWC=[(FW-DW)/(TW-DW)] x 100, where FW is the fresh weight, TW is the turgid weight measured after 6 h (time previously determined) of saturation on distilled water in Petri dishes at 4ºC in the dark, and DW is the dry weight determined after 72 h in an oven at 70ºC.

Photosynthetic pigments content

Chlorophyll and carotenoids were extracted according to Chapelle and Kim (1992) with minor modifications. Briefly, 25 mg FW of frozen cotyledons were placed in a test tube containing 2 ml of dimethyl sulfoxide, incubated at 45ºC for 12 h in the dark and then the absorbance was monitored using a UV-visible spectrophotometer (Metrolab 1700, Argentina) at wavelengths of 665, 649 and 480 nm. Pigment concentrations calculated according to Wellburn (1994) procedure were expressed as μg g^{-1} DW.

Soluble sugars, proline, glycinebetaine, and lipids content

Soluble sugars were extracted from 0.5 g FW of frozen cotyledons by homogenisation in 4 ml of 80% (v/v) ethanol with a mortar and pestle. The homogenate was heated in a water bath at 75ºC for 10 min and the insoluble fraction removed by centrifugation at 5000 × *g* for 10 min. After a second extraction with 4 ml of 80% (v/v) ethanol supernatants were pooled and dried under a stream of hot air. The dry residue was resuspended in 1 ml of distilled water and desalted by filtration through an ion-exchange column (Amberlite MB3, BDH, England). Total soluble sugar was determined according to Dubois et al. (1956), sucrose by the protocol of Cardini et al. (1955), and fructose by the method of Roe and Papadopoulos (1954). Glucose was determined using a glucose oxidase-peroxidase coupled assay according to Jorgensen and Andersen (1973). Aliquots of the extract without desalting were used for proline determination according to Ting and Rouseff (1979). Glycinebetaine was extracted using the method of Ladyman et al. (1983) with minor modifications. Briefly, 0.1 g FW of frozen cotyledons were homogenised in 2 ml of distilled water with a mortar and pestle, and then sulphuric acid added to make a final concentration of 0.5 M. The homogenate

was shaken for 18 h at room temperature and centrifuged at 3000 × *g* for 10 min. The precipitate was resuspended in 1 ml of 0.5 M sulphuric acid and centrifuged again. This process was repeated three times. Pooled supernatants were concentrated to a small volume under a stream of hot air, and added 0.3 ml of cold KI-I_2 reagent. After leaving overnight at 4ºC the extract was centrifuged at 3000 × *g* for 10 min and the supernatant discarded. The precipitate was rinsed with 0.2 ml of cold 1 N sulphuric acid, dissolved by agitating with 2 ml of dichloroethane, and then the absorbance was read at 365 nm. Lipids were extracted according to the procedure of Zenoff et al. (1994). Briefly, 1 g FW of frozen cotyledons were homogenised in 4 ml of methanol with a mortar and pestle. The homogenate was kept at 0ºC for 12 h, added 4 ml of chloroform and 1 ml of distilled water and then centrifuged at 3000 × *g* for 10 min. The upper phase was discarded and lipids concentrated by evaporating the solvent through nitrogen bubbling. Phospholipids were determined according to the protocol of Ames (1966), sterols were determined using the method of Lynch et al. (1963), and glycolipids were estimated by the phenol sulphuric acid method according to Roughan and Batt protocol (1967). Soluble sugars, proline, glycinebetaine, and lipids concentrations were expressed as μmol g^{-1} DW.

Protein content

Soluble protein was extracted from 1 g FW of frozen cotyledons using 4 ml of 50 mM phosphate buffer, pH 7.4 containing 1 mM β-mercaptoethanol and 5 μM $MnSO_4$ according to Rosa et al. (2004). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard, and expressed as mg g^{-1} DW.

Ion analysis

Sodium and potassium were extracted from 0.5 g of powdered dry cotyledons with 0.5 M HCl for 2 days according to the method of Hunt (1982), and measured by atomic emission flame photometry. Chloride was estimated on frozen material using the ferric ammonium sulphate and mercuric thiocyanate colorimetric method according to Guerrier and Patolia (1989). Ion concentrations were expressed as μmol g^{-1} DW.

Statistical analysis

Data correspond to four independent experiments that exhibited a high coincidence. Mean values of each parameter were compared between treatments using the SAS general linear model procedure (PROC GLM) one-way analysis of variance (ANOVA) and Tukey's Studentized Range test ($P \leq 0.05$) (SAS 1989).

Results

Germination

Final germination percentage of quinoa seeds was not affected by salt (Fig. 1). However, the presence of 250 mM NaCl in the test solution caused a delay of 2 h in the commencement of seed germination. Similar germination percentages were obtained with 300 and 400 mM NaCl but delay times were longer than with 250 mM salt (González and Prado 1992).

Growth parameters and RWC

Table 1 shows the time course of growth parameters and water status in salt-treated and control seedlings. Cotyledon area showed a progressive increase during the experimental period, but there were no significant

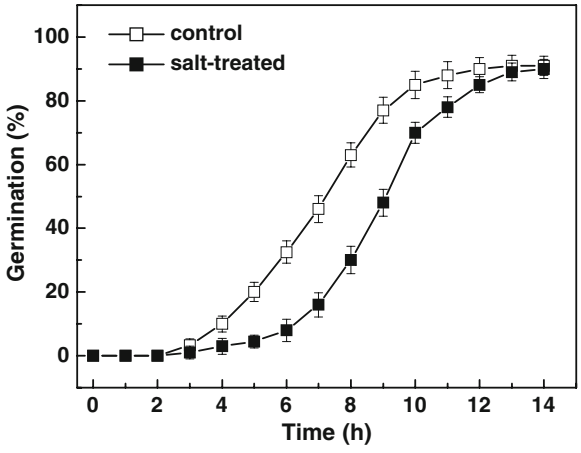

Fig. 1 Effect of 250 mM NaCl on germination of *C. quinoa* seeds. Percentage of germinated seeds was calculated each hour during a 14 h period. Values are means ± SD of four different experiments (n=5). Vertical bars represent ± SD

Table 1 Cotyledon area, seedling height, fresh weight (FW), dry weight (DW), DW/FW ratio and RWC in control and salt-treated cotyledons during the early developmental stages of *C. quinoa* seedlings

	Days		
	6	12	21
Control cotyledons			
Cotyledon area (mm^2)	$21.23 \pm 2.1_a$	$32.12 \pm 3.5_b$	$43.68 \pm 4.2_c$
Seedling height (mm)	$40.12 \pm 4.3_a$	$65.79 \pm 5.1_b$	$83.23 \pm 5.2_c$
FW (mg)*	$14.2 \pm 2.0_a$	$18.3 \pm 2.1_b$	$19.1 \pm 1.7_b$
DW (mg)*	$1.61 \pm 0.20_a$	$1.68 \pm 0.30_a$	$1.69 \pm 0.31_a$
DW/FW ratio	$0.11 \pm 0.01_a$	$0.09 \pm 0.01_a$	$0.09 \pm 0.01_{a,}$
RWC (%)	$87.34 \pm 5.2_a$	$88.56 \pm 6.6_a$	$86.32 \pm 4.3_a$
Salt-treated cotyledons			
Cotyledon area (mm^2)	$22.30 \pm 2.6_a$	$35.12 \pm 3.5_b$	$44.78 \pm 4.2_c$
Seedling height (mm)	$38.24 \pm 4.1_a$	$62.09 \pm 5.1_b$	$72.14 \pm 6.2_c$
FW (mg)*	$19.1 \pm 2.0_b$	$27.2 \pm 2.1_c$	$39.1 \pm 2.7_d$
DW (mg)*	$1.92 \pm 0.21_b$	$2.23 \pm 0.22_b$	$2.48 \pm 0.41_b$
DW/FW ratio	$0.10 \pm 0.01_a$	$0.09 \pm 0.01_a$	$0.08 \pm 0.01_a$
RWC (%)	$72.16 \pm 4.7_b$	$70.50 \pm 5.2_b$	$71.12 \pm 6.1_b$

* Data correspond to 5 pairs of cotyledons

Values are means±SD of four different experiments (n=5, for cot. area n=20). Within each column and for each measurement a different letter indicates that any difference between control and saline treatment is significant. Within each row and for each measurement a different letter indicates that any difference between harvest days is significant. Both analyses according to the Tukey's Studentized Range test ($P \leq 0.05$)

differences between treatments. Seedling height showed a similar trend, but at the end of experiment it was approximately 15% higher in control than in salt-treated seedlings. However, there were no appreciable morphological differences between seedlings during the experimental period. Salinity significantly increased both FW and DW during the experimental period. FW showed a progressive increase in control and salt-treated cotyledons, but the change was more pronounced in the latter than the former. DW did not show significant variations in control cotyledons, while in salt-treated ones DW progressively increased until the end of experiment. There were no significant differences in DW/FW ratio between treatments. The RWC, an indicator of the plant water status, was significantly lower in salt-treated than in control cotyledons (Table 1).

Photosynthetic pigments

Total chlorophyll (Chl), chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid concentrations on a dry weight basis were significantly lower in salt-treated than in control cotyledons; however, in the middle of the experimental period (12 days) the difference was smaller than observed at 6 and 21 days (Table 2). Pigment concentrations showed strong increases up to 12 days and then decreased strongly up to 21 days. Under salinity the content of Chl *b* was reduced faster than in control cotyledons. For example at the end of the experiment in salt-treated cotyledons Chl *b* content decreased 29%, while in control cotyledons the decrease was only 12%. By contrast there were no significant differences in Chl *a* content. It is worth noting that no difference was observed between treatments in the pigment content profile during the experimental period (distribution pattern). Chlorophyll *a*/*b* ratio in salt-treated cotyledons showed a progressive increase until the end of experiment (although differences from the control were significant only at 12 and 21 days), whereas in control cotyledons the chlorophyll *a*/*b* ratio did not show significant variations during the experimental period. Chlorophyll/carotenoid, (Chl/Car), ratio between treatments showed significant differences at 6 and 21 days, but not at 12 days.

Table 2 Total chlorophyll, chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), Chl *a*/*b* ratio, carotenoids, and Chl/Car ratio in control and salt-treated cotyledons during the early developmental stages of *C. quinoa* seedlings

	Days		
	6	12	21
Control cotyledons			
Total Chl (μg g^{-1} DW)	$210.3\pm17.3_a$	$320.7\pm21.2_b$	$180.6\pm12.2_c$
Chl *a* (μg g^{-1} DW)	$160.6\pm10.1_a$	$195.6\pm18.1_b$	$144.3\pm10.6_c$
Chl *b* (μg g^{-1} DW)	$50.50\pm6.22_a$	$75.6\pm12.2_b$	$44.9\pm5.21_a$
Chl *a*/*b* ratio	$3.2\pm0.13_a$	$3.0\pm0.21_a$	$3.2\pm0.11_a$
Carotenoids (μg g^{-1} DW)	$56.3\pm2.31_a$	$69.4\pm3.71_b$	$48.7\pm4.41_c$
Chl/Car ratio	$3.73\pm0.2_a$	$4.62\pm0.15_b$	$3.71\pm0.21_a$
Salt-treated cotyledons			
Total Chl (μg g^{-1} DW)	$125.7\pm5.2_b$	$235.6\pm15.1_c$	$110.3\pm6.1_d$
Chl *a* (μg g^{-1} DW)	$96.1\pm5.1_b$	$125.9\pm12.2_c$	$89.8\pm5.4_d$
Chl *b* (μg g^{-1} DW)	$29.4\pm2.1_b$	$35.1\pm4.1_b$	$22.8\pm2.1_c$
Chl *a*/*b* ratio	$3.3\pm0.1_a$	$3.5\pm0.12_b$	$3.9\pm0.1_c$
Carotenoids (μg g^{-1} DW)	$44.6\pm2.2_b$	$50.6\pm4.4_c$	$43.1\pm4.4_b$
Chl/Car ratio	$2.81\pm0.2_b$	$4.66\pm0.13_c$	$2.89\pm0.16_b$

Values are means ± SD of four different experiments ($n=5$). Within each column and for each measurement a different letter indicates that any difference between control and saline treatment is significant. Within each row and for each measurement a different letter indicates that any difference between harvest days is significant. Both analyses according to the Tukey's Studentized Range test ($P\leq0.05$)

Soluble carbohydrates and lipids

Carbohydrate patterns showed progressive increases in both treatments during the experimental period, but they were more pronounced under salinity, reaching at the end of experiment maxima values of 265, 75, and 107 μmol g^{-1} DW for soluble sugars, sucrose, and glucose, respectively. By contrast, fructose concentration was decreased by salinity (Table 3). Salinity decreased the concentration of phospholipids, sterols, and glycolipids during the experimental period, but this was greater (~ 30%) in the former than the latter. Lipid concentration in control and salt-treated cotyledons increased up to 12 days and thereafter declined until the end of experiment. Phospholipid/glycolipid, (PL/GL), ratio between treatments did not show significant variations during the experimental period (Table 3).

Soluble protein, proline and glycinebetaine

Soluble protein concentration was significantly lower in salt-treated than in control cotyledons (Table 4). In control cotyledons, the distribution pattern showed a decrease during the experimental period, whereas in salt-treated cotyledons there were no significant differences. Over time, there was a progressive increase in proline concentration in the salt-treated cotyledons up to 12 days, and thereafter a strong increase (87%) relative to the controls was observed at the end of experiment. In control cotyledons, proline concentration showed only a slow increase during the experimental period. Glycinebetaine concentration was also increased significantly under salinity, but its change profile over time was different from proline content. Maximum increase in glycinebetaine concentration under salinity was 38%.

Ion content

As expected, the endogenous Na^+ concentration in cotyledons of *C. quinoa* was significantly higher in the presence of salt. By contrast, K^+ concentration decreased slightly under salinity (Table 4). Salinity also induced a significant increase of the internal Cl^- concentration. K^+/Na^+ and $[Na^+ + K^+]/Cl^-$ ratios were significantly lower in salt-treated than in control cotyledons.

Table 3 Total soluble sugars, glucose, fructose, sucrose, phospholipids (PL), glycolipids (GL), sterols (ST), and PL/GL ratio in control and salt-treated cotyledons during the early developmental stages of *C. quinoa* seedlings

	Days		
	6	12	21
Control cotyledons			
Total soluble sugars (μmol equiv. glucose g^{-1} DW)	$134.4\pm8.1_a$	$140.6\pm9.1_a$	$200.8\pm9.3_b$
Glucose (μmol g^{-1} DW)	$32.8\pm3.2_a$	$36.4\pm3.5_a$	$54.3\pm5.1_b$
Fructose (μmol g^{-1} DW)	$23.5\pm3.0_a$	$27.3\pm2.0_a$	$35.4\pm2.3_b$
Sucrose (μmol g^{-1} DW)	$30.2\pm2.1_a$	$40.4\pm2.5_b$	$60.4\pm3.2_c$
PL (μmol g^{-1} DW)	$19.5\pm2.1_a$	$28.6\pm2.0_b$	$24.2\pm2.1_b$
GL (μmol g^{-1} DW)	$39.1\pm2.5_a$	$53.9\pm3.1_b$	$49.0\pm2.0_b$
ST (μmol g^{-1} DW)	$12.4\pm1.3_a$	$18.1\pm1.8_b$	$16.4\pm1.5_b$
PL/GL ratio	$0.50\pm0.1_a$	$0.52\pm0.1_a$	$0.50\pm0.1_a$
Salt-treated cotyledons			
Total soluble sugars (μmol equiv. glucose g^{-1} DW)	$138.5\pm8.1_a$	$159.5\pm7.7_b$	$265.3\pm9.8_c$
Glucose (μmol g^{-1} DW)	$38.7\pm2.3_b$	$58.1\pm3.1_c$	$107.4\pm7.7_d$
Fructose (μmol g^{-1} DW)	$16.2\pm1.5_b$	$17.4\pm2.0_b$	$20.3\pm2.1_c$
Sucrose (μmol g^{-1} DW)	$39.4\pm3.0_b$	$58.4\pm2.0_c$	$75.1\pm5.0_d$
PL (μmol g^{-1} DW)	$13.7\pm1.8_b$	$20.1\pm2.2_c$	$17.1\pm1.7_d$
GL (μmol g^{-1} DW)	$30.2\pm3.1_b$	$43.8\pm3.1_c$	$36.8\pm2.1_d$
ST (μmol g^{-1} DW)	$10.0\pm0.9_b$	$14.3\pm1.4_c$	$12.4\pm1.0_d$
PL/GL ratio	$0.45\pm0.1_a$	$0.46\pm0.07_a$	$0.46\pm0.1_a$

Values are means±SD of four different experiments ($n=5$). Within each column and for each measurement a different letter indicates that any difference between control and saline treatment is significant. Within each row and for each measurement a different letter indicates that any difference between harvest days is significant. Both analyses according to the Tukey's Studentized Range test ($P\leq0.05$)

Discussion

In saline environments the vulnerability of plants is determined by salt concentrations, so adaptation to salinity is crucial for the establishment of species (Koyro and Sayed 2008). During the seedling establishment, salt modifies many biological processes such as growth, osmotic homeostasis, photosynthesis, carbon partitioning, carbohydrate and lipid metabolism, protein synthesis, and gene expression (Prado et al. 2000; Munns 2002). Data presented here show that salinity did not affect germination percentage of quinoa (*Chenopodium quinoa*) seeds; however, physiological and metabolic parameters of cotyledons in young seedlings were altered. Seedling height was reduced by salinity whereas there were no differences in cotyledon area. Growth parameters during the experimental period showed a similar pattern under both treatments. However, increases in cotyledon area, seedling height, FW and DW between 12 and 21 days were less pronounced than between 6 and 12 days (Table 1). This fact may be related with the senescence-dependent decline of cotyledon metabolism (Kim 2004). Chlorophyll and carotenoid contents also decreased in salt-treated cotyledons of quinoa seedlings, but in a smaller percentage than observed in other species (Khosravinejad et al. 2008; Meloni et al. 2008). Parida and Das (2005) suggest in their review that the decrease in chlorophyll and carotenoid contents of leaves in response to salt stress is a general phenomenon. When the chlorophyll content was analyzed during the experimental period a sustained increased up to 12 days and thereafter a pronounced decrease until the end of experiment was observed; however, the decrease was more pronounced in salt-treated than in control cotyledons. A fast reduction in chlorophyll under salinity has been related to a salt-induced premature senescence (Vieira

Table 4 Soluble protein, proline, glycinebetaine, Na^+, K^+, Na^+/K^+ ratio, Cl^-, and $[Na^+ + K^+]/Cl^-$ ratio in control and salt-treated cotyledons during the early developmental stages of *C. quinoa* seedlings

	Days		
	6	12	21
Control cotyledons			
Soluble protein (mg g^{-1} DW)	$125.3\pm8.1_a$	$110.6\pm7.1_b$	$102.2\pm6.3_b$
Proline (μmol g^{-1} DW)	$10.5\pm2.2_a$	$11.2\pm2.1_a$	$13.5\pm1.7_a$
Glycinebetaine (μmol g^{-1} DW)	$5.5\pm0.9_a$	$6.2\pm0.8_a$	$8.3\pm1.2_b$
Na^+ (μmol g^{-1} DW)	$50.7\pm4.3_a$	$51.4\pm5.1_a$	$49.9\pm4.2_a$
K^+ (μmol g^{-1} DW)	$75.3\pm3.1_a$	$75.0\pm3.9_a$	$74.3\pm4.0_a$
K^+/Na^+ molar ratio	$1.48\pm0.2_a$	$1.46\pm0.1_a$	$1.48\pm0.2_a$
Cl^- (μmol g^{-1} DW)	$195.3\pm7.5_a$	$188.4\pm9.1_a$	$196.0\pm7.2_a$
$[Na^+ + K^+]/Cl^-$ ratio	$0.64\pm0.05_a$	$0.67\pm0.04_a$	$0.63\pm0.06_a$
Salt-treated cotyledons			
Soluble protein (mg g^{-1} DW)	$68.5\pm4.1_c$	$60.0\pm3.7_d$	$65.9\pm4.8_c$
Proline (μmol g^{-1} DW)	$13.5\pm2.3_b$	$16.4\pm2.5_b$	$25.3\pm3.1_c$
Glycinebetaine (μmol g^{-1} DW)	$6.5\pm0.9_b$	$7.3\pm1.0_b$	$11.5\pm1.4_c$
Na^+ (μmol g^{-1} DW)	$62.1\pm5.0_b$	$63.0\pm4.8_b$	$61.9\pm5.0_b$
K^+ (μmol g^{-1} DW)	$70.8\pm4.2_b$	$69.4\pm3.1_b$	$70.0\pm3.8_b$
K^+/Na^+ molar ratio	$1.14\pm0.1_b$	$1.10\pm0.1_b$	$1.13\pm0.1_b$
Cl^- (μmol g^{-1} DW)	$295.2\pm9.4_b$	$305.2\pm10.2_b$	$303.6\pm9.1_b$
$[Na^+ + K^+]/Cl^-$ ratio	$0.45\pm0.04_b$	$0.43\pm0.05_b$	$0.43\pm0.04_b$

Values are means±SD of four different experiments ($n=5$). Within each column and for each measurement a different letter indicates that any difference between control and saline treatment is significant. Within each row and for each measurement a different letter indicates that any difference between harvest days is significant. Both analyses according to the Tukey's Studentized Range test ($P\leq0.05$)

Santos et al. 2001), but measurements of cotyledon senescence were not made so it was not possible to confirm this supposition. By contrast carotenoids, which are not, or are only incompletely broken down during senescence (Matile 2000), did not show great changes at the end of experiment. Chl *b* decreased at a higher rate than Chl *a* in cotyledons exposed to 250 mM NaCl, so the Chl *a*/*b* ratio increased significantly. Although this can be explained by the fact that the first step in chlorophyll senescence involves the conversion of Chl *b* to Chl *a* (Scheumann et al. 1999). The highest Chl *a*/*b* ratio observed in salt-treated cotyledons could also reflect an accelerated oxidative catabolism of the Chl *b* produced by reactive oxygen species (ROS) generated by saline stress (Hendry and Price 1993; Hernández et al. 1995). In addition, chlorophyll decrease was also attributed to suppression of specific enzymes involved in its synthesis, increase of degrading enzymes *e.g.* chlorophyllase, or salt-induced disruption of chloroplast structure with instability of pigment-protein complexes (Djanaguiraman and Ramadass 2004).

Salt stress also exerts some effects on water relations (*e.g.* RWC and WUE, water use efficiency) in both glycophytic and halophytic species (Aldesuquy and Ibrahim 2001; Djanaguiraman et al. 2006). Decreases in RWC have been communicated for many seedlings growing under salinity (Omami and Hammes 2006; Meloni et al. 2008). The RWC of quinoa cotyledons decreased at 250 mM NaCl when compared to the control. The RWC, although a convenient and widely used method of assessing plant water status, is not a useful indicator of turgor in salt-treated plants undergoing osmotic adjustment. In most plants, especially halophytes, the solute content of cells at high salinity is higher than in non-saline condition, due largely to accumulation of ions (*e.g.* Na^+ and Cl^-) and organic solutes. Therefore

during the rehydration to establish TW, the higher solute content in salt-treated than in untreated cotyledons causes a greater water uptake in the former than the latter. Thus, this fact results in an apparently low RWC under salinity (Munns et al. 2006). Our result agrees with this supposition and salt-treated cotyledons had a TW significantly higher than untreated ones. FW and DW were higher in salt-treated than in control cotyledons in a way consistent with previous results found in other halophytic species (Yeo and Flowers 1980; Ungar 1996; Khan et al. 2000). However, in many halophytes growing under salinity the DW does not always reflect the organic dry mass gain due to the high intracellular accumulation of inorganic ions (Na^+ and Cl^-) (Yeo and Flowers 1980).

Salt stress has been generally considered to exert both osmotic and ionic effects. The excessive accumulation of ions in plants, principally Na^+ and Cl^-, often claimed as toxic, is the main cause of growth inhibition induced by salinity (Khan et al. 2000; Malcolm et al. 2003). Salinity tolerance also depends on limiting Na^+ accumulation and maintaining K^+ content in the cytosol in order to achieve the preservation of ion homeostasis (Flowers et al. 1977; Hasegawa et al. 2000). The content of Na^+ and Cl^- in cotyledons of *C. quinoa* significantly increased in the presence of 250 mM NaCl, whereas K^+ content showed a slower decrease. Decreases of endogenous K^+ levels induced by high external NaCl concentrations have previously been communicated for other species, and attributed to a transmembrane competition between K^+ and Na^+ fluxes (Khan et al. 2000; Song et al. 2005; Djanaguiraman et al. 2006; Meloni et al. 2008). In our study the endogenous K^+ content decreased slightly at 250 mM NaCl and so quinoa seedlings are able to maintain a relatively high K^+ level in their cotyledons. Although a low decrease in K^+ content could reflect a reduced cytosolic K^+ efflux and/or an increased K^+ translocation from root to shoot (Maathuis and Amtmann 1999; Maathuis 2007; Flowers and Colmer 2008); we only measured total ion content but not fluxes themselves, therefore, it is not possible to confirm such possibilities. Nevertheless, according to our results, K^+ seems act as the major monovalent osmoticum during the growth of quinoa seedlings in presence of high NaCl concentrations. The K^+/Na^+ ratio decreased in salt-treated quinoa cotyledons due to the high external concentration of NaCl; however, Koyro and Sayed (2008) communicated an inverse trend for quinoa seedlings growing under a high KCl concentration. Chloride accumulation was higher in salt-treated than in control cotyledons, however, the $[Na^+ + K^+]/Cl^-$ ratio was lower in the former than the latter. This result disagrees with previous findings obtained with other halophytes (Koyro 2006; Flowers and Colmer 2008), and could reflect a different mechanism of Cl^- uptake and transport. However, this subject requires further elucidation.

Soluble sugars are osmolytes frequently found in plants subject to drought and salinity conditions (Flowers et al. 1977, Hasegawa et al. 2000; Flowers and Colmer 2008). Therefore, it is logical to think that the increase observed in sucrose and glucose concentration in salt-treated cotyledons was due to the saline stress. However, such an increase could have been a result of the reduction in growth rate or by a salt-induced decrease of invertase activity (Prado et al. 1995). Since our study also showed a high glucose concentration in salt-treated cotyledons, this hypothesis must be discarded. Thus, the high glucose content observed under salinity can be attributed to increase of perispermic starch hydrolysis as was demonstrated in quinoa cotyledons under low temperature stress (Rosa 2006) as well as the interconversion of sugars (Pfeiffer and Kutschera 1996). Fructose, another soluble sugar present in plants, decreased in salt-treated cotyledons. This fact might reflect for this sugar another metabolic role rather than its role as an osmoprotectant compound. Although the accumulation and compartmentalization of the ions to osmotic adjustment requires energy (Yeo 1983), it has been demonstrated that this osmotic adjustment is less energy and carbon demanding than the adjustment by organic solutes (Raven 1985).

Proline and glycinebetaine are also important osmolytes found in many species subjected to saline and drought stress (Di Martino et al. 2003; Meloni et al. 2008). Glycinebetaine and other betaine derivatives have been recognized as major osmolytes in species of the Chenopodiaceae and Amaranthaceae families (Flowers et al. 1977, 1986; Flowers and Colmer 2008). In the present study, however, we demonstrated that cotyledons of salt-treated quinoa seedlings accumulate more proline than glycinebetaine. The proline levels (7–13 μmol g^{-1} FW) accumulated in salt stressed cotyledons mean that proline

concentration in cells could reach 7–13 mM by a simple supposition that 1 g of FW tissue is equivalent to 1 ml total volume and that vacuoles are absent in cotyledons. By contrast, glycinebetaine concentration only reachs 3–6 mM. Hence, contrary to its generalized osmoprotectant role accepted in many species, glycinebetaine does not seem to play an important role in the mechanism of salt tolerance in quinoa seedlings. However, it is still unknown if age and growth conditions are factors that can affect the accumulation of glycinebetaine in *C. quinoa* and therefore further investigations are needed. Other metabolic parameters such as lipids and soluble proteins were also affected by salinity. Because salt-induced senescence severely affects protein and lipid metabolisms (Vieira Santos et al. 2001; Kim 2004) additional studies will be necessary to clarify saline stress and senescence involvements into lipid and protein metabolism of cotyledons.

In conclusion our results confirm the hypothesis that the high adaptability to soil salinity that growing quinoa seedlings exhibit, is a consequence of better metabolic control than non-halophytic species, based on cotyledon's functionality, of ion absorption, osmolyte accumulation, and osmotic adjustment.

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