*Full Length Research Paper*

# Physiological responses of *Chenopodium quinoa* to salt stress

## A. Jason Morales[1,2], Prabin Bajgain[1], Zackary Garver[1], Peter J. Maughan[1] and Joshua A. Udall[1]*

[1]Department of Plant and Wildlife Science, Brigham Young University, Provo, UT, 84602, USA.
[2]Department of Agronomy, Purdue University, West Lafayette, IN, 47907, USA.

Physiological responses to salt stress were measured in *Chenopodium quinoa*, a regionally important halophytic staple crop of Andean South America. In a greenhouse experiment, salt (NaCl) was applied to quinoa varieties, Chipaya and KU-2, and to the model halophyte *Thellungiella halophila* to assess their relative responses to salt stress. Height and weight data from a seven-week time course demonstrated that both *C. quinoa* cultivars exhibited greater tolerance to salt stress than the model plant *T. halophila* in these specific conditions. In a separate growth chamber experiment, two quinoa cultivars (chipaya and ollague) adapted to saline soils and one quinoa cultivar (CICA-17) adapted to a lower elevation were grown hydroponically and evaluated for physiological responses to four salt stress treatments. Tissues collected from the growth chamber experiments were used to obtain leaf water content, tissue ion concentrations, compatible solute concentrations, and RNA for real-time PCR. High levels of trigonelline, a known osmoprotectant, were found to accumulate in the high salt treatment suggesting a key role in salt tolerance of quinoa. The expression profiles of genes involved in salt stress, showed constitutive expression in leaf tissue and up-regulation in root tissue in response to salt stress. These data suggest that quinoa tolerates salt through a combination of salt exclusion and accumulation mechanisms.

**Key words:** Salt stress, quinoa, *Thellungiella halophila*, trigonelline, osmoprotectant.

## INTRODUCTION

Intense irrigation and poor resource management has resulted in a widespread, gradual decrease of crop productivity on agricultural lands because of an accumulation of salt (Munns and Tester, 2008). Approximately 800 million hectares or 20% of all arable land is currently affected by high soil salinity (Flowers, 2004; Rengasamy, 2010). High soil salinity negatively affects the yield of traditional glycophytic crops that can only tolerate low levels of soil salinity (<50 mM) without retarded growth (Orcutt and Nilsen, 2000). This salt sensitivity of major food crops combined with gradual global climate change requires investigation of stress tolerant, alternative food crops such as quinoa (*Chenopodium quinoa*) (Wilson et al., 2002; Jacobsen et al., 2003; Tro gnitz, 2003; Koyro and Eisa, 2008).

*C. quinoa* is an important regional grain crop grown throughout Andean South America. Evolutionarily, quinoa is a domesticated halophytic member of Amaranthaceae, a widely distributed botanical family rich in halophytic genera (44%). Quinoa varieties were anciently and are currently cultivated across a range of extreme environ-ments, including varieties uniquely adapted to thrive in the southern altiplano (high plains) of South America near the Salar (or salt flat) of Uyuni (elevation 10,582 $km^2$). Despite the high soil salinity, altiplano ecotypes thrive in these conditions (Sanchez et al., 2003). Indeed, the altiplano cultivar "Kancolla" was reported to have a germination rate of 75% at salt concentration of 57 dS $m^{-1}$ (Christiansen et al., 1999; Jacobsen et al., 1999), a con-siderable feat considering that the electrical conductivity of seawater is 50 dS $m^{-1}$, or ~600 mM NaCl. Such

observations suggest that quinoa is a halophyte and may utilize unique physiological mechanisms to tolerate soil salinity. Halophytic plants have adapted physiological

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*Corresponding author. E-mail: jaudall@byu.edu.

mechanisms that provide osmoregulation in saline environments of approximately 300 mM NaCl or more (Orcutt and Nilsen, 2000). Plants regulate the osmotic potential across their membranes by secreting excess salt through glands or by tolerating high levels of ions within the plant. Salt tolerance within the plant generally uses ion sequestration, the use of compatible solutes, or a combination of both mechanisms. Molecular mechanisms such as ion transporters, (for example, salt overly sensitive 1 (*SOS1*), $Na^+/H^+$ Exchanger 1 (*NHX1*), and tonoplast intrinsic protein 2 (*TIP2*)) play a role in osmoregulation within *A. thaliana*.

These genes have also been found to be active in *Thellungiella halophila* (salt cress), the model halophytic plant, in response to salt stress (Vera-Estrella et al., 2005). *SOS1* is a $H^+ / Na^+$ antiporter in the plasma membrane that removes salt from the cytoplasm (Shi et al., 2000). *NHX1* is an antiporter located in the tonoplast that sequesters $Na^+$ into the central vacuole (Apse et al., 1999). *TIP2* encodes an aquaporin protein that moves water from the vacuole to the cytoplasm in response to salt stress (Boursiac et al., 2005; Zhang et al., 2008). For a recent, comprehensive review of $Na^+$ transport and salt tolerance mechanism in plants see Kronzucker and Britto (2011). Homologous genes within the *C. quinoa* genome may have an important role in its salt stress tolerance. For example, Maughan et al., (2009) recently reported active expression of *SOS1* after identification, cloning and sequencing of two homoeologous *SOS1* gene sequences in the quinoa genome. Documentation of gene expression levels and tissue specificity will aide in our understanding of the mechanisms present in *C. quinoa*.

Compatible solutes (also known as osmoprotectants) can buffer the effect of salt by maintaining the osmotic balance of water levels across plant membranes (Trinchant et al., 2004; Chen et al., 2007). When accumulated in high amounts, compatible solutes can offset the osmotic imbalance caused by a high accumulation of salt in the intercellular space without disrupting normal physiology in the cytosol. Compatible solutes also provide enzymatic protection and maintain membrane integrity under salt stress (Sakamoto and Murata, 2002).

Compatible solutes previously identified include pinitol (Adams et al., 1998), sorbitol, trehalose (Rontein et al., 2002), glycinebetaine (referred to as betaine), and proline (Ashraf and Foolad, 2007). Novel betaines have been found in quinoa, though the levels have not been quantified under stress (Dini et al., 2006). Perhaps, quinoa has a very active *BADH* (Betaine aldehyde dehydrogenase) that encodes an enzyme to convert betaine aldehyde into the compatible solute gylcine betaine.

Our objectives in this research were to i) compare the growth response of quinoa relative to the model halophyte, *T. halophila*, under varying salt treatments using a large replicated greenhouse experiment conducted over a seven-week period; ii) measure physiological changes of quinoa to salt stress in a carefully controlled hydroponic growth chamber through root fresh weight, shoot fresh weight, stomatal conductance, and osmoprotectant accumulation; and iii) examine transcriptional responses at several candidate salt tolerance genes (*SOS1*, *BADH*, *NHX1* and *TIP2*) during salt stress in leaf and root tissue.

## MATERIALS AND METHODS

### Plant materials

Four quinoa ecotypes were used in the experiments (growth chamber and greenhouse), including the altiplano salares accessions Chipaya and Ollague, the Peruvian valley accession CICA -17, and the Chilean coastal accession KU-2 (Mason et al., 2005; Christensen et al., 2007).

In the greenhouse experiment, Chipaya and KU-2 seeds were planted in 36-cell flats and germinated in Sunshine Basic Mix 2 soil (Sun Gro, Vancouver, British Columbia, Canada) under ambient greenhouse conditions. After one week of growth, plants were transferred to open, four-inch square pots for the duration of the experiment. *T. halophila*, Shangdong variety, was grown using the growth protocol provided by the University of Illinois, Urbana-Champaign (www.thellungiella.org) and then transferred to open, 3.5-inch propagation pots. Salt treatments were initiated at the eight-leaf stage of development to synchronize the physiological development of quinoa and *T. halophila*.

All plants were grown in greenhouses at Brigham Young University, Provo, Utah (40° 23" N, 111° 66 W) with an average daily maximum temperature of 37°C, an average daily minimum temperature of 16.5°C, and an average day length of 14 h. After three weeks, soil was supplemented with Osmocote (Scotts, Marysville, OH) slow release fertilizer and Marathon 1% granular insecticide (OHP Mainland, PA).

### Greenhouse salinity treatments

At the eight-leaf stage of development, two quinoa cultivars (Chipaya and KU-2) and *T. halophila* grown in 4-inch pots were randomly assigned a bench position and one of five salt treatments in a full factorial completely randomized design.

A total of 525 plants (175 of each type) were used in each of three replicates. Treatments consisted of tap water with NaCl added to reach concentrations of 150, 300, 450, and 600 mM with a 0 mM concentration serving as the control treatment. Plants were only watered with treatment solution as needed to avoid over-watering. Stomatal conductance in quinoa was measured on five plants from each treatment-type combination. Plants were harvested every seven days for height and above-soil fresh weight measurements.

### Growth chamber salinity treatments and tissue collection

Three quinoa cultivars (Ollague, CICA 17, and Chipaya) were grown in a hydroponic growth chamber as described by Camp et al. (1987) with a day temperature of 29.5°C, a night temperature of 19°C, and a day length of 13 h. *T. halophila* was not included in the growth chamber experiment because its roots were too small for the hydroponic system. Instead, we chose to include two purportedly salt tolerant lines (Ollague and Chipaya) adapted to the Bolivia Altiplano to identify potential cultivar differences in „salt-adapted" material. CICA -17 is purportedly an un-adapted line for saline soils from the lower elevations of Peruvian valleys. A randomized block design with four blocks and three replications (four blocks/

replication) was used. Each block contained four randomized treatments namely, „high-salt" (HS), „low-salt" (LS), „recovery treatment" (RT), and „recovery control" (RC). Each treatment was applied to a single bucket that contained one representative from each cultivar (four buckets/block). The hydroponic media was supplemented with NaCl as determined by the treatment.

The HS treatment was defined as a 50 mM incremental daily dosage of NaCl added to the hydroponic media from 50 mM to a final concentration of 450 mM. The LS treatment maintained a NaCl concentration of 50 mM. 48 h after the final concentration was reached the high salt and low salt treatments were harvested. The RT consisted of a 50 mM daily incremental increase in NaCl to a maximal concentration of 450 mM (simultaneously with the HS treatment to peak level of $Na^+$) followed by a 50 mM daily incremental decrease to a final concentration of 50 mM. The recovery control treatment consisted of a constant concentration of 50 mM NaCl for the duration of the stress treatment.

Plants from the recovery and recovery control treatments were harvested simultaneously. Stomatal conductance was measured every 3 to 4 days. Root and shoot fresh weights measured at the time of harvest. All plant tissue was harvested 48 to 72 h after reaching their final treatment concentration, flash frozen using liquid nitrogen, and stored at -80°C.

**Stomatal conductance and osmoprotectants**

Stomatal conductance of quinoa in both experiments was measured using a steady state leaf porometer (Decagon Devices, pullman, WA) that calculated stomatal conductance by measuring vapor concentration at two distinct points in the diffusion path. Measurements were taken over a 30 s period and reported in mmol $m^{-2}$ $s^{-1}$. Inductively coupled plasma (ICP) analysis on an IRIS Intrepid II XSP (Thermo Electron Corporation, Franklin, MD) was used to estimate inorganic ion concentrations including calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, sulfur, and zinc concentrations in lyophilized plant root and leaf tissue were determined using standard nitric-perchloric acid tissue digestion (Johnson and Ulrich, 1959). Tissue samples were bulked across reps, thus only a single measurement was available for each accession/tissue combination.

Concentrations of compatible solutes were determined for the growth chamber experiment using HPLC protocols developed by Naidu (1998) and performed on an Agilent 1100 HPLC platform (Agilent, Santa Clara, CA) using RI detection and a Waters Sugar-Pak I 6.5 X 300 mm column (Waters, Milford, MA) maintained at 80°C with a mobile phase of 5 mg $L^{-1}$ Ca-EDTA (Sigma-Aldrich, St. Louis, MO) and a flow rate of 0.6 ml $min^{-1}$ with 20 µl injection volume. For these concentrations, a single measurement was made for each rep (tissue bulked across blocks), but only a single rep of data was collected for CICA-17. HPLC standards were purchased from Sigma Aldrich (St. Louis, MO), including betaine, pinitol, proline, sorbitol, trehalose, and trigonelline.

**RNA extraction and quantitative PCR**

RNA was extracted from leaf and root tissue using an RNeasy® Plant Mini Kit (Qiagen, Valencia, CA) according to the supplied protocol. The RNA was cleansed of contaminating DNA using a TURBO DNase Kit (Ambion, Austin, TX) and quantified using a bioanalyzer (Agilent, Santa Clara, CA). Primers for quantitative PCR were developed from gene sequences found in genbank. Betaine-aldehyde dehydrogenase (*BADH*) primers were created from a consensus sequence generated from *Beta vulgaris* (AB221006), *Atriplex micrantha* (EF208902), and *Spinacia oleracea* (FJ595952). *NHX1* primers were generated in conserved domains

using a consensus sequence generated by accessions *Mesembryanthemum crystallinum* (AM746985), *Atriplex gmelini* (AB038492), and *Chenopodium glaucum* (AY371319). *TIP2* primers were created using a consensus sequence created from *Brassica napus* (AF118381), *A. thaliana* (NM_113559), *Gossypium hirsutum* (AY821911), and *Oryza sativa* (D25534). *SOS1* (*Salt overly sensitive1*) primers were developed from the *C. quinoa* SOS1 gene reported by (Maughan et al., 2009). *GAPDH* primers were previously reported by (Balzotti et al., 2008).

All primer sequences are reported in Additional Table 1. RNA was converted to cDNA using a high capacity RNA-to-cDNA master mix reagents Kit (ABI, foster city, CA), and real-time PCR was run using fast SYBR green master mix (Roche, Indianapolis, IN) on a 7300 real time PCR system (Applied Biosystems, Foster City, CA) according the manufacture"s recommendations. Relative quantifycation of gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

*GAPDH* served as an endogenous control gene and RNA extracted from leaf and root control tissues (low salt or recovery control) served as baseline calibrators. All RT-PCR reactions consisted of two technical replicates for each of three biological replicates. Primer sequences are shown in Additional Table 1.

## RESULTS

### Comparative halophyte analysis

The salt tolerances of quinoa accessions Chipaya and KU-2 and *T. halophila* were assessed in a single greenhouse experiment. Relative plant weight was calculated as a ratio between treated plants and their non-stressed respective control (Figure 1). All three plant types (two types of quinoa and *T. halophila*) had an adverse weight response to salt at all treatment levels. The sharper decline of relative weight in *T. halophila* than in quinoa indicated that quinoa is more resilient to salt stress under these specific conditions than *T. halophila*. At 300 mM NaCl *T. halophila* averaged a 10-fold decrease in fresh weight compared to its control, but Chipaya and Ollague only averaged a 2-fold decrease in fresh weight. Remarkably for a food crop, quinoa survived nearly four weeks at 600 mM NaCl and nearly six weeks at 450 mM NaCl, the approximate life cycle of the control plants, although chlorosis of the bottom leaves was pronounced in high-salt treated plants (Additional Figure 1).

While there was an expected pronounced difference between quinoa and *T. halophila*, there were only slight differences between the two quinoa types. Only one treatment level had a significant difference between plant weight means (150 mM). Thus, both types appeared to have equal tolerance to high salt concentrations in the soil. Slow but steady plant growth was observed at intermediate (150 mM) and high (300 mM) levels of salt (Additional Figure 2).

Increases in plant height and weight were halted or paused at the highest treatment levels (450 mM and 600 mM) for the duration of the salt treat-ments. The arrest of plant growth (and a corresponding delay of flowering time) suggested that plants at the highest treatment
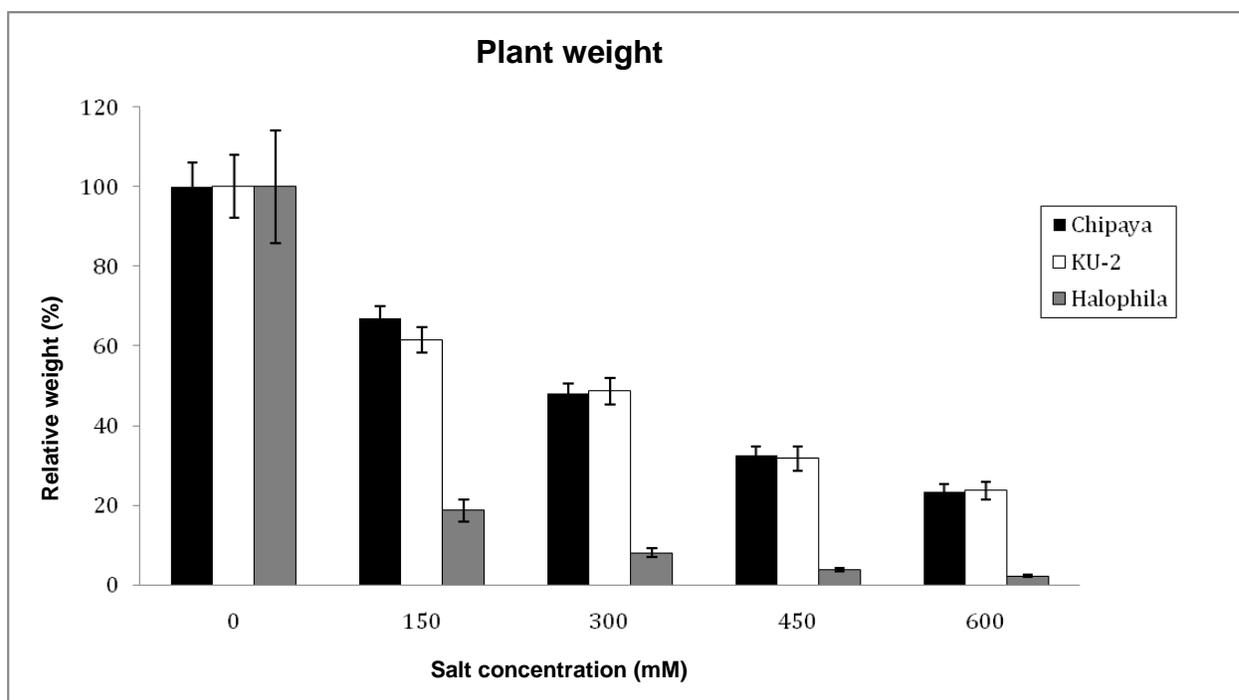
**Plant weight**



**Figure 1.** Quinoa and *T. halophila* height and weight relative to their respective control. Plants height and weight were combined over all time points and the average weight relative to the control was calculated. Bars represent standard error for each treatment/plant combination n=35.

levels entered into a stress induced dormant state. After prolonged treatment (plus 4 weeks), high-salt treated quinoa plants began to shed their chlorotic leaves, perhaps as a final mechanism of salt exclusion. Instead of the slight difference that was observed, we had expected a greater difference between the two cultivars since Chipaya was adapted to the highly saline environments of the southern Altiplano while KU-2 was adapted to the temperate, costal environments of Chile. Perhaps, salt tolerance is characteristic of the *Chenopodium* genus, rather than a specialized trait of individual cultivars.

**Physiological assessment of salt-stressed quinoa in hydroponics**

Further characterization of quinoa (Chipaya, Ollague, and CICA-17 cultivars) as a halophyte was performed using plants grown hydroponically under one of four salt treatments HS, LS, RT, and RC. As was observed in the greenhouse experiment, all three cultivars exhibited significant growth reduction with high salt treatment. A reduction of approximately 60% of fresh root and shoot weight was observed when treated samples were compared to their corresponding controls (Figure 2). At the end of treatment, RT plants exhibited shoot and root fresh weights equal to or exceeding their controls. The RT plants resumed growth after stress-induced dormancy while the recovery-controls (RC) had already passed their peak growth. Again, no significant differences were

observed in biomass between the salares and valley ecotypes under high salt conditions. Stomatal conductance measurements for all cultivars in the hydroponic experiment followed a pattern inversely proportional to that of the salt concentration and a different degree of stress was observed between the salares and valley ecotypes. At the peak of salt stress (450 mM) the salares ecotypes, Chipaya and Ollague, maintained 48 and 51% of the control transpiration rate, respectively, while the valley ecotype, CICA -17, maintained 39% of its control transpiration rate indicating a modest increase in stress in the valley type (Table 1).

Furthermore, the average control transpiration rates were 210 mm $m^2s^{-1}$ the valley type and 168 mm $m^2s^{-1}$ for both salares types. This transpiration difference may be a result of artificial selection resulting in greater water retention in the salares ecotypes than in the valley type.

**Biochemical assessment of salt-stressed quinoa in hydroponics**

ICP analysis of Chipaya leaf tissue revealed that the $Na^+$ concentration in the high salt treatment was almost twice that of the low salt treatment, increasing from 26.69 mg g $DW^{-1}$ to 43.68 mg g $DW^{-1}$. Analysis of Chipaya root tissue revealed a similar pattern with a 2.6-fold increase of $Na^+$ from 15.95 mg g $DW^{-1}$ in the low salt treatment to 42.05 mg g $DW^{-1}$ in the high salt treatment (Additional Figure 3). A similar pattern was observed in Ollague and CICA -17 leaf and root tissue. $K^+$ levels were generally reduced

## Root Fresh Weight
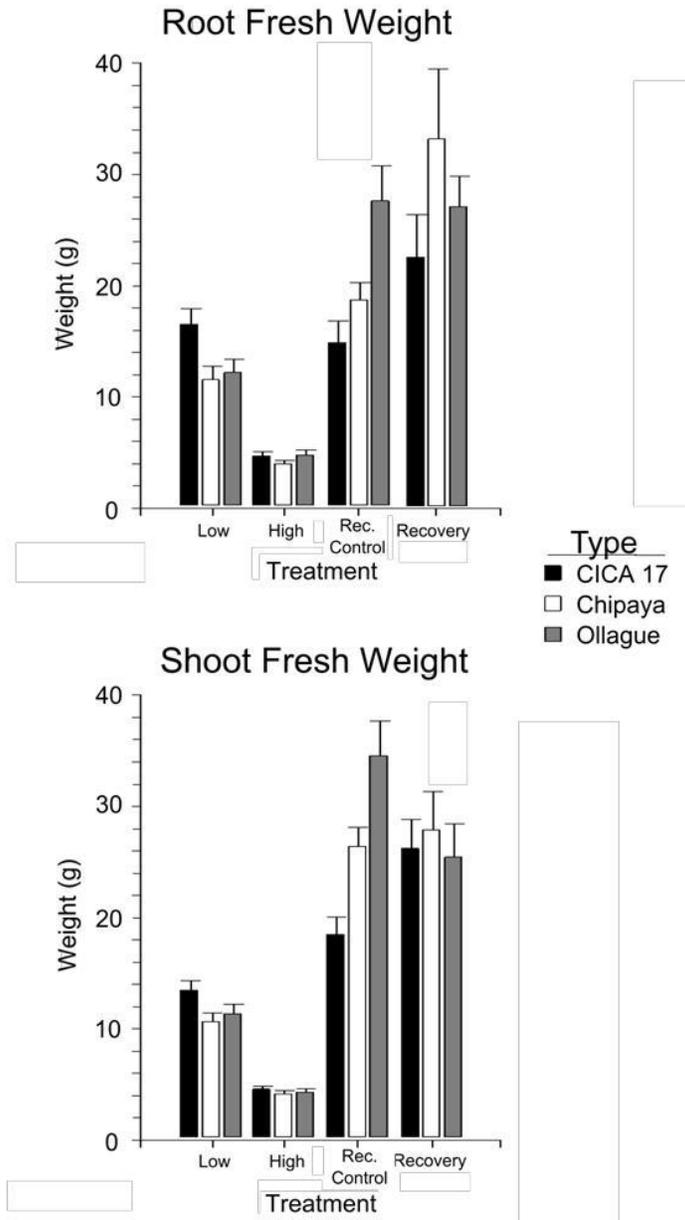


## Shoot Fresh Weight



**Figure 2.** Root and shoot fresh weight of Chipaya, Ollague, and CICA -17 quinoa cultivars grown in a growth chamber in hydroponics under high salt (gradual increase to 450 mM NaCl), low salt (50 mM for duration, harvested with high salt), recovery (gradual increase to 450 mM NaCl followed by gradual decrease to 50 mM NaCl), or recovery control (50 mM for duration harvested with recovery). Measurements were taken at the time of harvest for each treatment. For each cultivar/treatment combination n=16. Bars are mean ± SE.

in stressed tissues compared to the corresponding control (2 to 40%). Ollague leaves had the highest concentrations of both $Na^+$ and $K^+$ ions in the HS treatment (Additional Figure 1). In all of the cultivars, the leaf sodium content did not significantly decrease in the recovery treatment despite the decrease in sodium concentration in the growth media. However, root sodium content in the recovery treated plants decreased to normal levels. A decrease in the concentration of calcium was also observed in both leaf and root tissue in all three cultivars under salt stress. Concentrations of other ions were also measured, but only modest changes were found. Since plants are known to use compatible solutes to maintain osmotic balance with their environment, we

**Table 1.** Stomatal conductance of Chipaya, Ollague, and CICA -17. Stomatal conductance (mmol m$^{-2}$s$^{-1}$) was taken 24 h after treatment was administered and was measured twice on each plant for a total of eight measurements per cultivar-treatment combination per day.

| Na$^+$ Trt (mM) | 50 | 200 | 450 | 200 | 50 |
|---|---|---|---|---|---|
| (Day) | (1) | (4) | (11) | (16) | (21) |
| Chipaya HS | 217.8 | 123.0 | 93.3 | 99.3 | 153.4 |
| Chipaya LS | 193.9 | 184.8 | 191.7 | 146.9 | 128.8 |
| Difference | -23.9 | 61.8 | 98.4* | 47.6 * | -24.6 |
| Ollague HS | 199.7 | 106.4 | 92.6 | 109.3 | 165.9 |
| OllagueLS | 179.4 | 164.1 | 182.4 | 149.6 | 160.0 |
| Difference | -20.3 | 57.7* | 89.8* | 40.3 | -5.9 |
| CICA-17 HS | 222.3 | 146.4 | 79.1 | 108.0 | 167.6 |
| CICA-17 LSl | 218.1 | 229.6 | 203.2 | 186.4 | 213.6 |
| Difference | -4.2 | 83.2* | 124.1* | 78.4* | 46.0 |

\* Significantly different between the control and treatment ($p < 0.05$).

**Table 2.** Compatible solutes concentrations ($\mu$mol g$^{-1}$ DW) with significant differences between treatment and control.Trigonelline, betaine, and trehalose were measured in Chipaya and Ollague leaves and roots under high salt (HS; 450 mM), low salt (LS; 50 mM harvested simultaneously with HS), recovery treatement (RT; salt incrementaly reduced from 450 mM by 50 mM), or recovery control (RC; 50 mM harvested simultaneously with RT) salt treatments. For each chipaya and ollague, cultivar-treatment combination n=3. Asterisks indicate significant mean contrasts between treatment and its corresponding control (* indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, [§]indicates n=2).

| Tissue | Variety | Treatment | Trigonelline | Trehalose | Betaine |
|---|---|---|---|---|---|
| Leaf | Chipaya | HS | 4846 ± 106** | 75±34 | 356 ± 36 |
| | | LS | 1718 ± 515 | 48±31 | 204 ± 112 |
| | | RT | 5262 ± 1080*** | 47±14 | 257 ± 28 |
| | | RC | 1702 ± 921 | 50±19 | 316 ± 205 |
| | Ollague | HS | 5343 ± 1162** | 60±3 | 431 ± 81* |
| | | LS | 3478 ± 554 | 38±17 | 66 ± 43 |
| | | RT | 2039 ± 396 | 41±10 | 139 ± 58 |
| | | RC | 2398 ± 196 | 34±4 | 189 ± 39 |
| Root | Chipaya | HS | 6712 ± 208*** | 92 ± 3*** | 192 ± 26** |
| | | LS | 3478 ± 554 | 38±17 | 66 ± 43 |
| | | RT | 3065 ± 346 | 43±9 | 84 ± 5 |
| | | RC | 2346 ± 766 | 40±3 | 39 ± 27 |
| | Ollague | HS | 8239 ± 673[§]*** | 84±7 | 189 ± 151 |
| | | LS | 4224 ± 1010 | 59±31 | 127 ± 45 |
| | | RT | 3873 ± 259 | 72±23 | 120 ± 16 |
| | | RC | 3261 ± 848 | 73±19 | 105 ± 20 |

also measured concentrations of betaine, pinitol, proline, sorbitol, trehalose, and trigonelline in quinoa root and leaf tissue from the plants grown in the hydroponic experiment. In these plants, large quantities of betaine, trehalose, and trigonelline (Table 2) and negligible quantities of sorbitol, pinitol, and proline were detected (data not shown). Significant changes in betaine, trehalose, and trigonelline were observed in response to salt stress.

In particular, trigonel- line accumulated to very high concentrations ranging from 800 to 7000 $\mu$mol g$^{-1}$ DW depending on the tissue and treatment regime. This concentration in both leaves and roots far exceeds that reported in other crop species such as 4.0 $\mu$mol g$^{-1}$ DW in salt stressed soybean (Wood, 1999), 2.4 $\mu$mol g$^{-1}$ FW in sweet corn (Rhodes et al., 1989), and 12.3 $\mu$mol g$^{-1}$ DW in salt stressed tomato (Rajasekaran et al., 2001).

In leaf tissue, large differences in trigonelline concentration were found between high salt and low salt treatments. Both Chipaya and Ollague had a relatively similar response of trigonelline production in the HS compared to their respective controls (Table 2). However, these two varieties had different responses to the recovery treatment where there was a significant difference in quantities of trigonelline between RT and RC treatments in Chipaya, but the difference between treatment and control was not significant for Ollague. This suggests that distinct varieties of quinoa adapted to the Altiplano respond differently to changes in saline environments. It is also an interesting finding because $Na^+$ concentrations did not decrease in leaf tissue for any variety after the treatment of reduced salt was applied. Indeed, significant variation exists between Altiplano lines and long-term breeding projects have been created to select high performing varieties from the residual natural variation (Bonefacio, personal communication). Perhaps, the reduced levels of trigonelline are an indication of actively growing quinoa instead of a salt stressed induced dormancy. Alternatively, this could be a mal-adaptive trait in Chipaya where continued trigonelline production was at the expense of seed yield.

In root tissue, compatible solute concentrations mirrored $Na^+$ concentration. There were highly significant differences between HS and LS treatments, but in the RT treatments solutes were detected in much lower concentrations (similar to $Na^+$) and they were not significantly different than their respective controls. Presumably, compatible solutes were not longer produced once the $Na^+$ had left the root tissue, though residuals of compatible solute production were perhaps detected in gene expression through qPCR.

**Homologous gene expression**

Transcriptional changes at several candidate salt-tolerance genes (*BADH*, *SOS1*, *NHX1* and *TIP2*) were measured during salt stress in quinoa using qRT-PCR (Figure 3). In leaves, an ANOVA of real-time PCR data indicated that there were no differences in expression between cultivars of any of the genes tested (p-value <0.05). The relatively stable expression of *BADH*, *SOS1*, *NHX1*, and *TIP2* in leaves suggested that the mechanisms associated with these genes were in a constant active state regardless of the degree of salt stress (that is, our control had 50 mM NaCl). Expression of NHX1 suggested that sequestration of sodium from the cytoplasm to the vacuole also occurred in quinoa. *TIP2* expression in quinoa (particularly in Chipaya) suggested that water transport between the vacuole and the cytoplasm is increasingly important and may play a role in osmotic homeostasis (Zhang et al., 2008). Since there was negligible variation of plant weight at nearly all time points (i.e. growth rates) among the quinoa accessions,

the lack of expression variation of these selected genes also suggested that other genes may be involved in the mechanisms of salt stress response.

In roots, there were some notable differences between salares and valley ecotypes. GAPDH-normalized expression values of *BADH* and *SOS1* were up-regulated in the salt stressed roots of salares types compared to the control (that is, expression values >1). In these types, there was also more expression in high salt treated roots than in recovery treated roots. The up-regulation of *BADH* in roots suggests that betaine plays an important role in counteracting salt stress in roots. Indeed, expression of *BADH* may represent residual gene expression of compatible solute synthesis enzymes after the salt had been gradually reduced in the saline growth media. Alternatively, multiple copies of *BADH* may be present in the quinoa genome. Perhaps, the copy we assayed responded to stress-induced expressed in roots, but not leaves. Two copies of *SOS1* are present in the quinoa genome, likely the result of a historical genome-duplication event (Maughan et al., 2009). To ensure that all *SOS1* activity was observed, primers were designed which would amplify both copies of *SOS1* that are present in the quinoa genome. The expression level of *SOS1* in the roots of salares quinoa suggests that cytoplasmic $Na^+$ was moving out of the roots, particularly in the salares types.

**DISCUSSION**

**Halophyte comparison**

Our comparison of *C. quinoa* and *T. halophila* demonstrated that *C. quinoa,* a South American staple crop, exhibited greater salt tolerance than the model halophyte in a greenhouse environment. This study was not intended to identify the „most extreme salt tolerance" plant (quinoa or *T. halophila*) in every environment. Rather, it was to provide perspective to future research efforts on halophytic quinoa and to justify extrapolation of identified *T. halophila* molecular physiology of salt stress to quinoa. While the negative effects of increased salt concentration were more pronounced in *T. halophila* than in quinoa (Figure 1), these findings suggested that quinoa entered into stress-induced dormancy under saline conditions even while buds were evident on the main stems. This state of dormancy was not seen in *T. halophila,* perhaps because its commitment to flowering during our germi-nation procedure was greater than that possessed by both types of quinoa. *T. halophila* has been used as a model plant for abiotic stress studies (Inan, 2004), in part due to its native range where cold temperatures and poor poor soils are common. The difference in salt tolerance and dormancy between *T. halophila* and quinoa could be that quinoa is a specialized halophyte of saline soils while *T. halophila* is not. It could also be explained if
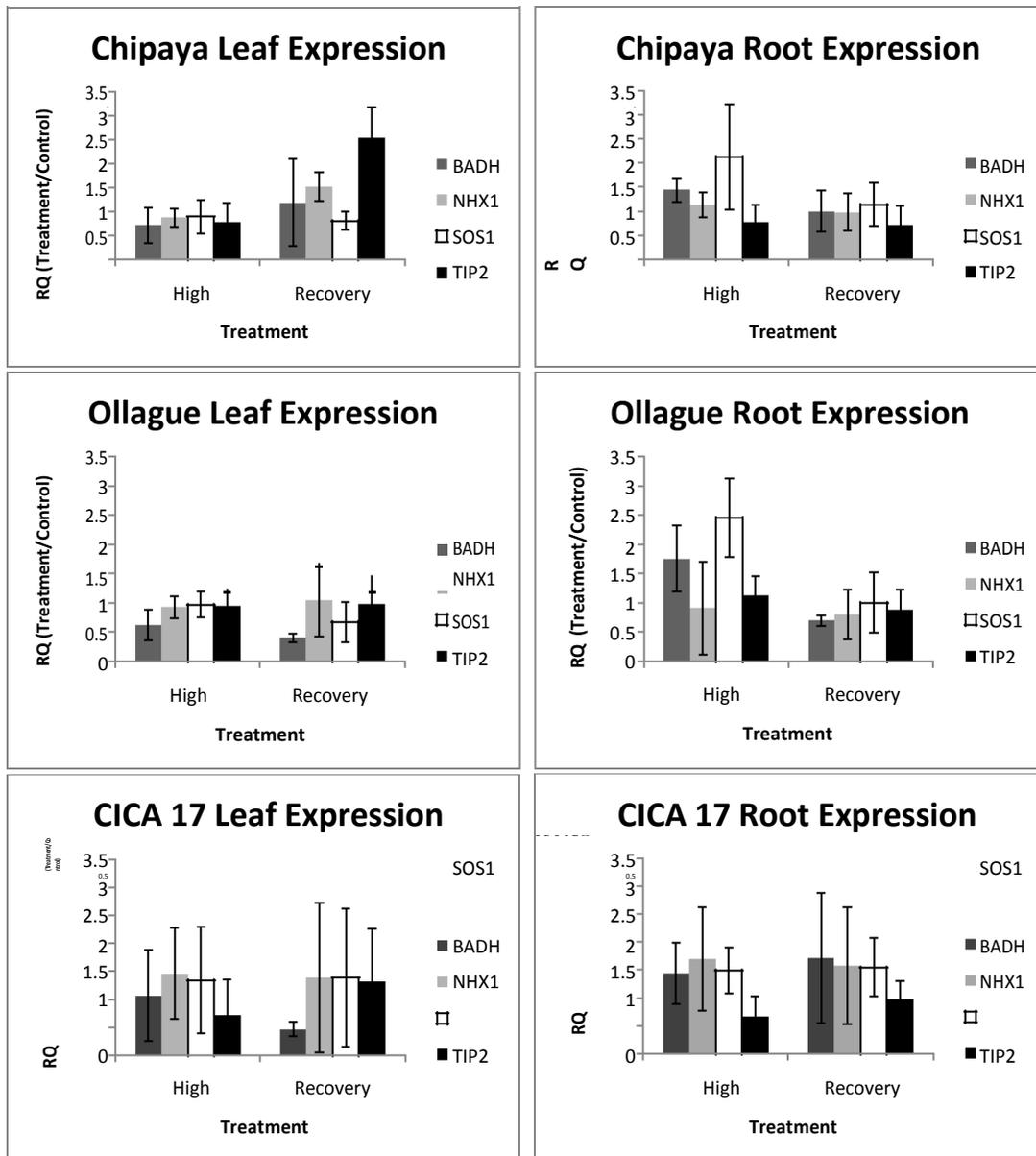
**Figure 3.** Gene expression of *BADH*, *NHX1*, *SOS1*, and *TIP2* in Chipaya, Ollague, and CICA -17 leaf and root tissue using real-time PCR. Relative quantification is expressed as a ratio of treatment to its corresponding control. GAPDH (not shown) was used as an endogenous control and all expression values were first normalized to GAPDH prior to forming the treatment/control ratio. Slight differences in GAPDH levels between leaves and roots made comparisons between tissues into general approximations. Bars represent standard deviation.

*T. halophila* was less suited for our greenhouse conditions than quinoa. With this side-by-side comparison, we have shown a similar loss in plant weight between quinoa and halophila, relative tolerances to very high salt conditions in a single environment, and a difference in stress-induced dormancy of salt stress in mature quinoa. Retardation of growth under salt stress has been well documented in many organisms including *T. halophila* (Inan et al., 2004), *A. thaliana* (Attia et al., 2008), and a

distantly related crop to quinoa, sugar beet (Ghoulam et al., 2002). Inhibition of growth, or dormancy, as a mechanism for salt tolerance was described by He et al. (2002).

They identified a dormancy-related gene expressed in salt tolerant varieties of rice during salt stress that was homologous to *PsDRM1*, a gene associated with dormancy in peas (Stafstrom et al., 1998). In this study, the high-salt treated and recovery-treated quinoa appeared

to enter a dormant state under salt stress.

The days-to-flower of the untreated quinoa closely resembled the days-to-flower in salt stressed quinoa once the days under salt stress (days in dormancy) were subtracted. After the experiment, quinoa plants that survived the 450 and 600 mM treatments were given time to recover by watering with tap water. They eventually resumed plant growth and developed fertile seed heads; however differences in seed yield between treated and untreated plants was not measured.

## Tolerance mechanisms in quinoa

In this experiment, compatible solute production was a mechanism that quinoa used to manage salt stress and osmotic potentials across cellular membranes.

A recent study assessed compatible solute concentrations in cotyledons of quinoa seedlings at 250 mM and suggested these solutes play a minor role in osmotic adjustment because of the low levels of glycinebetaine (3–6 $\mu$mol $g^{-1}$ FW) and proline (7–13 $\mu$mol $g^{-1}$ FW) (Ruffino et al., 2010). Trigonelline levels were not measured in that experiment, neither were measurements made in mature leaves.

A different experiment has suggested that osmotic adjustments of quinoa are primarily the result of balanced levels of inorganic ions across cellular membranes (Hariadi et al., 2011). Indeed, noticeable increases in shoot sap osmolality were found from $Na^+$ and $K^+$ ion levels.

However, concentrations compatible solutes were not measured in mature leaves. We detected a large increase in both inorganic ions and compatible solute concentrations when quinoa plants are exposed to stressful saline environments.

Our data suggests that both tolerance mechanisms are necessary to confer normal dormancy under stressful conditions. Potential genetic knock-out experiments could uncouple the two mechanisms and potentially identify which mechanism would be the most effective target for breeding strategies of salt tolerance.

Betaine, trehalose, and trigonelline were shown to increase in response to salt as a coping mechanism in quinoa with the later appearing to have the greatest correlation. Trigonelline production in quinoa was very high (5000-7000 $\mu$mol $g^{-1}$ DW) compared to other reported studies and it likely had a significant impact on the soil osmotic gradient across the membrane.

While betaine may not have accumulated to concentrations high enough to alter osmotic balance, its increased presence may have been involved in other forms of stress protection (directly or indirectly) (Sakamoto and Murata, 2002). Given that trigonelline can be a betaine derivative, it is possible that the lower betaine concentrations were the result of trigonelline synthesis. There was no apparent mechanism for salt removal in leaf tissue (for example, ICP $Na^+$ levels of recovery

plants) and the continued presence of compatible solutes was likely required in leaf tissue even after the salt stress was reduced.

Varietal comparisons of salt tolerance have been reported in other organisms. Significant differences in growth were observed between varieties of rice (Moons et al., 1995) and barley (Chen et al., 2007) although salt stress impaired growth to a large extent in all of the varieties tested. Betaine accumulation was also reported to vary significantly between varieties of wheat under salt stress and high betaine levels were correlated with high salt tolerance (Zhao et al., 2005).

In this experiment, the Altiplano ecotypes exhibited modest, but significant, differences from the valley ecotype in compatible solute accumulation. Fresh weight harvests of both ecotypes after treatment in hydroponics showed similar results. CICA-17, the valley ecotype, under 450 mM salt stress showed a 66% decrease in weight relative to its control while Chipaya and Ollague showed a 61 and 62% decrease respectively (Figure 2). The greatest difference among quinoa comparisons was in recovery treatment after high extreme salt stress.

For example, CICA-17 exhibited a more pronounced drop in stomatal conductance as the salt concentration increased, its transpiration rate was not able to recover from stress as well as the altiplano ecotypes, and the expression of candidate salt-tolerance genes showed increased expression after „recovery" (that is, a higher degree of continued perceived stress) compared to the salares types (Table 1).

Quinoa was able to accumulate salt using water retention mechanisms such as decreased stomatal conductance, sequestration, and compatible solutes. It was also able to exclude salt in roots and employed *SOS1* and SOS-related mechanisms to prevent salt stress as suggested by the gene expression data.

While different tolerance mechanisms have been identified in this experiment, individually they require investigation and the potential to discover novel mechanisms or alleles requires a more effective method of exploration. Salt stress genes have been identified through the use of a DNA microarray in many organisms including *Arabidopsis* (Gong et al., 2005), *T. halophila* (Taji et al., 2004), maize (Qing et al., 2009), sunflower (Fernandez et al., 2008) and cotton (Hall, personal communication).

A microarray or RNA-seq experiment comparing the expression profiles of quinoa under high salt, low salt, and recovery conditions would provide a comprehensive assessment of the genes involved in salt stress and improve our understanding of the physiological mechanisms used by quinoa to tolerate high saline soils.

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**Additional Table 1.** Primer information used for real-time gene expression analysis.

| Primer design | | | |
|---|---|---|---|
| Gene | Length | Forward/reverse sequence | T$_m$ (°C) |
| SOS1 | 24 | 5'-TAGCATCAGTGTTFTGGCTCGGAT-3' | 60.3 |
|  | 24 | 5'-AAAGTCATCACGGTCAGGACACCA-3' | 60.2 |
| NHX1 | 24 | 5'-ATCAGTTTACGAGGTCAGGGCACA-3' | 60.1 |
|  | 24 | 5'-GAGGCTTTGTCAGCAACCCAAACA-3' | 60.3 |
| TIP2 | 24 | 5'-CGCACCAATCGCCATAGGTTTCAT-3' | 60 |
|  | 24 | 5'-AGTCCACCACCGATAAGAGGACCA-3' | 61.3 |
| BADH | 28 | 5"- CCACATCCATGCAAATATGGAAAGAGGA-3" | 59.0 |
|  | 25 | 5"-TGAGCAATTAACCCAAACAGCTCCA-3" | 59.1 |
| GAPDH | 25 | 5'-GGTTACAGTCATTCAGACACCATCA-3' | 56.7 |
|  | 21 | 5'-AACAAAGGGAGCCAAGCAGTT-3' | 57.6 |



**Additional Figure 1.** Five pots of *T. halophila*plants (top row) and five pots quinoa plants, cultivar KU-2 (bottom row) two weeks after the start of treatment. Each pot represents a different salt concentration treatment. In both rowsof pots, the concentration of salt treatments increase from left to right with 0mM (white tag) on the left followed 150 mM (blue), 300 mM (green), 450 mM (yellow), and 600 mM (red) on the right.
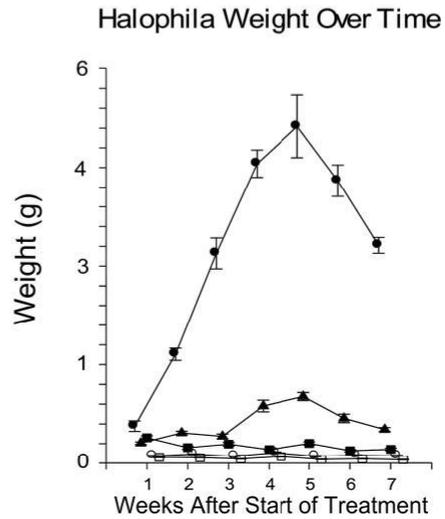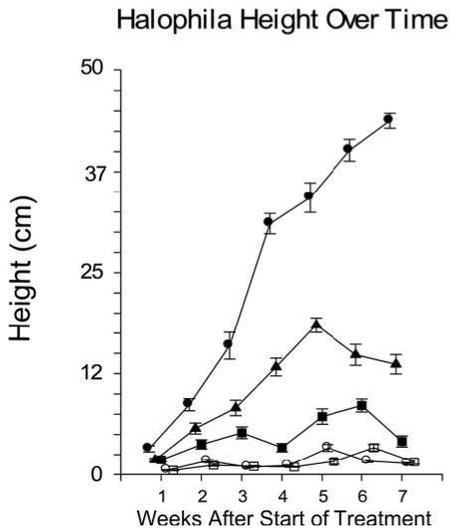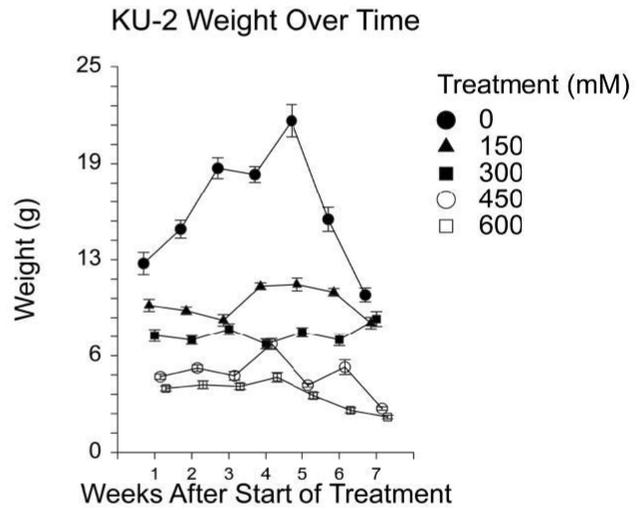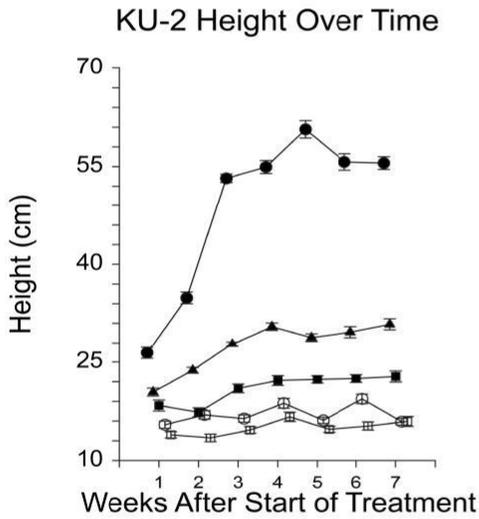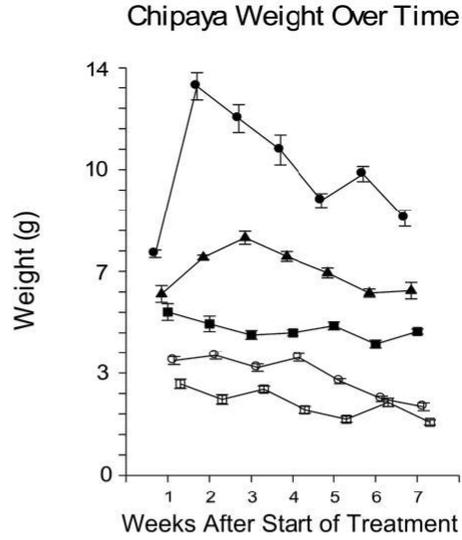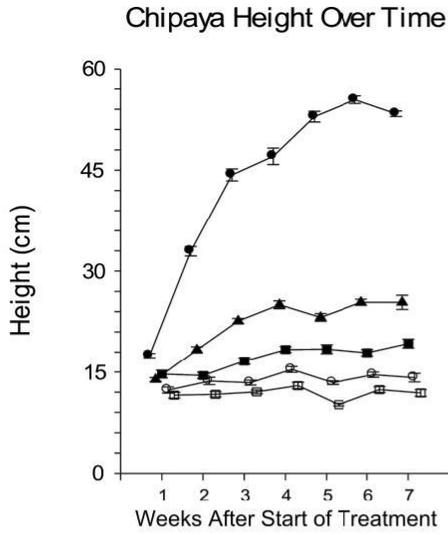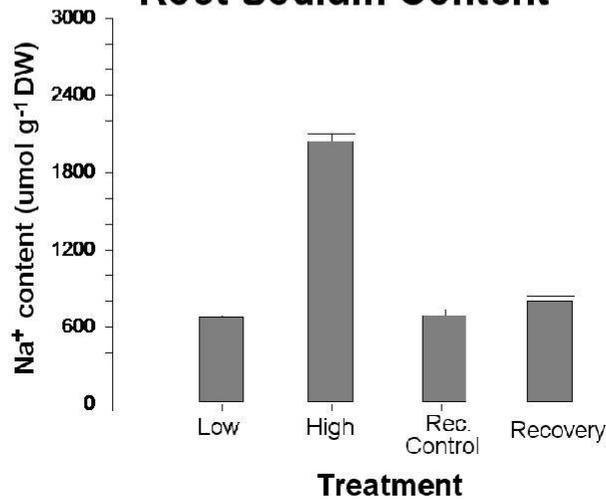
**Additional Figure 2.** Chipaya, KU-2, and *T. halophila* height and weight over time. Plant height and weight were measured weekly after the start of treatment. All treatments are represented and n=5 for each data point. Bars represent standard error.

## Leaf Sodium Content
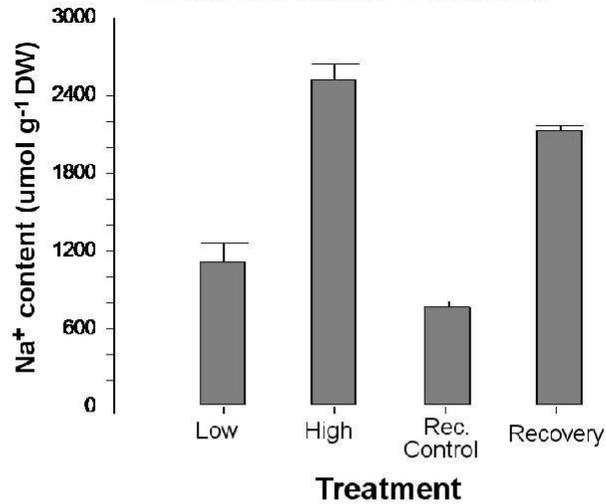


## Root Sodium Content



**Additional Figure 3.** ICP Analysis on quinoa to quantify sodium concentration in leaf and root tissue. Samples were collected from leaf and root tissue under high salt (450 mM), low salt (50 mM harvested with high salt), recovery (450 mM followed by 50 mM), or recovery control (50 mM harvested with recovery) salt treatments. Equal amounts of fresh weight tissue from 16 samples was combined and desiccated (n=16). Following desiccation one ICP analysis was run on each bulked sample. Results were averaged across cultivars (n=3). Bars represent standard error.