plant biology

Akaline, saline and mixed saline-alkaline stresses induce physiological and morfo-anatomical changes in Lotus tenuis shoots.

Journal:	*Plant Biology*
Manuscript ID:	Draft
Manuscript Type:	Research Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Paz, Rosalía; Unidad de Biotecnología 1, IIB-IINTECH/UNSAM-CONICET, Chascomús, Buenos Aires, Argentina, Reinoso, Herminda Espasandin, Fabiana González Antivilo, Francisco SANSBERRO, PEDRO; Facultad de Ciencias Agrarias UNIVERSIDAD DEL NORDESTE, 3Instituto de Botánica del Nordeste Rocco, Ruben; Unidad de Biotecnología 1, IIB-IINTECH/UNSAM-CONICET, Chascomús, Buenos Aires, Argentina, RUIZ, OSCAR; INTECH-CONICET, MENÉNDEZ, ANA; UNIVERSITY OF BUENOS AIRES, DEPARTMENT BIODIVERSITY AND EXPERIMENTAL BIOLOGY
Keyword:	salinity, alkalinity, shoot anatomy, proline, osmotic potential, transpiration

Akaline, saline and mixed saline-alkaline stresses induce physiological and morfo-anatomical changes in *Lotus tenuis* shoots.

Abridged title: Long term responses to neutral and alkaline stress

Abstract

Saline, alkaline and mixed, saline-alkaline conditions frequently co-occur in soil. In this work, we compared these plant stresses sources on the legume species *Lotus tenuis*, regarding their effects on shoot growth, and leaf and stem anatomy. In addition, we aimed at gaining insight on the plant physiological status of stressed plants. We performed pot experiments with four treatments, control without salt addition (pH=5.8; EC=1,2 dS m^{-1}) and three stress conditions: saline (100 mM NaCl, pH=5.8; EC=11 dS m^{-1}), alkaline (10 mM $NaHCO_3$, pH=8.0, EC= 1.9 dS m^{-1}), and mixed salt–alkaline (10 mM $NaHCO_3$ plus 100 mM NaCl, pH=8.0, EC= 11 dS m^{-1}). Neutral and alkaline salts produced a similar level of growth inhibition on *L. tenuis* shoots, whereas their mixture exacerbated their detrimental effects. Our results showed that none of the analyzed morpho-anatomical parameters categorically differentiated one stress from the other. However, NaCl and $NaHCO_3$-derived stresses could be discriminated by different extents and/or directions of changes in some of the anatomical traits. For example, alkalinity led to increased ostiolar opening area, conversely to NaCl-treated plants, where a stomatal aperture reduction was observed. Likewise, plants from the mixed saline-alkaline treatment characteristically lacked of a palisade mesophyll in their leaves. The stem cross-section and vessels areas, as well as the number of vascular bundles in the sectioned stem were reduced in all treatments. A rise in the number of vessel elements in the xylem was registered in NaCl-treated plants, but not in those treated exclusively with $NaHCO_3$.

Keywords: salinity, alkalinity, shoot anatomy, proline, osmotic potential, transpiration

Authors:

Paz, Rosalía Cristina 1,5

E-mail: rosaliapaz@gmail.com

Reinoso, Herminda 2

E-mail: hreinoso@exa.unrc.edu.ar

Fabiana Daniela Espasandin 4

E-mail: fabidani1@hotmail.com

González Antivilo, Francisco Alberto 5

E-mail: fgonzalezantivilo@fca.uncu.edu.ar

Sansberro, Pedro Adolfo 4

E-mail: sansber@agr.unne.edu.ar

Rocco, Ruben Anibal 1

E-mail: rubenrocco@intech.gov.ar

Ruiz, Oscar Adolfo 1#

E-mail: ruiz@intech.gov.ar

Menéndez, Ana Bernadina 1,3, #*

E-mail: anamenendez@intech.gov.ar

#A. Menéndez and O. Ruiz contributed equally and should be considered co-senior authors.

1 Unidad de Biotecnología 1, IIB-IINTECH/UNSAM-CONICET, Chascomús, Buenos Aires, Argentina

2 Laboratorio de Morfología Vegetal, Departamento de Ciencias Naturales, Universidad Nacional de Río Cuarto, 5800, Rio Cuarto, Córdoba, Argentina.

3 Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina

4 Facultad de Ciencias Agrarias (UNNE), Instituto de Botánica del Nordeste (IBONE-CONICET), Sargento Cabral 2131, Casilla de Correo 209, W3402BKG Corrientes, Argentina.

5 Facultad de Ciencias Agrarias (FCA), Instituto de Biotecnología Agrícola de Mendoza (IBAM), Alte. Brown 500, Chacras de Coria, Lujan de Cuyo, 5505, Mendoza, Argentina.

*Complete correspondence address

Dra. Ana B. Menéndez

Departamento de Biodiversidad y Biología Experimental

Facultad de Ciencias Exactas y Naturales,

Universidad de Buenos Aires.

Piso 4 Pab II Ciudad Universitaria (1428)

Buenos Aires

Argentina

Tel: (05411) 45763300-09 int 202

Tel/Fax: (05411) 47872706

email: anamen@bg.fcen.uba.ar

Introduction

Saline stress refers to the presence of neutral salts such as NaCl or Na_2SO_4 in soil, whereas alkaline stress is only related to the occurrence of alkaline salts (Na_2CO_3 or $NaHCO_3$, Yang *et al*., 2007). Saline and alkaline stresses affect more than 10% of world arable lands, limiting agricultural production (Lauchli and Luttge, 2002). Both conditions often co-occur in nature, with variable neutral to alkaline salt proportions according to the soil (Shi and Wang, 2005; Li *et al*., 2010).

Saline soils affect plant growth by inducing osmotic inhibition of water absorption (Munns 2002). Therefore, cells need to adjust osmotically and reestablish the ion balance (Li *et al.* 2003). Saline stress also induces ion injury in plants, inhibiting the activity of several enzymes, and hampering protein synthesis, photosynthesis and energetic metabolism (Tester and Davenport, 2003). Alkalinity can disrupt the balance of ions (Shi and Zhao 1997), cause micronutrient deficiency due to alteration of micronutrients availability in soil (Alam, *et al*., 1999) and change antioxidant enzymes, amino acids and carbohydrate composition (Zhang *et al*., 2012; Kukavika *et al*., 2013).

At the morphological level, NaCl alters morphological and anatomical characteristics of leaves and stems such as leaf thickness, stem cross section area or size and number of xylem vessel (Kiliç *et al.*, 2007; Boughalleb *et al*., 2009; Dolatabadian *et al*., 2011). Whereas the relationship between salinity and morpho-anatomical plant responses has been explored to some extent, reports on the effects of alkaline or mixed salt-alkaline stresses are limited to few plant species such as aspen (Mandre *et al*., 2012), *Pisum sativum* (Gharsalli *et al.* 2001), *Catharanthus roseus* (Cartmill *et al.* 2008), and *Phaseolus vulgaris* (Valdez-Aguilar and Reed 2008).

Lotus tenuis (Waldst. and Kit., syn. *L. glaber*) is a glycophytic forage legume, well adapted to the lowlands of the Buenos Aires Province (the most important cattle production region in Argentina). This region is characterized by the presence of soil Na_2CO_3 and $NaHCO_3$ (Costa and García, 1998), the main sources of high soil alkalinity (pH > 9.0). In a previous work, the response

of *L. tenuis* plants to saline, alkaline and mixed saline-alkaline stresses were compared in relation to plant growth, key nutrients accumulation, and root architecture and anatomy (Paz *et al*., 2012). However, the morpho-anatomical responses of *L. tenuis* shoots to these stresses remained unexplored.

With the purpose of increasing current knowledge on differences and similarities between the above mentioned stresses, in this work we proposed to compare saline, alkaline and mixed saline-alkaline stresses on *L. tenuis,* regarding their effects on shoot growth, and leaf and stem anatomy. In addition, we have prospected leaf proline content, gas exchange, transpiration and osmotic potential in order to gain insight on the plant physiological status of *L. tenuis* plants grown under these stress conditions.

Materials and Methods

Plant growth conditions.

Seeds of *L. tenuis* cv. Esmeralda were scarified with sulphur acid (100%), washed in distilled water and sown in Petri plates containing water-agar (0.8%). Plates were incubated during 7 days in a growth chamber, with a 16/8 h photoperiod at (day/night) 24°C/19°C and 60/80±5 % relative humidity. Light intensity (200 µmol m^{-2} s^{-1}) was provided by daylight and Grolux fluorescent lamps (F 40W). One seedling was transferred to each 5.8 cm (diam) × 20 cm (length) cylindrical pot (one replicate, n=1)) containing washed sand (pH 7.0 and EC= 0.05 $dS.m^{-1}$) and irrigated with 0.5 × Hoagland's nutrient solution containing 3 mM KNO_3; 2 mM $Ca(NO_3)_2$•$4H_2O$; 1 mM SO_4Mg•$7H_2O$; 0.5 mM $NH_4H_2PO_4$; 0.5 EDTA $NaFeO_8$•$2H_2O$ and 0.5 mM of each of the following micronutrients sources: $MnCl_2$•$4H_2O$, H_3BO_3, $CuSO_4$•$5H_2O$, $ZnSO_4$•$7H_2O$ and MoO_4Na_2•$2H_2O$. Sand was kept at container capacity during the time-lapse experiment. A drip irrigation system (9001 Digital Watering Timer Weekly Program, ELGO®, www.elgo.co.il) was

123 used in order to avoid variations in pH and salt accumulation due to water evaporation throughout
124 the experiment. This system allowed a homogeneous distribution of nutrients within the pot and a
125 daily replacement, by percolation, of an amount of nutrient solution equivalent to ¾ of the substrate
126 field capacity.
127
128 Experimental design and stress treatment.
129 Experiments were performed according to a completely randomized design of one factor
130 (stress) and four levels: control, saline, alkaline and mixed salt-alkaline. The alkaline condition in
131 the pot was created by adding 10 mM $NaHCO_3$ to 0.5 × Hoagland´s solution, whereas for the saline
132 condition, 100 mM NaCl were used as the salt stress source. For the mixed saline-alkaline
133 treatment, we employed the same 0.5 × Hoagland´s solution additioned with 90 mM NaCl plus 10
134 mM $NaHCO_3$, thus obtaining a stress solution with the same Na^+-derived EC, but a higher alkalinity
135 than that of the saline stress treatment. Control treatment consisted of plants irrigated with 0.5 ×
136 Hoagland´s solution without NaCl or $NaHCO_3$ addition. The pH and EC of irrigation solutions were
137 monitored every 3 days with a combined pH meter/conductimeter (HI 255, Hanna Instrument) and
138 maintained at pH-EC (dS m^{-1}) 5.8-1.2; 5.8-11; 8.0-1.9 and 8.0-11, for control, saline, alkaline and
139 mixed saline-alkaline treatments, respectively. In order to avoid any osmotic shock in the saline and
140 mixed saline-alkaline treatments, 8-day-old seedlings initially received 30 and 20 mM NaCl plus 10
141 mM $NaHCO_3$ concentrations, respectively. Salt concentrations were then stepwise increased during
142 one week (acclimatation) until reaching final concentrations. In the alkaline treatment, 8-day-old
143 plants received the final $NaHCO_3$ concentration. After acclimation, plants were further grown under
144 their respective treatments during 20 days longer.
145
146 Growth and morphological parameters

147 Ten plants per treatment were used for dry matter, length and number of stem nodes and leaf
148 area determinations (n=10). For dry matter, plants were dried at 60°C until constant weight. For leaf
149 area, fully extended leaves were scanned with a 600 ppi resolution (HP PSC 1510 Hewlett Packard
150 Development Company, LP, USA). Leaf area was measured on digitalized images with the Image-
151 ProPlus V 4.1 software.
152
153 Analysis of leaf and stem anatomy
154 Anatomical variations caused by the three different stresses were analyzed in tissues
155 developed at the end of the experiment in eight plants per treatment (n=8). A 1 cm long stem
156 sample was sectioned from the internode immediately below the apical bud, whereas the leaf from
157 the basal portion of this internode was dissected. The micro morphology of leaf epidermis was
158 analyzed according to D´Ambrogio de Argüeso (1986). Briefly, FAA
159 (Formaldehyde:Alcohol:Acetic Acid, 10%:50%:5% + 35% water) fixed leaves were incubated in
160 5% KOH at 35°C, overnight and then stained with 5% Safranin. Abaxial and adaxial epidermis
161 were removed from mesophyll with dissecting needles, mounted on slides with water-glycerin (1:1),
162 and observed at 400× (Nikon-Eclipse E-600 microscope attached to a computer and a digital camera
163 Nikon DS Qi1Mc). The ostiolar opening area (or stomatal aperture) and the number of stomata and
164 ordinary epidermal cells per mm^2 were registered in the central leaflets. Data from abaxial and
165 adaxial faces were averaged. The stomatal index was calculated according to Salisbury (1927): SI
166 (%)= stomatal density/(stomatal density + epidermal cell density) x 100. Leaf and internode
167 samples were fixed with FAA, dehydrated and embedded according to the procedures outlined in
168 Johansen (1940). A series of transversal cross sections 10 μm thick were obtained from the sample
169 blocks using a Minot rotary microtome. Cross sections were observed under the microscope and
170 photographed. Digitalized images were analyzed with the Image-ProPlus V 4.1 software. The
171 cuticle-epidermis height in both leaf faces, and the morphology and thickness of each mesophyll

(spongy and palisade) were measured on digitalized images of leaf transversal cross sections. The cross-section areas of stem, cortex, bundle xylem and phloem, vessel elements and pith, as well as the stem epidermal thickness and number of bundles, were also measured on digitalized images.

Analytical determinations

Leaf proline content was estimated spectrophotometrically by the ninhydrin reaction method (Troll and Lindsley, 1955) with modifications (Magné and Larher, 1992). Data was collected from six plants for each treatment (n=6).

Gas-exchange measurement

Fifteen days after the stress treatment was initiated, transpiration rate (E, mmol m^{-2} s^{-1}) and mean stomatal conductance (*g*) were measured in intact, fully expanded leaves, which were basal to the internode immediately below the apical bud. This measurement was performed with an infrared gas analyzers built into a leaf cuvette in an open-flow gas exchange system (LiCor 6400, USA). Measurement conditions were: PPFD, 1500 µmol m^{-2} s^{-1}; airstreams, 350 µmol CO_2 mol^{-1}; leaf temperature, 27°C; leaf-to-air vapor pressure deficit: 1.6 kPa was also measured with a LI-6400. Data was collected at mid-day, from ten plants for each treatment (n=10).

Osmotic potential (ψ_π^{100}) measurement.

At the en of the experiment, leaves from the same region as above were re-hydrated to constant fresh weight by placing them in a beaker of distilled water under controlled environmental conditions. Fully hydrated leaves were then introduced in a syringe, frozen in liquid N_2 and kept at -80°C, pending for further analysis. Syringes were thawed until samples reached room temperature, and the ψ_π^{100} of leaked sap was measured with a C-52 thermocouple. Data was collected from three plants for each treatment (n=3).

197

198 Statistical analysis.

199 Data was subjected to one-way analysis of variance (ANOVA), and comparisons using
200 Duncan´s test. A $y=\log_{10}x$ transformation was used to correct the lack of normality in biomass data.

201

202 **Results**

203

204 Plant growth and morphology

205 The three stress treatments substantially reduced total plant biomass, stem length and
206 number of nodes (Fig 1A, B and C). The amount of growth reduction induced by NaCl or $NaHCO_3$
207 as a sole stress source was similar, whereas the combination of both salts led to a maximum growth
208 reduction.

209 Our results also showed that leaf area was negatively affected by alkalinity, in either
210 presence or absence of the neutral salt (Fig 1D). In contrast, the leaf area did not change in plants
211 treated exclusively with NaCl.

212

213 Effect of salt treatments on leaf anatomy.

214 The leaf epidermal cells density was drastically reduced by soil alkalinity (Table 1),
215 indicating an enlargement of the epidermal cell size. Opposite to the former result, this parameter
216 did not change in NaCl-treated plants. Alkalinity also led to reduced stomatal density, being this
217 reduction more obvious in the mixed salts treatment. However, the SI was not affected by any
218 stress. On other hand, plants treated with NaCl (either alone or mixed with the alkaline salt),
219 showed reduced ostiolar opening area compared with control plants. Interestingly, plants treated
220 with $NaHCO_3$ as a sole stress source presented a higher ostiolar opening area than control plants.

L. tenuis leaves have a dorsiventral mesophyll, consisting of one layer of palisade and one layer of spongy tissue (Fig 2). Leaf thickness was increased by NaCl treatment, as result of augmented cuticle thickness, higher epidermal height and increased thickness of mesophyll cells (Table 1, Fig 2). Alkalinity also led to augmented leaf thickness derived from increments in cuticle and spongy parenchyma thickness, and in epidermal cells heights. However, the most striking effect of alkalinity on leaf anatomy was the dorsiventral mesophyll loss, because palisade parenchyma cells became more isodiametric and larger, losing their shape, and preventing the differentiation between palisade and spongy parenchyma. This phenomenon was observed in 50% and 100% of analyzed leaves in the alkaline and mixed saline-alkaline treatments, respectively (data not shown), and whenever palisade parenchyma was recognizable in the alkaline treatment, their cells were highly vacuolated and thicker, compared with the control.

Effect of salt treatments on stem anatomy.

The stem cross-section area was reduced in all cases, because of decreased pith, phloem and xylem cross-section areas, and reduced epidermal height (Table 2). In plants treated exclusively with the alkaline salt, a diminution of the cortex cross-section area was also observed, in addition to the above-mentioned effects, leading to a maximal stem cross-section area contraction in this treatment (Table 2).

The number of vascular bundles in the sectioned stem was also reduced by the three saline treatments, although the effect was more obvious under elevated alkalinity (Table 2). On other hand, a noteworthy rise in the number of vessel elements in the xylem was registered in NaCl-treated plants, but not in those treated exclusively with $NaHCO_3$. In contrast, the area of each vessel element was diminished in all cases. Moreover, both salts showed a synergistic effect on vessel cross-section area when applied simultaneously.

Effect of salt treatments on leaf proline content, gas exchange, transpiration and osmotic potential.

The leaf proline content was increased in leaves of NaCl-treated plants, whereas $NaHCO_3$ did not affect this content (Fig 3).

Reductions of *g* and E were observed in *L. tenuis* plants treated with NaCl regardless the alkalinity level (Fig 4). Contrarily, there were not changes in *g* or E in plants confronted exclusively with $NaHCO_3$ (Fig 4).

At the time of harvest, the leaf ψ_π^{100} was not significantly altered by separately applied NaCl or $NaHCO_3$, but in plants treated with the mixture of the neutral and alkaline salts, the ψ_π^{100} was significantly augmented (Fig 5).

Discussion

The present work was aimed at exploring and comparing the morpho-anatomical responses of *L. tenuis* shoots to alkaline, saline and mixed saline-alkaline stresses. Our results showing that separately applied NaCl and $NaHCO_3$ salts induced similar growth reductions (Fig 1) is in contrast with previous studies where alkaline salts created a significantly higher level of plant toxicity than neutral salts (Shi and Wang 2005; Shi and Sheng 2005; Wang *et al.* 2008; Yang *et al.* 2008). Such seeming disparity may be explained by the fact that at the $NaHCO_3$ concentration used in our work (10 mM, a several-fold lower concentration than those used by other authors), the ionic and osmotic components of saline stress are avoided and the growth reduction effect can be fundamentally attributable to alkalinity (Yang *et al.*, 2008). Whereas neutral and alkaline salts produced a similar level of growth inhibition on *L. tenuis* shoots, their mixture exacerbated their detrimental effects.

Such synergism is in line with previous observations reported for other glycophytes (Shi and Wang 2005; Shi and Sheng 2005; Wang *et al*., 2008; Yang *et al*., 2008).

Salt treatments effects on leaf and stem anatomy.

The anatomical analysis revealed that leaves and stems exhibited a plastic response to the three evaluated stress conditions (Tables 1 and 2). Interestingly, an enlargement of the epidermal cell size was found in plants grown under alkaline conditions. To our knowledge, there are no previous reports showing an alkalinity-induced enlarging effect on plant cells, beyond the documented relationship between alkalinity and cell extension in growing lily pollen tube (Lovy-Wheeler *et al*, 2006), and therefore, additional specific experimentation in this regard would be worthwhile of further attention.

The fact that the NaCl addition (regardless the alkalinity level) reduced the ostiolar opening area of *L. tenuis* leaves is in accordance with the concept that osmotic stress (either elicited by water deficit or high salt concentration) leads to stomatal closure. Similarly, our finding that $NaHCO_3$, as sole stress source, induced higher ostiolar opening area is compatible with previous results indicating that the stomatal aperture of isolated abaxial epidermis (incubated on simple buffers) increased with the external alkalinity (Wilkinson and Davies, 1997).

On other hand, despite the cell size augmentation observed in plants treated with $NaHCO_3$, leaf area was reduced, suggesting that alkalinity decreased new cells production by leaf meristem. The reduced leaf area evidenced in our alkalinized *L. tenuis* plants (either in presence or absence of NaCl) is in accordance with previous results obtained from several ornamental species such as rose, vinca, chrysanthemum and hibiscus treated with bicarbonate (HCO_3^-, Valdez-Aguilar, 2004, Cartmill *et al*., 2007, 2008). In addition, the neutral effect of NaCl, as sole stress source, observed on the *L. tenuis* leaf area agrees with former results obtained on soybean (Dolatabadian *et al*., 2011).

295 Our finding that plants treated with NaCl presented thicker leaves is in line with descriptions
296 in several other plant species (e.g.: Qadir and Shams, 1997; De Vos *et al*., 2010), suggesting the
297 occurrence of osmolytes accumulation and osmotic adjustment. Plants treated exclusively with
298 $NaHCO_3$ also presented thickened leaves, although in a much lower extent compared with NaCl
299 addition, but this effect should not be assigned to an osmotic effect, since a low salt concentration
300 (10 mM) was used in this treatment.

301 The observed loss of leaf mesophyll dorsiventrality in plants grown under high alkalinity
302 highlights a different stress sensitivity between palisade and spongy parenchyma mesophylls. As far
303 as we know, mesophyll dorsiventral loss has been reported formerly only in plants subjected to low
304 temperature stress (Kadohama *et al*., 2013). It has been postulated that since palisade mesophyll is
305 the site for most of the plant photosynthetic activity, an increased palisade: spongy mesophyll ratio
306 positively increases photosynthesis (Kulkarni *et al*., 2008). Thus, our results showing the absence of
307 palisade mesophyll in most of the analyzed leaves of $NaHCO_3$-treated plants could have possibly
308 contributed with the noticeable growth diminution registered in these plants.

309 The reduction in the stem cross-section area observed in *L. tenuis* plants stressed exclusively
310 with NaCl, is in line with previous studies performed on diverse plant species subjected to this
311 saline condition (Qadir and Shams, 1997; Habba *et al*., 2013; Guo *et al*., 2013; dos Santos *et al*,
312 2013; Soltekin *et al*, 2012). In the case of plants treated with the mixed salts, the registered smaller
313 leaf area could have led to further reduction of the stem cross-section area. However, maximal stem
314 cross-section reduction was registered in *L. tenuis* plants treated exclusively with $NaHCO_3$. In these
315 plants, the observed reduction could be related to other sources of growth constraint not measured
316 in this work, such as diminished cell division and expansion, besides a smaller leaf area. In turn,
317 slender stems could affect bending, tensile and shearing properties of *L. tenuis* plants. These
318 properties could be significant from the economical viewpoint, given the importance of *L. tenuis* as
319 forage species, in several cattle production areas worldwide.

Fewer vascular bundles, along with smaller vessel cross-section area was observed in salt-treated *L. tenuis* plants. These anatomical changes may lead to hampered water and solutes transport capacity. Therefore, our result displaying a rise in the number of vessel elements due to NaCl treatment could be interpreted as an adaptive response of *L. tenuis* plants to withstand the osmotic constraint imposed by NaCl, as it was shown for other crop species (Cachorro *et al.*, 1993; Nawaz *et al*., 2013).

Effect of salt treatments on leaf proline content, gas exchange, transpiration and osmotic potential.

The amino acid proline is one of the compatible osmolytes that most commonly build up in the cytoplasm as response to osmotic imbalance (Hasegawa *et al*. 2000). The increased leaf proline content detected in plants treated with NaCl (Fig 3), put forward that they had experienced some extent of osmotic stress. Furthermore, the fact that the proline level was not affected in plants treated exclusively with 10 mM $NaHCO_3$ indicates that no salt-derived osmotic effect intervened and hence, the observed growth and morphological detrimental effects should be assigned to alkalinity itself. This result helps to explain the apparent incongruence between our finding (no difference between alkaline and neutral salts in their effect on plant growth) and those obtained by other authors, who used a several-fold higher $NaHCO_3$ concentration, and reported a higher level of plant toxicity in plants treated with the alkaline, compared with the neutral salt (Shi and Wang 2005; Shi and Sheng 2005; Wang *et al.* 2008; Yang *et al.* 2008).

Diminutions in *g* and E parameters noted in NaCl-treated plants (Fig 4) are congruent with the observed decrease in the ostiolar opening area and could have accounted for the decline in plant growth registered in both NaCl treatments (Table 1; Fig 1). Since enhanced transpirational volume flow tends to augmentate salt accumulation and hence, cell damage (Munns, 1985), the observed contracted ostiolar opening area may be interpreted as an adaptive *L. tenuis* response to the osmotic component of saline stress. On other hand, our results from plants treated exclusively with $NaHCO_3$

showed no changes in *g* or E. This result would advocate a possible compensation effect between an increase in the ostiolar opening area and a reduction in the stomata density in these plants. A similar salinity-induced reduction in stomata density was recently reported for the halophyte species *Chenopodium quinoa* (Orsini *et al.*, 2011; Shabala *et al.*, 2012) and was interpreted as a fundamental mechanism by which quinoa plants may improve water use efficiency under saline conditions. Since the waxy cuticle of leaves allows water and small amounts of CO_2 to pass through (Scott, 1964,1966; Norris and Bukovac, 1968; Leon and Bukovac, 1978; Boyer *et al*., 1997), one may also hypothesize that the increase in cuticle thickness observed in plants from this treatment could have contributed to the E balance.

Another outcome of our work was the increased leaf ψ_π^{100} observed in *L. tenuis* plants subjected to the mixed saline-alkaline treatment (Fig 5). However, the proline level reached by plants in this treatment (0.4 $\mu mol.g^{-1}$ fresh weight, Fig 3) seems to be too low to act as osmolyte, compared with those needed for conventional osmotic adjustment (Marcum, 2006). On other hand, adverse environmental factors may induce a rapid production of reactive oxygen species (ROS), leading to oxidative burst and cell damage (Mittler, 2002). In turn, proline may significantly reduce ROS-induced K^+ effluxes (Cuien and Shabala, 2005, 2007). It has been postulated that a number of functions played by proline could come into the effect of reducing the extent of the ROS-induced K^+ efflux, such as free radicals scavenging (Smirnoff and Cumbes, 1989), reduction of ROS generation (Hong *et al.*, 2000), osmoprotection (Delauney and Verma, 1993) and protein stabilization (Shah and Dubey, 1998). Therefore, it is possible that the increased proline level could have indirectly contributed to the osmotic adjustment of *L. tenuis* leaves in the mixed saline-alkaline treatment.

Conclusions

Neutral and alkaline salts produced a similar level of growth inhibition on *L. tenuis* shoots, whereas their mixture exacerbated their detrimental effects. On other hand, common and distinct effects of the three stresses on *L. tenuis* shoot growth and anatomy were evidenced, according to the analyzed parameter. Our results showed that none of the analyzed parameters categorically differentiated the three types of stress from each other. However, NaCl and $NaHCO_3$-derived stresses could be discriminated by different extents and/or directions of changes in some of the anatomical traits. For example, alkalinity itself presented increased ostiolar opening area, conversely to NaCl-treated plants, where a reduction of the stomatal aperture was observed. Likewise, plants from the mixed saline-alkaline treatment may be recognized by the lack of a palisade mesophyll in their leaves. The conspicuous lack of information regarding the effect of alkalinity on plant cell growth prevents us from further discussing our results related with high pH conditions, while invites to conduct research in this direction. Further studies are required to ascertain the unambiguous cause-and-effect relationships between the observed anatomical traits changes in *L. tenuis* and each of the factors intervening in the three studied stresses (e.g.: osmotic, toxicity, pH homeostasis, etc.). Such studies could help to design mechanistic models for predicting the whole-plant response to stresses derived from different salt sources.

Acnowledgements

This work was supported by the following Argentine grants: PICT, Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad de San Martin (UNSAM) and Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC). Authors acknowledge grants in-aid from COST-Action FA0605. We also thank Sr. Jorge Luis Paz for their valuable help with the irrigation system set up. RCP expresses her gratitude to CONICET for doctoral fellowships.

394 **References**

395 Alam S.M., Mehdi Naqvi S.S., Ansari R. (1999) Impact of Soil pH on Nutrient Uptake by Crop
396 Plants. In: "Handbook of Plant and Crop Stress" M. Pessarakli (Ed) pp 51–60.
397 doi:10.1201/9780824746728.ch3

398 Boughalleb F., Denden M., Tiba B.B. (2009) Anatomical changes induced by increasing NaCl
399 salinity in three fodder shrubs, *Nitraria retusa, Atriplex halimus* and *Medicago arborea. Acta*
400 *Physiologiae Plantarum* **31,** 947-960. doi:10.1007/s11728-009-0310-7

401 Boyer J.S., Wong S.C., Farquhar C.D. (1997) CO, and Water Vapor Exchange across Leaf Cuticle
402 (Epidermis) at Various Water Potentials. *Plant Physiology* **114,** 185-191.

403 Cachorro P., Ortiz A., Barcelo A.R., Cerda A. (1993) Lignin deposition in vascular tissues of
404 *Phaseolus vulgaris* roots in response to salt stress. *Phyton-Annales Rei Botanicae* **33,** 33-40.

405 Cartmill A., Alarcón A., Valdez-Aguilar L.A. (2007) Arbuscular mycorrhizal fungi enhance
406 tolerance of *Rosa multiflora* cv. Burr to bicarbonate in irrigation water. *Journal of Plant Nutrition*
407 **30,** 1517–1540.

408 Cartmill A.D., Valdez-Aguilar L.A., Bryan D.L., Alarcón A. (2008) Arbuscular mycorrhizal fungi
409 enhance tolerance of vinca to high alkalinity in irrigation water. *Scientia Horticulturae* **115**, 275–
410 284. doi:10.1016/j.scienta.2007.08.019

411 Costa J.L., García F.O. (1998) Respuesta de un pastizal natural a la fertilización con fósforo y
412 nitrógeno en un natracuol. *Revista de Investigaciones Agropecuarias* **28**, 31-39.

413 Cuin A.T, Shabala S. (2005) Exogenously supplied compatible solutes rapidly ameliorate NaCl-
414 induced potassium efflux from barley roots. *Plant Cell Physiology* **46**, 1924-1933.

415 Cuin A.T, Shabala S. (2007) Compatible solutes reduce ROS-induced potassium efflux in
416 *Arabidopsis* roots. *Plant Cell and Environment* **30**, 875-885.

417 D'ambrogio de Argüeso A. (1986) Manual de Técnicas en Histología Vegetal. Hemisferio Sur. Pp.
418 50-67. (Buenos Aires Publisher)

419 Delauney A.J., Verma D.P.S. (1993) Proline biosíntesis and osmoregulation in plants. *Plant Journal*
420 **4**, 215-223.
421 De Vos A.C., Broekman R., Groot M.P., Rozema J. (2010) Ecophysiological response of *Crambe*
422 *maritima* to airborne and soil-borne salinity. *Annals of Botany* **105**, 925-937.
423 doi:10.1093/aob/mcq072
424 Dolatabadian A., Modarressanavy S.A.M., Ghanati F. (2011) Effect of salinity on growth, xylem
425 structure and anatomical characteristics of soybean. *Notulae Scientia Biologicae* **3,** 41-45.
426 dos Santos J.B., dos Santos D.B., de Azevedo C.A.V., Rebequi A.M., Cavalcante L.F., Cavalcante
427 I.H.L. (2013) Comportamento morfofisiológico da mamoneira BRS energia submetida à irrigação
428 com água salina | [Morphophysiological behavior of castor bean brs energia submitted to irrigation
429 with saline water]. *Revista Brasileira de Engenharia Agrícola e Ambiental* **17,** 145–152.
430 Gharsalli M., Zribi K., Hajji M. (2001) Physiological responses of pea to iron deficiency induced by
431 bicarbonate. In "Plant nutrition- food security and sustainability of agro-ecosystems". IN Horstet
432 (Ed) pp. 606-607. doi: 10.1007/0-306-47624-X_294
433 Guo X.P., Tackmore M., Obai K., Salahou M.K. (2013) The combined effects of salinity and water
434 stress on the growth and yield quality of tomato. *Applied Mechanics and Materials* **295,** 2265-
435 2273. doi:10.4028/www.scientific.net/AMM.295-298.2265
436 Habba I.E., Abd El Aziz N.G., Metwally .SA., Mazhar A.A.M. (2013) Response of growth and
437 chemical constituents in *Khaya sengalensis* to salinity and gypsum under calcareous soil
438 conditions. *World Applied Sciences Journal* **22**, 447-452.
439 Hasegawa P.M., Bressan R.A., Zhu J.K., Bohnert H.J. (2000) Plant cellular and molecular
440 responses to high salinity. *Annual Review of Plant Physiology* **51**, 463–499.
441 Hong Z.L., Lakkineni K., Zhang Z.M., Verma D.P.S. (2000) Removal of feedback inhibition of Δ^1-
442 pyrroline-5-carboxylated synthetase results in increased proline accumulation and protection of
443 plants from osmotic stress. *Plant Physiology* **122**, 1129-1136.

444 Johansen D.A. (1940) Plant microtechnique. McGraw-Hill (Ed) pp. 1-523. (New York)

445 Kadohama N., Goh T., Ohnishi M., Fukaki H., Mimura T. (2013) Sudden collapse of vacuoles in
446 *Saintpaulia* sp. palisade cells induced by a rapid temperature decrease. *PLoS ONE* **8**, e57259.
447 doi:10.1371/journal.pone.0057259

448 Kiliç S., Cavuşoğlu K., Kabar K. (2007) Effects of 24-epibrassinolide on salinity stress induced
449 inhibition of seed germination, seedling growth and leaf anatomy of barley. *Journal of Science* **2**,
450 41-52.

451 Kukavica B., Morina F., Janjiæ N., Boroja N., Jovanoviæ L., Veljoviæ-Jovanoviæ D. (2013) Effects
452 of mixed saline and alkaline stress on the morphology and anatomy of *Pisum sativum*: the role of
453 peroxidase and ascorbate oxidase in growth regulation. *Archives of Biological Sciences* **65**, 265-
454 278. doi:10.2298/ABS1301265K

455 Kulkarni M**.,** Borse T**.,** Chaphalkar S. (2008) Mining anatomical traits: a novel modeling approach
456 for increased water use efficiency under drought conditions plants. *Czech Journal of Genetics and*
457 *Plant Breeding* **44,** 11-21.

458 Läuchli A**.,** Lüttge U. (2002) Salinity: Environment — Plants — Molecules. (Ed XIV) pp. 552.

459 Leon J.M., Bukovac M.J. (1978) Cuticle development and surface morphology of olive leaves with
460 reference to penetration of foliar-applied chemicals. *Journal of the American Society of*
461 *Horticultural Science* **103**, 465-472.

462 Li P.H., Zhang H., Wang B.S. (2003) Ionic homeostasis of plant under salt stress. *Acta Botanica*
463 *Boreal-Occident Sin* **23**, 1810-1817.

464 Li R., Shi F., Fukuda K. (2010) Interactive effects of salt and alkali stresses on seed germination,
465 germination recovery, and seedling growth of a halophyte *Spartina alterniflora* (Poaceae). *South*
466 *African Journal of Botany* **76**, 380–387. doi:10.1016/j.sajb.2010.01.004

Lovy-Wheeler A., Kunkel J.G., Allwood E.G., Hussey P.J., Hepler P.K. (2006) Oscillatory increases in alkalinity anticipate growth and may regulate actin dynamics in pollen tubes of lily. *Plant Cell* **18,** 2182–2193. doi:10.1105/tpc.106.044867

Magné C., Larher F. (1992) High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Analytical Biochemistry* **200,** 115-118.

Mandre M., Klõšeiko J., Lukjanova A., Tullus A. (2012) Hybrid aspens responses to alkalization of soil: growth, leaf structure, photosynthetic rate and carbohydrates. *Trees* **26**, 1847–1858. doi:10.1007/s00468-012-0754-z

Marcum K.B. (2006) Saline tolerance physiology in grasses. In: "Ecophysiology of High Salt Tolerant Plants" M.A. Khan, D.J. Weber (Eds) pp 157-172.

Mittler R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7**, 405-410.

Munns R. (2002) Comparative physiology of salt and water stress. *Plant, Cell & Environment* **25**, 239-250.

Munns, R. (1985) Na^+, K^+ and $C1^-$ Xylem sap flowing to shoots of NaCl-treated barley. *Journal of Experimental Botany* **36,** 1032-1042.

Nawaz T., Hameed M., Ashraf M., Batool S., Naz N. (2013) Modifications in root and stem anatomy for water conservation in some diverse blue panic (*Panicum antidotale* Retz.) ecotypes under drought stress. *Arid Land Research and Management* **27**, 286-297. doi:10.108715324982.2012.727947

Norris R.R., Bukovac M.J. (1968) Structure of the pear leaf cuticle with special reference to cuticular penetration. *American Journal of Botany* **55,** 975-983.

Orsini F., Accorsi M., Gianquinto G., Dinelli G., Antognoni F., Ruiz Carrasco K.B., Martínez E.A., Alnayef M., Marotti I., Bosi S., Biondi S. (2011) Beyond the ionic and osmotic response to

491 salinity in *Chenopodium quinoa*: functional elements of successful halophytism. *Funtional Plant*
492 *Biology* **38**, 818-831.
493 Paz R.C., Rocco R.A., Reinoso H., Menéndez A.B., Pieckenstain F.L., Ruiz A.O. (2012)
494 Comparative study of alkaline, saline, and mixed saline-alkaline stresses with regard to their
495 effects on growth, nutrient accumulation, and root morphology of *Lotus tenuis*. *Journal of Plant*
496 *Growth Regulation* **31**, 448-459. doi:10.1007/s00344-011-9254-4
497 Qadir M., Shams M. (1997) Some agronomic and physiological aspects of salt tolerance in cotton
498 (*Gossypium hirsutum* L.) *Journal of Agronomy and Crop Science* **179**, 101-106.
499 Salisbury E.J. (1927). On the causes and ecological significance of stomatal frequency, with special
500 reference to the woodland flora. *Philosophical Transactions of the Royal Society of London* **B216,**
501 1–65.
502 Scott F.M. (1964) Lipid deposition in the intercellular space. *Nature* **203,** 164-165.
503 Scott F.M. (1966) Cell wall surface of the higher plants. *Nature* **210**, 1015-1017.
504 Shabala L., Mackay A., Tian Y., Jacobsen S., Zhou D., Shabala S. (2012. Oxidative stress
505 protection and stomatal patterning as components of salinity tolerance mechanism in quinoa
506 (*Chenopodium chinoa*) . *Physiologia Plantarum* **146,** 26-38.
507 Shah K., Dubey R.S. (1998) Effect of cadmium on proline accumulation and ribonuclease activity
508 in rice seedlings: role of proline as a possible enzyme protectant. *Biologia Plantarum* **40**, 121-130
509 Shi D., Sheng Y. (2005) Effect of various salt-alkaline mixed stress conditions on sunflower
510 seedlings and analysis of their stress factors. *Environmental and Experimental Botany* **54,** 8-21.
511 doi:10.1016/j.envexpbot.2004.05.003
512 Shi D., Zhao K. (1997) Effects of sodium chloride and carbonate on growth of *Puccinellia*
513 *tenuiflora* and on present state of mineral elements in nutrient solution. *Acta Pratacult Sinica* **6**,
514 51–61.

Shi D.C., Wang D. (2005) Effects of various salt–alkaline mixed stresses on *Aneurolepidium chinense* (Trin.) Kitag. *Plant and Soil* **271,** 15–26. doi:10.1007/s11104-004-1307-z

Smirnoff N., Cumbes Q.J. (1989) Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry* **28**, 1057-1060.

Soltekin O., Tüzel Y., Öztekin G.B., Tüzel I.H. (2012) Response of pepino (*Solanum muricatum* Aiton) to salinity. *Acta Horticulturae* **960,** 425-431.

Taiz, L., Zeiger, E. (2010) Plant Physiology, Fifth Edition, M.A. Sunderland (Ed). pp. 1970. (Sinauer Associates)

Tester M., Davenport R. (2003) Na^+ tolerance and Na^+ transport in plants. *Annals of Botany* **91,** 503–527.

Troll W., Lindsley J. (1995) The photometric methods to determination of proline. *The Journal of Biological Chemistry* **215,** 655-660.

Valdez-Aguilar L.A., Reed D.W. (2008) Influence of potassium substitution by rubidium and sodium on growth, ion accumulation, and ion partitioning in bean under high alkalinity. *Journal of Plant Nutrition* **31**:867-883. doi:10.1080/01904160802043231 3

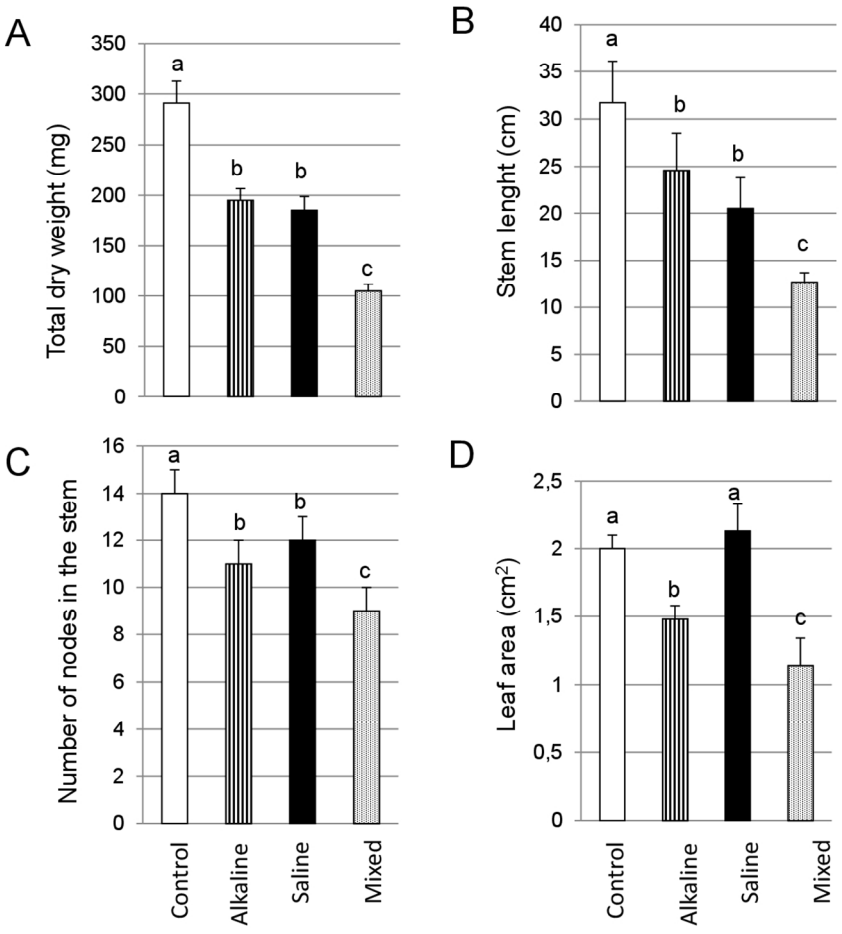
Valdez-Aguilar L.A. Effect of alkalinity in irrigation water on selected greenhouse crops. PhD thesis in Horticulture, Texas A&M; 2004, University, 500014.

Wang Y., Guo J.X., Meng Q.L., Cui X.Y. (2008) Physiological Responses of Krishum (*Iris lactea* Pall. var. chinensis Koidz) to neutral and alkaline salts. *Journal of Agronomy and Crop Science* **194**, 429–437. doi:10.1111/j.1439-037X.2008.00337.x

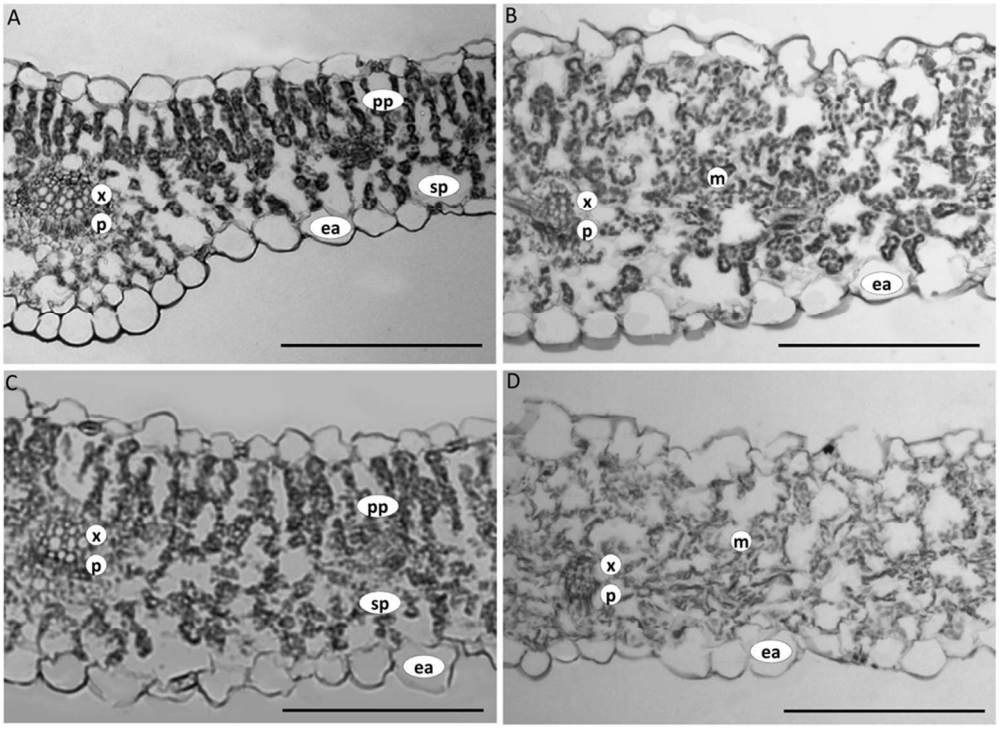
Wilkinson S., Davies W.J. (1997) Xylem sap pH increase: a drought signal received at the apoplastic face of the guard cell which involves the suppression of saturable ABA uptake by the epidermal symplast. *Plant Physiology* **113**, 559-573.

538 Yang C., Chong J., Changyou L., Kim C., Shi D., Wang D. (2007) Osmotic adjustment and ion
539 balance traits of an alkali resistant halophyte *Kochia sieversiana* during adaptation to salt and
540 alkali conditions. *Plant and Soil* **294**:263-276. doi:10.1007/s11104-007-9251-3
541 Yang C., Shi D., Wang D. (2008) Comparative effects of salt and alkali stresses on growth, osmotic
542 adjustment and ionic balance of an alkali-resistant halophyte *Suaeda glauca* (Bge.). *Plant Growth*
543 *Regulation* **56,** 179–190. doi:10.1007/s10725-008-9299-y
544 Zhang P., Fu J., Hu L. (2012) Effects of alkali stress on growth, free amino acids and carbohydrates
545 metabolism in Kentucky bluegrass (*Poa pratensis*). *Ecotoxicology* **21,** 1911-1918.
546 doi:10.1007/s10646-012-0924-1
547
548
549 **Legends**
550
551 Figure 1. Total dry weight (A), stem length (B), number of nodes in the stem (C) and leaf area (D)
552 of *L. tenuis*. Fifteen-day-old plants were watered with nutrient solution containing or lacking salt
553 addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$
554 respectively, were added to 0.5 × Hoagland's solution. For the mixed salt–alkaline stress treatment,
555 this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; n = 10) with the
556 same letter are not significantly different (Duncan, $P<0.001$).
557
558 Figure 2. Leaf anatomical response of *L. tenuis*. A) Control; B) Alkaline; C) Saline and D) Mixed
559 saline-alkaline. Scale bars = 200 μm. *pp*, palisade parenchyma; *sp*, spongy parenchyma; *ea*, abaxial
560 epidermis; *x*, xylem; *p*, phloem. Transversal cross-section of *L. tenuis* leaves. Fifteen-day-old plants
561 were watered with nutrient solution containing or lacking salt addition over 20 days. For saline and
562 alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$ respectively, were added to 0.5 ×

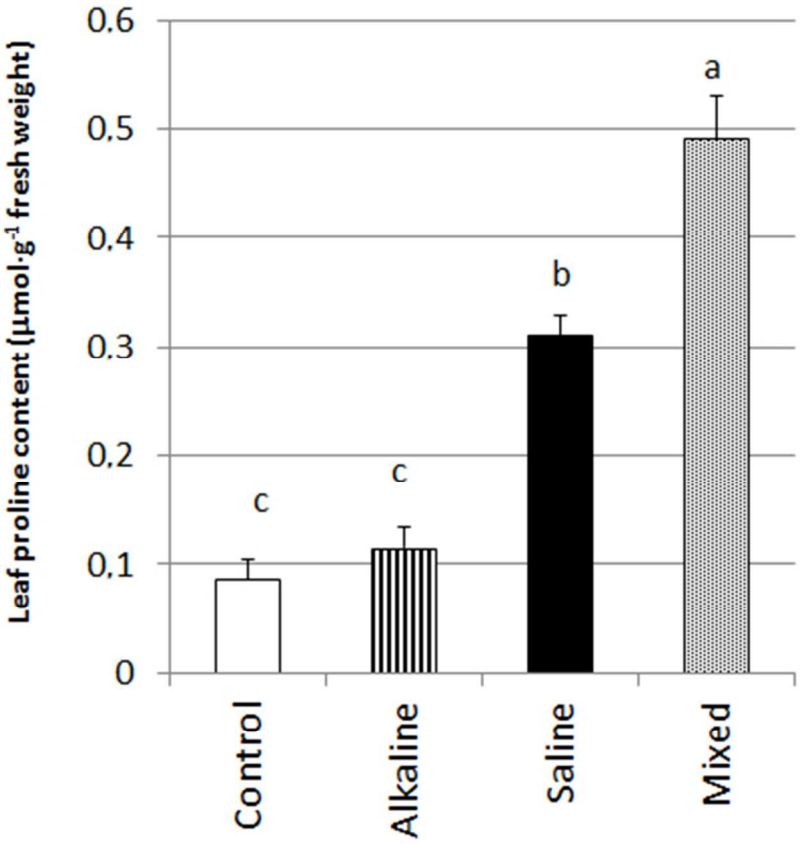
563 Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM
564 NaCl and 10 mM $NaHCO_3$.
565
566 Figure 3. Leaf proline contents (μmol g^{-1} fresh weight) in *L. tenuis*. Fifteen-day-old plants were
567 watered with nutrient solution containing or lacking salt addition over 20 days. For saline and
568 alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$, respectively, were added to 0.5 ×
569 Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM
570 NaCl and 10 mM $NaHCO_3$. Average data (±SE; n = 6) with the same letter are not significantly
571 different (Duncan, $P<0.01$).
572
573 Figure 4. Mean stomatal conductance (A) and transpiration rate (B) in *L. tenuis*. Fifteen-day-old
574 plants were watered with nutrient solution containing or lacking salt addition over 20 days. For
575 saline and alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$, respectively, were added
576 to 0.5 × Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90
577 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; n = 10) with the same letter are not
578 significantly different (Duncan, $P<0.01$).
579
580 Figure 5. Leaf osmotic potential (Ψ_π^{100} MPa). Fifteen-day-old plants were watered with nutrient
581 solution containing or lacking salt addition over 20 days. For saline and alkaline stress treatments,
582 100 mM NaCl and 10 mM $NaHCO_3$, respectively, were added to 0.5 × Hoagland's solution. For the
583 mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$.
584 Average data (±SE; n = 3) with the same letter are not significantly different (Duncan, $P<0.01$).
585



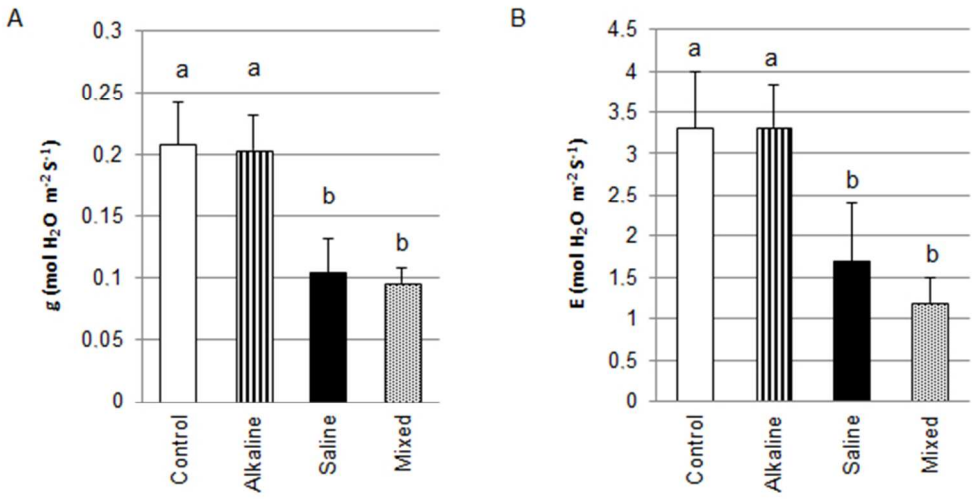
215x279mm (150 x 150 DPI)



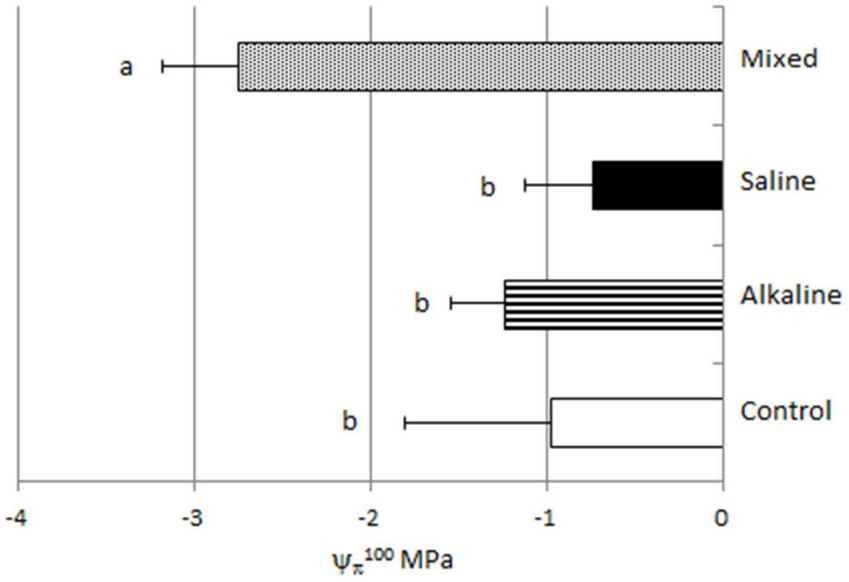
179x131mm (150 x 150 DPI)



148x153mm (72 x 72 DPI)



216x110mm (72 x 72 DPI)



170x114mm (72 x 72 DPI)

Table 1. Leaf micromorphological and anatomical parameters measured in *L. tenuis*. Fifteen-day-old plants were watered with nutrient solution containing or lacking salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$, respectively, were added to 0.5x Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; n = 8) with the same letter are not significantly different (Duncan, P<0.01).

Variable	Control	Alkaline	Saline	Mixed
Epidermal cells density (number of cells mm^2)	30.8 ± 0.9 **a**	24.9 ± 0.9 **b**	29.5 ± 0.9 **a**	20.7 ± 0.8 **c**
Stomata density (stomata number mm^2)	115 ± 6 **a**	93 ± 6 **b**	108 ± 6 **a,b**	75 ± 5 **c**
Stomatal index (SI %)	29 ± 3 **a b**	30 ± 1 **a**	29 ± 3 **b**	28 ± 1 **b**
Ostiolar opening area (μm^2)	15 ± 1 **b**	20 ± 1 **a**	12 ± 1 **c**	13 ± 1 **c**
Leaf thickness (µm)	99 ± 12 **b**	156 ± 11 **a**	178 ± 15 **a**	173 ± 16 **a**
Cuticle thickness (µm)	0.9 ± 0.0 **b**	1.6 ± 0.1 **a**	1.7 ± 0.1 **a**	1.8 ± 0.1 **a**
Adaxial epidermis height (µm)	9 ± 1.0 **c**	16 ± 1.0 **b**	21 ± 2.0 **a**	22 ± 2.0 **a**
Abaxial epidermis height (µm)	15 ± 2.0 **c**	24 ± 1.0 **b**	31 ± 2.0 **a**	36 ± 1.0 **a**
Thickness of palisade parenchyma (µm)	47 ± 4 **c**	69 ± 6 **b**	94 ± 6 **a**	-
Thickness of spongy parenchyma (µm)	52 ± 7 **b**	66 ± 9 **a**	92 ± 9 **a**	82 ± 4.0 **a**

Table 2. Stem anatomical parameters measured in *L. tenuis*. Fifteen-day-old plants were watered with nutrient solution containing or lacking salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$, respectively, were added to 0.5x Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; n = 8) with the same letter are not significantly different (Duncan, P<0.01).

Variable	Control	Alkaline	Saline	Mixed
Stem cross-section area (mm^2)	0.8 ± 0.01 **a**	0.3 ± 0.03 **c**	0.51 ± 0.02 **b**	0.53 ± 0.02 **b**
Epidermis thickness (µm)	25.7 ± 0.3 **a**	18.1 ± 1.0 **c**	22.4 ± 0.5 **b**	21.2 ± 0.7 **b**
Pith cross-section area (mm^2)	0.19 ± 0.01 **a**	0.05 ± 0.01 **d**	0.08 ± 0.01 **c**	0.11 ± 0.01 **b**
Cortex thickness (µm)	119 ± 2 **a**	90 ± 5 **b**	119 ± 3 **a**	79 ± 4 **c**
Phloem cross-section area (×100 mm^2)	4.31 ± 0.04 **a**	1.55 ± 0.19 **b**	1.96 ± 0.24 **b**	0.23 ± 0.01 **c**
Xylem cross-section area (×100 mm^2)	1.22 ± 0.01 **a**	0.49 ± 0.09 **b**	1.04 ± 0.08 **b**	0.41 ± 0.02 **c**
Number of vascular bundles	10.0 ± 0.0 **a**	8.0 ± 0.0 **c**	9.0 ± 0.0 **b**	8 ± 0.1 **c**
Total number of vessels in xylem	97 ± 1 **b**	82 ± 13 **b**	147 ± 8 **a**	146 ± 5 **a**
Vessel cross-section area (μm^2)	126 ± 2 **a**	62 ± 5 **b**	62 ± 6 **b**	28 ± 1 **c**