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

R. C. Paz1,2, H. Reinoso3, F. D. Espasandin4, F. A. González Antivilo2, P. A. Sansberro4, R. A. Rocco1, O. A. Ruiz1 & A. B. Menéndez1,5

1 Unidad de Biotecnología 1, IIB-INTECH/UNSAM-CONICET, Chascomús, Buenos Aires, Argentina
2 Facultad de Ciencias Agrarias (FCA), Instituto de Biotecnología Agrícola de Mendoza (IBAM), Mendoza, Argentina
3 Laboratorio de Morfología Vegetal, Departamento de Ciencias Naturales, Universidad Nacional de Río Cuarto, Córdoba, Argentina
4 Facultad de Ciencias Agrarias (UNNE), Instituto de Botánica del Nordeste (IBONE-CONICET), Corrientes, Argentina
5 Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

**Keywords**
Alkalinity; osmotic potential; proline; salinity; shoot anatomy; transpiration.

**Correspondence**
A. B. Menéndez, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Piso 4 Pab II Ciudad Universitaria, Buenos Aires 1428, Argentina.
E-mail: anamen@bg.fcen.uba.ar

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

**Editor**
T. Elzenga

Received: 10 November 2013; Accepted: 19 December 2013
doi:10.1111/plb.12156

**ABSTRACT**
Saline, alkaline and mixed saline–alkaline conditions frequently co-occur in soil. In this work, we compared these plant stress sources on the legume *Lotus tenuis*, regarding their effects on shoot growth and leaf and stem anatomy. In addition, we aimed to gain insight on the plant physiological status of stressed plants. We performed pot experiments with four treatments: control without salt (pH = 5.8; EC = 1.2 dS m⁻¹) and three stress conditions, saline (100 mM NaCl, pH = 5.8; EC = 11.0 dS m⁻¹), alkaline (10 mM NaHCO₃, pH = 8.0, EC = 1.9 dS m⁻¹) and mixed salt–alkaline (10 mM NaHCO₃ + 100 mM NaCl, pH = 8.0, EC = 11.0 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 analysed morpho-anatomical parameters categorically differentiated one stress from the other. However, NaCl- and NaHCO₃-derived stress could be discriminated to different extents and/or directions of changes in some of the anatomical traits. For example, alkalinity led to increased stomatal opening, unlike NaCl-treated plants, where a reduction in stomatal aperture was observed. Similarly, plants from the mixed saline–alkaline treatment characteristically lacked palisade mesophyll in their leaves. The stem cross-section and vessel 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 recorded in NaCl-treated plants, but not in those treated exclusively with NaHCO₃.

**INTRODUCTION**
Saline stress refers to the presence of neutral salts such as NaCl or Na₂SO₄ in soil, whereas alkaline stress is only related to the occurrence of alkaline salts (Na₂CO₃ or NaHCO₃; Yang *et al.* 2007). Saline and alkaline stresses affect more than 10% of world’s arable land, limiting agricultural production (Läuchi & Lütgte 2002). Both conditions often co-occur in nature, with variable neutral to alkaline salt proportions according to the soil (Shi & 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 re-establish 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 energy metabolism (Tester & Davenport 2003). Alkalinity can disrupt the balance of ions (Shi & Zhao 1997), cause micronutrient deficiency due to alteration of micronutrient availability in soil (Alam *et al.* 1999) and change antioxidant enzyme, amino acid and carbohydrate composition (Zhang *et al.* 2012; Kukavica *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 vessels (Kiliç *et al.* 2007; Bougailleb *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 & Reed 2008).

*Lotus tenuis* (Waldst. and Kit., syn. *L. glaber*) is a glycophytic forage legume, well adapted to the lowlands of Buenos Aires Province (the most important cattle production region in Argentina). This region is characterised by the presence of soil Na₂CO₃ and NaHCO₃ (Costa & 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 nutrient accumulation and root architecture and anatomy (Paz et al. 2012). However, the morpho-anatomical responses of *L. tenuis* shoots to the above stresses remains unexplored.

With the purpose of increasing current knowledge on differences and similarities between the above-mentioned stresses, in this work we 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 examined 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.

**MATERIAL AND METHODS**

**Plant growth conditions**

Seeds of *L. tenuis* cv. Esmeralda were scarified with sulphuric acid (100%), washed in distilled water and sown in Petri dishes containing water agar (0.8%), incubated for 7 days in a growth chamber, with a 16 h/8 h photoperiod at (day/night) 24 °C/19 °C and 60/80 ± 5% relative humidity. Light (200 μmol m−2 s−1) was provided with daylight and GroLux fluorescent lamps (F 40 W). One seedling was transferred to each 5.8 cm (diameter) × 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 KNO3, 2 mM Ca(NO3)2, 4H2O, 1 mM SO4Mg, 7H2O, 0.5 mM NH4H2PO4, 0.5 mM EDTA NaFeO8, 2H2O and 0.5 mM of each of the following micronutrients: MnCl2, 4H2O, H3BO3, CuSO4.5H2O, ZnSO4.7H2O and MoO4Na2.2H2O. 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 used in order to avoid variations in pH and salt accumulation due to water evaporation throughout the experiment. This system allowed homogeneous distribution of nutrients within the pot and daily replacement, by percolation, of an amount of nutrient solution equivalent to three-quarters of substrate field capacity.

**Experimental design and stress treatment**

Experiments were performed according to a completely randomised design of one factor (stress) and four levels: control, saline, alkaline and mixed salt–alkaline. The alkaline condition in a pot was created with addition of 10 mM NaHCO3 to 0.5 × Hoagland solution; whereas for the saline condition, 100 mM NaCl were used as the salt stress source. For the mixed saline–alkaline treatment, we employed the same 0.5 × Hoagland solution with 90 mM NaCl + 10 mM NaHCO3, thus obtaining a stress solution with the same Na+–derived EC but a higher alkalinity than that of the saline stress treatment. Control treatment consisted of plants irrigated with 0.5 × Hoagland solution without NaCl or NaHCO3. The pH and EC of irrigation solutions were monitored every 3 days with a combined pH meter/conductimeter (HI 255; Hanna Instruments, Padova, Italy) and maintained at pH–EC (dSm−1) 5.8–1.2, 5.8–11.0, 8.0–1.9 and 8.0–11.0, for control, saline, alkaline and mixed saline–alkaline treatments, respectively. In order to avoid any osmotic shock in the saline and mixed saline–alkaline treatments, 8-day-old seedlings initially received 30 and 20 mM NaCl + 10 mM NaHCO3, respectively. Salt concentrations were then step-wise increased during 1 week (acclimation) until reaching the final concentrations. In the alkaline treatment, 8-day-old plants received the final NaHCO3 concentration. After acclimation, plants were further grown under their respective treatments for a further 20 days.

**Growth and morphological parameters**

Ten plants per treatment were used for dry matter, length, number of stem nodes and leaf area determination (n = 10). For dry matter, plants were dried at 60 °C until constant weight. For leaf area, fully extended leaves were scanned with a 600 ppi resolution (HP PSC 1510; Hewlett Packard Development Co., Houston, TX, USA). Leaf area was measured on digitised images with the Image-ProPlus version 4.1 software (Media Cybernetics, Inc. Rockville, USA).

**Analysis of leaf and stem anatomy**

Anatomical variations caused by the three different stresses were analysed in tissues that had developed by the end of the experiment in eight plants per treatment (n = 8). A 1-cm long stem sample was sectioned from the internode immediately below the apical bud, whereas the leaf from the basal portion of this internode was dissected. The micro-morphology of the leaf epidermis was analysed according to D’Ambrogio de Argüeso (1986). Briefly, leaves fixed in FAA (formaldehyde:acetic acid, 10%:50%:5% + 35% water) were incubated in 5% KOH at 35 °C overnight, and then stained with 5% safranin. The abaxial and adaxial epidermis were removed from the mesophyll using dissecting needles, mounted on slides with water-glycerine (1:1), and observed at 400× (Nikon–Eclipse E–600 microscope attached to a computer and a digital camera Nikon DS Q1MC; Nikon, Tokyo, Japan). Stomatal aperture, number of stomata and ordinary epidermal cells per mm2 were recorded in the central leaflets. Data from abaxial and adaxial faces were averaged. The stomatal index was calculated according to Salisbury (1927): SI (%) = stomatal density/(stomatal density + epidermal cell density) × 100. Leaf and internode samples were fixed with FAA, dehydrated and embedded according to the procedures outlined in Johansen (1940). A series of transverse cross-sections 10-μm thick were obtained from the sample blocks using a Minot rotary microtome. Cross-sections were observed under the microscope and photographed. Digitised images were analysed with the Image-ProPlus version 4.1 software. The cuticle–epidermis height in both leaf faces, and the morphology and thickness of each mesophyll layer (spongy and palisade) were measured on digitised images of leaf transverse 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 digitised images.

**Analytical determinations**

Leaf proline content was estimated spectrophotometrically using the ninhydrin reaction method (Troll & Lindsley 1955).
with modifications (Magné & 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 analyser with a built-in leaf cuvette in an open-flow gas exchange system (LiCor 6400; LiCor, Lincoln, NE, USA). Measurement conditions were: PPFD, 1500 μmol m⁻² s⁻¹; airflow, 350 μmol CO₂ mol⁻¹; leaf temperature, 27 °C; leaf-to-air vapour pressure deficit: 1.6 kPa, all measured with a LI-6400. Data was collected at midday, from ten plants for each treatment (n = 10).

Osmotic potential (\(\psi_{w100}\)) measurement

At the end 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 into a syringe, frozen in liquid N₂ and kept at −80 °C pending further analysis. Syringes were thawed until samples reached room temperature, and the \(\psi_{w100}\) of leaked sap measured with a C-52 thermocouple. Data was collected from three plants for each treatment (n = 3).

**RESULTS**

Plant growth and morphology

The three stress treatments substantially reduced total plant biomass, stem length and number of nodes (Fig. 1A–C). The reduction in growth induced by NaCl or NaHCO₃ as a sole stress source was similar, whereas a combination of both salts led to maximum growth reduction. Leaf area was negatively affected by alkalinity in either the presence or absence of the neutral salt (Fig. 1D); in contrast, leaf area did not change in plants treated exclusively with NaCl.

Effect of salt treatments on leaf anatomy

The leaf epidermal cell density was drastically reduced with soil alkalinity (Table 1), indicating an enlargement of epidermal cell size. Unlike the above result, this parameter did not change in NaCl-treated plants. Alkalinity also led to a reduced stomatal density, being more obvious in the mixed salts treatment. However, stomatal index was not affected by any stress. On other hand, plants treated with NaCl (either alone or mixed with the alkaline salt) showed reduced stomatal opening

Statistical analysis

Data was subjected to one-way ANOVA, and comparisons using Duncan’s test. A \(y = \log_{10}x\) transformation was used to correct for lack of normality in biomass data.
compared with control plants. Interestingly, plants treated with NaHCO₃ as sole stress had higher stomatal opening than control plants.

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

Effect of salt treatments on stem anatomy

The stem cross-sectional area was reduced in all cases because of decreased pith, phloem and xylem cross-sectional area and reduced epidermal cell height (Table 2). In plants treated exclusively with the alkaline salt, there was a reduction in cortex cross-section area, in addition to the above-mentioned effects, leading to the largest contraction in stem cross-section area in this treatment (Table 2). The number of vascular bundles in the sectioned stem was also reduced by all three saline treatments, although the effect was more obvious under elevated alkalinity (Table 2). On other hand, a noteworthy rise in number of vessel elements in the xylem was recorded in NaCl-treated plants but not in those treated exclusively with NaHCO₃. In contrast, the area of each vessel element was reduced

**Table 1.** Leaf micro-morphological and anatomical parameters measured in Lotus tenuis. Fifteen-day-old plants were watered with nutrient solution with or without salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM NaHCO₃, respectively, were added to 0.5× Hoagland solution. For the mixed salt–alkaline stress treatment, the solution contained 90 mM NaCl and 10 mM NaHCO₃.

<table>
<thead>
<tr>
<th>variable</th>
<th>control</th>
<th>alkaline</th>
<th>saline</th>
<th>mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>epidermal cells density (number of cells mm⁻²)</td>
<td>30.8 ± 0.9 a</td>
<td>24.9 ± 0.9 b</td>
<td>29.5 ± 0.9 a</td>
<td>20.7 ± 0.8 c</td>
</tr>
<tr>
<td>stomata density (stomata mm⁻²)</td>
<td>115 ± 6 a</td>
<td>93 ± 6 b</td>
<td>108 ± 6 a,b</td>
<td>75 ± 5 c</td>
</tr>
<tr>
<td>stomatal index (SI %)</td>
<td>29 ± 3 a b</td>
<td>30 ± 1 a</td>
<td>29 ± 3 b</td>
<td>28 ± 1 b</td>
</tr>
<tr>
<td>stomatal opening area (μm²)</td>
<td>15 ± 1 b</td>
<td>20 ± 1 a</td>
<td>12 ± 1 c</td>
<td>13 ± 1 c</td>
</tr>
<tr>
<td>leaf thickness (μm)</td>
<td>99 ± 12 b</td>
<td>156 ± 11 a</td>
<td>178 ± 15 a</td>
<td>173 ± 16 a</td>
</tr>
<tr>
<td>cuticle thickness (μm)</td>
<td>0.9 ± 0.0 b</td>
<td>1.6 ± 0.1 a</td>
<td>1.7 ± 0.1 a</td>
<td>1.8 ± 0.1 a</td>
</tr>
<tr>
<td>adaxial epidermis height (μm)</td>
<td>9 ± 1.0 c</td>
<td>16 ± 1.0 b</td>
<td>21 ± 2.0 a</td>
<td>22 ± 2.0 a</td>
</tr>
<tr>
<td>abaxial epidermis height (μm)</td>
<td>15 ± 2.0 c</td>
<td>24 ± 1.0 b</td>
<td>31 ± 2.0 a</td>
<td>36 ± 1.0 a</td>
</tr>
<tr>
<td>thickness of palisade parenchyma (μm)</td>
<td>47 ± 4 c</td>
<td>69 ± 6 b</td>
<td>94 ± 6 a</td>
<td>—</td>
</tr>
<tr>
<td>thickness of spongy parenchyma (μm)</td>
<td>52 ± 7 b</td>
<td>66 ± 9 a</td>
<td>92 ± 9 a</td>
<td>82 ± 4.0 a</td>
</tr>
</tbody>
</table>

Average data (±SE, n = 8) with the same letter are not significantly different (Duncan, P < 0.01).

![Fig. 2](image-url) Leaf anatomical response of Lotus tenuis. (A) Control, (B) Alkaline, (C) Saline and (D) Mixed saline–alkaline. Scale bars = 200 μm. pp, palisade parenchyma; sp, spongy parenchyma; ea, abaxial epidermis; x, xylem; p, phloem. Transverse cross-section of L. tenuis leaves. Fifteen-day-old plants were watered with nutrient solution with or without salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM NaHCO₃, respectively, were added to 0.5× Hoagland solution. For the mixed salt–alkaline stress treatment, the solution contained 90 mM NaCl and 10 mM NaHCO₃.
Table 2. Stem anatomical parameters measured in Lotus tenuis. Fifteen-day-old plants were watered with nutrient solution with or without salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM NaHCO3, respectively, were added to 0.5 × Hoagland solution. For the mixed salt–alkaline stress treatment, the solution contained 90 mM NaCl and 10 mM NaHCO3.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Alkaline</th>
<th>Saline</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>stem cross-section area (mm²)</td>
<td>0.8 ± 0.01</td>
<td>0.3 ± 0.03</td>
<td>0.51 ± 0.02</td>
<td>0.53 ± 0.02</td>
</tr>
<tr>
<td>epidermis thickness (µm)</td>
<td>25.7 ± 0.3</td>
<td>18.1 ± 1.0</td>
<td>22.4 ± 0.5</td>
<td>21.2 ± 0.7</td>
</tr>
<tr>
<td>pith cross-section area (mm²)</td>
<td>0.19 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>cortex thickness (µm)</td>
<td>119 ± 2</td>
<td>90 ± 5</td>
<td>119 ± 3</td>
<td>79 ± 4</td>
</tr>
<tr>
<td>phloem cross-section area (× 100 mm²)</td>
<td>4.31 ± 0.04</td>
<td>1.55 ± 0.19</td>
<td>1.96 ± 0.24</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>xylem cross-section area (× 100 mm²)</td>
<td>1.22 ± 0.01</td>
<td>0.49 ± 0.09</td>
<td>1.04 ± 0.08</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>number of vascular bundles</td>
<td>10.0 ± 0.0</td>
<td>8.0 ± 0.0</td>
<td>9.0 ± 0.0</td>
<td>8.0 ± 1.0</td>
</tr>
<tr>
<td>total number of vessels in xylem</td>
<td>97 ± 1</td>
<td>82 ± 13</td>
<td>147 ± 8</td>
<td>146 ± 5</td>
</tr>
<tr>
<td>vessel cross-section area (µm²)</td>
<td>126 ± 2</td>
<td>62 ± 5</td>
<td>62 ± 6</td>
<td>28 ± 1</td>
</tr>
</tbody>
</table>

Average data (±SE, n = 8) with the same letter are not significantly different (Duncan, P < 0.01).

in all cases. Moreover, both salts showed a synergistic effect on vessel cross-sectional area when applied simultaneously.

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

The leaf proline content was increased NaCl-treated plants, whereas NaHCO3 had no effect (Fig. 3). Reductions in g and E were observed in L. tenuis plants treated with NaCl regardless the alkalinity level (Fig. 4), but there were no changes in g or E in plants confronted exclusively with NaHCO3 (Fig. 4). At the time of harvest, the leaf Ψp was not significantly altered by separately applied NaCl or NaHCO3, but in plants treated with the mixture of neutral and alkaline salts, the Ψp was significantly increased (Fig. 5).

DISCUSSION

The present work aimed to explore and compare the morpho-anatomical responses of L. tenuis shoots to alkaline, saline and mixed saline–alkaline stresses. Our results showing that separately applied NaCl and NaHCO3 salts induced similar growth reductions (Fig. 1) is in contrast with previous studies where alkaline salts were significantly more toxic than neutral salts (Shi & Sheng 2005; Shi & Wang 2005; Wang et al. 2008; Yang et al. 2008). Such seeming disparity may be explained by the fact that at the NaHCO3 concentration used in our work (10 mM, several-fold lower than those used by other authors), the ionic and osmotic components of saline stress are avoided and the growth reduction effect can be fundamentally attributed 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 the detrimental effects. Such synergism is in line with previous observations reported for other glycophytes (Shi & Sheng 2005; Shi & Wang 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, enlargement of epidermal cell size was found in plants grown under alkaline conditions. To

Fig. 3. Leaf proline content (µmol g⁻¹ fresh weight) in Lotus tenuis. Fifteen-day-old plants were watered with nutrient solution with or without salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM NaHCO3, respectively, were added to 0.5 × Hoagland solution. For the mixed salt–alkaline stress treatment, the solution contained 90 mM NaCl and 10 mM NaHCO3. Average data (±SE, n = 6) with the same letter are not significantly different (Duncan, P < 0.01).

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 tubes (Lovy-Wheeler et al. 2006) and, therefore, additional specific experimentation in this regard would be fruitful.

The fact that NaCl addition (regardless of alkalinity level) reduced stomatal opening 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 NaHCO3 as sole stress source, induced higher stomatal opening is compatible with previous results indicating that stomatal aperture of isolated abaxial epidermis (incubated on simple buffers) increased with the external alkalinity (Wilkinson & Davies 1997).

On the other hand, despite the cell size augmentation observed in plants treated with NaHCO3, leaf area was reduced, suggesting that alkalinity decreased new cell production by the leaf.
Long-term responses to neutral and alkaline stress

Paz, Reinoso, Espasandin, González Antivilo, Sansberro, Rocco, Ruiz & Menéndez

Page 6

Fig. 4. Mean stomatal conductance (A) and transpiration rate (B) in Lotus tenuis. Fifteen-day-old plants were watered with nutrient solution with or without salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM NaHCO3, respectively, were added to 0.5× Hoagland solution. For the mixed salt–alkaline stress treatment, the solution contained 90 mM NaCl and 10 mM NaHCO3. Average data (±SE; n = 10) with the same letter are not significantly different (Duncan, P < 0.01).

Fig. 5. Leaf osmotic potential (ψw)MPa. Fifteen-day-old plants were watered with nutrient solution with or without salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM NaHCO3, respectively, were added to 0.5× Hoagland solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM NaHCO3. Average data (±SE; n = 3) with the same letter are not significantly different (Duncan, P < 0.01).

The reduced leaf area evidenced in our alkalinised Lotus tenuis plants (either in the 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 (HCO3−; Valdez-Aguilar 2004; Cartmill et al. 2007, 2008). In addition, the neutral effect of NaCl, as sole stress source, observed on L. tenuis leaf area agrees with former results obtained on soybean (Dolatabadian et al. 2011).

Our finding that plants treated with NaCl had thicker leaves is in line with descriptions in several other plant species (e.g. Qadir & Shams 1997; De Vos et al. 2010), suggesting the occurrence of osmolyte accumulation and osmotic adjustment. Plants treated exclusively with NaHCO3 also had thickened leaves, although to a much lower extent compared with NaCl addition, but this should not be assigned to an osmotic effect, since a low salt concentration (10 mM) was used in this treatment.

The observed loss of leaf mesophyll dorsiventrality in plants grown under high alkalinity highlights a difference in stress sensitivity between palisade and spongy parenchyma mesophyll. As far as we know, dorsiventral mesophyll loss has been reported only in plants subjected to low temperature stress (Kadokama et al. 2013). It has been postulated that since palisade mesophyll is the site for most plant photosynthetic activity, an increase in palisade:spongy mesophyll ratio positively increases photosynthesis (Kulkarni et al. 2008). Thus, our results showing the absence of palisade mesophyll in most of the analysed leaves of NaHCO3-treated plants could possibly have contributed to the noticeable growth reduction seen in these plants.

The reduction in stem cross-sectional area observed in L. tenuis plants stressed exclusively with NaCl is in line with previous studies performed on diverse plant species subjected to this saline condition (Qadir & Shams 1997; Soltekin et al. 2012; Guo et al. 2013; Habba et al. 2013; dos Santos et al. 2013). In the case of plants treated with the mixed salts, the smaller leaf area could have led to further reductions in stem cross-sectional area. However, the highest stem cross-section reduction was recorded in L. tenuis plants treated exclusively with NaHCO3. In these plants, the observed reduction could be related to other growth constraints not measured in this work, such as reduced cell division and expansion, besides a smaller leaf area. In turn, slender stems could affect bending, tensile and shearing properties of L. tenuis plants. These properties could be significant from the economic viewpoint, given the importance of L. tenuis as a forage species in several cattle production areas worldwide.

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

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

The amino acid proline is the compatible osmolyte that most commonly builds up in the cytoplasm as response to osmotic imbalance (Hasegawa et al. 2000). The increased leaf proline

Plant Biology © 2014 German Botanical Society and The Royal Botanical Society of the Netherlands
content detected in plants treated with NaCl (Fig. 3) suggested that they had experienced some 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 could 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 salts (Shi & Sheng 2005; Shi & Wang 2005; Wang et al. 2008; Yang et al. 2008).

A reduction in g and E noted in NaCl-treated plants (Fig. 4) is congruent with the observed decrease in stomatal opening and could have accounted for the decline in plant growth recorded in both NaCl treatments (Table 1; Fig. 1). Since enhanced transpiration tends to augment salt accumulation and hence cell damage (Munns 1985), the observed contracted stomatal opening may be interpreted as an adaptive L. tenuis response to the osmotic component of saline stress. On the other hand, our results from plants treated exclusively with NaHCO$_3$ showed no changes in g or E. This would advocate a possible compensation effect between an increase in stomatal opening and a reduction in the stomatal density in these plants. A similar salinity-induced reduction in stomatal density was recently reported for the halophyte Chenopodium quinoa (Orsini et al. 2011; Shabala et al. 2012), and was interpreted as a fundamental mechanism by which halophytic 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 & Bukovac 1968; Leon & Bukovac 1978; Boyer et al. 1997), one may also hypothesise 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 increase in leaf $\psi_{100}$ observed in L. tenuis plants subjected to the mixed saline–alkali treatment (Fig. 5). However, the proline level in the plants of this treatment (0.4 μmol·g$^{-1}$ fresh weight; Fig. 3) seems to be too low to act as osmolyte, compared with that needed for conventional osmotic adjustment (Marcum 2006). On the other hand, adverse environmental factors may induce rapid production of reactive oxygen species (ROS), leading to an oxidative burst and cell damage (Mittler 2002). In turn, proline may significantly reduce ROS-induced K$^+$ efflux (Cui & Shabala 2005, 2007). It has been postulated that a number of functions of proline could be involved in reducing the extent of ROS-induced K$^+$ efflux, such as free radical scavenging (Smirnoff & Cimbres 1989), reduction of ROS generation (Hong et al. 2000), osmoprotection (Delauney & Verma 1993) and protein stabilisation (Shah & Dubey 1998). Therefore, it is possible that the increase in proline level could have indirectly contributed to the osmotic adjustment of L. tenuis leaves in the mixed saline–alkali treatment.

CONCLUSIONS
Neutral and alkaline salts produced a similar level of growth inhibition on L. tenuis shoots, whereas their mixture exacerbated the detrimental effects. On the other hand, common and distinct effects of the three stresses on L. tenuis shoot growth and anatomy were evidenced, according to the analysed parameter. None of the analysed parameters categorically differentiated the three types of stress. However, NaCl- and NaHCO$_3$-derived stresses could be discriminated to different extents and/or directions of changes in some anatomical traits. For example, alkalinity itself increased stomatal opening, converse to NaCl-treated plants, where a reduction in stomatal aperture was observed. Likewise, plants from the mixed saline–alkali treatment lacked palisade mesophyll in the leaves. The conspicuous lack of information regarding the effect of alkalinity on plant cell growth prevents us from further discussing our results related to high pH conditions, while invites future research in this direction. Further studies are required to ascertain the unambiguous cause-and-effect relationships between the observed anatomical trait changes in L. tenuis and each of the factors intervening in the three studied stresses (e.g. osmotic, toxic, pH homeostasis, etc.). Such studies could help to design mechanistic models for predicting the whole-plant response to stresses derived from different salt sources.

ACKNOWLEDGEMENTS
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 Martín (UNSAM) and Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC). The authors acknowledge grants-in-aid from COST-Action FA0605. We also thank Jorge Luis Paz for valuable help with the irrigation system set up. RCP expresses gratitude to CONICET for doctoral fellowships.

REFERENCES
Long-term responses to neutral and alkaline stress

Paz, Reinoso, Espasandin, González Antivilo, Sansberro, Rocco, Ruiz & Menéndez


