plant biology
Plant Biology ISSN 1435-8603

RESEARCH PAPER

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

R. C. Paz[1,2], H. Reinoso[3], F. D. Espasandin[4], F. A. González Antivilo[2], P. A. Sansberro[4], R. A. Rocco[1], O. A. Ruiz[1] & A. B. Menéndez[1,5]

1 Unidad de Biotecnología 1, IIB-IINTECH/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 $NaHCO_3$, pH = 8.0, EC = 1.9 dS·m^{-1}) and mixed salt–alkaline (10 mM $NaHCO_3$ + 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 $NaHCO_3$-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 $NaHCO_3$.

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's arable land, limiting agricultural production (Läuchli & Lüttge 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; 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 & 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 Na_2CO_3 and $NaHCO_3$ (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 $\mu mol \cdot m^{-2} \cdot s^{-1}$) 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 $dS \cdot m^{-1}$) and irrigated with 0.5 × Hoagland's nutrient solution containing 3 mM KNO_3, 2 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1 mM $SO_4Mg \cdot 7H_2O$, 0.5 mM $NH_4H_2PO_4$, 0.5 EDTA $NaFeO_8 \cdot 2H_2O$ and 0.5 mM of each of the following micronutrients $MnCl_2 \cdot 4H_2O$, H_3BO_3, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$ and $MoO_4Na_2 \cdot 2H_2O$. 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 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 $NaHCO_3$ 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 $NaHCO_3$, 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 $NaHCO_3$. 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 ($dS \cdot m^{-1}$) 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 $NaHCO_3$, 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 $NaHCO_3$ 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 digitalised 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: alcohol: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 Qi1Mc; Nikon, Tokyo, Japan). Stomatal aperture, number of stomata and ordinary epidermal cells per mm^2 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·CO_2·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 (ψ_π^{100}) 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 N_2 and kept at −80 °C pending further analysis. Syringes were thawed until samples reached room temperature, and the ψ_π^{100} of leaked sap measured with a C-52 thermocouple. Data was collected from three plants for each treatment (n = 3).

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.

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 $NaHCO_3$ 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

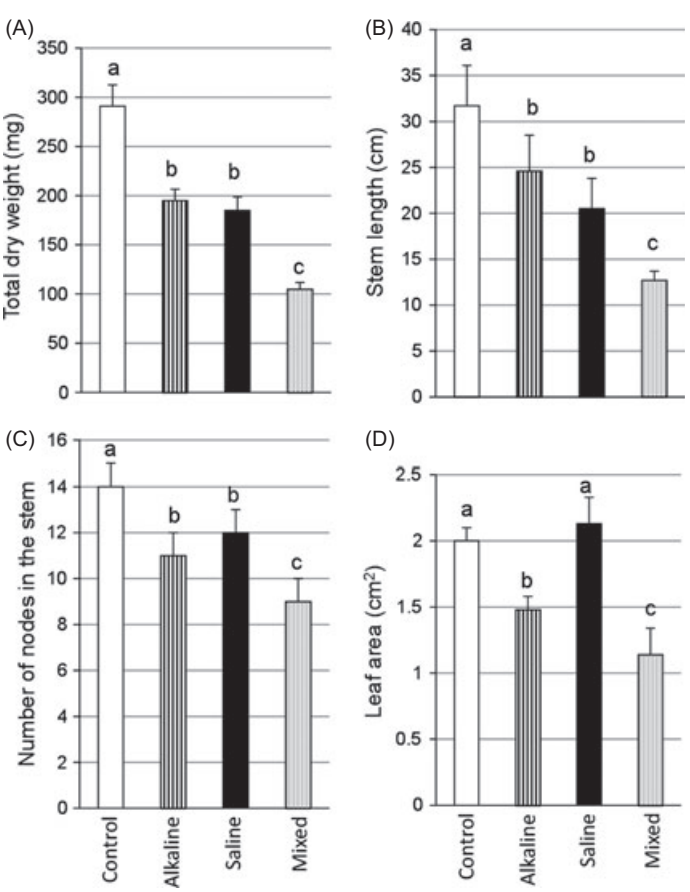

Fig. 1. Total dry weight (A), stem length (B), number of nodes in the stem (C) and leaf area (D) of *Lotus tenuis*. Fifteen-day-old plants were watered with nutrient solution with or without salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$ respectively, were added to 0.5× Hoagland solution. For the mixed salt–alkaline stress treatment, the solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE, n = 10) with the same letter are not significantly different (Duncan, $P < 0.001$).

compared with control plants. Interestingly, plants treated with $NaHCO_3$ 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 $NaHCO_3$. 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 $NaHCO_3$, respectively, were added to 0.5× Hoagland solution. For the mixed salt–alkaline stress treatment, the solution contained 90 mM NaCl and 10 mM $NaHCO_3$.

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 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
stomatal 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

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

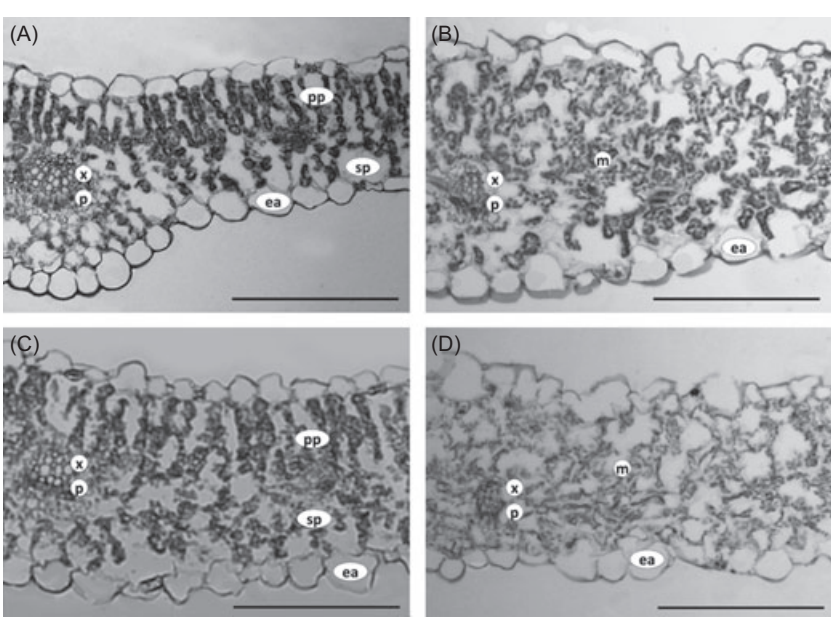

Fig. 2. Leaf anatomical response of *Lotus tenuis*. (A) Control, (B) Alkaline, (C) Saline and (D) Mixed saline–alkaline. Scale bars = 200 μm. *pp*, palisade parenchyma; *sp*, spongy parenchyma; *ea*, abaxial epidermis; *x*, xylem; *p*, phloem. Transverse cross-section of *L. tenuis* leaves. Fifteen-day-old 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 $NaHCO_3$, respectively, were added to 0.5× Hoagland solution. For the mixed salt–alkaline stress treatment, the solution contained 90 mM NaCl and 10 mM $NaHCO_3$.

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 $NaHCO_3$, respectively, were added to 0.5× Hoagland solution. For the mixed salt–alkaline stress treatment, the solution contained 90 mM NaCl and 10 mM $NaHCO_3$.

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

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 $NaHCO_3$ 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 $NaHCO_3$ (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 neutral and alkaline salts, the ψ_π^{100} was significantly increased (Fig. 5).

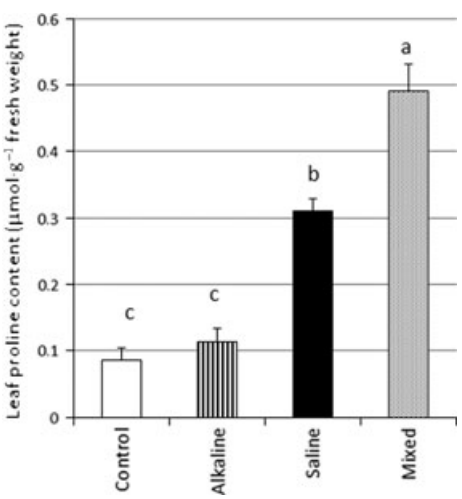

Fig. 3. Leaf proline content (μmol·g^{-1} fresh weight) in *Lotus tenuis*. Fifteen-day-old plants were watered with nutrient solution with or without salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$, respectively, were added to 0.5× Hoagland solution. For the mixed salt–alkaline stress treatment, the solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE, n = 6) with the same letter are not significantly different (Duncan, $P < 0.01$).

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 $NaHCO_3$ 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 $NaHCO_3$ 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 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 $NaHCO_3$, 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 other hand, despite the cell size augmentation observed in plants treated with $NaHCO_3$, leaf area was reduced, suggesting that alkalinity decreased new cell production by the leaf

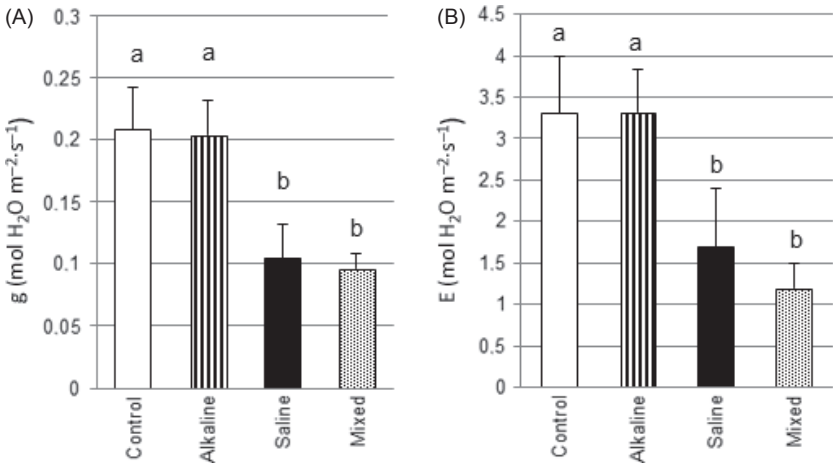

Fig. 4. Mean stomatal conductance (A) and transpiration rate (B) in *Lotus tenuis*. Fifteen-day-old plants were watered with nutrient solution with or without salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$, respectively, were added to 0.5× Hoagland solution. For the mixed salt–alkaline stress treatment, the solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE, n = 10) with the same letter are not significantly different (Duncan, $P < 0.01$).

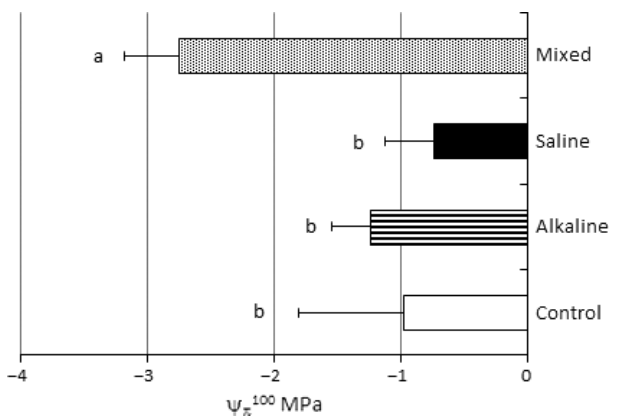

Fig. 5. Leaf osmotic potential (ψ_π^{100} MPa). Fifteen-day-old plants were watered with nutrient solution with or without salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$, respectively, were added to 0.5× Hoagland solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE, n = 3) with the same letter are not significantly different (Duncan, $P < 0.01$).

meristem. The reduced leaf area evidenced in our alkalinised *L. 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 (HCO_3^-; 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 $NaHCO_3$ 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 (Kadohama *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 $NaHCO_3$-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 $NaHCO_3$. 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

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 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 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 & 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 ψ_π^{100} observed in *L. tenuis* plants subjected to the mixed saline–alkaline treatment (Fig. 5). However, the proline level in the plants of this treatment (0.4 $\mu mol \cdot g^{-1}$ 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 (Cuin & 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 & Cumbes 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–alkaline 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–alkaline 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 Martin (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

Alam S.M., Mehdi Naqvi S.S., Ansari R. (1999) Impact of soil pH on nutrient uptake by crop plants. In: Pessarakli M. (Ed.), *Handbook of plant and crop stress*. CRC Press, Boca Raton, FL, USA, pp 51–60.

Boughalleb F., Denden M., Tiba B.B. (2009) Anatomical changes induced by increasing NaCl salinity in three fodder shrubs, *Nitraria retusa, Atriplex halimus* and *Medicago arborea*. *Acta Physiologiae Plantarum*, **31**, 947–960.

Boyer J.S., Wong S.C., Farquhar C.D. (1997) CO, and water vapor exchange across the leaf cuticle (epidermis) at various water potentials. *Plant Physiology*, **114**, 185–191.

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

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

Cartmill A.D., Valdez-Aguilar L.A., Bryan D.L., Alarcón A. (2008) Arbuscular mycorrhizal fungi enhance tolerance of *Vinca* to high alkalinity in irrigation water. *Scientia Horticulturae*, **115**, 275–284.

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

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

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

D'Ambrogio de Argüeso A. (1986) *Manual de Técnicas en Histología Vegetal. Hemisferio Sur*. Hemisferica Sur, Buenos Aires, Argentina, pp 50–67.

De Vos A.C., Broekman R., Groot M.P., Rozema J. (2010) Ecophysiological response of *Crambe mariti-*

ma to airborne and soil-borne salinity. *Annals of Botany*, **105**, 925–937.

Delauney A.J., Verma D.P.S. (1993) Proline biosynthesis and osmoregulation in plants. *The Plant Journal*, **4**, 215–223.

Dolatabadian A., Modarressanavy S.A.M., Ghanati F. (2011) Effect of salinity on growth, xylem structure and anatomical characteristics of soybean. *Notulae Scientia Biologicae*, **3**, 41–45.

Gharsalli M., Zribi K., Hajji M. (2001) Physiological responses of pea to iron deficiency induced by bicarbonate. In: Horst W., Schenk M.K., Bürkert A., Claassen N., Flessa H., Frommer W.B., Goldbach H.E., Olfs H.-W., Römheld V., Sattelmacher B., Schmidhalter U., Schubert S., von Wirén N., Wittenmayer L. (Eds), *Plant nutrition - food security and sustainability of agro-ecosystems*. Springer, Berlin, Germany, pp 606–607.

Guo X.P., Tackmore M., Obai K., Salahou M.K. (2013) The combined effects of salinity and water stress on the growth and yield quality of tomato. *Applied Mechanics and Materials*, **295**, 2265–2273.

Habba I.E., Abd El Aziz N.G., Metwally S.A., Mazhar A.A.M. (2013) Response of growth and chemical constituents in *Khaya sengalensis* to salinity and gypsum under calcareous soil conditions. *World Applied Sciences Journal*, **22**, 447–452.

Hasegawa P.M., Bressan R.A., Zhu J.K., Bohnert H.J. (2000) Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology*, **51**, 463–499.

Hong Z.L., Lakkineni K., Zhang Z.M., Verma D.P.S. (2000) Removal of feedback inhibition of Δ^1-pyrroline-5-carboxylated synthetase results in increased proline accumulation and protection of plants from osmotic stress. *Plant Physiology*, **122**, 1129–1136.

Johansen D.A. (1940) *Plant microtechnique*. McGraw-Hill, New York, USA, pp 1–523.

Kadohama N., Goh T., Ohnishi M., Fukaki H., Mimura T. (2013) Sudden collapse of vacuoles in *Saintpaulia* sp. palisade cells induced by a rapid temperature decrease. *PLoS ONE*, **8**, e57259.

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

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

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

Läuchli A., Lüttge U. (2002) *Salinity: Environment – Plants – Molecules*. Springer, Berlin, Germany, pp 552.

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

Li P.H., Zhang H., Wang B.S. (2003) Ionic homeostasis of plants under salt stress. *Acta Botanica Boreal-Occident Sinica*, **23**, 1810–1817.

Li R., Shi F., Fukuda K. (2010) Interactive effects of salt and alkali stresses on seed germination, germination recovery, and seedling growth of a halophyte *Spartina alterniflora* (Poaceae). *South African Journal of Botany*, **76**, 380–387.

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. *The Plant Cell*, **18**, 2182–2193.

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 aspen responses to alkalization of soil: growth, leaf structure, photosynthetic rate and carbohydrates. *Trees*, **26**, 1847–1858.

Marcum K.B. (2006) Saline tolerance physiology in grasses. In: Khan M. A., Weber D. J. (Eds), *Ecophysiology of high salinity tolerant plants*. Springer, Berlin, Germany, pp 157–172.

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

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

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

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.

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 salinity in *Chenopodium quinoa*: functional elements of successful halophytism. *Functional Plant Biology*, **38**, 818–831.

Paz R.C., Rocco R.A., Reinoso H., Menéndez A.B., Pieckenstain F.L., Ruiz A.O. (2012) Comparative study of alkaline, saline, and mixed saline-alkaline stresses with regard to their effects on growth, nutrient accumulation, and root morphology of *Lotus tenuis*. *Journal of Plant Growth Regulation*, **31**, 448–459.

Qadir M., Shams M. (1997) Some agronomic and physiological aspects of salt tolerance in cotton (*Gossypium hirsutum* L.). *Journal of Agronomy and Crop Science*, **179**, 101–106.

Salisbury E.J. (1927) On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. *Philosophical Transactions of the Royal Society of London B*, **216**, 1–65.

dos Santos J.B., dos Santos D.B., de Azevedo C.A.V., Rebequi A.M., Cavalcante L.F., Cavalcante I.H.L. (2013) Comportamento morfofisiológico da mamoneira BRS energia submetida à irrigação com água salina [Morphophysiological behavior of castor bean brs energia submitted to irrigation with saline water]. *Revista Brasileira de Engenharia Agrícola e Ambiental*, **17**, 145–152.

Scott F.M. (1964) Lipid deposition in the intercellular space. *Nature*, **203**, 164–165.

Scott F.M. (1966) Cell wall surface of the higher plants. *Nature*, **210**, 1015–1017.

Shabala L., Mackay A., Tian Y., Jacobsen S., Zhou D., Shabala S. (2012) Oxidative stress protection and stomatal patterning as components of salinity tolerance mechanism in quinoa (*Chenopodium chinoa*). *Physiologia Plantarum*, **146**, 26–38.

Shah K., Dubey R.S. (1998) Effect of cadmium on proline accumulation and ribonuclease activity in rice seedlings: role of proline as a possible enzyme protectant. *Biologia Plantarum*, **40**, 121–130.

Shi D., Sheng Y. (2005) Effect of various salt–alkaline mixed stress conditions on sunflower seedlings and analysis of their stress factors. *Environmental and Experimental Botany*, **54**, 8–21.

Shi D.C., Wang D. (2005) Effects of various salt–alkaline mixed stresses on *Aneurolepidium chinense* (Trin.) Kitag. *Plant and Soil*, **271**, 15–26.

Shi D., Zhao K. (1997) Effects of sodium chloride and carbonate on growth of *Puccinellia tenuiflora* and on present state of mineral elements in nutrient solution. *Acta Pratacult Sinica*, **6**, 51–61.

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.

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

Troll W., Lindsley J. (1955) The photometric methods for determination of proline. *Journal of Biological Chemistry*, **215**, 655–660.

Valdez-Aguilar L.A. (2004) Effect of alkalinity in irrigation water on selected greenhouse crops. Ph.D. thesis in Horticulture, Texas A&M University, Texas, USA.

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.

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.

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.

Yang C., Chong J., Changyou L., Kim C., Shi D., Wang D. (2007) Osmotic adjustment and ion balance traits of an alkali resistant halophyte *Kochia sieversiana* during adaptation to salt and alkali conditions. *Plant and Soil*, **294**, 263–276.

Yang C., Shi D., Wang D. (2008) Comparative effects of salt and alkali stresses on growth, osmotic adjustment and ionic balance of an alkali-resistant halophyte *Suaeda glauca* (Bge.). *Plant Growth Regulation*, **56**, 179–190.

Zhang P., Fu J., Hu L. (2012) Effects of alkali stress on growth, free amino acid and carbohydrate metabolism in Kentucky bluegrass (*Poa pratensis*). *Ecotoxicology*, **21**, 1911–1918.