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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*

Rosalía Cristina Paz · Ruben Anibal Rocco · Herminda Reinoso · Ana Bernadina Menéndez · Fernando Luis Pieckenstain · Oscar Adolfo Ruiz

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Abstract Both saline and alkaline conditions frequently coexist in nature; however, little is known about the effects of alkaline and salt–alkaline stresses on plants. We performed pot experiments with four treatments, control without salt addition and three stress conditions—neutral, alkaline, and mixed salt–alkaline—to determine their effects on growth, nutrient accumulation and root architecture in the glycophytic species *Lotus tenuis*. Neutral and alkaline salts produced a similar detrimental effect on *L. tenuis* growth, whereas the effect of their combination was synergistic. Neutral salt addition, alone or mixed with $NaHCO_3$, led to significant leaf Na^+ build up and reduced K^+ concentration. In contrast, in plants treated with $NaHCO_3$ only, Na^+ levels and the Na^+/K^+ ratio remained relatively unchanged. Proline accumulation was not affected by the high pH in the absence of NaCl, but it was raised by the neutral salt and mixed treatments. The total root length was reduced by the addition of NaCl alone, whereas it was not affected by alkalinity, regardless of the presence of NaCl. The topological trend showed that alkalinity alone or mixed with NaCl turned the root more herringbone compared with control roots, whereas no significant change in this index was observed in the treatment with the neutral salt only. The pattern of morphological changes in *L. tenuis* root architecture after the alkaline treatment (in the absence of NaCl) was similar to that found in the mixed salt–alkaline treatment and different from that observed in neutral salt. A unique root morphological response to the mixed salt–alkaline stress was the reduction in the ratio between xylem vessels and root cross-sectional areas.

Keywords *Lotus tenuis* · Alkaline stress · Saline stress · Growth · Carbon allocation · Root topology · Root anatomy · Ion homeostasis

R. C. Paz and R. A. Rocco contributed equally to this work

R. C. Paz · R. A. Rocco · F. L. Pieckenstain · O. A. Ruiz
Unidad de Biotecnología 1, IIB-IINTECH/UNSAM-CONICET, Chascomús, Buenos Aires, Argentina

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

A. B. Menéndez (✉)
Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina
e-mail: anamen@bg.fcen.uba.ar; anamenendez@intech.gov.ar

A. B. Menéndez
Unidad de Biotecnología 1, IIB-IINTECH/CONICET, Chascomús, Buenos Aires, Argentina

Introduction

Alkaline stress refers to the presence of alkaline salts (Na_2CO_3 or $NaHCO_3$) in the soil, whereas saline stress is related only to neutral salts such as NaCl or Na_2SO_4 (Yang and others 2007). Although saline stress has been addressed in numerous studies, alkalinity rather than salinity is the main constraint for cropping activity, given that the cultivated area of alkaline soils ($37\% = 0.56 \times 10^9$ ha) is greater than that of saline soils ($23\% = 0.34 \times 10^9$ ha; Tanji 1990).

Saline stress affects plant growth by inducing osmotic inhibition of water absorption (Greenway and Munns 1980; Munns 2002), whereas the intracellular accumulation of Na^+ or Cl^- may inhibit the activity of several enzymes and

diminish protein synthesis, affecting photosynthesis and energetic metabolism (Yeo 1998; Tester and Davenport 2003). Therefore, cells need to adjust osmotically and re-establish the ion balance (Li and others 2003).

Alkaline salts have a more severe effect on plant growth than neutral salts (Shi and Yin 1993; Tang and Turner 1999). When soil salinity is high and/or the pH is 8.5 or above, a number of micronutrients such as P, Fe, and Zn become deficient (Clark 1982; Marschner 1995). Both saline and alkaline conditions frequently coexist in nature, with the proportion of neutral salt to alkaline salt varying in different soils (Shi and Wang 2005; Li and others 2010). However, little is known about the effects of alkaline and salt–alkaline stresses on plants. These facts have led to the emergence of investigations addressing the problem of alkaline (Cartmill and others 2008; Valdez-Aguilar and Reed 2007, 2008) and salt–alkaline mixed (Shi and Wang 2005; Shi and Sheng 2005a, b) stresses over the last few years.

Root architecture has been directly related to plant productivity (Lynch 1995) because in no-till systems it can provide clues about resource cost, transport, and exploration efficiency, especially under limiting edaphic conditions. Root system architecture can be analyzed according to several measurable variables such as topology (Fig. 1), root length, and branching (Fitter 1987). Different levels of these variables represent attributes of the root system in terms of acquisition, storage of soil resources, anchorage, and cost of root production (Fitter 1987; Fitter and Stickland 1991). Salinity affects root developmental processes in different ways. Evident differences in the morphology of the root system as a function of salt stress treatment were found in cotton (Kurth and others 1986), rice (Bahaji and others 2002), and *Lotus tenuis* (Echeverria and others 2008). The occurrence of ion toxicity resulting from elevated NaCl concentration in a nutrient solution slowed down root extension (Cramer and others 1986; Neumann and others 1999), whereas lateral root formation was less altered (Waisel and Breckle 1987) or improved by high salt (Kramer 1980). Root anatomy traits are also directly implicated in the whole-plant functioning and may be altered by saline stress (Hummel and others 2007). However, very few reports have been published so far on the effect of high soil alkalinity on root architecture and anatomy (for example, Tang and Turner 1999).

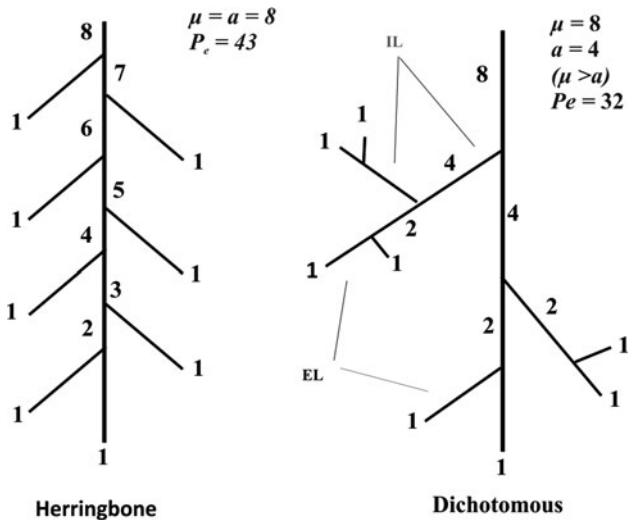

Fig. 1 Extreme topological root structures: μ magnitude, number of exterior links; a altitude, number of links in the longest unique path from the base link to an exterior link; P_e total external pathlength, sum of the number of links in all paths from all external links on the base link

The glycophyte *L. tenuis* (Waldst. and Kit., syn. *L. glaber*; Kirkbride 2006) is the best adapted legume forage in the lowlands of the Buenos Aires Province (the most important cattle production region in Argentina). The presence of Na_2CO_3 and $NaHCO_3$, the main sources of high soil alkalinity (pH >9.0), is a major characteristic of soils in that region (Costa and García 1998). These salts, alone or combined with NaCl, significantly decrease persistence and yield of common legumes (Mazzanti and others 1986).

Studies on abiotic stress in *L. tenuis* have been focused principally on NaCl tolerance (Mendoza and others 2005; Teakle and others 2006, 2007; Sannazzaro and others 2006, 2007; Echeverria and others 2008), whereas no study has so far addressed its tolerance level to alkaline and mixed salt–alkaline stresses.

The aims of the present research were (1) to compare *L. tenuis* responses to neutral salt versus alkaline salt stress with regard to their effect on growth, accumulation of key nutrients, and root morphology; (2) to study the response of *L. tenuis* to the mixed salt–alkaline stress; and (3) to identify common and distinctive features of the morphological and nutritional responses of *L. tenuis* roots to the mixed salt–alkaline stress with respect to the other two stresses.

Materials and Methods

Plant Material and Growth Conditions

Seeds of *L. tenuis* cv. Esmeralda were scarified with sulfuric acid (100%), washed in distilled water, and sown in Petri plates containing water–agar (0.8%). Plates were incubated for 7 days in a growth chamber, with a 16/8 h photoperiod at 24°C/19°C (day/night) and 60/80 ± 5% relative humidity. Light intensity (200 μmol m^{-2} s^{-1}) was provided by daylight and Grolux fluorescent lamps (F 40W). Two seedlings were transferred to each cylindrical

pot (5.8 × 20 cm; volume = 0.53 dm^3) containing washed sand (pH 7.0; E.C. = 0.05 mS cm^{-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 $MgSO_4{\cdot}7H_2O$; 0.5 mM $NH_4H_2PO_4$; 0.5 $NaFeO_8EDTA{\cdot}2H_2O$; 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 $Na_2MoO_4{\cdot}2H_2O$. Pots were kept at field capacity (200 ml) during the time lapse experiment. An ELGO® drip irrigation system (flow rate = 6.25 ml/h) was used to avoid variations in pH and salt accumulation due to water evaporation throughout the experiment. This system allowed a homogeneous distribution of nutrients within the pot and a daily replacement, by percolation, of an amount of nutrient solution equivalent to three quarters of the substrate field capacity.

Experimental Design

Experiments followed a completely randomized design of four treatments: control without salt addition and three stress conditions: neutral, alkaline, and mixed salt–alkaline. For morphometric and morphological determinations, the two plants growing within the pot were separated at harvest so that each plant was a replicate. For analytical determinations, plant material was pooled in groups of five individual plants, with each pool treated as one replicate. The experiment was repeated three times with similar results. Only results from the most representative experiment are shown.

Stress Treatments

Alkaline and saline stress conditions in the pot substrate were created by adding 10 mM $NaHCO_3$ and 100 mM NaCl, respectively, to 0.5× Hoagland's solution. For the mixed salt–alkaline stress treatment, we used a 0.5× Hoagland's solution containing 90 mM NaCl and 10 mM $NaHCO_3$. Control treatment consisted of plants irrigated with 0.5× Hoagland's solution without the addition of NaCl or $NaHCO_3$. The pH and E.C. of irrigation solutions were monitored every 3 days with a combined pH meter/conductimeter (HI 255, Hanna Instrument) and maintained at pH/E.C. (mS cm^{-1}) 5.8/1.2, 5.8/1.9, 8.0/11, and 8.0/11 for control, saline, alkaline, and mixed salt–alkaline treatments, respectively. To avoid any osmotic shock from the saline and mixed salt–alkaline treatments, 8-day-old seedlings initially received 30 and 20 mM NaCl concentrations, respectively. These concentrations were then stepwise increased until reaching final concentrations. In the alkaline treatment, plants received the final $NaHCO_3$ concentration when they were 8 days old. After acclimation, plants were grown under their respective treatments another 20 days. At harvest, plants were divided into shoots and roots for fresh and dry weight measurements and shoot/root ratio determined on a dry weight basis.

Root Architecture

Roots were stained with Trypan blue and then carefully extended over a 20-cm × 20-cm glass plate and scanned. Images were analyzed with the Image-ProPlus v4.1 software (Media Cybernetics, Bethesda, MD, USA). Taproot length, number and length of each type of lateral root (first or second order), and length of external and internal links (EL and IL, respectively, Fig. 1) were registered. The specific root length (SRL) was calculated as root length (cm)/dry weight (g).

Among the root parameters, root topology (Fitter 1987) was determined by measuring magnitude (μ), number of exterior links; altitude (a), number of links in the longest unique path from the base link to an exterior link; and total external pathlength (P_e), sum of the number of links in all paths from all external links on the base link.

To estimate the tendency of the root system architecture to one of the two extremes of topological structure, either herringbone or dichotomous pattern (Fig. 1), we used the topological trend (TT) (Trencia 1995), which ranges from 1 in the herringbone structure to 0 in the dichotomous one. The mathematical expression of is $TT = [P_e0 - P_e(\min)]/[P_e(\max) - P_e(\min)]$, where P_e0 is observed P_e and $P_e(\min)$ and $P_e(\max)$ are the possible minimum and maximum P_e values, respectively. Fitter (1987) proposed the following formulae for assessing those extreme values:

$$P_e(\max) = 1/2\left(a_{\max}^2 + 3a_{\max} - 2\right), \text{ where } a_{\max} = \mu$$

$$P_e(\min) = \mu(a_{\min} + 1) - 2a_{\min} - 1, \text{ where } a_{\min} = [\log_2(\mu - 1)] + 2$$

Young Root Anatomy Analysis

Salt stress-derived anatomical variations were analyzed in 5-mm segments obtained from the external link of first-order lateral roots. Segments were sectioned from two regions of these external links: (1) the root hair region and (2) the external link base. Segments were fixed in FAA (95% ethanol:glacial acetic acid:40% formaldehyde:water at 50:5:10:35 by volume). The tissues were dehydrated with a graded series of ethanol, then impregnated in pure xylol (8 h) and embedded in a graded series of pure xylol:Histowax™ (EMD Chemicals, Cincinnati, OH, USA) [3:1, 1:1, and 1:3 (v:v)] (Reinoso and others 2004). Embedded roots were cross-sectioned with a Minot rotative microtome. The 10-µm cross sections were stained with hematoxylin, safranin O, and Fast Green FCF (Johansen 1940) and mounted on slides with distilled water (98°C).

Histological sections were observed at ×100 and ×400 using a Nikon-Eclipse E-600 microscope attached to a computer and a digital camera (Nikon DS Qi1Mc). A total of ten root sections from each of the ten plants were analyzed by treatment. At least eight microscope fields per section were examined and photographed. Total cross-sectional area of root (CSAr), stele (CSAs), and xylem vessels (CSAxv) were measured from images using the Image-ProPlus v4.1 software.

Analytical Determinations

The Na^+ and K^+ contents were extracted from shoots and roots with 100 mM HCl and estimated by standard flame photometry (Chen and others 2001). Proline was estimated spectrophotometrically by the ninhydrin reaction method (Troll and Lindsley 1955) with modifications (Magné and Larher 1992). Chloride was determined by a thiocyanate-Hg-based colorimetric reaction. For this, 12.5 mg of powdered dry plant material was extracted in 0.5 ml of a solution containing H_2O_2 (30%):concentrated HNO_3:iso-amyl alcohol:H_2O at 1:1:0.08:7.9 (v/v), incubated at room temperature for 15 min, diluted to 5 ml with Milli-Q® water, and vigorously agitated in a Vortex. Then, 1.5 ml of the extraction mixture was centrifuged (10,000 rpm, 5 min) and the supernatant transferred to another Eppendorf tube. The colorimetric reaction solution contained polyethylene glycol dodecyl ether–water (Brij 35®, 4%):mercuric thiocyanate (4.17 g/l methanol):$(NO_3)_3Fe$ (202 g/l Milli-Q water plus 21 ml concentrated HNO_3): Milli-Q water at 0.05:15:15:70 (v/v). One milliliter of this reaction was added to 320 µl of the supernatant (control treatment). In the case of saline treatments, 50 µl of the supernatant was previously diluted to 320 µl with the extraction solution. Sample absorbance was determined at 450 nm with a spectrophotometer (Hitachi U-1100) and interpolated into a KCl calibration curve (0, 5, 10, 15, 20 ppm) to calculate Cl^- concentration. Leaf contents of the following ions were analyzed according to Benton and Case (1990): N by Kjeldahl; P, S, and B by inductively coupled plasma; and Ca, Mg, Na, K, Fe, Cu, Mn, and Zn by atomic absorption spectrometry.

Statistical Analysis

Data were subjected to one-way analysis of variance (ANOVA) and comparisons using Duncan's test. Transformations were used when needed to correct for lack of normal distribution or homogeneity of variances. Statistical analyses were performed with SAS software (SAS Institute, Cary, NC, USA).

Results

Plant Growth

Total plant biomass was appreciably reduced by the three types of salt stress treatments, but this effect was more pronounced in the presence of mixed neutral–alkaline salts, where plants reached only one third the biomass of non-stressed plants (Table 1). The shoot-to-root ratio varied according to the type of salt applied. Alkaline and mixed salt–alkaline stresses increased the root-to-shoot ratio as a result of augmented root biomass, whereas the addition of NaCl alone decreased this ratio through a pronounced diminution of root biomass.

Ion and Proline Analysis

Neutral salt addition, alone or mixed with $NaHCO_3$, led to a several-fold increase of the leaf Na^+/K^+ ratio as a consequence of significant leaf Na^+ buildup and K^+ titers reductions (Fig. 2a–c). In contrast, in plants treated with $NaHCO_3$ only, Na^+ levels and the Na^+/K^+ ratio remained relatively unchanged.

Roots accumulated several-fold higher amounts of Na^+ when treated exclusively with NaCl, compared with non-treated plants (Fig. 2d–f). When NaCl was mixed with $NaHCO_3$, this accumulation doubled relative to that found

Table 1 Total, shoot, and root dry weight and root/shoot ratio of *L. tenuis*

Treatment	Total biomass (mg/plant)	Shoot biomass (mg/plant)	Root biomass (mg/plant)	Root/shoot ratio
Control	291 ± 121 (0.58)[a]	241 ± 101 (0.86)[a]	49 ± 26 (1.37)[a]	0.21 ± 0.08 (0.70)[b]
Alkaline	195 ± 65 (0.74)[b]	150 ± 57 (0.66)[c]	45 ± 15 (1.37)[a]	0.33 ± 0.13 (0.51)[a]
Saline	185 ± 77 (0.77)[b]	164 ± 70 (1.13)[b]	21 ± 10 (1.71)[b]	0.13 ± 0.05 (0.89)[c]
Mixed	105 ± 38 (1.00)[c]	78 ± 31 (0.82)[a,b]	27 ± 9 (1.59)[a,b]	0.35 ± 0.08 (0.46)[a]
$F_{(39,9)}$	9.23*	10.31*	10.85*	20.64*

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 0.5× Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; $n = 10$) with the same letter within a column are not significantly different (Duncan, * $P < 0.001$)

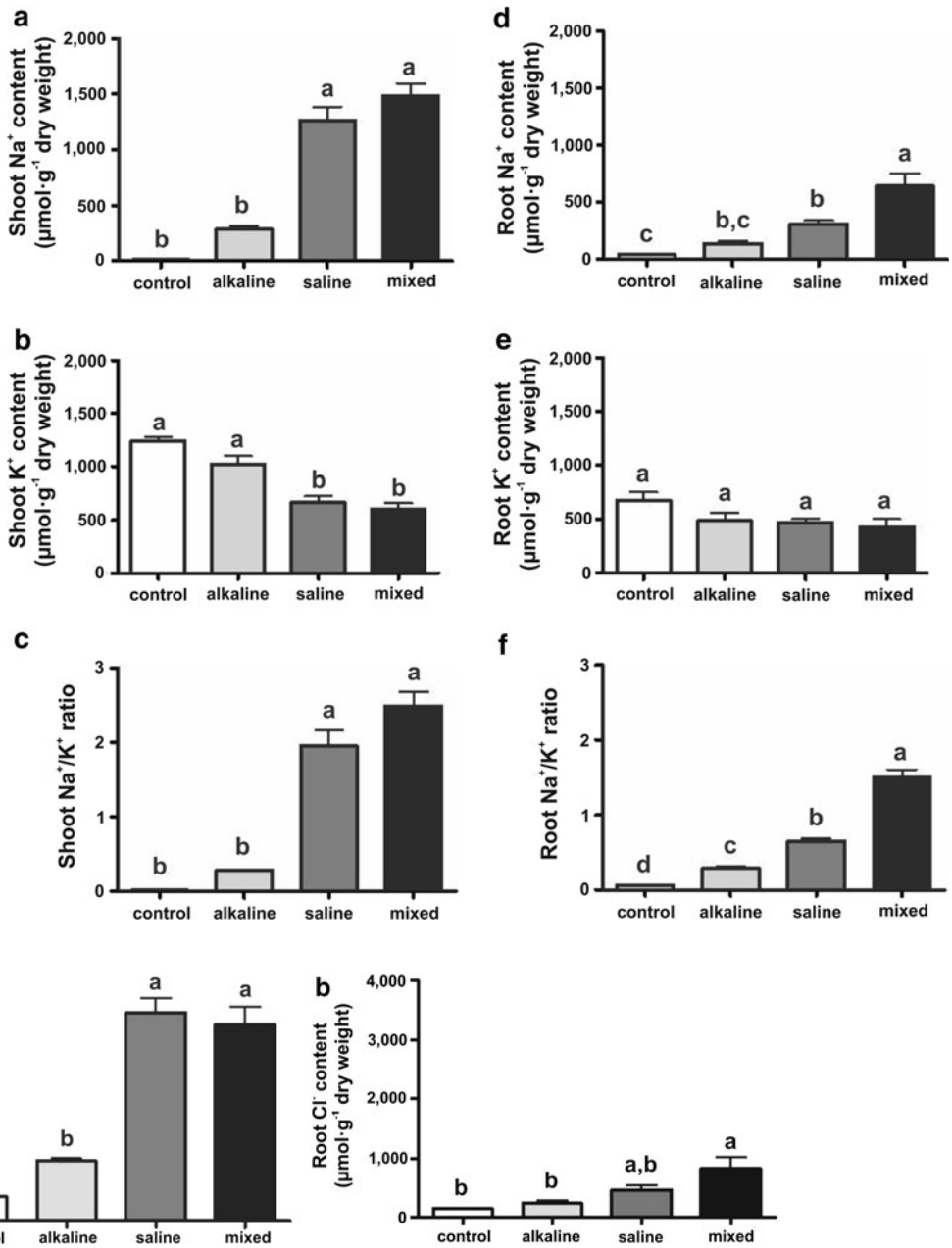

Fig. 2 Sodium (Na^+) and potassium (K^+) contents (µmol g^{-1} dry weight), and Na^+/K^+ ratio in leaf (**a–c**) and root (**d–f**). Fifteen-day-old plants were watered with nutrient solution containing or lacking salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$ respectively, were added to 0.5× Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; $n = 6$) with the *same letter* are not significantly different (Duncan, $P < 0.01$)

Fig. 3 Leaf (**a**) and root (**b**) chloride (Cl_2) contents in *L. tenuis* (µmol g^{-1} dry weight). 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 0.59 Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; $n = 6$) with the *same letter* are not significantly different (Duncan, $P < 0.01$)

in plants treated with NaCl alone. There were no significant differences in the root K^+ levels among treatments, whereas the root Na^+/K^+ ratio followed a similar pattern to that observed for root Na^+ accumulation.

No significant differences were registered in the Cl^- amount in plants treated solely with $NaHCO_3$ (Fig. 3). In leaves, NaCl and mixed salt–alkaline treatments produced similar several-fold increases of this ion compared to the untreated control, whereas in roots, these increments were of lower magnitude than those registered in the shoot.

Proline accumulation was not affected by the high pH in the absence of NaCl, but it was raised by the neutral salt (Fig. 4). Alkalinity diminished the leaf Zn content (especially when NaCl was incorporated in the treatment) without affecting the amount of the other ions (Table 2). The presence of NaCl as a unique salt source in the nutrient solution reduced the leaf Mg^{2+} content, whereas it diminished leaf Ca^{2+} and B amounts, whether combined with alkalinity or not. There were no differences in P, N, Cu, Fe, and Mn^{2+} among treatments (data not shown).

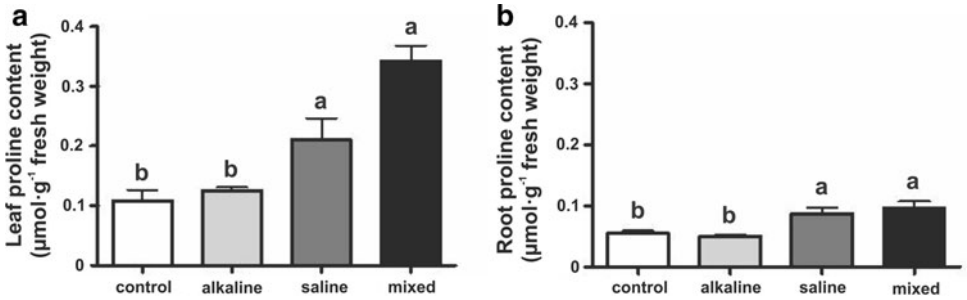

Fig. 4 Leaf (**a**) and root (**b**) proline contents (µmol g^{-1} fresh weight) in *L. tenuis*. Fifteen-day-old plants were watered with nutrient solution containing or lacking salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$, respectively, were added to 0.5× Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Data were transformed (−log 10). Average data (±SE; $n = 6$) with the *same letter* within a column are not significantly different (Duncan, $P < 0.01$)

Table 2 Calcium, magnesium, boron, and zinc contents in *L. tenuis* leaves

Treatment	Ca AAS (g/kg)	Mg AAS (g/kg)	B ICP (mg/kg)	Zn AAS (mg/kg)
Control	12.2 ± 1.4^{a}	$2.0 \pm 0.6^{a,b}$	53.2 ± 3.5^{a}	22.6 ± 3.0^{a}
Alkaline	12.6 ± 2.0^{a}	2.7 ± 0.3^{a}	57.1 ± 4.5^{a}	17.5 ± 2.2^{b}
Saline	7.5 ± 0.5^{b}	1.4 ± 0.5^{c}	3.7 ± 3.8^{c}	23.2 ± 3.0^{a}
Mixed	8.9 ± 0.5^{b}	2.2 ± 0.5^{b}	46.3 ± 3.2^{b}	13.1 ± 2.8^{c}
$F_{(15,3)}$	14.97*	12.99*	21.69*	23.62*

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 0.5× Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; $n = 4$) with the same letter within a row are not significantly different (Duncan, * $P < 0.01$)

ICP Inductively coupled plasma, *AAS* atomic absorption spectrometry

Table 3 Length of different root parts and number of first- and second-order lateral roots in *L. tenuis*

Treatment	Total root length (cm)	Taproot length (cm)	No./averaged length of individual 1st-order lateral roots (cm)	No./averaged length of individual 2nd-order lateral roots (cm)
Control	407 ± 128^{a}	25 ± 6^{a}	$33.2 \pm 3.4^{b}/8.1 \pm 2.3^{a}$	$52.2 \pm 14.2^{a}/1.9 \pm 0.6^{a}$
Alkaline	$359 \pm 70^{a,b}$	29 ± 3^{a}	$54.5 \pm 4.4^{a}/5.2 \pm 1.1^{b}$	$31.3 \pm 18.3^{b}/1.3 \pm 0.3^{b,c}$
Saline	257 ± 134^{b}	27 ± 7^{a}	$35.5 \pm 10.7^{b}/5.1 \pm 1.5^{b}$	$20.9 \pm 11.6^{b}/1.1 \pm 0.3^{c}$
Mixed	$312 \pm 99^{a,b}$	26 ± 5^{a}	$48.1 \pm 12.5^{a}/4.6 \pm 0.6^{b}$	$33.6 \pm 15.1^{b}/1.6 \pm 0.3^{b}$
$F_{(39,9)}$	3.34*	ns	12.81**/ns	6.87**/ns

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 0.5× Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; $n = 10$) with the same letter within a column are not significantly different (Duncan, * $P < 0.05$; ** $P < 0.001$)

Root Morphology

Salt treatments altered the root growth. The total root length was reduced by the addition of NaCl alone, whereas it was not affected by alkalinity, regardless of the presence of NaCl (Table 3). However, the individual lengths of first- and second-order lateral roots were reduced by all stress treatments. The last effect was more drastic in the case of second-order lateral roots of plants treated solely with NaCl.

The total number of lateral roots was significantly reduced by the addition of NaCl alone (100 ± 25 in the control vs. 57 ± 22 in the NaCl treatment, $P < 0.05$). Alkalinity increased the number of first-order lateral roots, whereas NaCl alone did not affect this number. In contrast, the number of second-order lateral roots was reduced by

Table 4 Length of external (EL) and internal (IL) root links and external links/total root lengths ratio in *Lotus tenuis*

Treatment	ELL (cm)	IEL (cm)	EL/IL
Control	290 ± 101^{a}	117 ± 34^{b}	0.7 ± 0.1^{b}
Alkaline	271 ± 48^{a}	82 ± 41^{a}	0.8 ± 0.1^{a}
Saline	176 ± 94^{b}	82 ± 43^{b}	0.7 ± 0.0^{b}
Mixed	$225 \pm 59^{a,b}$	63 ± 23^{a}	0.8 ± 0.1^{a}
$F_{(39,9)}$	4.04*	3.68*	3.87*

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 0.5× Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; $n = 10$) with the same letter within a column are not significantly different (Duncan, * $P < 0.05$)

Table 5 Topological parameters: magnitude (μ), altitude (a), and topological trend (TT) in *L. tenuis* root

Treatment	μ	a	TT
Control	100 ± 25^{a}	33 ± 5^{b}	0.21 ± 0.11^{c}
Alkaline	$94 \pm 23^{a,b}$	56 ± 5^{a}	0.54 ± 0.20^{a}
Saline	57 ± 22^{b}	36 ± 11^{b}	$0.34 \pm 0.13^{b,c}$
Mixed	$97 \pm 30^{a,b}$	52 ± 12^{a}	$0.48 \pm 0.15^{a,b}$
$F_{(39,9)}$	5.20*	16.68**	9.15**

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 0.5× Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; $n = 10$) with the same letter within a column are not significantly different (Duncan, * $P < 0.01$; ** $P < 0.001$)

the three salt stress conditions (Table 3). On the other hand, alkalinity increased the ratio of lengths of external and internal root links, whereas NaCl by itself did not affect this ratio (Table 4).

Changes in the root branching pattern due to stress treatments were accompanied by alterations in root topology (Table 5). The TT index showed that alkalinity alone or mixed with NaCl addition turned the root more herringbone compared with control roots, whereas no significant change in this index was observed in the treatment with the neutral salt only.

SRL was not affected by any of the three stress treatments (Fig. 5). However, alkalinity and salinity by themselves induced opposite trends in this parameter in relation to the control: plants treated with NaCl only presented longer roots per gram of biomass than those treated exclusively with $NaHCO_3$.

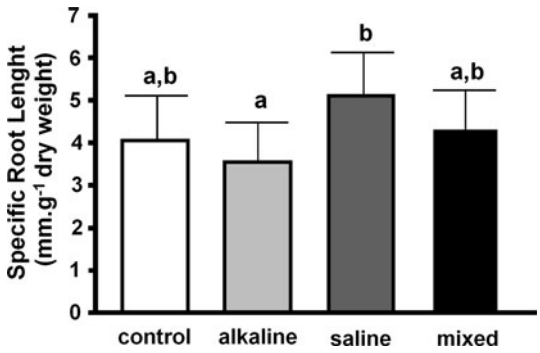

Fig. 5 Specific length of *L. tenuis* roots. Fifteen-day-old plants were watered with nutrient solution containing or lacking salt addition over 20 days. For saline and alkaline stress treatments, 100 mM NaCl and 10 mM $NaHCO_3$ respectively, were added to 0.5× Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; $n = 10$) with the *same letter* are not significantly different (Duncan, $P < 0.01$)

Young Root Anatomy

Sections obtained from the root hair region showed exclusively primary growth (absorption region), whereas those obtained at the base of all external links presented secondary tissue development; therefore, the latter sections were omitted from the present analysis. At the absorption region, the CSAr was diminished by alkalinity (Table 6). This trend was also observed in the CSAs, although it was statistically significant only when $NaHCO_3$ was combined with NaCl. There were no differences in the average number of xylem vessels among treatments (results not shown), but the mixed salt–alkaline treatment induced a strong decline in the CSAxv as well as in the CSAxv/CSAr

Table 6 Root (CSAr), stele (CSAs), and xylem vessels (CSAxv) cross-section areas and CSAxv/CSAr ratio in *L. tenuis* primary roots

Treatment	CSAr (mm^2)	CSAs (mm^2)	CSAxv (μm^2)	CSAs/CSAr	CSAxv/CSAr
Control	0.23 ± 0.07^{a}	0.012 ± 0.03^{a}	943 ± 246^{a}	0.054 ± 0.08	0.043 ± 0.014^{a}
Alkaline	0.13 ± 0.06^{b}	$0.008 \pm 0.05^{a,b}$	762 ± 599^{a}	0.054 ± 0.01	0.059 ± 0.036^{a}
Saline	$0.17 \pm 0.01^{a,b}$	0.011 ± 0.01^{a}	889 ± 792^{a}	0.043 ± 0.01	0.037 ± 0.011^{a}
Mixed	0.13 ± 0.06^{b}	0.006 ± 0.003^{b}	259 ± 133^{b}	0.046 ± 0.01	0.023 ± 0.017^{b}
$F_{(39,9)}$	5.44*	3.36*	14.36**	ns	9.56**

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 0.5× Hoagland's solution. For the mixed salt–alkaline stress treatment, this solution contained 90 mM NaCl and 10 mM $NaHCO_3$. Average data (±SE; $n = 10$) with the same letter within a column are not significantly different (Duncan, * $P < 0.05$; ** $P < 0.001$)

ratio. In contrast, NaCl addition as a sole stress source caused no change in the cross-sectional areas and ratio.

Discussion

L. tenuis Responses to Neutral and Alkaline Stresses

Growth and Nutritional Balance

The application of 10 mM $NaHCO_3$ as a single stress source (alkaline stress) diminished by one-third the biomass of *L. tenuis* plants, a similar level of growth reduction to that produced by NaCl without alkaline salt addition (saline stress, Table 1). This result differs from previous studies, where alkaline salts created a significantly higher level of plant toxicity than neutral salts (Shi and Wang 2005; Shi and Sheng 2005a, b; Wang and others 2008; Yang and others 2008). This apparent incongruence could 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 and others 2008).

Although the levels of growth reduction attained by neutral and alkaline treatments of *L. tenuis* plants were similar, the physiological and morphological parameters measured responded differently to both stress types. Patterns of Na^+, K^+, and Cl^- accumulation were clearly different in plants treated with the neutral and alkaline salts separately (Figs. 2, 3). Under NaCl treatment, *L. tenuis* plants accumulated excessive amounts of Na^+ at the expense of K^+. This led to passive Cl^- accumulation, probably due to increased membrane permeability (Greenway and Munns 1980). On the other hand, it was stated that Na^+ and Cl^- buildup in the shoot progressively inhibits plant growth (Munns and others 1995). Thus, with neutral saline stress, growth reduction of *L. tenuis* plants could be explained in part by increased leaf Cl^- accumulation and K^+/Na^+ imbalances.

Plants sense saline stress through both ionic (Na^+) and osmotic signals (Manchanda and Garg 2008). Beyond proline's role as a compatible osmolyte in plants (Hasegawa and others 2000) and its contribution to membrane stability (Gadallah 1999) and alleviation of the Na^+ effect on cell membrane disruption (Mansour 1998), it is considered a sensitive physiological index for osmotic stress (Shi 1995). In fact, proline correlated well with *L. tenuis* sensitivity to NaCl-derived stress (Sannazzaro and others 2007). On this basis, the low amount of proline accumulated in roots and leaves of *L. tenuis* plants treated solely with $NaHCO_3$ (Fig. 4) would indicate that in contrast with neutral saline treatment, where a significant increase was registered, osmotic stress did not contribute significantly to plant growth reduction in this treatment.

It has been widely described that elevated soil pH either diminishes the availability or hampers the uptake of several nutrients (Clark 1982; Marschner 1995; Yang and others 2007). On one hand, our results showed a 22% reduction in the Zn^{2+} content of plants treated with $NaHCO_3$ exclusively (Table 1). High soil pH and excess bicarbonate conditions (as imposed in the $NaHCO_3$ treatment) may immobilize Zn^{2+}, causing various plant disorders (Marschner 1995). Zinc is a crucial constituent of plants because it has several functions in carbohydrate, protein, and auxin metabolism, as well as in the protection of the membrane structure. Therefore, it is possible that impaired Zn^{2+} uptake might have contributed to the growth reduction observed in *L. tenuis* plants treated only with $NaHCO_3$. Besides Zn^{2+} limitation, increasing soil alkalinity brings about decreases in the availability of Fe, B, P, and Mn to the plant (Marschner 1995). However, no effect on these nutrients was found in *L. tenuis* plants upon alkaline treatment. On the other hand, in the absence of the alkaline salt, NaCl caused a significant decrease in leaf B, Ca^{2+}, and Mg^{2+} titers (Table 1), in line with several previous results (Dhingra and Varghese 1985; Alam 1993; Vigo and others 2005). These effects could also be related to the plant growth inhibition observed in this treatment, because the mentioned ions are key components of plant

functioning: Ca^{2+} is crucial in maintaining membrane integrity and counteracting the harmful effects of Na^{+} on crops (Lahaye and Epstein 1971), Mg^{2+} is an essential component of chlorophyll and proton pumping (Rea and Sanders 1987), and B plays a primary role in cell wall biosynthesis and structure and plasma membrane integrity (Shelp 1993).

Root Architecture and Anatomy

Root topology can change due to nutritional variations (Sorgonà and Cacco 2002) and it has been correlated with exploration efficiency (Fitter 1987; Fitter and Stickland 1991). Alkalinity (whether mixed with salinity or not) led to a more herringbone topological pattern, whereas salinity alone did not cause any effect on this parameter (Table 5). On the other hand, *L. tenuis* plants treated separately with neutral and alkaline salts showed contrasting effects on carbon partition: NaCl decreased but $NaHCO_3$ increased the root-to-shoot ratio (Table 1). These last results are in agreement with previous reports in *Pisum sativum* (Gharsalli and others 2001), *Catharanthus roseus* (Cartmill and others 2008), and *Phaseolus vulgaris* (Valdez-Aguilar and Reed 2008).

Such increases in the TT index and root-to-shoot ratio observed in *L. tenuis* grown under alkaline conditions indicates that they are part of a morphological plant response, whereby these plants acquire improved soil exploration and nutrient uptake capacities. In particular, the latter effects might be a plant response to the lower Zn^{2+} content observed in those plants, because Zn deficiency has been related to increased root-to-shoot ratio in wheat (Rengel and Römheld 2000) and *Phaseolus vulgaris* (Cakmak and others 1989).

There is agreement that only the younger portions of the root participate in inorganic ion uptake because they may access unexplored regions of soil. The fact that in *L. tenuis* roots, all the analyzed external links presented secondary tissues in their basal region indicates that absorption was restricted to their distal, nonsuberized portions. The result showing that alkalinity increased the EL/total root length ratio (Table 4) may also be interpreted as a more cost-effective allocation of available plant carbohydrates toward soil exploration and nutrient improvement acquisition, consistent with an increased root-to-shoot ratio and TT index.

Root length reduction in response to drought and salinity has been reported for several plant species (Garcia and others 1997; Bahaji and others 2002). Under NaCl-derived salinity, such reduction has been attributed to nuclear damage and vacuolar or mitotic abnormalities in root meristematic cells (Radić and others 2005). The negative effect of NaCl, applied as a sole stress source, on root length of *L. tenuis* (Table 3) is in agreement with previous results obtained by our group (Echeverria and others 2008). This effect was due to a decrease in the number of second-order lateral roots and to the shortening of first- and second-order lateral roots (Table 3). However, taproot length was not affected by salinity, showing that taproot and lateral roots have different sensitivities to saline stress, in accordance with previous observations in *Opuntia ficus-inidica* (Gersani and others 1993) and radish seedlings (Waisel and Breckle 1987). In contrast with the NaCl treatment, the total root length was not affected by alkalinity because reductions in the number and length of second-order lateral roots and the length of the first-order lateral roots were compensated for by an increase in the number of first-order laterals, which was the main contributor to the total root length (result not shown).

Information regarding the influence of pH on root development is very limited. In rice genotypes, which are able to uptake Zn efficiently, a strong enhancement of root growth by bicarbonate was found by Hajiboland and others (2003), but the authors did not report whether that effect was due to effective length enlargement of root links or to an increased number of lateral roots. In isolated pea roots, it was found that pH affected lateral root formation differently in apical versus basal segments (Torrey 1956). To date however, no general explanation has emerged on the pH effect on lateral root development, and more research in this area is highly desirable.

Mixed Salt–Alkaline Stress

With the purpose of detecting a possible synergistic effect of salinity and alkalinity in the mixed salt–alkaline treatment, we used a stress solution that had the same saline strength as that used for the neutral salt treatment and the same pH as the alkaline one. Our results showed that there was an almost twofold reduction in plant growth with the mixed salt–alkaline treatment (64%, Table 1) than with the other two stresses (33 and 36%). On the other hand, the similarity in the proline content between plants treated with NaCl alone and those treated with NaCl + $NaHCO_3$ indicates that they were subjected to equivalent osmotic constraints. Therefore, the difference in the level of plant growth reduction between these two treatments could be explained in part by the detrimental effect on plant nutrition produced by the 25% higher Na^{+} accumulation observed in the mixed salt–alkaline treatment. On the other hand, it has been reported that Zn and B micronutrients showed a reciprocally enhancing action on plant growth in mustard (*Brassica nigra*, Sinha and others 2000) and corn (Hosseini and others 2007). On this basis, one could speculate that Zn and B deficiencies observed in plants treated exclusively with $NaHCO_3$ and exclusively with

NaCl, respectively (Table 2), could have converged in mixed salt–alkaline-treated plants, thus deteriorating their nutritional status. Our plant growth data confirm previous reports on *Aneurolepidium chinense* (Shi and Sheng 2005b), sunflower (Shi and Sheng 2005a), and *Chloris virgata* (Yang and others 2008), where deleterious effects of a high pH value or salinity alone on survival and several growth parameters were significantly less evident than those of both stresses combined.

It is interesting that in plants subjected to the mixed salt–alkaline stress, values of most morphological root parameters were more like those attained by the alkaline stress than the neutral saline stress. However, roots of mixed salt–alkaline-stressed plants presented a unique response, that is, a reduction of the CSAxv/CSAr ratio at the absorption region (Table 6). It has been generally observed that the xylem of plants grown under stressful saline environments present xylem vessels with smaller diameters than nonstressed plants (Baum and others 2000; Junghans and others 2006). If such a response is an adaptation to high salinity, it is intriguing that a reduction in the CSAxv/CSAr ratio was not also observed in plants treated exclusively with NaCl. It has been shown that *L. tenuis* is able to exclude a certain amount of Na^+ (and Cl^-) from the xylem when treated with 200 mM NaCl (Teakle and others 2007) and that it can normally grow in saline soils (Montes 1988). When soil salinity is high and/or pH is 8.5 or more, the ion homeostasis of plant cells may alter intracellular cation distribution in cells (Niu and others 1995). Our hypothesis is that at elevated pH, the ion homeostasis capacity of *L. tenuis* was altered. Hence, plants were no longer able to avoid excess Na^+, which accumulated above a threshold where saline stress effects became exacerbated and just then the CSAxv reduction was triggered.

Overall, our results indicate that phenotypic parameters of *L. tenuis* roots respond in a plastic manner when subjected to alkaline and mixed salt–alkaline stresses. Previously, it was suggested that root phenotypic plasticity could be part of the general mechanism of *L. glaber* tolerance to NaCl-derived salinity (Echeverria and others 2008). These reports may partly explain the high adaptability shown by this species in fields characterized by soil patches, offering different types of salt stresses such as those found in the lowlands of the Buenos Aires Province.

Conclusions

Neutral and alkaline salts produced a similar level of growth inhibition on *L. tenuis*, whereas their combination mutually enhanced their effects. Such enhancement could be due in part to the detrimental effect on plant mineral nutrition derived from the 25% higher total Na^+ accumulation and the convergence of nutrient deficiencies produced by neutral and alkaline salts. The pattern of morphological changes in *L. tenuis* root architecture and carbon allocation upon the alkaline treatment (in the absence of NaCl) was similar to that from the mixed salt–alkaline treatment and different from that of the neutral one.

Root morphological features here discussed are central factors in plant nutrient acquisition. In contrast with the neutral saline condition, root response under the alkaline conditions involved the improvement of soil exploration capacities. A unique root morphological response to the mixed salt–alkaline stress was the reduction of the CSAxv/CSAr ratio. However, the lack of previous studies demonstrating a straight relationship between these features and nutrient uptake prevents us from analyzing this more deeply. Future studies are required to ascertain the unambiguous cause-and-effect relationships between form and function in *L. tenuis*. In addition, organic and inorganic anions accumulated for the sustainment of the intracellular ionic balance should be also assessed. Such studies would allow the design of mechanistic models for predicting the whole-plant response to stresses derived from different salt sources.

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