Journal of Plant Physiology 167 (2010) 382–392

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Mucilage and polysaccharides in the halophyte plant species *Kosteletzkya virginica*: Localization and composition in relation to salt stress

Michel Edmond Ghanem [a,*,1], Rui-Ming Han [a,1], Birgit Classen [b], Joëlle Quetin-Leclerq [c], Gregory Mahy [d], Cheng-Jiang Ruan [e], Pei Qin [f], Francisco Pérez-Alfocea [g], Stanley Lutts [a]

[a] *Groupe de Recherche en Physiologie Végétale, Université catholique de Louvain (UCL), Croix du Sud 5 Bte 13, 1348 Louvain-la-Neuve, Belgium*
[b] *Pharmaceutical Institute, Department of Pharmaceutical Biology, Christian-Albrechts-University of Kiel, Gutenbergstr. 76, 24118 Kiel, Germany*
[c] *Unité d'analyse chimique et physico-chimique des médicaments et pharmacognosie, Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCL), Avenue Mounier, 72, Bte 7230, à 1200, Bruxelles, Belgium*
[d] *Laboratoire d'Ecologie–Unité Sol-Ecologie-Territoire-Faculté Universitaire des Sciences agronomiques de Gembloux (FUSAGx)-Passage des Déportés 2, 5030 Gembloux, Belgium*
[e] *Key Laboratory of Biotechnology and Bio-resources Utilisation, State Ethnic Affairs Commission and Ministry of Education, Dalian National University, Dalian 116600, China*
[f] *Halophyte Research Laboratory, School of Life Science, Nanjing University, Nanjing 210093, China*
[g] *CEBAS-CSIC–Campus de Espinardo, E-30100, Espinardo, Murcia-Spain*

ARTICLE INFO

Article history:
Received 31 August 2009
Received in revised form
1 October 2009
Accepted 2 October 2009

Keywords:
Halophyte
Kosteletzkya virginica
Mucilage
Polysaccharides
Salt tolerance

ABSTRACT

Mucilage is thought to play a role in salinity tolerance in certain halophytic species by regulating water ascent and ion transport. The localization and composition of mucilage in the halophyte *Kosteletzkya virginica* was therefore investigated. Plants were grown in a hydroponic system in the presence or absence of 100 mM NaCl and regularly harvested for growth parameter assessment and mucilage analysis with the gas liquid chromatography method. NaCl treatment stimulated shoot growth and biomass accumulation, had little effect on shoot and root water content, and reduced leaf water potential (Ψ_w), osmotic potential (Ψ_s) as well as stomatal conductance (g_s). Mucilage increased in shoot, stems and roots in response to salt stress. Furthermore, changes were also observed in neutral monosaccharide components. Levels of rhamnose and uronic acid increased with salinity. Staining with a 0.5% alcian blue solution revealed the presence of mucopolyssacharides in xylem vessels and salt-induced mucilaginous precipitates on the leaf abaxial surface. Determination of ion concentrations showed that a significant increase of Na^+ and a decrease of K^+ and Ca^{2+} simultaneously occurred in tissues and in mucilage under salt stress. Considering the high proportion of rhamnose and uronic acid in stem mucilage, we suggest that the pectic polysaccharide could be involved in Na^+ fixation, though only a minor fraction of accumulated sodium appeared to be firmly bound to mucilage.

Introduction

Mucilage is a polysaccharide mixture commonly found in various organs of many higher plant species. Due to its high variability in terms of chemical constituents, mucilage probably assumes a multitude of physiological functions in plants. It is found in rhizomes, roots and seed endosperms, where it may act primarily as energy reserves (Franz, 1979). Foliar mucilages also play a role in wound responses (Clarke et al., 1979), plant host–pathogen interactions (Davis et al., 1986), water transport (Zimmermann et al., 1994) and responses to abiotic stresses (Goldstein and Nobel, 1991; Lipp et al., 1994; Zimmermann et al. 1994, 2001).

The high water-binding capacity of hydroxyl groups in the polysaccharide core allows mucilage to hydrate and thus store huge reserves of water, which may offer plants the ability to resist physiological drought (Clarke et al., 1979). There is growing evidence that extracellular mucilages in particular may play an important role in the drought resistance of certain plant species (Goldstein and Nobel, 1991; Morse, 1990; Nobel et al., 1992). In *Hemizonia luzifolia*, extracellular mucilages have been demonstrated to buffer leaf water status against environmental fluctuation during the middle of the day (Morse, 1990). By acting as an apoplastic capacitor (Nobel et al., 1992), mucilages can also enable leaves to maintain low water potential when soil water deficits develop (Robichaux and Morse, 1990).

The rise of xylem sap in mangroves and other halophytes rooting in seawater (or high-salinity groundwater) has puzzled plant physiologists for many decades. Zimmermann et al. (1994) have shown that gel-like structures made up of acid polysaccharides (mucopolysaccharides) are apparently involved in water

* Corresponding author. Tel.: +32 10 47 20 50; fax: +32 10 47 34 35.
E-mail address: Michel.Ghanem@uclouvain.be (M. Edmond Ghanem).
[1] MEG and R-MH contributed equally to this work.

0176-1617/$ - see front matter © 2009 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2009.10.012

lifting in the xylem conduit of *Rhizophora mangle*. Furthermore, negatively charged mucopolysaccharides are major constituents of the xylem vessels of tall trees that root in high-salinity water. Xylem mucilage has also been found in lianas (Benkert et al., 1995) and in mammoth trees such as *Sequoia sempervirens* and *Sequoiadendron giganteum* (Zimmermann et al., 2004). Some trees growing on fog-laden coasts contain extremely high concentrations of mucilage in the bark and the needles. Zimmermann et al. (2004, 2007) recently hypothesized that mucilage is involved in moisture uptake from the atmosphere and that it plays a crucial role in the water supply of apical leaves of high-salinity tolerant trees. To the best of our knowledge, however, the possible involvement of mucilage in sodium sequestration, and thus regulation of ion nutrition, in salt-treated plants has not been addressed.

Mucilaginous substances have been reported occurring widely in the Malvaceae, including the genera *Althea* and *Abelmoschus* (Medina-Torres et al., 2000). *Kosteletzkya virginica*, a Malvaceae commonly known as seashore mallow, is a perennial dicot and halophytic species that is native to brackish portions of coastal tidal marshes of the mid-Atlantic and southeastern United States and is considered an obligate wetland species. It is found as far north as Long Island, New York, south into Florida, and along the coastal plain in east Texas (Godfrey and Wooten, 1981). Previous reports demonstrated the presence of mucilage in seeds (Gallagher, 1985; Islam et al., 1982) or in callus maintained under salt stress conditions (Hasson and Poljakoff-Mayber, 1995), but issues such as the abundance, localization, chemical characteristics and potential role of mucilage in this genus have not been reported. The presence of mucilage in vegetative parts of the plant has been suspected, but its precise distribution and physiological functions remain elusive.

This paper examines the location of mucilage in *K. virginica* and studies the effects of salt stress treatment on changes in abundance and chemical composition of crude mucilage extracts in relation to its putative ability to fix and retain Na^+ ions. The physiological implications of mucilages and associated polysaccharides in *K. virginica* are discussed.

Materials and methods

Plant material and culture conditions

Seeds of *Kosteletzkya virginica* (L.) Presl. (Malvaceae) were collected from a field plantation at Jinhai Agricultural Experimental Farm in Yancheng City of Jiangsu Province, China in 2005. Seeds were sown in trays filled with a perlite vermiculite mix (1:3, v/v) moistened regularly with a half-strength modified Hoagland nutrient solution (Went, 1957) and seedlings were allowed to grow in a phytotron under a 12 h photoperiod (mean light intensity (PAR)=150 μmol m^{-2} s^{-1} provided by Osram Sylvania (Danvers, MA) fluorescent tubes (F36W/133-T8/CW) with 27 °C/23 °C day/night temperatures and 70%/50% atmospheric humidity). 15 d after sowing, seedlings were transferred into a greenhouse and fixed on polyvinyl chloride plates floating on aerated half-strength modified Hoagland nutrient solution. The nutrient solution contained the following chemicals (in mM): 2.0 KNO_3, 1.7 $Ca(NO_3)_2$, 1.0 KH_2PO_4, 0.5 NH_4NO_3, 0.5 $MgSO_4$, and (in μM) 17.8 Na_2SO_4, 11.3 H_3BO_3, 1.6 $MnSO_4$, 1.0 $ZnSO_4$, 0.3 $CuSO_4$, 0.03 $(NH_4)_6Mo_7O_{24}$ and 14.4 μM Fe-EDDHA. Solutions were readjusted every 2 d and renewed every week. Minimum temperatures were 12–14 °C and daily maxima were 24–28 °C. Relative humidity was kept at 75 ± 5%. Natural light was supplemented by PHILLIPS lamps (Phillips Lighting S.A., Brussels, Belgium) (HPLR 400 W) in order to maintain a light irradiance of 240 μmol m^{-2} s^{-1} (PAR) at the top of the canopy.

After 10 d of acclimatization in control conditions (25 d after sowing), the seedlings were exposed to 0 or 100 mM NaCl (EC=0.98 and 10.82 dS ms^{-1}, respectively) and added to the nutrient solution for 68 d.

Vegetative growth assessment

Ten plants per treatment were harvested on days 15, 33 and 51 after the beginning of the stress period. For each plant, shoots and roots were separated and weighed to determine the fresh weight (FW). Roots were quickly rinsed in sterilized deionized water for 30 s to remove ions from the free space and gently blotted dry with a paper towel. Samples were then incubated in an oven at 70 °C for 72 h. After estimation of the dry weight (DW), data were used to calculate the water content (WC) and the root to shoot ratio. The water potential (Ψ_w) of the top expanded leaves was measured on three plants per treatment with a pressure chamber (PMS Instruments Co., Corvallis, Oregon, USA).

For osmotic potential (Ψ_s) determination, leaves were quickly collected from five plants, cut into small segments, then placed in eppendorf tubes perforated with small holes and immediately frozen in liquid nitrogen. Samples were then thawed at ambient temperature to rupture the membranes. After three freezing-thaw cycles, each tube was then encased in a second intact eppendorf tube and centrifuged at 9000 × *g* for 10 min at 4 °C. The osmolarity of the collected sap was analyzed with a vapor pressure osmometer (Model 5500, WESCOR, Logan, Utah, USA) and converted to Ψ_s according to the Van't Hoff equation. Stomatal conductance (g_s) was determined at the mid-photoperiod on ten mature leaves per treatment with a porometer (AP4-UM-3, Delta-T Devices Ltd, Burwell, Cambridge, England).

Xylem sap sampling

Xylem sap was obtained from three plants per treatment immediately after severing the shoot about 2–3 cm above the root system. The root system was placed into a Scholander pressure chamber, and samples obtained by applying a nitrogen pressure similar to the leaf water potential (–0.5 MPa for control plants and about –0.9 MPa for stressed ones) in order to maintain sap flow rates as close as possible to whole plant transpiration rate (Albacete et al., 2008). The foremost efflux was discarded to avoid the contamination that results from the radial pressure applied by a collecting sleeve. Samples were then collected for 2 min by lightly touching the cut stump with a glass capillary tube. The sap was immediately transferred to a pre-weighed eppendorf tube and weighed. The pressure-induced sap flow rate (μL min^{-1}) was determined by dividing the sap volume (μL) by the collection time.

Ion concentration

For K^+, Na^+, Ca^{2+} and Mg^+ quantification in dry matter, plant tissues harvested from five plants per treatment were oven-dried at 70 °C for 48 h and 50 mg dry weight (DW) were digested in 35% (v/v) HNO_3 at 80 °C. Minerals were then dissolved in 0.1 M HCl. For ion quantification in xylem saps, samples extracted from three plants per treatment after 33 d of treatment were first centrifuged for 10 s at 10,000 *g*. The supernatants were then thoroughly mixed with a vortex before a dilution in 0.1 M HCl solution for the analysis. Ionic concentrations were determined using an inductively coupled argon plasma emission spectrophotometer (YJ48; Jobin Yvon, Edison, NJ) calibrated with certified standard

solutions. All measurements were performed in triplicate. Ion concentrations in mucilage were determined according to the same procedure with tissue samples. The xylem ionic transport rate was determined by multiplying sap flow rate and sap ionic concentration.

Localization of mucilage

Mucilage staining experiments were always performed on freshly collected plant material. Transverse and longitudinal sections were made using a cryo-microtome as follows: pieces of tissues were cut with a razor blade and immediately frozen at −20 °C on stubs to make sections with a fixed thickness of 25 μm (Reichert, Vienna, Austria). A 0.5% aqueous solution of alcian blue 8GX (Sigma-Aldrich) at pH 3 was used for localization of polyanionic mucopolysaccharides (Clifford et al., 2002). Sections were then viewed and photographed in air or under immersion oil for higher magnification.

For demonstration of mucopolysaccharides on leaf abaxial surfaces, pieces of the lamina were cut and immediately incubated in 0.5% alcian blue solution for 2 h. The leaf pieces were then rinsed very carefully with distilled water or with a 3% acetic acid solution to remove excess alcian blue before observation.

For woody stem segments with a diameter ranging around 1–1.5 cm, expelling of xylem sap was performed by compression followed by gas bubble formation upon rapid decompression (so-called jet-discharge) according to Zimmermann et al. (2004). Branch segments about 20 cm long were wrapped immediately after collection with several layers of Parafilm (in order to avoid evaporation). After removal of the Parafilm, the branch pieces were quickly cut into 5 cm specimens; the end pieces were discarded. The samples were placed vertically into a cylindrical plastic chamber of appropriate height and diameter (Zimmermann et al., 2007). Wood pieces were pressurized to 4 MPa for 40 s. Pressure was then instantaneously released, resulting in bubble formation in the vessels expelling the xylem sap without contamination by cell sap or cell damage.

The lower cut ends of woody stem specimens were put into alcian blue solutions immediately after the jet-discharge procedure. This so-called "dye refilling" of the emptied vessels (Zimmermann et al., 2007) determines whether the xylem sap mucopolysaccharides originates from the xylem elements. In addition, cross-sections and longitudinal sections of untreated woody specimens were hand-cut using a cryo-microtome in order to precisely analyze the spatial and tissue-specific distribution pattern of the mucopolysaccharides.

Mucilage analysis

Leaves, stems, and roots of *K. virginica* were harvested from ten plants per treatment at day 33 after the beginning of the stress period. Tissues were oven-dried at 40 °C for 1 week and then ground into a fine powder using a ball mill and passed through a 0.315 mm mesh sieve.

The swelling index of tissue powder was determined according to the PE method (Pharmacopée européenne, 2007). Half a gram of homogenized powdered material was placed in a 25 mL ground glass stoppered cylinder graduated over a height of 120 to 130 mm in 0.5 mL divisions. The powder was moistened with 1 mL of ethanol (96%), distilled water was added up to 25 mL, and the cylinder was closed. It was shaken vigorously every 10 min for 1 h and then allowed to stand for 3 h. The volume occupied by the disintegrating agent including adhering mucilage was recorded.

Crude mucilage was extracted with water (500 mL) from homogenized leaves, stems and roots (5 g) under stirring for 40 h at room temperature (Classen and Blaschek, 1998). After centrifugation (20,000*g*, 4 °C, 5 min), the supernatant was removed and stored at 4 °C. The pellet was re-extracted once more using the same procedure, and the aqueous supernatants combined and concentrated to 150 mL by evaporation. The mucilage solution was poured into 600 mL of a mixture of 96% ethanol and 1% acetic acid. The precipitate was washed with cold ethanol and lyophilized. The dry weight of the lyophilized crude mucilage was determined.

Quantitative analysis of neutral monosaccharides in the mucilage was performed by gas liquid chromatography (GLC method) as described in Blakeney et al. (1983) after conversion of hydrolyzates into alditol acetates. To produce alditol acetates, the polysaccharide was hydrolyzed with trifluoroacetic acid (TFA, 2 mol/L) at 121 °C. After evaporation of TFA, monosaccharides were converted to alditole acetates by reduction and acetylation and analyzed by gas liquid chromatography (GLC) on a fused silica capillary column (Optima-OV 225–0.25 μm, L:25 m, ID:0.25 mm, Macherey-Nagel) using a gas chromatograph (HP 6890 Plus Series; Hewlett Packard) with a flame ionization detector. The helium flow rate was 0.5 mL/min and the oven temperature increased from 180 °C (5 min) to 210 °C with a rate of 1 °C/min; the temperature of the injector and detector was 240 °C. For quantitative analysis, a defined amount of myo-inositol was added to the samples as internal standard. Uronic acid content was determined colorimetrically using the *m*-hydroxy biphenyl method (Blumenkrantz and Asboe-Hansen, 1973). Identification of Galacturonic acid (GalA) and Glucoronic acid (GlcA) was achieved by High Performance Thin-Layer Chromatography (HPTLC) as described previously (Classen et al., 2000).

Statistical analysis

Growth parameters, water status and xylem sap associated parameters data were subjected to an analysis of variance (ANOVA II, treatment and duration of treatment as levels of classification) using SAS software (SAS System for Windows, version 8.02). The statistical significance of the results was analyzed using the Student–Newman–Keuls test at the 5% level. Mucilage-related parameters and ion concentrations in plant tissues data were subjected to an analysis of variance (ANOVA I) using the same SAS software. The statistical significance of these results was analyzed by the Student's *t*-test at the 5% level.

Results

Plant growth and water status

No treated plants died during the course of the experiment. The leaf appearance remained unaffected by salt stress (Fig. 1A). After the application of 100 mM NaCl, however, a consistent but slight increase in the number of leaves compared to that of control plants (1–2 more leaves) was observed. Salt stress had little effect on shoot elongation during the first 15 d (Fig. 1B) and significantly increased it by more than 20 cm after 33 d of treatment. Salt stress also increased shoot dry weight and root dry weight as well as the root/shoot ratio after 33 d (Fig. 1B and C), which indicated a modified biomass distribution in salt-stressed plants compared to controls.

Salt stress had no impact on shoot and root water content (data not shown). Leaf water potential was lower in NaCl-treated

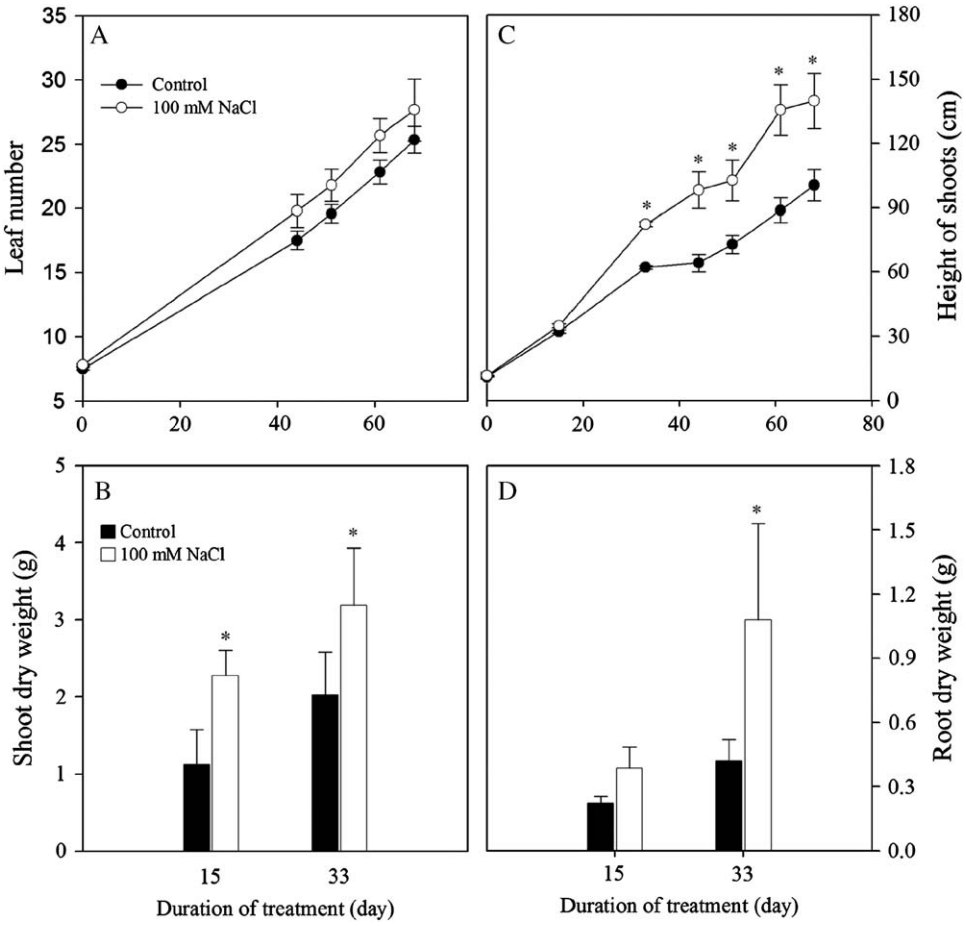

Fig. 1. Leaf number (A), shoot height (C), and dry weight (DW) of shoot (B), root (D) of *Kosteletzkya virginica* seedlings exposed for 68 d in the absence or in the presence of 100 mM NaCl. Data points and vertical bars represent means (n=10) and SE, respectively (∗, significant at $P < 0.05$ according to the Student–Newman–Keuls test).

plants than in control plants after 15 or 33 d of treatment (Fig. 2A). Values of leaf water potential in both control and stressed plants slightly increased with the age of the plant. Salt stress reduced both Ψ_w and Ψ_s after 15 and 33 d of treatment. Stomatal conductance (Fig. 2A and B) significantly decreased in salt-treated plants after 15 and 33 d of stress exposure. In addition to a non-significant decrease at the fifth day, the xylem sap flow rate significantly declined in salt stress conditions after either 15 d or 33 d of salt treatment (Fig. 3).

Ion concentration

Sodium concentrations sharply increased in leaves, stems and roots (Fig. 4A). It is, however, noteworthy that no significant differences were recorded between roots and shoots for Na^+ accumulation. In contrast, K^+ concentrations declined (Fig. 4B) in all plant tissues, as did Ca^{2+} concentrations (Fig. 4C) in leaves and stems. Magnesium concentrations (Fig. 4D) were reduced in leaves but increased slightly in roots. In the xylem sap, Na^+ concentrations (Fig. 4E) increased steadily in salt-treated plants, while K^+ concentrations (Fig. 4F) remained unaltered. Calcium and Mg^{2+} concentrations (Fig. 4G and H) increased markedly in salt-stressed plants, especially after 15 and 33 d of treatment. However, when considering the fluctuation of sap flow rate, ion transport rate from root to shoot via xylem sap (Table 1) demonstrated modified ion net transport efficiency. In salt stress conditions, Na^+ transport was strongly elevated, while K^+ transport was inhibited; Ca^{2+} transport rate declined on day 15 and Mg^{2+} transport rate did not change markedly.

Salt stress induced a conspicuous increase in Na^+ concentration in mucilage; for control plants, Na^+ was not detectable in mucilage extracted from leaves or stems, while concentrations of 240 and 100 μmol g^{-1} were recorded for mucilage extracted from salt-treated plants for these two organs, respectively (Fig. 5A). Potassium concentrations (Fig. 5B) decreased in mucilage from all stressed organs compared to controls, while Ca^{2+} concentrations (Fig. 5C) decreased in mucilage of stressed leaves and stems, but increased in roots. Salt stress slightly increased Mg^{2+} concentration in leaf and stem mucilage, but led to a decrease in root mucilage (Fig. 5D).

Leaf mucilage content and composition

The swelling index of dry tissue powders increased in leaves under salt stress conditions, which was not the case in roots (Fig. 6A). Mucilage content expressed on a dry weight basis also increased in response to 100 mM NaCl in leaves (Fig. 6B).

Acid hydrolysis of crude mucilage extract in *K. virginica* revealed that the main neutral monosaccharide constituents were rhamnose, galactose, and glucose (Table 2). The most interesting observation was the extremely high rhamnose proportion in both control and salt-stressed stems compared to roots and leaves. The galactose proportion in crude mucilage from stems significantly decreased under salt stress. Uronic acid content was much higher

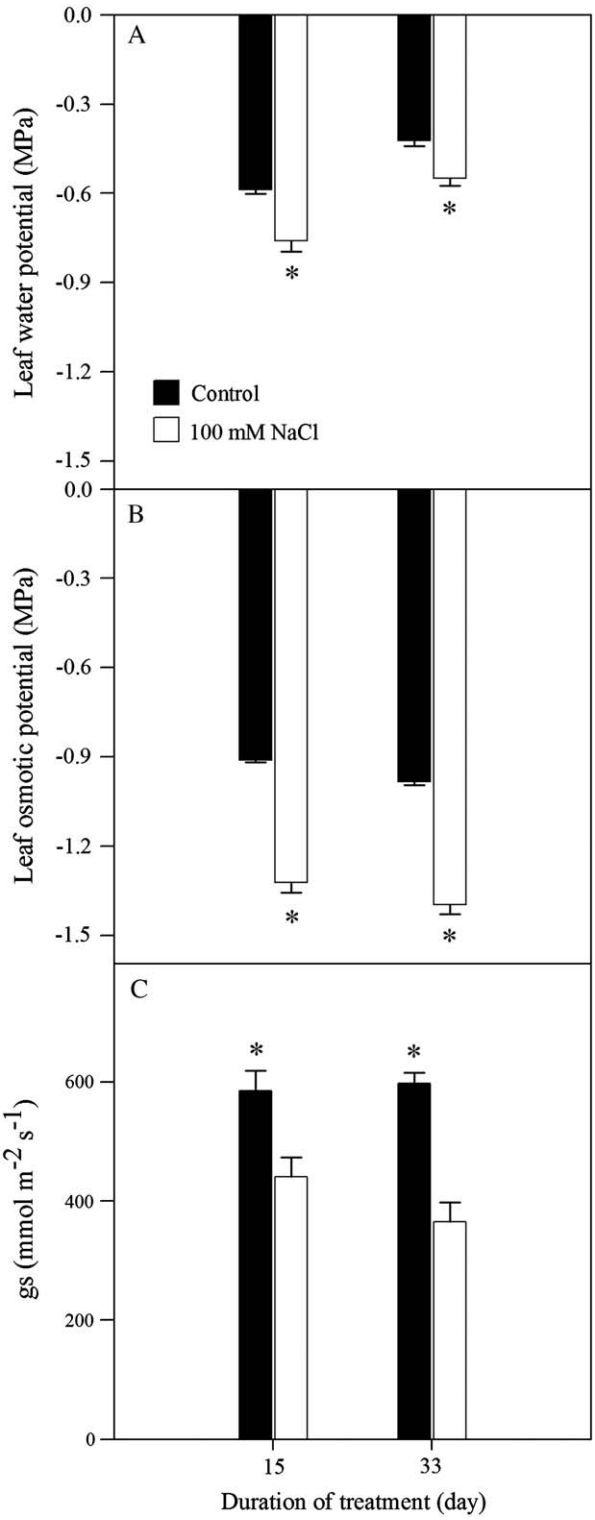

Fig. 2. Leaf water potential (A), leaf osmotic potential (B), and stomatal conductance (C) of *Kosteletzkya virginica* seedlings exposed for 15 and 33 d in the absence or in the presence of 100 mM NaCl. Data points and vertical bars represent means (n=5) and SE, respectively (∗, significant at $P < 0.05$ according to the Student–Newman–Keuls test).

for stems in comparison to roots and leaves, and did not show notable changes in mucilage samples extracted from NaCl-treated organs. Uronic acids were identified as galacturonic and glucuronic acid by HPTLC analyses.

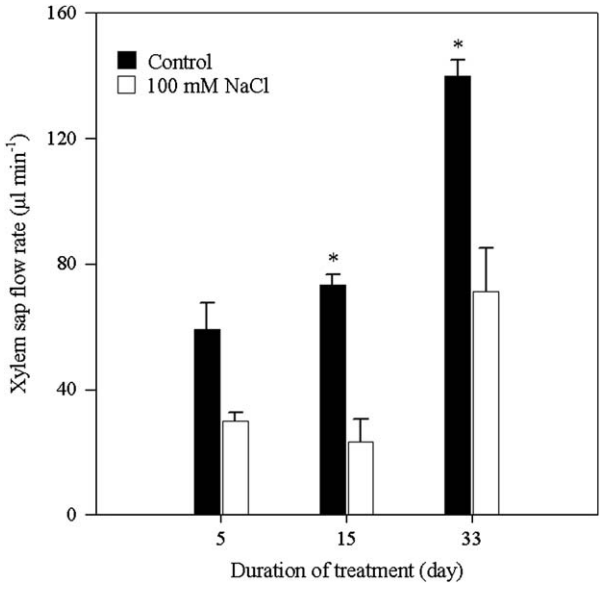

Fig. 3. Xylem sap flow rate of *Kosteletzkya virginica* seedlings exposed for 5, 15 and 33 d in the absence or in the presence of 100 mM NaCl. Data points and vertical bars represent means of triplicates and SE, respectively (∗, same as in Fig. 1).

Localization of mucilage

A transverse section of a fresh mature control leaf showed blue colorized epidermal cells and surrounding conductive tissues (Fig. 7A). The salt-stressed leaf exhibited a growing number of trichomes (Fig. 7B) as well as an intense blue coloration, especially in epidermal cells, where abundant mucilage appeared. On the other hand, palisade parenchyma cells and spongy parenchyma cells in both control and salt-stressed leaves remained green, suggesting that mucilage preferentially accumulated in the leaf epidermis.

A cross-section of a fresh stem revealed the presence of mucopolysaccharides in xylem vessels in control (Fig. 7C) and salt-stressed (Fig. 7D) plants. Salt stress (100 mM NaCl) did not modify the pattern of mucilage distribution. Longitudinal sections of control stems before (Fig. 7E) and after (Fig. 7F) a cycle of the compression procedure confirmed the existence of abundant mucilage in xylem vessels and its adherence on walls.

Cross-cuttings in roots indicated the presence of mucilage in xylem vessels in both control (Fig. 7G) and salt-stressed (Fig. 7H) plants. The stressed roots showed an identical blue colorized xylem vessel.

Mucilage was not detected at the leaf surface of control plants (Fig. 8A), while the abaxial leaf surface exhibited a dramatic precipitation of mucilage in epidermis cells under salt stress conditions (Fig. 8B). This phenomenon was more obvious in mature than in young leaves.

Discussion

Salt stress stimulates growth of K. virginica without affecting its water content

The present study confirms the halophytic nature of *K. virginica*; salt stress indeed had no detrimental impact on plant growth, and even increased plant height as well as root and shoot dry weights (Fig. 1). These observations, together with the fact that root to shoot ratio and internode length were increased, suggest that NaCl may affect the hormonal balance within plant

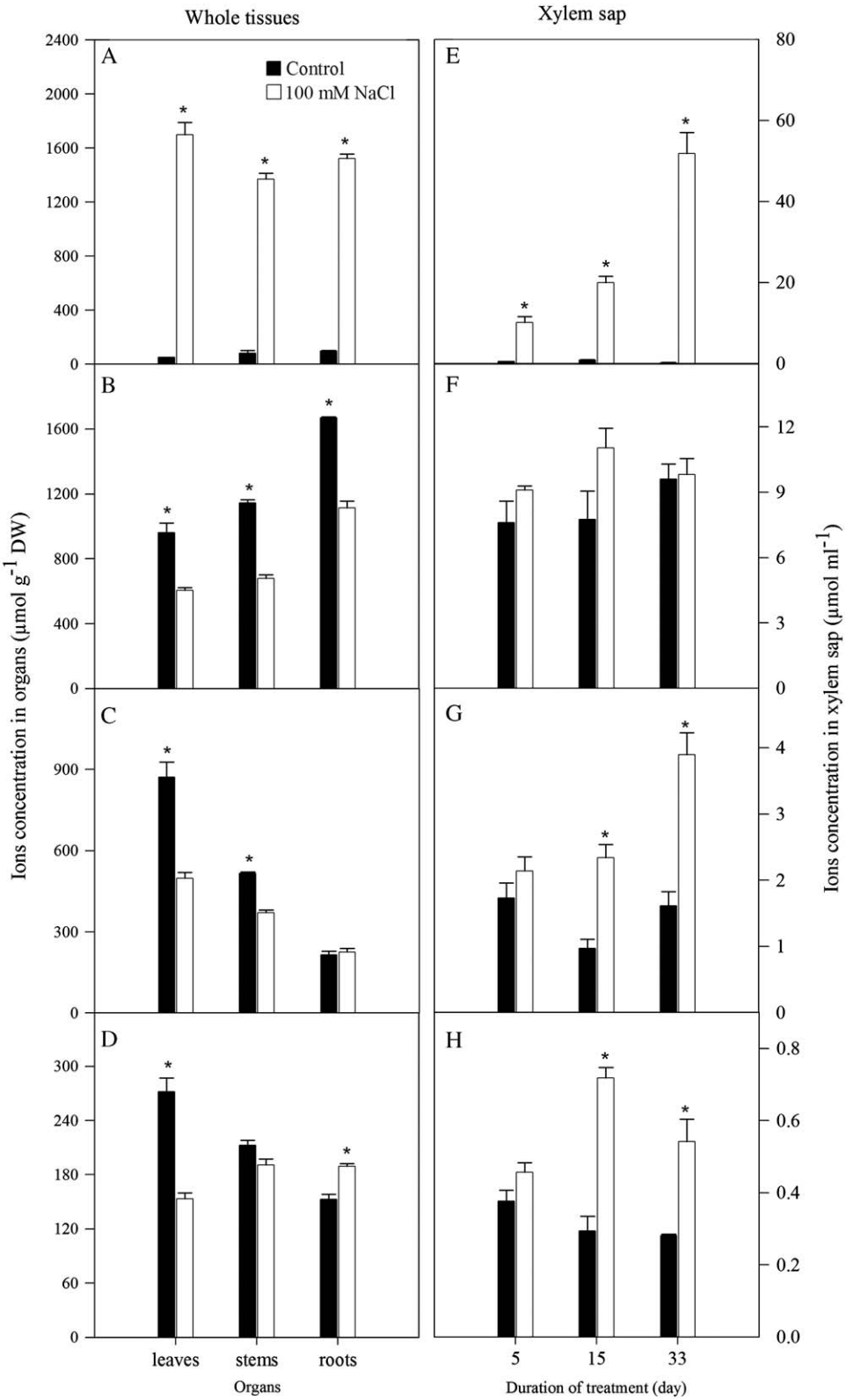

Fig. 4. Ions concentrations in µmol g^{-1} DW of whole plant tissues (A—Na^+, B—K^+, C—Ca^{2+}, D—Mg^{2+}) of *Kosteletzkya virginica* seedlings exposed for 26 d, and in xylem saps (E—Na^+, F—K^+, G—Ca^{2+}, H—Mg^{2+}) for 5, 15 and 33 d in the absence or in the presence of 100 mM NaCl, respectively. Data points and vertical bars represent means of triplicates and SE, respectively (∗, same as in Fig. 1).

tissues. Salinity was indeed reported to induce numerous changes in hormone synthesis and translocation, which may have an effect on biomass partitioning and shoot metabolism (Albacete et al., 2008; Ghanem et al., 2008).

Salinity resistance in *K. virginica* is related to its ability to cope with both ionic and osmotic components of salt stress. Plants were able to maintain constant water content, even after long-term exposure to 100 mM NaCl. Such a property may be at least

Table 1
Transport rate (in μl min^{-1}) of Na, K, Ca, and Mg from root to shoot via xylem vessels in *Kosteletzkya virginica* seedlings exposed for 5, 15 and 33 d in the absence or in the presence of 100 mM NaCl.

Treatment	Na (μg min^{-1})	K (μg min^{-1})	Ca (μg min^{-1})	Mg (μg min^{-1})
	5 d exposure			
Control	0.75 ± 0.14	17.63 ± 2.73	4.08 ± 0.51	1.23 ± 0.17
100 mM NaCl	6.98 ± 1.00*	10.69 ± 0.48	2.55 ± 0.17	0.75 ± 0.043
	15 d exposure			
Control	1.59 ± 0.27	21.92 ± 2.95*	2.83 ± 0.28*	1.17 ± 0.13
100 mM NaCl	10.59 ± 3.35*	9.56 ± 2.55	2.25 ± 0.79	0.91 ± 0.28
	33 d exposure			
Control	0.95 ± 0.08	52.80 ± 4.91*	9.00 ± 0.82	2.17 ± 0.08
100 mM NaCl	82.29 ± 8.83*	26.64 ± 3.05	10.85 ± 1.34	2.05 ± 0.20

* Significant at $P < 0.05$ according to the Student–Newman–Keuls test.

partly related to the ability of the plant to perform osmotic adjustment, which plays an important role in the response to salt and water stress (Teixeira and Pereira, 2007). The stress-induced decrease in g_s (Fig. 2C) nevertheless suggests that stressed plants also reduced water losses through transpiration stream, which is supported by the recorded stress-induced decrease in xylem sap flow (Fig. 3). Hence, it must be noted that such a decrease in water loss occurred concomitantly with growth stimulation, suggesting that water use efficiency was strongly improved in stressed plants. Such behavior is of special interest for a wetland halophyte plant species, since increased water use efficiency is more frequently reported for xero-halophyte species exhibiting an adaptive water saving strategy (Ben Hassine et al., 2008).

K. virginica did not limit Na^+ accumulation in the shoot

In addition to regulation of its water status, *K. virginica* also appears able to cope with high levels of internal accumulated Na^+. *K. virginica* behaves as a typical "includer", as Na^+ was equally distributed among organs and was not retained within roots, as reported for salt-tolerant glycophyte species (Munns and Termaat, 1986). Sodium concentration in the xylem sap strongly increased with the age of the plant (Fig. 4) as did ion transport rate (Table 1), suggesting that there was no attempt to limit Na^+ accumulation within roots. This implies that tolerance mechanisms should operate at the shoot level in order to allow maintenance of efficient metabolism capable of sustaining growth. Several halophytes are able to excrete sodium in bladder hairs and trichomes present at the leaf surface (Lefèvre et al., 2009; Levizou et al., 2005). Until now, *K. virginica* was considered as a hairless species (Gallagher, 1985). Our observation nevertheless suggested that the development of trichomes may be somehow "induced" by NaCl (data not shown). Although the quantitative involvement of these structures in Na^+ removal from the leaves was not determined in the present work, it could not explain salt tolerance in *K. virginica*, since trichome density, even if increased, remained relatively low.

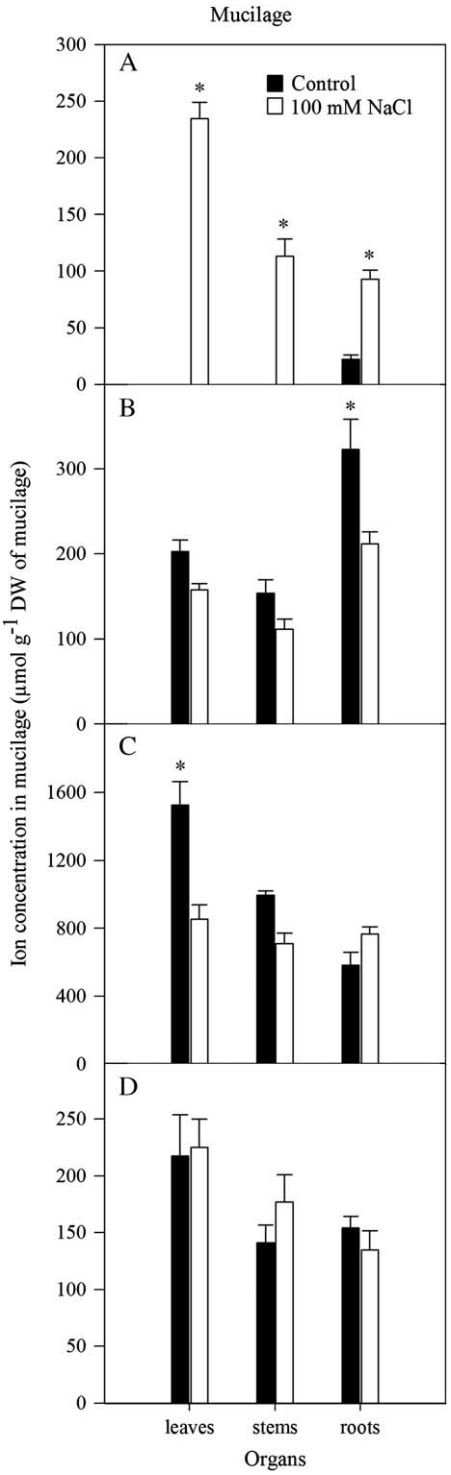

Fig. 5. Ions concentrations in mucilage (in μmol g^{-1} DW of mucilage; A—Na^+, B—K^+, C—Ca^{2+}, D—Mg^{2+}) of *Kosteletzkya virginica* seedlings exposed for 26 d in the absence or in the presence of 100 mM NaCl (*, same as in Fig. 1).

Salt stress increases leaf mucilage content and changes its composition

The presence, localization and composition of mucilage suggest that it may assume key functions in the plant response to salt stress and that it may be involved in the management of both the osmotic and the ionic component of stress. The existence of hydrogel-forming mucopolysaccharides in the plant tissues, which are a three-dimensional network of hydrophilic polymer chains that are crosslinked through either chemical or physical bonding, will give tissues particular characteristics in the presence of water (Nobel et al., 1992). Because of the hydrophilic nature of polymer chains, hydrogels absorb water to swell in the presence of abundant water. We found that the powders were able to retain water and that the holding capacity increased with salt stress in powders extracted from leaves and stems (Fig. 6A).

The use of the swelling index to determine relative amounts of mucilage in the dried powder tissues has the basic problem that in the salt-treated plants, the presence of Na^+ and other ions might affect the capacitance of the mucilage, as noted by Nobel et al. (1992). In order to check this possible effect on the water-holding capacity, which might bias these semi-quantitative results, we extracted the mucilage from different parts of the plant and determined its quantity on a dry matter basis. These results indicate that salt stress causes increased mucilage content independent of the effects of ion on tissue powders' water-holding capacity.

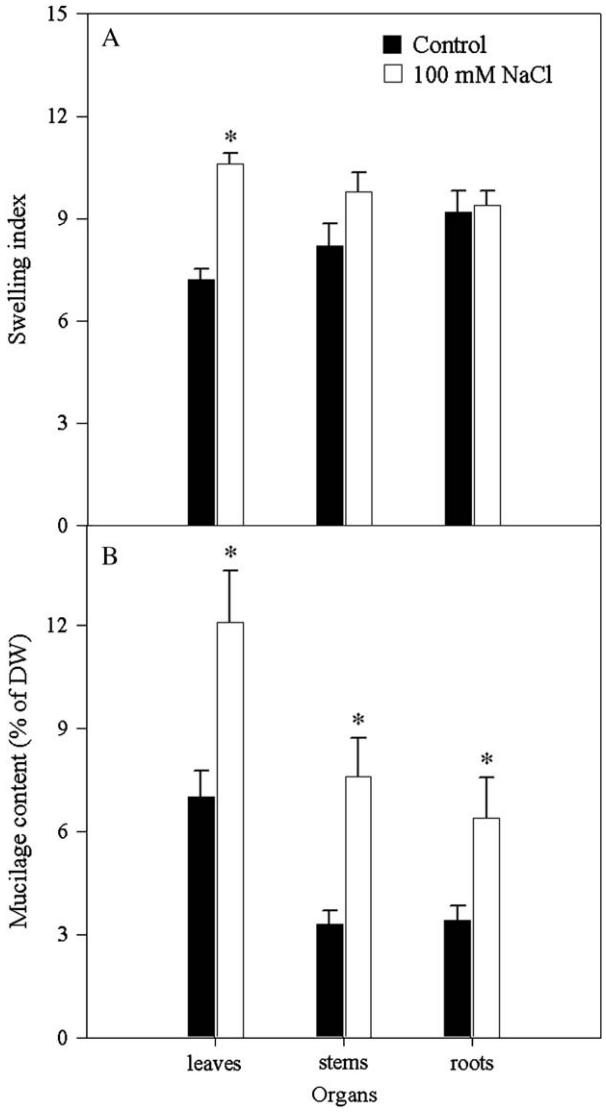

Fig. 6. Swelling index (A) and mucilage content (B) in *Kosteletzkya virginica* seedlings exposed for 26 d in the absence or in the presence of 100 mM NaCl (∗, same as in Fig. 1).

The analytical results also appear to show a "gradient" of growing mucilage content from the roots to the leaves for plants subjected to 100 mM NaCl (Fig. 6). According to Zimmermann et al. (2004), the mucopolysaccharides reduce the chemical activity of water and the mucilage gradients could balance or even offset the weight of the water column (due to the force of gravity) at a constant hydrostatic pressure. In other words, the effect of mucilage on the ascent of water can be regarded as equivalent along with the osmotic potential gradients (due to low molecular weight solutes), which together with the capillary forces and tensions in short distance related to transpiration, could play an important role in the ascent of water in halophytes.

The presence of filamentary precipitates of natural mucopolysaccharide in the xylem sap extracted by several methods (data not shown) enabled us to reject the hypothesis that the mucilage observed in the vessels would be an artifact due to the cutting technique, as reported previously (Schill et al., 1996). In addition, the dye refilling confirms the xylem as the origin of mucilage in *K. virginica* by the accession of precipitates to the walls of some xylem vessels. According to Zimmermann et al. (2007), such deposits could be involved in the "packaging" of vessels undergoing embolism (cavitation), ensuring the maintenance of continuous flow of water.

The precise contribution of mucilage to salt stress resistance is still speculative. One possible explanation is that mucilage may play a significant role in regulating water transport in the plant. A reverse transpiration has already been shown in various plant species (Burgess and Dawson, 2004; Koch et al., 2004; Tognetti et al. 2001), and it has been hypothesized that water would be supplied to the plant not only by root uptake, but also by foliar absorption of moisture in the air. The foliar absorption of water would be facilitated by the presence of mucilage on the surface of leaves. Some acidic mucopolysaccharides, such as pectin, can absorb 10 to 50 times their weight in water (Morse, 1990) and this capacity can be greatly increased in the presence of di-(multi)-equivalent gelling agent cations (such as Ca^{2+}) (Nobel et al., 1992; Zimmermann et al., 2007). Experiments on the vacuolar mucilage of *Valonia utricularis* showed that mucilage is soluble in distilled water, but that a gelling occurs with the

Table 2
Neutral monosaccharides composition (in mol % of total neutral monosaccharides) and uronic acid content (in % of dry weight of mucilage) of mucilage extracted from *Kosteletzkya virginica* seedlings exposed for 26 d in the absence or in the presence of 100 mM NaCl.

Monosaccharide constituent	Root		Stem		Leaf	
	Control	100 mM NaCl	Control	100 mM NaCl	Control	100 mM NaCl
Rha	21.9 ± 0.1	21.9 ± 1.9	51.8 ± 0.8	57.7 ± 10.7	28.8 ± 0.3	30.4 ± 4.4
Fuc	3.0 ± 2.0	1.9 ± 1.1	0.7 ± 0.3	2.3 ± 1.7	0.6 ± 0.4	0.9 ± 0.2
Ara	11.1 ± 2.9	11.7 ± 1.4	8.1 ± 2.9	5.4 ± 2.7	15.9 ± 3.2	15.9 ± 3.2
Xyl	4.5 ± 0.6	5.3 ± 0.7	3.5 ± 0.5	4.0 ± 1.0	1.7 ± 0.4	2.5 ± 0.5
Man	9.3 ± 0.8	11.0 ± 0.0	3.8 ± 0.2	6.7 ± 1.3	10.9 ± 2.9	8.5 ± 0.6
Gal	28.0 ± 7.0	30.3 ± 2.3	17.7 ± 0.3	10.3 ± 0.8*	23.9 ± 0.1	22.8 ± 0.3
Glc	20.4 ± 1.4	16.0 ± 1.0	12.5 ± 4.5	12.2 ± 1.8	16.4 ± 3.4	16.8 ± 2.8
Uronic acid	7.4	7.4	25.7	29.2	7.7	13.1

Values represent means ± SE, $n=2$.
Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose. Uronic acid content in mucilage is examined once without statistical analysis.

* Significant at $P < 0.05$ according to the Student's *t*-test.

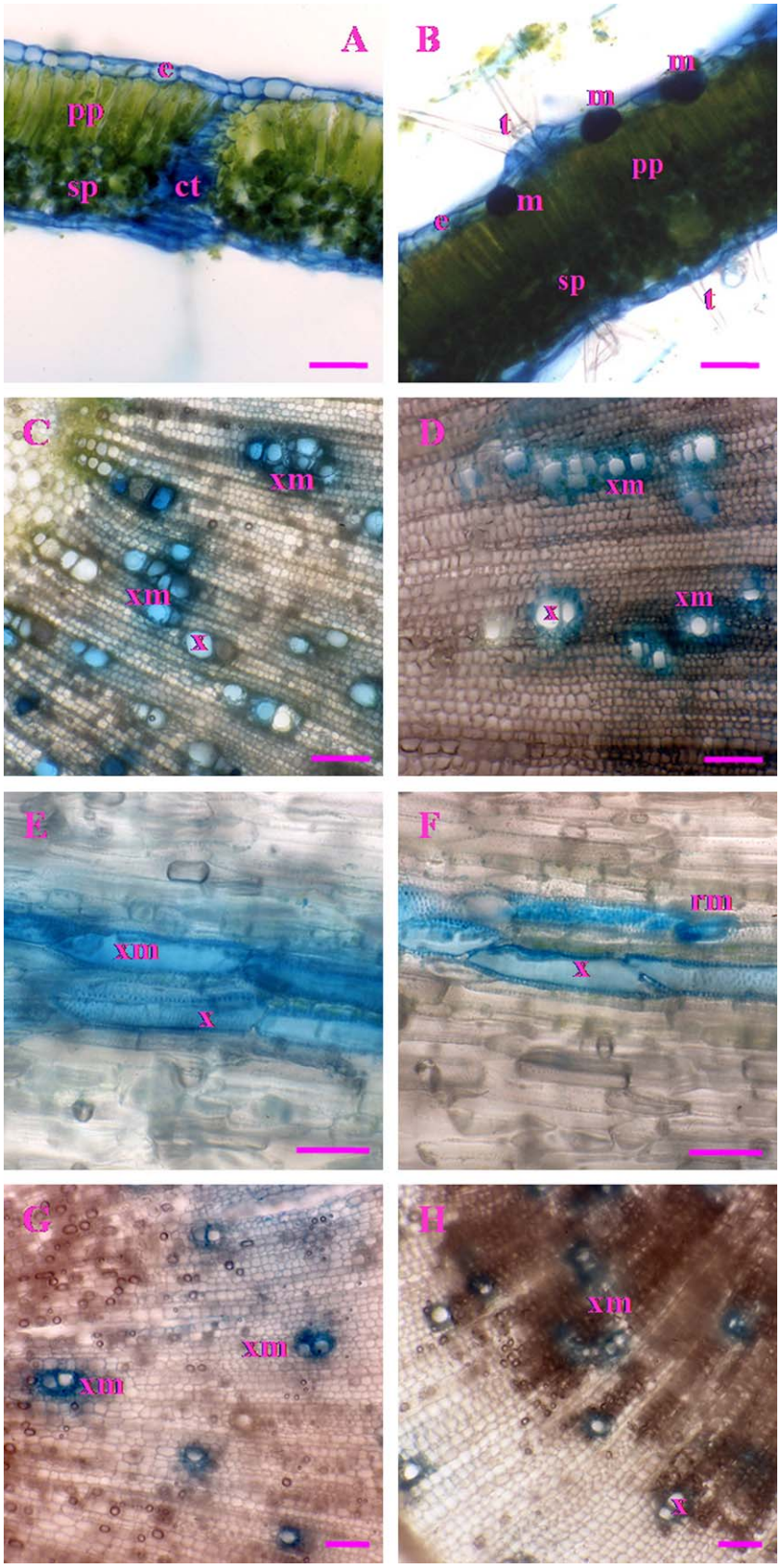

Fig. 7. Fresh sections of leaf (A and B), stem (C–F), and root (G and H) of *Kosteletzkya virginica* after 51 d treatment, stained with alcian blue. Scale bar represents 50 μm. (A) Transverse leaf section of a control plant. Epidermis (e) and the conductive tissues (ct) are clearly stained. Palisade parenchyma cells (pp) and spongy parenchyma cells (sp) appear green. (B) Transverse leaf section of a salt-stressed plant. Both abaxial and adaxial leaf surface are densely pubescent (t, trichome). Intensely staining tracts of mucilage (m) are present in the epidermis and surrounding tissues. (C and D) Transverse stem sections of (C) a control and (D) a salt-stressed plants, respectively. Mucilage is present in xylem vessels (xm). (E) Longitudinal section of a control stem that has not undergone a cycle of compression–decompression. Xylem vessels accumulate abundant mucilage (xm). (F) Longitudinal section of a control stem that has undergone a cycle of compression–decompression. Residue of mucopolysaccharide material appears in xylem vessel walls (rm). (G and H) Transverse root section of a (G) control and a (H) salt-stressed plant showing the presence of mucilage in xylem vessels (xm).

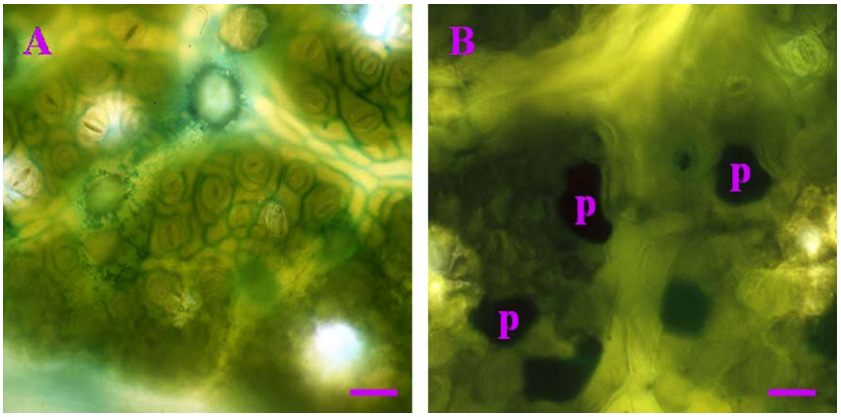

Fig. 8. The abaxial view of leaf laminas at 51 d of treatment, stained in an alcian blue solution for 2 h. Scale bar represents 200 μm. The presence of mucilaginous substances is highlighted by a blue color. (A) Absence of mucilaginous precipitates (p) on the surface of a control leaf. (B) Abundant mucilage in epidermis cells stains an intense blue, and presence of mucilage-blue precipitates (p) on the surface of a salt-stressed leaf.

following addition of 20 mM NaCl, which shows the importance of ions in the formation of gels (cited in Zimmermann et al., 2002a).

Mucilage composition

Monosaccharide analysis suggests that, in *K. virginica*, the mucilage is a mixture of pectic polysaccharide substances rich in anionic groups. Even if salt stress increased mucilage content in all organs (Fig. 6B), it had only a limited impact on mucilage composition (Table 2). However, this mucilage composition strongly differed among organs, and a very high proportion of rhamnose and uronic acids characterized the mucilage extracted from stem tissues. A high proportion of rhamnose was previously reported in salt-exposed diatoms (Abdullahi et al. 2006) and in tobacco cell cultures (Iraki et al. 1989). A high amount of pectic rhamnogalacturonanes could be of special interest in salt-treated *K. virginica*. Pectins are known for their ability to bind cations (mainly calcium) because of the presence of galacturonosyl non-methyl ester residues (Pellerin and O'Neill, 1998). Given their anionic character, pectins can be used as cationic exchangers for fixing the metal cations in aqueous solutions (Dronnet et al., 1996). Divalent cations of pectin can then be exchanged against other divalent cations (Cu^{2+}, Pb^{2+}, Cd^{2+}) or against monovalent ions such as Na^+ or K^+ (Pellerin and O'Neill, 1998). Our present work indeed showed that Ca^{2+} increased in the xylem sap of salt-treated plants, but that its concentration did not increase at the whole plant level. This suggests that it may be released from binding sites in mucilage, and this hypothesis is reinforced by the analysis of mucilage ion content, since a salt-induced decrease in Ca^{2+} and a salt-induced increased in Na^+ were recorded in stem mucilage. It must, however, be noted that, although mucilage from stems contained the highest proportion of rhamnose, it did not sequester the greatest amounts of Na^+, and an important concentration of "free" Na^+ was still detected in the xylem sap. From a quantitative point of view, the highest concentration of mucilage was found in leaves, where the highest amounts of mucilage-bound Na^+ in mucilage were also recorded. This observation strengthens the hypothesis that mucilage may act not only as a water capacitor, but also as a Na^+ sequestering compound in order to protect photosynthetically active tissues. However, considering the mucilage content (Fig. 6B), the total amount of Na^+ recorded in leaves (Fig. 4A) and in mucilage (Fig. 5A), one may assume than less than 10% of quantified Na^+ is retained by pectic polysaccharides. Because of the washing steps used during mucilage extraction, Na^+ recorded during mucilage analysis should be regarded as the most tightly bound fraction and should therefore be considered as a minimal value. If the contribution of mucilage to the total ion balance under salt stress conditions cannot be completely ruled out despite the low mucilage-bound fraction of ions, its role in water retention in plant tissues can be further stressed.

Although this study provides evidence of increasing mucilage production under salt stress conditions for the first time in higher plants, dissecting the role of mucilage in controlling water and ion status requires more direct evidence. Other experiments are being sought out using different mucilage localization techniques (different stains and antibodies) as are precise qualitative and quantitative non-destructive localizations of ions within the biological tissues through techniques like scanning electron microscopy coupled to energy-depressive X-ray microanalysis (SEM-EDX) or proton-induced X-ray emission (PIXE).

Acknowledgements

We thank Mrs. Marie-Christine Fayt (CHAM, UCL) for technical assistance, Dr. Luc Bachérius (IMAP, UCL) for valuable help in ion quantification, Dr. Malcolm O'Neill (UGA, CCRC) for precious help in monosaccharide analysis and Dr. Alexis de Lespinay (GRPV, UCL) for stimulating discussion of many aspects covered. This work was supported by the Belgian Science Policy (BELSPO): Chinese–Belgian Cooperation Project (200441505). R-M H. is grateful to the Université catholique de Louvain (UCL) for the award of a research fellowship.

References

Abdullahi AS, Underwood GJC, Gretz MR. Extracellular matrix assembly in diatoms (bacillariophyceae). V. Environmental effects on polysaccharide synthesis in the model diatom, *Phaeodactylum tricornutum*. J Phycol 2006;42:363–78.

Albacete A, Ghanem ME, Martínez-Andújar C, Acosta M, Sánchez-Bravo J, Martínez V, Lutts S, Dodd IC, Pérez-Alfocea F. Hormonal changes in relation to biomass partitioning and shoot growth impairment in salinized tomato (*Solanum lycopersicum* L.) plants. J Exp Bot 2008;65:142–52.

Ben Hassine A, Ghanem ME, Bouzid S, Lutts S. An inland and a coastal population of the Mediterranean xero-halophyte species *Atriplex halimus* L. differ in their ability to accumulate proline and glycinebetaine in response to salinity and water stress. J Exp Bot 2008;59:1315–26.

Blakeney AB, Harris PJ, Henry RJ, Stone BA. A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydr Res 1983;113:291–9.

Benkert R, Zhu JJ, Zimmermann G, Türk R, Bentrup F-W, Zimmermann U. Long-term xylem pressure measurements in the liana *Tetrastigma voinierianum* by means of the xylem pressure probe. Planta 1995;196:804–13.

Blumenkrantz N, Asboe-Hansen G. New method for quantitative determination of uronic acids. Anal Biochem 1973;54:484–9.

Burgess SSO, Dawson TE. The contribution of fog to the water relations of *Sequoia sempervirens* (D. Don): foliar uptake and prevention of dehydration. Plant Cell Environ 2004;27:1023–34.

Clarke AE, Andreson RL, Stone BA. Form and function of arabinogalactans and arabinogalactan-proteins. Phytochemistry 1979;18:521–40.

Classen B, Blaschek W. High molecular weight acidic polysaccharides from *Malva sylvestris* and *Alce rosea*. Planta Med 1998;64:640–4.

Classen B, Witthohn K, Blaschek W. Characterization of an arabinogalactan-protein isolated from pressed juice of *Echinacea purpurea* by precipitation with the ß-glucosyl Yariv reagent. Carbohydr Res 2000;327:497–504.

Clifford SC, Arndt SK, Popp M, Jones HG. Mucilages and polysaccharides in *Ziziphus* species (Rhamnaceae): localization, composition and physiological roles during drought-stress. J Exp Bot 2002;53 (366):131–8.

Davis KR, Darvill AG, Albersheim P, Dell A. Host-pathogen interactions. 29. Oligogalacturonides released from sodium polypectate by endopolygalacturonic acid lyase are elicitors of phytoalexines in soybean. Plant Physiol 1986;80:568–77.

Dronnet VM, CMGC Renard, Axelos MAV, Thibault JF. Characterisation and selectivity of divalent metal ions binding by citrus and sugar beet pectins. Carbohydr Polymers 1996;30:253–63.

Franz G. Metabolism of reserve polysaccharides in tubers of *Orchis morio* L. Planta Med 1979;36:68–73.

Gallagher JL. Halophytic crops for cultivation at sea water salinity. Plant Soil 1985;89:323–36.

Ghanem ME, Albacete A, Martínez-Andújar C, Acosta M, Romero-Aranda R, Dodd IC, Lutts S, Pérez-Alfocea F. Hormonal changes during salinity-induced leaf senescence in tomato (*Solanum lycopersicum* L.). J Exp Bot 2008;59:3039–50.

Godfrey RK, Wooten JW. In: Aquatic and wetland plants of the Southeastern United States: Dicotyledons. Athens, GA: University of Georgia Press; 1981 p. 944.

Goldstein G, Nobel PS. Changes in osmotic pressure and mucilage during low-temperature acclimation of *Opuntia ficus-indica*. Plant Physiol 1991;97:954–61.

Hasson E., Poljakoff-Mayber A. Callus culture from hypocotyls of *Kosteletzkya virginica* (L.) seedlings—its growth, salt tolerance and response to abscisic acid. Plant Cell Tissue Organ Cult 1995; 43: 279–285.

Iraki NM, Singh N, Bressan RA, Carpita C. Cell walls of tobacco cells and changes in composition associated with reduced growth upon adaptation to water and saline stress. Plant Physiol 1989;91:48–53.

Islam MN, Wilson CA, Watkins TR. Nutritional evaluation of seashore mallow seed, *K. virginica*. J Agric Food Chem 1982;30:1195–8.

Koch GW, Sillett SC, Jennings GM, Davis SD. The limits to tree height. Nature 2004;428:851–4.

Lefèvre I, Marchal G, Meerts P, Corréal E, Lutts S. Chloride salinity reduces cadmium accumulation by the Mediterranean halophyte species *Atriplex halimus* L. Environ Exp Bot 2009;65 142–142.

Levizou E, Drilias P, Psaras GK, Manetas Y. Nondestructive assessment of leaf chemistry and physiology through spectral reflectance measurements may be misleading when changes in trichome density co-occur. New Phytol 2005;165:463–72.

Lipp CC, Goldstein G, Meinzer FC, Niemczura W. Freezing tolerance and avoidance in high-elevation Hawaiian plants. Plant Cell Environ 1994;17:1035–44.

Medina-Torres L, Brito-De La Fuente E, Torrestiana-Sanchez B, Katthain R. Rheological properties of the mucilage gum (*Opuntia ficus indica*). Food Hydrocolloids 2000;14:417–24.

Morse SR. Water balance in *Hemizonia luzulifolia*: the role of extracellular polysaccharides. Plant Cell Environ 1990;13:39–48.

Munns R, Termaat A. Whole-plant responses to salinity. Aust J Plant Physiol 1986; 13:143–60.

Nobel PS, Cavelier J, Andrade JL. Mucilage in cacti—its apoplastic capacitance associated solutes, and influence on tissue water relations. J Exp Bot 1992; 43:641–8.

Pellerin P, O'Neill MA. The interaction of the pectic polysaccharide Rhamnogalacturonan II with heavy metals and lanthanides in wines and fruit juices. Analysis 1998;26:32–6.

Pharmacopée européenne, 6th edition. 2007. Chapter 2.8.4, p. 267.

Robichaux RH, Morse SR. Extracellular polysaccharide and leaf capacitance in Hawaiian bog species *Argyroxiphium grayanum* (Compositeae, Madiinae). Am J Bot 1990;77:134–8.

Schill V, Hartung W, Orthen B, Weisenseel MH. The xylem sap of maple (*Acer platanoides*) trees-sap obtained by a novel method shows changes with season and height. J Exp Bot 1996;47:123–33.

Teixeira J, Pereira S. High salinity and drought act on an organ-dependent manner on potato glutamine synthetase expression and accumulation. Environ Exp Bot 2007;60:121–6.

Tognetti R, Longobucco A, Raschi A, Jones MB. Stem hydraulic properties and xylem vulnerability to embolism in three co-occuring Mediterranean shrubs at a natural CO_2 spring. Aust J Plant Physiol 2001;28:257–68.

Went FW. In: The experimental control of plant growth. Mass: Waltham: Chronica Botanica Company; 1957 p. 343.

Zimmermann U, Zhu JJ, Meinzer FC, Goldstein G, Schneider H, Zimmermann G, et al. High molecular weight organic compounds in the xylem sap of mangroves: implications for long-distance water transport. Bot Acta 1994;107:218–29.

Zimmermann U, Thürmer F, Jork A, Weber M, Mimietz S, Hillgärtner M, et al. A novel class of amitogenic alginate microcapsules for long-term immunoisolated transplantation. In: Hunkeler D, editor. Bioartificial organs III: tissue sourcing, immunoisolation, and clinical trials. Annals of the New York academy of science. New York: New York Academy of Science; 2001. p. 199–215.

Zimmermann U, Schneider H, Wegner LH, Haase A. Water ascent in tall trees: does evolution of land plants rely on a highly metastable state?. New Phytol 2004;162:575–615.

Zimmermann D, Westhoff M, Zimmermann G, Geßner P, Gessner A, Wegner LH, et al. Foliar water supply of tall trees: evidence for mucilage facilitated moisture uptake from the atmosphere and the impact on pressure bomb measurements. Protoplasma 2007;232:10–34.

Zimmermann U, Schneider H, Wegner LH, Wegner H-J, Szimtenings M, Haase A, et al. What are the driving forces for water lifting in the xylem conduit? Physiol Planta 2002a;114:327–35.