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RESEARCH PAPER

Why are *Chloris gayana* leaves shorter in salt-affected plants? Analyses in the elongation zone

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Abstract

Reduced hydraulic conductance calculated from growth data was suggested to be the main reason for reduced leaf expansion in salt-stressed *Chloris gayana* (Rhodes grass). In this work, xylem vessel cross-sections and wall enzyme activities were analysed to re-examine the effects of salinity on leaf growth in this species. Maximal segmental growth rates were 20% lower and the growth zone was 23% shorter in leaves from salinized plants than in controls; however, growth rates between 0 mm and 15 mm from the ligule were similar in both types of leaves. Xylem cross-sectional areas in this region were about 65% smaller in leaves of salinized plants, suggesting that hydraulic restrictions in the leaves of salinized plants were much higher than overall growth reductions. Extractable xyloglucan endotransglucosylase activity in this zone was twice as high in leaves of salinized plants as in leaves of controls. Nevertheless, the activity of the extracted enzyme was not affected by up to 1 M NaCl added to the reaction medium. Therefore, increased xyloglucan endotransglucosylase activity under salinity may be due to a promotion of transcription of *XTH* (xyloglucan endotransglucosylase/hydrolases) genes and/or translation of preformed transcripts. These results suggest that, as in drought stress, increased activity of cell wall enzymes associated with wall loosening may contribute to the maintenance of growth under saline conditions despite hydraulic restrictions.

Key words: *Chloris gayana*, peroxidase, Rhodes grass, salt stress, wall autolysis, xylem, xyloglucan endotransglucosylase.

Introduction

Rhodes grass (*Chloris gayana* Kunth) is a widely used forage grass, recognized for its salt tolerance (Bogdan, 1969); nevertheless, its yield in saline soils is significantly reduced (Pérez *et al.*, 1999), owing to reductions in leaf area expansion and an increased proportion of dead leaves (de Luca *et al.*, 2001). In grasses, leaf blade growth is restricted to the expanding zone (EZ) in the lamina base. Very early in leaf development the intercalary meristem subdivides into two meristems that give rise, first to the lamina and, later, to the sheath (Fricke, 2002). The profile of growth rates along the blade EZ remains constant during the linear phase of leaf elongation (Muller *et al*., 2001). Salt-affected *C. gayana* plants had shortened blade growth zones and decreased growth rates within it (Ortega and Taleisnik, 2003). As elongation growth results from irreversible cell enlargement determined by the rate of water uptake and the plastic properties of the cell wall, the present work examines how these aspects are affected by salinity in this species.

Salinity and water stress often result in reduced hydraulic conductance in plants (Peyrano *et al.*, 1997; Steudle, 2000). When this parameter was calculated from growth data, it was suggested to be, among other causes, the main reason for reduced leaf expansion in salt-stressed *C. gayana* (Ortega and Taleisnik, 2003). This conclusion seems logical; nevertheless, additional information from

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Abbreviations: AZ, accelerating growth region in the blade expanding zone; CL, leaves from non-salinized plants; DZ, decelerating growth region in the blade expanding zone; EZ, expanding zone; SL, leaves from salinized plants; XET, xyloglucan endotransglucosylase; XTH, xyloglucan endotransglucosylase/hydrolase.

growth-independent variables is required to validate it. Axial hydraulic conductivity can be calculated from the cross-sectional area of xylem vessels, and these estimations are consistent with the actual measured hydraulic conductivity (Martre *et al.*, 2000). In this work, the cross-section of the xylem vessels was used to re-examine the effect of salinity on hydraulic conductance in *C. gayana*.

If hydraulic constraints are the main cause for salinity-associated leaf growth reductions in *C. gayana*, it is expected that sustained leaf elongation under such conditions would require increased cell wall-loosening activities. Conceptually, cell elongation requires a positive balance between loosening and tightening of the wall polysaccharide matrix. That balance defines regions of accelerating and decelerating growth within the grass leaf growth zone (Muller *et al.*, 2001), and the resulting typical tailed-bell shape of growth rate distribution within it (Volenec and Nelson, 1981; Bernstein *et al.*, 1993). While the effects of environmental stress on cell wall tightening have been the subject of many studies (Cramer and Bowman, 1991; Botella *et al.*, 1994; Cosgrove, 1997; Peyrano *et al.*, 1997; Wang *et al.*, 1997; Ma *et al.*, 2004), information relating salinity and wall loosening mechanisms is relatively scarce.

Cell wall loosening and tightening results from the action of enzymes and reactive oxygen species (Cosgrove, 1999; Schopfer, 2001; Fry, 2004). Several proteins have been directly implicated in cell wall loosening, among them xyloglucan endotransglucosylase/hydrolases (XTHs) (Fry *et al.*, 1992; Rose *et al.*, 2003; Nishitani, 2005), expansins (McQueen-Mason and Cosgrove, 1995; Cosgrove, 1999), and yieldins (Okamoto-Nakazato *et al.*, 2001), while others, like glucanases, which mediate the hydrolytic degradation of the hemicellulose mixed-linkage β-glucan (Huber and Nevins, 1982) have been postulated to have a synergistic role for the action of the former ones (Cosgrove, 1997; Peña *et al.*, 1999). XTH proteins are responsible for xyloglucan endotransglucosylase (XET) activity, catalysing the cutting and rejoining of xyloglucan chains (Fry *et al.*, 1992), and thus facilitating the slippage of microfibrils within the polysaccharide matrix of the cell wall as cells expand in volume. The expression pattern of an *XTH* transcript followed the distribution of growth rates in the growing zone of *Festuca pratensis* and the resulting XET activity was proposed to be involved in cell wall modification processes during cell elongation (Reidy *et al.*, 2001). XET activity was enhanced in the apical region of maize roots from plants grown under low water potentials (Pritchard *et al.*, 1993; Wu and Cosgrove, 2000; Wu *et al.*, 2005), and suggested to be necessary for maintaining elongation under these conditions. This activity is strongly dependent on the ionic environment and the presence of anionic polysaccharides (Takeda and Fry, 2004). Thus, it is likely to be affected by the apoplastic ionic environment prevailing in plants grown under salinity.

Processes related to maturation and arrest of growth prevail in the growth-decelerating region of the EZ. Cell wall peroxidase activity (Fry, 1986; McDougall, 1992; Fry *et al.*, 2000) and action (Kerr and Fry, 2004; Encina and Fry, 2005) have often been associated with cell wall tightening. In *Lolium temulentum* under drought stress, shortening of the EZ and the retardation of leaf growth were associated with increased ionically bound peroxidase activity (Bacon *et al.*, 1997). In a gibberellin-unresponsive dwarf mutant of maize, leaf elongation zones are shorter than in the wild type because of reduced final cell length, and apoplastic peroxidase activity and isoforms were very closely related to this profile (Souza and MacAdam, 2001).

Results from the present work confirm the relevance of hydraulic constraints to leaf growth in saline conditions and show that salinity may stimulate the production of cell wall-loosening XET activity.

Materials and methods

Plant material and growth conditions

Rhodes grass (*Chloris gayana* K. cv. Boma) caryopses were soaked overnight in running tap water and later sown on moist vermiculite in plastic trays. Upon germination, plantlets were transferred to hydroponic trays (3.5 l) with half-strength Hoagland solution, in a naturally illuminated greenhouse. The solution was changed twice a week, and it was not aerated. When two leaves were visible above the sheath whorl, the solution in the trays was gradually supplemented with NaCl (in three successive increments, every 3–4 d) until a concentration of 200 mM was reached. Leaf 5, which was visible above the sheath whorl 3 d after reaching the final salt concentration and, thus, had elongated under salinity in stress treatments, was chosen for this study. Leaves from non-salinized plants are called CL and those from treated plants, SL.

Growth rates along the EZ

The anatomical method for inferring growth rates has been proposed as a useful alternative to the analysis of the displacement of marks in organs, such as monocot leaves (enclosed within the whorl of older leaves), where marking may perturb the normal growth pattern (Silk *et al.*, 1989), and it has been suggested that it provides a more accurate description of growth distribution. Five leaves from each treatment, at the linear elongation phase, were selected on the second day after appearance from the whorl, and freed from older enclosing leaves under a stereomicroscope. A transparent negative film of the abaxial epidermis was obtained from a thin nail varnish layer spread on the rolled leaf surface. Films were carefully removed with forceps, placed between a glass slide and cover slip and examined under a microscope at a magnification of ×400. Digital images were analysed with image-processing software (Optimas 6.1; Optimas Corporation, Bothell, WA, USA). Ten to thirty cells on both sides of the midrib were measured at 5 mm intervals in each leaf. Measurements were performed on interstomatal cells where transverse cell walls were most easily identified. Growth parameters were calculated from cell length according to Silk *et al.* (1989). Cellochron (the time interval during which a new cell is added to a cell file at the base of the EZ) was calculated as the ratio of mature cell length to leaf elongation rate for the day of leaf sampling. The reciprocal of this value, termed cell flux (f), i.e. the number of cells passing a given point per unit time, along with cell lengths (l), were used to compute local

velocity of displacement (v) in the formula: $v=f \times l$. A three-parameter sigmoid function was fitted to individual plots of cell lengths versus position along the leaf blade, and differentiated using a five-point derivative formula (Erickson and Sax, 1956) to obtain the strain rate $R = f \times (\delta l/\delta x)$, where x is the distance from the ligule.

Leaf zones sampled in this study

The leaf blade extension zone (EZ) comprised the 70 mm from the ligule in control plants, and 50 mm in salinized ones. The zone of accelerating growth (AZ) spanned a maximum of 25 mm from the ligule in controls and 15 mm in salinized plants. The decelerating growth zone (DZ) was the 20 mm segment starting at either 30 mm from the ligule in control plants or 20 mm in salinized ones. Expanded leaf blade samples were obtained beyond 70 mm in both control and salinized plants.

Xylem lumen measurements

Three representative plants of each treatment were excised at the root–shoot junction, the EZ of leaf 5 was exposed and free-hand cross-sections were obtained with a razor blade at 5, 10, 20, and 70 mm above the ligule. The lumen area of all vessels was measured in digital images taken under a fluorescence microscope at a magnification of ×400 as described by Martre *et al.* (2000).

Cell wall autofluorescence

Cell wall autofluorescence under UV light is indicative of wall phenolics, such as ferulic or *p*-coumaric acid or of lignin (Harris and Hartley, 1976; Fukazawa and Imagawa, 1981). Free-hand cross-sections of the EZ, obtained at various distances above the ligule, were mounted on microscope slides with 50% glycerol (pH 6.5). The intensity of blue autofluorescence was measured as bright intensity by luminance determination on digitized images using Adobe Photoshop© software.

Peroxidase activity determination and isoform isoelectrofocusing

Samples for peroxidase activity were processed according to Quiroga *et al.* (2000) with modifications. Segments (20 mm) from the AZ and DZ regions of the EZ (100 mg) were ground with a mortar and pestle, on ice, with 50 mM K-phosphate buffer, pH 6, and 0.2% polyvinylpyrrolidone. After 30 min centrifugation at 3500 *g* the supernatant was assayed for peroxidase activity using *o*-dianisidine (Quesada *et al.*, 1990). The pellet was extensively washed with more K-phosphate buffer until no peroxidase activity was detected in the supernatant. Ionically bound peroxidases were extracted by incubating this pellet in 50 mM phosphate buffer, pH 6, containing 1 M KCl for 2 h. The supernatant was dialysed overnight against 25 mM K phosphate buffer, pH 6. As for the soluble peroxidase extract, activity was assayed with *o*-dianisidine. Protein content was determined according to Bradford (1976) with bovine serum albumin as standard. Isoelectric focusing of these ionically bound peroxidase isozymes was performed by non-denaturing 7.5% PAGE; samples were run for 150 min at 2 mA on a pH range of 3.0–10 on vertical polyacrylamide gel slabs (Robertson *et al.*, 1987). Gels were stained for peroxidase activity with benzidine as described by Forchetti and Tigier (1990).

XET activity

This assay was performed according to Fry *et al.* (1992). Ten to three hundred milligrams of freeze-dried AZs or expanded laminae were homogenized in 300 mM succinate buffer, pH 5.5, containing 10 mM $CaCl_2$ and 10% glycerol, and centrifuged in a bench-top centrifuge. Ten microlitres of the resulting supernatant were added to a reaction mixture (20 µl) containing tamarind xyloglucan (0.5%, w/v), 0.5% chlorobutanol, and 50 kBq ml^{-1} [^{3}H]XXXGol (radiolabelled xyloglucan oligosaccharide; for nomenclature, see Fry *et al.*, 1993). The reaction was stopped by addition of 30 µl 30% formic acid. This mixture was dried on Whatman 3MM paper (3×3 cm) and washed with running tap water overnight. After drying, incorporated [^{3}H]XXXGol was assayed by scintillation counting.

Cell wall autolytic activity

Frozen isolated EZs (200–300 mg FW) were processed according to Inouhe and Nevins (1991) and transferred to chromatography columns. Wall autolytic activity was measured as sugars released from walls incubated in 20 mM K-citrate buffer at 37 °C. Controls were done by immersion of columns in boiling water for 5 min to inactivate any enzyme activity. Total soluble carbohydrates released into the buffer in the columns were determined by the phenol–sulphuric acid method (Dubois *et al.*, 1956).

Results and discussion

Salinity effects on growth rates in the leaf elongation zone

Growth of leaf 5 in *C. gayana* seedlings was followed on a daily basis until complete leaf expansion. In control plants, final leaf length was 28.9±0.9 cm by day 8 after appearance from the enclosing sheaths, and, in salinized plants, it reached 20±1.4 cm 1–2 d after leaves from control plants had ceased growing (Fig. 1). The phyllochron, the time interval between two successive leaves, was increased from 2.3 d in controls to 4.7 d in salinized plants, indicating that fewer leaves were expanding simultaneously in the latter.

Figure 2A shows the spatial distribution of abaxial epidermal cell lengths for leaves from both treatments. Cell lengths increased to a final value of 100.3±5.9 µm in CL and 86.3±3.2 µm in SL. Thus, final leaf length was more severely affected than final cell length, suggesting that cell division is more affected than cell elongation, as shown

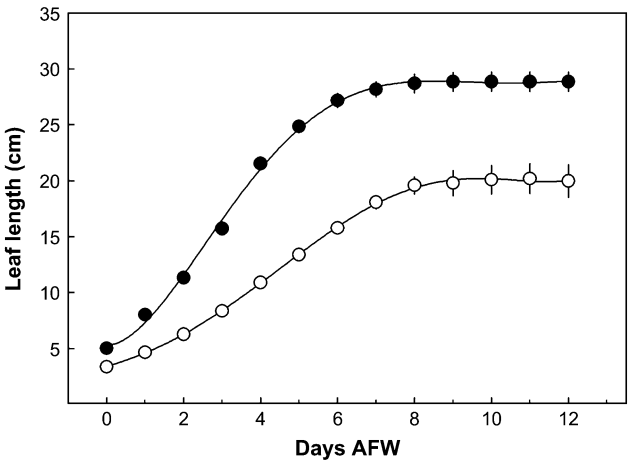

Fig. 1. Salinity effects on length of leaf 5 of *C. gayana* seedlings on successive days. Differences between control and salinized plants were significant for the whole period. Results are means ±standard error for 15 blades. AFW, After leaf tip appearance above the sheath whorl. Filled circles, control, open circles, NaCl.

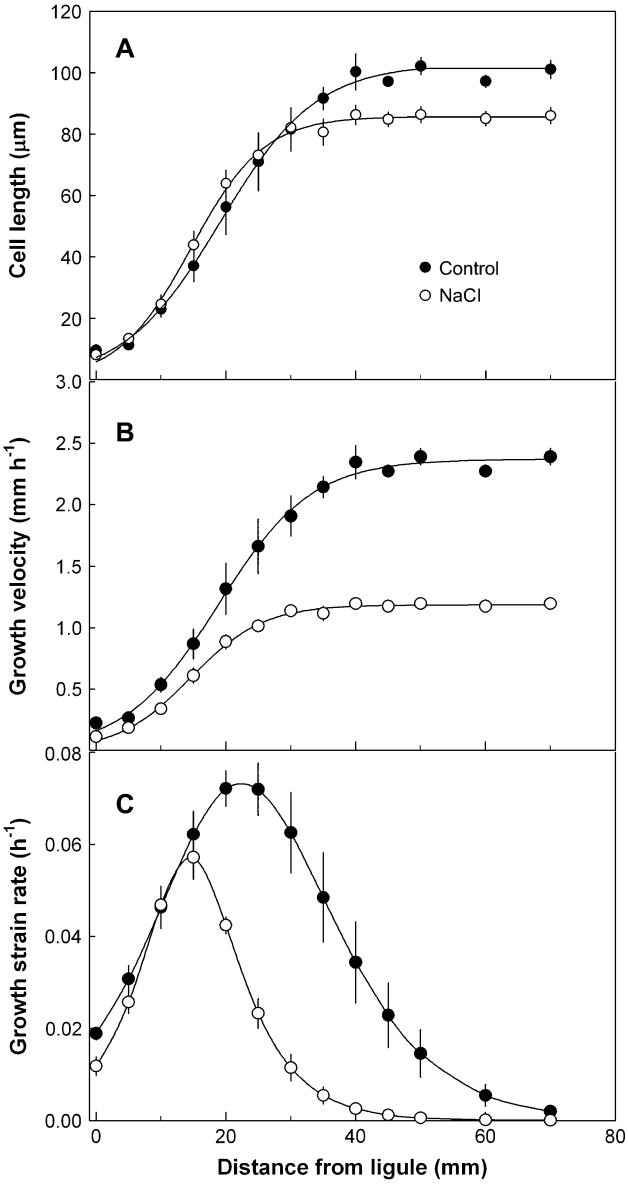

Fig. 2. (A) Spatial distribution of abaxial interstomatal cell lengths in leaf 5 from plants grown under control or 200 mM NaCl conditions. Measurements were taken on day 3 after leaf tip appearance above the sheath whorl, and are means ±standard error of 10–30 cells from four to six different leaves. (B) Velocity of tissue displacement, based on data from (A). (C) Distribution of growth rates, based on (B); notice maximum elongation rates and EZ length differ in SL and CL.

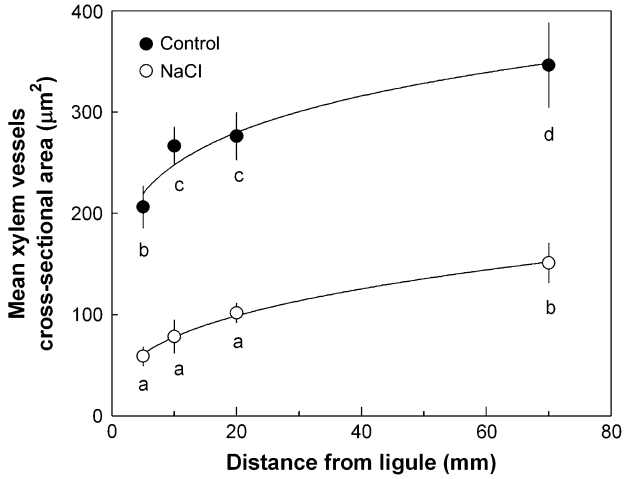

Fig. 3. Xylem conduit areas in the EZ from control and salinized plants at various distances from the ligule. Different letters indicate significant differences ($P \leq 0.05$) between treatments for that position. Each point is the mean ±standard error of 8–18 vessels measured in three different leaves.

by Beemster and Masle (1996) for mechanical impedance stress. Cellochron, the time interval during which a new cell is added to a cell file in the elongation region, was 0.0428 h in CL and 0.0722 h in SL, a very significant 70% increase caused by the salt treatment. In addition, the velocities of displacement (Fig. 2B), which indicate the rate at which tissues located at a given distance from the ligule are pushed away by the growing cells in the preceding segments, were also lower in the distal part of the EZ, in leaves from the salt treatment. Reduced cell division and elongation both appear to contribute to the reduction in displacement velocity in salinized plants.

Mean cell growth rates obtained from the slope of change of velocities of displacement are shown in Fig. 2C. The distribution of growth rates in the elongation zone was approximately symmetrical as a function of distance, and growth rates between 0 mm and 15 mm from the ligule were not affected by salinity. The maximal growth rate in CL was 0.07 h^{-1}, and 0.057 h^{-1} in SL (significantly different at $P < 0.05$), a reduction of about 20% in the stressed leaves. Maximal growth rates divide the regions of accelerating (AZ) and decelerating growth (DZ) and were registered at 20–25 mm and 15 mm from the ligule in CL and SL, respectively. The length of the EZ zone was reduced from 70 mm in CL to 50 mm in SL.

The effect of salinity on growth rates in the elongation zone of *C. gayana* seedlings found in this study is very similar to that previously reported for tillers of this species (Ortega and Taleisnik, 2003) and maize (Neves-Piestun and Bernstein, 2001, 2005). However, the distribution of growth rates deduced from anatomical data is more homogeneous than that obtained previously from pricking studies. This difference may be ascribed mainly to the long time interval between pricking and harvest in that study (Erickson, 1976).

Xylem conduits area

Xylem cross-sectional area in the region spanning from 5 mm to 70 mm from the ligule was significantly reduced in salt-treated plants (Fig. 3). The Hagen–Poiseuille formula for calculating fluxes along ideal capillaries indicates that fluxes are proportional to the fourth power of the vessels' diameter, and hydraulic conductance estimates also take

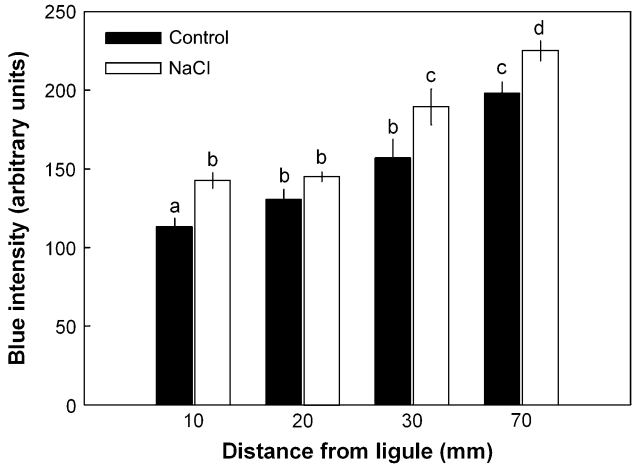

Fig. 4. Blue autofluorescence intensity measured on digitized images from leaf preparations observed under a UV microscope. Data are means ±standard error of three measurements collected from cross-sections of four blades. Black columns, CL; white solumns, SL.

Table 1. *XET activity on DW basis in AZ and expanded regions from leaves of* C. gayana *plants grown under control or salinized conditions*

Data are means ±standard error of seven independent measurements.

Leaf segment	XET activity (^{3}H incorporation min^{-1} mg^{-1})	
	Control	200 mM NaCl
AZ	2.07±0.33	4.34±0.57
Expanded	0.123±0.01	0.126±0.01

into account the water potential gradient along the vessel. In the hypothetical case that those gradients were similar for control and salinized plants, theoretical axial laminar flows based on xylem vessel diameters would be *c*. 83% lower in SL than in CL. This difference between control and salinized plants is much higher than the difference in turgor pressure (Ortega and Taleisnik, 2003) and elongation rates in those plants. It is possible that phloem water supply may be counteracting part of the restriction resulting from decreased xylem water conductivity (Martre *et al.*, 2000). Physical properties of the cell wall determine growth differences along the EZ in the presence of constant turgor (Pritchard *et al.*, 1993). Physical properties of the wall can change in response to salt stress (Cramer and Bowman, 1991; Lu and Neumann, 1999), and could also contribute to the explanation of why hydraulic restrictions do not match growth reductions. This aspect was studied next.

Cell wall autofluorescence and peroxidase activity

As cell walls mature, the cross-linking of phenolic compounds forms a hydophobic meshwork that bonds tightly to cellulose and prevents wall enlargement. Cell wall fluorescence under UV light is considered to be a good indicator of its phenolic compound content (Fukazawa and Imagawa, 1981). This parameter (measured as blue intensity in digitized images; Fig. 4) increased as a function of distance from the ligule, and was generally higher in salinized plants, suggesting a higher content of integrated phenolics in SL. These would strengthen the association among cell wall polysaccharides and thus contribute to wall tightening.

Phenolic compounds are oxidized in place by apoplastic phenol oxidases, and the association between peroxidase activity and cessation of cell expansion has been shown in several studies (MacAdam *et al.,* 1992*a*, *b*; Bacon *et al.*, 1997; Souza and MacAdam, 2001). In CL, cell wall peroxidase activities were similar throughout the EZ (Fig. 5A); however, a significant increase in peroxidase activity was observed in the DZ in SL. The analysis of the isozyme electrophoretic pattern indicates different isozyme bands in the AZ and DZ, and effects of salinity on band intensity. In the AZ of SL (Fig. 5B), decreased staining intensity was observed in bands located at pH 7 and 9 while, in the DZ (Fig. 5C), increased staining was observed in bands located between pH 6.5 and 7. Leaves from salinized plants showed shortened EZ and increased wall tightening and peroxidase activity in the DZ. However, to find out if changes in band distribution and intensity associate with changes in polymer cross-linking requires substrate-specificity tests (Quiroga *et al.*, 2000).

XET and cell wall autolytic activity

By contrast to the clear growth reductions observed in the DZ of SL (Fig. 2C), data from the AZ do not show a significant inhibitory effect of salinity on growth. Growth rates in the 20 mm leaf segment closest to the ligule were similar in CL and SL, despite significant differences in calculated hydraulic conductance. As sustaining growth under such conditions would require especially loose cell walls, the effects of salinity on some wall-loosening enzymes were assessed next.

XET activity was significantly higher in the AZ of the EZ than in expanded laminae (Table 1), in accordance with its proposed role in cell wall loosening and maintenance of growth. Activity was twice as high in SL as in CL. Apoplastic salt concentration is higher in salinized plants, and salt has been reported to stimulate XET activity (Takeda and Fry, 2004). Therefore, to evaluate salt influence on XET activity, NaCl was added to the reaction mixture. NaCl (0.25 M), at a concentration compatible with what has been reported in the EZ of *C. gayana* (de Luca *et al.*, 2001), caused both CL and SL XET activity to increase by the same increment (6 cpm×10^2); though smaller than that reported by Takeda and Fry (2004), probably because the extracts used in the present work already contained some endogenous salts and polyanions. However, activity of neither CL or SL was further enhanced by the addition of up to 1 M NaCl to the reaction medium

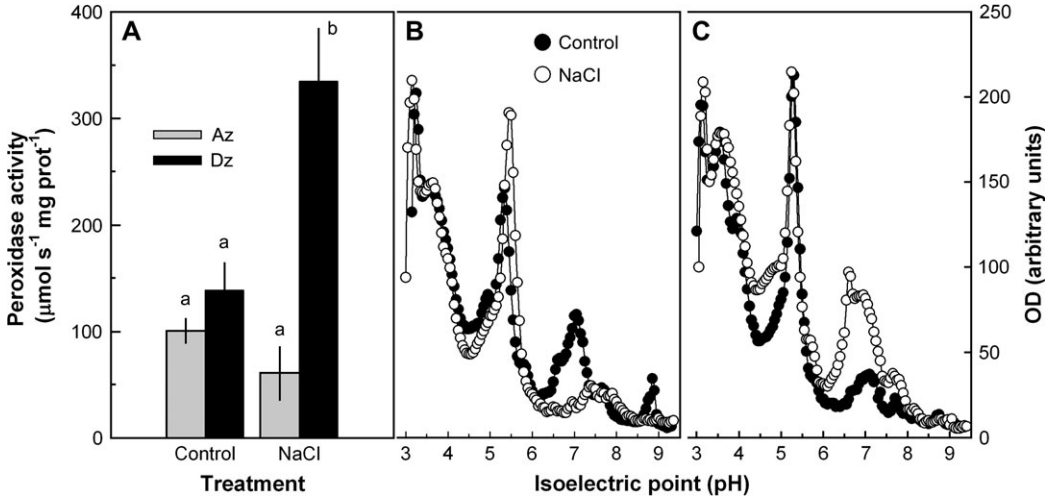

Fig. 5. (A) Specific peroxidase activity in accelerating (AZ) and decelerating (DZ) zones of EZ of *C. gayana* blades grown in control and salinized conditions. Data are means ±standard error of three independent determinations. (B) Electrophoretic isozyme pattern from the AZ in the same leaves. (C) Electrophoretic isozyme pattern from the DZ in the same leaves.

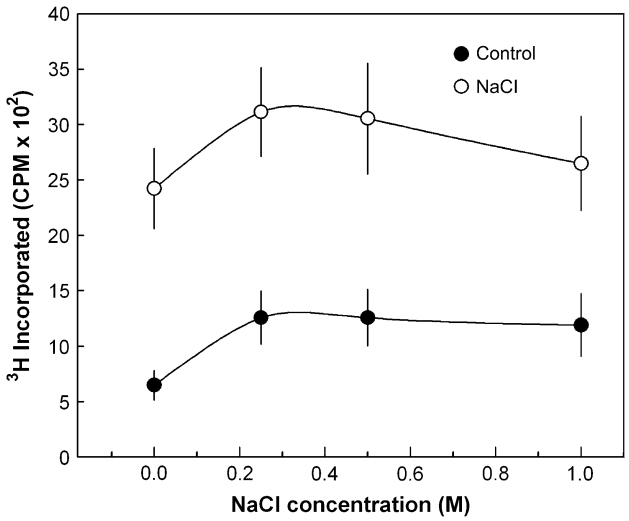

Fig. 6. Effect of NaCl on XET activity from control or salinized (200 mM NaCl) plants. NaCl was added to the reaction medium at the concentrations shown in the figure. Data are means ±standard error of seven independent determinations. The *y*-axis shows ^{3}H incorporated per unit time, a measure of XET activity.

(Fig. 6). Since the addition of NaCl did not strongly stimulate XET activity, it is suggested that the higher activity in SL may be due to a promotion of transcription and/or translation of preformed transcripts by salinity, rather than to the higher NaCl content of the cell wall. In a study of genes regulated specifically by salinity in *Arabidopsis*, Ma *et al.* (2006) mention a putative XET gene among those that were only or most strongly up-regulated in salt-stressed roots.

Glucanases participate in cell wall loosening in an indirect manner, by modulating expansin and XET-mediated creep. Contrary to the effects observed on XET activity, no differences between CL and SL were found in cell wall hydrolytic activities (Fig. 7). These measurements were performed in the absence of added salts; however, the addition of 200 mM NaCl, inhibited sugar release by 50% (Fig. 7). This result may be significant in the context of the interaction between this and other cell wall-loosening activities such as expansins and XET.

The results from the present study indicate that while reduced xylem vessel dimensions suggest hydraulic constraints throughout the EZ of SL, lamina growth dynamics in salinized plants may be influenced by different processes operating in the AZ and the DZ. The analysis of the DZ indicates growth reduction was substantiated by a higher phenolic compound content and increased peroxidase activity. Nevertheless, in the AZ, XET activity was significantly higher in SL than in CL, suggesting that, *in vivo*, this activity may render walls more extensible, compensating for the lower water supply.

On the other hand, it has been suggested that XET may have a role in restructuring primary walls at the time when secondary wall layers are deposited, by creating and reinforcing the connections between the primary and secondary wall layers (Bourquin *et al.*, 2002). In *Chloris gayana* leaves, as in soybean roots (Hilal *et al.*, 1998), salinity led to a premature maturing of xylem vessels. Whether XET activity participates in this context remains to be evaluated. It must also be borne in mind that wall extensibility depends on the type of xyloglucan substrate for XET activity as reported by Takeda *et al.* (2002), who found that pea hypocotyl extensibility varied as a function of the size of xyloglucans incorporated by XET activity. To find out if salinity affects XET and glucanase substrate availability and characteristics will be the object of another study.

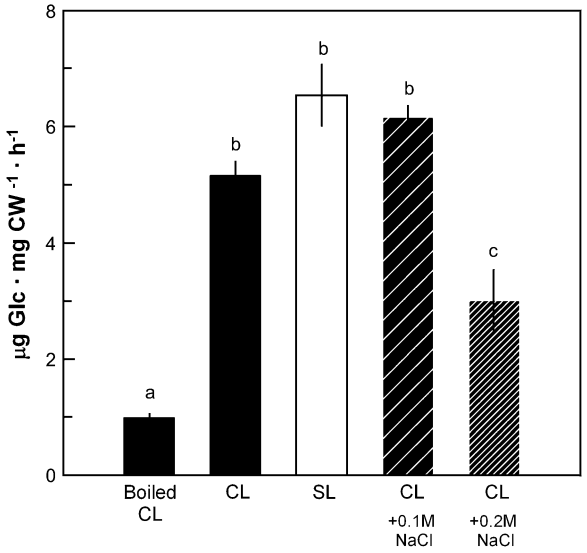

Fig. 7. Cell wall autolytic activity in entire EZ extracts from *C. gayana* leaves obtained from plants grown under control (CL) or salinized (SL) conditions, expressed per milligram cell walls (CW). Boiled samples are inactive controls and the effects of added NaCl to CL walls are also shown. Results are means ±standard error of three to five samples and different letters indicate significant differences at $P < 0.05$.

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