

## Reductions in Maize Root-tip Elongation by Salt and Osmotic Stress do not Correlate with Apoplastic $O_2^{\bullet-}$ Levels

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Received: 1 May 2008 Returned for revision: 28 May 2008 Accepted: 3 July 2008 Published electronically: 15 August 2008

- **Background and Aims** Experimental evidence in the literature suggests that  $O_2^{\bullet-}$  produced in the elongation zone of roots and leaves by plasma membrane NADPH oxidase activity is required for growth. This study explores whether growth changes along the root tip induced by hyperosmotic treatments in *Zea mays* are associated with the distribution of apoplastic  $O_2^{\bullet-}$ .
- **Methods** Stress treatments were imposed using 150 mM NaCl or 300 mM sorbitol. Root elongation rates and the spatial distribution of growth rates in the root tip were measured. Apoplastic  $O_2^{\bullet-}$  was determined using nitro blue tetrazolium, and  $H_2O_2$  was determined using 2', 7'-dichlorofluorescein.
- **Key Results** In non-stressed plants, the distribution of accelerating growth and highest  $O_2^{\bullet-}$  levels coincided along the root tip. Salt and osmotic stress of the same intensity had similar inhibitory effects on root elongation, but  $O_2^{\bullet-}$  levels increased in sorbitol-treated roots and decreased in NaCl-treated roots.
- **Conclusions** The lack of association between apoplastic  $O_2^{\bullet-}$  levels and root growth inhibition under hyperosmotic stress leads us to hypothesize that under those conditions the role of apoplastic  $O_2^{\bullet-}$  may be to participate in signalling processes, that convey information on the nature of the substrate that the growing root is exploring.

**Key words:** Root tip growth, *Zea mays*, salt stress, reactive oxygen species, ROS.

### INTRODUCTION

Production of reactive oxygen species (ROS) appears to be a general characteristic of expanding plant cells and organs (Foreman *et al.*, 2003; Peleg-Grossman *et al.*, 2007; Potocky *et al.*, 2007). A requirement for ROS for elongation of tip-growing cells has been demonstrated for root hairs using NADPH oxidase mutants (Foreman *et al.*, 2003; Carol and Dolan, 2006); infection threads in symbiotic relationships (Peleg-Grossman *et al.*, 2007) and pollen tubes (Potocky *et al.*, 2007) also require ROS for active growth. Expanding organs such as embryonic axes (Puntarulo *et al.*, 1988), growing roots (Jon *et al.*, 2001), and germinating seeds (Schopfer *et al.*, 2001) can produce and extrude ROS. In maize, pharmacological treatments that reduce  $O_2^{\bullet-}$  levels indicate that growth of leaves (Rodríguez *et al.*, 2002) and roots (Liszak *et al.*, 2004) depends on active apoplastic ROS production. ROS production occurs in almost all cell compartments (Mittler *et al.*, 2004), including the apoplast, where it has been repeatedly associated with NADPH oxidase activity as a major source. Similar experiments have shown that elongation of *Arabidopsis* roots depends on the generation of  $OH^{\bullet}$  by cell wall peroxidases and this reaction, in turn, depends on the apoplastic supply of  $H_2O_2$  resulting from the dismutation of  $O_2^{\bullet-}$ , which is produced by an NADPH-oxidase-like enzyme in the plasma membrane (Dunand *et al.*, 2007). Ten NADPH-oxidase catalytic subunit genes exist in the *Arabidopsis* genome (Torres *et al.*, 1998; Foreman *et al.*,

2003) and *atrbohF* null mutants of *Arabidopsis* have reduced root elongation as compared to wild types (Kwak *et al.*, 2003), providing functional evidence for the need of NADPH-oxidase-generated ROS for root elongation.

On the other hand, increased oxidative damage is often the result of stress conditions and, specifically in roots, it has been reported for drought (Porcel and Ruiz-Lozano, 2004; Sofo *et al.*, 2004), salinity (Katsuhara *et al.*, 2005) and heavy metals (Schutzendubel and Polle, 2005; Singh *et al.*, 2007). Stimulated NADPH-oxidase-mediated ROS production occurs under both hyper-osmotic (Kawano *et al.*, 2001; Beffagna *et al.*, 2005) and hypo-osmotic stress conditions (Cazalé *et al.*, 1998; Rouet *et al.*, 2006), and it is known to be the result of complex regulatory mechanisms including changes in cytosolic free  $Ca^{2+}$  concentration (Foreman *et al.*, 2003), pH (Beffagna *et al.*, 2005) and phospholipids with signal functions. The plant NADPH-oxidase complex can be activated by phosphatidylinositol 3-phosphate (PI3P), the product of phosphatidylinositol 3-kinase (PI3K; Joo *et al.*, 2005). Under salt stress, enhanced PI3K activity has been found to lead to an endocytosis-dependent intracellular activation of NADPH oxidase (Leshem *et al.*, 2007). In *Arabidopsis*, phospholipase Da1 (PLDa1) and phosphatidic acid (PA) have also been implicated in increasing NADPH oxidase activity and ROS production (Sang *et al.*, 2001).

The association between changes in ROS production and growth reductions under salt and osmotic stress has not been thoroughly explored. Decreased apoplastic  $O_2^{\bullet-}$  production, resulting from ion-specific effects on plasma membrane NADPH oxidase (Rodríguez *et al.*, 2007), was

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observed in the growing region of leaf blades from salt-treated maize plants (Rodríguez *et al.*, 2004), where it was shown to contribute to the reduction in leaf elongation. The current study explores whether growth changes along the root tip and the distribution of apoplastic  $O_2^{\bullet-}$  are associated in maize (*Zea mays*) roots subject to growth inhibition by hyper-osmotic stress treatments. A detailed analysis is presented of salt and osmotic stress effects on the spatial distribution of  $O_2^{\bullet-}$  production along the root tip, and its correlation with the pattern of distribution of relative growth rate. The results indicate that root elongation reductions by NaCl or sorbitol occur under low and high apoplastic  $O_2^{\bullet-}$  production, respectively.

## MATERIAL AND METHODS

### Root elongation measurements

Maize (*Zea mays* L.) 'Prozea 30', (Produce, Pergamino, Argentina) was used for this study. Seeds were imbibed on moist filter paper, and after 3 d germinated seeds were transferred to plastic pouches (SCS®) containing half-strength Hoagland solution (Hoagland and Arnon, 1950). Two small styrofoam cubes inserted half way down the pouch prevented the plastic surface from sticking to the moist paper lining and helped air from the upper portion (where seeds separated the plastic and the lining) reach the roots. Pouches were kept at 21 °C under a 16-h photoperiod of 65  $\mu\text{E m}^{-2} \text{s}^{-1}$ .

For the short-term treatments, the root medium was changed to one containing 150 mM NaCl or 300 mM sorbitol, and root elongation rates were measured for 3 h immediately before the change and in the following 3-h treatment period. To assess the effects of stress release, the salt or sorbitol treatments were initiated when most emerged roots in the pouch were at least 2 cm long. In this case, NaCl or sorbitol were added on two consecutive days to final concentrations 150 mM (NaCl) or 300 mM (sorbitol). Two days after the final concentration was reached, the root medium was changed to half the concentration of salt or sorbitol (controls were changed to a medium of the same composition). Elongation rates were assessed by measuring the displacement over a 3- or 4-h interval using marks indicating the position of the root apices on the pouch surface. There were at least 20 seeds per experiment, and experiments were repeated up to four times. Only roots selected for uniformity of elongation rate (within +15 % of the mean per pouch) were included in the final analysis.

In order to estimate the distribution of growth rates along the root tip, a flap was opened in the pouch and marks were made on the root surface with an ultra-fine ball pen ('Uni-Ball', Faber Castell, Germany), at approx. 1-mm intervals for 12 mm from the root apex, as described by Sharp *et al.* (1988). Photographs were taken under a stereoscopic microscope with a NIKON DS camera (DS Camera Control Unit DS-L1, DS Camera Head DS-5M and DS Cooled Camera Head DS-5Mc) immediately after marking (time 0) and 3 h later. Spatial growth patterns were obtained by computing distances between marks

using image-processing software (Optimas 6-1, Optimas Corporation, Bothell, WA). Segmental growth was quantified as a relative growth rate (Hunt, 1978)

$$\text{RGR} = (\ln L_f - \ln L_i) / \Delta t$$

where  $L_i$  and  $L_f$  denote the initial and final segment length, respectively, and  $\Delta t$  is the measurement period. For statistical analysis, data were grouped into 0.5-mm intervals and subject to ANOVA followed by Tukey's test, using InfoStat (InfoStat/Profesional ver. 2007p, Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina).

### Histochemical analyses

*O<sub>2</sub><sup>•-</sup> detection.* Presence of  $O_2^{\bullet-}$  in root tips was determined with nitro blue tetrazolium (NBT), which reacts with  $O_2^{\bullet-}$  to produce a blue formazan precipitate. Excised root tips (approx. 1 cm long) were placed in an Eppendorf tube containing 0.01 % NBT solution of the medium in which the roots had been growing and incubated in darkness for 15 min. Note that NBT was always dissolved in the appropriate root medium, as hyper- or hypo-osmotic shocks alter  $O_2^{\bullet-}$  levels in the tissue. Staining the root tips directly on glass slides was found to produce uneven staining and was therefore avoided; shorter and longer incubation periods were also tested. Stained roots were mounted on glass slides, in the same medium but devoid of NBT, and colour images were obtained under a stereoscopic microscope, as described above. Control staining reactions included 10 mM  $\text{MnCl}_2$ , a highly effective  $O_2^{\bullet-}$  dismutating catalyst agent (Hernández *et al.*, 2001); this was used rather than superoxide dismutase because it readily penetrates cell walls.

The distribution of stain intensity was measured as luminance (Lu, scale 0–255 from darkest to lightest) with the image processing software, and transformed to optical density,  $\text{OD} = \log(\text{Lu})^{-1}$ .

*H<sub>2</sub>O<sub>2</sub> detection.* Roots were treated with 10  $\mu\text{M}$  2', 7'-dichlorofluorescein (DCFH-DA) for 5 min. Epifluorescence was observed with an Axiophot microscope (Zeiss, Germany) with excitation filter BP 450–490 and emission filter LP 520. Images were taken with the camera described above, using identical exposure settings for each root zone (tip or root-hair zone).

## RESULTS

### Elongation rates and apoplastic $O_2^{\bullet-}$ distribution in root tips under hyper-osmotic stress

Maize roots elongated at a rate of approx. 1 mm h<sup>-1</sup> in Hoagland solution, and in plants treated for 3 h with 150 mM NaCl or 300 mM sorbitol elongation rates were 0.51 and 0.56 mm h<sup>-1</sup>, respectively. Thus, these treatments decreased elongation rates by approx. 50 % and there were no statistical difference between them; however, both differed from the rate in control plants ( $P < 0.05$ ).

Reductions in elongation rates were even higher in roots of plants subjected to longer (4-d) stress treatments.

The distribution of elongation rates within root segments is shown in Fig. 1. In non-stressed roots, elongation rates increased up to approx. 3 mm from the root tip and then decreased. In the growth-acceleration zone, elongation rates were similar in controls and in roots stressed for 3 h, but peaked closer to the tip in the latter. Elongation rates in the decelerating zone were lower in both stress treatments than in control roots ( $P < 0.05$ ), and the elongation zone was approx. 1 mm shorter in stressed roots compared with control roots. The distribution of growth rates in this analysis, which were determined over 3-h intervals, may be shifted towards the tip, as would be expected when intervals between marking and measurement depart from being instantaneous (Erickson and Silk, 1980). Nevertheless, the data show that stress treatments significantly affected elongation rates in the growth-decelerating zone, but not closer to the tip as previously reported by Sharp *et al.* (1988) and Fan and Neumann (2004) for roots under water stress.

The level of  $O_2^{\bullet-}$  was high up to 1 mm from the root tip and then decreased (Fig. 2). Staining intensity increased in sorbitol-treated roots and was decreased by NaCl treatments (Fig. 2A–C). Beyond the initial 2 mm, the stress treatments produced either a relatively high or reduced apoplastic presence of ROS. This is clearly seen when densitometry

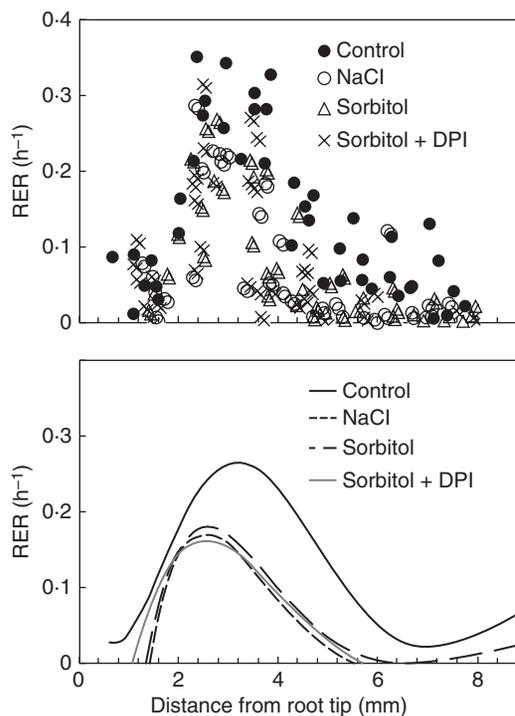


FIG. 1. Effect of iso-osmotic NaCl or sorbitol treatments on segmental relative elongation rates (RER) in maize roots. Plants were treated for 3 h with either 150 mM NaCl, 300 mM sorbitol or 300 mM sorbitol + 50  $\mu$ M DPI. (A) RER for each segment plotted against the initial position of the mark on the root closest to the apex. (B) Profiles of RER obtained by fitting sixth-order polynomials to the data points. Data from six control roots and from seven samples for each stress treatment.

measurements of formazan distribution along the roots are compared (Fig. 2G–I; to correctly interpret these figures it must be kept in mind that the OD units are presented on a logarithmic scale). These results indicate that alterations of the root growth profile by osmotic and salt stresses of similar magnitude were significant beyond 3 mm from the root tip. Growth changes, however, were not associated with changes in apoplastic  $O_2^{\bullet-}$  levels, not only because the treatments had different effects on  $O_2^{\bullet-}$  levels, but also because those effects became evident closer to the apex, where growth was essentially not altered. DPI reduced  $O_2^{\bullet-}$  levels in sorbitol-treated roots (Fig. 2C, inset); however, it had no effect on the growth of such roots (Fig. 1).

#### SOD activity and $H_2O_2$ in stressed roots

Superoxide dismutase (SOD) activity and increased internal ROS generation were assessed as possible causes for differences in  $O_2^{\bullet-}$  levels between salt- and sorbitol-treated roots. In order to determine stress-treatment effects on SOD, roots were treated with Na-diethyldithiocarbamate (DDC), an inhibitor of SOD activity (Ogawa *et al.*, 1996); results are included in Fig. 2. DDC slightly increased stain intensity, indicating SOD activity in all cases (in Fig. 2, compare each panel in the second row, which included DDC, with its control above; corresponding densitometry measurements are below). However, in NaCl-treated roots NBT staining still remained distinctly lighter than in non-stressed roots when DDC was added (Fig. 2D vs. E), suggesting that changes in SOD activity were not responsible for the observed low apoplastic levels of  $O_2^{\bullet-}$ .

Internalization of NADPH oxidase activity could also have reduced apoplastic  $O_2^{\bullet-}$  levels in salt-treated roots, while increasing internal ROS generation (Leshem *et al.*, 2007). However, fluorescence microscopy of DCFH-DA-treated samples did not show increased ROS levels in the growing region of root tips for either stress treatment (Fig. 3A–C). Only further from the tip, in the root-hair region, did sorbitol-treated roots show increased fluorescence (Fig. 3D–F). These results indicate that the differential effect of these stresses on  $O_2^{\bullet-}$  levels in the roots did not seem to be related to SOD activity, and apparently also not to increased generation of internal  $H_2O_2$  in the growth zone.

#### Growth stimulation and $O_2^{\bullet-}$ production in roots after stress relief

Stressed roots can be stimulated to grow by increasing the water potential in the medium. This was used to determine whether different apoplastic oxidative conditions generated by salt or osmotic stress may influence root elongation when it is stimulated by relief from stress. Plant roots were treated for 2 d with either 150 mM NaCl or 300 mM sorbitol and root elongation was then stimulated by decreasing the concentration of the growth medium. Elongation rates were measured immediately before and 4 h after the change to the root medium (Fig. 4). Root elongation rates nearly doubled when stressed roots were transferred to a medium with higher water potential

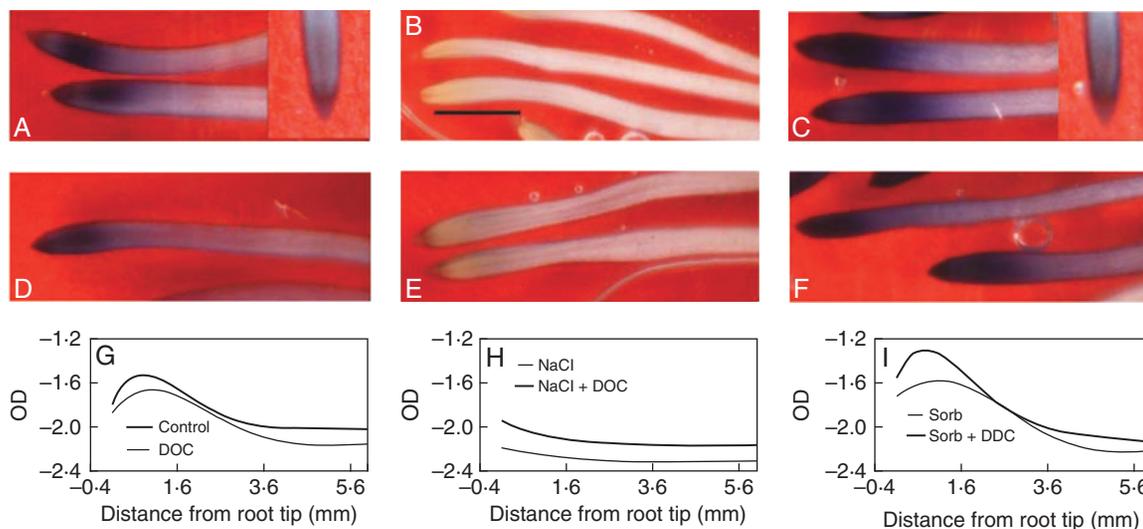


FIG. 2. Effect of a 3-h NaCl (150 mM) or sorbitol (300 mM) treatment on  $O_2^{\bullet-}$  levels in root tips. (A–F) NBT staining in those roots; (A, D, G) non-stressed controls; (B, E, H) NaCl treatment; (C, F, I) sorbitol treatment. (D–F) Roots treated with 1 mM DDC to inhibit SOD activity. The inset image in (A) is a control with 10 mM  $MnCl_2$ , and the inset in (C) is a root treated with sorbitol + 50  $\mu M$  DPI. Scale bar in (B) = 2 mm. (G–I) Densitometry measurements of stain intensity; curves are fitted with sixth-order polynomials on average measurements,  $n = 5$ .

(Fig. 4A: NaCl, 150 vs. 150 → 75; Fig. 4B: sorbitol, 300 vs. 300 → 150). Growth stimulation clearly depended on water uptake and was decreased by the aquaporin inhibitor  $HgCl_2$  (data not shown). The addition of DPI and of the  $O_2^{\bullet-}$  scavengers  $MnCl_2$  and Tiron reduced growth in these experiments, indicating that  $O_2^{\bullet-}$  production is required for

growth stimulation under these conditions. However, the addition of oxidative reagents such as  $H_2O_2$  or a Fenton reaction mixture to the NaCl step-down experiments also inhibited root elongation (these oxidative treatments were not applied to sorbitol-treated roots as ROS levels were already high in them). The Tiron concentration used in

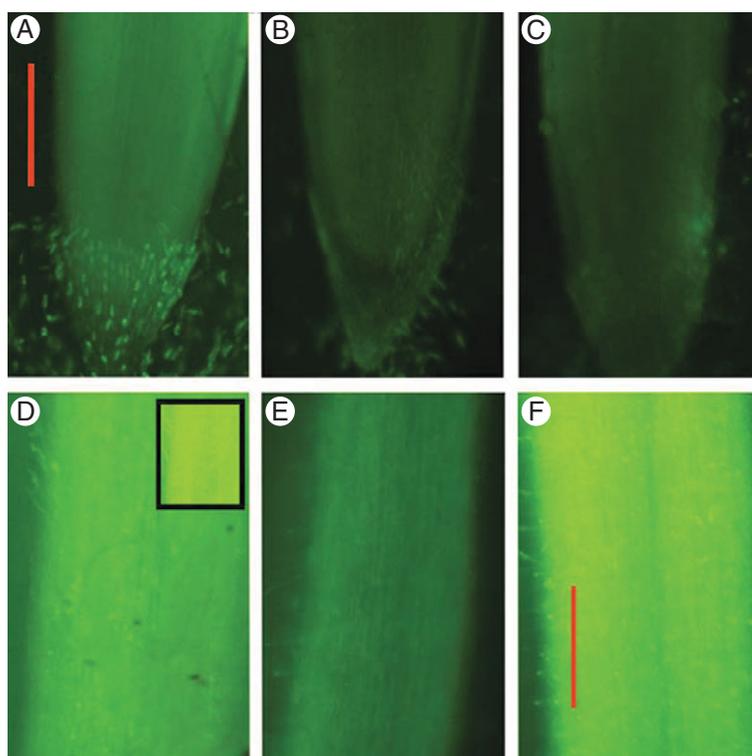


FIG. 3. Intracellular ROS detection by DCFH-DA fluorescence microscopy. (A, D) control; (B, E) 150 mM NaCl for 3 h; (C, F) 300 mM sorbitol treatment for 3 h. (A–C) Root tips, exposure time 1 s; (D–F) root hair region, exposure time 0.5 s; inset in (D) indicates fluorescence intensity in the same roots when exposure was 1 s. Scale bars = 1 mm.

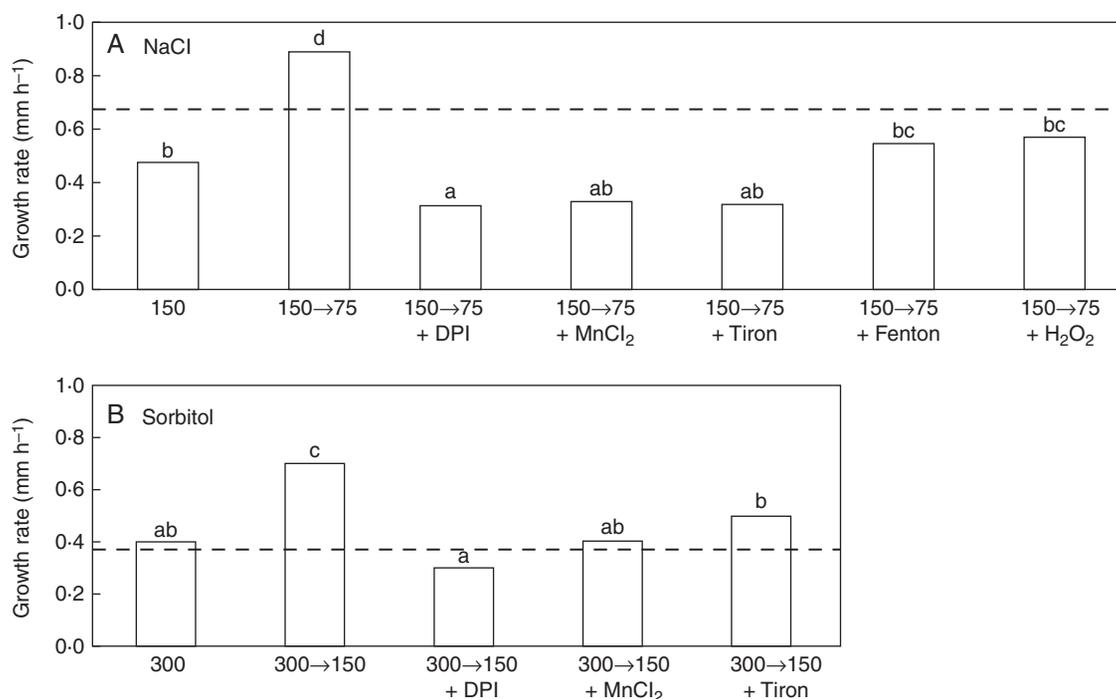


FIG. 4. Root elongation rates in stress-relief experiments. Plants were grown in Hoagland solution for 4 d and gradually treated with either 150 mM NaCl or 300 mM sorbitol. Average elongation rates (4 h) were measured on day 6, before and after transfer to a hypo-osmotic medium. The dashed lines across the figures indicate the mean elongation rate before transfer, the bars represent elongation rates after transfer; results are means of 34–119 roots. (A) Roots kept in 150 mM NaCl, or transferred to medium containing 75 mM NaCl (150 → 75) with the following additions as indicated: 50  $\mu$ M DPI, 10 mM MnCl<sub>2</sub>, 50 mM Tiron, a Fenton mixture (0.2 mM ascorbate + 0.2 mM CuCl<sub>2</sub> + 0.2 mM H<sub>2</sub>O<sub>2</sub>) or 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>. (B) Roots kept in 300 mM sorbitol, or changed to medium containing 150 mM sorbitol (300 → 150) with the following additions as indicated: 50  $\mu$ M DPI, 10 mM MnCl<sub>2</sub>, 50 mM Tiron. Different letters indicate significant differences among elongation rates after transfer within each stress treatment ( $P < 0.05$ ).

these experiments (assuming it did not ionize) would have added 50 mM to the 75 mM NaCl and the 150 mM sorbitol medium. In the NaCl treatment, it would have reduced the water potential gradient that it was intended to produce, but in the case of sorbitol the gradient would have still existed as the medium would be at 67 % of its original osmolarity.

Stimulated root elongation in the osmotic step-down experiments was due to increases in growth rates in the region beyond 1 mm from the tip, but these were statistically higher only between 2.5 and 3 mm from the tip (Fig. 5B). Increased  $O_2^{\bullet-}$  was detected in the growth region and beyond after reducing the NaCl concentration in the growth medium (Fig. 5A). In sorbitol-treated roots, NBT staining was also higher after transfer but it was not significantly different from the initial state, which was already high in these roots. Thus, independent of the initial oxidative status of the apoplast, the stimulation of root elongation growth by stress relief was accompanied by  $O_2^{\bullet-}$  production.

## DISCUSSION

### $O_2^{\bullet-}$ levels and root growth under stress

Cell elongation depends on the hydrostatic pressure, derived from water uptake in response to a water-potential gradient generated by solute accumulation, and the yielding properties of cell walls.  $OH^{\bullet}$ , derived from apoplastic

metabolism of  $O_2^{\bullet-}$  (Schopfer *et al.*, 2001), is involved in wall-polymer cleavage (Fry, 1998, 2004) and the control of ion- (Demidchik and Maathuis, 2007) and water-channel (Henzler *et al.*, 2004) activity. These functions, in part, provide the basis for the observation that  $O_2^{\bullet-}$  is required for the elongation of plant cells, and consequently participates in root elongation regulation. Evidence from various sources points to a role of ROS in root growth (Gapper and Dolan, 2006): *atrbohF* null mutants of *Arabidopsis* had reduced root elongation as compared to wild types (Kwak *et al.*, 2003); in maize roots, inhibitor studies have indicated that that  $OH^{\bullet}$  formation resulting from apoplastic  $O_2^{\bullet-}$  produced by NADPH oxidase activity is essential for normal root growth (Liszky *et al.*, 2004). In the current study, in non-stressed plants the distribution of accelerating growth and highest  $O_2^{\bullet-}$  levels coincided along the root tip, supporting the association between growth and ROS. Root elongation stimulation after NaCl stress relief also supports this idea, as increased  $O_2^{\bullet-}$  levels and stimulated growth coincided. However, salt and osmotic stress of the same intensity, which had similar inhibitory effects on root elongation, were not associated with consistent changes in apoplastic  $O_2^{\bullet-}$  levels. The alterations of the root growth profile by NaCl and sorbitol hyper-osmotic stress were observed beyond 3 mm from the tip, as has been reported for water-stressed roots by Sharp *et al.* (1988) and Fan and Neumann (2004).  $O_2^{\bullet-}$  levels, however, increased in sorbitol-treated roots and were decreased by NaCl

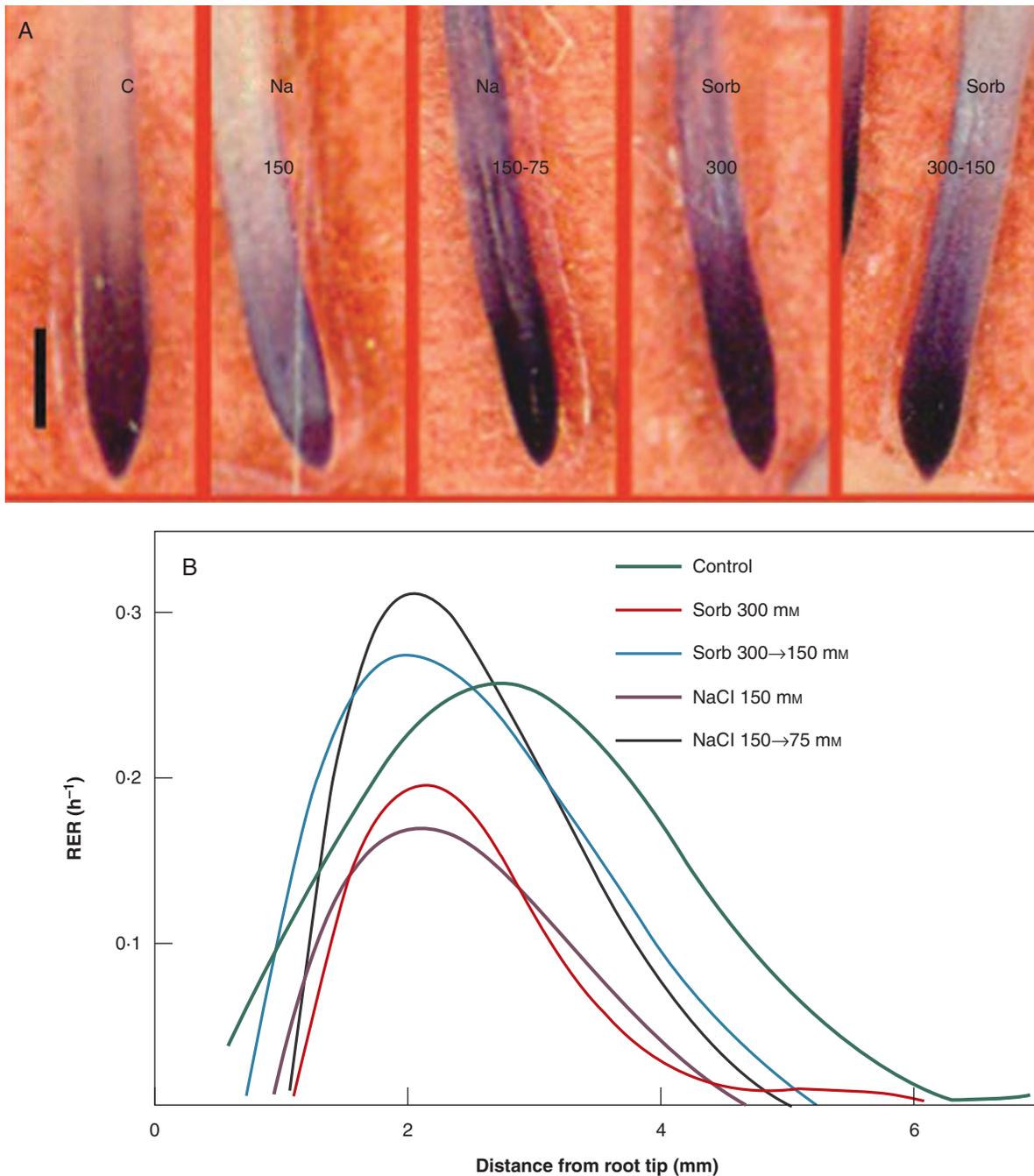


FIG. 5. (A) NBT staining and (B) segmental elongation rates in stress-relief experiments. Plants were grown in Hoagland solution for 4 d and gradually treated with either 150 mM NaCl or 300 mM sorbitol; elongation rates were measured on day 6, before and after transfer to a hypo-osmotic medium (from 150 to 75 mM NaCl, or from 300 to 150 mM sorbitol); C indicates roots from non-stressed plants. Scale bar = 1 mm. (B) Segmental elongation rates measured immediately before and during the period following stress relief, both for 3 h. RER for each segment is plotted against the initial position of the mark on the root closest to the apex. Data are from 4–8 roots from each treatment and were fitted with sixth- or seventh-order polynomials.

treatments. Furthermore, these effects were also observed closer to the tip, in the region where growth was essentially not altered by stress, highlighting that changes in other factors dominated the growth response to stress conditions.

In the case of water stress, it has been suggested that spatial differences in proton pumping into expanding cell walls (Fan and Neumann, 2004) and cell wall metabolism

(Wu *et al.*, 2001; Zhu *et al.*, 2007) contribute to the growth response of roots to this stress. Hardening of cell walls (Neumann *et al.*, 1994), but not reduced proton-pumping activity (Zidan *et al.*, 1990), have been associated with reduced maize root elongation under salinity. Growth stimulation after stress reduction implies that the cells involved had not yet experienced permanent changes in

wall stiffening. Wu *et al.* (1996) have shown higher abundance of expansin and susceptibility to expansin action in the apical 5-mm of roots grown at low compared with high water potential, in agreement with the suggestion that there is increased yielding ability of the cell walls in this region previously made by Spollen and Sharp (1991). In leaves of salt-treated *Chloris gayana* plants increased wall tightening and peroxidase activity were found in the growth-decelerating zone (Ortega *et al.*, 2006). Peroxidase activity has been detected beyond the zone of accelerated growth in maize roots (L. Ortega, IFFIVE-INTA, Argentina, pers. comm.) and it may be associated with the observed growth reductions.

While external supply of apoplastic ROS has been shown to stimulate growth in leaf segments of salt-treated plants (Rodríguez *et al.*, 2004), the same treatment was ineffective in roots, and prevented recovery after stress relief. Oxidative damage was not detected in salt-treated roots in these experiments, indicating that this level of salinity was not sufficient to produce oxidative stress; however, supplementary ROS may have produced it, and a differential sensitivity of root and leaf tissues may explain a different response to ROS. Increased oxidative damage has been documented in roots under several stress conditions (Porcel and Ruiz-Lozano, 2004; Sofo *et al.*, 2004; Schutzendubel and Polle, 2005; Singh *et al.*, 2007), including salinity (Shalata *et al.*, 2001; Katsuhara *et al.*, 2005), and antioxidants have been implicated in stimulated growth under stress (Córdoba-Pedregosa *et al.*, 2005), highlighting that in root tips ROS balance must be very tightly controlled.

#### *Differential effects of NaCl and sorbitol stress treatments on apoplastic $O_2^{\bullet-}$ levels*

$O_2^{\bullet-}$  abundance is the result of the balance of production and dismutation activity, each one subject to complex regulation (Mittler *et al.*, 2004). The dual role of ROS in growth stimulation and inhibition by oxidative damage is now well established, and the balance between stimulatory and inhibitory activities depends on the type of ROS and its concentration (Knight, 2007). NADPH oxidases, pH-dependent cell wall peroxidases, germin-like oxalate oxidases and amine oxidases have been proposed to generate ROS at the apoplast. ROS-scavenging mechanisms include enzymes such as superoxide dismutase, ascorbate peroxidase, catalase, glutathione peroxidase and peroxiredoxin, which together with the antioxidants ascorbic acid, glutathione and Mn provide alternative and convergent ways to control ROS concentration. In addition, membranes are protected by  $\alpha$ -tocopherol, which is kept in its reduced state by the pool of reduced ascorbic acid. Elements of these mechanisms are located in almost all plant organs (Blokina *et al.*, 2003) and cell compartments, including the apoplast (Pignocchi and Foyer, 2003), and their concerted action is responsible for the fine tuning of ROS-specific activities. Both increased (Arbona *et al.*, 2003; Pang *et al.*, 2005; Sekmen *et al.*, 2007) and reduced  $O_2^{\bullet-}$  dismutating activity under salinity has been reported (Hernández *et al.*, 2001; Aktas *et al.*, 2005). Drought and salinity had different effects on SOD activity when assayed in lupin leaves (Yu

and Rengel, 1999). If decreased  $O_2^{\bullet-}$  levels under salinity had resulted from increased SOD activity, DDC inhibition would be expected to increase NBT staining in the NaCl treatment, but not in sorbitol. Alternatively, if salt and sorbitol had exerted differential effects on  $O_2^{\bullet-}$  production (and not on SOD activity), DDC would not be expected to differentially increase NBT staining in either of the stress treatments, and the presence of  $H_2O_2$ , a product of  $O_2^{\bullet-}$  dismutation would have been expected to be higher in sorbitol than in NaCl. Our results are consistent with the second alternative. The inhibition of SOD activity increased staining in both treatments, suggesting that SOD activity was not differentially affected by the stress treatments we imposed. Increased SOD protein was detected in apoplastic fluid from the growth-accelerating zone of water-stressed maize roots (Zhu *et al.*, 2007), and the results from both sorbitol-treated and NaCl-treated roots also suggest increased SOD activity in that region. The metabolic transformation of  $O_2^{\bullet-}$  by SOD would have resulted in increased levels of  $OH^\bullet$  and  $H_2O_2$ . Verifying the level of those ROS along the root would also indicate whether alterations in growth profile by stress that do not produce oxidative damage are related to other ROS (Knight, 2007) and, in this context, whether the action of sorbitol as an effective  $OH^\bullet$  scavenger (Smirnoff and Cumbes, 1989) could also be related to reduced growth of roots under that treatment. This analysis would also shed light on the perplexing finding that growth was maintained in NaCl-stressed roots whilst apoplastic levels of  $O_2^{\bullet-}$  were drastically reduced.

Salt, but not osmotic stress, increases internalization of NADPH oxidase activity in *Arabidopsis* roots (Leshem *et al.*, 2007). Internalization would result in lowering apoplastic  $O_2^{\bullet-}$  while increasing internal ROS generation, as reported by Leshem *et al.* (2007). However, in our work increased DCFH-DA fluorescence in stressed roots was observed beyond the elongation zone, and only in sorbitol-treated roots. The differences between our results and those of Leshem *et al.* (2007) could perhaps be due to the different species studied and the duration of the treatments (3 h in our case). Direct effects of NaCl on plasma membrane NADPH oxidase activity, as suggested by Rodríguez *et al.* (2007), could provide an alternative explanation for those results.

Root growth rates appear to be specifically limited by the inner tissues of the stele, rather than by the epidermis (Pritchard, 1994; Fan *et al.*, 2006). However, in the growing part of maize root tips,  $O_2^{\bullet-}$  formation is greatest in the epidermis and vascular tissue (Liszky *et al.*, 2004). In this context, the lack of association between growth inhibition by salt and osmotic stress and epidermal apoplastic  $O_2^{\bullet-}$  levels may be interpreted as indicating that ROS produced in the apoplast of the epidermis of the expanding zone could participate in an early 'alert' system, conveying information on the substrate quality that the new growing root is exploring, a role that has been suggested for ROS in *Arabidopsis* root hairs under ion-deficiency conditions (Shin *et al.*, 2005). Cells closer to the tip, whose growth is apparently unaffected by stress, will then progress onto regions beyond it, where both osmotic and NaCl stress result in inhibited growth. The signals originating from the region closer to the tip

may convey specific information under various environmental conditions, and have an influence on the adaptation of the plant to them. Clues to the differential components of this system, for the case of salinity, may reside in the genes commonly regulated by both salt and sorbitol + DPI; such analysis is under currently being undertaken.

#### ACKNOWLEDGMENTS

ET, HRL, ES, DB and LV are fellows of CONICET (Consejo de Investigaciones Científicas y Técnicas de la República Argentina). Funding is gratefully acknowledged from Agencia Nacional de Promoción Científica y Técnica (PICT 00270/2 to ET); Instituto Nacional de Tecnología Agropecuaria (AEEV1513 to ET) and Fundación Antorchas.

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