Journal of Experimental Botany, Vol. 55, No. 401, pp. 1383–1390, June 2004
DOI: 10.1093/jxb/erh148 Advance Access publication 21 May, 2004

Journal of Experimental Botany

RESEARCH PAPER

Decreased reactive oxygen species concentration in the elongation zone contributes to the reduction in maize leaf growth under salinity

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Received 27 November 2003; Accepted 5 March 2004

Abstract

Reactive oxygen species (ROS) in the apoplast of cells in the growing zone of grass leaves are required for elongation growth. This work evaluates whether salinity-induced reductions in leaf elongation are related to altered ROS production. Studies were performed in actively growing segments (SEZ) obtained from leaf three of 14-d-old maize (*Zea mays* L.) seedlings gradually salinized to 150 mM NaCl. Salinity reduced elongation rates and the length of the leaf growth zone. When SEZ obtained from the elongation zone of salinized plants (SEZ_s) were incubated in 100 mM NaCl, the concentration where growth inhibition was approximately 50%, $O_2^{\bullet-}$ production, measured as NBT formazan staining, was lower in these than in similar segments obtained from control plants. The NaCl effect was salt-specific, and not osmotic, as incubation in 200 mM sorbitol did not reduce formazan staining intensity. SEZ_s elongation rates were higher in 200 mM sorbitol than in 100 mM NaCl, but the difference could be cancelled by scavenging or inhibiting $O_2^{\bullet-}$ production with 10 mM $MgCl_2$ or 200 μM diphenylene iodonium, respectively. The actual ROS believed to stimulate growth is $^{\bullet}OH$, a product of $O_2^{\bullet-}$ metabolism in the apoplast. SEZ_s elongation in 100 mM NaCl was stimulated by a $^{\bullet}OH$-generating medium. Fusicoccin, an ATPase stimulant, and acetate buffer pH 4, could also enhance elongation in these segments, although both failed to increase ROS activity. These results show that decreased ROS production contributes to the salinity-associated reduction in grass leaf elongation, acting through a mechanism not associated with pH changes.

Key words: Leaf elongation, maize, monocot growth, reactive oxygen species, salt stress.

Introduction

Reactive oxygen species (ROS) participate in development, hormone action, and responses to biotic and abiotic stresses (Alvarez *et al*., 1998; Mittler, 2002). While oxidative damage, a 'negative' consequence of ROS presence, is a common response to many environmental stresses and has led to extensive studies of the antioxidant system (Mittler, 2002), more recently, growing evidence supports a 'positive' role for ROS in signalling events that regulate ion channel activity (Foreman *et al*., 2003) and gene expression (Neill *et al*., 2002).

In plant cells, ROS, mainly H_2O_2, superoxide anion ($O_2^{\bullet-}$), and hydroxyl radical ($^{\bullet}OH$) are generated in the cytosol, chloroplasts, mitochondria, and the apoplastic space (Bowler and Fluhr, 2000; Mittler, 2002). ROS produced in the apoplast of cells in the growing zone of maize leaf blades are required for elongation growth (Rodríguez *et al*., 2002). Apoplastic ROS can participate in the regulation of cell expansion through a modulation of Ca^{2+} channel activity (Demidchik *et al*., 2003; Foreman *et al*., 2003). Apoplastic ROS can also regulate growth by affecting the rheological properties of cell walls (Cosgrove, 1999), contributing both to wall loosening (Miller, 1986; Fry, 1998; Schweikert *et al*., 2000; Schopfer, 2001) and stiffening (Ogawa *et al*., 1997; Schopfer, 1996).

Salinity is a common constraint to agricultural productivity (Rains, 1991), and the reduction of leaf expansion is

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Abbreviations: ROS, reactive oxygen species; SEZ, segments from the leaf elongation zone; SEZ_c or SEZ_s, SEZ from non-salinized or salinized plants, respectively; DPI, diphenylene iodonium; FC, fusicoccin; *REGR*, relative elongation growth rate; NBT, nitro blue tetrazolium; XTT, Na, 3′-[1-[(phenylamino)-carbonyl]-3, 4-tetrazolium](4-methoxy-6-nitro) benzene sulphonic acid hydrate; SOD, superoxide dismutase.

one of the primary effects of salt stress (Munns and Termaat, 1986). In grasses, stress conditions that decrease leaf elongation rates affect the blade elongation zone, located at the base of the growing leaf blade (Langer, 1979), reducing the length of the zone and elemental growth rates within it (Bernstein *et al*., 1993; Neves-Piestun and Bernstein, 2001; Ortega and Taleisnik, 2003; Thomas *et al*., 1999). Increased ROS formation was observed after exposure of *Arabidopsis* roots to high salt (Demidchik *et al*., 2003), and apoplastic increases in $O_2^{\bullet-}$ were reported in pea plants under salt stress (Hernández *et al*., 2001). Cations, both divalent and monovalent, induced $O_2^{\bullet-}$ production in tobacco cell suspension cultures through the activity of NADPH oxidase (Kawano *et al*., 2001), and a major portion of the $O_2^{\bullet-}$ was released extracellularly. If changes in ROS concentration also occur in the leaf expansion zone in response to saline treatments, it is expected they would contribute to regulating leaf elongation under these conditions. Such a possibility has not yet been experimentally tested. In this work it was examined whether salinity affects ROS production in the leaf elongation zone and if this action contributes to regulating leaf elongation under this condition.

Materials and methods

Plant material

Seeds of maize (*Zea mays* cv. Prozea 30, Produsem, Pergamino, Argentina) were sown on moist vermiculite contained in plastic net frames placed over 4.5 l black plastic trays containing aerated water. Trays were kept at 25 °C under a light panel of fluorescent and incandescent lights providing 95 µmol photons m^{-2} s^{-1} illumination, with a 12 h photoperiod. When the second leaf emerged, 6 d after sowing, the water was changed to one of half-strength Hoagland solution (Hoagland and Arnon, 1950), which included 25 mM NaCl in the saline treatment. This solution was changed daily, increasing the NaCl concentration from 25 mM to 50 mM, then to 100 mM, and then to 150 mM NaCl. Solutions were then replaced every 2 d, until day 14 when plants were processed.

Growth measurements and Na^+ content

The third leaf, used as the experimental system, began to appear above the sheath whorl when plants were at 100 mM NaCl, and was at least 10 cm long on day 14. At this stage the sheath was about 2 mm long, thus its contribution to whole-leaf growth was negligible. Relative elongation growth rates (*REGR*s) within the blade expansion zone were calculated from the displacement of pinpricks in a 24 h interval according to Schnyder *et al*. (1987), as previously described for this system (Rodríguez *et al*., 2002). Briefly, the basal zone of the seedling was pricked through the sheath whorl with needles placed 5 mm apart, and, after 24 h, the sheath whorl was opened and the third leaf exposed and examined under a stereoscopic microscope to determine the distance between marks. Segmental growth was quantified as a relative growth rate (Hunt, 1978), termed relative elemental growth rate, $REGR=(\ln L_f - \ln L_i)(\Delta t)^{-1}$ where L_i and L_f denote the initial and final segment length, respectively, and Δt stands for the duration of the experiment.

REGR was plotted versus average segment midpoint position (Peters and Bernstein, 1997) to yield profiles of relative elemental elongation rates. Pricking reduced the velocity of leaf elongation by 50% in control plants, but not in salt-treated plants. The data shown are for pricked plants.

Elongation in isolated segments from leaf three was measured as described in detail previously (Rodríguez *et al*., 2002). Segments (SEZ), comprising the region from 10–20 mm from the leaf base (ligule), were gently vacuum infiltrated for 1.5 min and incubated for 3 h in various treatment solutions. Digital images of each segment were obtained before and after the incubation period, by means of a scanner (AGFA Snapscan Touch, Agfa-Gevaert Group, Morstel, Belgium), and length measurements were obtained with an image processing software (Optimas 6.1, Optimas Corporation, Bothell, WA). Growth was expressed as percentage length increase in that period. In some cases, images were also obtained at regular intervals (10 min) within that period to calculate growth kinetics.

To determine Na^+ concentration, plant samples were weighed (fresh and dry weight), boiled in distilled water and solution aliquots used for ion content determination by flame photometry (digital flame analyser 2655-00, Cole-Parmer Instrument Co., Chicago, Illinois).

Detection of localized accumulation of $O_2^{\bullet-}$ in leaf segments

$O_2^{\bullet-}$ accumulation in tissues was determined with nitro blue tetrazolium (NBT), which reacts with $O_2^{\bullet-}$, producing a blue formazan precipitate. Control reactions included 10 mM $MnCl_2$, a highly effective $O_2^{\bullet-}$ dismutating catalyst agent (Hernández *et al*., 2001). Segments were gently infiltrated (1.5 min) with a 0.01% NBT solution in water or other solutions (100 mM NaCl or 200 mM sorbitol), depending on the experiment, and incubated in the dark in the same solution, for 2 h at 30 °C, with very slow shaking. Stained segments were mounted on a glass slide, and scanned. Colour images were first inverted to obtain a negative, transformed to black and white 8-bit images, and formazan colour intensity (in the negative corresponding to the lighter tones of grey), determined by an image processing software (Image J 1.29 Wayne Rasband, National Institutes of Health, USA).

Determination of $O_2^{\bullet-}$ release

$O_2^{\bullet-}$ release to the medium was determined spectrophotometrically, using Na, 3′-[1-[(phenylamino)-carbonyl]-3, 4-tetrazolium](4-methoxy-6-nitro) benzene sulphonic acid hydrate [XTT (Frahry and Schopfer, 2001)]. Groups of 10 SEZ were gently infiltrated and incubated 2 h in 1.5 ml of aqueous solution containing 500 mM XTT, with/without 100 mM NaCl, with/without 3.5 units ml^{-1} superoxide dismutase (SOD). Aliquots were read at 470 nm in a spectrophotometer (Beckman DU Series 600, Beckman Instruments, Fullerton, CA). Specific absorbance due to the presence of $O_2^{\bullet-}$ was calculated as the difference in A_{470} between samples without and with SOD.

Verification of the effect of NaCl on the formazan formation reaction

The effect of NaCl on the formazan reaction was determined *in vitro*. $O_2^{\bullet-}$ was generated by wounding SEZ in an aqueous medium. The medium was immediately divided into two fractions, one of which was salinized with 100 mM NaCl, and 0.01% NBT was added to both fractions. A_{560} was measured after 30 min incubation at 30 °C in the dark. Alternatively, $O_2^{\bullet-}$ was generated by illuminating a riboflavin solution in the presence or absence of 100 mM NaCl.

Statistical analysis

Data were analysed by one-way or two-way ANOVA and DGC tests (Di Rienzo *et al*., 2002), using InfoStat (InfoStat 2002. Grupo InfoStat. Facultad de Ciencias Agropecuarias. Universidad Nacional de Córdoba. Ver. 1.1. Córdoba, Argentina)

Results and discussion

Experimental system

In monocots, leaf growth reflects events taking place at the elongation zone located at the base of the leaf, and the analysis of the spatial distribution of growth can provide initial information on the effects of stress. In this zone, the distribution of *REGR*s exhibits a typical tailed bell shape (Bernstein *et al*., 1993; Volenec and Nelson, 1981), which can be altered by stress in various ways: growth rates may be generally decreased, maximum growth rates may be reduced or shifted proximally, and the elongation zone may be shortened (Fricke, 2002).

In maize leaf three from control plants, highest *REGR*s were registered between 10 and 25 mm from the ligule (Fig. 1). Salinity (150 mM NaCl) reduced the highest *REGR* values, similar to the effects reported on leaf three of salt-stressed barley (Fricke and Peters, 2002). Significant differences in *REGR* between control and salinized plants were observed at and beyond 10 mm from the ligule, and the growth zone was shorter in salt-treated plants.

Segments spanning from 10–20 mm from the ligule were chosen for the following experiments, aimed at evaluating the relationship between salinity, ROS production, and elongation growth. Such segments were termed SEZ, and it had been previously shown that SEZ obtained from non-salinized plants and incubated in aqueous solutions reproduced the ROS production pattern of intact leaves (Rodríguez *et al*., 2002).

NaCl effects on ROS production by segments from the leaf elongation zone (SEZ)

To evaluate ROS production in segments obtained from salt-treated plants (SEZ_s), the incubation medium contained 100 mM NaCl, a salt concentration where growth was approximately 50% of that achieved by segments from control plants (SEZ_c) incubated in water (Fig. 2), and similar to the average *REGR* reduction in homologous segments from intact leaves, caused by providing 150 mM NaCl to the growth medium (Fig. 1). When SEZ_s were incubated in media with higher water potential, elongation was either greatly stimulated (in water) or less inhibited, as in 50 mM NaCl.

In non-salinized maize plants, the presence of apoplastic $O_2^{\bullet-}$ was detected in cells from the blade elongation zone (Rodríguez *et al*., 2002). In the current experiments, $O_2^{\bullet-}$ presence was determined by nitro blue tetrazolium (NBT) formazan staining.

SEZ_c, incubated for 2 h in water containing 0.01% NBT showed intense formazan staining (Fig. 3A), which was inhibited by adding $MnCl_2$, a $O_2^{\bullet-}$ dismutating catalyst agent (Hernández *et al*., 2001). When either SEZ_c or SEZ_s were incubated in 100 mM NaCl, much lower formazan deposition was observed. Various salts of Na^+ or Cl^- ($NaNO_2$, $Na_2(SO_4)$, and KCl), in 100 mM concentration of the ions in question, exerted similar inhibitory effects on formazan deposition (Fig. 4), thus, both ions seemed to be capable of affecting $O_2^{\bullet-}$ production. Incubation in phosphate salts at the same osmotic potential also exerted a similar effect.

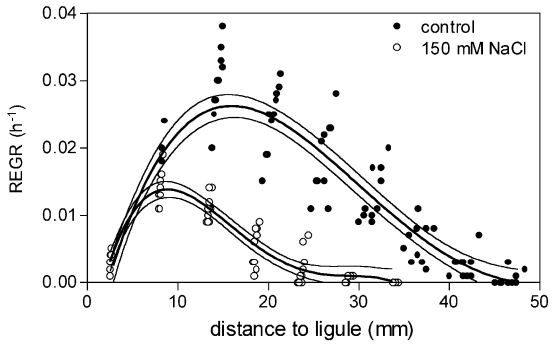

Fig. 1. Effect of salinity on the distribution of *REGR* along the elongation zone in maize leaf three. Plants were 14-d-old and had been gradually salinized to 150 mM NaCl and processed for 3–5 d after reaching the final salinity level. Each point is segmental *REGR* versus average segment midpoint position. Curves are fourth order polynomials with 95% confidence intervals.

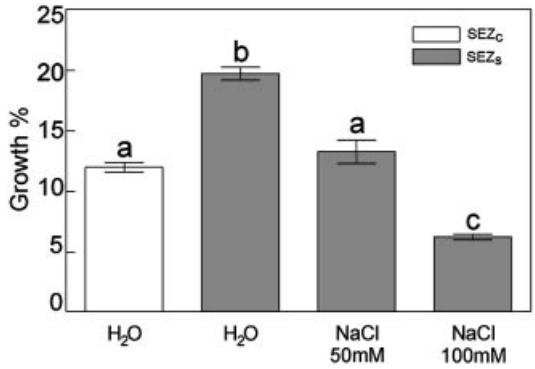

Fig. 2. Growth of leaf segments in media with various NaCl concentrations. Segments were obtained from non-salinized (SEZ_c, white bar) or salinized plants (SEZ_s, grey bars). Percentage length increase during a 3 h incubation period was measured. Bars represent the average ±SE of 20 samples. Bars with different letters are significantly different (P <0.05). Absolute growth rate for SEZ_c was 0.402 ± 0.011 mm h^{-1}.

To rule out possible effects of NaCl on formazan formation, the reaction was checked *in vitro*. Leaf segments were wounded in water to induce $O_2^{\bullet-}$ production (Orozco-Cárdenas *et al*., 2001), and aliquots were immediately assayed for NBT formazan formation in the presence or absence of 100 mM NaCl. A_{560} values were 0.0459 and 0.0406 respectively, and not significantly different (t-test, P=0.39, n=5), in accordance with the common practice of including NaCl in reaction mixtures leading to formazan formation (see, for example, Lopukhina *et al*., 2001). Similar effects were obtained when $O_2^{\bullet-}$ was generated *in vitro* by illuminating a

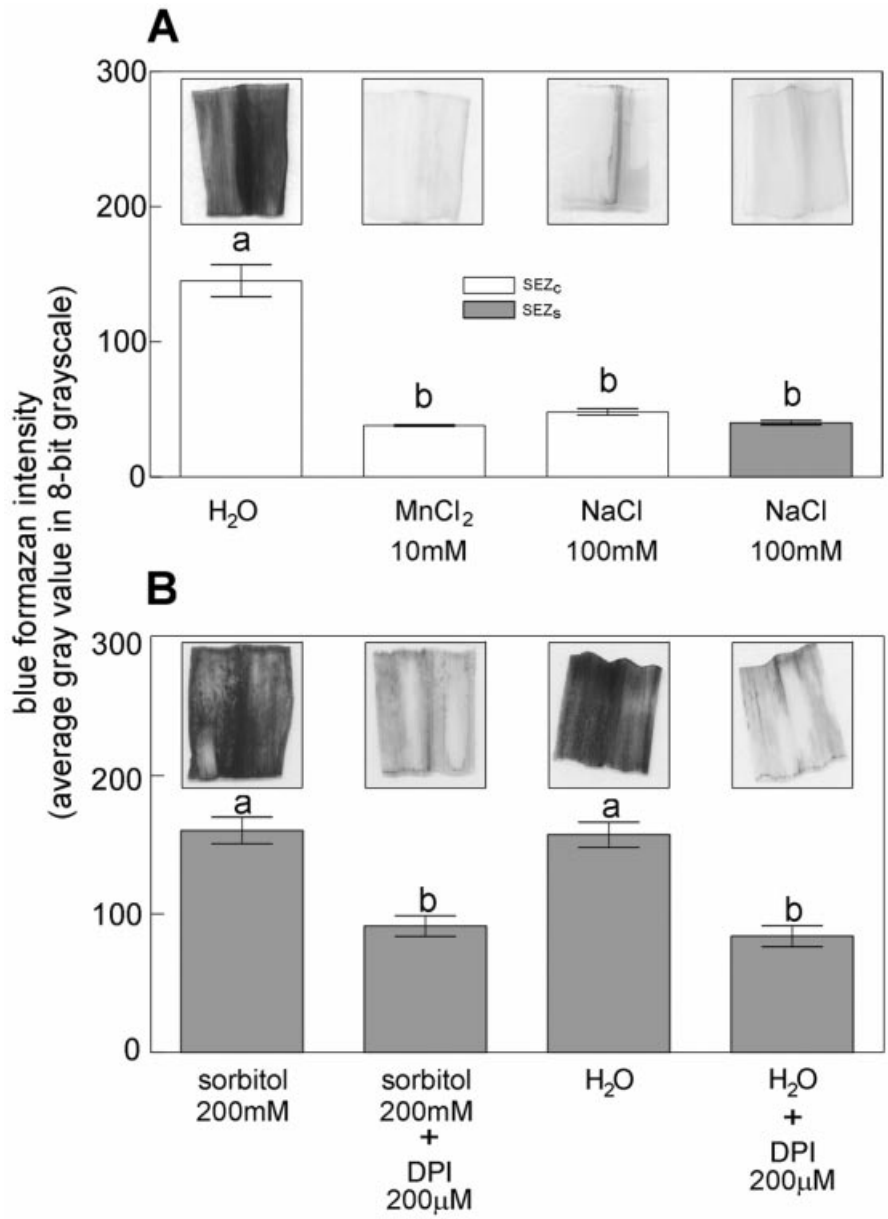

Fig. 3. $O_2^{\bullet-}$ concentration in SEZ, as determined from blue formazan staining. Segments obtained from non-salinized (SEZ_c, white bar) or salinized plants (SEZ_s, grey bars), were incubated for 2 h in various media containing 0.01% NBT. Colour images (insets) were inverted to obtain negatives, and transformed to black and white 8-bit images, where the average grey value in an 8-bit greyscale indicates blue formazan intensity. Bars represent average ±SE of 10 samples. Bars with different letters are significantly different (P <0.05).

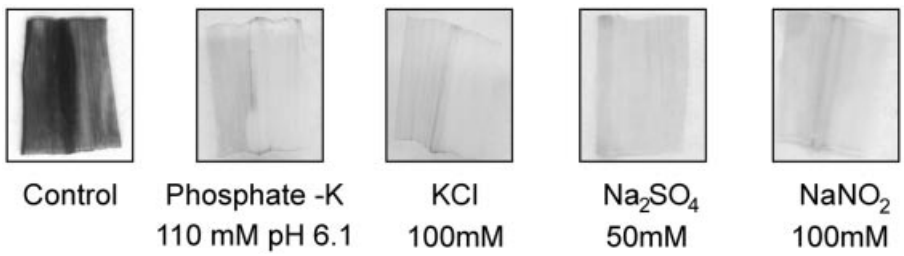

Fig. 4. Effects of various salts on blue formazan staining. Segments were obtained from the leaf elongation zone of non-salinized plants, and were incubated in the solutions indicated in the figure (controls in water), all containing 0.01% NBT.

riboflavin solution in the presence or absence of NaCl (data not shown). Further evidence that $O_2^{\bullet-}$ production could be visualized under saline conditions was provided by wounding: when SEZ_s were firmly pressed with forceps, and incubated in a salinized NBT solution, formazan deposition could be distinctively seen in the wounded areas (Fig. 5). Therefore, the inhibitory effect of NaCl on formazan staining in the elongation zone of intact leaves, was due to salinity-associated changes in $O_2^{\bullet-}$ concentration and not to an artefact associated with formazan generation.

The inhibitory effect of NaCl on formazan staining described above could have resulted from osmotic or salt-specific effects. To distinguish between these alternatives, formazan staining was compared in SEZ_s incubated in 100 mM NaCl and in 200 mM sorbitol, a solution iso-osmotic with the former. SEZ_s incubated in 200 mM sorbitol and 0.01% NBT had intense staining (Fig. 3B), indicating that osmotic effects failed to reduce formazan deposition, and suggesting that the ionic, and not the osmotic component of NaCl was inhibiting $O_2^{\bullet-}$ formation. Intense staining was also observed when SEZ_s were incubated in water. Since whole tissue SEZ_s Na^+ concentration (183 mM on a water basis) decreased after segments were incubated for 2 h in water or sorbitol (to 54 and 140 mM, respectively), a decrease expected to result mainly from the washout of the apoplastic free space (Lüttge and Higinbotham, 1979), those results may indicate that infiltration and incubation with either water or sorbitol decreased apoplastic NaCl concentration, and, thus, reverted the inhibition on $O_2^{\bullet-}$ production exerted by the salt.

The addition of DPI was effective for reducing formazan staining in SEZ_s incubated in sorbitol or water (Fig. 3B), indicating that the source of $O_2^{\bullet-}$ was affected. $O_2^{\bullet-}$ released by the segments to the incubation medium was measured by Na, 3′-[1-[(phenylamino)-carbonyl]-3, 4-tetrazolium](4-methoxy-6-nitro) benzene sulphonic acid hydrate (XTT). A_{470} values were much lower in the presence of NaCl after 2 h incubation (Table 1). Since $O_2^{\bullet-}$ is not readily transported across membranes, XTT formazan in the incubation medium could have been formed only from apoplastic $O_2^{\bullet-}$. The results from those measurements support the idea that salinity exerted a negative effect on apoplastic $O_2^{\bullet-}$ concentration.

Together, these results indicate incubation in NaCl decreased apoplastic $O_2^{\bullet-}$ concentration, and treatments that reduced Na^+ concentration, restored it, possibly by exerting effects from the apoplastic side.

The salt-specific reduction of apoplastic $O_2^{\bullet-}$ concentration by NaCl contrasts with other recent reports of increased $O_2^{\bullet-}$ production in response to saline stress, where $O_2^{\bullet-}$ production was measured shortly (minutes) after subjecting plants (Demidchik *et al*., 2003) or suspension cultures (Kawano *et al*., 2001) to ion shocks.

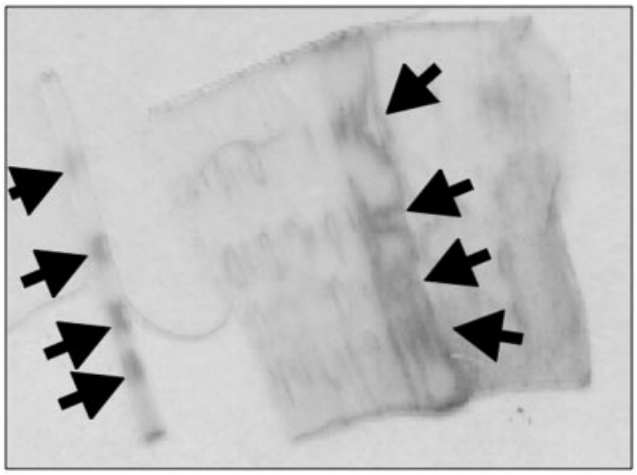

Fig. 5. Formazan deposition in wounded areas of leaf segments. Segments obtained from the leaf elongation zone of salinized plants (SEZ_s) were pressed with forceps (arrows indicate the pressed points) and incubated in 100 mM NaCl containing 0.01% NBT. A folded segment is shown on the left, and an unfolded one on the right.

The $O_2^{\bullet-}$ production peak lasted less than 1 min in suspension cultures (Kawano *et al*., 2001), possibly suggesting that it is transient, while the observations from this study reflect steady-state conditions. Hernández *et al*. (2001), reported increased apoplastic $O_2^{\bullet-}$ concentration under a steady-state salinity condition, but in expanded leaf blades, and in relation to the appearance of necrotic lesions, while changes are reported here in tissues that are actively expanding and not senescent.

Changes caused by salinity in apoplastic $O_2^{\bullet-}$ concentration in the growing zone of leaf blades could have resulted from either an inhibition of ROS-generating mechanisms, or from the activation of antioxidant defence elements (Hernández *et al*., 2001) or both, and such alternatives must be tested with further experiments. Preliminary results from SOD activity measurements in apoplastic fluid do not indicate increased activation of this enzyme in salinized plants.

Relation of ROS to elongation growth under salinity

Whether the change in ROS concentration under salinity was relevant for modulating elongation under that condition was assessed next. Incubation in 200 mM sorbitol, iso-osmotic with 100 mM NaCl, was less inhibitory to SEZ_s elongation than the salt (Fig. 6). Since $O_2^{\bullet-}$ content in SEZ_s was higher in 200 mM sorbitol than in 100 mM NaCl (Fig. 3B), it was asked whether that relative elongation stimulation was ROS-dependent. The addition of either 200 μM DPI or 10 mM $MnCl_2$ to the sorbitol incubation medium (effective for reducing $O_2^{\bullet-}$ content, Fig. 3B), inhibited elongation by approximately 50% (Fig. 6). Therefore, in sorbitol-incubated SEZ_s, where both growth and $O_2^{\bullet-}$ content were higher than in 100 mM NaCl,

Table 1. *Formazan production from XTT by SEZ from control or salinized plants*

SEZ were incubated for 2 h in the absence or presence of 100 units ml^{-1} SOD (means ±SE, *n*=4).

SEZ and incubation medium	A_{470}	ΔA_{470} ($O_2^{\bullet-}$ specific formazan absorbance)
Control		
Water	0.069±0.007	
Water +SOD	0.013±0.006	0.057±0.007
NaCl 100 mM	0.030±0.006	
Salinized		
NaCl 100 mM+SOD	0.027±0.006	0.006±0.005

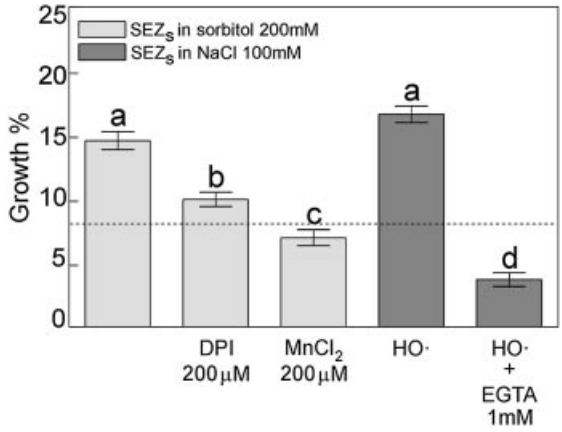

Fig. 6. Elongation of maize SEZ_s in media that modify apoplastic ROS concentration. SEZ_s were incubated for 3 h in 100 mM NaCl (dark bars) or 200 mM sorbitol (light bars), with the additions shown in the figure, and the percentage length increase in that period was measured. For comparison purposes, average SEZ_s elongation in 100 mM NaCl (without additions) for this experiment is depicted by the dashed line across the figure, the absolute growth rate was 0.221±0.008 mm h^{-1}. Bars represent the average ±SE of 20 samples. Bars with different letters are significantly different (P <0.05).

reducing $O_2^{\bullet-}$ content reduced elongation rates. As expected, 200 μM DPI had no effect on growth of SEZ_s incubated in 100 mM NaCl (results not shown).

Conversely, growth in SEZ_s could be stimulated by supplying apoplastic ROS. The actual ROS exerting the growth-stimulating action is believed to be •OH (Foreman *et al*., 2003; Schopfer, 2001). •OH can be generated in the apoplast in the presence of H_2O_2, ascorbate, and Cu^{2+} or Fe^{2+} (Fry *et al*., 2001; Halliwell and Gutteridge, 1990), in a reaction requiring, first, the generation of $O_2^{\bullet-}$, which is then dismutated to H_2O_2 by apoplastic superoxide dismutase (Ogawa *et al*., 1997). When SEZ_s were incubated in a solution of 100 mM NaCl containing 0.2 mM ascorbate, 0.2 mM $CuCl_2$ and 0.2 mM H_2O_2, elongation was higher than in NaCl alone, and similar to the level attained in 200 mM sorbitol (Fig. 6). The kinetics of SEZ_s elongation in that medium was very similar to that of SEZ_c in water. Thus, adding •OH to the SEZ_s saline incubation medium, reverted the inhibition in elongation, providing support to the idea that salinity-decreased ROS production negatively affected elongation, and that the growth-stimulating action of ROS is •OH-dependent.

It has been demonstrated that ROS can regulate cell expansion through the activation of Ca^{2+} channels (Foreman *et al*., 2003). Exposure of *Arabidopsis* roots to NaCl caused a rapid reduction of cytosolic Ca^{2+} concentration (Cramer and Jones, 1996), and a strong positive correlation was found between Ca^{2+} concentration and root elongation rates. Thus, it was asked whether the observed ROS effects on SEZ_s elongation were also mediated by Ca^{2+}. The Ca^{2+} chelating agent, ethylene glycol *bis* (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), added to the salinized ·OH-generating medium, in a concentration of 1 mM, completely reversed the growth stimulation (Fig. 6), and depressed elongation to values even lower than those attained with 100 mM NaCl. This result, while acknowledging that EGTA may affect membrane permeability by removing Ca^{2+}, suggests that the stimulating action of ROS on SEZ_s elongation requires an external source of Ca^{2+}. This ion is part of signal transduction chains and it is also involved in the modulation of actin dynamics and exocytosis, but the relationship between cytosolic Ca^{2+} and altered growth rates is not completely understood (Bush, 1995).

SEZ_s elongation could also be stimulated by fusicoccin (FC) (Neves-Piestun and Bernstein, 2001), an enhancer of plasmalemma H^+-ATPase activity (Marré, 1979). The effect of FC on SEZ_s elongation is accompanied by the lowering of apoplastic pH (Neves-Piestun and Bernstein, 2001). Both low pH and FC stimulated elongation in SEZ_s incubated in 100 mM NaCl (Fig. 7A), although both treatments failed to increase ROS activity (Fig. 7B). It has been shown that pH-dependent elongation cannot be reduced by adding ROS scavengers to the incubation medium (Schopfer, 2001), which is consistent with the idea that ROS and pH activate elongation through different mechanisms. Elongation stimulated by FC could only be partially inhibited by including EGTA in the incubation medium (Fig. 7A). The partial reversion by EGTA of FC-stimulated elongation indicates that (a) FC is also stimulating elongation through a Ca^{2+}-related mechanism, but (b) that the effect of FC is not entirely dependent on external Ca^{2+}, in accordance with the report that FC does not stimulate Ca^{2+} uptake in plasmalemma vesicles (Zocchi and Rabotti, 1993).

Taken together, these results indicate that ROS play a role in the regulation of leaf elongation under salinity, possibly by a Ca^{2+}-requiring mechanism, however, they also show that ROS are not necessarily a common feature in all mechanisms that participate in the regulation of elongation under such conditions, since low pH stimulation of elongation was not accompanied by higher ROS production.

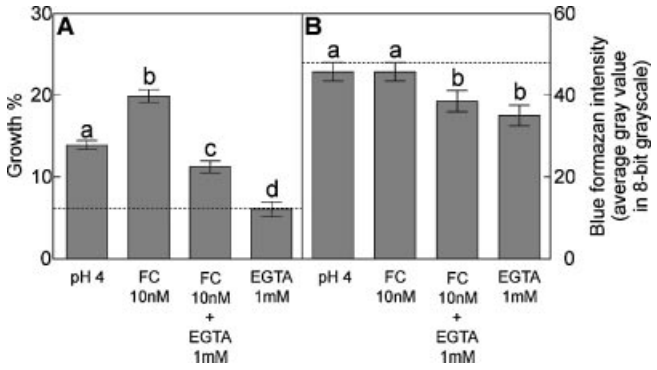

Fig. 7. Effect of fusicoccin (FC) on elongation and $O_2^{\bullet-}$ concentration in maize leaf segments from salinized plants. (A) SEZ_s were incubated for 3 h in 100 mM NaCl dissolved water (pH 6.5) with the additions shown in the figure, and the percentage length increase during in that period was measured. Growth in 30 mM acetate buffer pH 4 containing 100 mM NaCl is also shown. For comparison purposes, average SEZ_s elongation in 100 mM NaCl (without additions) is depicted by the dashed line across the figure. Bars represent the average ±SE of 20 samples. (B) Blue formazan intensity in the same segments, incubated for 2 h in various media containing 0.01% NBT. Blue formazan intensity was determined as explained in Fig. 5. Mean colour intensity of segments incubated in 100 mM NaCl without additions is depicted by the dashed line across the figure. Bars represent average ±SE of 10 samples. Bars with different letters are significantly different (P <0.05).

Salinity and ROS modulation of leaf elongation

This paper shows that leaf elongation can be stimulated by ROS under saline conditions. Apoplastic ROS have been implied in the control of growth through effects on cell wall properties (Fry *et al*., 2001; Fry, 1998) and ion balance (Demidchik *et al*., 2002). Salinity was shown to inhibit root hair elongation via alterations in the tip-focused Ca^{2+} gradient that regulates root hair growth (Halperin *et al*., 2003), and by a reduction in cytosolic Ca^{2+} in *Arabidopsis* roots (Cramer and Jones, 1996). Similar mechanisms are likely to operate in the leaf expansion zone mediated by ROS. ROS in the apoplast may be generated by the activity of several sources such as plasmalemma NAD(P)H oxidases, apoplastic peroxidases, amine and oxalate oxidases (Bolwell and Wojtaszek, 1997). The experiments of this study indicate that $O_2^{\bullet-}$ levels in the apoplast change in response to salinity, thus, the participation of NAD(P)H oxidase, an enzyme complex generating superoxide in the apoplast, may be expected. However, since the DPI concentration used in this case was high enough to inhibit both NAD(P)H oxidase and peroxidases (Bolwell *et al*., 1998), the elucidation of this point must await results from experiments with isolated membranes, which are currently underway.

Salinity may restrict leaf elongation growth by affecting rates of water or osmolytes uptake, turgor generation, and wall properties. Many of these effects are common consequences to both osmotic and ionic stress. Toxic salinity effects may also result from the build-up of excessive ion concentration and from ion imbalance, which, in turn may affect signalling chains. These are salt-specific effects, and the effects of salinity on apoplastic ROS concentration are of this kind. In an evaluation of the expression levels for 127 proteins in different tissues of rice plants subject to phytohormone, biotic and abiotic stress treatments, Cooper *et al*. (2003) showed that, although for many genes expression changed similarly in response to the treatments, in a few cases, salt and osmotic stress resulted in significantly different levels of expression.

Acknowledgements

The authors thank Dr Samuel Taleisnik for critically reading the manuscript and the technical assistance of Paola Suarez is gratefully acknowledged. This work was supported by the Agencia de Promoción Científica y Tecnológica of Argentina (FONCYT grant 6869) and Fundación Antorchas, and it is part of AAR's doctorate.

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