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RESEARCH PAPER

Polyamine oxidase activity contributes to sustain maize leaf elongation under saline stress

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Abstract

The possible involvement of apoplastic reactive oxygen species produced by the oxidation of free polyamines in the leaf growth of salinized maize has been studied here. Salt treatment increased the apoplastic spermine and spermidine levels, mainly in the leaf blade elongation zone. The total activity of polyamine oxidase was up to 20-fold higher than that of the copper-containing amine oxidase. Measurements of H_2O_2, $\cdot O_2^-$, and HO· production in the presence or absence of the polyamine oxidase inhibitors 1,19-*bis*-(ethylamine)-5,10,15 triazanonadecane and 1,8-diamino-octane suggest that, in salinized plants, the oxidation of free apoplastic polyamines by polyamine oxidase by would be the main source of reactive oxygen species in the elongation zone of maize leaf blades. This effect is probably due to increased substrate availability. Incubation with 200 μM spermine doubled segment elongation, whereas the addition of 1,19-*bis*-(ethylamine)-5,10,15 triazanonadecane and 1,8-diamino-octane to 200 μM spermine attenuated and reversed the last effect, respectively. Similarly, the addition of $MnCl_2$ (an $\cdot O_2^-$ dismutating agent) or the HO· scavenger sodium benzoate along with spermine, annulled the elongating effect of the polyamine on the salinized segments. As a whole, the results obtained here demonstrated that, under salinity, polyamine oxidase activity provides a significant production of reactive oxygen species in the apoplast which contributes to 25–30% of the maize leaf blade elongation.

Key words: Growth, maize, polyamine oxidase, polyamines, reactive oxygen species, salinity.

Introduction

Reactive oxygen species (ROS), namely the superoxide radical ($\cdot O_2^-$), the hydroxyl radical (HO·), and H_2O_2 are the major apoplastic ROS (aROS) in plants (Schopfer *et al.*, 2001). aROS are necessary in many plant developmental processes (Foreman *et al.*, 2003; Demidchik and Maathuis, 2007), particularly in the elongation zone (EZ) of maize leaves during leaf extension (Rodríguez *et al.*, 2002). In these plants, the salt-induced decrease of aROS contributes to the reduction of leaf elongation (Rodríguez *et al.*, 2004). On the other hand, the diminution of the aforementioned aROS has been attributed to the inhibitory effect of NaCl on the NADPH oxidase (NOX) complex (Rodríguez *et al.*,

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Abbreviations: $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+}; $\cdot O_2^-$, superoxide radical; 1,8-DO, 1,8-diamino-octane; 4-AAP, μM 4-aminoantipyrine; AES, atomic emission spectrophotometry; AOs, amine oxidases; aPA, apoplastic extract; aROS, apoplastic ROS; BZ, sodium benzoate; cPA, cell extract; CuAO, copper-containing amine oxidase; Dap, 1,3-diaminopropane; DCHBS, 3,5-dichloro-2-hydroxybenzenesulphonic acid; DPI, diphenylene iodonium; EGTA, ethylene glycol *bis* (β-aminoethylether)-*N*, *N*, *N′*, *N′*-tetra-acetic acid; EZ, elongation zone; FZ, ferrozine; H_2O_2, hydrogen peroxide; HO·, hydroxyl radical; NBT, nitro-blue tetrazolium; NC, neocuproine; NOX, NADPH oxidase; NSCCs, non-selective cation channels; PA, polyamines; PAO, polyamine oxidase; PCA, perchloric acid; POX, peroxidase; Put, putrescine; ROS, reactive oxygen species; SL-11061, 1,19-*bis*-(ethylamine)-5,10,15 triazanonadecane; SOD, superoxide dismutase; Spd, spermidine; Spm, spermine; XTT, Na, 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium](4-methoxy-6-nitro) benzenesulphonic acid hydrate.

2007). It has been shown that non-enzymatic processes involving reactive oxygen species (ROS) cause wall polysaccharide scission *in vitro* (Miller, 1986; Fry, 1998; Schweikert *et al.*, 2000; Fry *et al.*, 2001) and *in vivo* (Schopfer, 2001). It has also been suggested that a delicate equilibrium between cleavage and cross-linking activities by ROS may take place in the apoplast (Cosgrove, 1999). Under optimal conditions, NOX is the main source of apoplastic $\cdot O_2^-$ (Schopfer *et al.*, 2001), which dismutes to H_2O_2 through superoxide dismutase (SOD) activity. Remarkably, despite the fact that NaCl inhibits NOX activity, plants continue producing aROS in low concentrations and growing at a reduced rate (Rodríguez *et al.*, 2004). Up to now, the origin of those aROS is unknown, and whether such a low aROS amount may still contribute to plant growth under salt stress conditions is uncertain.

Polyamines (PA) are small organic polycations, naturally found in eukaryotic and prokaryotic cells, which have been associated with cell growth and development (Bais *et al.*, 1999; Steiner *et al.*, 2007). In plant cells, the most abundant PA are putrescine (Put), spermidine (Spd), and spermine (Spm, Kaur-Sawhney *et al.*, 2003). Although PA are detected in both symplastic and apoplastic compartments (Torrigiani *et al.*, 1986; Pistocchi *et al.*, 1988; Slocum, 1991; Tiburcio *et al.*, 1997), their biosynthesis takes place only in symplastic subcellular localizations (Slocum, 1991; Borrell *et al.*, 1995; Tiburcio *et al.*, 1997). In turn, PA cross the plant cell membrane towards the apoplast via a still unknown mechanism (Cona *et al.*, 2006*a*), where they are catabolized by amine oxidases (AOs), enzymes associated with apoplastic compartments (Federico and Angelini, 1991; Angelini *et al.*, 1995; Tavladoraki *et al.*, 1998; Cona *et al.*, 2006*a*). The copper-containing amine oxidase (CuAO) catabolizes the oxidation of lower PA, such as Put and cadaverine, on primary amino groups, whereas plant polyamine oxidase (PAO) oxidizes higher PA, Spd, and Spm on their secondary amino groups (Federico and Angelini, 1991). PA oxidation produces hydrogen peroxide (H_2O_2) among other products (Lim *et al.*, 2006). Interestingly, some reports have demonstrated that, unlike NOX, PAO activity is stimulated by NaCl in *Brassica campestris* (Das *et al.*, 1995). Therefore, in the present work, the possibility is addressed that maize AOs maintain or even increase their activity under saline conditions, thus contributing to keep the basal ROS level needed to uphold leaf growth under saline stress. For this purpose, PA levels and AOs activities upon salinization were determined, as well as the effect of PA concentration on growth of the most actively elongating region of the salinized maize leaf.

Materials and methods

Plant material

Maize seeds (*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 with aerated water. Trays were kept at 25 °C under a light panel of fluorescent and incandescent light bulbs providing 95 μmol photons m^{-2} s^{-1} illumination, with a 16 h photoperiod. When the second leaf emerged, 6 d after sowing, the water was changed to half-strength Hoaglands 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, 100 mM, and finally, 150 mM NaCl. Solutions were thereafter refreshed every 2 d. At harvest, 14 d after seeding, the region spanning 10–20 mm from the ligule was sectioned. This segment was used throughout the experiments.

Segment elongation measurements

The elongation of the leaf blade segments was measured as previously described by Rodríguez *et al.* (2004). Segments were gently vacuum infiltrated for 1.5 min and incubated for 7 h in different solutions. Digital images of segments were obtained before and after the incubation period, using a scanner (HP PSC 1510, Hewlett Packard Company, Palo Alto, CA). Segment length was measured with image processing software (Optimas 6.1, Optimas Corporation, Bothell, WA) and segment growth was expressed as a percentage of length increase, with respect to the control in that period.

Extraction of free PA, 1,3-diaminopropane (Dap), and apoplastic Na^+

To extract free PA from the cell extracts (cPA), 30 leaf blade segments were frozen in liquid N_2 and homogenized. The homogenate (300 mg) was resuspended in 1 ml of PCA 5% (v/v), incubated in ice for 30 min and centrifuged at 15 000 *g* (15 min). The pellet was discarded and the supernatant was kept at –20 °C (solution A). Maize apoplastic fluid extraction was performed according to Rodríguez *et al.* (2002) with modifications. Segment pools were introduced within a net bag, which was, in turn, placed inside a plastic tube. To extract free PA and Dap from apoplastic extract (aPA and aDap, respectively), tubes were centrifuged for 10 min at 2000 *g* and the fraction collected was lyophilized, resuspended in 200 μl perchloric acid (PCA) 5% (v/v) (solution B) and used for free PA extraction according to Marina *et al.* (2008). For apoplastic Na^+ extraction, plastic tubes were centrifuged for 1 min at 1000 *g* to discard the washing solution and centrifuged again for 10 min at 2000 *g* to collect the apoplastic fluid. All centrifugations were done at 4 °C and the apoplastic fluids obtained were kept at –20 °C. Thirty segments from 30 leaves were used for free aPA and aDap extractions, and 120 segments from 24 leaves for Na^+ extraction. In addition, 30 leaf blade segments from 10 unsalinized plants were pooled, washed, transferred to net bags, and gently vacuum infiltrated for 2 min with water or 100 mM NaCl. The resulting apoplastic fluid was used to determine the apoplastic peroxidase (POX) levels and to check for variations in free aPA contents

derived from the presence of salt in the apoplastic environment. Glucose 6-phosphate dehydrogenase activity, a marker of cytosolic contamination, was determined in every apoplastic fluid fraction according to Rodríguez *et al.* (2002).

Determination of free PA and Dap

Maize free PA were determined according to Jiménez-Bremont *et al.* (2007). For dansylation, 200 µl of solution A or B (see above) were added to 10 µl of 0.1 mM heptanodiamine (internal standard, ICN) plus 200 µl saturated Na_2CO_3 and 400 µl dansyl chloride-acetone 1% (w/v). After 16 h at 25 °C in the dark, 100 µl of proline 100% (w/v) was added to stop the reaction. Dansyl-derived PA were extracted with 500 µl toluene. The organic phase (400 µl) was evaporated under vacuum and resuspended in 400 (cPA) or 200 (aPA and aDap) µl acetonitrile. Dansyl-derived PA were separated by HPLC (ISCO 2350, ISCO Inc, Lincoln, NE) with a reverse phase column Sephasil C18 (Amersham Pharmacia) and detected with a spectrofluorometer (Variant Fluorichrom). The solvent mix was obtained with a gradient programmer ISCO 2360, with a flow of 1.5 ml min^{-1} as follows: 0–4.5 min, acetonitrile:H_2O 70:30 v/v; 4.5–9 min, acetonitrile 100; 9–15 min, acetonitrile:H_2O 70:30 v/v). Peak areas were integrated, normalized to heptanodiamine and interpolated into a PA standards calibration curve.

POX enzyme level

The reaction mixture (1 ml) contained 15 µl of apoplastic fluid, 20 µl 0.02 M guaiacol, and 0.1 M potassium phosphate pH 6.4. The reaction was started by adding 35 µl 88 mM H_2O_2 and activity was measured as an increase in A_{560} after 30 s with a spectrophotometer (Beckman DU Series 600, Beckman Instruments, Fullerton, CA). The specific activity calculation was based on the protein content of each sample, determined according to Bradford (1976).

Determination of the apoplastic Na^+ content

Apoplastic Na^+ concentration was determined by atomic emission spectrophotometry analysis of the apoplastic fluid fraction, using a Perkin-Elmer AA 100 spectrophotometer in emission mode.

H_2O_2 production by amine oxidase activity

The AO activity level was determined according to Cona *et al.* (2006*b*) with some modifications. Segments were washed in water (control) or 100 mM NaCl (salinized) for 6 min in order to remove symplastic contamination. For *in vivo* measurements, pools of five segments were introduced in 1 ml solutions containing 100 µM 4-aminoantipyrine (4-AAP), 1 mM 3,5-dichloro-2-hydroxybenzenesulphonic acid (DCHBS), 20 mM potassium phosphate pH 6.5, plus or minus 0.5 mM Spm or Put, and 100 mM NaCl for the saline treatment. Segments were subsequently infiltrated for 2 min and further incubated for 5 h at room temperature. Then 1 ml of the incubation medium was collected and the resultant pink adduct was measured at A_{515} with a spectrophotometer (HITACHI U-2000, Hitachi, Tokyo, Japan) and transformed into an H_2O_2 molar concentration with a molar extinction coefficient at 515 nm (2.6×10^4 M^{-1} cm^{-1}). PAO and CuAO activities were calculated as the difference in H_2O_2 produced between treatments containing and lacking substrate.

Extraction of apoplastic PAO

Extraction of apoplastic proteins was performed as described by Li (1993) with slight modifications (Maiale *et al.*, 2008). Plant material (40 g) was cut in 2 mm pieces, washed in distilled water and vacuum-infiltrated with 100 ml 5 mM potassium phosphate pH 6.5 added with 200 mM NaCl. The vacuum was broken and re-established every 5 min, for three successive times. The apoplastic fluid was collected, cooled at 4 °C and added with 1 vol. of pre-cooled (–20 °C) Me_2CO. The resulting solution was incubated at 4 °C, for 30 min and centrifuged at 15 000 *g* for 15 min. The supernatant was discarded and the pellet resuspended in 20 mM *bis-tris*-propane buffer pH 6.5 and applied to a DEAE-Sephacell column (1× 2 cm) equilibrated with the same solution. The eluted solution (Solution D) was kept at 4 °C until used.

In vitro *PAO activity assay*

For *in vitro* PAO activity measurement, pools of 20 segments were frozen with liquid N_2, homogenized in 1 ml of 0.1 M potassium phosphate pH 6.5 at 4 °C, and centrifuged at 15 000 *g* for 15 min. The pellet was discarded and the homogenate was kept at 4 °C (sSolution C). PAO activity was determined according to Cona *et al.* (2006*b*). Previous tests determined that the optimal pH for PAO activities was 6.5. The reaction mixture contained 1 ml, 50 µl solution C or 80 µl D (see above) plus 100 µM 4-AAP, 1 mM DCHBS, 0.06 mg ml^{-1} horseradish POX, and 100 mM potassium phosphate pH 6.5. The mixture was incubated at 30 °C for 2 min. The reaction was started by adding 5 µl of 10 mM Spm and the activity was measured for 1 min at A_{515} with a spectrophotometer and transformed into an H_2O_2 molar concentration. PAO K_i for SL-11061 was calculated (Lineweaver and Burk, 1934). The K_m value obtained for this preparation was K_m=17.7 µM.

Extraction and purification of plasma membrane for NOX activity determination

Plasma membrane was prepared according to Larsson (1985) with some variations. Leaves (70 g) from 7-d-old plants were homogenized with an omnimixer by giving three 20 s pulses at full speed. The extraction solution (200 ml) contained 50 mM TRIS-HCl pH 7.5, 0.33 M sucrose, 1 mM EDTA, 0.1 mM $MgCl_2$, 1 mM ascorbate, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride, and 0.6% (w/v) polyvinylpoly pyrrolidone. The homogenate was filtered

through four layers of cheesecloth, and the filtrate centrifuged at 10 000 *g* for 10 min. Microsomes were pelleted from the supernatant by centrifugation at 140 000 *g* for 45 min and resuspended in 10 ml 5 mM potassium phosphate pH 7.8 containing 0.33 M sucrose and 3 mM KCl. The suspension was fractionated by the aqueous two-phase partitioning method (Larsson, 1985). Phase separations were carried out in a series of 10 g phase systems with a final composition of 6.2% (w/w) dextran T500 (Sigma), 6.2% (w/w) polyethylene glycol 3350 (Sigma), 0.33 M sucrose, 5 mM potassium phosphate pH 7.8, and 3 mM KCl. Three successive partitioning rounds yielded an upper phase (U3) and a lower phase (L3). U3 was 3-fold diluted in 10 mM TRIS-HCl buffer (pH 7.4) containing 0.33 M sucrose. The solution was centrifuged at 140 000 *g* for 60 min and the resulting pellet resuspended in 2 ml 10 mM TRIS-HCl buffer pH 7.4 and 0.33 M sucrose. All procedures were carried out at 4 °C. The enrichment in plasma membranes of the upper phase was monitored by the percentage of V-ATPase inhibition (Serrano, 1978). U3 was enriched in plasma membrane up to 90%. U3 was kept at 4 °C and used for enzyme activity immediately.

NADPH oxidase activity

NADPH oxidase activity was assayed spectrophotometrically according to Sagi and Fluhr (2001). The reaction medium contained 50 µl U3, 0.3 mM Na,3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium](4-methoxy-6-nitro) benzenesulphonic acid hydrate (XTT) and 0.2 mM NADPH in 50 mM TRIS-HCl buffer pH 7.4. The reaction was initiated by adding NADPH. Data were transformed into an $\cdot O_2^-$ molar extinction coefficient at 470 nm (2.16×10^4 M^{-1} cm^{-1}).

H_2O_2 production by NADH-dependent POX

Reactions were carried out in 0.1 M potassium phosphate pH 4.5 containing 3 µg ml^{-1} horseradish peroxidase and 0.2 mM NADH (Frahry and Schopfer, 1998) with some modifications. Reactions were initiated by adding NADH and, 5 min later, aliquots of 500 µl were removed from the reaction mixture. NADH was eliminated with 0.1 M HCl followed by 0.1 M NaOH. H_2O_2 was measured by fluorescence of 55 µM homovanillic acid at 407 nm (EM) and 305 nm (EX), in the presence of 12 µg ml^{-1} horseradish peroxidase and 0.2 M potassium phosphate pH 4.5 in a final 1 ml volume. The calibration curve was linear in the range of 0.5–3 µM H_2O_2. The calibration curve was not affected by 100 µM or 200 µM SL-11061, 50 µM or 200 µM DPI, and 100 µM or 200 mM NaCl.

Detection of $\cdot O_2^-$ accumulation in the whole leaf

$\cdot O_2^-$ accumulation was detected by blue formazan precipitation (Hernández *et al.*, 2001). For this purpose, 0.01% (w/v) nitro-blue tetrazolium (NBT) was added with nutrient solution to control and salinized 13-d-old plants. One day later, plants were harvested and the third leaf was boiled in 80% (v/v) ethanol for 10 min. Leaves were mounted on a glass slide and scanned.

In vivo $\cdot O_2^-$ production

In blade segments, release of $\cdot O_2^-$ to the medium was determined through spectrophotometry, using XTT (Frahry and Schopfer, 2001). Pools of eight segments were gently vacuum infiltrated and incubated 7 h in 1 ml of aqueous solutions containing 0.5 mM XTT, 100 mM NaCl and the following potential PAO activity modulators: 100–800 µM Spm, 200 µM SL-11061: 200 µM 1,8-diamino-octane (1,8-DO): 200 mM Dap, 1 mM ferrozine (FZ), and 1 mM neocuproine (NC). Segments were removed and the medium centrifuged at 10 000 *g* for 10 min. 1 ml of the incubation medium was collected, measured with a spectrophotometer at A_{470} and data transformed into an $\cdot O_2^-$ molar extinction coefficient at 470 nm (2.16×10^4 M^{-1} cm^{-1}).

In vivo HO· production

HO· release to the medium was determined by the hydroxylation of sodium benzoate (BZ) by HO·. Hydroxyl BZ was detected by spectrofluorometry according to Schopfer *et al.* (2001) with modifications. Pools of six salinized segments were gently infiltrated and incubated for 7 h in 1 ml of aqueous solutions containing 2.5 mM BZ and 100 mM NaCl in the presence or absence of 100 µM SL-11061 at 30 °C in the dark. Fluorescence was determined at 407 nm emission after excitation at 305 nm in a spectrofluorometer (Bio-Tek Kontron SFM 25, Kontron Instruments, Zürich, Switzerland).

Statistical analysis

Data were analysed by one-way or two-way ANOVA and Tukey or DGC tests (Di Rienzo *et al.*, 2002), using InfoStat (InfoStat 2007. InfoStat Group. Facultad de Ciencias Agropecuarias. Universidad Nacional de Córdoba. Version 1.1. Córdoba, Argentina).

Results

Effect of NaCl on elongation and apoplastic Na^+ concentration and free PA levels in leaf segments

Previous results showed that elongation in unsalinized and salt-treated leaves is maximal at the second blade EZ segment, that is, the region spanning 10–20 mm from the ligule (Fig. 1A). Elongation of excised second blade segments from salt-treated plants incubated in 100 mM NaCl was 50% compared with unsalinized segments (Fig. 1B), confirming the previous results by Rodríguez *et al.* (2004). Atomic emission spectrophotometry (AES) analysis of the segment apoplastic fluids revealed a 76.4±2.4 and 1.4±0.5 mM Na^+ content in salinized and unsalinized leaf blades, respectively (no cytosolic contamination was detected in the apoplastic fluid; see Supplementary Table S1 at *JXB* online). Therefore, as the saline content of the incubation

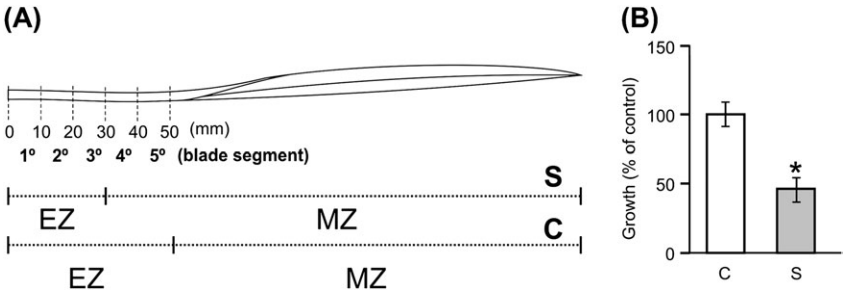

Fig. 1. Schematic representation of expanding and expanded regions in unsalinized and salt-treated maize leaf blades and the effect of NaCl on the elongation of second blade EZ segments. (A) Distribution of EZ and maturation zone (MZ) in unsalinized and salt-treated maize leaf blades, adapted from Rodríguez *et al.* (2004). (B) Effect of NaCl on the elongation of second blade EZ segments. Segments were incubated for 7 h in water (unsalinized) or in solutions containing 100 mM NaCl (salt-treated). Segments were scanned before and after the incubation period and their length measured with an image processing software. Results are the percentage of length increase during a 7 h incubation period with respect to the control unsalinized segment. Absolute growth rate for unsalinized second blade segments was 0.402±0.011 mm h^{-1}. The experiment was conducted twice, yielding similar results. Abbreviations: c, unsalinized; s, salt-treated. Results are means ±SE (n=20). Asterisks indicate a difference from the control (P <0.05).

solution was comparable with that existing in the apoplast of salinized leaves, it was decided to add 100 mM NaCl to the incubation mixture in the next *in vivo* experiments, in the case of salinized plants, as a means to avoid changes in the osmotic potential of the apoplastic environment. Free PA levels were measured by HPLC. Salinity lowered Put and increased Spd and Spm level of the cell extracts (Fig. 2A–C), whereas it greatly increased Spm and Spd and slightly increased Put in the apoplast (Fig. 2D–F), suggesting a role for PA accumulation in the elongation zone of the maize leaf blade under saline stress.

Evaluation of the mechanisms involved in aPA increment in salinized leaves

It has been hypothesized that after being synthesized in the cytoplasm, PA cross the plasma membrane towards the apoplast, where they are catabolized by AOs. Thus, the observed rise in free aPA in salinized plants is probably the result of: (i) promotion of PA passage towards the apoplast or (ii) a decrease in the amount of AOs enzymes (or AOs activities) leading to free aPA accumulation. Given that the mechanisms of PA passage to the apoplast are unknown, it was decided to assess the last possibility. For this, the effect of salt addition on maximal *in vivo* and *in vitro* AO activity was determined. In this approach, H_2O_2 production by PA oxidation was estimated through an oxidative POX-dependent reaction that produces a pink adduct measurable by spectrophotometry (Cona *et al.*, 2006*b*). Blade segments from salinized and unsalinized plants were infiltrated and incubated in the reaction mix with the addition of 0.5 mM of exogenous substrates Put and Spm, for CuAO and PAO determination, respectively (POX addition to the reaction mixture was not necessary since no variation was observed in the apoplastic oxidative POX activity between treatments, see Supplementary Fig. S1 at *JXB* online). As result, it was observed that (i) there were no differences in the maximal CuAO and PAO activities (achieved under saturating substrate conditions) due to salt treatment (Fig. 3A), (ii) PAO activity levels were up to 20-fold higher than those of CuAO (therefore further studies will be performed only on PAO activity). A second *in vitro* analysis using cell-free extracts from segment homogenates (and saturating substrate conditions) confirmed former results on PAO activity levels (Fig. 3B). Taken together, these results led us to: (i) reject the possibility of a negative saline effect on the total activities of AOs enzymes and (ii) to assume that salinity somehow promoted PA passage from the symplastic compartment towards the apoplast. Having in mind that aPA may interact with cell wall components, a third possibility is that the presence of NaCl in the apoplast causes the dissociation of pre-existent aPA from the cell wall. Blade segments from unsalinized plants were then infiltrated either with water or with 100 mM NaCl, resulting in the absence of any effect of this salt on free aPA contents (see Supplementary Table S2 at *JXB* online). Thus, any dissociating action of NaCl on aPA putatively associated to cell wall components of the apoplast was ruled out.

NaCl increases inherent PAO activity

So far, it has been demonstrated that aPA levels increased as a result of plant salinization (Fig. 2). This result encouraged us to examine whether the inherent PAO activity, which depends on the concentration of its endogenous substrate (aPA), correlates with that result. Therefore, PAO activity was evaluated by measuring the Dap content using HPLC. Dap is a product of the Spd and Spm oxidation, formed in the same molar quantities as H_2O_2 (Cona *et al.*, 2006*a*). The measurement of aDap levels indicated that salinity led to increased PAO activity in the leaf blade region under study (Fig. 4A). Alternatively, PAO activity *in vivo* was determined as previously described (Fig. 3A), without the exogenous Spm supplement. For this purpose, H_2O_2 levels were measured in the presence or absence of 1,19-*bis*-(ethylamine)-5,10,15 triazanonadecane (SL-11061), a tobacco PAO inhibitor (Marina *et al.*, 2008), which has also been found to inhibit

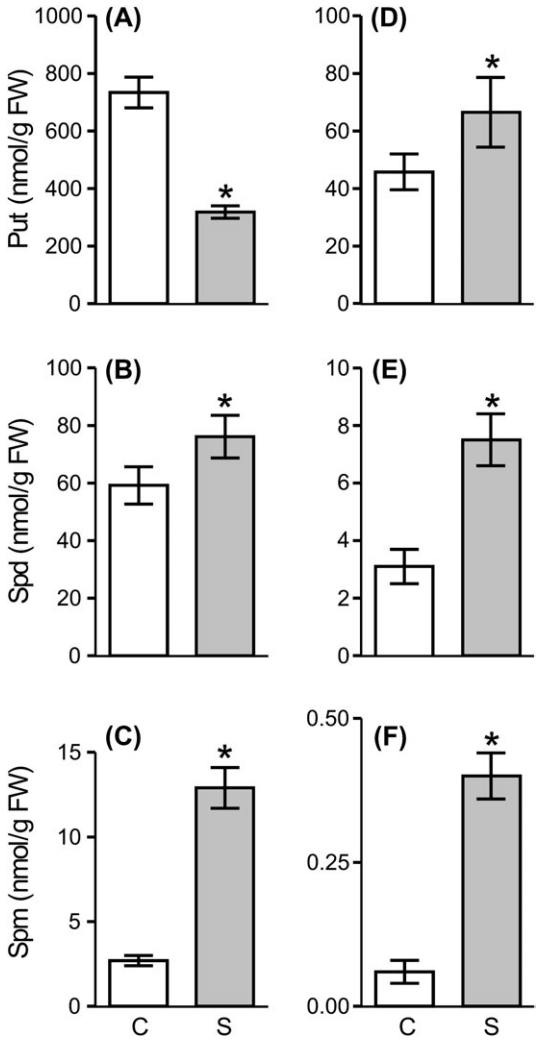

Fig. 2. Free PA levels in segments of unsalinized and salt-treated plants. (A–C) Pools of second blade segments were homogenized and cell extracts used for cPA measurements. (D–F) aPA were extracted from second blade segment pools by centrifugation and the collection of apoplastic fluid. PA were dansyl-derived according to Jiménez-Bremont *et al.* (2007) and determined by HPLC. The experiment was performed twice, yielding similar results. Abbreviations: c, unsalinized; s, salt-treated. Results are means ±SE (n=6). Asterisks indicate a difference from the control (P <0.05).

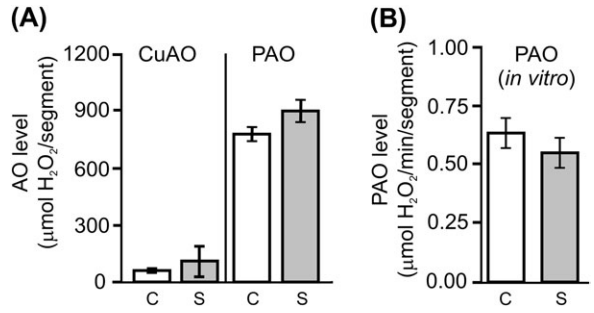

Fig. 3. PAO and CuAO activities under substrate saturating conditions. (A) *In vivo* AOs activities were determined according to Cona *et al.* (2006*b*). Pools of five segments were introduced in 1 ml of solution containing 100 µM 4-AAP, 1 mM DCHBS, 20 mM potassium phosphate pH 6.5 with and without 0.5 mM Spm or Put, and 100 mM NaCl for salinized segments. Segments were subsequently infiltrated for 5 min, incubated for 5 h and AOs activities determined by pink adduct production at A_{515}. Data were transformed into H_2O_2 molar concentrations with a molar extinction coefficient at 515 nm (2.6×10^4 M^{-1} cm^{-1}). PAO and CuAO levels were calculated as the difference in H_2O_2 amounts between treatments with and without substrate. (B) *In vitro* PAO measurement. Pools of 20 blade segments were homogenized in 1 ml of 0.1 mM potassium phosphate pH 6.5 at 4 °C, and centrifuged at 15 000 *g* for 15 min. PAO activity was determined according to Cona *et al.* (2006*b*). The experiment was conducted twice, yielding similar results. Abbreviations: c, unsalinized; s, salt-treated. Results are means ±SE (n=6).

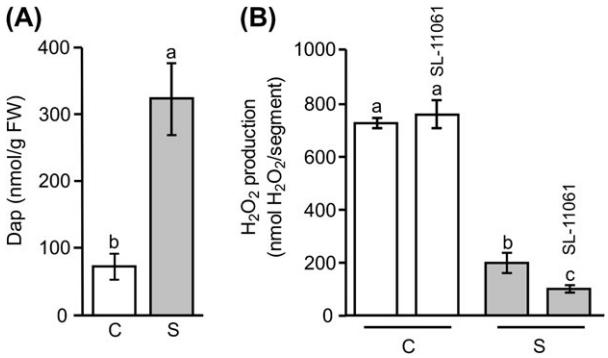

Fig. 4. Effect of salinity on PAO activity without exogenous substrate. (A) aDap was determined in apoplastic fluids obtained from pools of 30 second segments. Dap dansylation was performed according to Jiménez-Bremont *et al.* (2007) and dansyl-derived Dap was determined through HPLC. (B) H_2O_2 production in segments from unsalinized and salt-treated plants was measured as in Fig. 3A, although without exogenous Spm, after 7 h incubation in presence or absence of 100 µM SL-11061. The experiment was performed twice, yielding similar results. Abbreviations: c, unsalinized; s, salt-treated. Results are means ±SE (n=4). Bars sharing the same letter are not significantly different (P <0.05).

oat PAO *in vivo* (Maiale *et al.*, 2008). The results showed that SL-11061 had no effect on apoplastic H_2O_2 content in the absence of NaCl (Fig. 4B), showing that the contribution of PAO activity to the total apoplastic H_2O_2 level was negligible under control conditions. Conversely, a 50% lowered H_2O_2 content was found in SL-11061-treated segments under saline conditions. As a whole, these results showed that, under salt stress conditions, the contribution of PAO to the observed apoplastic H_2O_2 pool in the elongation zone of the maize leaf blade is relevant, whereas in the absence of salt treatment, the formation of the main apoplastic H_2O_2 would rely on mechanisms different from aPA oxidation.

On the other hand, under *in vitro* conditions NaCl did not affect PAO activity at all (Table 1), reinforcing that this enzyme keeps its activity in salinity. In addition, a low K_i

Table 1. Effects of inhibitors on apoplastic ROS-producing enzymes

PAO was extracted and purified from apoplastic fluids of leaf blade segments according to Li (1993) with some modifications. PAO activity was determined according to Cona *et al.* (2006*b*). NOX was extracted and purified according to Larsson (1985) with some variations. NOX activity was determined according to Sagi and Fluhr (2001). H_2O_2 production by NADH-dependent POX was determined according Frahry and Schopfer (1998) with modifications. Data are means ±SE (n=3). Asterisks indicate significantly different from control (P <0.05). 100% of control for PAO, NADH-dependent POX and NOX represent: 6.15±0.02 μmol H_2O_2 min^{-1}, 389±9 μmol H_2O_2 min^{-1} g^{-1}, and 20.05±0.62 pmol $\cdot O_2^-$ min^{-1}, respectively. PAO, NOX, and NADH-dependent POX substrate concentrations (Spm, NADH, and NADPH, respectively) were 200 μM in all cases.

Inhibitor treatment	PAO (% of control)	NOX (% of control)	NADH-dependent POX
Control	100.0±0.3	100.0±6.7	100.0±3.1
SL-11061 (μM)			
5	31.2±0.8*	–	–
20	9.9±0.3*	–	–
100	0.0±0.0*	105.5±1.0	106.3±11.0
200	0.0±0.0*	104.8±0.1	102.5±6.2
DPI (μM)			
20	99.7±2.7	23.3±0.5*[a]	6.0±6.0*[b]
200	95.8±0.1*	8.0±0.1*	–
NaCl (mM)			
100	101.1±0.2	37.8±1.6*[a]	41.5±4.6*
200	102.3±0.1	10.7±0.6*	28.9±0.6*

[a] From Rodríguez *et al.* (2007).
[b] From Frahry and Schopfer *et al.* (2007).

value (8.7×10^{-7} M) was found for the polyamine analogue SL-11061, indicating its high efficiency as a maize PAO inhibitor. This K_i is comparable to that found for oat PAO (K_i=1.5×10^{-9} M; Maiale *et al.*, 2008).

PAO activity provides $\cdot O_2^-$ and HO· radicals under saline stress

When leaves of salt-treated plants were stained with NBT, they showed a strong decrease in precipitate intensity as compared with control leaves (Fig. 5), demonstrating a reduction of the $\cdot O_2^-$ level due to the saline treatment. However, a certain amount of $\cdot O_2^-$ was still observable in these conditions. The fact that NaCl substantially decreases NOX activity, the main source of apoplastic $\cdot O_2^-$ and H_2O_2 in non-stressed maize plants (Schopfer *et al.*, 2001; Rodríguez *et al.*, 2007) as well as H_2O_2 production by NADH-dependent POX (Table 1), suggests the occurrence of some salt-tolerant mechanism for aROS production, alternative to POX and NOX. Interestingly, it has been proposed that AO activity is involved in the production of extracellular $\cdot O_2^-$ and HO· radicals (Kawano *et al.*, 2000*a*). In order to test whether this process takes place *in vivo* in the apoplast maize leaf under saline condition, $\cdot O_2^-$ formation was determined by incubating salt-treated blade segments in XTT solution, in the presence or absence of SL-11061 (Fig. 6A). Our results showed that the addition of the PAO

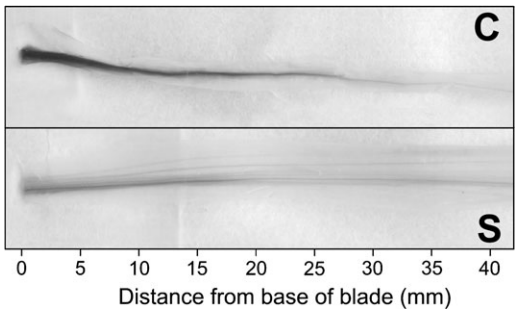

Fig. 5. $\cdot O_2^-$ production in the whole leaf blade. $\cdot O_2^-$ was detected by formazan precipitation. Control and salt-treated plants were incubated in the presence of 0.01% (w/v) NBT for 24 h. Plants were harvested and the third leaf was boiled in 80% (v/v) ethanol, mounted on a glass slide, and scanned. Abbreviations: c, unsalinized; s, salt-treated.

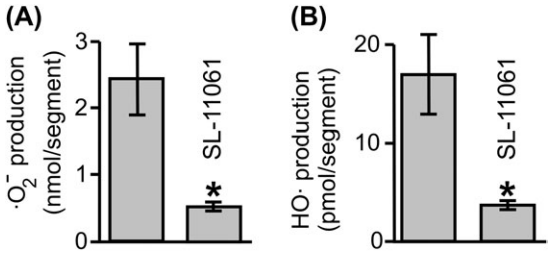

Fig. 6. *In vivo* PAO activity-derived $\cdot O_2^-$ and HO· production under salinity. (A) $\cdot O_2^-$ was detected by XTT according to Frahry and Schopfer (2001). Pools of eight salinized second blade segments were gently infiltrated and incubated for 7 h in the dark in 1 ml of aqueous solutions containing 0.5 mM XTT and 100 mM NaCl in the presence or absence of 100 μM SL-11061. The incubation medium (1 ml) was centrifuged at 10 000 *g* and the supernatant was subjected to measurement with a spectrophotometer at A_{470}. Data were transformed into $\cdot O_2^-$ molar extinction coefficient at 470 nm (2.16×10^4 M^{-1} cm^{-1}). (B) HO· was detected by hydroxylation of BZ according to Schopfer *et al.* (2001). Pools of six salinized segments were gently infiltrated and incubated for 7 h in 1 ml of aqueous solutions containing 2.5 mM BZ and 100 mM NaCl plus or minus 100 μM SL-11061 at 30 °C in the dark. Fluorescence was determined in a spectrofluorometer (EX: 305 nm EM: 407 nm). Experiment was conducted twice, yielding similar results. Results are means ±SE (n=4). Asterisks indicate a difference from the control (P <0.05).

inhibitor resulted in a highly diminished $\cdot O_2^-$ formation. These results (in addition to the fact that SL-11061 does not scavenge $\cdot O_2^-$ radicals produced by NOX; see Table 1) support the idea that PAO activity indirectly produces most of the $\cdot O_2^-$ in this zone under saline conditions. Similarly, when HO· was determined by the BZ method (Schopfer *et al.*, 2001), PAO activity represented around 70% of the production of that free radical in the salt-treated segments (Fig. 6B). These results reinforced the hypothesis that, under saline conditions, PA oxidation by PAO would be the main source for aROS production in the elongation zone of maize leaf blades.

In order to gain further insight into the involvement of higher polyamines catabolism by PAO on aROS generation, the effect was examined of adding different PAO modulators to the incubation buffer, on $\cdot O_2^-$ production in the elongation zone of salt-treated plants, detected by XTT (Fig. 7). The addition of 100 or 200 μM Spm increased the *in vivo* $\cdot O_2^-$ production, whereas treatment with SL-11061 (without Spm) or 1,8-DO, a commercial competitive PAO inhibitor (K_i=3×10^{-7} M; Cona *et al.*, 2004), either separately or in combination with Spm showed the opposite effect (Fig. 7). As expected, Dap addition did not change the $\cdot O_2^-$ levels. Similarly, $\cdot O_2^-$ production in segments treated with diphenylene iodonium (DPI), reported as a NOX (Schopfer *et al.*, 2001) and NADH-dependent POX inhibitor (Frahry and Schopfer, 1998) was similar to that of the control treatment, evidence that, under saline conditions, the enzymes mentioned were inhibited. Such *in vivo* inhibition was in line with that observed in the *in vitro* assay (Table 1) and in previous results concerning NOX (Rodríguez *et al.*, 2007). Based on these results, it is concluded that PAO is the main contributor to apoplastic $\cdot O_2^-$ production in salinized maize leaves.

Possible source for the observed $\cdot O_2^-$ *and* HO·

H_2O_2 may be consumed to generate $\cdot O_2^-$ and HO· through the Haber–Weiss reaction (Haber and Weiss, 1932) and POX activity in the Fenton-like reaction (Schopfer *et al.*, 2001; Liszkay *et al.*, 2004; Carol and Dolan, 2006). Therefore, the possibility that, under saline conditions, these free radicals originate in a reaction from the H_2O_2 produced by PAO (which remains active under salinity), through a chain reaction catalysed by Fe^{2+} or Cu^+ (Fry, 1998; Kawano *et al.*, 2000*b*) was tested. Salinized segments were treated with Spm plus the Fe^{2+}-specific chelator FZ (Kosegarten *et al.*, 1999) or the Cu^+-specific chelator NC (Kunapuli and Vaidyanathan, 1983). Results demonstrated that chelators significantly decreased $\cdot O_2^-$ production, indicating a probable involvement of a Fenton–Haber–Weiss-like reaction in this process.

Effect of PAO and ROS modulators on segment elongation of salinized plants

So far it has been shown that aPA oxidation is mostly responsible for the presence of certain aROS amounts in leaf blades of salinized maize plants. However, since the amount of aROS (H_2O_2 and H_2O_2-derived $\cdot O_2^-$ and HO·) produced in leaf segments is much lower in the presence of salt, compared with that of the control without NaCl, the question remains as to whether the observed amounts of these aROS may still contribute to leaf elongation. To answer this question, the effect of diverse PAO and ROS modulators on segment elongation of salinized plants was tested (Fig. 8). Incubation with 200 μM Spm doubled segment elongation, whereas the addition of SL-11061 attenuated and 1,8-DO reversed the last effect. Interestingly,

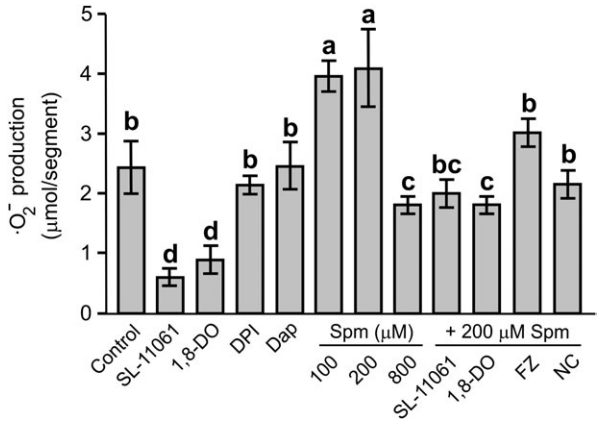

Fig. 7. *In vivo* effect of PAO activity modulators on $\cdot O_2^-$ production under salinity. Salinized second blade segments were used to detect $\cdot O_2^-$ by XTT. Pools of eight segments were gently infiltrated and incubated for 7 h in the dark in 1 ml of aqueous solutions containing 0.5 mM XTT, 100 mM NaCl, and modulators of PAO activity. When indicated, the following concentrations were used: 200 μM SL-11061, 200 μM 1,8-DO, 100–800 μM Spm, 200 μM DPI, 200 μM Dap, 1 mM FZ, or 1 mM NC. Incubation medium (1 ml) was centrifuged at 10 000 *g* and the supernatant subjected to measurement with a spectrophotometer at A_{470}. Data were transformed into $\cdot O_2^-$ molar extinction coefficient 470 nm (2.16×10^4 M^{-1} cm^{-1}). The experiment was performed twice, yielding similar results. Results are mean ±SE (n=8). Bars with the same letter are not significantly different (P <0.05).

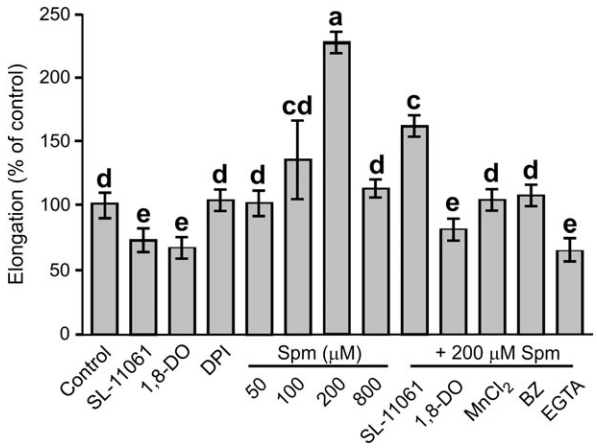

Fig. 8. Effects of PAO activity modulators, ROS scavengers, and a Ca^{2+} chelator on segment elongation. Second blade segments from salinized plants were incubated for 7 h in the dark with 100 mM NaCl. When indicated, the following concentrations were used: 200 μM SL-11061, 200 μM 1,8-DO, 50–800 μM Spm, 10 mM $MnCl_2$, 5 mM BZ, and 10 mM EGTA. Segments were scanned before and after the incubation period and their length measured with an image processing software. The results are the percentage of length increase, with respect to the control during a 7 h incubation period. Absolute growth rate for control salinized second blade segments was 0.221±0.008 mm h^{-1}. The experiment was conducted twice, yielding similar results. Results are means ±SE (n=20). Bars with the same letter are not significantly different (P <0.05).

800 μM Spm had no effect on segment elongation. In the absence of exogenous Spm addition, both PAO inhibitors diminished segment length, as compared with the control. Moreover, when 1,8-DO was added to the plant nutrient solution from the beginning of salt treatment, it produced reduced growth and Dap accumulation, as well as increased Spd and Spm contents of the entire maize leaf (Table 2), compared with plants not treated with the inhibitor. The addition of $MnCl_2$, a $\cdot O_2^-$ dismutating agent (Hernández *et al.*, 2001) or BZ along with Spm, nullified the elongating effect of the polyamine on salinized segments. Finally, the incorporation of the specific Ca^{2+} chelating agent ethylene glycol *bis* (β-aminoethylether)-*N*, *N*, *N′*, *N′*-tetra-acetic acid (EGTA), reduced segments length, even in the presence of 200 μM Spm.

Discussion

Evidence has accumulated over recent decades demonstrating that polyamines play an important role in many plant developmental processes (Evans and Malmberg, 1989; Walden *et al.*, 1997) and in plant responses to salinity and other abiotic stress conditions in diverse plant species (Krishnamurthy and Bhagwat, 1989; Galston and Sawhney, 1990; Aziz *et al.*, 1998; Bouchereau *et al.*, 1999; Simon-Sarkadi *et al.*, 2002; Sanchez *et al.*, 2005; Kusano *et al.*, 2007). Several biotechnological approaches like overexpressing PA-synthesizing enzymes (Kumria and Rajam, 2002; Capell *et al.*, 2004; Kasukabe *et al.*, 2004; Wi *et al.*, 2006) or antisense and mutant generation (Kasinathan and Wingler, 2004; Yamaguchi *et al.*, 2006), allowed the generation of plants with increased and decreased stress tolerance, respectively. Recent studies using transgenic plants overexpressing or downregulating apoplastic polyamine oxidase, revealed the importance of the H_2O_2 derived from aPA catabolism in the induction of either salinity-induced tolerance or programmed cell death in tobacco (Moschou *et al.*, 2008). The present work was focused on the possible involvement of the ROS produced from aPA oxidation in leaf growth processes of maize plants, grown under saline conditions. The first results showed that salt treatment reduced elongation in the region spanning 10–20 mm from the leaf ligule and, in parallel, it provoked a remarkable increment of higher apoplastic polyamines concentration in that region. This observation is in agreement with recent results showing that Spd, which is synthesized in the cytoplasm, is secreted into the apoplast upon salt treatment in tobacco (Moschou *et al.*, 2008). In the present work, any other possibility was ruled out in order to confirm that salinity stimulates the passage of these substrates from the cytoplasm to the apoplast in maize plants. Similarly, the salt-induced decrease observed in Put levels, concomitant with the increase of total Spm levels of cell-free extracts in the segments, are consistent with the Spm accumulation described by other authors in salinized rice (Maiale *et al.*, 2004), several vegetables (Zapata *et al.*, 2004), *Lotus glaber* (Sannazzaro *et al.*, 2007), and maize (Jiménez-Bremont *et al.*, 2007).

In vivo and *in vitro* measurements of H_2O_2 levels in the presence of saturating substrate conditions revealed the maximum feasible AO activity and showed, on the one hand, that PAO was the main enzyme contributing to the total PA oxidation level in maize leaves. Consequently, further studies were performed only on PAO activity, leaving CuAO activity aside. Biochemical, histochemical, and immunocytochemical studies allowed the localization of PAO, showing that it is specially abundant in the primary and secondary cell walls of xylem parenchyma, the endodermis, and epidermis of maize seedlings (Cona *et al.*, 2006*a*). On the other hand, it was shown that salt treatment does not affect maximal PAO activity, suggesting that the enzyme is tolerant to this stress. Furthermore, the results obtained without exogenous substrate (that is to say, based on the actual polyamine cell content in the tissue) via the detection of the PAO product, Dap (Fig. 4A), demonstrated that salinity enhanced PAO activity. The last result consistently reflected the high Spd and Spm levels in that region (Fig. 2D–F) and suggested that the observed increase of inherent PAO activity under saline stress was a consequence of the rise in its substrate. These results are in line with *in vitro* results obtained by Smith (1977). Interestingly, the fact that the aDap amount was two and three orders higher than those of Spd and Spm, respectively, suggests that higher PA were actively oxidized to Dap and H_2O_2 in the apoplast, once they crossed the plasma membrane.

Although out of the scope of the present work, the possibility that polyamine metabolism in the root (the first organ sensing salinity) behaves upon salt treatment in similar manner as the leaf blade is intriguing. As far as we know, the information regarding root PAO activity and salinity is limited to one report by Zhao *et al.* (2003), who reported that 0–200 mM NaCl increased Put, Spd, and PAO activity in the roots of barley seedlings. Unfortunately, this information was not discussed in terms of root growth or elongation.

Table 2. Effects of maize PAO inhibition on leaf growth and PA contents under salinity

Plants were treated with nutrient solution plus 300 μM 1,8-DO for 14 d, from the beginning of salinization. Length of the expanding third leaf was measured and Dap, Put, Spd, Spd, and 1,8-DO levels of homogenized EZ segments determined by HPLC according to Jiménez-Bremont *et al.* (2007). Data are means ±SE (n=10 and n=4 for length and PA, respectively). Asterisks indicate significantly different from control (P <0.05).

	Control	1,8-DO
Length (cm)	14.46±0.88	10.79±0.62*
Polyamines(nmol g^{-1} FW)		
Put	54.6±6.1	41.1±1.4*
Spd	134.9±4.6	209.3±37.6*
Spm	14.0±5.8	29.6±5.7*
Dap	238.2±0.6	116.5±4.8*
1,8-DO	0.0±0.0	13.3±1.92*

Evidence of reduced $\cdot O_2^-$ amounts in salt-treated leaves by NBT staining (Fig. 5), revealed the occurrence of some mechanism for its production, alternative to that of NOX and NADH-dependent POX activities, which (unlike PAO) was strongly inhibited by salinity. The remarkable increase in *in vivo* $\cdot O_2^-$ production by Spm addition, along with the substantially lowered *in vivo* $\cdot O_2^-$ and HO· generation in the salt-treated segments by both PAO inhibitors or the Fe^{2+} and Cu^+-specific chelators FZ and NC (Figs 6, 7), support the notion that $\cdot O_2^-$ and HO· generation could occur from H_2O_2 production through PA oxidation and a further reaction catalysed by Fe^{2+} or Cu^+, such as a Fenton–Haber–Weiss chain reaction (Kawano *et al.*, 2000*a*, *b*).

It is noteworthy that *in vitro*, cadaverine, putrescine, spermidine, and spermine do not scavenge superoxide radicals, but were found to be scavengers of hydroxyl radicals (Das and Misra, 2004) and unpublished results from our group have confirmed those results. However, such a ROS-scavenger effect was observed only when polyamines were used in concentrations of 0.5 mM or higher. As in the present work, polyamine concentration has been always much lower than that amount, we may discard any ROS-scavenging effect in our results.

AO activity has formerly been related either to cell elongation in roots and hypocotyls of soybean seedlings (Delis *et al.*, 2006) or to cell wall maturation in tobacco (Paschalidis and Roubelakis-Angelakis, 2005; Cona *et al.*, 2006*a*). The purpose of this work was to evaluate the possible involvement of ROS production by PA oxidation in the leaf growth of maize plants grown under saline stress conditions. Our results suggest that tetramine oxidation contributes 25–30% of segment elongation under salinity (Fig. 8). Furthermore, when applied systemically along with NaCl, the PAO inhibitor 1,8-DO caused a 25% reduction in the elongation of whole leaves, compared with the control treatment without the inhibitor. Bearing in mind that the blade region analysed has 90% of the leaf elongation, it is deduced that, under a salt-stress situation, the minor contribution of PAO activity could still mean a significant yield improvement from an agronomical viewpoint. As a whole, these facts generate the expectation that biotechnological approaches like overexpressing enzymes responsible for PA biosynthesis or catabolism may be used to overcome reductions in the productivity of maize plants caused by salinity. In turn, the elimination of Spm-stimulated elongation by the specific Ca^{2+} chelating agent EGTA (Fig. 8), suggests that such elongation could be mediated by the activation of non-selective cation channels (NSCCs), through the HO· produced by Spm oxidation (a possibility that should be addressed in future research). This proposal is supported by the bulk of the evidence that has appeared during the last decade, which showed transient increases in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) as a second messenger, suggesting that there are ROS/$[Ca^{2+}]_{cyt}$ signalling pathways in several developmental processes. For example, guard cells and stomatal closure has been reported in *Commelina communis* and *A. thaliana* (McAinsh *et al.*, 1996; Pei *et al.*, 2000), as well as (2)-catechin-induced ROS production followed by ROS-induced Ca^{2+} increases in *Centaurea diffusa* and *Arabidopsis thaliana* roots (Bais *et al.*, 2003) or the growth stimulation of *A. thaliana* roots (Foreman *et al.*, 2003) and pollen tubes (Demidchik and Maathuis, 2007) by the aROS activation of Ca^{2+}-permeable NSCCs that induce inward Ca^{2+} currents. Recently, it was shown that the lack of Spm in the *Arabidopsis acl5*/*spms* mutants caused hypersensitivity to NaCl, possibly due to impaired Ca^{2+}-homeostasis (Yamaguchi *et al.*, 2006) and that H_2O_2 generated by CuAO activates NSCCs in the abscisic acid-induced stomatal closure process in *Vicia faba* (An *et al.*, 2008).

ROS could also act on growth through a promotion of cell wall polysaccharide cleavage *in vivo* (Schopfer, 2001), such as that shown to operate *in vitro* (Miller, 1986; Fry, 1998; Schweikert *et al.*, 2000). However, the action of ROS in the apoplast should be viewed as a delicate balance between cleavage and cross-linking activities (Cosgrove, 1999). An increase in PAO immunolabelling was observed inside secretory cytoplasmic organelles, suggesting the need for the intraprotoplasmic production of H_2O_2 for polymer cross-linking in the secretory pathway (Fry *et al.*, 2000; Cona *et al.*, 2003). Also, the balance between cleavage and cross-linking activities may be associated with a differential activity of cell wall peroxidases because different soluble peroxidase isozymes characterize the expanding and expanded regions in maize leaves (de Souza and MacAdam, 2001) and in *Festuca arundinacea* (MacAdam *et al.*, 1992).

The effectiveness of SL-11061 as inhibitor towards PA oxidation was formerly demonstrated *in vivo*, in an experiment using leaf blade segments in the presence of Spd (Maiale *et al.*, 2008). In the present work, the inhibitory effect of DPI on NOX and NADH-dependent POX (Table 1) reported previously is also confirmed: 50 μM DPI inhibited NOX activity by 77% (Rodríguez *et al.*, 2007) and the H_2O_2-producing activity by NADH-dependent POX by 94% (Frahry and Schopfer, 2001). In addition, strong NOX inhibition by DPI is in congruence with the $K_i = 5.6 \times 10^{-6}$ M reported by O'Donnell *et al.* (1993). Reductions in $\cdot O_2^-$ production and elongation of salinized segments treated with SL-11061 *in vivo* (Figs 7, 8), in addition to the fact that this polyamine analogue is efficient as a PAO but not as a NOX or NADH-dependent POX *in vitro* inhibitor (Table 1), demonstrated that PAO is not repressed by salinity. These facts also supported the use of this inhibitor to distinguish the PAO contribution to aROS production from that of the other two enzymes, under saline conditions. On the other hand, DPI treatment did not diminish either $\cdot O_2^-$ production or elongation of salinized segments (Figs 7, 8), in agreement with *in vitro* observations (Table 1), showing that NOX and NADH-dependent POX activities are inhibited *in vivo* by salinity.

Apoplastic Na^+ concentration varies among and within plant species. Under salt treatment, apoplastic ion concentrations of 164 mM and 56 mM were reported in pea and spinach, respectively (Speer and Kaiser, 1991), whereas it approached 600 mM in salt-stressed rice plants (Flowers *et al.*, 1991). Dissimilar apoplastic Na^+ contents have been

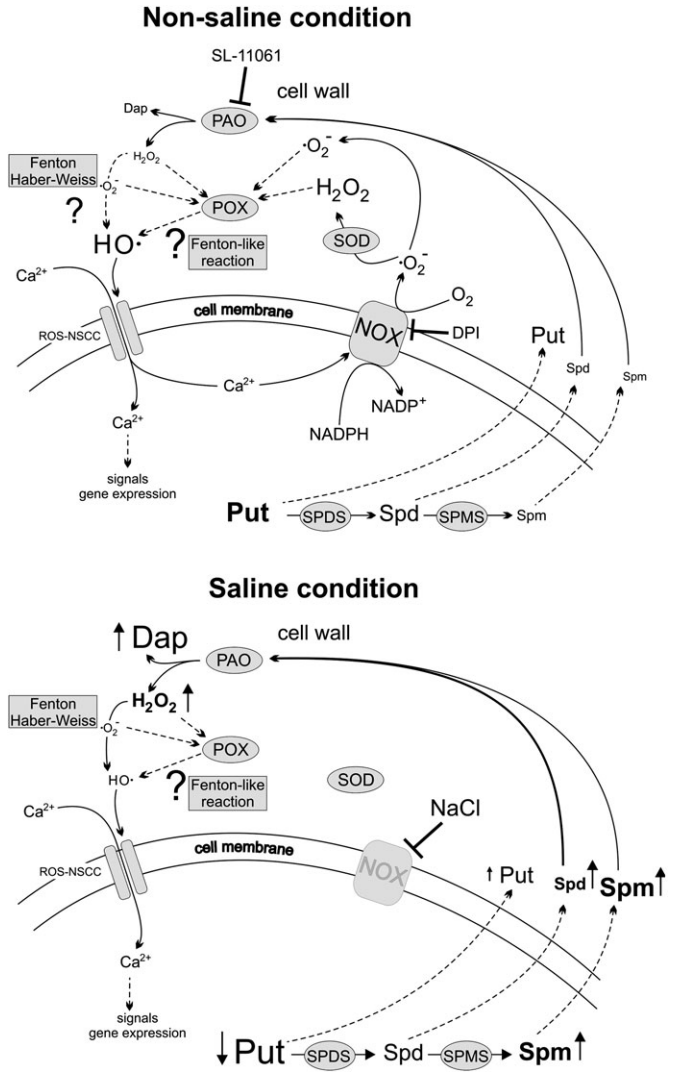

Fig. 9. Model of apoplastic ROS generation in a maize leaf blade grown under saline and non-saline conditions. The illustration integrates the models for probable apoplastic HO· production by POX (Schopfer *et al.*, 2001) and PAO (through the Fenton–Haber–Weiss reaction, own results), higher PA catabolism by PAO (own results), and the activation of ROS-NSCC by Ca^{2+} (Foreman *et al.*, 2003) in saline and non-saline conditions. SPDS: spermidine synthase; SPMS: spermine synthase.

reported in salt-stressed maize leaves: 4–5 mM (Lohaus *et al.*, 2000), 25 mM (Neves-Piestun and Bernstein, 2001), and 76 mM (own results). Compared with earlier reports, the higher apoplastic Na^+ content registered in the present work may be ascribed to a more concentrated NaCl solution used for salinization (150 versus 100 or 80 mM) or to variations in other experimental conditions. Such a diversity of results on apoplastic Na^+ contents highlights the importance of having assessed the actual apoplastic ion content in the salinized plant material under study in order to set a realistic experimental condition.

Finally, variations in the effect of different Spm concentrations on $\cdot O_2^-$ production and segment elongation (Figs 7, 8) gave evidence of a concentration-dependent Spm effect on ROS production and segment elongation. Unfortunately, previous reports describing changes in apoplastic PA levels have not measured cell elongation (Yoda *et al.*, 2003; Angelini *et al.*, 2008; Marina *et al*, 2008; Moschou *et al.*, 2008).

Taken together, our results demonstrated that, under saline stress, PAO might still provide the necessary H_2O_2 to generate $\cdot O_2^-$ through an increased substrate availability and thus sustain leaf elongation. These results allowed us to propose the model depicted in Fig. 9. Thus, in the scenario where NOX is inhibited by non-lethal NaCl stress and the ROS produced by this enzyme is substantially reduced, oxidation by PAO of Spm and Spd accumulated in the apoplast of the EZ would result in an alternative source to generate ROS, partially counteracting the growth-inhibiting effect caused by salinity.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Detection of apoplastic extracts contaminants.

Supplementary Table S2. Effect of the NaCl presence in the infiltration solution on the level of extracted apoplastic PA.

Supplementary Fig. S1. Apoplastic POX activity.

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