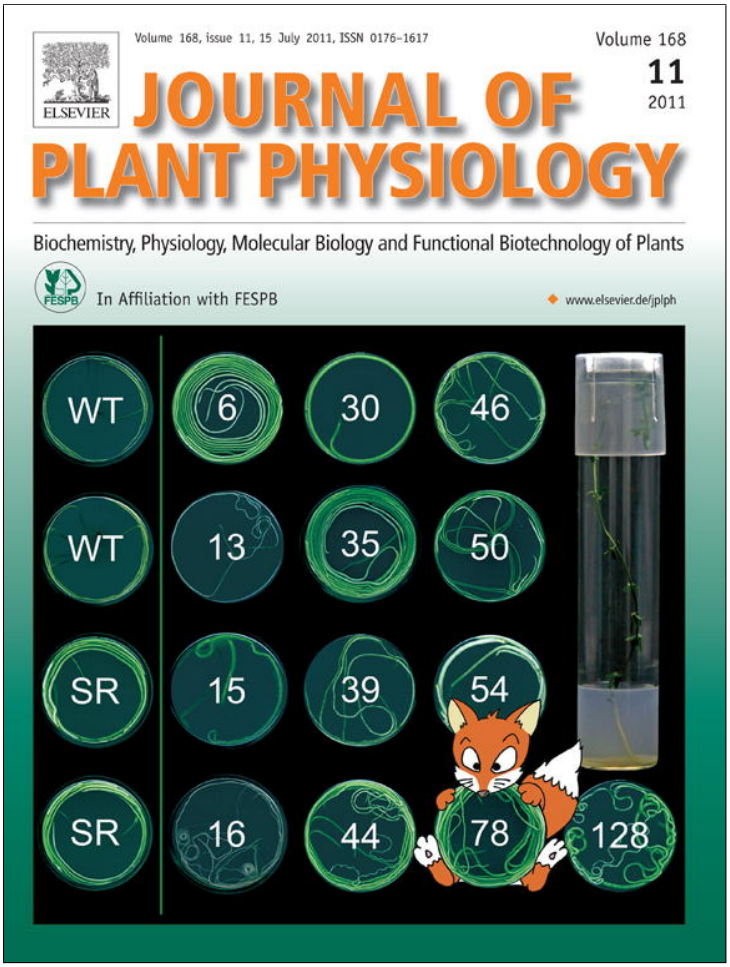

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Polyamine catabolism is involved in response to salt stress in soybean hypocotyls

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SUMMARY

The possible relationship between polyamine catabolism mediated by copper-containing amine oxidase and the elongation of soybean hypocotyls from plants exposed to NaCl has been studied. Salt treatment reduced values of all hypocotyl growth parameters. *In vitro*, copper-containing amine oxidase activity was up to 77-fold higher than that of polyamine oxidase. This enzyme preferred cadaverine over putrescine and it was active even under the saline condition. On the other hand, saline stress increased spermine and cadaverine levels, and the *in vivo* copper-containing amine oxidase activity in the elongation zone of hypocotyls. The last effect was negatively modulated by the addition of the copper-containing amine oxidase inhibitor N,N′-diaminoguanidine. In turn, plants treated with the inhibitor showed a significant reduction of reactive oxygen species in the elongation zone, even in the saline situation. In addition, plants grown in cadaverine-amended culture medium showed increased hypocotyl length either in saline or control conditions and this effect was also abolished by N,N′-diaminoguanidine. Taken together, our results suggest that the activity of the copper-containing amine oxidase may be partially contributing to hypocotyl growth under saline stress, through the production of hydrogen peroxide by polyamine catabolism and reinforce the importance of polyamine catabolism and hydrogen peroxide production in the induction of salt tolerance in plants.

Introduction

Plant cell expansion requires chemical loosening of cell walls, resulting in the relaxation of wall tension and concomitant water uptake (Cosgrove, 1999). It has been suggested that the action of reactive oxygen species (ROS) in the apoplast may involve a delicate balance between cleavage and cross-linking activities (Cosgrove, 1999). Recently, several authors reported that apoplastic ROS promote cellular elongation in maize coleoptile (Schopfer, 2001) maize leaf (Rodríguez et al., 2002) and *Arabidopsis* root (Demidchik et al., 2003; Foreman et al., 2003; Demidchik and Maathuis, 2007). Polyamines (PA) oxidation is a source of apoplastic H_2O_2 (Lim et al., 2006). These molecules are small organic polycations, naturally found in eukaryotic and prokaryotic cells, which have been associated with cell growth and development (Steiner et al., 2007). Putrescine (Put), spermidine (Spd) and spermine (Spm) are the most abundant PA in plant cells (Kaur-Sawhney et al., 2003). Cadaverine (Cad) is an uncommon polyamine in plants and it is mainly present in legumes (Smith and Wilshire, 1975). The biosynthesis of these compounds takes place only in symplastic subcellular localizations (Tiburcio et al., 1997). In turn, PA cross the plant cell membrane towards the apoplast *via* a still unknown mechanism (Cona et al., 2006a), where they are catabolized by amine oxidases (AO) found in this subcellular compartment (Šebela et al., 2001). The copper-containing amine oxidase (CuAO) catabolizes the oxidation of lower PA, such as Put and Cad on primary amino groups, whereas plant polyamine oxidase (PAO) oxidizes higher PA, Spd and Spm on their secondary amino groups (Federico and Angelini, 1991).

Salt stress hinders plant growth of several glycophytic species (Munns and Termatt, 1986) and induces changes in plant PA levels (Zapata et al., 2003; Maiale et al., 2004; Sannazzaro et al., 2007). Salinity provokes growth reduction in soybean hypocotyls (Aghaei et al., 2009). The hypocotyl is an amenable experimental system to study cell elongation mechanisms (Cosgrove and Durachko, 1994).

Abbreviations: 4-AAP, 4-aminoantipyrine; AO, amine oxidases; Cad, cadaverine; CuAO, copper-containing amine oxidase; DAG, N,N′-diaminoguanidine; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin-diacetate; DCHBS, 3,5-dichloro-2-hydroxybenzensulphonic acid; DW, dry weight; FW, fresh weight; PA, polyamines; PAO, polyamine oxidase; POX, peroxidase; Put, putrescine; ROS, reactive oxygen species; Spd, spermidine; Spm, spermine.

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Delis et al. (2006) described that the highest growth rate of etiolated hypocotyls in soybean was restricted to the apical segment of the hypocotyls. These authors also found a high activity level of enzymes involved in Put catabolism and expression of a tissue-specific gene coding for CuAO in this region. Soybean CuAO has an apoplastic localization and produces H_2O_2 by oxidation of Put and Cad (Gamarnik and Frydman, 1991) and a high concentration of the last polyamine Cad was found in hypocotyls of this species (Scoccianti et al., 1990). ROS production has shown to be involved in maize leaf elongation under salinity (Rodríguez et al., 2004; Taleisnik et al., 2009) and recently, it was reported that PA oxidation by PAO activity would be the main source contributing to that ROS production (Rodríguez et al., 2009). These authors showed that salt treatment increased the apoplastic Spm and Spd levels, mainly in the leaf blade elongation zone. Results suggested that in salinized plants, the oxidation of free apoplastic PA by PAO would be the main source of ROS in the elongation zone of maize leaf blades, probably due to increased substrate availability. In this work, we hypothesize that ROS produced as consequence of PA catabolism participate of the hypocotyl elongation of soybean plants, grown under salt-stress condition.

Materials and methods

Plant material and culture conditions

Seeds of soybean (*Glycine max* (L.) Merr cv DM 3700, Don Mario, Chacabuco, Argentina), kindly provided by Marcelo Benaglia, were sown on 250 mL pots containing perlite–sand mixture (1:1 v/v). One half of the pots were irrigated with half-strength Hoagland's nutrient solution (control treatment) and the remaining pots received Hoagland's solution amended with 80 mM NaCl (salt-stress treatment). Pots were kept at 25 °C under fluorescent and incandescent light bulbs providing 100 μmol photons m^{-2} s^{-1} illumination, with a 16 h photoperiod at 25 ± 2 °C and 65 ± 5% relative humidity.

Length measurements and determination of fresh and dry weights

The hypocotyl of each unsalinized and salinized 7 d old plant was isolated, scanned (AGFA Snapscan Touch, Agfa-Gevaert Group, Morstel, Belgium) and the resultant image digitalized. Hypocotyl length was measured with the software Optimas 6.1 (Optimas Corporation, Bothell, WA). Then, hypocotyls were weighed to determine fresh weight (FW) and dry weight (DW; 70 °C, 48 h). For the calculation of the hypocotyl growth rate, length measurements were obtained with a ruler in living plants, every 24 h during 9 d. Growth rate was calculated as the difference between two consecutive hypocotyl lengths measures.

Determination of Na^+ and K^+ contents

Hypocotyls sectioned from unsalinized and salinized 7 d old plants were frozen in liquid N_2 and homogenized. Homogenate (10 mg) was mixed with 1 mL HCl 0.1 N in an Eppendorf tube, thoroughly vortexed and incubated for 2 h at 60 °C. Then, the mixture was cooled during 20 min at 20 °C and centrifuged at (10,000 × *g*, 5 min). Pellet was discarded and 250 μL of the supernatant was diluted in 5 mL of distilled water. The resultant solution was used to determine Na^+ and K^+ content in a flame photometer (2655-00, Cole-Parmer Instrument Co., Chicago, IL).

Protein extraction for AO activity characterization

Pools of hypocotyls sectioned from unsalinized 7 d old plants were frozen in liquid N_2, homogenized in 1 mL extraction buffer (0.1 M potassium phosphate pH 7 and 1 mM phenylmethylsulphonyl fluoride) and centrifuged (15,000 x *g*, 15 min). Pellet was discarded and supernatant was kept at 4 °C. The resultant solution was additioned with NH_4SO_4 (114 g/L) and kept at 4 °C, 30 min. The solution was centrifuged (10,000 x *g*, 4 °C), the pellet discarded and the supernatant additioned with 340 g/L NH_4SO_4 and incubated during 30 min. The resultant solution was centrifuged once more (10,000 x *g*, 4 °C) and the supernatant discarded. The pellet was resuspended in 1 mL 0.1 M potassium phosphate pH 7 and dialyzed during 16 h at 4 °C (solution A).

Protein extraction to determinate the CuAO activity in the elongation zone

Proteins were extracted from segments of the elongation zone of hypocotyls isolated from unsalinized and salinized 5 d old plants. The hypocotyl elongation zone spans a 15 mm portion of the hypocotyl, starting 8 mm below the cotyledonary node (Bensen et al., 1988). Pools of segments from the elongation zone were frozen in liquid N_2, homogenized in 1 mL extraction buffer (0.1 M potassium phosphate pH 7 and 1 mM phenylmethylsulphonyl fluoride) and centrifuged (15,000 × *g*, 15 min). Pellet was discarded and supernatant was kept at 4 °C (solution B).

In vitro AO activity assay

AO activity was determined according to Maiale et al. (2008). The reaction mixture (1 mL) contained 50 μL of solution A or 20 μL of solution B (see above) and substrate/reactive solution (100 μM 4-aminoantipyrine (4-AAP), 1 mM 3,5-dichloro-2-hydroxybenzensulphonic acid (DCHBS), 0.06 mg mL^{-1} horseradish peroxidase (POX, EC 1.11.1.7) and 100 mM potassium phosphate pH 7.3). The mixture was incubated at 30 °C for 2 min and the reaction was started by adding Spd, Spm, Cad or Put. The activity was measured during 1 min at A_{515} with a spectrophotometer. Data was transformed into H_2O_2 molar concentration with molar extinction coefficient at 515 nm (2.6×10^4 M^{-1} cm^{-1}). The specific activity was calculated on the base of the protein content of each sample, determined according to Bradford (1976).

Extraction of free PA

To extract free PA, pools of hypocotyls isolated from unsalinized and salinized 7 d old plants were frozen in liquid N_2 and homogenized. The homogenate (300 mg) was resuspended in 1 mL of perchloric acid 5% (v/v), incubated on ice during 30 min and centrifuged at 15,000 × *g* for 15 min. Pellet was discarded and the supernatant kept at −20 °C (solution C).

Determination of free PA

Soybean free PA were derivatized with dansyl chloride and determined by HPLC according to Jiménez-Bremont et al. (2007). For the dansylation reaction, 200 μL of solution C (see above) was 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 and the dansyl-derived PA extracted with 500 μL toluene. Then, the organic phase (400 μL) was evaporated under vacuum and resuspended in 400 μL acetonitrile. 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, flow 1.5 mL/min as follows: 0–4.5 min, acetonitrile–H_2O 70:30; 4.5–9 min, acetonitrile 100; 9–15 min, acetonitrile–H_2O (70:30).

Peak areas were integrated, normalized to heptanodiamine and interpolated into a PA standards calibration curve.

In vivo CuAO activity

The CuAO (EC 1.4.2.22) activity level was determined in the elongation zone of hypocotyls isolated from unsalinized and salinized 5 d old plants, according to Cona et al. (2006b) with some modifications. Hypocotyl segments isolated from unsalinized and salt-treated plants were washed respectively in water or 80 mM NaCl during 6 min, in order to remove symplastic contamination. Pools of segments were introduced in 1 mL solution containing 100 μM 4-AAP, 1 mM DCHBS, 0.06 mg mL^{-1} horseradish POX and 20 mM potassium phosphate pH 7.3, plus or minus 25 μM Cad, 250 μM N,N′-diaminoguanidine (DAG) and 80 mM NaCl for saline treatment. Segments were subsequently infiltrated 2 min and further incubated 7 h at 30 °C. 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 H_2O_2 molar concentration with molar extinction coefficient at 515 nm (2.6×10^4 M^{-1} cm^{-1}). CuAO activities were calculated as the difference in produced H_2O_2 between treatments containing and lacking substrate.

Measurement of ROS release

Soybean plants were cultivated as explained before but half of the unsalinized and half of the salt-stress plants were subjected to 1 mM DAG. Then, 5 d old plants were embedded in DCFH-containing agar at 30 °C. Those plants that had been cultivated with DAG were embedded in agar additioned with 1 mM DAG. *In vivo* determination of ROS release along the hypocotyl was performed by a modification of the agar technique described by Schopfer et al. (2001). 2′,7′-dichlorofluorescin (DCFH)-containing agar was prepared by adding an appropriate volume of a 25 mM 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) ethanol solution to a 1% (w/v) agar solution in 20 mM phosphate buffer pH 6, to obtain a 50 μM DCFH-DA mixture. The mixture was heated to solubilize the agar. Epifluorescence was observed after 30 min with a microscope (Axiophot, Zeiss, Jena, Germany) with excitation filter BP 450-490 and emission filter LP 520. Images were taken with a video camera (SONY DXC-950P, Sony, Tokyo).

RNA extraction and cDNA synthesis

Total RNA was extracted from frozen segments of the elongation zone of hypocotyls isolated from unsalinized and salinized 5 d old plants. For this, a TRI reagent (Sigma) was used according to the manufacturer's instructions. First strand cDNA was obtained by employing the following mixture: 2 mg of total RNA, 0.5 mM dNTPs, 1 μL of moloney murine leukemia virus RT (200 units mL^{-1}; Promega), 5 mL 5× reaction buffer (250 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT, pH 8.3), 10 pmol of oligo(dT) primer (Biodynamics SRL), and distilled water up to a total volume of 25 μL. The reaction mixture was incubated at 37 °C for 1 h.

Analysis of gene expression by semi quantitative RT-PCR

PCR amplification was performed with 2 μL of the RT reaction as template, 4 μL of 10× Taq polymerase buffer (500 mM KCl, 100 mM Tris–HCl, 15 mM $MgCl_2$, pH 9), 0.05 mM dNTPs, 0.2 μL Taq polymerase (EC 2.7.7.7, 5 units mL^{-1}; Promega), and 12.5 pmol of specific primers in a total volume of 40 μL. The primers used were those described by Delis et al. (2006) for soybean copper amine oxidase 1 gene (*GmCuAO1* gene) Gen-Bank accession number CAE47488.1: GmCuAO1 (sense) 5′-CAGCATTGGTGTTTACCACG-3′, GmCuAO1 (antisense) 5′-GGCAGTTTCAACCTCAGTTG-3′ and for soybean ubiquitin gene (*GmUBQ* gene) GenBank accession number X13251: GmUBQ (sense) 5′-GGGTTTTAAGCTCGTTGT-3′, GmUBQ (antisense) 5′-GGACACATTGAGTTCAAC-3′. Amplification conditions for both genes were 25 and 28 cycles respectively (94 °C, 45 s; 60 °C and 55 °C respectively, 1 min; and 72 °C, 45 s) with final elongation at 72 °C for 7 min. Amplification products were visualized in ethidium bromide stained gels and equal loading of RNAs was confirmed by monitoring *GmUBQ* gene levels.

Table 1
Hypocotyl fresh weight (FW), dry weight (DW) and length in soybean plants, subjected to 0 or 80 mM NaCl.

Treatment (mM NaCl)	FW (g)	DW (g)	Length (mm)
0	0.18 ± 0.01a	0.021 ± 0.002a	46.1 ± 0.7a
80	0.12 ± 0.01b	0.011 ± 0.001b	39.2 ± 0.6b

Results are means ± SE ($n = 20$). Means with the same letter are not statistically different (Student's test; $P < 0.05$).

Analysis of gene expression by quantitative RT-PCR

To perform amplification reactions with each of the specific primers pairs described above, 1 μL of cDNA was used as template and 7.5 μL of FastStart Universal SYBR Green Master with ROX (ROCHE USA) per reaction. The reference GmUBQ gene was used as internal standard to normalize differences in template quantity. Quantitative PCR was carried out using a Stratagene Mx3005P Real Time qPCR System (LaJolla, Ca), with PCR cycling conditions as follows: 10 min at 95 °C, followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min. All reactions were checked for their dissociation curves. Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method according to Livak and Schmittgen (2001). The mean ΔCt from control (hypocotyls from unsalinized plants) was used to calculate ΔΔCt in all cases. Primer sequences were as described in the analysis of gene expression by semiquantitative RT-PCR.

Statistical analysis

Data were analyzed by one-way or two-way ANOVA and Tukey's test (Di Rienzo et al., 2002), using InfoStat (InfoStat 2007. InfoStat Group. Facultad de Ciencias Agropecuarias. Universidad Nacional de Córdoba. Version 1.1. Córdoba, Argentina). Data sets for which the parametric analysis could not be applied were subjected to Student's test.

Results

Growth measurement and Na^+ and K^+ contents

Saline stress (80 mM NaCl) reduced values in all growth parameters, FW, DW and length of soybean hypocotyls (Table 1). As expected, NaCl addition to the nutrient solution increased Na^+ accumulation and the Na^+/K^+ ratio in the hypocotyl (Fig. 1).

In vitro AO activity characterization

Hypocotyl cell-free extracts from unsalinized plants were used to determine H_2O_2 produced by PA oxidation *in vitro*, through an oxidative POX-dependent reaction that produces a pink adduct, measurable by spectrophotometry (Maiale et al., 2008). In the presence of Cad, AO activity was 77-fold higher than those observed when Spd or Spm were present, and 70% superior to that observed when Put was added as a substrate (Fig. 2A), indicating that CuAO (and not PAO) was the main active AO. To corroborate the CuAO involvement in H_2O_2 production, the competitive inhibitor of CuAO

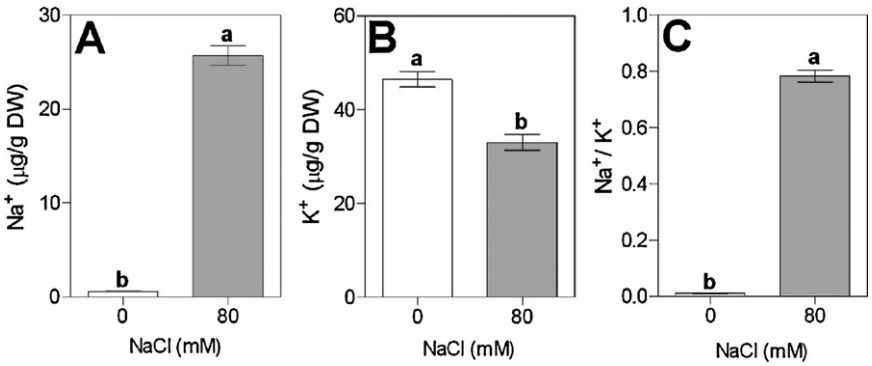

Fig. 1. Na^+ and K^+ contents and Na^+/K^+ ratio in soybean hypocotyls. White and grey bars represent unsalinized and salt treatment, respectively. The experiment was performed three times, yielding similar results. Results are means ± SE ($n = 20$). Bars with the same letter are not statistically different ($P < 0.05$).

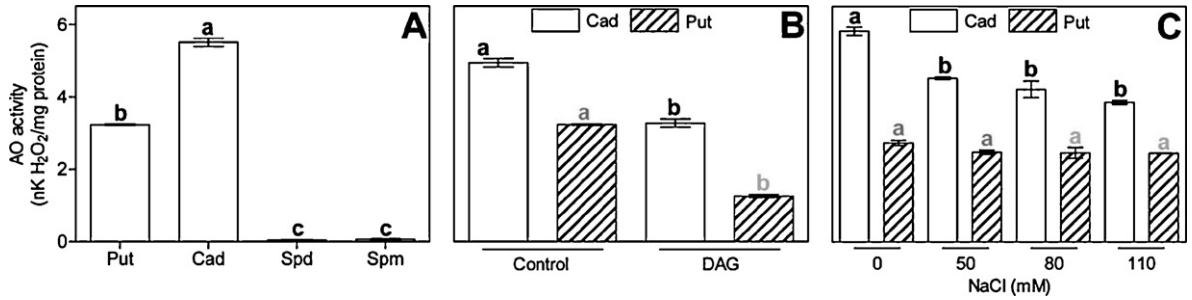

Fig. 2. *In vitro* AO activity characterization. AO were extracted from hypocotyls obtained from unsalinized plants according to the protein extraction protocol for AO activity characterization (see Materials and methods section). (A) CuAO and PAO activities were determined according to Maiale et al. (2008) in the presence of the AO substrates Put, Cad, Spd or Spm (1 mM), (B) CuAO activity in the presence of the CuAO inhibitor DAG (1 mM) and 1 mM Cad or Put. (C) CuAO activity under different NaCl concentrations, in presence of Cad or Put. The experiments were performed three times, yielding similar results. Results are means ± SE ($n = 4$). Bars with the same letter are not statistically different ($P < 0.05$).

DAG (Xing et al., 2007), was incorporated to the reaction mixture. As expected, there was a significant reduction in the AO activity in the presence of either Put or Cad (Fig. 2B). On the other hand, measurements of CuAO activity showed Km values equal to 0.19 and 0.02 mM for Put and Cad, respectively, evidencing that CuAO would have a higher affinity for Cad than for Put. Determinations of CuAO activity in presence of different NaCl concentrations showed that this enzyme remained active even at the highest NaCl concentration, with either of the two substrates (Put or Cad; Fig. 2C).

NaCl effects on free PA levels

Salinity caused a substantial reduction in Put and Spd levels in hypocotyls, being Put almost undetectable under stress conditions. In contrast, important increases of the Spm and Cad levels were originated by the saline treatment (Fig. 3). On the base that Cad was the most abundant PA under the saline condition, along with the fact that this substrate was preferred over Put by CuAO, Cad was used for the following experiments.

Salinity effect on in vitro and in vivo CuAO activities in the elongation zone

Hypocotyl growth rate was maximal at the fifth day after seeding, independently of the saline treatment. At that moment, a detrimental effect of salt on growth rate was detected (Fig. 4). To determine the relationship between the NaCl effect on hypocotyl growth and CuAO activity, proteins were extracted from the hypocotyl elongation zone of unsalinized or salinized 5 d old plants and CuAO activity was assayed *in vitro*. Results showed a 65% increased CuAO activity in extracts obtained from the elongation zone of saline-stressed hypocotyls, compared to that observed in the corresponding unsalinized control (Fig. 5A). CuAO activity was determined also *in vivo* by H_2O_2 production in presence of Cad, using segments from the elongation zone under the same conditions as before. Spectrophotometric measurement of pink adduct revealed an increase of CuAO activity due to the saline condition, in agreement with *in vitro* measurements. On the other hand, the addition of DAG to the reaction mixture, negatively modulated CuAO activity in the elongation zone of hypocotyl, regardless the saline condition (Fig. 5B).

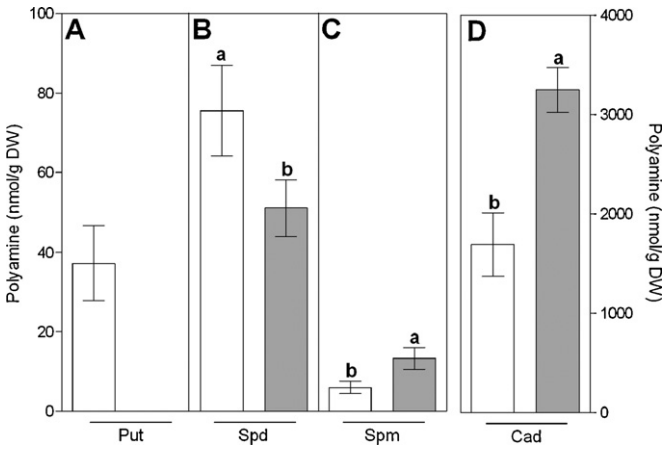

Fig. 3. Effect of NaCl on free PA levels in soybean hypocotyls. Pools of hypocotyls obtained from unsalinized (white) and salinized (grey) plants were homogenized and cell extracts used for PA measurements (A) Put, (B) Spd, (C) Spm and (D) Cad by HPLC. The experiment was performed three times, yielding similar results. Results are means ± SE ($n = 6$). Bars with the same letter are not statistically different ($P < 0.05$).

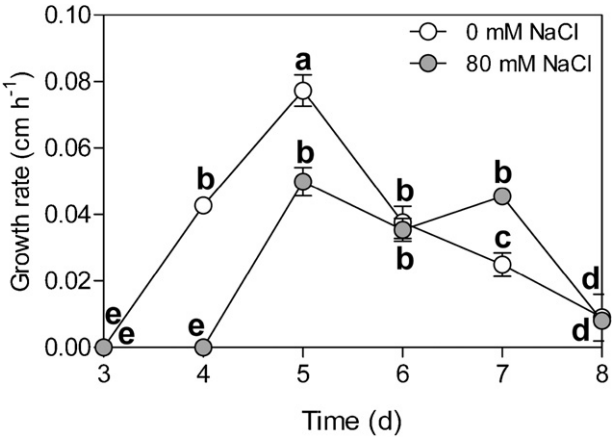

Fig. 4. Growth rate of soybean hypocotyls. Growth rate was calculated as the difference between two consecutive hypocotyl lengths measured every 24 h. The experiment was conducted three times, yielding similar results. Results are means ± SE ($n = 25$). Points with the same letter are not statistically different ($P < 0.05$).

ROS production in the elongation zone and hypocotyl elongation under saline stress

A commonly used reagent to detect ROS is DCFH, which is oxidized by ROS to the highly fluorescent 2′,7′-dichlorofluorescein (DCF; Schopfer et al., 2001). DCFH exhibits selectivity for H_2O_2 over other free radicals (Allan and Fluhr, 1997). The hypocotyl elongation zone from unsalinized plants showed DCF fluorescence evidencing ROS presence (Fig. 6), whereas the addition of DAG to the mixture reaction substantially reduced fluorescence, in accordance with the inhibition of H_2O_2 production observed *in vivo* (Fig. 5B). The same effect of DAG was observed on ROS production in the elongation zone of salt-treated hypocotyls (data not shown). It is worth mentioning the observation that the most intense fluorescence in the hypocotyl coincided with the elongation zone. In another experiment, DAG and different Cad concentrations were incorporated to the plant nutrient solution to test their effects on the hypocotyl length of intact plants. Interestingly, plants grown in cultures supplemented with 25–100 mM (unsalinized) or 25 mM (salinized) Cad, presented increased hypocotyl length (Fig. 7), whereas DAG abolished the elongation caused by this polyamine (Cad 25 μM + DAG treatment). Former results are in line with those obtained from CuAO activity (Fig. 5) and ROS production measurements in the elongation zone (Fig. 6B).

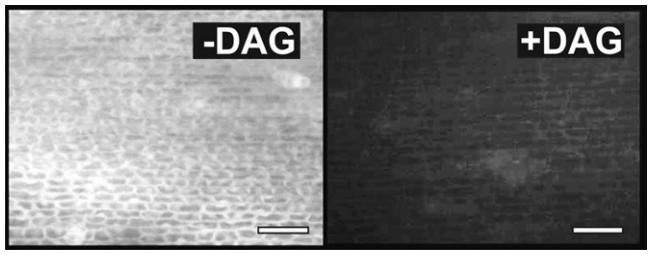

Fig. 6. Effect of DAG on ROS production in the elongation zone of the hypocotyl. Soybean seedlings were cultivated in absence or presence of 1 mM DAG. ROS presence is indicated by DCF fluorescence in brighter areas of epidermal tissue sections. White bars represent 50 μm.

Discussion

On the last decade, more evidence has been accumulated indicating that PA play an important role in plant response to abiotic and biotic stresses (Walters, 2003; Alcázar et al., 2006; Rodríguez-Kessler et al., 2008). On the other hand, CuAO and PAO activities modulate plant PA levels generating H_2O_2 among other products (Lim et al., 2006; Marina et al., 2008; Rodríguez et al., 2009). Since H_2O_2 functions as a signal molecule activating many of the plant responses deployed to cope with stress, it is believe that its generation from PA oxidation (along with PA depletion) might orchestrate (at least partially) plant adaptation to these conditions (Moschou et al., 2008; Rodríguez et al., 2009). Our first result showed that salt treatment reduced 15% the hypocotyls growth, in accordance with the observed higher Na^+ level and Na^+/K^+ ratio (Table 1, Fig. 1). The fact that CuAO activity was 77-fold higher that PAO activity (Fig. 2A), indicated that the first enzyme was the main active AO in soybean hypocotyl. This result agrees with Cona et al. (2006a), who reported that plant CuAO occurs at high levels in dicots, particularly in soybean seedlings and other legumes. Our data from the *in vitro* AO assessment, showing that CuAO remained active even under high salt concentration (Fig. 2C), suggests that this enzyme might be also active in soybean plants cropped under saline soil conditions. Such insensitivity to NaCl was previously observed in maize PAO (Rodríguez et al., 2009), moreover, this enzyme was stimulated by salinity in *Brassica campestris* (Das et al., 1995). In the present work, it was tested the hypothesis that ROS generated as consequence of PA catabolism participate of the hypocotyl elongation of salinized soybean plants. Four lines of evidence obtained from our data support that hypothesis: (1) salinity increased free Cad level, the preferred CuAO substrate (Fig. 3), (2)

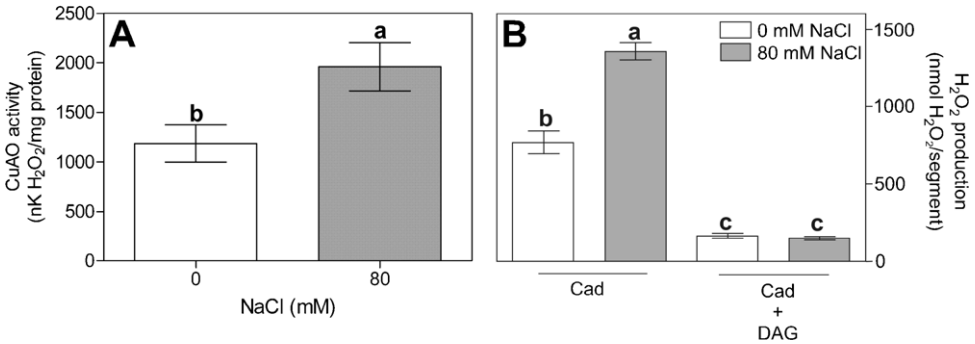

Fig. 5. *In vitro* and *in vivo* CuAO activities. (A) CuAO was extracted from segments of the elongation zone of untreated (white) and salt-treated (grey) hypocotyls according to the protein extraction protocol for determination of CuAO activity in the elongation zone (see Materials and methods section). *In vitro* CuAO activity was determined according to Maiale et al. (2008). (B) *In vivo* CuAO activity was measured in pools of segments of the elongation zone from untreated and treated hypocotyls and incubated in the presence of 25 μM Cad plus or minus 250 μM DAG and 80 mM NaCl (saline treatment) according to Cona et al. (2006b). The experiments were conducted three times, yielding similar results. Results are means ± SE ($n = 4$ in A; $n = 9$ in B). Bars with the same letter are not statistically different ($P < 0.05$).

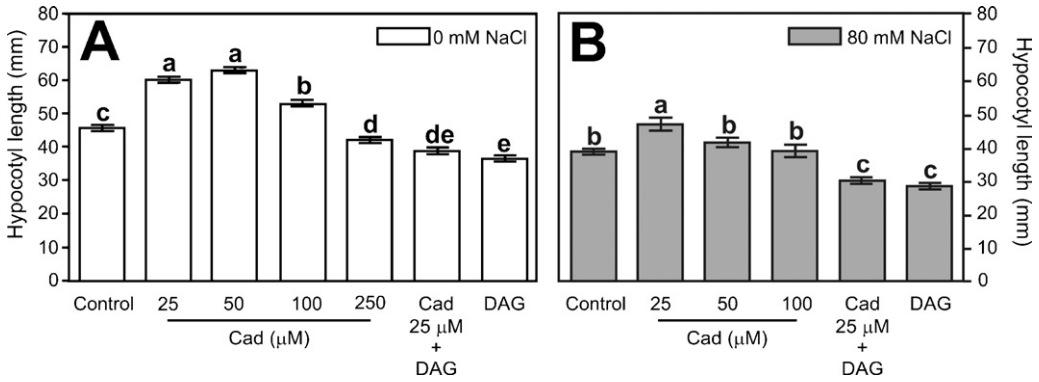

Fig. 7. Effects of different Cad concentrations and 1 mM of the CuAO inhibitor DAG on the hypocotyl elongation of intact soybean plants. Unsalinized (A) and salinized plants (B). The experiment was conducted three times, yielding similar results. Results are means ± SE ($n = 65$). Bars sharing the same letter are not statistically different ($P < 0.05$).

NaCl increased CuAO activity in segments of the hypocotyl elongation zone, measured as H_2O_2 production in the presence of Cad, in *in vitro* and *in vivo* experiments (Fig. 5), (3) ROS production in the hypocotyl elongation zone in intact plants was abolished when plants were grown in the presence of DAG, (Fig. 6) and (4) Cad (25 μM) induced an increment in the hypocotyl length of intact salinized plants, which was further abolished by DAG (Fig. 7B). The Cad-induced increment of the hypocotyl length of intact unsalinized plants and its abolition by DAG (Fig. 7A), confirmed previous studies, where most of the elongation rate in the hypocotyl was ascribed to a higher CuAO activity in that region (Delis et al., 2006). In addition, the difference in hypocotyl length observed between the untreated control and DAG treatment (either in presence or absence of Cad, Fig. 7) could be reflecting an inhibitory action of DAG on the oxidation of endogenous CuAO substrates, which would lead to reduced ROS production and hypocotyl elongation. Besides, our results showed that hypocotyl reaches the highest growth rate at day 5. These results, in addition to the fact that CuAO activity also reaches a maximum between the fourth and fifth day of germination (Gamarnik and Frydman, 1991), reinforces the view that cell elongation and AO activity are concomitant events, as suggested by several authors (Delis et al., 2006; Rodríguez et al., 2009). In soybean, two genes codify for CuAO, *GmCuAO1 and GmCuAO2* (Delis et al., 2006). *GmCuAO1* gene expression is predominantly found in tissues which are characterized by rapid extension growth, such as the apical segments of etiolated hypocotyls, while *GmCuAO2* gene seems not to be expressed in a tissue-specific manner. In our work, the expression of *GmCuAO1* gene was detected in the elongation zone but no change in the expression level of this gene was observed under salinity conditions (Supplemental data, Fig. S1). This result indicates that the higher CuAO activity observed under saline condition should not be attributed to a higher expression of this gene. Our results showed that salinity caused a significant increase not only in Cad but also in Spm level (Fig. 3) suggesting that the last polyamine could play a role in soybean response to saline treatment as well. The salt-induced decrease observed in Put and Spd levels, in parallel with the increase in total Spm level in soybean hypocotyls is in line with the Spm accumulation described by other authors in salt-treated rice (Maiale et al., 2004), several vegetables (Zapata et al., 2003), *Lotus glaber* (Sannazzaro et al., 2007), and maize (Jiménez-Bremont et al., 2007; Rodríguez et al., 2009). Altogether, our results reinforce the importance of PA catabolism and H_2O_2 production in the induction of salt tolerance in plants. This research area could be relevant, given the current scenario of expansion of soybean agriculture frontier to regions characterized by unfavorable soil conditions and the possibility of generating transgenic plants overexpressing or downregulating apoplastic polyamine oxidase (Moschou et al., 2008).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2011.01.007.

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