Environmental and Experimental Botany 145 (2018) 75–86

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Research Paper

Ethylene triggers salt tolerance in maize genotypes by modulating polyamine catabolism enzymes associated with H_2O_2 production

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ARTICLE INFO

Keywords:
Ethylene metabolism
Hydrogen peroxide
Polyamines
Salt stress
Zea mays

ABSTRACT

The current study was undertaken to investigate if there is a relationship between metabolisms of ethylene and polyamines in the processes of salinity acclimation of salt-tolerant and salt-sensitive maize genotypes. Biphasic ethylene production (at 5.5 and 12.5 h) was registered only in salt-sensitive plants during NaCl exposure. In the salt-tolerant genotype, the unique ethylene peak at 5.5 h was closely related to increased polyamine accumulation (a polyamine-dependent H_2O_2 signalling process), whereas the same did not occur in the salt-sensitive genotype. The absence of H_2O_2 signalling at 5.5 h in the salt-sensitive genotype was related to a burst in ethylene production at 12.5 h, known as 'stress ethylene', as well as a concomitant decrease in total polyamine content by salinity. The lack of stress ethylene synthesis in the salt-tolerant genotype was attributed to down-regulation of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) activity and *ZmACO5b* gene expression. Our findings suggest that ethylene is intimately involved in salt stress acclimation through activation of a complex pathway of signalling by H_2O_2 that is polyamine catabolism-dependent.

1. Introduction

Separately, ethylene and polyamines can act as regulators during plant growth and development as well as inducers of resistance against environmental stresses (Gupta et al., 2013; Lasanajak et al., 2014; Müller and Munné-Bosch, 2015). Ethylene and polyamines are biochemically related, since they share a common precursor in biosynthetic pathways, suggesting that alteration in ethylene production can affect polyamine homeostasis (Grzesiak et al., 2013).

Ethylene (C_2H_4) is a gaseous hormone that regulates diverse aspects of plant growth and development; it is considered a senescence hormone (Van de Poel and Van Der Straeten, 2014; Van de Poel et al., 2015). Recent evidence has shown that ethylene plays an important role in plant responses to abiotic stress, such as oxidative, ozone, salt and drought stress (Moeder et al., 2002; Wi et al., 2010; Habben et al., 2014). Contribution of ethylene to salt acclimation processes can vary with respect to type of response, including enhanced ethylene production and/or improved expression of ethylene receptors (Wu et al., 2008; Jiang et al., 2013; Zhai et al., 2013; Shi et al., 2015). However, how ethylene signalling and production are involved in the plant response to salinity is poorly understood.

Deletion of the *soil salinity tolerant1* (*sst1*) allele improved soil salinity tolerance of *Arabidopsis thaliana* via loss of ethylene overproducer1 (ETO1, whose normal function is to reduce ethylene production by degrading ethylene biosynthetic enzymes) function and enhancement of ethylene production (Jiang et al., 2013). In a different way, overexpression of the ethylene response factor (ERF) protein in tobacco transcriptionally regulates several genes responsive to osmotic and oxidative stress by a reactive oxygen species (ROS)-mediated regulatory pathway, alleviating harmful salt effects on photosynthetic carbon

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; ADC, arginine decarboxylase; DAO, diamine oxidase; MACC, 1-malonylaminocyclopropane-1-carboxylic acid; PAO, polyamine oxidase; Put, putrescine; ROS, reactive oxygen species; Spd, spermidine; Spm, spermine

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http://dx.doi.org/10.1016/j.envexpbot.2017.10.022
Received 26 December 2016; Received in revised form 28 October 2017; Accepted 29 October 2017
Available online 31 October 2017
0098-8472/

assimilation/metabolism (Wu et al., 2008). Also, tobacco plants over-expressing ERF7 from *Glycine max* displayed enhanced salt tolerance by low oxidative damage and increased levels of both chlorophyll and soluble carbohydrates (Zhai et al., 2013).

Although increased ethylene synthesis has been cited as a determinant for salt tolerance, other findings have shown that the reduction in ethylene production can display a positive role in salt tolerance of plants. Transgenic tobacco plants with poor ethylene biosynthesis showed elevated salt tolerance, which seems to occur through decreased ROS accumulation by up-regulating gene expression and activity of ROS-detoxifying enzymes (Wi et al., 2010). In the same way, an *A. thaliana ACS7*-knockdown mutant displayed reduced ethylene synthesis and improved salinity tolerance (Dong et al., 2011).

Some reports have suggested that ethylene and H_2O_2 may act as synergistically and self-amplifying signalling molecules in feed-forward loop regulation. Salt-tolerant *A. thaliana* callus and plants exhibited a close relationship between ethylene and H_2O_2 by inducing a cyanide-resistant alternative pathway (alternative oxidase), highlighting that ethylene could act mutually with H_2O_2 (Wang et al., 2010). In concordance with *A. thaliana* mutants, ethylene alleviated the harmful salt effects on germination by modulating the endogenous concentration of H_2O_2 in germinating seeds (Lin et al., 2012).

Polyamine catabolism-generated H_2O_2 also has been proposed to play an important role in plant defence against abiotic stress, functioning as a signalling molecule in crosstalk regulation (Campestre et al., 2011; Guo et al., 2014). In polyamine metabolism, H_2O_2 is originated from a reaction catalysed by diamine oxidase (DAO) and polyamine oxidase (PAO) localized mainly in the apoplast (Groppa and Benavides, 2008; Alcázar et al., 2010; Tavladoraki et al., 2012). In addition, others studies has shown that PAO can be found in vacuole, cytoplasm and peroxisomes (Cervelli et al., 2004; Tavladoraki et al., 2006; Moschou et al., 2008c). *Nicotiana tabacum* plants overexpressing the *pao* gene from *Zea mays* displayed a decreased polyamine content associated with H_2O_2 generation, which, in turn, up-regulated the antioxidant genes necessary for cell survival/adaptation (Moschou et al., 2008a). Nevertheless, the same transgenic plants showed ROS accumulation and oxidative damage higher than those of wild-type plants when subjected to abiotic stress. This response was attributed to polyamine secretion from cells to the apoplast and, after catabolism by PAO, higher ROS levels leaded to programmed cell death. Yet, in salt-stressed tobacco plants over-expressing (*S-pao*) or under-expressing (*A-pao*) PAO, Moschou et al. (2008b) reported an improved abundance of all transcripts involved in polyamine biosynthetic pathway in *S-pao* plants and a delay in up-regulation of the respective genes in *A-pao* plants. The authors suggested that the apoplastic ROS generation may induce either the expression of stress-responsive genes or programmed cell death syndrome, depending on the specific threshold.

Polyamine metabolism does not only increase H_2O_2 production to signalling levels but also provides a polyamine pool for defence purposes. Gong et al. (2014) demonstrated that tomato plants over-expressing S-adenosyl-L-methionine synthetase (*SlSAMS1*) displayed enhanced tolerance to alkali stress due to spermidine (Spd) and spermine (Spm) accumulation, ROS scavenging and greater photosynthetic performance compared to wild-type lines. In a later study, the authors found that the PAO-originated H_2O_2 is a downstream signal of *SlSAMS1*-conferred alkali stress tolerance (Gong et al., 2016).

Despite such clear demonstration of a crucial role of polyamines, ethylene and H_2O_2 in plant growth, development and stress responses, crosstalk between polyamines and ethylene under salt stress as well as ethylene-mediated signalling in salinity tolerance are poorly understood. In this study, we explored the ethylene and polyamine metabolisms in two salt-contrasting maize genotypes to investigate their relationship in salinity tolerance of plants. We found that the ethylene production acts as a signal of salt tolerance/sensitivity in maize plants. Our data point to a role of polyamine catabolism-dependent H_2O_2 as a link between ethylene and polyamine metabolism in plant salt responses.

2. Materials and methods

2.1. Plant material and growth conditions

Zea mays L. seeds of salt-sensitive (BR5011) and salt-tolerant (BR5033) genotypes (Azevedo Neto et al., 2004) were germinated in distilled water-moistened filter paper in a growth chamber with a 12-h photoperiod (25 ± 3 °C night/day) for 7 days. Thereafter, ten uniform seedlings were transferred to 10-L trays containing half-strength Hoagland nutrient solutions (Hoagland and Arnon, 1950). After thirteen days, the plants were transferred to 5-L plastic pots (one plant per pot) and subjected to saline treatment with NaCl at 0 (control) and 80 mM NaCl (salt stress). Fresh leaf and root (for ethylene measurement) tissues were harvested for analysis in different times (from 0 to 72 h) depending on assay. The experiment was carried out in a greenhouse and the environmental conditions were as follows: a mean air temperature of 28 ± 3 °C, a mean air relative humidity of 65 ± 5%, an average midday photosynthetic photon flux density of 1200 μmol m^{-2} s^{-1}.

2.2. Ethylene measurement

Ethylene production was determined by gas chromatography coupled to mass spectrometer (GCMS) (QP2010, Shimadzu, Tokyo, Japan). Leaf or root samples of control and salt-treated plants were enclosed in vials containing water-moistened filter papers for 1 h. Thereafter, 1.0 mL of headspace gas was injected in split mode (1:500), using helium as a carrier gas at a constant flow of 0.86 mL min^{-1} on an RTX-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Restec, Bellefonte, USA). The oven temperature was overall constant at 60 °C for 3 min, and the temperatures of the injector, interface and ion source were, respectively, 150, 200 and 200 °C. Each sample was run at least three times by GCMS, and the ethylene concentrations were quantified with reference to a standard curve.

2.3. Ethylene metabolites analyses

Total and free 1-aminocyclopropane-1-carboxylic acid (ACC) contents were extracted from fresh leaves and quantified by GCMS according to the Lizada and Yang (1979) procedure, which was recently updated by Bulens et al. (2011). The amount of conjugated ACC (MACC) was calculated by subtracting the amount of free ACC from that of the total ACC.

2.4. Ethylene biosynthesis enzyme activities

ACC synthase (ACS) and ACC oxidase (ACO) activities in leaves were measured using GCMS as described by Bulens et al. (2011). Incubation time, optimum temperature, optimum pH and substrate concentration were optimised to provide linear rates. Enzyme activities were expressed as nanomoles of ethylene per hour per milligramme of protein. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

2.5. Measurement of free, conjugated and bound polyamines

For extraction of polyamines, samples from fresh leaves were homogenised in 5% (v/v) perchloric acid (PCA) at 4 °C on an orbital shaker for 1 h and then centrifuged at 27,000 × *g* at 4 °C for 30 min. The same procedure was repeated twice. Free polyamines were derived with benzoyl chloride and directly measured in the homogenate by high performance liquid chromatography (HPLC) (Shimadzu, Tokyo, Japan). In parallel, the homogenate and pellet were individually hydrolysed with 12 M hydrochloric acid (HCl) and heated at 110 °C for 18 h in

flame-sealed glass ampules to liberate soluble and insoluble bound polyamines, respectively. After acid hydrolysis, the homogenate was filtered through microfilters (PVDF, 22 mm, 0.22 μm); the HCl was evaporated by heating at 70 °C and the precipitate resuspended in 5% PCA. Polyamines recovered from the non-hydrolysed supernatant (free polyamines), the hydrolysed supernatant (free + soluble conjugated polyamines) and the pellet (bound polyamines) were derived with benzoyl chloride (Duan et al., 2008) and further measured by HPLC (Shimadzu, Tokyo, Japan). Polyamines benzoyl-derivatives were separated on a reverse-phase C18 column at 30 °C (Shim-pack, CLC−ODS, 150 mm × 4.6 mm) with a mobile phase composed of methanol:water (64:36) at a flux rate of 1.0 mL min^{-1}. Putrescine (Put), Spermidine (Spd) and Spermine (Spm) peaks were detected at 240 nm in a SPD-20A detector (Shimadzu, Tokyo, Japan) and determined from reference standard curves. Soluble conjugated polyamine contents were determined by subtracting soluble polyamines from free polyamines.

2.6. Polyamine enzyme activities

Arginine decarboxylase (ADC) activity was determined according to methods of Duan et al. (2008), with some modifications. Fresh leaves were homogenised in 100 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM phenylmethylsulphonyl fluoride, 40 μM pyridoxal phosphate (PLP), 5 mM dithiothreitol (DTT), 5 mM ethylene diamine tetraacetic acid (EDTA), 20 mM ascorbic acid and 0.1% polyvinylpyrrolidone at 4 °C. The homogenate was filtered through cheesecloth and centrifuged at 12,000 × *g* for 40 min at 4 °C. The reaction (containing 100 mM tris-HCl buffer (pH 7.5), 40 μM PLP, 5 mM DTT, 5 mM EDTA, 40 mM L-arginine and the sample) was carried out at 37 °C for 1 h and stopped with 0.5 mL of 20% (v/v) trichloroacetic acid. The reaction mixture was then centrifuged at 12,000 × *g* for 10 min. ADC activity was assayed by measuring agmatine (Agm) produced in the reaction, which was initially derived with N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) and further determined by GCMS. One microliter of silyl-derivative samples was injected in split mode (1:25) using helium as a carrier gas at a constant flow of 1.48 mL min^{-1} on an RTX-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Restec, Bellefonte, USA). The oven temperature was programmed to change in the following way: maintain at 150 °C for 2 min, raised 4 °C min^{-1} to 165 °C, raised 2 °C min^{-1} to 220 °C and, finally, raised 4 °C min^{-1} to 280 °C and hold for 7 min. The temperatures of the injector, interface and ion source were, respectively, 150, 200 and 200 °C. Each sample was run at least three times by GCMS.

Diamine oxidase (DAO) and polyamine oxidase (PAO) activities were estimated spectrophotometrically based on the colourimetric assay of Δ-pyrroline by reading the absorbance at 430 nm (Holmstedt et al., 1961). Fresh leaves were homogenised in 100 mM potassium phosphate buffer (pH 7.0) containing 5 mM DTT at 4 °C. The homogenate was centrifuged at 16,000 × *g* for 20 min at 4 °C. The supernatant was used to measure the activities of soluble DAO and PAO. To assay the conjugated DAO and PAO activities, the enzymes were first solubilised with 100 mM potassium phosphate buffer (pH 7.0) containing 20 mM EDTA for 10 min at 4 °C and then centrifuged at 16,000 × *g* for 20 min at 4 °C. The reaction mixture was composed of the sample and 50 mM potassium phosphate buffer (pH 7.5 for DAO or 6.0 for PAO), 50 units of catalase and 0.1% 2-aminobenzaldehyde. Substrates used in the activity reactions were 10 mM putrescine for DAO and 5 mM spermidine + 5 mM spermine for PAO. The reaction was carried out at 36 °C for 1 h, stopped with 1.0 mL of 10% PCA and centrifuged at 5,000 × *g* for 15 min. Control reactions were conducted with inactivated enzymes prepared by heating for 20 min at 70 °C. PAO and DAO activities were also measured in presence of 100 μM aminooxyacetic acid (AOA) (a pharmacological inhibitor of ethylene synthesis). Protein contents were determined according to the Bradford method (Bradford, 1976).

2.7. H_2O_2 production

Hydrogen peroxide (H_2O_2) content was extracted according to methods of Cheeseman (2006) and determined by monitoring the absorbance of potassium iodide at 390 nm following the method Sergiev et al. (1997). The H_2O_2 content in maize leaves was calculated using a standard curve of known as H_2O_2 concentrations.

2.8. Detection of H_2O_2 by confocal laser scanning microscope (CLSM)

Free hand sections of fully-expanded leaves from maize plants grown under control and 80 mM-NaCl stress, in absence and presence of 100 μM AOA, were incubated on a solution of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma Aldrich) at 50 μM on PBS buffer for 10 min. Then, the sections were washed with PBS buffer and immediately observed under illumination Ar laser (λ = 488 nm). Excitation signals were collected between 500 and 580 nm. Control images were taken only with chlorophyll B auto fluorescence under illumination with the HeNe laser (λ = 633 nm), collecting the excitation signals between 638 and 721 nm. Digital images were acquired using a 20 × magnification objective lens and 3D reconstruction of the serial images was obtained by CLSM with Carl Zeiss LSM software (ZEN 2012).

2.9. RNA extraction

Plants were harvested at 0, 2.5, 5.5, 7.5, 10.5 and 12.5 h after 80 mM NaCl exposure. Total RNA was extracted from leaves using a commercial kit (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol. To avoid genomic DNA contamination, an additional on-column DNase digestion step was performed using an RNase-Free DNase Set (Qiagen, Hilden, Germany). Effectiveness of the DNase treatment was confirmed in real-time quantitative PCR (qPCR) analysis using RNA samples as templates. Total RNA was quantified in a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). The RNA purity and integrity were checked by analysing the $A_{260}/_{280}$ and $A_{260}/_{230}$ ratios, respectively, as well as visualising the ribosomal RNA bands on 1.5% agarose gel stained with ethidium bromide. First-strand cDNA was synthesised employing the ImpromII kit (Promega, Madison, USA) according to the manufacturer's protocol.

2.10. Gene annotation and classification

The genes encoding ACS, ACO, ADC, DAO and PAO were first identified through an extensive search in the available public databases, the National Center for Biotechnology Information (NCBI) and Phytozome, for the maize genome. Gene annotation (identification of exons/introns or open reading frames) was done manually for all sequences, comparing the identified sequences against annotated ACS, ACO, ADC, DAO or PAO cDNAs from other species (for instance, *A. thaliana* and *Oryza sativa*). Also, a phylogenetic classification was performed by comparing the maize genes/proteins with those of other crops (data not shown).

2.11. qPCR assay

Real-time quantitative PCR (qPCR) was carried out on a Mastercycler ep realplex 4S (Eppendorf, Hamburg, Germany) using as an indicator the Power SYBR-Green PCR Master Mix (Applied Biosystems). The relative expression was normalised through qbase PLUS software version 1.5 (Biogazelle) (Vandesompele et al., 2002), using three reference genes (*EF1α*, *GAPDH* and *PP2A*) selected from eight tested genes (*EF1α*, *EF1β*, *EF1d*, *GAPDH*, *GTPB*, *PP2A*, *SAND* and *UBC18*). Specific primers for qPCR analyses were designed based on sequences deposited in NCBI and Phytozome databases (Supplementary

Table S1) using variable regions corresponding to exon–exon junctions, where possible. Nevertheless, we presented the relative expression only from the genes highly expressed in leaves and responsive to studied treatments (Supplementary Table S2).

2.12. Statistical analysis

Experimental design was completely randomised in a 2 × 2 factorial scheme, consisting of two maize genotypes (salt-tolerant BR5033 and salt-sensitive BR5011) and two salinity levels (0 and 80 mM NaCl). The number of replications varied depending on analysis, comprising three replications for ethylene measurement and qPCR assays; and five replications for biochemical and enzymatic assays. For all case, an individual plant was considered one repetition. The data were subjected to a two-way analysis of variance (ANOVA), and the mean values were compared through Tukey's test at $p \leq 0.05$, using SigmaPlot® software (version 11.0, Systat Software, Inc., London, UK). All data are given as means + standard error (SE).

3. Results

3.1. Ethylene production under salinity

Stimulation of ethylene biosynthesis in response to salt stress treatment in plants is well documented. To investigate whether ethylene could be involved in acclimation of *Z. mays* plants to salinity, patterns of ethylene production in leaves and roots of salt-tolerant and salt-sensitive genotypes were monitored. Ethylene production was induced in a biphasic manner only in the leaves of the salt-sensitive genotype (Fig. 1A). Firstly, ethylene production significantly increased 0.5 fold at 5.5 h of salinity and then declined to a basal level (phase I). Secondly, after 12.5 h of salt exposure, a massive increase of 4.6 fold was detected (phase II), after which ethylene levels gradually declined (Fig. 1A). In the salt-tolerant genotype, phase I of ethylene synthesis occurred in a similar manner as the salt-sensitive genotype, while phase II was not observed (Fig. 1B). In the roots, salt stress did not alter ethylene synthesis in the salt-tolerant genotype, but it induced biphasic ethylene production in the salt-sensitive genotype (Fig. 1C, D), with a much smaller intensity than that in the leaves (Fig. 1A).

3.2. Ethylene metabolism

To investigate how ethylene production is differentially affected in the salt-contrasting maize genotypes, the accumulations of ACC and MACC and the activities of ACS and ACO in the leaves were measured at 5.5 and 12.5 h of salinity. In general, ACC content in the salt-sensitive genotype was higher than that in the salt-tolerant genotype (Fig. 2A). Moreover, the enhancement of salt-induced ACC content was greater at 5.5 h, irrespective of maize genotype (Fig. 2A). The MACC content was slightly diminished by salt stress in the salt-tolerant genotype (Fig. 2B), whereas in the salt-sensitive genotype it decreased by 91% at 5.5 h, suggesting that MACC could be mobilised back into ACC (Fig. 2A, B).

Under salt stress, ACS activity increased at 5.5 h in both genotypes; a phenomenon similar to that was observed regarding ACC content (Fig. 2A, C). At 12.5 h, an increase was observed only for the salt-sensitive genotype (Fig. 2C). In the salt-tolerant genotype, the ACO activity increased in response to salinity at 5.5 h but decreased remarkably (32%) at 12.5 h (Fig. 2D). On the other hand, in the salt-sensitive genotype, salt stress did not affect the ACO activity at 5.5 h but did promote a sharp increase at 12.5 h. In addition, the activity of ACO was more prominent at 12.5 h compared to 5.5 h (Fig. 2D), a time point corresponding to the synthesis of 'stress ethylene'.

In order to evaluate if biphasic ethylene biosynthesis during salt stress is under transcriptional regulation, expression profiles of all *ACS* and *ACO* gene members in *Z. mays* were studied by qPCR (Fig. 3). The *Z. mays* genome has four *ACS* genes (named *ZmACS1a*, *ZmACS1b*, *ZmACS2* and *ZmACS3*), but only the *ZmACS1a* isoform was found to be expressed in leaves. Yet, ACO is encoded by a large family of 13 gene

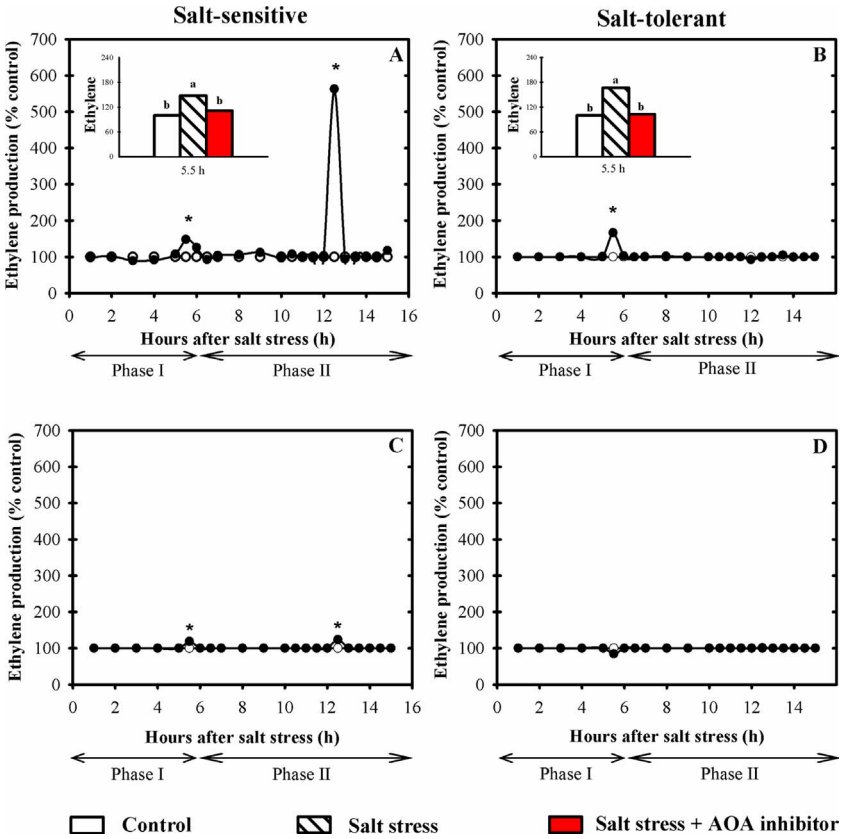

Fig. 1. Kinetics of ethylene production in leaves (A and B) and roots (C and D) of salt-sensitive and salt-tolerant maize genotypes grown in nutrient solutions in the absence (open circle) and presence (close circle) of 80 mM NaCl stress. Harvests were done at 30-min intervals for 72 h after salt exposure, using three replications (one plant per repetition) for each treatment. The values are given as the means of at least six independent experiments. An asterisk indicates a significant difference between the control and salt treatments ($p \leq 0.05$), according to Tukey's test. More statistical details in Supplementary Table S3. The inset figures in A and B show the ethylene production in presence of 100 µM aminooxyacetic acid (AOA), an ethylene biosynthesis inhibitor. In this case, significant differences due to inhibitor treatment are denoted by different lower case letters using Tukey's test ($p \leq 0.05$).

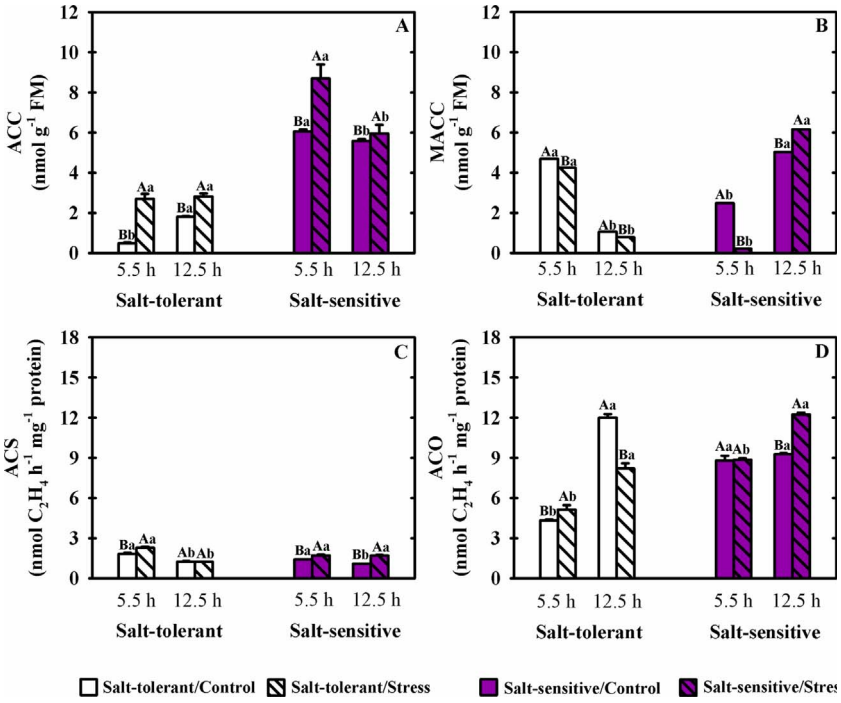

Fig. 2. Ethylene biosynthetic metabolism in leaves of salt-sensitive and salt-tolerant maize genotypes grown in nutrient solutions in the absence (control) and presence (stress) of 80 mM NaCl. (A) Measurements of free and (B) conjugated ACC (MACC) content as well as (C) ACS and (D) ACO activities were done at the times corresponding to phase I (5.5 h) and phase II (12.5 h) of ethylene production. The data are the means + SE of five replications (n = 5). In the same maize genotype and harvest time, significant differences due to salt stress (control × stress) are indicated by different capital letters; whereas, in the same salt treatment, significant differences due time of salinity (5.5 h × 12.5 h) are denoted by different lowercase letters, according to Tukey's test ($p \leq 0.05$).

members (named *ZmACO1a*, *ZmACO1b*, *ZmACO1c*, *ZmACO1d*, *ZmACO2a*, *ZmACO2b*, *ZmACO3*, *ZmACO4a*, *ZmACO4b*, *ZmACO5a*, *ZmACO5b*, *ZmACO5c* and *ZmACO5d*); however, only the *ZmACO5b* gene was responsive to saline treatment.

ZmACS1a gene expression was down-regulated by salinity at 5.5, 7.5 and 12.5 h in the salt-tolerant genotype, whereas it was down-regulated at 2.5 and 12.5 h in the salt-sensitive one (Fig. 3A). Remarkably, *ZmACS1a* transcript abundance was strongly up-regulated by NaCl stress at 5.5 and 10.5 h in the salt-sensitive genotype (Fig. 3A). These results were partially consistent with the increases of ACC content and ACS activity at 12.5 h (Fig. 2A, C).

At 2.5 h, salt stress strongly up-regulated *ZmACO5b* gene expression by 33 fold in the salt-tolerant maize genotype, whereas expression was down-regulated at 10.5 h. Interestingly, the first burst of *ZmACO5b* expression was delayed about 3 h in phase I of ethylene production, suggesting the occurrence of transcriptional regulation. In the salt-sensitive genotype, salinity did not change the *ZmACO5b* transcript levels at 5.5 h but did induce an up-regulation at 12.5 h (Fig. 3B), which was consistent with phase II of ethylene production (Fig. 1A).

3.3. Polyamine metabolism and polyamine-dependent H_2O_2-generation process

To further investigate a possible connection between polyamine metabolism and ethylene production, the contents of Put, Spd and Spm (free, soluble conjugated and insoluble bound), activities of ADC, DAO and PAO and corresponding gene expression were measured in salt-contrasting maize genotypes. Herein, Put was the most abundant free polyamine, irrespective of salinity and genotype analysed (Table 1). Salt stress significantly increased the free Spd and Spm polyamines in the salt-tolerant genotype, whereas it reduced the free polyamine accumulation in the salt-sensitive genotype, with the exception of Spd (at 5.5 h) and Spm (at 12.5 h) (Table 1). Soluble conjugated polyamine accumulation varied according to salinity and genotype studied. At 5.5 h, the soluble conjugated Spm significantly increased in the salt-tolerant genotype, while Put and Spd showed a strong decrease in the salt-sensitive one. On the other hand, salt stress increased all soluble conjugated polyamines in both genotypes at 12.5 h, with the exception of Put of the salt-tolerant genotype (Table 1). Also, with exception of insoluble bound Spm, which increased due to salinity, no significant difference by NaCl stress was observed for insoluble bound Put and Spd contents in the salt-tolerant genotype, irrespective of analysed time. In general, the insoluble bound polyamines of the leaves of the salt-sensitive genotype significantly increased under salt stress.

In the salt-tolerant genotype, salinity caused a significant increase of 31% in the total polyamine content (Put + Spd + Spm) at 5.5 h, while no significant alteration was observed at 12.5 h (Table 1). In contrast, salinity caused a significant decrease in the salt-sensitive genotype at both time points. Under salinity, the salt-tolerant genotype exhibited a total polyamine content greater than that of the salt-sensitive genotype (Table 1), suggesting that not only the individual form but also the balance among free, conjugated and bound polyamines might be crucial

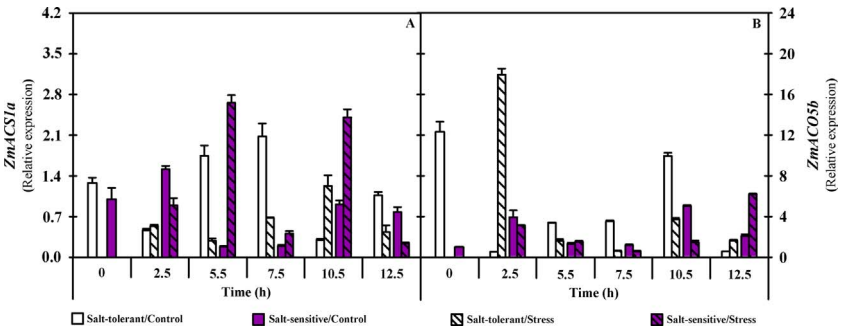

Fig. 3. Relative mRNA expression levels of genes involved in ethylene biosynthesis in leaves of salt-sensitive and salt-tolerant maize genotypes grown in nutrient solutions in the absence (control) and presence (stress) of 80 mM NaCl. (A) The relative expression of *ZmACS1a* and (B) *ZmACO5b* was quantified by qPCR using as reference three housekeeping genes (*EF1α*, *GAPDH* and *PP2A*). Values represent the means + SE of three biological replications. Statistical details are shown in Supplementary Table S4.

Table 1
Free, soluble conjugated and insoluble bound polyamine accumulation (putrescine − Put, spermidine − Spd and spermine − Spm), total polyamine content and (Spd + Spm)/Put ratio in leaves of salt-sensitive and salt-tolerant maize genotypes grown in nutrient solutions in absence (control) and presence (stress) of 80 mM NaCl. Analyses were done at the times corresponding to phase I (5.5 h) and phase II (12.5 h) of ethylene production. The data are the means of five replications (n = 5). In each column and maize genotype, in the same harvest time, significant differences due to salt stress (control × stress) are indicated by different capital letters; whereas, in the same salt treatment, significant differences due time of salinity (5.5 h × 12.5 h) are denoted by different lowercase letters, according to Tukey's test ($p \leq 0.05$).

Time of salinity	Treatment	Salt-tolerant			Salt-sensitive		
		Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
				Free (μmol g^{-1} FM)			
5.5 h	**Control**	$26,24^{Aa}$	$5,62^{Ba}$	$1,23^{Ba}$	$24,09^{Aa}$	$3,09^{Ab}$	$0,67^{Aa}$
	Stress	$27,86^{Aa}$	$14,65^{Aa}$	$1,64^{Aa}$	$15,06^{Ba}$	$3,10^{Aa}$	$0,50^{Ba}$
12.5 h	**Control**	$21,38^{Ab}$	$4,78^{Bb}$	$0,24^{Bb}$	$24,09^{Aa}$	$7,68^{Aa}$	$0,39^{Ab}$
	Stress	$16,10^{Bb}$	$7,53^{Ab}$	$0,82^{Ab}$	$16,43^{Ba}$	$3,05^{Ba}$	$0,38^{Ab}$
				Soluble conjugated (μmol g^{-1} FM)			
5.5 h	**Control**	$0,98^{Ab}$	$0,12^{Bb}$	$0,01^{Bb}$	$1,61^{Aa}$	$1,01^{Ab}$	$0,02^{Aa}$
	Stress	$1,00^{Ab}$	$0,18^{Ab}$	$0,02^{Aa}$	$1,22^{Bb}$	$0,23^{Bb}$	$0,02^{Ab}$
12.5 h	**Control**	$1,63^{Aa}$	$0,69^{Ba}$	$0,01^{Ba}$	$1,36^{Bb}$	$1,31^{Ba}$	$0,01^{Bb}$
	Stress	$1,39^{Ba}$	$1,89^{Aa}$	$0,02^{Ab}$	$1,50^{Aa}$	$1,66^{Aa}$	$0,03^{Aa}$
				Insoluble bound (μmol g^{-1} FM)			
5.5 h	**Control**	$1,07^{Aa}$	$0,76^{Ab}$	$0,03^{Ba}$	$0,51^{Ba}$	$0,29^{Bb}$	$0,04^{Bb}$
	Stress	$1,04^{Aa}$	$0,76^{Ab}$	$0,04^{Aa}$	$1,06^{Aa}$	$0,46^{Ab}$	$0,09^{Aa}$
12.5 h	**Control**	$0,89^{Ab}$	$1,04^{Aa}$	$0,02^{Bb}$	$0,49^{Ba}$	$0,45^{Ba}$	$0,05^{Aa}$
	Stress	$0,95^{Ab}$	$1,04^{Aa}$	$0,02^{Ab}$	$0,86^{Ab}$	$0,56^{Aa}$	$0,01^{Bb}$
	Total polyamine (μmol g^{-1} FM)		**(Spd + Spm)/Put**		**Total polyamine** (μmol g^{-1} FM)	**(Spd + Spm)/Put**	
5.5 h	**Control**	$36,05^{Ba}$	$0,27^{Ba}$		$31,83^{Ab}$	$0,21^{Bb}$	
	Stress	$47,23^{Aa}$	$0,58^{Ab}$		$20,75^{Bb}$	$0,27^{Ab}$	
12.5 h	**Control**	$30,03^{Ab}$	$0,28^{Ba}$		$35,94^{Aa}$	$0,39^{Aa}$	
	Stress	$29,78^{Ab}$	$0,61^{Aa}$		$24,59^{Ba}$	$0,31^{Ba}$	

for stress tolerance in maize. Notably, the (Spd + Spm)/Put ratio in the salt-tolerant genotype was significantly higher under salt stress than the control, while no conspicuous effects were observed for the salt-sensitive genotype (Table 1).

In plants, although Put can be synthesised by ornithine (ODC) and arginine decarboxylase (ADC), ADC is considered the main enzyme of biosynthesis in maize plants under salt stress. Thus, to establish a link between polyamine content and tolerance to salt stress in *Z. mays*, ADC activity and gene expression were measured. In the salt-tolerant genotype, salt stress both decreased and increased the ADC activity at 5.5 and 12.5 h, respectively (Fig. 4), although the stress did not result in a direct effect on the free Put content (Table 1). In contrast, in the salt-sensitive genotype, no significant difference in ADC activity (Fig. 4) was observed by salinity treatment, while the free Put content decreased at both analysed times (Table 1).

Two *ADC* genes are found in the *Z. mays* genome (*ZmADC1* and

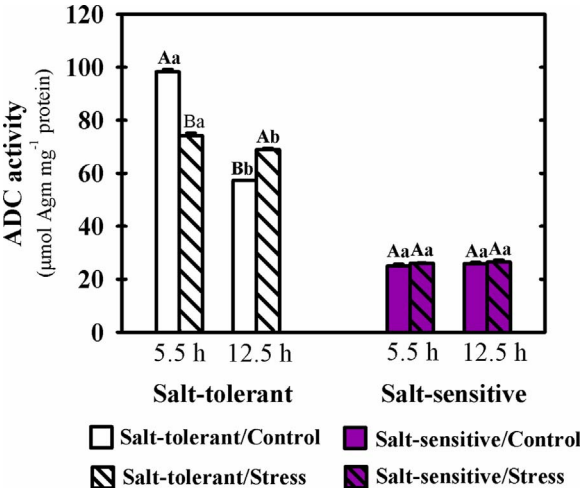

Fig. 4. Activity of arginine decarboxylase (ADC) in leaves of salt-sensitive and salt-tolerant maize genotypes grown in nutrient solutions in the absence (control) and presence (stress) of 80 mM NaCl, at 5.5 and 12.5 h after salt exposure. Statistical details same as in Fig. 2.

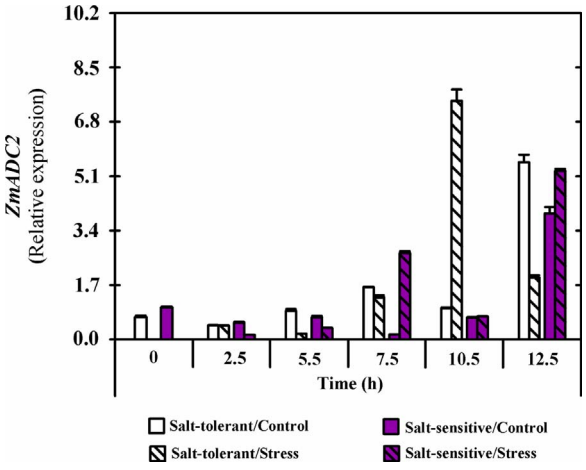

Fig. 5. Relative expression levels of polyamine biosynthetic genes in leaves of salt-sensitive and salt-tolerant maize genotypes grown in nutrient solutions in the absence (control) and presence (stress) of 80 mM NaCl. The relative expression of *ZmADC2* was quantified by qPCR, using as reference three housekeeping genes (*EF1α*, *GAPDH* and *PP2A*). Values represent the means + SE of three biological replications. Statistical details are shown in Supplementary Table S4.

ZmADC2), but only *ZmADC2* is highly expressed in maize leaves. Under salinity, a drastic up-regulation of *ZmADC2* was observed at 10.5 h for the salt-tolerant genotype (Fig. 5), which was consistent with elevation of ADC activity at 12.5 h (Fig. 4). In contrast, although the *ZmADC2* transcript abundance was up-regulated in the salt-sensitive genotype at 12.5 h, it was not correlated with the ADC activity.

We also performed a functional and transcriptional trait involving DAO and PAO to investigate whether polyamine catabolism contributes to intra- and/or intercellular signalling through ROS generation and whether this mechanism triggers the initial responses of salt stress acclimation. Soluble and conjugated DAO activities of the salt-tolerant genotype were significantly higher under salt stress than the respective controls, with the exception of conjugated DAO activity at 12.5 h (Fig. 6A, B). Surprisingly, at 5.5 h, the increased enzyme activity was closely correlated with increased H_2O_2 production (Fig. 7). Otherwise,

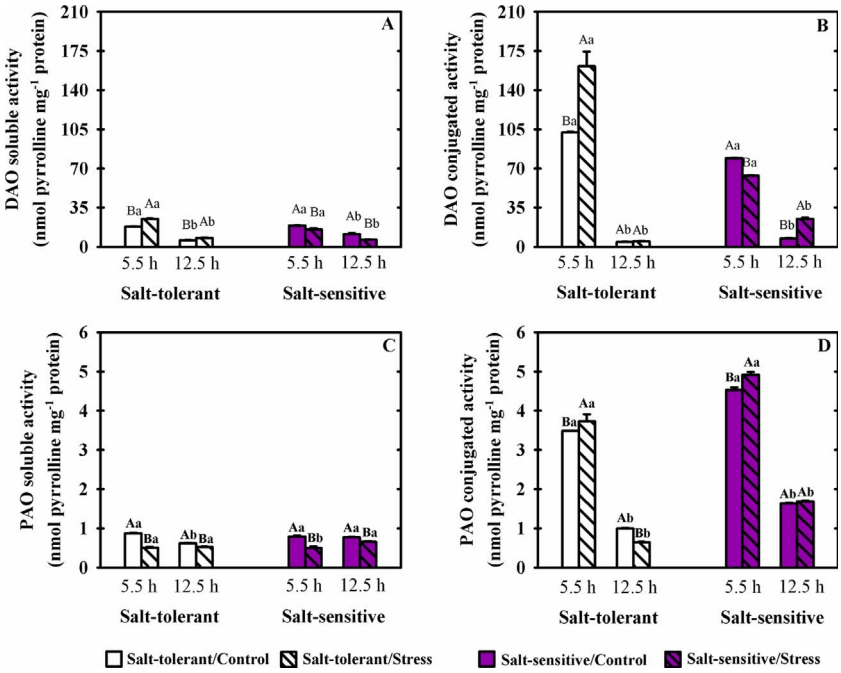

Fig. 6. NaCl stress-induced modulation of polyamine catabolism enzymes in leaves of salt-sensitive and salt-tolerant maize genotypes grown in nutrient solutions in the absence (control) and presence (stress) of 80 mM NaCl. (A) Activities of soluble and (B) conjugated DAO as well as (C) soluble and (D) conjugated PAO were measured at the times corresponding to phase I (5.5 h) and phase II (12.5 h) of ethylene production. Statistical details same as in Fig. 2.

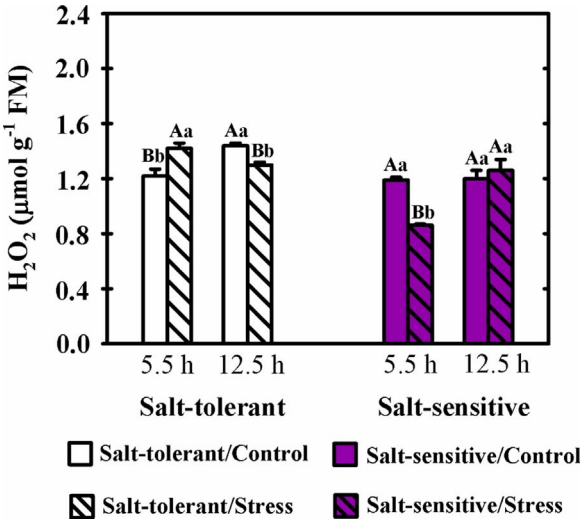

Fig. 7. Alterations in hydrogen peroxide (H_2O_2) production in leaves of salt-sensitive and salt-tolerant maize genotypes grown in nutrient solutions in the absence (control) and presence (stress) of 80 mM NaCl, at 5.5 and 12.5 h after salt exposure. Statistical details same as in Fig. 2.

the salt-sensitive genotype displayed a marked decrease in DAO activities, excluding the conjugated DAO activity at 12.5 h (Fig. 6A, B), a finding that was also correlated with a decrease in H_2O_2 production at 5.5 h (Fig. 7). Our data suggest that polyamine catabolism directly contributes, in addition to other processes, to generation of H_2O_2.

The *Z. mays* genome has three gene members of *DAO*, specifically *ZmDAO1*, *ZmDAO2* and *ZmDAO3*; however, only *ZmDAO1* and *ZmDAO3* were expressed in the leaves (Fig. 8A, B). In the salt-tolerant genotype, salinity promoted a marked increase in *ZmDAO1* gene expression, with values of stressed plants found to be 0.4-, 15.5- and 58.4-fold higher than the respective controls at 7.5, 10.5 and 12.5 h (Fig. 8A). Additionally, we observed up-regulation in *ZmDAO3* expression at 5.5 and 10.5 h (Fig. 8B), suggesting that the DAO enzyme is under transcriptional control (Fig. 6A, B). In the salt-sensitive genotype, the transcript levels of *ZmDAO1* and *ZmDAO3* were enhanced by salinity from 5.5 to 12.5 h, and the activity of conjugated DAO enzyme at 12.5 h was directly correlated with expression levels.

In NaCl presence, soluble PAO activity was reduced in both genotypes, a response that was accompanied by increases of Spd and Spm forms, regardless of analysed time for the salt-tolerant genotype (Table 1 and Fig. 6C). Under salt stress, the conjugated PAO activity increased at the first time point for both genotypes, while a reduction was registered at 12.5 h in the salt-tolerant genotype (Fig. 6D). These results were consistent with the increased free and soluble conjugated Spd and Spm contents (Table 1), indicating a reduced catabolism associated with improved synthesis.

Based on the genome sequence, maize has nine genes encoding PAO, but only two genes are intensively expressed in leaves, *ZmPAO4a* and *ZmPAO4b*, with *ZmPAO4b* showing higher transcript levels than *ZmPAO4a* (Fig. 8C, D). In the salt-tolerant genotype, salinity markedly up-regulated *ZmPAO4a* by 11 fold at 2.5 h and *ZmPAO4b* by 1 fold at 10.5 h (Fig. 8C, D). However, in the salt-sensitive genotype, both gene transcripts increased at 7.5 h in response to salt stress (Fig. 8C, D).

3.4. Pharmacological inhibitor assays

To clarify the closely relationship between ethylene synthesis and polyamine catabolism enzymes in H_2O_2 production, assays with pharmacological inhibitor aminooxyacetic acid (AOA) of ethylene synthesis were performed (Fig. 1A, B, inset, 9). For all cases, the ethylene production for signalling purposes (phase I − 5.5 h) was completely inhibited in the presence of AOA in both salt-sensitive and salt-tolerant maize genotypes (inset in Fig. 1A, B). Surprisingly, in salt-tolerant genotype, the absence of ethylene in phase I resulted in a higher production of H_2O_2 under salinity (Fig. 9C, D, K), a process catalyzed by enhanced DAO activity (Fig. 9I), but not PAO (Fig. 9J). On the other hand, in salt-sensitive maize genotype, the ethylene inhibition (Fig. 1A, inset) coincided with an amplified H_2O_2 synthesis (Fig. 9G, H, K) catalyzed by both polyamine catabolism enzymes, with more prominent action of DAO in comparison to PAO (Fig. 9I, J).

4. Discussion

4.1. Biphasic ethylene production is a signal of salt sensitivity

Ethylene causes paradoxical effects on plant metabolism (Stearns

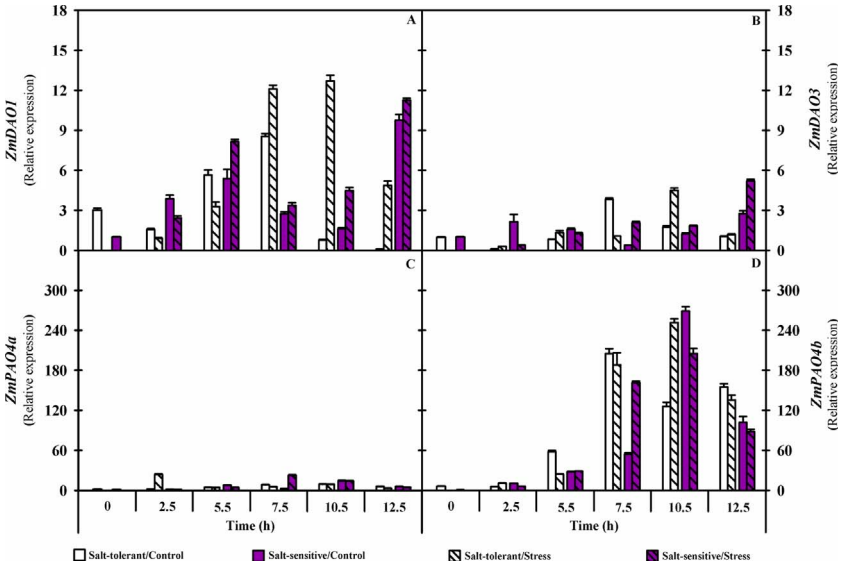

Fig. 8. Relative expression levels of genes involved in polyamine catabolism in leaves of salt-sensitive and salt-tolerant maize genotypes grown in nutrient solutions in the absence (control) and presence (stress) of 80 mM NaCl. (A) The relative expression of *ZmDAO1*, (B) *ZmDAO3*, (C) *ZmPAO4a* and (D) *ZmPAO4b* was quantified by qPCR, using as reference three housekeeping genes (*EF1α*, *GAPDH* and *PP2A*). Values represent the means + SE of three biological replications. Statistical details are shown in Supplementary Table S4.

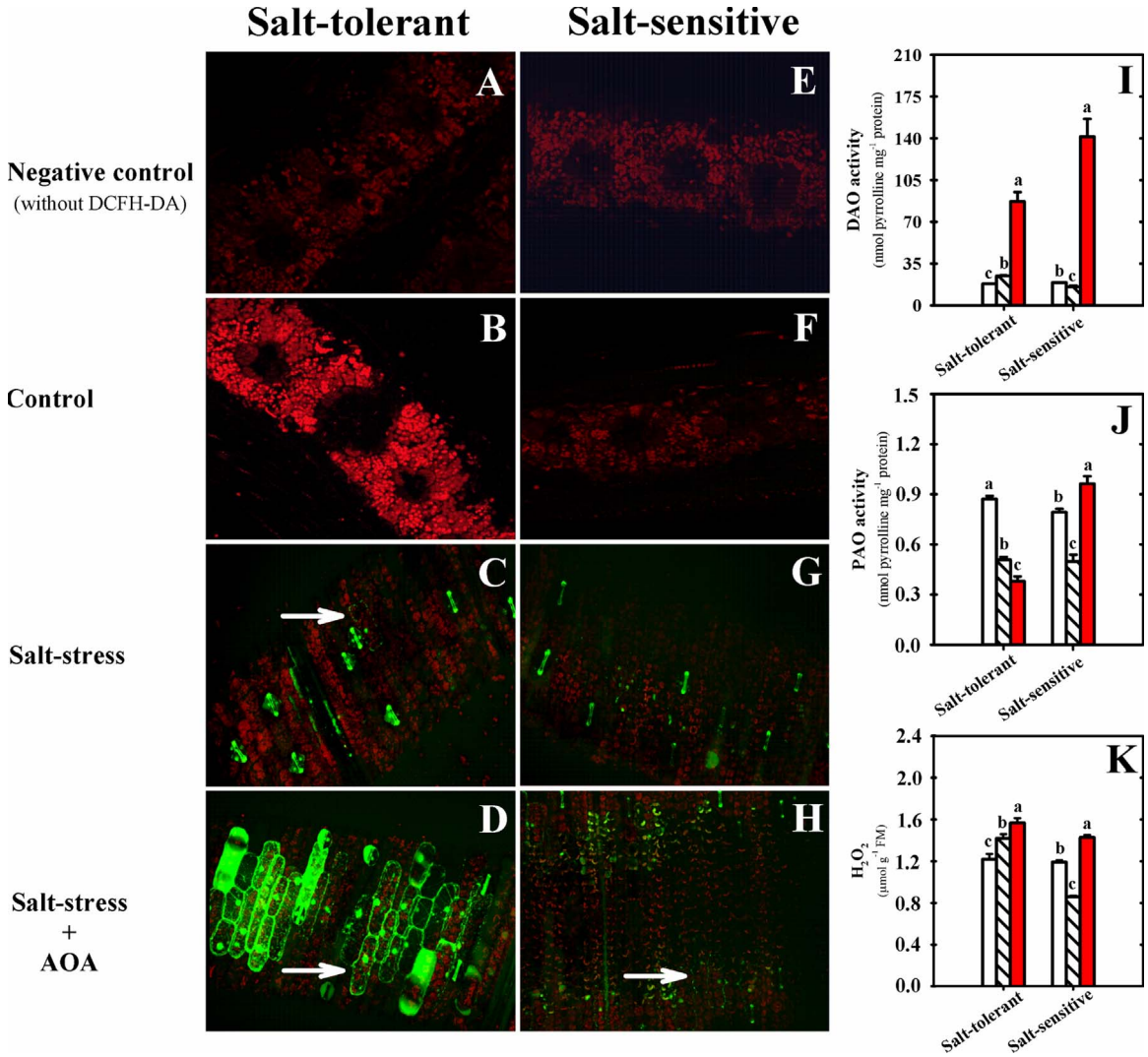

Fig. 9. Effects of 100 μM aminooxyacetic acid (AOA) ethylene biosynthesis inhibitor in generation of H_2O_2 in cell wall and polyamine catabolism enzymes in leaves of salt-sensitive and salt-tolerant maize genotypes. Leaf section images were obtained by confocal microscopy after incubation with 2′,7′-dichlorofluorescein diacetate (DCFH-DA). (A, E) Negative control (without DCFH-DA), (B, F) control, (C, G) salt stress and (D, H) salt stress + AOA. The H_2O_2 synthesis is indicated by white arrow and the chlorophyll B auto fluorescence is showed in red. (I) DAO and (J) PAO activity and (K) H_2O_2 content in leaves were measured at the time corresponding to phase I (5.5 h) of ethylene production. Values represent the mean of five biological replications + SE. Significant differences due to inhibitor treatment are denoted by different lower case letters using Tukey's test ($p \leq 0.05$).

and Glick, 2003). Although ethylene is well known as an important mediator in regulating ionic homeostasis and plant salt tolerance (Yang et al., 2013; Wang et al., 2011; Jiang et al., 2013), it impairs the early vegetative phase in some species (Zapata et al., 2007). Previously, Stearns and Glick (2003) reported that there is an initial small peak of ethylene production (phase I) soon after the onset of stress and a second larger peak later (phase II). The authors argued that the phase I production can play a protective signalling role in response to stress, whereas phase II usually coincides with inhibitory processes of plant survival, such as foliar senescence, chlorosis and abscission.

In agreement, we observed that the biphasic ethylene production matched with salt-sensitive maize plants (Fig. 1A, C). The salt-sensitive genotype exhibited enhanced ethylene peaks at 5.5 h (phase I) and 12.5 h (phase II) (Fig. 1A). Thus, the lack of biphasic ethylene production in the salt-tolerant genotype (Fig. 1B, D) explains, at least in part, the better performance of the BR5033 maize genotype in saline environments (Azevedo Neto et al., 2004). Our argument was further corroborated by studies with *N. tabacum* plants expressing the antisense *ACS* gene of carnation (*Dianthus caryophyllus*) (Wi et al., 2010). Transgenic plants did not exhibit phase II of ethylene production, and they were more tolerant to H_2O_2 stress. Therein, the greater tolerance was attributed to a lower ROS accumulation associated with an up-regulation of activity and gene expression of ROS-detoxifying enzymes (MnSOD, CuZnSOD and catalase) (Wi et al., 2010).

ACC content is widely recognised as a limiting factor for ethylene production (Abts et al., 2014; Bulens et al., 2014). In the current study, ACC synthesis seemed to not impair the ethylene production during phase I (Figs. 1 A, B, 2 A, C). For both maize genotypes, the increased ACC content under salinity (Fig. 2A) may arise from the reaction catalysed by ACS enzyme activity (Fig. 2C) and conversion of MACC to ACC (5.5 h) (Fig. 2B). Similarly, the high ACC content in the embryonic axis of chickpea seeds could not rely solely on the products of ACS but relied also on an improved ACC due to MACC hydrolase activity (Martín-Remesal et al., 2000).

Unlike the reports of Abts et al. (2014) and Bulens et al. (2014), our results are consistent with the idea that the activity of ACO, but not ACS, is the rate-limiting step in ethylene biosynthesis phase II (Figs. 1 A, 2 C). Here, the second peak of ethylene synthesis in the salt-sensitive genotype was fully concordant with a marked increase in ACO activity (Fig. 2D); in the salt-tolerant genotype, the decrease in ACO activity coupled with reduced MACC concentrations increased ACC accumulation at 12.5 h and coincided with the absence of phase II (Figs. 1 B, 2 A, B, D). Our hypothesis is confirmed by the recent study of Chen et al. (2014). The constitutively expressed *ACO1* gene resulted in *A. thaliana* plants more sensitive to salinity stress. The authors also reported that the salt sensitivity of transgenic plants might have originated from a down-regulation of stress-responsive genes (such as C-repeat binding factors *cbf1* and *cbf6*) involved in the signal transduction.

Expression levels of all *ACS* and *ACO* genes found in the *Z. mays* genome were monitored by qPCR in order to investigate if biphasic ethylene production involves transcriptional regulation. We observed that the major genes responsive to salt stress were *ZmACS1a* and *ZmACO5b* (Fig. 3A, B). qPCR analysis showed that transcripts for ACC synthase (*ZmACS1a*) were up-regulated in the salt-sensitive genotype, with a slight increase in the activity of ACS, suggesting the existence of a post-transcriptional/translational modulation (Figs. 2 C, 3 A). The down-regulation of the *ZmACO5b* gene expression during subsequent salinity exposure for the salt-tolerant genotype, together with the fact that ACO activity is determinant for phase II of ethylene synthesis, clearly suggests that the *ACO* gene plays an important role in the regulation of ethylene synthesis under salt stress (Stepanova and Alonso, 2009; Chen et al., 2014). Taking biochemical and gene expression data together, we can speculate that phase II of ethylene production under salinity is under intricate functional and transcriptional control of the *ACO* gene.

4.2. Ethylene displays critical role in control of H_2O_2 generation catalysed by polyamine catabolism enzymes in stress signalling

Many researchers have indicated that polyamine catabolism plays an important role in plant responses to abiotic and biotic stresses, but the specific crosstalk between maize polyamine catabolism *versus* ethylene signalling remains misunderstood, particularly under salinity. Our findings suggest that ethylene contributes to H_2O_2 generation fine-tuning for signalling events involving the regulation of polyamine catabolism enzymes during the early response to salt stress in maize genotypes. This idea was supported by the assays in presence and absence of AOA, a potent inhibitor of ethylene synthesis (Amrhein and Wenker, 1979) (Figs. 1, 6, 7, 9). Under salinity, the phase I of ethylene production (at 5.5 h) in salt-tolerant genotype was accompanied by a slight increase in H_2O_2 content (Fig. 7) together with up-regulation of DAO activity and *ZmDAO3* gene expression (Figs. 6 A, B, 8 B). Nevertheless, when the phase I of ethylene was abolished (inset in Fig. 1A,), a higher production of H_2O_2 (Fig. 9B–D, K) and strong DAO activity were observed (Fig. 9I), while PAO activity decreased remarkably (Fig. 9J). Concordantly, Zarza et al. (2017) showed that PAO contribution to the H_2O_2 pool in *Arabidopsis* during early stages of salinity is rather limited.

Another interesting finding was also registered in salt-sensitive genotype. Maize plants failed to activate the polyamine-dependent H_2O_2 generation during phase I of ethylene synthesis (Figs. 1, 7); but an H_2O_2 overproduction (Fig. 9F, G, H, K) coupled to strong increase of polyamine catabolism enzymes (DAO and PAO) (Fig. 9I, J) was noticed without signalling ethylene (inset in Fig. 1B). These results suggest that the signalling ethylene controls the balance of polyamine enzyme-catalysed H_2O_2 production, which in turn acts as a second messenger activating downstream targets to confer the salt tolerance in maize plants, a genotype-dependent response at least during short-term salinity stress.

4.3. Interplay between polyamine metabolism versus salt tolerance in maize genotypes

The salt-tolerant maize genotype exposed to 5.5 h of salinity did not show obvious changes in all Put forms compared to the control but did exhibit marked increases in the free and conjugated forms of Spd and Spm (Table 1). Moreover, down-regulation in ADC activity and *ZmADC2* gene expression at 5.5 h was detected (Figs. 4, 5). Thus, the low ADC activity and expression without alterations in the free Put suggest a complex mechanism for balancing the forms of Put in an ADC-independent pathway. It is likely that at 5.5 h, the maintenance of free Put resulted from its *de novo* synthesis rather than conversion of conjugated and bound Put to free Put, as evidenced by unaltered concentrations of conjugated and bound Put coupled to DAO catabolism (Fig. 6A, B and Table 1).

After longer exposure to salinity (12.5 h), in the salt-tolerant genotype, the increase of ADC activity and *ZmADC2* overexpression (~660% at 10.5 h) did not result in an overaccumulation of Put (Figs. 4, 5 and Table 1). Additionally, the enzyme involved in oxidative degradation of Put (DAO) showed only a slight increase. Taken together, these results indicate that Put may have been combined with aminopropyl groups in order to produce Spd via Spd synthase and/or Spm by transferring a second aminopropyl group through Spm synthase (Gupta et al., 2013), as evidenced by the improved Spd and Spm contents (Table 1). Concordantly, the increase of Spd and Spm has been cited during exposure to salinity (Rodríguez-Kessler et al., 2006; Jiménez-Bremont et al., 2007; Sudhakar et al., 2014). Our results showed that, under salinity, the increase of free and soluble conjugated Spd and Spm was pronounced in the salt-tolerant genotype rather than in the salt-sensitive one, suggesting their possible role in the processes of acclimation to salt stress. In a similar way, an increase in Spd and Spm forms was required for tolerance of plants to osmotic, salt and drought stresses (Jiménez-Bremont et al., 2007; Wang et al., 2007; Wi

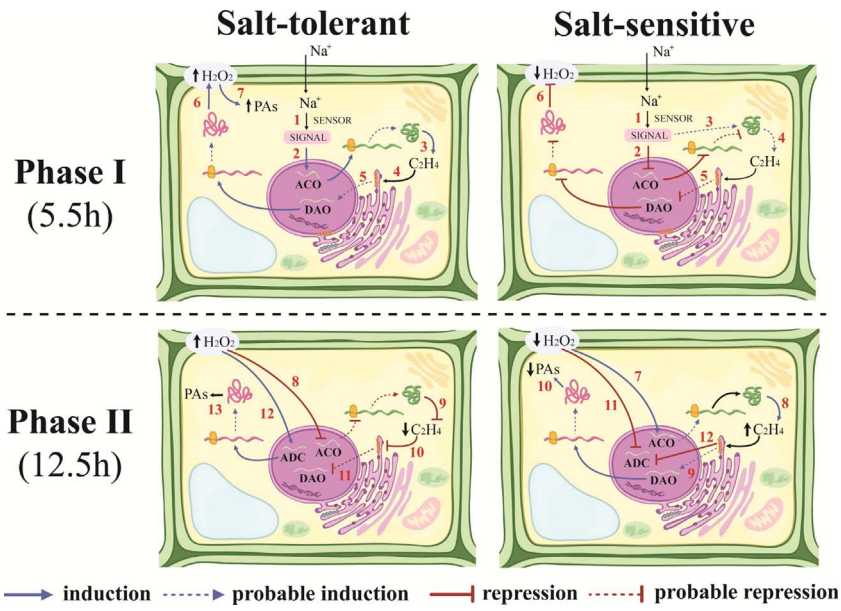

Fig. 10. Model proposed for crosstalk regulation among ethylene, polyamines and H_2O_2 (as a byproduct of polyamine catabolism) in maize genotypes with salt-contrasting tolerances. In the salt-tolerant maize genotype, NaCl stress is first perceived (**1**) by unknown sensors, inducing a signal cascade that up-regulates ACO gene expression (**2**) and activity (**3**) and culminates in an initial ethylene production peak at 5.5 h (phase I). Then, ethylene binds to receptors (**4**), eliciting DAO gene expression (**5**) and activity (**6**), which, in turn, lead to H_2O_2 production. The H_2O_2 synthesised during phase I acts as a signalling molecule for the synthesis of polyamines (**7**) as well as for the inhibition of 'stress ethylene' production at 12.5 h (phase II). Its signalisation is composed of down-regulation of the ACO expression and activity and, subsequently, the DAO enzyme (**8**-**11**). Simultaneously, during phase II, H_2O_2 strongly activates the polyamine anabolic pathway by up-regulating ADC expression and activity (**12**-**13**). In the salt-sensitive genotype, salinity perception (**1**) also culminates in phase I of ethylene production; however, this was not due to *ACO* gene expression improvement (**2**) but likely is due to functional regulation of the ACO protein (**3**-**4**). Surprisingly, the first peak of ethylene production does not promote H_2O_2 synthesis by polyamine catabolism (**5**-**6**), which coincides with the production of 'stress ethylene' in phase II by positive ACO modulation (**7**-**8**). During phase II, ethylene up-regulates DAO expression and activity (**9**-**10**) and, afterward, reduces polyamine content. Finally, the absence of H_2O_2 synthesis during phase I matches with the repression of polyamine anabolism through negative regulation of the ADC pathway. As a conclusion, metabolic alterations due to the absence of 'stress ethylene' in the salt-tolerant genotype are correlated with its better performance under salt stress. **Abbreviations**: **ACO** – ACC oxidase; **ADC** – arginine decarboxylase; **C_2H_4** – ethylene; **DAO** – diamine oxidase; **H_2O_2** – hydrogen peroxide; **PAs** – polyamines.

et al., 2014; Yin et al., 2014).

In contrast, in the salt-sensitive genotype, the polyamine content was likely regulated by conversion of the free form to the conjugated and bound forms (Table 1). Thus, different from what occurred at 5.5 h (modulation of biosynthetic and catabolic pathways) (Figs. 4, 6), the conjugation reactions might be essential for regulating the polyamine pool at 12.5 h (Table 1) (Groppa and Benavides, 2008; Alcázar et al., 2010; Tavladoraki et al., 2012).

The total polyamine pool and relationships among individual forms may vary in response to a variety of abiotic stresses, with an increased pool of polyamines found to be closely related to salt tolerance (Sudhakar et al., 2014). Similarly, the total polyamine content of the salt-tolerant genotype was markedly enhanced at 5.5 h (~31%) and unaltered at 12.5 h under salt stress (Table 1), while it decreased by 35% and 32% at 5.5 and 12.5 h, respectively, in the salt-sensitive genotype.

Elevating the (Spd + Spm)/Put ratio also has been critical in improving plant tolerance to several environmental stresses (Groppa and Benavides, 2008; Autar et al., 2010; Sudhakar et al., 2014). However, Wen et al. (2011) found that the exogenous application of Spd alleviated the negative effects of both salt and cadmium stress, irrespective of the (Spd + Spm)/Put ratio. The authors discussed that, in addition to the optimal polyamine accumulation, there is a desirable (Spd + Spm)/Put ratio that may vary depending on the plant species and type of stress. In the present study, the (Spd + Spm)/Put ratio significantly increased (~110%) in the salt-tolerant genotype at both time points of salt stress (Table 1), whereas it increased and decreased at 5.5 and 12.5 h, respectively, in the salt-sensitive genotype. It is tempting to speculate that the (Spd + Spm)/Put ratio in the salt-sensitive genotype was not desirable and might have affected negatively the defence response against salt stress.

5. Conclusions

In conclusion, our results provide a novel insight into how ethylene and polyamine metabolisms are related to salt tolerance, involving at least three mechanisms: (1) the first ethylene production peak triggers a signal for activating salt responses, and the second peak points out to the salt sensitivity; (2) the DAO protein increases H_2O_2 production for signalling the powerful plant defence responses; and (3) the polyamine pool might play an important role against harmful salt effects. Taken together, we propose a model to illustrate the crosstalk regulation among ethylene, polyamines and H_2O_2 (as a byproduct of polyamine catabolism) in salt tolerance of maize plants (Fig. 10).

Conflict of interest

No conflicts of interest declared.

Author contributions

This study was accomplished with the collaboration of all authors under the supervision of E. Gomes-Filho, who also helped in drafting of the manuscript. V.S. Freitas and R.S. Miranda were the masterminds, planning and conducting the research, performing the biochemical and molecular determinations, analyzing the data and drafting the manuscript. J.H. Costa helped with both the bioinformatics and molecular traits. D.F. Oliveira and S.O. Paula helped with pharmacological inhibitor assays. E.C. Miguel and R.S. Freire contributed with confocal microscopy studies. J.T. Prisco contributed by scientific advices during experiments and by revise some parts of the manuscript.

Acknowledgments

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Instituto Nacional de Ciência e Tecnologia em Salinidade (INCTSal) and Central Analítica-UFC/CT-INFRA/MCTI-SISNANO/Pró-Equipamentos CAPES. V.S. Freitas thanks Instituto Federal de Educação, Ciência e Tecnologia do Ceará (IFCE) for support during her PhD studies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.envexpbot.2017.10.022.

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