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In vivo inhibition of polyamine oxidase by a spermine analogue, MDL-72527, in tomato exposed to sublethal and lethal salt stress

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Abstract. The spermine analogue N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527), an effective inhibitor of polyamine oxidases (PAOs), triggers a systemic response in tomato (*Solanum lycopersicum* L.) exposed to sublethal (100 mM) and lethal (250 mM) NaCl concentrations. The accumulation of free polyamines (PAs), the terminal oxidation of PAs by diamine oxidases (DAOs) and PAOs, and the production of H_2O_2 by PA oxidases depends on the intensity of salt stress. Spermidine and spermine content increased significantly under sublethal salt concentrations, but remained low under lethal salt stress. Along with increased expression of the selected *SlDAO1* and *SlPAO1* genes in the leaves and roots, respectively, DAO and PAO activities and their product, H_2O_2, increased and initiated cell death by irreversible loss of electrolytes at 250 mM NaCl. MDL-72527 significantly increased spermine, spermidine and/or putrescine contents as a result of reduced activity of PA oxidases; furthermore, it inhibited H_2O_2 and NO production during salt treatment. These results indicate that PAO contributed to H_2O_2 and NO production under salt stress, and the terminal activities of DAO and PAO play a role in cell death induction at 250 mM NaCl. However, the inhibition of PAO by MDL-72527 does not increase the salt tolerance of plants, since electrolyte leakage increased significantly in the presence of the inhibitor.

Additional keywords: diamine oxidase, electrolyte leakage, nitric oxide, reactive oxygen species, programmed cell death.

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Introduction

Soil salinity is one of the major abiotic stresses, which reduces plant productivity by inducing strong ionic, osmotic and oxidative stress. Production of reactive oxygen species (ROS), the failure of ROS-scavenging mechanisms and disturbance in the redox status caused by supraoptimal salt concentrations result in the degradation of proteins, lipids and DNA, which can finally lead to the death of plant cells (Munns and Tester 2008). Programmed cell death (PCD) initiated by high salinity was mainly a result of ion disequilibrium caused by a high cytoplasmic Na^+ : K^+ ratio, which is accompanied by intensive ROS generation in the apoplast (Demidchik *et al*. 2010; Demidchik *et al*. 2014; Maathuis *et al*. 2014).

Polyamines (PAs) (putrescine (Put), spermidine (Spd) and spermine (Spm)) are polycationic compounds that can regulate salt stress acclimation or high salinity-induced PCD in plant tissues (Gill and Tuteja 2010). In most cases, the accumulation of PAs is positively correlated with the salt stress tolerance of plants and externally applied PAs can mitigate salt stress symptoms. This is because by binding to negative charges, PAs can stabilise membranes and, indirectly or directly, they play an essential role in membrane transport and in the maintenance of the K^+ : Na^+ ratio in plant tissues (Pottosin and Shabala 2014).

Shabala *et al*. (2007) reported that exogenous application of PAs at micromolar concentrations prevented NaCl-induced K^+ efflux from pea (*Pisum sativum* L.) leaf mesophyll cells by the inhibition of nonselective cation channels. Similarly, externally applied PAs inhibited voltage-independent nonselective cation channels, the most important mediators of Na^+ influx in the roots and in this way, they blocked excessive Na^+ uptake in root tissues (Shabala *et al*. 2006). Zhao *et al*. (2007) demonstrated that PAs were able to act on nonselective cation channels from the cytoplasmic side of the plasma membrane (PM) as well but the effect proved to be indirect. PAs were also able to activate vacuolar proton pumps, the V-type H^+-ATPase and the proton-translocating vacuolar pyrophosphatase, which is a prerequisite for vacuolar sequestration of Na^+ and for the maintenance of the K^+ : Na^+ ratio in the cytoplasm. They can also inhibit the slow and fast activating vacuolar cation channels and, as a consequence, the leakage of Na^+ from vacuoles (Pottosin and Shabala 2014). PAs may preserve the ion balance of the cells not only by stabilisation of the cell membranes but by inducing ROS-scavenging mechanisms (Gill and Tuteja 2010).

However, PAs may function as pro-oxidants, since catabolism of PAs generates H_2O_2, which, depending on the strength of ROS signal, potentiates plant survival or cell death (Moschou and Roubelakis-Angelakis 2014; Pottosin *et al*. 2014*a*). Degradation of PAs is catalysed by copper-containing diamine oxidases (DAOs, EC 1.4.3.6) and FAD-dependent polyamine oxidases (PAOs, EC 1.5.3.3). DAOs localised in cell walls catalyse the oxidation of the primary amino group of Put and the apoplastic

PAOs catalyse the terminal oxidation of Spd, generating H_2O_2 at the external surface of the PM (Moschou and Roubelakis-Angelakis 2014). Spd and Spm can also be substrates for PAOs (AtPAO2–4) that catalyse the sequential back-conversion of Spm to Spd and Spd to Put in *Arabidopsis thaliana* (L.) Heynh. peroxisomes and cytoplasm. This process is subjected to feedback inhibition by its end product, Put. Put can be oxidatively deaminated by DAOs not only in the cell wall but also in the cytosol (e.g. by AtCuAO1–3), avoiding excessive accumulation of Put inside the cell (Planas-Portell *et al*. 2013). Since PA degradation is an essential process in the maintenance of steady-state PA levels, DAO and PAO activities are key elements in biotic and abiotic stress responses.

High salinity results in PA exodus to the cell wall and, by activating apoplastic DAO and PAO, a rapid increase in the levels of ROS ($\bullet O_2^-$, H_2O_2 and •OH). This activates Ca^{2+} influx across the PM and increases cytosolic free $[Ca^{2+}]_{cyt}$. The other source of apoplastic ROS is PM NADPH oxidase (NOX) (Pottosin *et al*. 2014*a*), which is activated by salt stress-induced cytosolic Ca^{2+} signals within seconds (Shabala *et al*. 2015). However, supraoptimal NaCl inhibits the activity of NOX in maize (*Zea mays* L.) leaves, thus oxidation of PAs in the apoplast can be an alternative source of ROS (mainly H_2O_2 and •OH) production under salt stress (Rodriguez *et al*. 2009). Apoplastic ROS triggered increased conductance in the PM, which was stimulated by PAs and induced membrane depolarisation by initiating massive K^+ and anion efflux (Zepeda-Jazo *et al*. 2011; Velarde-Buendía *et al*. 2012; Pottosin *et al*. 2014*a*, 2014*b*). The kinetics of ROS production was similar to the kinetics of the increase in K^+ efflux in response to the same stress. (Demidchik *et al*. 2010). Thus the tissue- and time-dependent accumulation of various PA species during salt stress may determine the outcome of the process.

Besides the accumulation of ROS, Spd and Spm can induce nitric oxide (NO) biosynthesis as well (Tun *et al*. 2006). Along with H_2O_2 production, NO synthesis can be connected with the catabolism of PAs by DAO and PAO (Wimalasekera *et al*. 2011). NO can interplay with ROS in a variety of ways and participates in defence signalling or cell death responses under salt stress (Siddiqui *et al*. 2011). In cucumber (*Cucumis sativus* L.) plants, sodium nitroprusside, an NO donor, reduced the accumulation of free Put and Spd as well as PAO activity under salt stress, but it increased the Spm content, which improved the salt tolerance of plants (Fan *et al*. 2013). NO promotes or suppresses cell death through selective protein *S*-nitrosylation and its reaction product with the superoxide anion radical, peroxinitrite, contributes to PCD initiation through increased tyrosine nitration (Wang *et al*. 2010). However, a comparison of the effects of sublethal- or lethal salt stress on PA oxidation along with changes in ROS production, NO accumulation and PA patterns has not been established up to now in time-course experiments.

Natural PAs or synthetic PA analogues are frequently applied to modify endogenous PA levels, and thus the growth and development of plants. Several PA analogues have been reported to inhibit PAO activity, including agmatine, *N*-prenylagmatine, guazatine and N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527 or MDL) (Bianchi *et al*. 2006). MDL has proven to be a selective inactivator of the FAD-dependent PAOs and has been used in numerous animal experiments for studying the biochemical and physiological consequences of PAO inhibition *in vitro* and *in vivo* (Hu and Pegg 1997; Seiler *et al*. 2002), but only a few studies have used MDL in plant systems. MDL could bind to the central part of the catalytic tunnel and has proven to be a competitive inhibitor of maize PAO (Bianchi *et al*. 2006). However, under prolonged incubation, inhibition by MDL resulted in the irreversible loss of PAO activity resulting from the formation of a covalent adduct (Bellelli *et al*. 2004). Although both activation and prevention of PCD caused by PA depletion by PAOs have been reported for animal cell lines (Seiler *et al*. 2002), similar information relating to plant physiological research are fragmentary. Although the impact of exogenous PAs on endogenous PA metabolism and PA-induced oxidative and nitrosative stress has been investigated in detail in *Citrus* plants exposed to salt stress (Tanou *et al*. 2014), much less attention has been devoted to the putative effects of synthetic PA analogues on these processes.

The aim of the present study was to analyse and compare the effects of sublethal and lethal salt stress on PA catabolism (DAO and PAO expression and activity), and on the accumulation of the most important free PAs in the presence or in the absence of the PAO enzyme inhibitor MDL *in vivo*. It was also of interest whether the inhibition of PA catabolism by MDL affects ROS and NO production along with DAO and PAO activity. Furthermore, we would like to reveal whether the inhibition of terminal oxidation of PAs by the PAO-inhibitor MDL could mitigate salt stress injury in tomato (*Solanum lycopersicum* L.).

Materials and methods

Plant material and growth conditions

Seeds of tomato (*Solanum lycopersicum* L. cv. Rio Fuego) were purchased from a seed-producing company (Beviseed Vető magkereskedelmi Kft), which is a dealer of seeds certified according the European Union standard. For all experiments 100–100 seeds were germinated on moistened filter paper on trays at 26°C for 3 days in the dark, and the seedlings were subsequently transferred to perlite for 2 weeks. Two plants were then transferred into 0.5-L pots containing a modified Hoagland solution (Poór *et al*. 2011). The nutrient solution was changed three times a week. The plants were then grown in a hydroponic system for 6 weeks in a controlled environment under 200 µmol m^{-2} s^{-1} PPFD (F36W/GRO lamps, OSRAM SYLVANIA), with 12 : 12-h light : dark period, a day : night temperatures of 24 : 22°C and a relative humidity of 55–60%.

Ten minutes before salt exposure, plants were pretreated with 50 µM N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527), the inhibitor of PAO, through the root system and the inhibitor remained in the hydroponic culture throughout the whole experiment. Although guazatine is the most powerful inhibitor of plant PAOs, the well-known toxicity of this compound, which is commonly used as a fungicide in agriculture, makes it unsuitable for physiological experiments. This is the reason why MDL was chosen for our studies (Šebela *et al*. 2007). Plants were then treated with 100 or 250 mM NaCl supplied in the nutrient solution. Salt concentrations were chosen in our previous studies. It was shown that tomato could acclimate to 100 mM NaCl: the plants maintained membrane

integrity and a high K^+ : Na^+ ratio in the root cells. In plants exposed to 250 mM NaCl, the initiation of cell death occurred within 6 h after salt treatment, and high electrolyte leakage, a reduced K^+ : Na^+ ratio and the activation of cysteine proteases could be detected in the root tissues (Poór *et al*. 2014; Poór *et al*. 2015). The samples were prepared from the second fully expanded young leaves or roots from three replicates at 0.5, 1, 2, 3 and 6 h after the salt or MDL treatments. The experiments were conducted from 0900 to 1500 hours and were repeated three times. Data are means ± s.e. from at least three independent biological samples.

DAO and PAO activity assay

The terminal activity levels of DAO (EC 1.4.3.6) and PAO (EC 1.4.3.4) were determined according to Takács *et al*. (2016). Briefly, 200 mg of excised leaf and root tissues were homogenised in liquid N_2 in 0.6 mL of an extraction buffer. The extraction buffer contained 0.2 M Tris (pH 8.0), 10% glycerol, 0.25% Triton X-100, 0.5 mM phenylmethanesulfonyl fluoride and 0.01 mM leupeptin in a 100-mM K^+ phosphate buffer (pH 6.6). The homogenates were left on ice for 20 min and centrifuged for 10 min at 7000*g* at 4°C. The reaction mixture contained 0.15 mL of the tissue extract, 0.6 mL of the 100-mM K^+ phosphate buffer (pH 6.6), 50 U of catalase in 50 µL volume, 50 µL of 2-aminobenzaldehyde (0.1%), and 150 µL of 20 mM Put for DAO or 150 µL of 20 mM Spd for PAO activity measurements. The reaction mixture was incubated for 1.5 h at 37°C and after incubation, the reaction was stopped by adding 50 µL of 20% (w/v) trichloroacetic acid. The absorbance was measured at 430 nm (KONTRON). The enzyme activity was expressed as specific activity (U g^{-1} FW), where one unit (U) represents the amount of enzyme catalysing the formation of 1 µmol of Δ^1-pyrroline min^{-1}. *In vitro* measurement of DAO and PAO activity was carried out with the same leaf and root extracts prepared from plants that were exposed to 250 mM NaCl for 1 h without MDL. The effect of MDL on PAO activities was determined in 50–150 µM concentration intervals.

All reagents were purchased from Sigma-Aldrich unless otherwise specified.

RNA extraction and gene expression analyses with real-time quantitative reverse transcription–PCR

RNA was extracted from leaf and root samples according to Horváth *et al*. (2015). DNA digestion was performed by DNase I (Fermentas UAB). One µg of total RNA was used as a template for first-strand cDNA synthesis using MMLV reverse transcriptase (Fermentas UAB). Tomato genes were mined from the Sol Genomics Network database (http://solgenomics.net/, accessed 19 January 2017). Primers were designed using National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/, accessed 19 January 2017) and Primer 3 software (http://frodo.wi.mit.edu/, accessed 19 January 2017) on the selected genes. We selected one of the *SlDAO* genes (*SlDAO1*, *Solyc05g013440*) and one of *SlPAO* genes, (*SlPAO1*, *Solyc01g087590*). The selection was based on the multiple sequence alignment of the *A. thaliana*, *S. lycopersicum* and *Z. mays* amino acid sequences, which was carried out by the CLUSTALW (Multiple Sequence Alignments) (http://www.ebi.ac.uk/Tools/msa/clustalw2/, accessed 21 January 2017) and BioEdit Sequence Alignment (http://www.mbio.ncsu.edu/bioedit/bioedit.html, accessed 21 January 2017) programs. The gene specificity of the designed primer pairs was controlled *in silico* using the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 19 January 2017) and primer pairs were synthesised in the Nucleic Acid Synthesis Laboratory of Biological Research Center, Szeged, Hungary. The expression rate of *SlDAO1* (forward: 5'-AATACGGGTTCGGGTTACAA-3'; reverse: 5'-TGCCACAAATACACCATCCATA-3') and *SlPAO1* (forward: 5'-CCGTCAACTCCAAAAACACC-3'; reverse: 5'-TCCTCTTTCATCAGCAACCA-3') was monitored by quantitative real-time PCR (RT qRT-PCR) (BioRad) using SYBR green dye. The reaction mixture (20 µL) contained 10 µL of Maxima SYBR Green qPCR Master Mix (2×) (Thermo Scientific), 0.25 µM of the forward primers and 0.25 µM of the reverse primers, 10 ng of cDNA template and nuclease-free water. The two-step qPCR was started with denaturation at 95°C for 10 min, followed by 41 cycles of denaturation at 95°C for 15 s and an annealing extension at 60°C for 1 min. The specificity of the PCR amplification procedure was checked with a melting curve between 55°C and 90°C (0.2°C s^{-1}) after the final cycle of the PCR. Data analysis was performed using Opticon monitor software (BioRad). The tomato *18S* rRNA and *elongation factor-1α* subunit genes were used as reference genes. Each reaction was repeated at least three times and data from the qPCR were calculated using the $2^{(-\Delta\Delta Ct)}$ formula (Livak and Schmittgen 2001).

Analysis of free polyamines

Free PA contents were determined by Takács *et al*. (2016) via analysis of the benzoyl derivatives of free PAs separated by HPLC (JASCO HPLC System). The applied standards were Put, Spd and Spm in the form of hydrochlorides. The results are means of three independent biological samples expressed in nmol g^{-1} FW.

H_2O_2 determination

H_2O_2 was measured according to Horváth *et al*. (2015). One hundred mg of leaf and root samples were homogenised in 0.5 mL of ice-cold 0.1% trichloroacetic acid. After centrifugation (10 000*g*, 20 min), 0.25 mL of the supernatant was incubated with 0.25 mL of a 50-mM potassium phosphate buffer (pH 7.0) and 0.5 mL of 1 M potassium iodide (KI) (prepared in the 50-mM potassium phosphate buffer, pH 7.0) for 10 min at 25°C. The absorbance values were recorded at 390 nm. The results are expressed as µmol H_2O_2 g^{-1} FW.

Determination of ROS and NO production

A Zeiss Axiowert 200M-type fluorescence microscope (Carl Zeiss Inc.) equipped with a high-resolution digital camera (Axiocam HR, Carl Zeiss Inc.) and Filter Set 10 (excitation: 450–495 nm; emission: 515–565 nm) was used for the analysis of ROS and NO accumulation in tomato tissues. For the detection of ROS, leaf discs 10 mm in diameter were infiltrated under a vacuum with 10 µM 2',7'-dichlorodihydrofluorescein diacetate dye (prepared in a 10 mM MES-50 mM KCl buffer, 1 : 1, (v : v), pH 6.15). The discs and 1-cm-long root apices were stained for

20 min at 37°C in the dark. The incubation solution was removed and the samples were washed twice with a MES-/KCl buffer solution. NO production in leaf disks and root tips was visualised using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate dye. The samples were incubated for 30 min at room temperature in the dark in a 10-μM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate dissolved in a10-mM TRIS–HCl buffer (pH 7.4). After staining, the samples were rinsed twice with the 10-mM TRIS–HCl buffer (pH 7.4) (Poór *et al*. 2015). The intensity of ROS- and NO-dependent fluorescence was measured in digital images using Axiovision Rel. 4.8 software (Carl Zeiss Inc.). Pixel intensity in leaf disks was determined in circles 600 μm in diameter and in the root tips in circles 200 μm in diameter at 500 μm from the apex. The measurements were performed in 10 replicates.

Determination of electrolyte leakage

Electrolyte leakage was determined as described earlier (Poór *et al*. 2014). Briefly, 100 mg of the leaf or root tissue was transferred to 25 mL of double-distilled water. After 2 h of incubation at 25°C, the conductivity of the bathing solution (C_1) was determined with a conductivity meter (OK-102/1, Radelkis). The samples were then heated at 95°C for 40 min and the total conductivity (C_2) of the cooled samples was measured. Relative electrolyte leakage (*EL*) was expressed as a percentage of total conductivity:

$$EL = \frac{C_1}{C_2} \times 100.$$

Statistical analysis

The data represent one of three independent experiments with similar tendencies. In each experiment, at least three independent biological samples were measured. ANOVA followed by Fischer's LSD post-test was performed using SigmaPlot ver. 12.0 software (SYSTAT Software Inc. SPSS) to determine statistically significant differences between the control and the two NaCl concentrations with and without MDL treatments at each time point. Differences were considered significant at $P \leq 0.001$. In some cases, Duncan's post-test was applied ($P \leq 0.05$).

Results

In vitro *effect of MDL on DAO and PAO activity*

To detect the specific inhibitory effect of MDL on PAO activity in plants, *in vitro* measurements were carried out. DAO activities did not change significantly *in vitro* at low concentrations of MDL; however, PAO activity could be inhibited by as low as 50 μM MDL in the leaf and root tissue extracts of plants exposed to 250 mM NaCl for 1 h (Fig. S1, available as Supplementary Material to this paper).

In vivo *effect of MDL on DAO and PAO activity and on* H_2O_2 *levels*

DAO and PAO activities were investigated as a function of time in the leaves and roots of intact tomato in the presence or absence of MDL after treatments with sublethal and lethal concentrations of NaCl. DAO and PAO activity did not change in the leaves after treatment with 100 mM NaCl but the enzyme activity increased significantly within 1 h under lethal salt stress (Fig. 1*a–f*). H_2O_2 contents in the leaf samples changed in parallel with DAO and PAO activity and exhibited two peaks at 0.5 and 2 h after the treatment with 250 mM NaCl (Fig. 1*g*, *h*, *i*). In contrast to the *in vitro* experiments, both DAO and PAO were inhibited by MDL in the salt-stressed samples. Furthermore, in the presence of MDL, the double peaks of DAO and PAO activity along with H_2O_2 accumulation disappeared in leaves exposed to lethal salt stress (Fig. 1*c*,*f*,*i*). Changes in the activity of DAO and PAO were similar and negligible in the roots exposed to 100 mM NaCl (Fig. 2*a*,*b*,*d*,*e*). The enzyme activity and H_2O_2 contents were also characterised by two smaller peaks at 0.5 and 2 h after 250 mM NaCl treatment (Fig. 2*c*,*f*,*i*); however, the second peak was more pronounced in the roots. In the presence of MDL, DAO and PAO activity as well as H_2O_2 content decreased significantly in the root samples at both concentrations of NaCl compared with the samples without the inhibitor.

Effect of MDL on transcript levels of SlDAO1 *and* SlPAO1 *genes*

The selection of *SlDAO* and *SlPAO* amine oxidase coding sequences was based on the amino acid sequence similarity of *SlDAO1* (*Solyc05g013440*) with known DAOs. *SlDAO1* exhibited 77% similarity to *AtCuAO3* (*At2G42490* in The *Arabidopsis* Information Resource database) and *SlPAO1* (*Solyc01g087590*) showed 75% similarity to the *AtPAO1* of *A. thaliana* (*At5G13700*; The *Arabidopsis* Information Resource database). We found that the amino acid sequence similarities between *NsDAO1* (*XP009802912*; NCBI) from *Nicotiana sylvestris* Speg. & Comes and *SlDAO1* (*Solyc05g013440*) from tomato and between *NsPAO1* (*XP009758563*; NCBI) and SlPAO1 (*Solyc01g087590*) were 93% and 94% respectively. Furthermore, the amino acid identity shared by *SlPAO1* and the *ZmPAO1* enzyme is 46%, which is higher than the identity it shares with *SlPAO2* (30%), *SlPAO3* (18%), *SlPAO4* (23%) and *SlPAO5* (23%).

It has to be mentioned that *SlDAO1* and *SlPAO1* genes were sensitive to high salinity. The transcript levels of the chosen *SlDAO1* in the leaves were upregulated under lethal NaCl treatments after 6 h (Fig. S2*c*). The expression of *SlPAO1* exhibited a diurnal decline after 6 h in control leaves (Fig. S2*d*) and its expression declined in the first 3 h of salt stress compared with the untreated control (Fig. S2*e*, *f*). In the presence of MDL, the transcript levels of *SlDAO1* increased or remained generally unchanged, whereas the expression of *SlPAO1* was inhibited in the control samples and it was upregulated under salt stress.

Beside small fluctuations, the transcript levels of *SlDAO1* did not differ significantly between the untreated (control, sublethal and lethal salt concentrations) and MDL-treated roots during the 6 h of the analysis (Fig. 3*a–c*). On the other hand, *SlPAO1* showed a substantial downregulation in control roots as a function of time. Although the transcript levels of *SlPAO1* remained almost constant during the 100 mM NaCl treatment, the gene was upregulated under 250 mM NaCl in the roots. Unexpectedly, in the presence of PAO inhibitor, the transcript

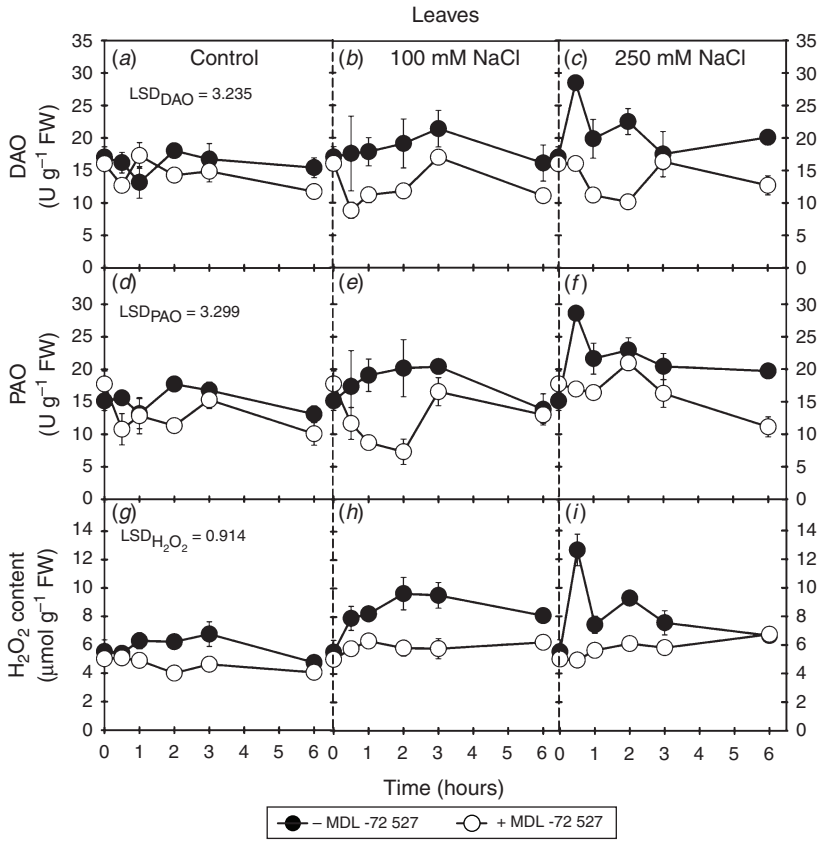

Fig. 1. Changes in the specific activity of (*a*, *b*, *c*) diamine oxidase (DAO) and (*d*, *e*, *f*) polyamine oxidase (PAO) (U g^{-1} FW), and (*g*, *h*, *i*) H_2O_2 concentration (µmol g^{-1} FW) as a function of time in the leaves of (*a*, *d*, *g*) control tomato plants or in plants exposed to (*b*, *e*, *h*) 100 mM or (*c*, *f*, *i*) 250 mM NaCl in the presence or absence of 50 µM N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527) (black circles, without MDL; white circles, with MDL). Means ± s.e. ($n = 3$). The differences between means were compared using Fisher's LSD ($P \leq 0.001$).

levels of *SlPAO1* were upregulated both in control and salt-stressed roots, especially at 100 mM NaCl (Fig. 3*d*–*f*).

Effect of MDL on free PA contents

Time-dependent changes in free Put, Spd and Spm contents revealed that the concentration of Put remained nearly constant in the leaves within 6 h after the 100 mM NaCl treatment, but it exhibited two small peaks at 0.5 and 2 h under the 250 mM NaCl treatment (Fig. 4*a*–*c*). Though Spd and Spm accumulated under sublethal salt stress (Fig. 4*e*, *h*), their concentrations, especially that of Spd, remained much lower in the leaves exposed to 250 mM NaCl (Fig. 4*f*, *i*). After MDL treatment, the control leaves enhanced the Put level at 3 h and increased Spd content at 0.5 h, but Spm content changed only slightly (Fig. 4*a*, *d*, *g*). In the presence of MDL, the Spd level increased fivefold (Fig. 4*e*) and twofold (Fig. 4*f*) in plants treated with 100 or 250 mM NaCl, respectively. The free Spm levels also increased significantly at 100 mM NaCl but this tendency was much more pronounced at 250 mM NaCl in the presence of the inhibitor (Fig. 4*h*, *i*).

In the root tissues, free Put levels decreased as a function of time in both the control and stressed plants (Fig. 5*a*–*c*). Spd content displayed a maximum in 1-h samples at 100 mM and 250 mM NaCl (Fig. 5*e*, *f*). Furthermore, after treatment with 250 mM NaCl, Spd content decreased below that of the control (Fig. 5*f*), whereas the plants at 100 mM NaCl could maintain the Spd level of the roots after 6-h-long treatment (Fig. 5*e*). Spm contents were very low in the roots of the control and salt-stressed plants (Fig. 5*g*–*i*). In the presence of the PAO inhibitor, Spm content increased fivefold in controls (Fig. 5*g*) and more than threefold under sublethal salt stress (Fig. 5*h*). However, under lethal salt stress, the Spm levels exhibited only small, transient increases in the roots in the presence of MDL (Fig. 5*i*). This result suggests that the reduction of PAO activity by the inhibitor led to a significant increase in Put, Spd and Spm contents, which was more pronounced in control roots and in plants exposed to 100 mM NaCl.

Effect of MDL on ROS and NO production

In the leaves and roots, the production of ROS and NO did not change under the control conditions in the presence or absence of the PAO inhibitor (Figs 6*a*, *d*; 7*a*, *d*). Although the accumulation

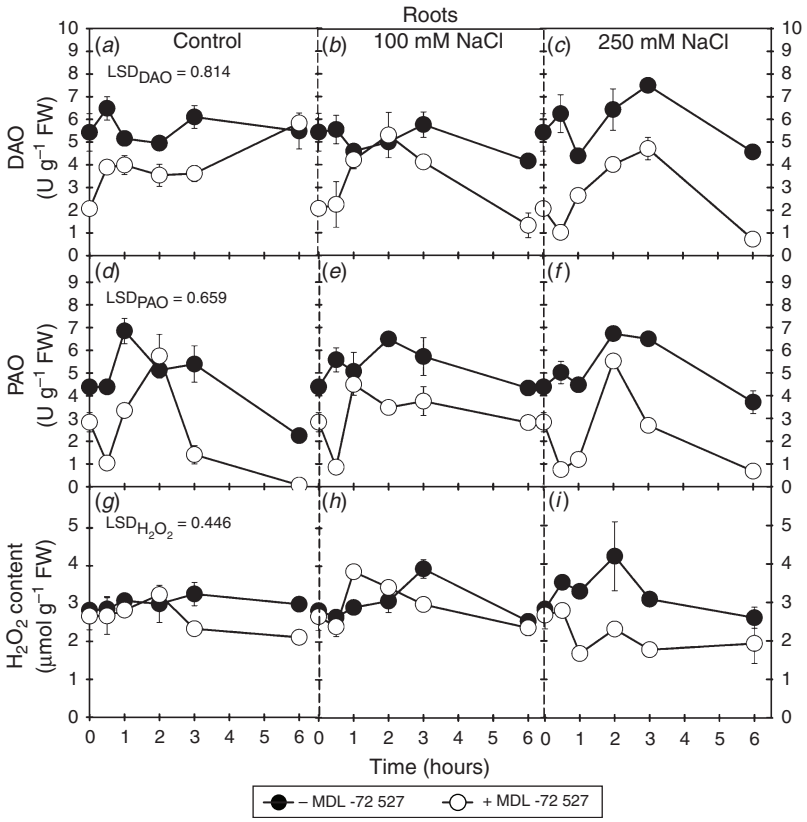

Fig. 2. Changes in the specific activity of (*a*, *b*, *c*) diamine oxidase (DAO) and (*d*, *e*, *f*) polyamine oxidase PAO (U g^{-1} FW) and (*g*, *h*, *i*) H_2O_2 concentration (µmol g^{-1} FW) as a function of time in the roots of (*a*, *d*, *g*) control tomato plants or in plants exposed to (*b*, *e*, *h*) 100 mM or (*c*, *f*, *i*) 250 mM NaCl in the presence or absence of 50 µM N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527) (black circles, without MDL; white circles, with MDL). Means ± s.e. ($n = 3$). The differences between means were compared using Fisher's LSD ($P \leq 0.001$).

of ROS was induced by both salt treatments (Fig. 6*b*, *c*), the lethal salt concentration induced a higher and more significant increase, which, in parallel with H_2O_2, exhibited two peaks in the leaves. Similarly, 100 mM NaCl induced a significant increase in NO production with a maximum at 3 h (Fig. 6*e*) but there were only slight changes under lethal salt stress (Fig. 6*f*). In the presence of MDL, leaves of salt-treated plants accumulated a significantly lower amount of ROS and NO than those without the PAO inhibitor (Fig. 6*b*, *c*, *e*, *f*). However, the first peak in ROS production induced by 250 mM NaCl could be eliminated by the PAO inhibitor only partially, which supports the role of other enzymes in oxidative burst (Fig. 6*c*). These results suggest that the generation of ROS and NO is time-dependent during salt stress and that the significant increase in ROS production was not accompanied by an accumulation of NO under lethal salt stress in the leaves.

Enhanced ROS production was also detected in the root tips exposed to low and high salinity after 1 h, which proved to be transient (Fig. 7*b*, *c*). The accumulation of NO began after 3 h in the case of the sublethal salt stress, but it exhibited two maxima under lethal salt stress (Fig. 7*e*, *f*). The maximum of ROS coincided with first NO peak at 250 mM NaCl treatment, whereas NO accumulation followed the ROS burst in the root apices exposed to 100 mM NaCl. The PAO inhibitor could eliminate ROS and NO accumulation under both salt treatments (Fig. 7*b*, *c*, *e*, *f*). Thus it can be supposed that PA catabolism by PAO contributed not only to the accumulation of H_2O_2 but also indirectly to the production of NO in tomato tissues.

Effect of MDL on electrolyte leakage

Although MDL increased the PA contents of leaf tissues – Put and Spd accumulated in control leaves, and Spd and Spm in the leaves of salt stressed plants – it enhanced the leakage of solutes to a small but significant extent compared with the respective controls. These changes seem to be within a tolerable level of membrane injury (Fig. 8*a*–*c*) (Poór *et al.* 2012).

In the root tissues, MDL induced a substantial accumulation of the higher PAs, Spd and Spm, leading to a high Spm+Spd : Put ratio, and the electrolyte leakage remained close to that of untreated control values in the absence of NaCl. At 100 mM NaCl, more Put accumulated in the first 3 h of salt stress and the higher PAs accumulated later in the presence of MDL. These tissues exhibited a significant increase in the ion efflux on the effect of MDL. Under the lethal salt treatment, root tissues were not able to accumulate much more PA in the presence of MDL, but

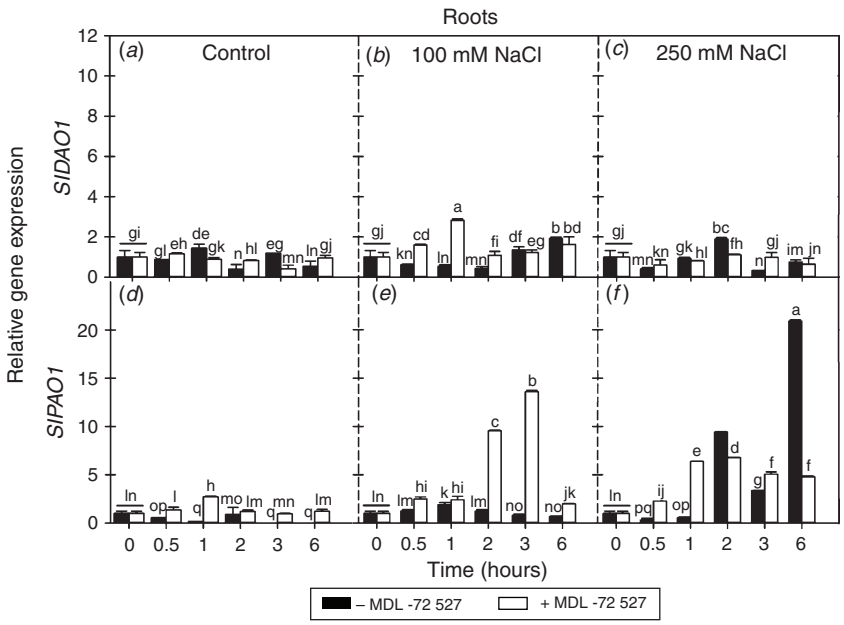

Fig. 3. Changes in the relative expression levels of the (*a*, *b*, *c*) *SlDAO1* and (*d*, *e*, *f*) *SlPAO1* genes as a function of time in the roots of (*a*, *d*) control tomato plants or in plants exposed to (*b*, *e*) 100 mM or (*c*, *f*) 250 mM NaCl in the presence or absence of 50 µM N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527) (black bars, without MDL; white bars, with MDL). Means ± s.e. ($n = 3$). Bars denoted by different letters indicate significant differences at $P \leq 0.5$ (Duncan's multiple range test).

the electrolyte leakage was a little bit higher than in the respective salt-stressed control (Fig. 9*a–c*).

Discussion

PA analogues and 'specific' inhibitors of the enzymes participating in PA biosynthesis (cyclohexylamine, an inhibitor of Spd synthase) or in PA degradation (aminoguanidine, an inhibitor of DAO, and guazatine, an inhibitor of PAO) are frequently used in PA research (Moschou and Roubelakis-Angelakis 2014). The Spm analogue MDL has been applied *in vivo* as a potent inhibitor of PAO in several animal and human cell lines (Duranton *et al*. 2002; Seiler *et al*. 2002) but this is the first paper in which this compound has been applied in an intact plant system through the roots to investigate its effect on salt stress-induced PA metabolism. Our results proved that at 50 µM, MDL decreased PAO activity in homogenised leaf and root tissue extracts but it had no effect on the activity of DAO *in vitro*.

If tomato plants were treated with MDL through the root system, this triggered a systemic response, suggesting the generation of mobile signalling compounds.

PAOs and DAOs may have an important physiological role in stress-induced defence or cell death-inducing mechanisms (Moschou *et al*. 2008*a*). It was found that the terminal oxidation of PAs by DAO and PAO as well as H_2O_2 content changed only slightly after a 6-h-long exposure to sublethal NaCl treatment. Interestingly, under lethal salt stress, especially in the leaves, the induction of DAO and PAO activity, along with the respective H_2O_2 maxima could be a crucial signal to the induction of cell death. Similar results were obtained by Moschou *et al*. (2008*b*) with transgenic tobacco (*Nicotiana tabacum* L.) plants overexpressing the *ZmPAO* gene of maize, the enzyme participating in terminal oxidation of Spd. Under high salinity, these lines exhibited higher H_2O_2 levels and the induction of PCD.

In the present study, when plants were exposed to 100 mM or 250 mM NaCl in the presence of MDL, the activity of DAO and PAO and the concentration of H_2O_2 decreased significantly in both leaf and root tissues. These results suggested that the terminal oxidation of PAs by DAO and PAO during salt stress contributed to the generation of H_2O_2 in this system. However, at 250 mM NaCl, the first peak of ROS was not eliminated in the leaf tissues in the presence of MDL, which suggests that other enzymes (e.g. NOX) may also be involved in this early phase of apoplastic ROS production. It has to be mentioned that although MDL is a specific inhibitor of PAO *in vitro*, DAO activity was also inhibited *in vivo* in tomato. This phenomenon may be caused by the control of DAO activity by an unknown post-translational modification.

The transcription of DAO and PAO isoenzymes can also be controlled by several factors, such as H_2O_2, NO or PAs. The expression of *DAO* but not that of *PAO* was induced by external Spd and Spm in salinised *Citrus* leaves (Tanou *et al*. 2014). The salt stress-induced expression of DAO and PAO was not affected by 100 mM H_2O_2, but 100 µM sodium nitroprusside resulted in significant enhancement in the transcript abundance of both enzymes in the leaves (Tanou *et al*. 2012). In our system, the expression of the selected *SlDAO1* gene exhibited a small diurnal fluctuation in the leaves after salt exposure but, with the exception of the 6-h sample, it was only slightly affected

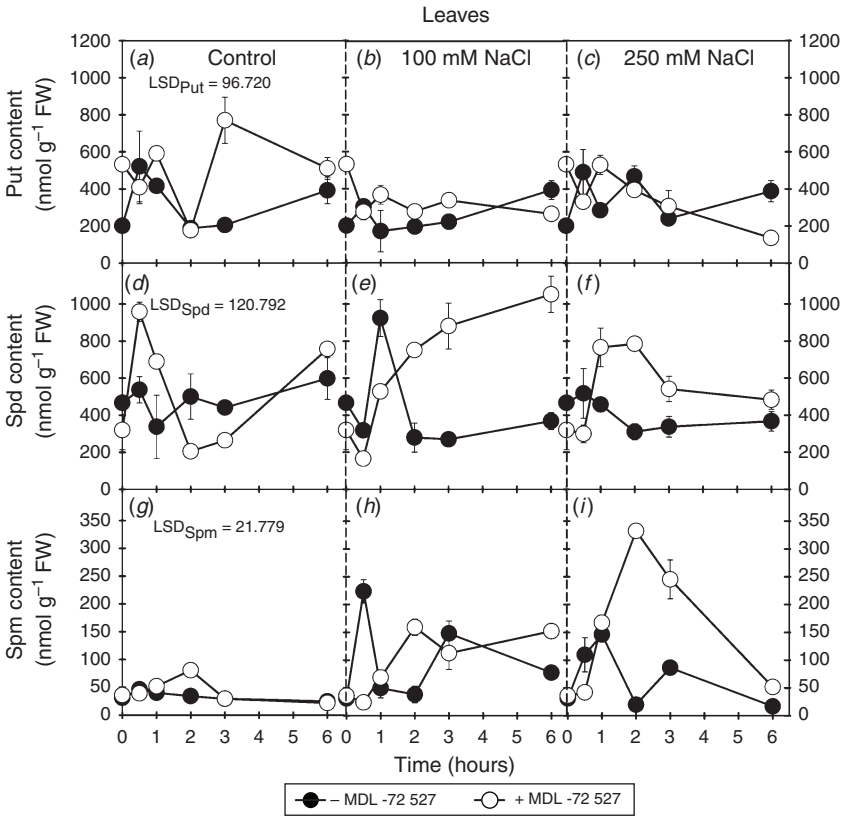

Fig. 4. Changes in free polyamine contents as a function of time in the leaves of (*a*, *d*, *g*) control tomato plants or in plants exposed to (*b*, *e*, *h*) 100 mM or (*c*, *f*, *i*) 250 mM NaCl in the presence or absence of 50 µM N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527) (black circles, without MDL; white circles, with MDL). (*a*, *b*, *c*) Putrescine (Put); (*d*, *e*, *f*) spermidine (Spd); (*g*, *h*, *i*) spermine (Spm). Means ± s.e. ($n = 3$). The differences between means were compared using Fisher's LSD ($P \leq 0.001$).

by salt stress. *SlPAO1* expression was inhibited in the leaves when the plants were exposed to high salinity. This suggests that the inhibition of PAO expression in the leaves is an early defence response to salt stress. Unlike *SlDAO1*, the expression of *SlPAO1* was induced significantly by salt treatments in the roots, as was also found in *A. thaliana* (Cona *et al*. 2006) or *Citrus aurantium* L. plants (Tanou *et al*. 2014). MDL had a time- and salt concentration-dependent effect on the expression of the selected *SlDAO1* and *SlPAO1* genes in the leaves, but the expression *of SlPAO1* was significantly induced in the control and low salt samples in the roots, suggesting that the increased Spd or Spm concentrations in these MDL-treated tissues may control the expression of *PAO*. This is in accordance with the data of Liu *et al*. (2014), who found that *OsPAO1* expression was markedly upregulated by treatment with 0.1 mM Spm but not by either Put or Spd in rice (*Oryza sativa* L.) roots.

PA biosynthesis and accumulation represent an important aspect of plants' response to high salinity. Higher PAs, especially Spd and Spm, reached much higher levels under the 100 mM NaCl treatment than under lethal salt stress in the leaf tissues, which can be crucial in the acclimation to high salinity. In the same tissues, the accumulation of Spd and Put can be observed in the controls, whereas Spm and Spd accumulated at both salt concentrations in the presence of the inhibitor. MDL treatment resulted in high Spm, Spd and Put levels in control roots and under 100 mM NaCl, but free PA concentrations remained low under 250 mM NaCl. This means that the inhibitor triggered the tissue-, time- and NaCl concentration-dependent accumulation of various PA species.

It has been revealed by several authors that external application of H_2O_2 or its production inside the cells may control NO generation in plant tissues (reviewed by Pottosin and Shabala 2014). NO is the other important molecule in the defence responses against salt stress. NO release into the reaction medium was significantly induced when *A. thaliana* seedlings were treated with 1 mM Spd or Spm (Siddiqui *et al*. 2011). Based on our results, 250 mM NaCl induced the ROS burst in parallel with an early NO accumulation in the roots, which could initiate cell death. However, 3 h after the exposure to 100 mM NaCl, NO accumulation in the roots may contribute to salt stress acclimation. To best of our knowledge, this is the first demonstration that maximal ROS and NO generation occurred at different time points during sublethal and lethal salt stress, and cell death was initiated when oxidative and nitrosative stress operated synergistically at 250 mM NaCl. Nevertheless, NO production can be eliminated by MDL, which suggests that

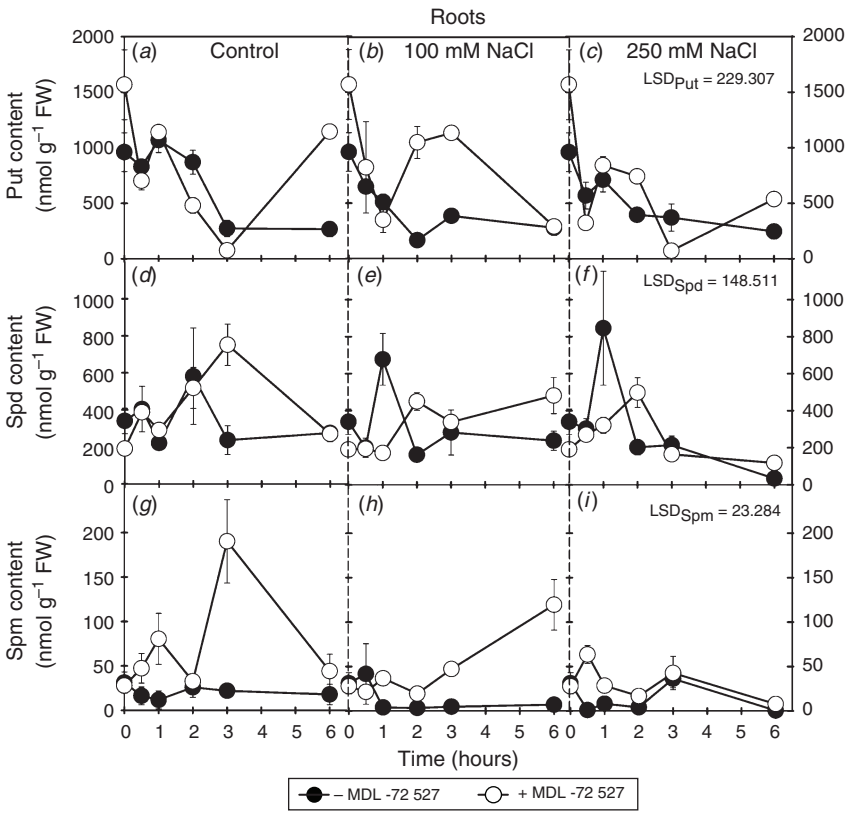

Fig. 5. Changes in free polyamine contents as a function of time in the roots of (*a*, *d*, *g*) control tomato plants or in plants exposed to (*b*, *e*, *h*) 100 mM or (*c*, *f*, *i*) 250 mM NaCl in the presence or absence of 50 μM N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527) (black circles, without MDL; white circles, with MDL). (*a*, *b*, *c*) Putrescine (Put); (*d*, *e*, *f*) spermidine (Spd); (*g*, *h*, *i*) spermine (Spm).Means ± s.e. ($n = 3$). The differences between means were compared using Fisher's LSD ($P \leq 0.001$).

the PAO inhibitor can modulate NO accumulation independently of ROS accumulation under sublethal salt stress.

PA catabolism-related ROS and NO synthesis is a good hypothesis for explaining PA-mediated stress responses. It has been proposed that PCD is strictly regulated by the ratio of PA anabolism to catabolism, whereas ROS generation or accumulation is a nodal point in the cell fate decision (Moschou *et al*. 2008*b*). Our results revealed that 250 mM NaCl induced a double peak in H_2O_2 production in leaf and root tissues, which was eliminated by the use of the PAO inhibitor MDL, suggesting that this process is controlled at least partially by DAO and PAO activity under salt stress. Since MDL inhibited both ROS and NO accumulation significantly, it can be supposed that PA catabolism by PAO contributes not only to H_2O_2 accumulation but also, at least indirectly, to NO production in the tissues exposed to lethal salt stress. This idea was supported by a report demonstrating that PAO can be inhibited by a nitric oxide synthase (NOS) inhibitor L-nitroarginine methyl ester (L-NAME) and might be a candidate for PA-based NO production, via a yet unknown reaction (Tun *et al*. 2006). On the basis of the different kinetics of ROS and NO accumulation in root apices under 100 mM NaCl, it can be concluded that the control of NO generation at sublethal salt stress is more complex.

As a potent inhibitor of PAO, MDL was expected to alleviate salt stress injury. In accordance with its function, the inhibition of the PA-degrading enzymes DAO and PAO resulted in selective accumulation of PAs. Earlier, the effect of NaCl on electrolyte leakage was attributed to a general loss of membrane integrity, but in several plant species, it has been found that salt-induced K^+ efflux may be a regulated process mediated by K^+-permeable channels (Demidchik 2014; Demidchik *et al*. 2014). PAs at appropriate concentrations are able to depolarise the PM, leading to increased PM conductance (Pottosin and Shabala 2014). Thus supraoptimal PA levels created by MDL may contribute to the moderate increment of ion efflux from tissues in control and salt-stressed leaves.

In contrast to control roots, the MDL treatment caused significant increases in electrolyte leakage in the roots exposed to salt stress. This is in accordance with earlier results that PAs potentiated K^+ efflux in intact roots under salt stress, although they were incapable of inducing any K^+ efflux in root tissues by themselves (Velarde-Buendía *et al*. 2012). Since outward-rectifying K^+ channels can be directly inhibited by *S*-nitrosylation (Pottosin and Shabala 2014), the elimination of NO production by MDL may interfere with this regulatory step by enhancing K^+ efflux from the tissues. These results support the finding that

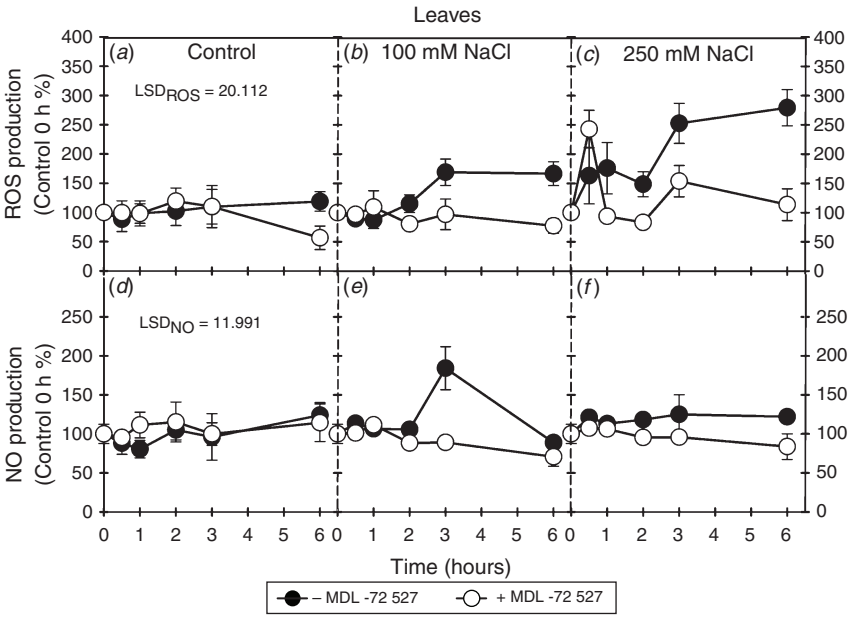

Fig. 6. Changes in (*a*, *b*, *c*) reactive oxygen species (ROS) and (*d*, *e*, *f*) NO accumulation normalised to 0 h samples as a function of time in the leaf disks of (*a*, *d*) tomato plants under control conditions, or in plants exposed to (*b*, *e*) 100 mM or (*c*, *f*) 250 mM NaCl in the presence or absence of 50 µM N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527) (black circles, without MDL; white circles, with MDL). Means ± s.e. ($n = 10$). The differences between means were compared using Fisher's LSD ($P \leq 0.001$).

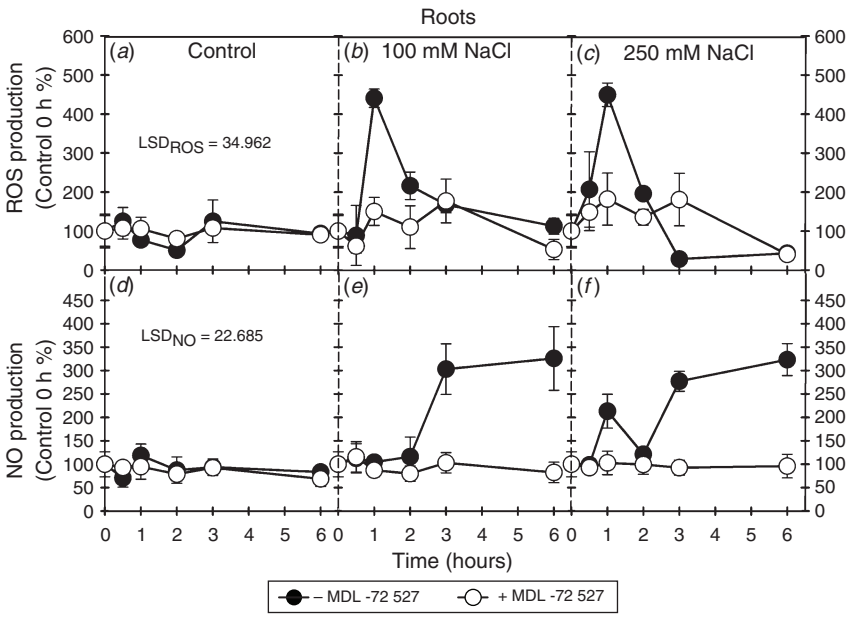

Fig. 7. Changes in (*a*, *b*, *c*) reactive oxygen species (ROS) and (*d*, *e*, *f*) NO accumulation normalised to 0 h samples as a function of time in the root tips of (*a*, *d*) tomato plants under control conditions or in plants exposed to (*b*, *e*)_ 100 mM or (*c*, *f*) 250 mM NaCl (C, F) in the presence or absence of 50 µM N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527) (black circles, without MDL; white circles, with MDL). Means ± s.e. ($n = 10$). The differences between means were compared using Fisher's LSD ($P \leq 0.001$).

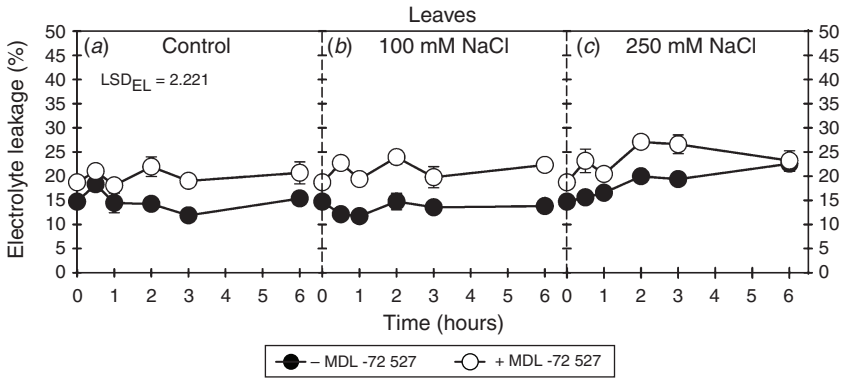

Fig. 8. Relative electrolyte leakage from the leaf disks of (*a*) tomato plants under control conditions or in plants exposed to (*b*) 100 mM or (*c*) 250 mM NaCl in the presence or absence of 50 µM N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527) (black circles, without MDL; white circles, with MDL). Means ± s.e. ($n = 6$). The differences between means were compared using Fisher's LSD ($P \leq 0.001$).

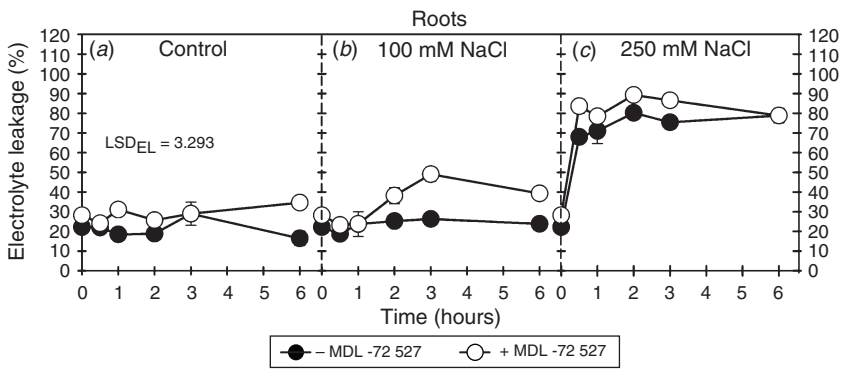

Fig. 9. Relative electrolyte leakage from the roots of (*a*) tomato plants under control conditions or in plants exposed to (*b*) 100 mM or (*c*) 250 mM NaCl in the presence or absence of 50 µM N^1,N^4-*bis*-(2,3-butadienyl)-1,4-butanediamine (MDL-72527) (black circles, without MDL; white circles, with MDL). Means ± s.e. ($n = 6$). The differences between means were compared using Fisher's LSD ($P \leq 0.001$).

in spite of PAO's involvement in PCD induction, the inhibition of PA degradation by specific inhibitors of PAO does not increase the salt stress tolerance of plants.

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