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H_2O_2 Involvement in Polyamine-Induced Cell Death in Tobacco Leaf Discs

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Abstract The response of tobacco (*Nicotiana tabacum* L.) wild-type SR1 leaf discs in terms of reactive oxygen species (ROS) formation and cell death occurrence was evaluated after exposure to the polyamines (PAs) putrescine (Put), spermidine (Spd), and spermine (Spm). Although NADPH oxidase-like enzyme activity was inhibited by all PAs at 3 or 21 h of treatment, H_2O_2 content increased significantly in a time- and concentration-dependent manner, suggesting that H_2O_2 accumulation was linked to the activity of other ROS-generating enzymes. Polyamine oxidase (PAO) activity, which increased markedly upon application of Spd or Spm, is a prime candidate for the increased H_2O_2 accumulation. Except for 0.1 mM Put, which maintained guaiacol peroxidase (GPOX) and catalase (CAT) activities at the same level as the control, the other PA treatments decreased CAT, ascorbate peroxidase, and GPOX activities at 21 h, contributing to the H_2O_2 increase. Esterase activity and Evans blue staining, two cell death parameters, were negatively affected at 3 h of treatment with 1 mM Spd and with both concentrations of Spm, whereas at 21 h there was an increase in cell death with both concentrations of the three PAs, except for 0.1 mM Put, which did not alter those parameters. The expression of the senescence-associated cysteine protease gene *CP1* was measured to monitor senescence, a physiological cell death process. Application of all PAs increased the expression of the gene, except for 0.1 mM Put, which decreased its expression at 21 h. This result was in agreement with the prevention of cell death exerted by Put and evidenced by Evans blue staining, esterase activity, and electrolyte release.

Keywords Cell death · Hydrogen peroxide · *Nicotiana tabacum* · Polyamines · Reactive oxygen species

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Introduction

Reactive oxygen species (ROS) are normally produced in the cells of all aerobic organisms under steady-state conditions and are scavenged by various antioxidative defense mechanisms (Foyer and Noctor 2005). However, ROS formation greatly increases when plants face environmental challenges. ROS affect plant growth and development, involving biochemical disturbances and causing an imbalance in the cell redox state commonly referred to as oxidative stress, that may even lead to cell death (Miller and others 2008). An uncontrolled increase in the production of the main ROS formed in plant cells triggers a complex detoxification mechanism that may involve nonenzymatic and enzymatic systems capable of preventing the cascades of unrestrained oxidation (Gill and Tuteja 2010). ROS are not only generated as by-products of other cell processes, they are also "deliberately" generated by enzymatic complexes (Apel and Hirt 2004). The main ROS formed in plant cells are the superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). The latter is moderately reactive with a relatively long half-life (1 ms) and may inactivate enzymes by oxidizing their thiol groups (Foyer and Noctor 2005). H_2O_2 plays a dual role in plants: at low concentrations it acts as a signal molecule triggering tolerance to various

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biotic and abiotic stresses (Tanou and others 2009), and at high concentrations it can lead to plant cell death (PCD) (Quan and others 2008).

Polyamines (PAs), including spermidine (Spd, a tri-amine), spermine (Spm, a tetra-amine), and their obligate precursor putrescine (Put, a diamine), are aliphatic amines widely present in living organisms. These molecules are involved in the regulation of many basic cellular processes, including DNA replication, transcription, translation, cell proliferation, modulation of enzyme activities, cellular cation–anion balance, and membrane stability (Groppa and Benavides 2008; Alcázar and others 2010). It has been illustrated that PAs also play pivotal roles in plant physiological and developmental processes such as the control of cell division and embryogenesis (Handa and Mattoo 2010), morphogenesis, pollen viability, senescence, fruit ripening, and responses to biotic and abiotic stresses (Takahashi and others 2003; Ziosi and others 2006; Cuevas and others 2008; Groppa and Benavides 2008; Alcázar and others 2010; Moschou and others 2012). Antioxidant and protective functions, including possible regulation of the structure and function of nucleic acids and maintenance of cellular pH and membrane integrity (Groppa and others 2008; Hussain and others 2011), have been attributed to PAs.

The intracellular pool of free PAs depends on their synthesis, but also on their degradation, transport, and conjugation (Igarashi and Kashiwagi 2000). PA catabolism is carried out by two classes of amine oxidases (AOs), the copper amine oxidases (CuAOs), with high substrate specificity toward diamines (Put), and the flavin-containing polyamine oxidases (PAOs) which oxidize Spd and Spm (Cohen 1998). Oxidation of PAs by CuAOs and PAOs contributes to regulation of PA homeostasis (Cona and others 2006; Angelini and others 2010), but, in addition, it generates catabolic products that have been linked to several biological functions ascribed to PAs. In particular, PA-derived H_2O_2 has been shown to play an important role in cell wall maturation and stress-induced stiffening, in signaling of stomata opening, and in PCD in both defense and development (Angelini and others 2010).

The role of PA-derived H_2O_2 is still controversial. It has been reported that exogenous application of PAs delays senescence and cell death-related processes in many plant species (Tassoni and others 2006), and PAs were also demonstrated to behave as antioxidants that protect against chilling or metal-derived oxidative stress when they were exogenously added (Shen and others 2000; Groppa and others 2001, 2008). However, it remains unclear how exogenous application of PAs is manifested as a signal that delays senescence or cell death. One hypothesis is that PAs are oxidized and the H_2O_2 produced acts as a signaling compound, which could activate processes that contribute to extension of life span (Moschou and others 2012). PAs have also been reported to be NADPH oxidase inhibitors (Shen and others 2000; Papadakis and Roubelakis-Angelakis 2005) or ROS scavengers (Groppa and others 2001), thus contributing to the protection of cell membranes against oxidative stress.

On the other hand, PAs have been postulated as cell death inducers in animals (Zahedi and others 2007) and plants (Yoda and others 2003; Tisi and others 2011). Natural senescence is a genetically determined cell death process, characterized by chlorophyll breakdown and upregulation of many senescence-associated genes (SAGs) (He and others 2008). These genes encode various hydrolytic enzymes such as proteases, ribonucleases, lipases, and enzymes involved in ethylene biosynthesis, which carry out tissue degradation processes and are involved in PCD (Xu and Chye 1999).

The aim of the current work was to evaluate whether PAs function as cell death inducers in tobacco leaf discs, while trying to elucidate if this process was mediated by H_2O_2, and whether PAs regulate de novo H_2O_2 generation by PAO or by modulating the activity of enzymes involved in ROS formation (such as NADPH oxidase) or ROS scavenging, such as guaiacol peroxidase (GPOX), ascorbate peroxidase (APOX), and catalase (CAT).

Materials and Methods

Plant Growth Conditions and Treatments

Seeds of *Nicotiana tabacum* var. Petit Havana SR1 wild type (kindly provided by Dr. F. Van Breusegem, Ghent University, Belgium) were germinated and grown as described previously (Iannone and others 2010). Experiments were performed using the fourth to fifth leaf (counting from the bottom) of 6–8-week-old plants. Leaf discs (8-mm diameter) were cut with a cork-borer, put in glass flasks containing 25 ml of the treatment solution (distilled water and 0.1, 1, or 5 mM Put, Spd, or Spm), and incubated in a rotary shaker, under continuous illumination, for 3 or 21 h to assess what occurs at short or long times of exposure. When indicated, tobacco leaf discs were incubated with 200 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-1-oxy-3-oxide (cPTIO, a NO scavenger); 10 or 100 mM sodium nitroprusside (SNP, $Na_2[Fe(CN)5NO]$, a NO donor); 1,500 U ml^{-1} CAT, 300 U ml^{-1} SOD, 5 mM $LaCl_3$ (Ca^{2+} channel blocker), 1,700 U ml^{-1} peroxidase, or 100 mM caffeine (Ca^{2+} chelator), before or together with the corresponding treatment. After that, leaf discs were washed with distilled water, dried with adsorbent paper, and used for analysis.

Hydrogen Peroxide Content Assay

Determination of H_2O_2 content was performed according to the method of Sergiev and others (1997). Leaf discs (300 mg) were ground with 0.1 % trichloroacetic acid and centrifuged at 12,000×g for 20 min. Then, 0.7 ml of the supernatant was mixed with 0.7 ml of 10 mM PBS (pH 7) and 1.4 ml of 1 M KI, and the absorbance of the solution was measured at 390 nm. H_2O_2 concentrations were calculated using a standard curve prepared with known concentrations of H_2O_2.

Cell Death Evaluation: Evans Blue Staining, Electrolyte Leakage, and Esterase Activity

To determine changes in cell viability, leaf discs were incubated with a 0.25 % (w/v) aqueous solution of Evans blue (Baker and Mock 1994) for 15 min at room temperature, then washed twice with distilled water and left in distilled water overnight. The samples were then incubated for 1 h at 50 °C with a methanol–SDS solution and the absorbance resulting from the release of the dye was measured at 595 nm. To assess the involvement of H_2O_2 in the cell death process, leaf discs were incubated with 1,500 U ml^{-1} CAT for 90 min before applying treatments. Cell death was also estimated by measuring ion leakage from leaf discs (Shou and others 2004). Initial conductivity was measured for each solution (T_0). The conductivity of the solution was then measured after incubation for 1 h at room temperature (RT) (T_1) and after heating at 100 °C for 1 h (T_2). The results were expressed as relative conductivity $[(T_1 - T_0)/(T_2 - T_0)] \times 100$. Esterase (EA; EC 3.1.1.x) activity was measured as another parameter of cell death, as described by Steward and others (1999).

Analysis of DNA Fragmentation

DNA extraction was performed using the ZR Plant/Seed DNA MiniPrep™ Kit (Zymo Research Corp., Irvine, CA, USA). DNA samples were separated on 2 % (w/v) agarose gels and visualized by staining with ethidium bromide. Gels were photographed with Fotodyne equipment (Hartland, WI, USA).

Thiobarbituric Acid Reactive Substances (TBARS) Determination

Lipid peroxidation was determined in the leaf discs and in the incubation medium as the amount of thiobarbituric acid reactive substances (TBARS), measuring the absorbance at 532 nm and subtracting the turbidity at 600 nm ($\varepsilon = 155\ mM^{-1}\ cm^{-1}$) according to Heath and Packer (1968).

Subcellular Fractionation and Assay of NADPH Oxidation

NADPH oxidase activity was measured in the microsomal fraction and in the cytosol by monitoring NBT reduction by NADPH at 530 nm for 3 min, according to Shen and others (2000). NADPH oxidation activity was calculated by taking the difference between the apparent reaction rates with or without SOD (75 U ml^{-1}) in the reaction mixture, using $\varepsilon = 12.8\ mM^{-1}\ cm^{-1}$.

Assay for Polyamine Oxidase

Proteins from 300 mg of tobacco leaf discs were used for PAO analysis. The extraction buffer consisted of 0.1 M phosphate buffer (pH 6.5), 10 µM pyridoxal phosphate, and 2 mM dithiothreitol. PAO activity for the oxidation of Spm was determined spectrophotometrically by following the formation of a pink adduct as a result of oxidation of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid catalyzed by horseradish peroxidase, and monitoring the increase in absorbance at 515 nm according to Smith and Barker (1988). The assays were performed using the same buffer containing 0.06 mg horseradish peroxidase, with 2 mM Spm as the substrate in 1 ml total volume. Enzyme activity was expressed in IUs (1 unit is the amount of enzyme that catalyzes the oxidation of 1 µmol substrate min^{-1}) on protein basis.

Antioxidant Enzyme Preparations and Assays

Homogenates for determination of APOX (EC 1.11.1.11), GPOX (EC 1.11.1.7), superoxide dismutase (SOD, EC 1.15.1.1), and CAT (EC 1.11.1.6) activities were prepared from 200 mg fresh weight (FW) of leaf tissues in 50 mM phosphate buffer (pH 7.8) containing 0.5 mM EDTA, 1 g PVP, and 0.5 % (v/v) Triton X-100. APOX activity was determined immediately by the decrease in absorbance at 290 nm due to oxidation of ascorbate ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$; Nakano and Asada 1981). GPOX activity was determined following the increase in absorbance at 470 nm due to the formation of tetraguaiacol ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$; Maehly and Chance 1954). SOD activity was assayed as described by Becana and others (1986). CAT activity was determined by measuring the decrease in absorbance at 240 nm due to consumption of H_2O_2 (Chance and others 1979).

Western Blot Analysis of CAT

Soluble protein extracts were prepared from leaf discs and 25 µg total protein were electrophoresed on 12 % SDS-PAGE in a Mini PROTEAN III (Bio-Rad, Hercules, CA, USA). The proteins were then electrotransferred to a

nitrocellulose membrane (Amersham Biosciences, GE Healthcare Biosciences Corp., Piscataway, NJ, USA). To immunodetect CAT, the membrane was incubated at 4–8 °C overnight with a polyclonal antibody raised in rabbit against a cottonseed CAT (kindly provided by Dr. Trelease from Arizona State University). Bands were detected with an anti-rabbit IgG peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO, USA) and revealed with DAB.

Analysis of Gene Expression

Total RNA was extracted from leaf tissue (100 mg) using a modified TRIzol (Invitrogen, Carlsbad, CA, USA) procedure and RNA was then treated with DNase I (Sambrook and others 1989). It was converted to cDNA with random primers using the RevertAid™ M-MuLV reverse transcriptase (Fermentas, ThermoFisherScientific, Waltham, MA, USA). Primers for the *NtrbohD* gene, a tobacco NADPH oxidase gene, were designed and optimized for PCR using the bioinformatics program Primer 3 and a nucleotide sequence available in DFCI Plant Gene Indices (forward primer: 5′-TGCTGCAGTTTCTCCATTTG-3′ and reverse primer: 5′-CCATTGCCTTCATGTTGTTG-3′). A senescence-related gene, *CP1*, was selected to test a natural cell death process as senescence. For *CP1* amplification (accession no. AY881011), primers described by Niewiadomska and others (2009) were used. Cycling conditions were 94 °C for 1 min; 32 or 36 cycles of denaturing at 94 °C for 0.5 min, annealing at 53 or 63 °C for 1 min, and extension at 72 °C for 1 min; and a final step of 72 °C for 7 min, for *NtrbohD* and *CP1*, respectively. Amplification of 18S ribosomal cDNA (forward primer: 5′-GGCTACCACATCCAAGGAA-3′; reverse primer: 5′-CTATTGGAGCTGGAATTACCG-3′) was used as an internal control of the amount of RNA. Each PCR reaction was amplified using an optimized number of cycles to ensure the linearity requirement for semiquantitative RT-PCR analysis. The PCR products were electrophoresed through 2 % agarose gel and visualized with ethidium bromide. Gels were photographed with FOTO/Analyst® Investigator/Eclipse systems (Fotodyne) and analyzed with GelPro Analyzer (Media Cybernetics, Rockville, MD, USA) software, and data were expressed as arbitrary units (assuming control value equal to 100) based on absolute integrated absorbance of each band.

Determination of Protein Concentration

Protein concentration for all samples was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistics

All data are presented as the mean value of three independent sets of experiments. Each value is presented as mean ± standard error (SE), with a minimum of three replicates. Statistical analysis was carried out by one-way ANOVA using the Tukey test to evaluate whether the means were significantly different, with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ as significant.

Results

Hydrogen Peroxide Formation in Leaf Tissues

Because PAs are postulated as antioxidants through the regulation of $O_2^{\cdot-}$ or H_2O_2 formation, but they are as well catabolized to produce H_2O_2, we first examined H_2O_2 formation in leaf tissues after addition of exogenous PAs. After 3 h of exposure, only 1 and 5 mM Spd or Spm increased H_2O_2 content, which increased from 50 % for 1 mM Spd to 101 % for 5 mM Spm over the controls (Fig. 1). At 21 h, the highest Put concentration and both 1 and 5 mM Spd or Spm produced a further increase in the H_2O_2 level, reaching values of 36 and 110 % over the controls for 5 mM of Put and Spd, respectively, whereas 1 and 5 mM Spm increased H_2O_2 content around 145 % compared to nontreated leaf discs (Fig. 1).

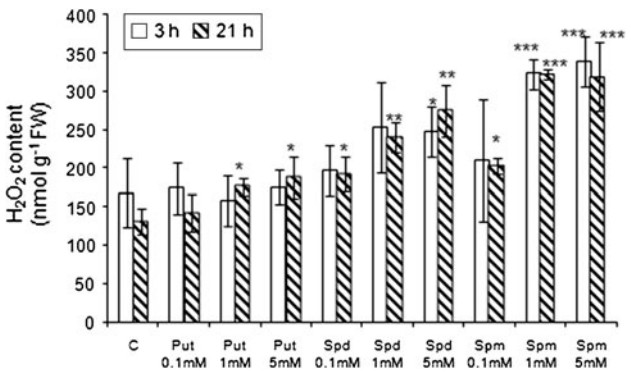

Fig. 1 Hydrogen peroxide formation in SR1 plants exposed to polyamines. Leaf discs exposed to distilled water and 0.1, 1, or 5 mM Put, Spd, and Spm for 3 or 21 h. Homogenates from exposed leaf discs were made as described in the "Materials and Methods" section. Hydrogen peroxide content is expressed in nmol g^{-1} FW. Values are the mean ± SE from three independent experiments with six replicated measurements. *Asterisks* indicate significant differences ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) according to Tukey's multiple-range test

NADPH Oxidase Activity and *NtrbohD* Transcript Level

To test whether H_2O_2 increased after an increase in the activity of NADPH oxidase induced by PAs, the specific enzyme activity was determined in microsomal preparations from tobacco leaves, as described in Materials and Methods. The activity of the enzyme was strongly inhibited by PAs, in a concentration-dependent manner, in a range from about 60 % (0.1 mM Put and Spd at 3 h) to 90 % (1 mM of the three PAs at 3 h). At 21 h, 0.1 mM Spm produced a 73 % decline in enzyme activity, with 1 mM Spm producing the greatest inhibition (Fig. 2a).

The expression of the native *NtrbohD* gene under PA exposure was analyzed using semiquantitative RT-PCR. The *NtrbohD* transcript was the expected size (340 bp) (Simon-Plas and others 2002). At 3 h, a slight increase in the *NtrbohD* transcript level was observed with 0.1 or 1 mM Spd and Spm treatments. At 21 h, 1 mM Put and 0.1 mM Spm did not alter the gene expression, whereas the other PA concentrations tested produced a decrease in gene expression of about 25–60 % with respect to the control (Fig. 2b).

PAO Activity

To ascertain whether the high levels of H_2O_2 were due to mainly increased PAO activity, we measured the enzyme activity using Spm as substrate. The effect appeared to be dose- and time-dependent. At 3 h, PAO activity levels, expressed on a protein basis (Fig. 3), increased from 31 to 88 % when tissues were exposed to increased Spd levels, and from 71 to 146 % when leaf discs were exposed to Spm, whereas at 21 h, the increase in the enzyme activity ranged from 72 to 123 % and from 112 to 183 % for Spd and Spm treatments, respectively, compared with non-treated controls (Fig. 3).

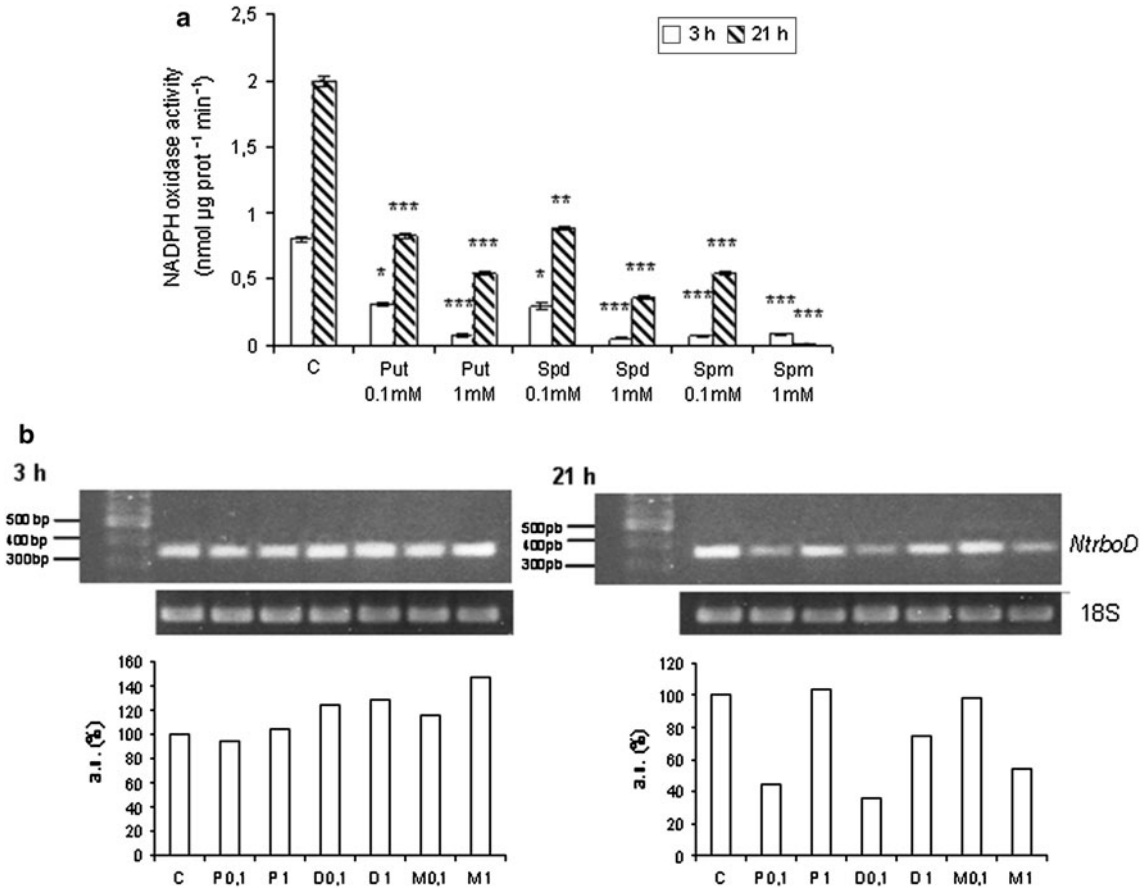

Fig. 2 NADPH-dependent superoxide production (**a**) and *NtrbohD* transcript level (**b**) in SR1 tobacco leaf discs. Leaf discs were exposed to 0.1 and 1 mM Put, Spd, or Spm for 3 or 21 h. NADPH-dependent oxidase activity and RT-PCR for *NtrbohD* were performed according to the description in the "Materials and Methods" section. **a** Values are the mean ± SE of three different experiments with three replicated measurements. *Asterisks* indicate significant differences ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) according to Tukey's multiple-range test. **b** Relative mRNA values were calculated as the ratio *NtrbohD/18S*. Data are expressed as percentage of arbitrary units (assuming the control value as equal to 100), based on the integration of the absolute optical density of each band. The fragment of *NtrbohD* corresponds to 340 bp. The figure shows results typical of those obtained in three independent experiments. Put (P), Spd (D), Spm (M)

Antioxidant Enzyme Activities

The activity of superoxide dismutase, an enzyme capable of converting $O_2^{\cdot-}$ to H_2O_2, increased with both concentrations of Put and Spd at 3 h, but did not show changes at 21 h, except for a reduction of 40 % with 1 mM Spd (Table 1). The status of the antioxidant enzymatic machinery capable of detoxifying H_2O_2 was evaluated through the measurement of CAT, APOX, and GPOX activities. At 3 h, the three PAs at both concentrations (except for 0.1 mM Spm) reduced CAT activity between 19 and 84 %, whereas at 21 h, the activity of the enzyme increased 45 and 25 % over the controls when leaf discs were treated with 0.1 and 1 mM Spm, respectively. APOX and GPOX activities were not affected by 3 h of treatment with PAs, whereas at 21 h, both concentrations of all PAs decreased APOX activity between 56 and 83 % below the control. By this time, Spd and Spm at both levels reduced GPOX activity between 30 and 63 %, respectively. In contrast, the activity of CAT and GPOX enzymes remained unaltered when leaf discs were exposed to 0.1 mM Put for 21 h (Table 1).

CAT protein content increased by 50 and 30 % at 3 h of treatment with 1 mM Put and 0.1 mM Spm, respectively. However, at 21 h, the protein content decreased markedly with 0.1 and 1 mM Spd or Spm and was maintained higher than controls only with 0.1 mM Put (Fig. 4).

Evidence of Oxidative Damage and Cell Death: TBARS Content, Electrolyte Leakage, Evans Blue Staining, Esterase Activity, and DNA Fragmentation

None of the tested PAs induced lipid peroxidation (measured in the tissues and in the incubation medium)

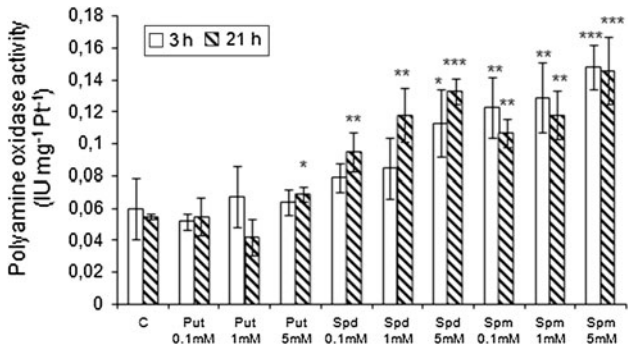

Fig. 3 Effect of PAs on PAO enzyme activity in tobacco leaf discs. The enzyme activity was assessed as described in the "Materials and Methods" section after supplying 2 mM Spm as substrate. PAO activity is in IUs (1 unit is the amount of enzyme that catalyzes the oxidation of 1 µmol substrate min^{-1}) on protein basis. Values are the mean ± SE from three independent experiments with three replicated measurements. *Asterisks* indicate significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) according to Tukey's multiple-range test

Table 1 Effect of 0.1 and 1 mM Put, Spd, or Spm on CAT, APOX, GPOX, and SOD activities at 3 or 21 h of treatment

		C	0.1 mM Put	1 mM Put	0.1 mM Spd	1 mM Spd	0.1 mM Spm	1 mM Spm
CAT	3 h	0.57 ± 0.04	0.31 ± 0.10^{b}	0.35 ± 0.07^{a}	0.45 ± 0.04^{a}	0.09 ± 0.00^{b}	0.56 ± 0.10	0.46 ± 0007^{a}
(pmol g^{-1} FW s^{-1})	21 h	1.01 ± 0.13	1.20 ± 0.02	0.72 ± 0.03	0.85 ± 0.02	0.65 ± 0.01^{a}	1.47 ± 0.01^{a}	1.27 ± 0.07^{a}
APOX	3 h	19.14 ± 1.29	17.58 ± 1.01	23.65 ± 0.30	24.28 ± 0.55	21.70 ± 0.60	22.28 ± 2.05	13.88 ± 0.58
(pmol g^{-1} FW s^{-1})	21 h	24.97 ± 3.73	8.83 ± 0.00^{d}	4.21 ± 0.13^{c}	6.87 ± 0.00^{c}	4.30 ± 0.33^{c}	14.15 ± 0.66^{a}	4.15 ± 0.01^{c}
GPOX	3 h	1933.90 ± 347.1	1839.25 ± 142.0	1895.02 ± 27.51	1873.50 ± 131.5	1891.94 ± 40.10	1879.33 ± 60.01	1866.73 ± 80.05
(µmol g^{-1} FW)	21 h	1634.91 ± 268.6	1330.98 ± 10.74	1354.17 ± 281.9	1142.22 ± 12.8^{a}	599.73 ± 50.24^{b}	731.97 ± 50.22^{b}	612.10 ± 40.11^{b}
SOD	3 h	17.95 ± 5.39	22.60 ± 0.52^{b}	21.96 ± 0.38^{a}	24.54 ± 0.10^{a}	21.68 ± 0.79^{a}	20.25 ± 1.11	20.34 ± 0.45
(U g^{-1} FW)	21 h	24.45 ± 4.23	27.72 ± 0.40	20.79 ± 2.27	25.77 ± 0.53	14.72 ± 0.31^{a}	20.98 ± 2.71	19.60 ± 0.66

Data are the mean ± SE of three independent experiments, with five replicates for each treatment. Different letters within rows indicate significant differences (^{a} $p < 0.05$; ^{b} $p < 0.01$; ^{c} $p < 0.001$), according to Tukey's multiple-range test. One unit of CAT is the amount of the enzyme that oxidized 1 µmol of H_2O_2 per minute under the assay conditions. One unit of APOX forms 1 mmol of ascorbate oxidized per minute under the assay conditions. One unit of GPOX is the amount of the enzyme that reduced 1 mmol of H_2O_2 per minute under the assay conditions. One unit of SOD is the amount of the enzyme that inhibits the reduction of NBT by 50 % under the assay conditions

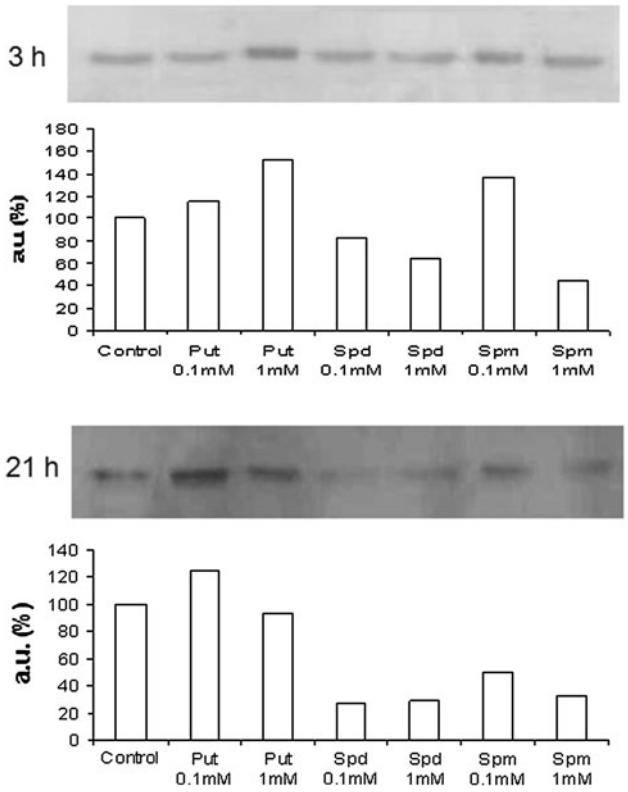

Fig. 4 Catalase protein expression in SR1 plants determined by Western blot protein and densitometry analysis. Leaf discs were exposed to 0.1 and 1 mM Put, Spd, or Spm for 3 or 21 h. CAT expression was determined as described in the "Materials and Methods" section. The experiment was repeated three times and a representative image is presented

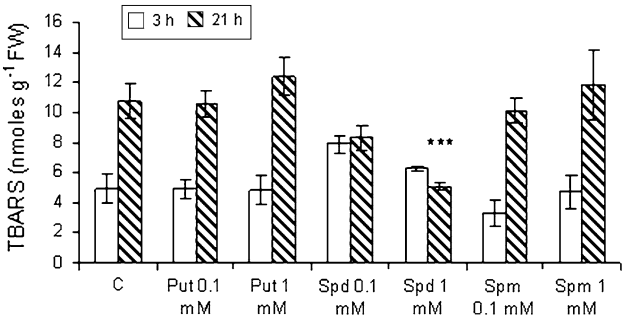

Fig. 5 Thiobarbituric acid reactive substance (TBARS) content of SR1 plants. Leaf discs were exposed to 0.1 and 1 mM Put, Spd, or Spm for 3 or 21 h as described in the "Materials and Methods" section. Values are the mean ± SE of two different experiments with three replicated measurements. *Asterisk* indicates significant differences (***$p < 0.001$) according to Tukey's multiple-range test

determined as the content of TBARS. In fact, 1 mM Spd decreased TBARS content after 21 h of exposure (Fig. 5).

Ion leakage was selected as one of the cell death markers. Except for 1 mM Spd, which doubled ion leakage over the controls (Fig. 6a), no symptoms of cell damage were observed after 3 h of treatment with any of the three PAs used. After 21 h of exposure, 1 mM Spd and Spm increased electrolyte leakage by 144 and 127 % over the controls, respectively. In contrast, 3 h of exposure to the higher concentration of Put still decreased electrolyte leakage and a similar result was observed after 21 h of treatment with 0.1 mM Put (Fig. 6a).

Cell death was also estimated using Evans blue (a dye that stains specifically dead cells). At 3 h of treatment, leaf discs exposed to 1 mM Spd or both concentrations of Spm showed an increase in cell death, whereas at 21 h, all PAs tested, except 0.1 mM Put, increased this parameter (Fig. 6b). To test the participation of H_2O_2 in cell death, leaf discs were preincubated with 1,500 U m^{-1} CAT, prior to treatment with 1 mM PAs for 21 h. The presence of this antioxidant enzyme resulted in a total rescue of the cell death that occurred after addition of Put and a partial recovery for Spd and Spm treatments, suggesting that H_2O_2 was one of the molecules that mediated PA-induced cell death (Fig. 7).

Measurement of esterase activity was used as another cell death parameter. This method is dependent not only on cell viability, but also on the metabolic state of the cell (Matanguihan and others 1994). A statistically significant decrease in cell death was observed after 3 h of treatment with 1 mM Spd and both concentrations of Spm, whereas at 21 h, cell death increased (EA activity decreased) along with increased concentration of applied PAs and with the number of amino groups in the PA molecules (Fig. 6c). Esterase activity was also determined after 48 h of incubation, with similar results. All PAs tested caused a marked decrease in cell viability, ranging from a decrease of 30–90 % with respect to the controls, with the exception of 0.1 mM Put, which did not alter EA activity at any studied time (Fig. 6c).

To corroborate the role of H_2O_2 in cell death induced by PAs, EA activity was measured in leaf discs treated for 21 h with the highest concentration of Put, Spd, or Spm, previously infiltrated with enzymes that can modify H_2O_2 status in the cell (1,500 U ml^{-1} CAT and 300 U ml^{-1} SOD). Superoxide dismutase pretreatment did not modify PA-induced decay in EA activity (Table 2A), whereas preincubation of the tissues with CAT partially (with Spd and Spm) or totally (with Put) prevented PA-induced decrease in EA activity (Table 2A). However, when CAT was infiltrated 6 h after initiation of treatment, cell death was not avoided (data not shown). Furthermore, addition of 1 and 10 mM H_2O_2 decreased EA activity 28 and 36 %, respectively (Table 2B), whereas simultaneous incubation with PAs and peroxidase (1,700 U ml^{-1}) produced a partial recovery (14 or 22 %) of EA activity with respect to the

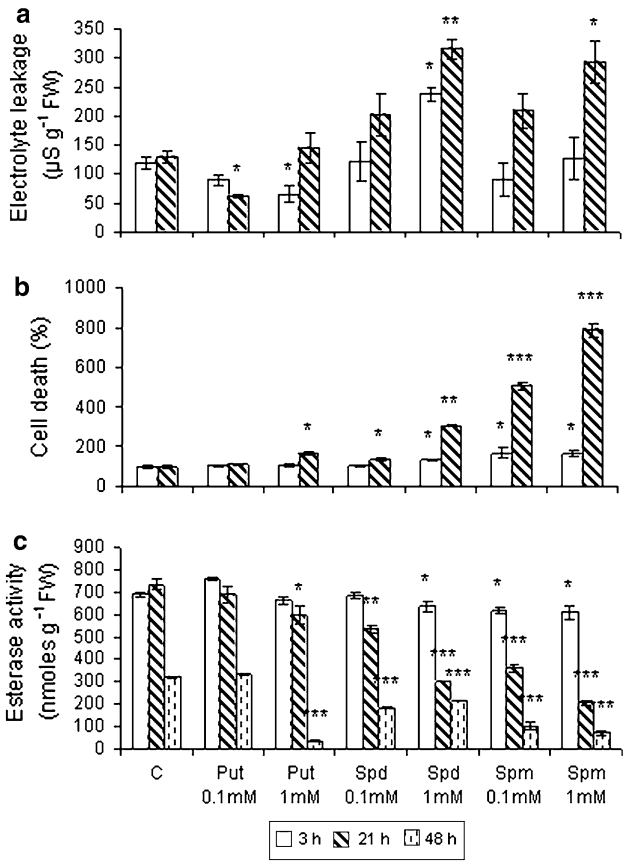

Fig. 6 Cell death measured as electrolyte leakage, Evans blue staining, and esterase activity in SR1 plants. Leaf discs were exposed to 0.1 and/or 1 mM Put, Spd, or Spm for 3, 21, or 48 h as described in the "Materials and Methods" section. Values are the mean ± SE of two different experiments with three replicated measurements. *Asterisks* indicate significant differences ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) according to Tukey's multiple-range test. **a** Electrolyte leakage expressed as relative conductivity. **b** Evans blue staining expressed as percentage of the controls. **c** Esterase activity expressed as nmol g^{-1} FW

control in leaf discs incubated with Spd and Spm, respectively (Table 2A).

We evaluated whether NO was involved in cell death by incubating leaf discs with 10 or 100 μM SNP. This NO donor did not alter cell viability by itself (Table 2B), and the simultaneous incubation of PAs with 200 μM cPTIO, a NO scavenger, produced results similar to those obtained with PAs used alone (Table 2A). These results suggested that NO was not contributing to PA-induced cell death in tobacco SR1 leaf discs.

Plant cell death activates nucleases that cleave DNA into multiple fragments of approximately 180 bp, recognized as a ladder when it is separated by agarose gel electrophoresis (Danon and Gallois 1998). To verify the existence of fragmentation, DNA was extracted from tobacco leaf discs exposed for 21 h to each PA and then electrophoresed on 2 % agarose gel. The gel visualization

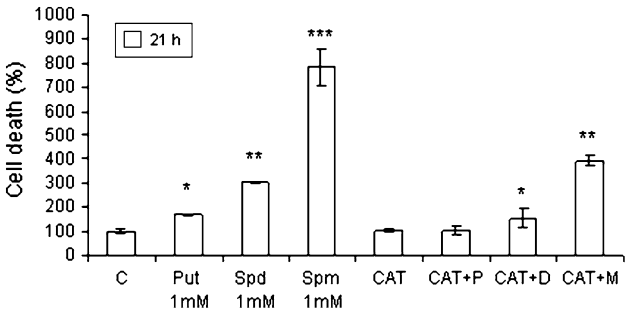

Fig. 7 Cell death measured by Evans blue staining expressed as percentage of the controls. Leaf discs were exposed to 1 mM Put, Spd, or Spm for 21 h. Some samples were preincubated with 1,500 U ml^{-1} CAT for 90 min and subsequently incubated with the corresponding PA as described in the "Materials and Methods" section. Values are the mean ± SE of two different experiments with three replicated measurements, and *bars* indicate SE. *Asterisks* indicate significant differences ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) according to Tukey's multiple-range test. Put (P), Spd (D), Spm (M)

showed that there was no DNA degradation in any of the conditions tested (data not shown).

Calcium as Mediator of Cell Death

Reactive oxygen species production can be compromised in the presence of Ca^{2+}-chelating or Ca^{2+} channel-inhibiting compounds (Scheel 1998). Therefore, we tested the effect of adding Ca^{2+} channel blockers or Ca^{2+} chelators on the induction of cell death by PA treatments. Leaf discs were incubated for 21 h with 5 mM $LaCl_3$, a Ca^{2+} channel blocker, or with 100 μM caffeine, a Ca^{2+}-releasing agent. The simultaneous incubation of $LaCl_3$ with PAs completely prevented cell death (Table 2A), whereas the simultaneous incubation of PAs with caffeine intensified the decrease in cell viability observed with the three PAs, tested as esterase activity, showing a maximum decline of 78 % with 1 mM Spm (Table 2A).

CP1 Transcript Level

To further characterize the events preceding cell death, we analyzed the expression of the SAG *CP1*. This gene, which encodes a tobacco cysteine protease, is considered a molecular marker of senescence. Induction of *CP1* expression was observed after 3 h of incubation with the three PAs at both concentrations (0.1 and 1 mM), with 0.1 mM Spm producing the largest increase in gene expression. At 21 h, an increase of the gene transcript level was observed with the highest concentration of the three PAs and with 0.1 mM Spm, whereas 0.1 mM Put decreased *CP1* expression by this time (Fig. 8).

Table 2 Cell death measured as esterase activity in leaf discs of tobacco SR1 plants

A: Leaf discs were preincubated for 3 h with different compounds (1,500 U ml^{-1} CAT, 300 U ml^{-1} SOD) and then incubated with PAs. In the case of 1,700 U ml^{-1} peroxidase, 200 μM cPTIO, 5 mM $LaCl_3$, and 100 μM caffeine, leaf discs were incubated simultaneously with the PAs

	Esterase activity (nmol g^{-1} FW)			
	C	1 mM Put	1 mM Spd	1 mM Spm
+H_2O	733.73 ± 22.02	598.01 ± 38.65[b]	303.03 ± 2.00[d]	205.23 ± 10.29[d]
+CAT	729.22 ± 8.13	705.13 ± 49.81[a]	565.32 ± 13.62[b]	555.77 ± 18.95[b]
+SOD	730.00 ± 9.56	537.72 ± 35.01[b]	326.32 ± 20.17[d]	201.08 ± 13.87[d]
+Peroxidase	725.14 ± 13.22	590.03 ± 31.14[b]	398.79 ± 9.65[d]	360.95 ± 14.41[d]
+cPTIO	735.00 ± 16.01	564.64 ± 6.77[b]	283.62 ± 13.62[d]	204.03 ± 8.84[d]
+$LaCl_3$	730.44 ± 3.55	771.87 ± 35.57[a]	762.45 ± 22.18[a]	819.40 ± 60.98[a]
+Caffeine	729.99 ± 6.55	478.55 ± 6.57[c]	262.41 ± 4.62[d]	161.36 ± 7.96[d]

B: Leaf discs were incubated for 21 h with 1 or 10 mM H_2O_2, 10 or 100 μM SNP. cPTIO (200 μM) was incubated simultaneously with 10 μM SNP. Values are the mean ± SE of three different experiments with three replicated measurements. Different letters within rows indicate significant differences ([b]$p < 0.05$; [c]$p < 0.01$; [d]$p < 0.001$), according to Tukey's multiple-range test

Esterase activity (nmol g^{-1} FW)					
C	1 mM H_2O_2	10 mM H_2O_2	10 μM SNP	100 μM SNP	cPTIO + SNP
733.7 ± 22.02	530.21 ± 3.08[b]	472.82 ± 5.00[b]	755.11 ± 3.07[a]	747.04 ± 5.49[a]	741.47 ± 5.01[a]

Leaf discs were exposed to 1 mM Put, Spd, or Spm for 21 h as described in "Materials and Methods" section

Discussion

Polyamines and ROS Formation

The relationship between PAs and ROS is questionable because it has been suggested that PAs protect cells against ROS by quenching them (Kumuda and Hara 2004) or by inhibiting their formation (Papadakis and Roubelakis-Angelakis 2005), but it is also postulated that they are ROS producers (H_2O_2) during their catabolism (Cona and others 2006; Moschou and others 2008a) or even protectors or effectors of PCD (Zahedi and others 2007; Hussain and others 2011). Despite exogenously added PAs, particularly Spd and Spm, increasing H_2O_2 formation in tobacco tissues with respect to the controls at 21 h, Put, Spd, and Spm significantly reduced NADPH oxidase activity, depending on PA concentration, at 3 and 21 h, which indicated that this enzyme was not the source of the superoxide anion that was then converted to H_2O_2 in the tissues. Papadakis and Roubelakis-Angelakis (2005) reported that 2.5 or 5 mM Put, Spd, and Spm induced a significant decline in $O_2^{\cdot-}$ accumulation, but also in NADPH oxidase activity in *N. tabacum* L. cv Xanthi protoplasts during the protoplast isolation procedure. The effect was positively correlated with the number of amino groups (Spm > Spd > Put), even though PAs did not affect $O_2^{\cdot-}$ or H_2O_2 accumulation in *Vitis vinifera* L. protoplasts.

The NADPH oxidases, also referred to as respiratory burst oxidases (RBO), have been implicated in development, biotic interactions, and abiotic stress responses in different plant species (Suzuki and others 2011). Spd and Spm inhibit NADPH oxidase activation and activity in human neutrophils (Ogata and others 1996), and Spd also completely inhibits the activity of microsomal NADPH oxidase during chilling injury of cucumber (Shen and others 2000). The decrease in the transcript level of *NtrbohD* in tobacco leaf discs correlated with the decay in the enzyme activity only after 3 h of PA exposure (except for 1 mM Put and 0.1 mM Spm). Before 3 h, the three PAs produced a slight increase in the expression of the gene that was not followed by an increase in the enzyme activity. This result strongly suggests that the observed H_2O_2 formed in tobacco leaf discs exposed to PAs was the result of PAO activity rather than NADPH oxidase activity.

PAO Activity and H_2O_2 Formation

It has been proposed that PAs are important substrates for H_2O_2 production, being degraded by copper-containing AOs or PAO (Yoda and others 2003). It is therefore highly probable that enzymatic degradation of PAs also directly contributes to ROS production and cell death in plants. As a result of Spd or Spm treatments, PAO activity increased significantly at 3 and 21 h, and, concomitantly, H_2O_2 increased markedly, mostly at 21 h, confirming that this increase was associated with PAO activity. This result was in line with findings in tobacco plants, where PAs accumulated after *Pseudomonas cichorii* infection, serving as

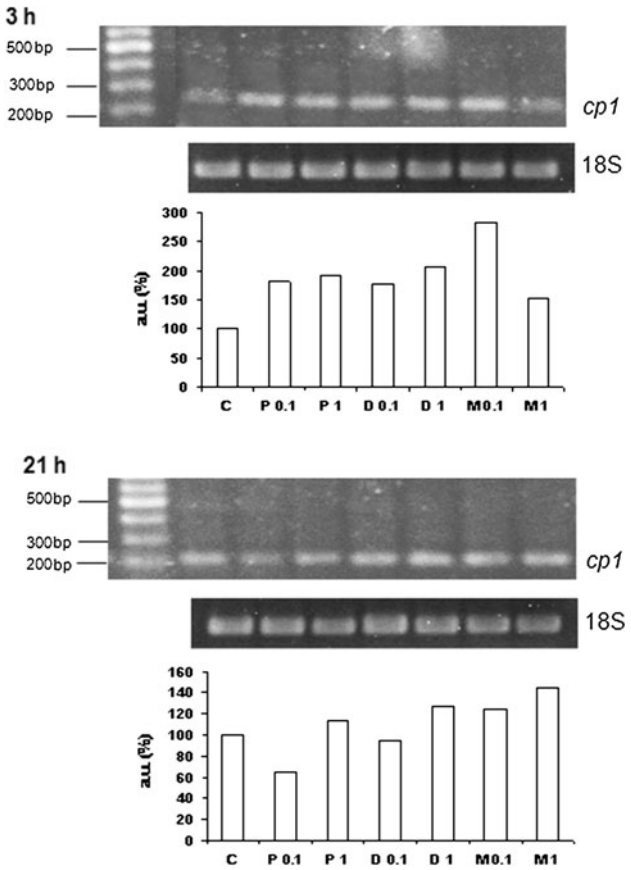

Fig. 8 The effect on *CP1* transcript level in SR1 leaf discs exposed to 0.1 and 1 mM Put, Spd, or Spm for 3 or 21 h. RT-PCR for *CP1* was performed as described in the "Materials and Methods" section. Relative mRNA values were calculated as the ratio *CP1/18S*. Data are expressed as percentage of arbitrary units (assuming the control value as equal to 100), based on the integration of the absolute optical density of each band. The fragment of cysteine protease (*CP1*) corresponds to 209 bp. The figure shows results typical of those obtained in three independent experiments. Put (P), Spd (D), Spm (M)

the source of H_2O_2 produced by PAO and inducing hypersensitive cell death at infected sites (Yoda and others 2009) or during tobacco protoplast culture, where PAO activity increased several-fold in Spd- and Spm-treated protoplasts, concomitant with higher H_2O_2 titers (Papadakis and Roubelakis-Angelakis 2005).

Polyamines and Oxidative Damage

Although H_2O_2 content was high in tobacco leaves exposed to PAs at almost all times tested, particularly at 21 h, lipid peroxidation was not observed, even at 21 h, and 1 mM Spd even reduced TBARS content with respect to the control. These results suggest that PAs participate in the regulation of physical and chemical membrane properties through the maintenance of membrane integrity (Groppa and others 2001; Hussain and others 2011 and references therein).

It has been demonstrated that cells undergoing H_2O_2-induced PCD fail to cope with oxidative stress due to the inhibition of the antioxidant defense system and the anti-PCD signaling cascades (Vannini and others 2012). PAs, despite their greater inhibitory effect on NADPH oxidase activity, altered the antioxidant enzymatic machinery by decreasing CAT, GPOX, and APOX activities along with increasing PAO activity.

These results suggested that accumulation of H_2O_2 produced by PAO in Spd- or Spm-treated tissues was still favored with the decline in ROS scavenger activity. Moschou and others (2008a) found that transgenic tobacco plants overexpressing PAO from *Zea mays* presented an induction of the antioxidant machinery and were expected to be able to cope with increased H_2O_2 generated by an additional abiotic stimuli. However, a further increase in the intracellular ROS by exogenous H_2O_2 resulted in oxidative stress. On the other hand, it was reported that transgenic tobacco plants with downregulated PAO accumulated less H_2O_2 and exhibited less PCD than did wild-type plants (Moschou and others 2008b).

H_2O_2 and Plant Cell Death

Changes in H_2O_2 homeostasis trigger genetic programs that promote stress acclimation or induce PCD (Gechev and Hille 2005; de Pinto and others 2012). Overexpression of the H_2O_2-detoxifying enzyme ascorbate peroxidase (APOX) suppresses H_2O_2-induced PCD (Murgia and others 2004), whereas a decrease in CAT activity causes perturbations of H_2O_2 homeostasis, inducing PCD (Dat and others 2003). In tobacco leaf discs, Spm increased CAT activity at 21 h of treatment, but this was not sufficient to remove the H_2O_2 generated and protect against the observed adverse effects on cell viability and membrane integrity, events that characterized cell death. Papadakis and Roubelakis-Angelakis (2005) suggested that despite the increase in CAT activity, the negative effects of Spd and Spm in tobacco protoplasts implied that CAT, with a high K_m for H_2O_2, is not sufficient to scavenge the H_2O_2 generated by PAO or that subcellular compartmentation of H_2O_2 and CAT is not compatible. However, the exogenous addition of CAT before PA treatments partially or totally prevented cell death in tobacco tissues, whereas exogenous H_2O_2 decreased EA activity greatly, corroborating the implication that H_2O_2 was involved in PA-induced cell death.

Only Spd at both concentrations and 1 mM Spm increased electrolyte leakage, whereas Put decreased this parameter. In accordance, Papadakis and Roubelakis-Angelakis (2005) demonstrated an increase in electrolyte release and DNA laddering of tobacco protoplasts incubated for 8 days with 2.5 mM Spd and Spm, whereas Put

prevented an increase. Moreover, Spd treatment induced nuclear condensation and DNA fragmentation in the maize primary root apex (Tisi and others 2011). In our experiment, using much lower concentrations of PAs than those used by Papadakis and Roubelakis-Angelakis (2005), DNA fragmentation was not observed, possibly as a result of a protective effect on nucleic acid integrity by PAs (Hussain and others 2011).

Polyamines and Signaling to Cell Death

There is an emerging concept that ROS-dependent cell death in plants is not only mediated through damage caused by indiscriminate oxidation, but it also occurs through interactions with other signaling pathways. Cell growth and cell death pathways modulated by PAs work in concert with other elements of signal transduction, such as NO or calcium. Several lines of evidence show that different cell death phenotypes induced by H_2O_2, alone or together with NO (Neill and others 2008), did or did not correlate with the accumulation of fatty acid hydroperoxides. Montillet and others (2005) showed that cell death activated by an increase in H_2O_2 levels was associated with an enhancement in membrane lipid peroxidation intensity, whereas when NO and H_2O_2 levels were moderate, cell death took place without massive cell peroxidation. In SR1 tobacco leaf discs, SNP did not cause changes in EA activity with respect to the controls. Although Spm or Spd are regarded as inducers of NO formation in *Arabidopsis* or wheat (Tun and others 2006; Rosales and others 2012), the simultaneous incubation of SR1 tobacco leaf discs with PAs and the NO scavenger cPTIO did not alter cell viability compared to the treatment with PAs alone, implying that NO was not a partner in PA-induced cell death in tobacco leaf discs.

On the other hand, evidence of interplay between cytosolic calcium transients and ROS production has been documented (Takeda and others 2008). In *Pisum sativum* root cells, Zepeda-Jazo and others (2011) observed that PAs interact with ROS to alter intracellular Ca^{2+} homeostasis by modulating both Ca^{2+} influx and efflux transport systems. The simultaneous incubation of tobacco leaf discs with PAs and $LaCl_3$ resulted in prevention of cell death caused by PAs, in agreement with the findings of McCabe and others (1997), who reported that $LaCl_3$ could block cell death in heat-treated carrot cells when it was added before applying a heat shock.

Senescence is a cell death process that reduces the expression of most genes but induces the expression of the generically designated SAGs (Lin and Wu 2004). One of the SAG genes, *CP1*, is a cysteine protease gene involved in the development of PCD (Xu and Chye 1999). In SR1 leaf discs exposed to PAs, an increase in *CP1* expression was observed after application of the three PAs, except 0.1 mM Put at 21 h, coincident with the reduction in electrolyte release and the unaltered cell death percentage indicated by Evans blue staining and EA activity. The increase in the expression of *CP1* observed in the shorter exposure time accompanied a marked accumulation of H_2O_2 in tobacco leaf discs, in line with that reported by Niewiadomska and others (2009), who suggested that H_2O_2 was responsible for the induction of SAG genes.

Conclusion

The results obtained in this work suggest that exogenous PAs exert an antioxidant role by inhibiting NADPH oxidase activity and by protecting cell membranes from lipid peroxidation, but they are also cell death inducers due to the increased formation of H_2O_2 produced by enhanced PAO activity under Spd or Spm exposure. Moreover, the results obtained with individual PAs are somewhat opposite. The diamine Put, which produced only small changes in H_2O_2 formation, protected against cell death and membrane damage, whereas the higher PAs, Spd and Spm, proved to be harmful to cell viability, depending on the concentration used and the exposure time. The reason for such a discrepancy may be differences in absorption, transport, and utilization among plant species. The question is open regarding the importance of amino groups in the molecule in relation to cell death induction or cell protection exerted by PAs, their behavior as signaling compounds by themselves or through H_2O_2 derived from their catabolism, or even the crucial role of molecules like calcium on the PA oxidation process.

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