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Cell death induced by ozone stress in the leaves of *Populus deltoides* × *maximowiczii*

G. BARTOLI[1]*, L.M.C. FORINO[1], A.M. TAGLIASACCHI[1], and M. DURANTE[2]

Department of Biology, University of Pisa, Pisa, I-56126, Italy[1]
Department of Agriculture, Food and Environment, University of Pisa, Pisa, I-56124, Italy[2]

Abstract

When exposed to an acute ozone stress, cell death occurred in leaves of the O_3 sensitive *Populus deltoides* × *maximowiczii* clone Eridano. After treatment (5 h fumigation and 24 h recovery), the damaged areas covered more than 50 % of the leaf surface. At cellular level, these lesions were preceded by some apoptotic hallmarks and by biochemical and physiological alterations evoked by the apoplastic O_3 dissociation. The cell death pattern was highly localized and involved an increase of membrane permeability, externalization of phosphatidylserine, DNA fragmentation, callose accumulation, polyphenol production, and a biphasic oxidative burst accompanied by NO overproduction. These results indicate a process of programmed cell death that could have the biological significance of limiting the spreading the oxidative burst triggered by ozone dissociation in apoplastic environment. Moreover, materials derived from cell dismantling could be remobilized toward developing structures which can conclude their ontogenetic program after the stress.

Additional key words: hypersensitive response, phosphatidylserine, oxidative burst, nitric oxide, TUNEL assay, DNA-laddering assay, DNA smear.

Introduction

Tropospheric ozone concentrations have increased markedly since the turn of the last century (Vingarzan 2004, Tamaoki 2008). Future scenarios predict worrying increases up to 0.08 mm^3 dm^{-3} (Pachauri and Reisinger, 2007) in ozone ground-levels and also episodic ozone peaks exceeding 0.15 mm^3 dm^{-3} and sometimes 0.20 mm^3 dm^{-3} (Strohm *et al.* 1999 and references therein, Richet *et al.* 2012). In the most ozone-sensitive plants, the deleterious effects of O_3 are numerous and vary with the concentration and duration of exposure (Pasqualini *et al.* 2003). Acute ozone stress, consisting in transient exposures to high O_3 doses, can quickly induce cell death events, macroscopically detectable as foliar lesions, and many physiological changes referable to a severe oxidative stress (Faoro and Iriti 2005, Dizengremel *et al.* 2009). O_3 enters into the apoplast through open stomata and reacts with different cellular symplastic or apoplastic components that can promote or quench the production and spreading reactive oxygen species (ROS) (Fiscus *et al.* 2005, Kangasjärvi *et al.* 2005). ROS, if not scavenged, can result in an additional ROS wave most probably through the activation of apoplastic NADPH oxidase (Laloi *et al.* 2004). The biphasic oxidative burst triggers a complex of downstream signalling cascade that involves a well orchestrated network of different factors (salicylic acid, ethylene, jasmonic acid, Ca^{2+}, nitric oxide, oxygen radicals, MAPK cascades, *etc*.) (Kangasjärvi *et al*. 2005, Ahlfors *et al.* 2009a) and results in the up- or down-regulation of genes involved in the defence, repair, and compensation

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Abbreviations: CTAB - cetyltrimethyl ammonium bromide; DAF-2DA - 4,5-diaminofluorescein diacetate; FITC - fluoresceine isothiocyanate; H_2DCFDA - 2',7'-dichlorodihydrofluorescein diacetate; HR - hypersensitive response; MES - 2-(N-morpholino)ethanesulfonic acid; PAL - phenylalanineammonia lyase; PBS - phosphate-buffered saline; PCD - programmed cell death; PI - propidium iodide; PS - phosphatidylserine; ROS - reactive oxygen species; TUNEL - terminal deoxynucleotidyl transferase (TdT)-mediated dUTPnick-end labeling.
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* Corresponding author; fax: (+50) 2211309, e-mail: g.bartoli@sssup.it

processes (Bernardi *et al.* 2004, Mahalingam *et al.* 2005, Rizzo *et al.* 2007). Recent findings suggest that nitric oxide modulates O_3-induced responses and cell death in particular (Neill *et al.* 2002, Ahlfors *et al.* 2009b, Di Baccio *et al.* 2012). In both herbaceous plants and trees, the ROS can lead to programmed cell death (PCD) (Koch *et al.* 2000, Pasqualini *et al.* 2003) and other reactions very similar to hypersensitive reactions (HR), a type of PCD showing some similarities to apoptosis (Lam 2004, Gadjev *et al.* 2008), occurring in plants when subjected to a wide range of biotic and abiotic stresses (Havel and Durzan 1999, Fan and Xing 2004, Overmyer *et al.* 2005, Günthardt-Goerg and Vollenweider 2007, Ahlfors *et al.* 2009b). PCD constitutes an active, genetically controlled process leading to selective elimination of unwanted or damaged cells. PCD is indispensable for plant survival, development, and interaction with the environment (Greenberg 1996, Pennell and Lamb 1997, Havel and Durzan 1999, Gechev 2006, Gadjev *et al.* 2008, Lenochová et al. 2009). The mechanisms of PCD share a certain conservation degree throughout eukaryotes in their execution and regulatory systems (Havel and Durzan 1996, Pennell and Lamb 1997). In animals, the PCD is characterized by a well defined array of morphological, biochemical, and molecular hallmarks that might or might not be detectable in each individual case of plant PCD (Gilchrist 1998, Havel and Durzan 1999, Xu and Roossinck 2000, Gadjev *et al.* 2008) because of the plant cell peculiarities in terms of cyto-architecture, specific organelles, and physiological traits.

The poplar, including highly O_3 sensitive genotypes (Nikula *et al* 2009), constitutes a useful tree-model for developing and testing hypotheses on short-term acute and long term chronic ozone stress (Taylor 2002). This study aimed at better characterizing the cell death experimentally induced by acute O_3 exposure in leaves of *Populus deltoides* × *P. maximowiczii*, clone Eridano, that exhibits an increased sensitivity to O_3 peaks (Nali *et al.* 1998, Diara *et al.* 2005, Rizzo *et al.* 2007, Bartoli *et al.* 2010). Special attention was paid to establishing whether the O_3-induced cell death can be referred to PCD processes; whether changes in cell-membrane asymmetry can occur as in animal apoptosis; whether important signal molecules, such as H_2O_2 and NO, can be involved in cell death events induced by O_3 in leaves.

Materials and methods

Rooted cuttings of the O_3 sensitive *Populus deltoides* × *P. maximowiczii* clone Eridano, obtained from plants growing in experimental fields of the Department of Agriculture, Food and Environment, University of Pisa, were grown for two months in a greenhouse in plastic pots containing a steam-sterilized substrate (soil:peat: *Perlite*, 1:1:1, v/v/v). The average temperature in the greenhouse ranged from 15 ° (night) to 26 °C (day), the relative humidity ranged from a minimum of 55 % to a maximum of 85 %, and the photosynthetic photon flux density (PPFD) was 500 μmol m^{-2} s^{-1} at plant height during a 16-h photoperiod. Uniform plants (about 60 cm high) were randomly selected and subjected to the different experimental procedures.

In fumigation chamber (0.48 m^3), O_3 was generated by electrical discharge in pure oxygen by a *Fisher 500* air-cooled generator (*Fisher Labor und Verfahrenstechnik*, Meckenheim, Germany) and its concentration in the fumigation chambers was continuously monitored with a *Monitor Laboratories Analyzer* (*Model 8810*, *Monitor Labs*, San Diego, CA, USA) operating on the principle of UV absorption and interfaced with a personal computer. The photosynthetic photon flux density (PPFD, 400 - 700 nm) at plant height was 530 μmol m^{-2} s^{-1} and was produced by an incandescent lamp. Ten uniform plants were placed in a fumigation chamber, pre-adapted to the chamber conditions for 48 h and then exposed to a single pulse of 0.15 mm^3 dm^{-3} O_3 for 5 h (from 09:00 to 14:00). During the adaptation period and the O_3 fumigation, the temperature in the chambers was maintained at 20 ± 1 °C and relative humidity at 85 ± 5 %. Two mature leaves per plant were sampled before the ozone generation (T0, the non fumigated leaves were considered as control), at 2 and 5 h after the fumigation start (T1 and T2, respectively), or 24 h after switching-off of O_3 generation (T3). These time-points were selected on the basis of the results of previous works (Nali *et al.* 1998, Bartoli *et al.* 2010).

For each experimental group, part of the collected leaves was immediately used for the analyses on fresh materials (macroscopical investigations, phenol autofluorescence assessment, H_2O_2 and NO *in situ* detection, and annexin-V test) and part was chemically fixed for histological investigations or fixed in liquid nitrogen and stored at -80 °C for biochemical or molecular analyses. Freshly excised leaves were weighed in order to determine fresh mass.

In order to evaluate the percentage of symptomatic areas on the total leaf surface, adaxial and abaxial blades of ten leaves from each experimental group were acquired by scanning (*Epson Perfection 1260*, *SEIKO Epson Corporation*, Nagano, Japan). Total and injured leaf areas were assessed by the image analysis software *SCION IMAGE*, release 4.0.2 (*Scion Corporation*, Frederick**,** MD, USA).

Two portions per sampled leaf from each experimental group were fixed in 4 % (v/v) formalin PBS-buffered (pH 7.4) for 24 h, dehydrated in a graded ethanol series, and embedded in *Paraplast Plus* (*Sigma Chemical Co.*, St. Louis, MO, USA) or in *LR-White*

medium grade (*London Resin Company*, Reading, Berkshire, UK). Thin sections (10 μm thick) were sliced from paraffin embedded samples, deparaffinized, and then double-stained with hematoxilin and eosin staining for general cytological investigations (O' Brien and Mc Cully 1981). Semi-thin sections (3 μm thick) were cut from resin embedded samples and then stained with aniline blue (5 %, m/v, in 0.1 M Na_2CO_3 buffer, pH 8.5 for 2 min), to detect callose deposits appearing green-blue stained if observed with UV filter (350 - 390 nm; Jensen 1962). At least 100 leaf thin and semi-thin sections for each experimental group were analyzed. The observations were performed with a *LEITZ DIAPLAN* light microscope (Wetzlar, Germany) or with a *LEICA DM LB* fluorescent microscope (Wetzlar, Germany) equipped with a filter set for group A (bandpass filter 340 - 380 nm, dichroic mirror 450 nm, barrier filter Lp 430 nm) for light and fluorescent microscopy, respectively. Images were captured by using a *Leica DFC 420* or an *Image Leica DC300F* digital cameras (*Leica Microsystems*, Heerbrugg, Germany) for light and fluorescence microscopy, respectively.

Two portions per sampled leaf from each experimental group were excised and immediately examined under blue radiation excitation (450 - 490 nm, emission at 515 nm) for the detection of green-yellow autofluorescing polyphenolic compounds; at the same excitation, the healthy cells exhibited the red chlorophyll primary fluorescence. The images were observed with a *LEICA DM LB* microscope equipped with a filter set for group A and were taken using an *Image Leica DC300F* digital camera.

Intact DNA was isolated from fumigated and non fumigated frozen leaves following the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). Samples were subjected to electrophoresis in 1 % (m/v) agarose gel at constant voltage (70 V). The gel was visualized under UV transilluminator in order to visualize DNA fragmentation patterns and captured using the *Digi Doc-It* imaging System (*Ultraviolet Products Limited*, Cambridge, UK). A 1 kbp ladder was used as a standard. According to Singer *et al.* (1979) and Durante *et al.* (1989), the average of DNA fragment lengths and percentages were determined by densitometric scanning analysis of gel electrophoretic pattern pictures performed by *Quantity One 4.1.1* software (*Bio-Rad*, Hercules, CA, USA). After confirming that the area under the peaks of the densitometer tracings was proportional to DNA concentration (Bird and Southern 1978), we determined the percentage of fragments on total DNA and the average length of DNA fragments as follows. First, we determined the axis bisecting each peak into equal areas by "cutting and weighing", then we found the corresponding value of the weight average length of DNA by use of a "standard curve" consisting of a semi-log plot of the migration distance of fragments of known size (the 1 kbp ladder used as a standard) subjected to electrophoresis on the same gel.

Two portions per sampled leaf from each experimental group were fixed at 4 °C in 4 % (m/v) buffered paraformaldehyde (PBS, pH 7.4) overnight; then they were dehydrated in ethanol, embedded in *Paraplast Plus* (*Sigma*) at 60 °C for 48 h, sliced in 10 μm sections, and finally collected on polylysine coated slides. Deparaffinized sections were rehydrated and subjected to terminal deoxynucleotidyl transferase (TdT)-mediated dUTPnick-end labeling (TUNEL) assay by using *TACS•XL Blue* label kit (*Trevigen*, Gaithersburg, MD, USA) according to manufacturer's instructions. A negative control was included in each experiment by omitting TdT from the reaction mixture and as a positive control, sections were incubated with DNase I (10 U cm^{-3}) for 10 min before TUNEL assay. The sections were air dried, mounted with DPX, observed with a *LEITZ DIAPLAN* light microscope, and then captured using a *Leica DFC 420* digital camera. At least 100 sections for each experimental group were analyzed.

Sterilized leaves from each experimental group were cut in small pieces (10 g of fresh mass for each sample) and incubated at 30 °C in 10 cm^3 of an enzyme mixture consisting of 0.1 % (v/v) pectinase, 2 % (v/v) cellulase, and 13 % (m/v) sorbitol in 10 mM MES/KOH (pH 5.5) for 120 min. Then the protoplast suspension was inserted in Eppendorf tubes and subjected to annexin V-FITC PI double staining by using the *MEBCYTO*^{R} apoptosis kit (*Medical and Biochemical Laboratories Co.*, Naka-ku, Nagoya, Japan) in order to discriminate between viable cells (annexin V-FITC negative and PI negative), apoptotic cells (annexin V-FITC positive and PI negative), and necrotic/late apoptotic cells (annexin V-FITC positive and PI positive). For each detection: 0.1 cm^3 of protoplast suspension was directly mixed with 0.18 cm^3 of binding buffer, 0.030 cm^3 of annexin V-FITC, and 0.015 cm^3 of PI (100 μg cm^{-3}) and incubated in the dark at room temperature for 25 min. Then, 0.05 cm^3 of protoplast suspensions from each experimental group were collected on a slide, covered by a coverslip, and immediately viewed by a *LEICA DM LB* microscope equipped with a filter set for group I3 (bandpass filter 450 - 490 nm, dichroic mirror 510 nm, barrier filter Lp 515 nm). For each experimental group, at least 100 leaf protoplasts were analyzed. The images were captured with an *Image Leica DC300F* digital camera.

Two portions (12 mm diameter, 14.5 mg) of sampled leaves from each experimental group were incubated at room temperature with 1 cm^3 of 20 μM H_2DCFDA (*Molecular Probes*, Eugene, OR, USA) in 5 mM MES/KOH buffer (pH 5.6) at the darkness for 30 min in order to detect ROS distribution in intact leaf tissues. After washing, they were collected on the slide, mounted with glycerin, and observed: the oxidation of the fluorogenic probe by ROS generates a green fluorescent derivative under blue radiation excitation (450 - 490 nm,

emission at 515 nm) viewed by at a *LEICA DM LB* microscope equipped with a filter set for group A. The images were captured using an *Image Leica DC300F* digital camera.

Frozen leaf samples (0.5 g) were homogenized in 2 cm^3 of sodium phosphate buffer (0.05 M NaH_2PO_4/ Na_2HPO_4, pH 6.8). The homogenate was centrifuged at 20 000 g for 10 min. Three volumes of supernatant were mixed with one volume of 5 % (m/v) titanium oxysulfate ($TiOSO_4$) (*Fluka*, Munich, Germany) in 20 % (v/v) H_2SO_4. The mixture was centrifuged at 6 000 g for 15 min and 1 cm^3 of the supernatant was analyzed by using a *UV-2101 PC UV-VIS* scanning spectrophotometer (*Shimadzu Corporation*, Tokyo, Japan). The peroxides contained in the homogenate generate in the presence of the titanium reagent stable and optically active complexes peroxides-$TiOSO_4$ that confer a yellow color to the supernatant. The absorbance of supernatant was measured at 410 nm and peroxide concentrations were calculated using a calibration curve prepared in a H_2O_2 concentration range 0 - 100 μM and expressed on the basis of tissue fresh mass. For the time-course of H_2O_2 production, the samples were collected at intervals of 1 h.

Two portions (12 mm diameter, 14.5 mg) per sampled leaf from each experimental group were incubated at room temperature in the darkness with 1 cm^3 of 20 μM DAF-2DA (*Callbiochem*, Darmstadt, Germany) in 5 mM MES/KOH (pH 5.6) for 1 h in order to detect NO distribution in intact leaf tissues. After washing, the samples were collected on a slide, mounted with glycerin, and then observed under blue light excitation by a *LEICA DM LB* microscope equipped with a filter set for group A. In presence of NO and O_2, the probe is converted in a green fluorescent derivative. The images were captured using an *Image Leica DC300F* digital camera.

All the experiments were performed at least in triplicate. The data were analyzed by one-way analysis of variance (*ANOVA*) and Student-Newman-Keuls *post hoc* test with values of $P < 0.01$ sufficient to reject the null hypothesis. Statistical analyses were performed by using statistical package *Primers of Biostatistics* (*S.A. Glantz, Statistical Software Program Version 6.0*, McGraw Hill 2005). Microphotographs were representative of at least 20 samples yielding similar results.

Results

Control leaves (T0) as well as the leaves during the O_3 treatments T1 and T2 did not display any symptomatic areas. However, dark-black regions in the interveinal parts of leaf surface were detectable at 24 h after the treatment T3. At this time-point, symptomatic areas covered more than 50 % of the total leaf surface (Table 1).

In contrast to control plant leaves that showed intact mesophyll (Fig. 1*A,A'*), the leaves after O_3-treatment showed cells with collapsed walls, disrupted tonoplast, degenerated organelles, and cytoplasmic debris displaced against the degenerating cell walls in the dark-black areas (Fig. 1*B,B'*).

In contrast to T0 leaves (Fig. *1C*), at T1 and T2 (Fig. 1*D*,*E*), green-yellow fluorescing cells (with an altered polyphenol composition) appeared within the red fluorescing healthy tissues and at T3 these cells delimited the damaged areas (Fig. 1*F*). At the same time-point, the walls of degenerating cells showed random deposits of callose (Fig. 1*H*) whereas callose in control plants was only in sieve plates (Fig. 1*G*).

The electrophoretic migration of genomic DNA extracted from poplar leaves sampled at the different

Table 1. Symptomatic leaf areas in the abaxial and adaxial sides of leaves from clone Eridano exposed to 0.15 mm^3 dm^{-3} O_3 for 5 h. Mean ± SE, $n = 20$. Values in rows marked with different letters are significantly different at $P < 0.01$.

Symptomatic leaf areas	T0	T1	T2	T3
Adaxial side [%]	0.10 ± 0.02^a	0.81 ± 0.08^b	1.20 ± 0.10^c	57.90 ± 1.03^d
Abaxial side [%]	0.09 ± 0.01^a	0.59 ± 0.07^b	0.85 ± 0.12^c	51.50 ± 1.87^d

Table 2. Fragment percentage and sizes of the DNAs extracted from Eridano clone leaves at the different experimental time-points. Mean ± SE, $n = 20$. Values in rows marked with different letters are significantly different from each other at $P < 0.01$.

Parameters	T0	T1	T2	T3
Fragment percentage [%]	8.7 ± 0.80^a	27.3 ± 1.30^c	42.6 ± 3.20^d	17.4 ± 1.10^b
Fragment size [kbp]	4.0 ± 0.38^c	1.9 ± 0.18^b	1.4 ± 0.29^a	2.0 ± 0.24^b

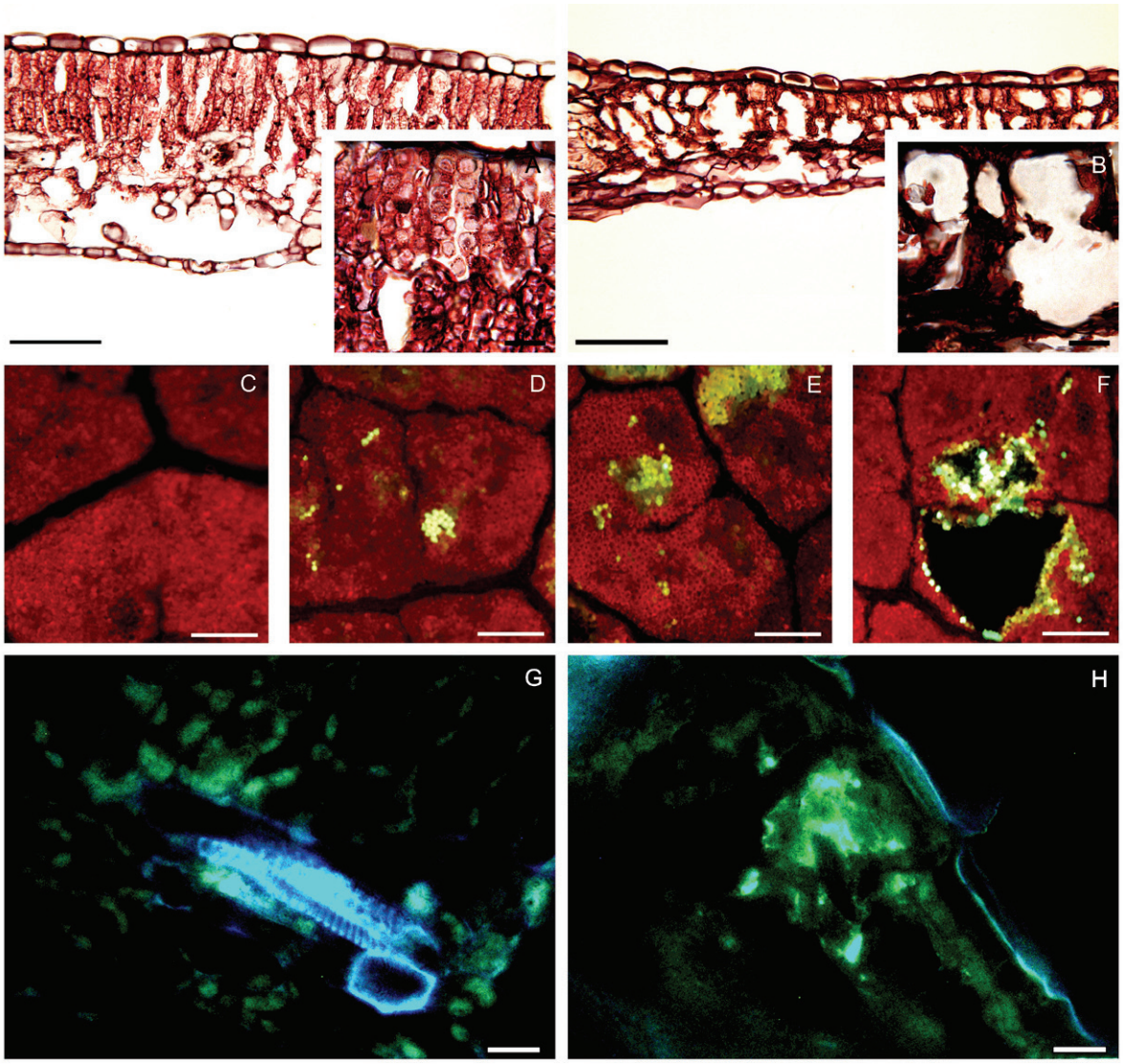

Fig. 1. Leaf injury and histological modifications induced by an acute ozone stress in Eridano leaves. *A*, *B* - Transverse sections stained with hematoxilin and eosin showing asymptomatic poplar leaf tissues at T0 (*A*) and damaged leaf zones at T3 (*B*) (*bar* = 30 μm). *A'*, *B'* - Mesophyll details (*bar* = 10 μm). *C*, *D*, *E*, *F* - Autofluorescence in poplar leaf samples at T0, T1, T2, and T3, respectively; red fluorescence derives from chlorophylls, yellow-green fluorescence indicates polyphenols in leaf cells (*D*, *E*, *F*), and dark-black spots (*F*) represent symptomatic areas (*bar* = 100 μm). *G*, *H* - Callose deposits (green-yellow fluorescing after aniline blue staining) in transverse sections at T0 (*G*) and at T3 (*H*) (*bar* = 20 μm).

Table 3. Annexin V-FITC and PI positivity evaluation in Eridano protoplasts calculated as the ratio of number of stained or unstained cells to total cell number. Mean ± SE, $n = 20$. Values marked with different letters are significantly different from each other at $P < 0.01$.

Protoplasts	T0	T1	T2	T3
annexin V+/PI–	0.26 ± 0.08^{a}	0.46 ± 0.09^{b}	0.48 ± 0.08^{b}	0.19 ± 0.06^{d}
annexin V+/PI+	0.24 ± 0.02^{a}	0.09 ± 0.05^{c}	0.51 ± 0.07^{e}	0.20 ± 0.07^{d}

especially in the ozone treated samples (mostly at T2) (not shown). Densitometric scanning analysis of electrophoretic patterns of genomic DNA followed by digital evaluation evidenced significant differences in

DNA fragmentation percentage as well as in the average size of the most abundant fragments in each sample (Table 2). In particular, the samples at T2 showed the highest fragmentation percentage with the smallest fragments (1.4 kbp; Table 2).

TUNEL assay (Fig. 2*A*-*D*) showed that at T0, no significant DNA fragmentation occurred (Fig. 2*A*) and at the T1, only rare TUNEL positive cells were observed (Fig. 2*B*). On the contrary, a consistent number of TUNEL positive cells, intermingled with TUNEL negative cells, were detectable at fumigation end (T2, Fig. 2*C*) mostly in mesophyll tissues. At T3, only a very small number of cells from epidermis and spongy parenchyma were TUNEL positive (Fig. 2*D*).

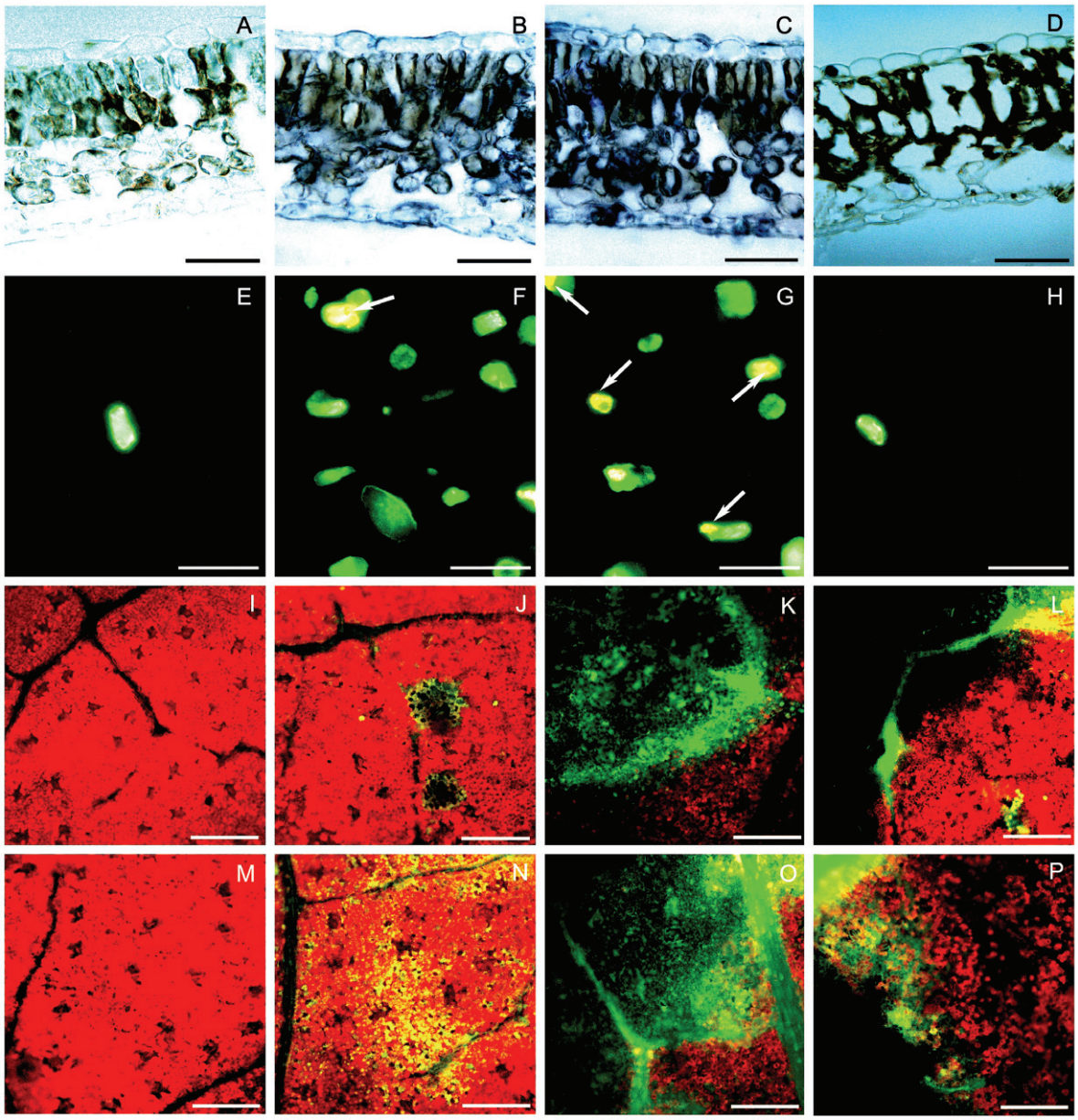

Fig. 2. PCD hallmarks, peroxides, and NO in Eridano leaves at the different experimental time-points: *A, B, C, D* - TUNEL positive cells with blue-stained nuclei occurred at T1, T2, and T3 (*B, C, D*), and no TUNEL positive cells at T0 (*A*) (*bar* = 30 µm). *E, F, G,* H - Leaf protoplasts double-stained by annexin V-FITC (green fluorescence) and PI (orange-red fluorescence; *arrows* in *F* and *G*) at T0, T1, T2, and T3, respectively (*bar* = 20 µm). *I, J, K, L* - H_2O_2 and peroxides *in situ* detections in leaf samples at T0, T1, T2, and T3, respectively, by using the fluorescent probe H_2DCFDA; red fluorescence derives from chlorophylls, green fluorescence indicates peroxides (*J*, *K*, *L*), and dark-black areas (*L*) represent the ozone induced lesions (*bar* = 200 µm). *M, N, O, P* - NO *in situ* detection in leaf samples at T0, T1, T2, and T3, respectively, by using the fluorescent probe DAF2DA; red fluorescence derives from chlorophylls, green fluorescence indicates nitric oxide occurrence (*N*, *O*, *P*), and black areas (*P*) represent the ozone induced lesions (*bar* = 200 µm).

Analyses of changes in membrane asymmetry and leakage in leaf protoplasts evidenced that phosphatidylserine (PS) residues were externalized on the outer layer of intact cell membranes at T1 as evidenced by the green fluorescence of annexin V-FITC complex specifically binding the externalized PS residues (Fig. 2*F*; Table 3). Later, at T2, PI positive nuclei appeared in some annexin V-FITC-positive protoplasts (Fig. 2*G*; Table 3) indicating that PS externalization was followed by an alteration in cell-membrane permeability, typical of dead cells. At T3 (Fig. 2*H*; Table 3) as well as before the ozone fumigation beginning (T0, Fig. 2*E*, Table 3), a very low number of annexin and/or PI-positive protoplasts were observed. In particular at T3, the observed protoplasts derived from healthy zones survived after the end of fumigation when cell death processes were terminated.

Table 4. Time-course of content of H_2O_2 and other peroxides [μmol g^{-1}(f.m.)] in Eridano leaves subjected to an acute ozone stress. Mean ± SE, $n = 20$. The means are significantly different at $P < 0.01$ from T0.

Time [h]	H_2O_2	Time [h]	H_2O_2
0 (T0)	0.25 ± 0.03	15	1.13 ± 0.02
1	1.06 ± 0.02	16	1.16 ± 0.03
2 (T1)	1.80 ± 0.04	17	1.17 ± 0.04
3	2.20 ± 0.10	18	1.18 ± 0.03
4	2.28 ± 0.15	19	1.21 ± 0.05
5 (T2)	2.31 ± 0.18	20	1.25 ± 0.06
6	2.34 ± 0.12	21	1.27 ± 0.04
7	1.75 ± 0.21	22	1.29 ± 0.07
8	1.47 ± 0.18	23	1.30 ± 0.05
9	0.75 ± 0.04	24	1.30 ± 0.06
10	0.87 ± 0.03	25	1.31 ± 0.31
11	0.91 ± 0.04	26	1.32 ± 0.15
12	1.09 ± 0.06	27	1.32 ± 0.25
13	1.10 ± 0.07	28	1.34 ± 0.18
14	1.12 ± 0.06	29 (T3)	1.35 ± 0.04

Histological ROS determination by using the green-fluorescing probe H_2DCFDA evidenced no appreciable ROS accumulation in poplar leaves at T0 (Fig. 2*I*). However, an increase in ROS content occurred at T1 and T2 (Fig. 2*J,K*). The green fluorescence was mainly observed in small and well localized zones around the stomatal pore in T1 (Fig. 2*J*) whereas in T2, it evolved in well defined and uniform areas of the leaf blades (Fig. 2*K*). At T3, green fluorescing cells were closely localized around the damaged areas (Fig. 2*L*). Spectrophotometric determination evidenced two waves of ROS production (Table 4). The first wave appeared just after the fumigation beginning (between 0 and 2 h) and reached the value maximum at 6 h. Then, the content declined up to 9 h. The second oxidative wave characterized by a gradual and prolonged increase of peroxides started at 10 h, and 29 h after fumigation end, the peroxide concentrations were 1.35 ± 0.04 μmol g^{-1}(f.m.).

The production and the spatial distribution of NO, evidenced in poplar leaf surface by the specific green-fluorescing probe DAF2DA, was not significant at T0 (Fig. 2*M*). However, a significant increase in NO production was detected at T1 (Fig. 2*N*) as evidenced by the distribution of the green fluorescing cells intermingled with the red fluorescing cells. Subsequently (T2, Fig. 2*O*), the green fluorescence was mainly distributed in specific areas of the leaf surface. At T3 (Fig. 2*P*), when the tissues were largely injured, significant NO production occurred around the damaged areas.

Discussion

The results of the present investigation evidenced an O_3-dependent programmed cell death in the leaves of poplar clone Eridano exposed to a short period of high ozone concentration. This process responsible of the broad foliar lesions involves a complex array of cell responses occurring very early during the fumigation and progressing beyond the end of the treatment. A fine-tuned overproduction of polyphenols, peroxides, and NO accompanies a significant inside-outside translocation of PS residues in cell membranes. All these events precede nuclear DNA fragmentation and the cell dismantling.

Notably, annexin positive cells were observed at 2 h from the fumigation beginning (T1), when Bartoli *et al.* (2010) reported the first cytological stress signs in palisade cells (*i.e.* shrunken cells and changes in vacuolar arrangement) that precede cell membrane leakage, nuclear changes, chloroplast degeneration, and cell-wall collapse. As it occurs in animal apoptosis (O'Brien *et al.* 1997), the externalization of PS residues is one of the earliest events of PCD elicited by ozone in Eridano. This hallmark was evidenced only in few other cases of plant cell death (O'Brien *et al.* 1997, 1998, Lei *et al.* 2003, Xu *et al.* 2004). The reason why plant cells surrounded by the wall can undergo PS externalization is unknown at present. However, this event might constitute part of a short distance signaling pathway involved in the spatial progression of the defensive responses in which ROS and NO play a pivotal role. As in the earliest events of HR or in some cases of animal apoptosis (Draper 1997, Lamb and Dixon 1997, Di Baccio *et al.* 2008, 2012), a biphasic oxidative burst was evidenced in ozone exposed leaves. The first massive wave spread during the ozone

exposition and can be related to the apoplastic O_3 disintegration. The second wave, less pronounced than the first, appeared during the post-treatment periods and is probably a part of the protective response exerted by the plant against the harmful effects of this pollutant. The O_3-induced peroxides were distributed in discrete leaf areas as well as the successive lesions. This is consistent with different findings on both tree and herbaceous plants subjected to ozone stress (Schraudner *et al.* 1998, Wohlgemuth *et al.* 2002, Pasqualini *et al.* 2003, Diara *et al.* 2005, Di Baccio *et al.* 2008, 2012, Guo *et al.* 2009).

Several authors evidenced an involvement of NO in PCD processes (Pennel and Lamb 1997, Pedroso *et al.* 2000, Wang *et al.* 2010, Di Baccio *et al.* 2012). Accordingly, a dynamic NO production was evidenced simultaneously with the two oxidative peaks in the O_3-treated poplar leaves. Moreover, NO was found to modulate the transcription of different categories of genes, such as those involved in signal transduction or in plant defence against different kinds of abiotic or biotic stresses (Garces *et al.* 2001, Qiao and Fan 2008). The early NO production was almost evenly distributed on the whole leaf surface, but the subsequent NO production at the fumigation end (T2) was restricted in well defined leaf regions according to the temporal and spatial pattern of peroxides and cell injuries. This localization of NO can be due to the differential spatial distribution of ROS and also to its cooperation with oxygen radicals in inducing cytological and biochemical PCD hallmarks (Pedroso *et al.* 2000, Delledonne *et al.* 2001, De Pinto *et al.* 2002).

The same pattern and timing of both peroxides and NO is also observed for the secondary compounds, mainly phenols. These compounds originate from activation of the phenylpropanoid pathway. Phenylalanineammonia lyase (PAL) seems to be induced by O_3 in plants (Pääkkönen *et al.* 1997, Vollenweider *et al.* 2003). Accordingly, in our preliminary results on differently O_3-susceptible poplar clones, significant changes in the expression of the genes encoding not only PAL2 but also polyphenol oxidase, glutathione S-transferase, metallothioneins, and pathogenesis-related proteins, are potentially involved in the defensive responses elicited by the O_3 (Bartoli *et al.* 2009).

At the end of fumigation, significant genomic DNA fragmentation occurred in poplar leaf tissues in accordance with the spatial and the temporal pattern of the cytological and biochemical events showed in this study and in a previous research (Bartoli *et al.* 2010). Nevertheless, well detectable oligonucleosomal DNA fragments were not generated. Accordingly, in plants, there is no consistency regarding the size of DNA fragments being more or less 50 kb in some cases (Mittler and Lam 1997) and smaller than 50 kb in others (Cohen 1994, O'Brien *et al.* 1998). The absence of the DNA ladder could be the consequence of the high heterogeneity of analysed tissues (living cells, dead cells, and cells committed to die), that may be related to the different intrinsic sensitivity to the O_3 stress of the different cell kinds (Bethke *et al.* 1999, Pasqualini *et al.* 2003, Gunawardena *et al.* 2004, Kolodziejek *et al.* 2007, Lombardi *et al.* 2007, Xie *et al.* 2008). Alternatively, as proposed by Faoro and Iriti (2009) about the HR-like cell death and lesion formation in bean leaves after acute O_3 exposure, necrotic and apoptotic processes can coexist in the same tissue. Presumably, molecular signals from cells that undergo rapid necrotic cell death due to the apoplastic O_3 dissociation may trigger the neighbouring cells to die by PCD and consequently both necrosis and PCD may co-exist adjacently to undamaged cells (Heath 2008, Faoro and Iriti 2009). During post-exposure periods, conspicuous changes consisting both in wall collapse and random callose accumulation took place in the more affected tissues. Callose can contribute to detoxification of ozone entering the cell surface (Moldau *et al.* 1997) and consequently the observed random deposition can be considered an active physiological response against O_3 stress, as occur in other plant systems exposed to biotic and abiotic stressors including O_3 (Fincher and Stone 1981, Paolacci *et al.* 2001, Pasqualini *et al.* 2003, Gravano *et al.* 2003, Ljubešić and Britvec 2006).

In light of the obtained results, the O_3-induced foliar PCD can assume two significant roles in the clone Eridano. The death of the mesophyll cells experiencing an oxidative stress can trigger a well orchestrated response resulting in a specific gene expression and in a series of physiological and biochemical events counteracting ROS spreading and limiting the damage. Consequently, the tissues preserved from the oxidative damage can continue their physiological processes. The evidenced asynchronous progress of PCD in the mesophyll cells might be assumed to be beneficial for the whole plant allowing a gradual remobilization and reallocation of nutrients and other factors. The substances derived from the mesophyll dismantling could be addressed toward young and developing structures (*i.e.* new leaves, fruits) that consequently can complete their differentiation process. Alternatively, the remobilized substances could be addressed to sink organs. In accord with this hypothesis, the conductive bundles remained long time unaffected by the damage (Bartoli *et al.* 2010) and able to transport elsewhere the substances derived from the most injured tissues.

In this way, O_3-sensitive poplars and probably all the broad-leaved O_3-sensitive plants that are not able to fully prevent or escape the oxidative power of ozone through intrinsic morpho-anatomical or biochemical traits can partially counteract its phytotoxic effect by displaying PCD processes as extreme defensive/adaptive strategy in the short-term.

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