Cell death induction and nitric oxide biosynthesis in white poplar (Populus alba) suspension cultures exposed to alfalfa saponins

Alma Balestrazzi, Valentina Agonia, Aldo Tava, Pinarosa Avato, Elisa Biazzi, Elena Raimondi, Anca Macoveia and Daniela Carbonera

The present work reports on the biological activity of alfalfa (Medicago sativa) saponins on white poplar (Populus alba, cultivar ‘Villafranca’) cell suspension cultures. The extracts from alfalfa roots, aerial parts and seeds were characterized for their saponin content by means of thin layer chromatography (TLC) and electrospray ionisation coupled to mass spectrometry. The quantitative saponin composition from the different plant extracts was determined considering the aglycone moieties and determined by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) analyses. Only soyasapogenin I was detected in the seed extract while several other saponins were found in the root and leaf extracts. Actively proliferating white poplar cell cultures were challenged with the different saponin extracts. Only alfalfa root saponins, at 50 μg ml⁻¹, induced significant cell death rates (75.00 ± 4.90%). Different cell subpopulations with peculiar cell death morphologies were observed and the programmed cell death (PCD)/necrosis ratio was reduced at increasing saponin concentrations. Enhancement of nitric oxide (NO) production was observed in white poplar cells treated with root saponins (RSs) at 50 μg ml⁻¹ and release of reactive oxygen species (ROS) in the culture medium was also demonstrated. Saponin-induced NO production was sensitive to sodium azide and NG-monomethyl-L-arginine, two specific inhibitors of distinct pathways for NO biosynthesis in plant cells.

Introduction

Programmed cell death (PCD) represents an essential physiological process required during specific stages of plant development, such as xylogenesis, embryogenesis and seed development, but also involved in the plant response to environmental changes and pathogen attack (Williams and Dickman 2008). PCD can be defined as a highly regulated cellular suicide, distinct from necrosis which is generally recognized as an unorganized cell death event resulting from exposure to severe stress (Reape et al. 2008, Reape and McCabe 2010). Specific genetic programmes, activated by intra/extracellular signals, are switched on in PCD-committed cells, which

Abbreviations – CCD, Charge-coupled device; CPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-2DA, 4,5-diaminofluorescein diacetate; DAPI, 4',6-diamidino-2-phenylindole; ESI/MS, electrospray ionization/mass spectrometry; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HS, heat shock; iNOS, inducible nitric oxide synthase; l-NMMA, N⁶-monomethyl-L-arginine; NMR, nuclear magnetic resonance; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; NT, untreated cells; PCD, programmed cell death; ROS, reactive oxygen species; RS, root saponin; TLC, thin layer chromatography.
use endogenous biochemical pathways to fulfill their duty. Within this context, reactive oxygen species (ROS) play different regulatory roles, depending on type and localization (Doyle and McCabe 2010) while there is evidence that, as in animals, the execution of PCD in plants is mediated by cysteine proteases (caspase-like proteins) which perform cellular destruction (Elbaz et al. 2002). The occurrence of PCD in plants is associated with a typical morphology which includes protoplast shrinkage, that is cytoplasm condensation away from the cell wall, and DNA fragmentation (Reape et al. 2008). A key regulatory role is played by mitochondria that release critical apoptogenic proteins such as cytochrome c (Balk et al. 1999). Plants, which share all these PCD features with animal apoptosis, also display some unique aspects related to the presence of chloroplasts. Under stress conditions, chloroplasts produce ROS which contribute, as signalling molecules, to PCD regulation in green tissues (Doyle et al. 2010).

In recent years, the key role played by the highly reactive molecule nitric oxide (NO) in PCD regulation has emerged in plants (Besson-Bard et al. 2008). The free radical NO can directly modulate gene expression by interacting with specific transcription factors. The latter are subjected to redox-dependent modulations among which is NO-mediated S-nitrosylation occurring on the thiol side chain of cysteine residues (Stamler et al. 2001). It is worth noting that redox signalling, a process which relies on free radicals (reactive oxygen and nitrogen species) acts mainly through modification of cysteine residues. As for PCD regulation, it has been reported that in Arabidopsis S-nitrosylation control cell death by maintaining inactive the protein prometacaspase 9, a putative cell death executioner (Belenghi et al. 2007). Notwithstanding this advancement, the complexity of NO-related signalling networks still requires extensive investigation together with the description of novel NO-mediated processes.

As apoptotic and necrotic cells can coexist within the same population, it is essential to detect and quantify the PCD events to better understand the molecular mechanisms underlying the plant response to environmental stresses and/or developmental signals. To this purpose, morphological hallmarks such as protoplast shrinkage and nuclear condensation are combined with molecular parameters such as DNA laddering resulting from extensive endonuclease-mediated cleavage of nuclear DNA. Biochemical hallmarks, e.g. the release of cytochrome c from mitochondria (leading to caspase activation), are also used (McCabe and Leaver 2000).

Within the genus Medicago, the triterpene saponins represent a relevant class of secondary metabolites involved in the defence response against herbivores, soil-borne pathogens and pests (Tava and Avato 2006). Saponins from Medicago sativa, commonly known as alfalfa, have been extensively investigated, mainly because of the importance of this species for animal feeding (Humphreys 2005), and for their biological properties (Tava and Avato 2006, Avato et al. 2008), in particular, their antitumor activity which has been tested in vitro (Avato et al. 2008).

In animal cells, the anticancer function played by saponins relies on very complex mechanisms and apoptosis seems to be the outstanding pathway. It has been reported that saponins can specifically impair the proliferation of cancer cells by inducing apoptosis, without affecting the surrounding healthy tissues (Gu and Belury 2005, Zhang et al. 2008). These compounds, which might be highly valuable anticancer agents, can selectively induce cell cycle arrest at the G1 phase, thus preventing cancer cells from entering into the S phase (Gu and Belury 2005). Saponins might then represent an interesting way to trigger and explore some uncovered aspects of PCD in plants. Despite the huge literature from animal systems, no information is currently available concerning the cytotoxic effect of saponins towards plant cells. It is worth noting that some aspects of the plant defense response, involving saponins, have been highlighted by Bouarab et al. (2002) who demonstrated that these antimicrobial compounds are hydrolysed by a fungal saponin-detoxifying enzyme. The resulting product suppresses the plant defense response by interfering with the related signal transduction pathways.

In animal cells, exposure to saponins induces NO production. It has been reported that saponins from Panax ginseng can stimulate NO production in murine macrophage cell lines (Kim et al. 2005). In contrast, Suh et al. (2007) demonstrated that a triterpenoid saponin from the edible plant Aralia elata inhibits NO production in murine macrophage cells by downregulating the NF-κB transcription factor, which controls the inducible nitric oxide synthase (iNOS) gene expression. In a previous work, Haridas et al. (2001) found that avicin-dependent inhibition of NF-κB binding to DNA was reversed by dithiothreitol, suggesting that avicin may modify a sulfhydryl group critical for activation of NF-κB.

To date, there are no reports describing the response, in terms of NO production, of plant cells exposed to saponins. According to recent literature, NO production in plants depends on different pathways, requiring the cytosolic nitrate reductase (NR) (Yamasaki and Sakihama 2000), a root-specific plasma membrane nitrite reductase (Ni-NOR) (Stohr et al. 2003) and a nitric oxide synthase (NOS)-like enzyme whose identity is still controversial (Besson-Bard et al. 2008).
The white poplar (Populus alba) cultivar ‘Villafranca’ used in this study has been engineered with relevant agronomic traits (Balestrazzi et al. 2006) and tested with innovative marker-free gene-transfer technologies (Balestrazzi et al. 2009a, Zelasco et al. 2007) while previous investigations have been carried out with cell suspension cultures (Balestrazzi et al. 2009b, Zelasco et al. 2006).

In the present work, the effect of alfalfa saponins on plant cell viability has been investigated using white poplar suspension cultures and the death response evaluated by means of morphological and molecular hallmarks. The ability of white poplar cells to produce NO in response to these metabolites and the possible involvement of the two most relevant NO biosynthetic pathways were also assessed.

Materials and methods

Extraction, purification and characterization of M. sativa saponins

Alfalfa (M. sativa cultivar ‘Equipe’) plants were grown at C.R.A.-F.L.C. The aerial parts (collected at plant anthesis) and roots (collected in autumn) were used for saponin extraction. Seeds were kindly supplied by Dr Luciano Pecetti (C.R.A.-F.L.C.). Saponins used in this study were newly extracted and purified following general procedures reported previously (Bialy et al. 2004, 2006, Tava et al. 2005). Purified mixtures of saponins were obtained as whitish powders in pure grade (90–95%) and analysed by silica gel 60H ready-to-use thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany) as described (Pecetti et al. 2006). Extracted and purified saponin mixtures were characterized for their quantitative aglycone composition by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) analyses of derivatized sapogenins, obtained after acid hydrolysis as already reported (Pecetti et al. 2006). Detailed structural elucidation was obtained by nuclear magnetic resonance (NMR) and electrospray ionization/mass spectrometry (ESI/MS) analysis (Bialy et al. 2004, 2006, Tava et al. 2005, Tava and Avato 2006). The purified saponin mixtures were dissolved in 50% ethanol and added at the indicated concentration to white poplar cell suspension cultures.

Cell cultures and treatments with alfalfa saponins

The white poplar (P. alba) cultivar ‘Villafranca’ used in this study was kindly supplied by Dr Stefano Bisoffi (C.R.A.-Unità di Ricerca per le Produzioni Legnose fuori Foresta, Casale Monferrato, Alessandria, Italy). Cell suspension cultures of ‘Villafranca’ were obtained and maintained in vitro as previously described (Zelasco et al. 2006). Exponentially growing (4-day-old) cell suspension cultures were exposed to saponins (25 and 50 μg ml⁻¹, respectively) and subsequently monitored at 0, 0.5, 1, 2 and 3 h following treatment. Sodium azide (100 μM NaN₃, Sigma-Aldrich S.r.l., Milan, Italy) and NG-monomethyl-L-arginine (500 μM L-NAME, Sigma-Aldrich S.r.l.) were added to cell cultures 24 h before the treatment with saponins. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) (500 μM, Sigma-Aldrich) was added 10 min before treatments. Untreated cells (NT) and cells exposed to the same volume of 50% ethanol used to dissolve saponins were used as control. Three independent experiments were performed. Each experiment included the treatments with the saponin mixtures from the three plant organs and three replicated samples were used for each dose treatment.

Cell viability and cellular and nuclear morphology

Cell viability was determined by Evans Blue assay as described by Carimi et al. (2003). As a positive control, the percentage of dead cells in each sample was determined based on the absorbance rate of intact healthy and dead cells after HS treatment (55°C, 10 min). The morphology of dead cells in suspension cultures was checked using a light microscope (Reichert Biovar, Austria). Nuclear morphology was analysed as described by Callard et al. (1996) using 4',6-diamidino-2-phenylindole (DAPI) staining (BioChemika, Sigma-Aldrich S.r.l., Milan, Italy) and a ZEISS Axioplan microscope equipped with a Charge-coupled device (CCD) videocamera (Photometrics); image collection, merge and analysis were performed with the IPLAB SPECTRUM software (Digital Pixel Advanced Imaging System, Brighton, UK). For each treatment, 500 cells were examined. Three independent experiments were carried out, which included saponin-treated cells, ethanol-treated and NT.

H₂O₂ detection

The release of H₂O₂ in the cell suspension medium was measured as described by Bellincampi et al. (2000). One ml of cell culture was harvested by centrifugation (10 000 g, 20 s, 25°C) and H₂O₂ was measured in the supernatant. An aliquot of supernatant (500 μl) was added to 500 μl of assay reagent (500 μM ferrous ammonium sulphate, 50 mM H₂SO₄, 200 μM xylenol orange, 200 mM sorbitol). After 45 min of
incubation, the peroxide-mediated oxidation of Fe$^{2+}$ to Fe$^{3+}$ was determined by measuring the A$_{560}$ of the Fe$^{3+}$-xylenol orange complex. Catalase (Sigma-Aldrich) was added to cell suspension cultures at the final concentration of 0.15 mg ml$^{-1}$, 30 min before treatment with saponins.

**Determination of extracellular nitrite content**

Aliquots (1 ml) of cultured cell suspensions were collected, centrifuged and 0.5 ml of the culture medium were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid) (Sigma-Aldrich) (Green et al. 1982). Samples were incubated at room temperature for 15 min and nitrite was measured by spectrophotometric analysis at 540 nm. NO content was calculated by comparison with a standard curve of NaNO$_2$. Standard solutions of NaNO$_2$ were prepared in cell culture medium.

**NO production**

The intracellular NO was detected using the fluorophore 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma-Aldrich) which was added to cell suspension cultures at the final concentration of 10 $\mu$M (Lamotte et al. 2004). Cells were incubated for 1 h in the dark on a rotary shaker (150 rpm) and then rinsed twice with fresh medium in order to remove excessive fluorophore. Subsequently, the white poplar cells were treated with saponins, aliquots (1 ml) were collected at the indicated times and analysed using a ZEISS Axioplan microscope equipped with a CCD videocamera (Photometrics); image collection, merge and analysis were performed with the IPLAB SPECTRUM software (Digital Pixel Advanced Imaging System). Quantitative analysis of fluorescence was carried out using the IMAGEJ software available at: http://rsb.info.nih.gov/ij/.

**Statistical analyses**

Experiments were repeated three times and carried out in triplicate. Data are expressed as means ± standard deviation (SD) values. Differences observed in cell viability and NO production in saponin-treated cells compared with the NTs were statistically evaluated. Similarly, differences in NO production in saponin-treated cells exposed to inhibitors compared with saponin-treated cells not exposed to these compounds were analysed. Statistical significance of differences was determined using Student’s t-test (*$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001).

**Results**

**Saponin composition from the different plant organs**

The chemical structures of the most abundant sapogenins typical of the alfalfa saponins are reported in Fig. 1. Compositional differences between leaf and RSs can be ascribed to the different content and ratio of the dominant sapogenins, principally medicagenic and zanhic acid. As shown in Table 1, alfalfa leaf saponins used in this study are characterized by the presence of a higher amount of medicagenic acid, quoted as 45.1% of the total sapogenins, followed by zanhic acid (23.5%) and soyasapogenol B (11.1%). Saponins from alfalfa roots are almost totally characterized by medicagenic acid glycosides (medicagenic acid 60.1%), followed by hederagenin accounting for the 19.2% of the total. In this plant material, zanhic acid is present in very low amount, accounting for 0.9%. Soyasapogenol B, which amounts to 11.1 and 4.4% in the leaf and root extracts, respectively, is instead almost the only one saponin (92.2%) detected in the seed extract. The saponin composition of the three extracts from the different alfalfa organs was investigated by means of ESI/MS analyses of the crude saponin mixtures (data not shown). Differences in the saponin composition were also confirmed by TLC analyses (Fig. 2) and the most abundant saponins identified by comparison with pure compounds, previously characterized by NMR and ESI/MS analyses (Bialy et al. 2004, 2006, Tava et al. 2005, 2009). Only one saponin was detected in the seed extract (Fig. 2A) and identified as soyasapogenol I, a saponin of soyasapogenol B, while several other saponins were found in both root and leaf extracts. The root sample (Fig. 2B), showed the presence of two constituents, identified as 3-O-$\alpha$-L-rhamnopyranosyl medicagenic acid and 3-O-$\alpha$-L-glucopyranosyl medicagenic acid and 3-O-$\alpha$-L-glucopyranosyl-28-O-$\beta$-D-glucopyranoside medicagenic acid, (1 and 2, respectively) that were absent in the leaf extract. A series of medicagenic acid and hederagenin glycosides were detected in both root and leaf saponin mixtures (Fig. 2B, C), with 3-O-$\beta$-D-glucuronopyranosyl-28-O-[$\beta$-D-xylopyranosyl(1→4)-$\alpha$-L-rhamnopyranosyl(1→2)-$\alpha$-L-arabinopyranoside] medicagenic acid (Fig. 2B, 9) as one of the most abundant compounds in both extracts, as also confirmed by the ESI/MS analyses (data not shown). Moreover, the leaf sample also contained a higher amount of zanhic acid saponins (Fig. 2C, 11). These results are in agreement with the sapogenin evaluation performed by GC analyses.
Fig. 1. Chemical structure of the most abundant sapogenins (R = R1 = H) identified in alfalfa. Saponins: R = sugar or sugar chain, R1 = H: monodesmosides; R = R1 = sugar or sugar chain: bidesmosides.

Table 1. Percentage composition of the most abundant sapogenins from saponin extracts in the alfalfa organs.

<table>
<thead>
<tr>
<th>Plant organs</th>
<th>Medicagenic acid</th>
<th>Zanhic acid</th>
<th>Hederagenin</th>
<th>Bayogenin</th>
<th>Soyasapogenol A</th>
<th>Soyasapogenol B</th>
</tr>
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<tbody>
<tr>
<td>Leaves</td>
<td>45.1</td>
<td>23.5</td>
<td>2.3</td>
<td>1.9</td>
<td>1.3</td>
<td>11.1</td>
</tr>
<tr>
<td>Roots</td>
<td>60.1</td>
<td>0.9</td>
<td>19.2</td>
<td>2.9</td>
<td>0.2</td>
<td>4.4</td>
</tr>
<tr>
<td>Seeds</td>
<td>--</td>
<td>--</td>
<td>92.2</td>
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</table>

Induction of cell death in white poplar suspension cultures exposed to alfalfa saponins

Two different concentrations (25 and 50 μg ml⁻¹, respectively) of alfalfa saponin obtained from the plant aerial parts, roots and seeds have been used in the analysis with white poplar cells. Saponins were added to 4-day-old cultures, exponentially growing. Cell death was evaluated 24 h following treatments, using the Evans Blue spectrophotometric assay. In the absence of saponins, the fraction of dead cells did not exceed 10–12% (Fig. 3, NT) and in cultures exposed to 50% ethanol, at the same volume used to dissolve saponins, the rate of cell death did not significantly vary (Fig. 3, E). Treatments with the seed saponins did not show cytotoxic effect (Fig. 3A, NT, 25 and 50). In contrast, a significant increase in the dead cells ratio (75.00 ± 4.90%) was detected 24 h following the exposure to the highest concentration of RSs (Fig. 3B, 50). When saponins from the aerial part were assayed (Fig. 3C, 25 and 50), the percentage of dead cells was reduced, compared with saponins from alfalfa roots. The highest cell death rate, close to 100%, was observed following HS treatment used as a positive control in spectrophotometric assays (Fig. 3, HS).

Cell death morphology, chromatin condensation and DNA fragmentation in white poplar suspension cultures exposed to alfalfa RSs

Evans Blue staining was absent in viable cells (Fig. 4A), while 24 h following treatment with RSs (50 μg ml⁻¹) different cell death morphologies were evidenced. Within the cell population showing protoplast shrinkage, two subpopulations were further identified: (1) cells in which the empty space between the cell wall and the detached protoplast was very thin (see arrow in Fig. 4B, early protoplast shrinkage) and (2) cells in which protoplast shrinkage was more pronounced and the cellular content looked as a round-like body inside the empty space surrounded by the cell wall (see arrows in Fig. 4C, late protoplast shrinkage). A subpopulation with no apparent protoplast shrinkage but with a uniformly blue cytoplasm (hallmark of necrosis) was detected (Fig. 4D). The percentage of cells belonging to each subpopulation is reported in Table 2. In cultures exposed to the lower dose of RS (25 μg ml⁻¹) the cells showing absence of protoplast shrinkage and uniform cytosol staining were 42.70% of the total Evans Blue-positive population while moderate and pronounced protoplast shrinkage
saponins from roots, the estimated level of NO was not shown. Twenty four hours after treatment with RSs (25 and 50 μg ml⁻¹), total genomic DNA was extracted and analysed on agarose gels. Extensive DNA fragmentation was invariably observed in the saponin-treated samples (Fig. 5C, 25 and 50), while no DNA laddering was detected. Finally, the saponin-treated cells did not show cytochrome c release from mitochondria to cytosol (data not shown).

Saponin-induced NO generation in white poplar suspension cultures

NO production in suspension cultures treated with alfalfa RSs was measured using different approaches. First, the amount of NO released into the culture medium was quantified using the Griess reagent which measures a stable product derived from NO oxidation. Two different concentrations (25 and 50 μg ml⁻¹) were tested and cell cultures were monitored for NO production at different times (0, 0.5, 1, 2 and 3 h) (Fig. 6A). In the untreated suspension cultures, the amount of NO ranged from 1.93 ± 0.12 to 2.67 ± 0.20 μmol l⁻¹ while exposure to ethanol did not significantly affect the response of white poplar cells (Fig. 6A). With the lowest concentration of saponins from roots, the estimated level of NO was 5.03 ± 1.22 μmol l⁻¹ at the beginning of the treatment.
and 7.03 ± 1.12 μmol l⁻¹ at 0.5 h (Fig. 6A). One hour after treatment, the values slightly increased and they did not change until the end of the experiment. The highest dose of RSs produced a significant increase of the amount of NO, 39.51 ± 4.73 μmol l⁻¹, 0.5 h after treatment. Subsequently the values gradually decreased, reaching 30.21 ± 4.42 μmol l⁻¹ at 1 h. The NO level further decreased at 2 h and remained constant until the end of the experiment.

NO production in response to RSs was detected using DAF-2DA, a fluorescent indicator which allows direct NO measurement (Fig. 6B). The fluorimetric assays demonstrated that RSs were able to induce NO production with a peak at 0.5 h following treatment (Fig. 6B, RS). When RSs were added to cell cultures previously incubated with the specific NO scavenger CPTIO, the NO burst was completely suppressed, thus confirming that the enhancement of fluorescence was due to NO production (Fig. 6B, RS + CPTIO). In the absence of RSs, the white poplar cells did not show significant changes in fluorescence (Fig. 6B, NT). Quantitative image analyses allowed estimation of the intensity of the fluorescent signal produced by the saponin-treated cells as fivefold (Fig. 6C, RS), compared with the untreated (Fig. 6C, NT) and CPTIO-treated (Fig. 6C, CPTIO) samples.

To investigate the possible involvement of the NOS-like- and NR-dependent pathways in the biosynthesis of NO observed in response to saponins, the white poplar cultures were treated with 100 μM NaN₃ and with 500 μM L-NMMA, 24 h before the addition of 50 μg ml⁻¹ RSs. No significant variation in NO production was observed when the cells were monitored.

Table 2. Relative frequency of different cell death morphologies observed within the white poplar suspension cultures (4-day-old) exposed to RSs and to HS Cell cultures were stained with Evans Blue for 24 h following treatments and cell morphology was scored under a light microscope. For each treatment, 500 cells were examined. n. d., not detected. a The total Evans Blue positive population has been considered as 100% in order to better evidence the different rates of cell death morphologies.

<table>
<thead>
<tr>
<th>Cell death morphology</th>
<th>Necrosis</th>
<th>PCD</th>
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<tbody>
<tr>
<td></td>
<td>Protoplast shrinkage</td>
<td>Presence of protoplast shrinkage. Detachment from cell wall:</td>
</tr>
<tr>
<td>Treatment</td>
<td>Evans Blue</td>
<td>Moderate</td>
</tr>
<tr>
<td>RSs (25 μg ml⁻¹)</td>
<td>42.79% a</td>
<td>27.09%</td>
</tr>
<tr>
<td>RSs (50 μg ml⁻¹)</td>
<td>67.68%</td>
<td>16.15%</td>
</tr>
<tr>
<td>HS (65°C, 10 min)</td>
<td>90.00%</td>
<td>10.00%</td>
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</table>

Fig. 4. Morphology of dead cells in white poplar suspension cultures exposed to alfalfa RSs and stained with Evans Blue, 24 h following treatment. (A) Viable white poplar cell. (B) Cell showing evidence of PCD, as demonstrated by the moderate protoplast shrinkage (arrow). (C) Cell showing evidence of PCD, in this case pronounced protoplast shrinkage can be observed. (D) Cell showing evidence of necrosis, as indicated by the uniform cytosol staining. p, protoplast; cw, cell wall. Bar = 20 μm.

Fig. 5. Analysis of nuclear morphology and DNA fragmentation in white poplar cell cultures exposed to alfalfa RSs. (A) Nucleus of an untreated cell, showing uniformly stained chromatin and a large nucleolus (arrow). (B) Irregularly stained granular nucleus of a saponin-treated cell, resulting from chromatin condensation and redistribution. The arrow indicates a peripheral chromatin granule. Bar = 5 μm. (C) Agarose gel electrophoresis of total DNA from untreated and saponin-treated white poplar cell suspension cultures. NT, untreated cells. E, cells treated with the same volume of 50% ethanol used to dissolve saponins. 25 and 50, cells exposed to 25 and 50 μg ml⁻¹ saponin, respectively. M, λ DNA/EcoRI+HindIII Marker, 3 (MBI Fermentas, Cornaredo, Italy).
only in the presence of NaN₃ and L-NMMA (Fig. 7A). In contrast, the peak in NO production induced at 0.5 h by RSs was absent in cells exposed to sodium azide (Fig. 7B), indicating that this inhibitor totally suppressed NO production. L-NMMA caused a reduction of NO production to approximately 75% of the level observed in the untreated sample exposed only to saponins. Similar results were observed when NO production was measured using DAF-2DA (data not shown). In the absence of RSs, the fraction of dead cells, evidenced by Evans Blue spectrophotometric assay, did not exceed 10–12%, even in the presence of inhibitors (Fig. 7C). RSs resulted into cytotoxic effects (Fig. 7D, 1). The presence of NaN₃ and L-NMMA reduced the cell death rates caused by saponins (from 75% to 45 and 57%) (Fig. 7D, 2 and 3) while the PCD/necrosis ratio did not change (data not shown).

**ROS production in response to RS treatment**

As the occurrence of oxidative burst typically anticipates the entry of cells into PCD, the H₂O₂ release in the culture medium of white poplar cells treated with the RS extract (50 μg ml⁻¹) was evaluated at different times as shown in Table 3. As for the NT, the release of H₂O₂ was in the range 0.10–0.25 μM during the tested period. In cells exposed to RSs, H₂O₂ release increased up to 0.77 μM at 15 min from the beginning of the treatment, reaching the maximum value (2.14 μM) at 45 min. A similar response was also observed with the lowest dose (25 μg ml⁻¹) of RS extract (data not shown). The occurrence of an oxidative burst suggests that the PCD pathway is activated in the white poplar cells exposed to RSs. Prior to exposure to saponins, cell suspension cultures were also treated with catalase (0.15 mg ml⁻¹) in order to remove any saponin-induced H₂O₂ production and verify the specificity of the response. As shown in Table 3, catalase nearly completely removed H₂O₂. This finding demonstrates that the observed oxidative burst results from treatments with saponins.

**Discussion**

The increasing body of literature currently available on animal cells has allowed to hypothesize the possible role of saponins within the complex apoptotic network while very limited studies have so far been carried out in plants. As a further step towards the better understanding of the mechanisms induced in plant cells by exposure to saponins, an investigation was performed to assess the biological activity of alfalfa saponins on white poplar cell suspension cultures.

The aim of the present work was to explore the possible occurrence of apoptotic vs necrotic cell death response and the involvement of well known signalling molecules, such as ROS and NO in saponin-mediated cytotoxicity.

Among the tested alfalfa extracts, only the RSs caused a significant cell death response in white poplar cell suspension cultures. This can likely be explained with the different composition of RSs if compared with saponins from the other plant parts. In fact, at least two saponins are present in alfalfa roots, namely 3-O-β-D-glucopyranosyl medicagenic acid and 3-O-β-D-glucopyranosyl-28-O-β-D-glucopyranoside medicagenic acid, which are not found in the leaf extract and which are known to possess a strong biological activity (Tava and Avato 2006). Moreover, the lack of toxicity of saponins from aerial parts against white poplar cells might be due to the almost unique presence of more complex sugar chain bidesmosides of medicagenic and zanhic acid, which are reported to be less bioactive (Tava and Avato 2006). Additionally, the presence of a higher amount of hederagenin glycosides in the root extract may contribute to improve the cytotoxic effects. As glycosylation of secondary metabolites represents a common mechanism of self-protection against phytotoxic compounds, stored into the cell vacuole (Martinoia et al. 2007), this might be the main explanation for the lack of planta toxicity of alfalfa RSs. Further studies are,
Fig. 7. Effects of inhibitors on NO production in white poplar 4-day-old cell suspension cultures exposed to alfalfa RSs. NO release into the culture medium was quantified using the Griess reagent. (A) Without RSs: (○) untreated cells, (■) 100 μM NaN3, (△) 500 μM L-NMMA. (B) With RSs: (○) 50 μg ml⁻¹ saponin, (●) 50 μg ml⁻¹ saponin + 100 μM NaN3, (△) 50 μg ml⁻¹ saponin + 500 μM L-NMMA. (C) Percentage of dead cell induced by exposure of untreated cells to inhibitors of NO synthesis. (D) Percentage of dead cell induced by exposure of saponin-treated cells to inhibitors of NO synthesis. Values are expressed as means ± SD of three independent experiments. Statistical significance was determined (**P < 0.01; ***P < 0.001 compared with saponin-treated cells not exposed to inhibitors).

However, required to investigate whether the observed toxicity has to be directly ascribed to one or more saponins of the root extract.

As for the cell death response occurring in white poplar suspension cultures exposed to saponins, both morphological and molecular markers were considered in this study. Protoplast shrinkage has been recognized as a morphological PCD hallmark in plant cells while, in contrast, the absence of cytoplasm retraction is an indicator of necrosis (McCabe and Leaver 2000). It is generally believed that necrosis depends on high stress and that moderate stress activates PCD in most of the treated cells (Balestrazzi et al. 2010, Reape et al. 2008). Furthermore, the screening for cell death morphologies and the consequent evaluation of the PCD/necrosis ratio is a helpful element in understanding whether a specific cytotoxic treatment interferes with the cell death programme and its related signalling pathways.

According to the reported data, it seems that the white poplar cell response to increasing saponin doses is consistent with this hypothesis. The lower saponin dose is responsible for moderate levels of stress because PCD is recorded in almost half of the total cell population. In contrast, an increase in saponin concentration causes significant changes in the PCD/necrosis ratio with a strong enhancement of necrosis. In our opinion, this
is a significant finding because the reported data on the bioactivity of alfalfa RSs will help define the more convenient range of stress treatments to be used in future studies aimed at dissecting the PCD signal transduction routes activated in plant cells by these metabolites and their target genes.

As a subsequent step, the possible involvement of ROS as signalling molecules in the cell death response activated by white poplar cells exposed to alfalfa saponins was investigated. The finding that ROS are produced in response to RSs seems to confirm that PCD is triggered in a white poplar cell subpopulation. Wang et al. (2007) reported that the saponin dioscin extracted from the roots of Polygonum zanlansciasense induces apoptosis in human myeloblast leukemia cells by stimulating $\text{H}_2\text{O}_2$ and $\text{O}_2^{-}$ accumulation within 1.5 h of treatment. Similarly, the tomato saponin $\alpha$-tomatine induces ROS-mediated PCD in the fungal pathogen Fusarium oxysporum (Ito et al. 2007). In this case, the ROS burst occurred at 30 min after treatment and the fungicidal action of $\alpha$-tomatine was significantly blocked when ROS production was inhibited. Thus, also in plant cells, as already demonstrated in animal cells and fungi, exposure to saponins seems to trigger ROS accumulation and, possibly those signalling events leading to PCD.

Together with ROS, NO is involved in the stress response as signal molecule in both animal and plant cells and the reported data suggest that the bioactive NO molecule is also a part of the signalling events triggered by saponins. This is not surprising because it is known that reactive oxygen and nitrogen species are the major regulators of key cellular functions. Furthermore, it is worth noting that NO production in white poplar cells challenged with saponins occurs within the same time frame with that of $\text{H}_2\text{O}_2$. This is a distinctive feature, already evidenced for other stress conditions (Delledonne et al. 2001), which underlines how the critical balance between these redox molecules is the essential factor responsible for cell survival.

Another interesting question raised concerns the enzymes involved in NO production in white poplar cells. This is an intriguing aspect because the enzymatic sources of NO participating in the plant response to stress are still a matter of discussion (Besson-Bard et al. 2008).

As for the possible source of NO production in white poplar cells challenged with alfalfa saponins, the effect of sodium azide, an inhibitor of NR (Neill et al. 2003, Yamasaki and Sakihama 2000) is outstanding. In white poplar cells, the possible involvement of NR in NO biosynthesis is also supported by the fact that sodium tungstate, a specific inhibitor of the enzyme, was able to block NO production (Balestrazzi et al. 2009a). On the other hand, the limited specificity of mammalian NOS inhibitors in plants has been discussed by Besson-Bard et al. (2008). In our experiments, NO biosynthesis was affected when the white poplar cells were exposed to L-NMMA, thus indicating that a consistent part of the NO released into the culture medium was derived from the NOS-like-mediated pathway. This data differs from that obtained with the same white poplar cells challenged with heavy metals (Balestrazzi et al. 2009a).

The block of NO production caused by inhibitors in the saponin-treated white poplar cell cultures was associated with a significant reduction in cell death rates, in agreement with the report from Balestrazzi et al. (2009b), while the PCD/necrosis ratio was not influenced. This finding seems, however, difficult to explain, because NO inhibitors are presumed to interfere with the signalling pathway leading to PCD and, thus, an increase in the percentage of necrotic events should be expected. On the other hand, it could be also hypothesized that signalling routes other than those relying on NO, might contribute to limit the necrotic response.

To our knowledge, the present paper is the first report dealing with the effects of alfalfa saponins on the viability of plant cell suspension cultures. The experimental work carried out on the white poplar cultures adds novel and useful information concerning the effects of natural saponins in plant cells which can be summarized as follows: (1) saponin-triggered PCD events are accompanied by the concomitant accumulation of both reactive oxygen and nitrogen species, (2) a significant part of the NO produced in response to saponins derives from the NOS-like-mediated pathway.

On the basis of these premises, the several components of the root extract need to be isolated and tested separately to better define their contribution to the cell death response. The availability of purified saponin molecules will enable more detailed molecular studies and will possibly help focus on the target genes involved into this specific cell response.

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