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Cell wall integrity, genotoxic injury and PCD dynamics in alfalfa saponin-treated white poplar cells highlight a complex link between molecule structure and activity

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ABSTRACT

In the present work, eleven saponins and three sapogenins purified from *Medicago sativa* were tested for their cytotoxicity against highly proliferating white poplar (*Populus alba* L.) cell suspension cultures. After preliminary screening, four saponins with different structural features in terms of aglycone moieties and sugar chains (saponin **3**, a bidesmoside of hederagenin; saponins **4** and **5**, monodesmoside and bidesmoside of medicagenic acid respectively, and saponin **10**, a bidesmoside of zanhic acid) and different cytotoxicity were selected and used for further investigation on their structure–activity relationship. Transmission Electron Microscopy (TEM) analyses provided for the first time evidence of the effects exerted by saponins on plant cell wall integrity. Exposure to saponin **3** and saponin **10** resulted into disorganization of the outer wall layer and the effect was even more pronounced in white poplar cells treated with the two medicagenic acid derivatives, saponins **4** and **5**. Oxidative burst and nitric oxide accumulation were common hallmarks of the response of white poplar cells to saponins. When DNA damage accumulation and DNA repair profiles were evaluated by Single Cell Gel Electrophoresis, induction of single and double strand breaks followed by effective repair was observed within 24 h. The reported data are discussed in view of the current issues dealing with saponin structure–activity relationship.

1. Introduction

Saponins are biologically active plant-derived glycosides consisting of a sugar moiety linked to a hydrophobic aglycone (sapogenin) with a triterpenoid or a steroid structure (Hostettmann and Marston, 1995). In the genus *Medicago*, saponins are a complex mixture of triterpenic pentacyclic glycosides with medicagenic acid, hederagenin, bayogenin, zanhic acid and soyasapogenols A and B as the dominant aglycones. Sugars or sugar chains are then attached at the triterpenic core of the molecule in selected positions to give monodesmosidic or bidesmosidic saponins: in monodesmosides, sugars are linked at the 3-*O* position, while glycosylation at 3-*O* and 28-*O* positions gives bidesmosidic saponins. An additional glycosylation at the 23-*O* position of the triterpenic core gives tridesmosidic saponins, detected until now only in *Medicago sativa* (alfalfa) and *Medicago truncatula* (Tava and Avato, 2006). Detailed stage as well as organ or tissue in which they are synthesized (Pecetti et al., 2006; Tava and investigations on saponin content and chemical identification have been carried out in several *Medicago* species (Tava and Avato, 2006; Tava et al., 2009, 2011a,b). Saponin content and composition can change depending on environmental conditions, plant genotype, growth Avato et al., 2006; Tava et al., 1999). Tava and Avato (2006) showed evidenced that medicagenic acid glycosides are found in roots and aerial parts, while hederagenin glycosides are more dominant in roots while leaves are abundant in zanhic acid glycosides.

It has been shown that saponins from the genus *Medicago* display fungicidal, molluscicidal, nematicidal, antibacterial, antiviral and antitumoral properties (D'Addabbo et al., 2011; Tava and Avato, 2006). The biological activity of saponins extracted from

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M. sativa root and aerial system has been extensively investigated to acquire information on their structure–activity relationship (Argentieri et al., 2008; Avato et al., 2006; D'Addabbo et al., 2011; Tava and Avato, 2006). The chemical structure certainly plays a key role in the bioactivity of saponins, and the current investigations aim to unravel the specific role of aglycone, as well as the nature and position of the sugar(s) in the molecule. However, these studies have provided contrasting results so far. In some cases there is evidence that sugar moieties play a relevant role, while according to other authors sapogenins are more active (Avato et al., 2006). The observed antimicrobial activity of alfalfa root saponins against *Trichoderma viride* indicated that the monodesmoside derivatives of medicagenic acid were more effective than related bidesmosides, even though a direct correlation between number of sugar residues and saponin bioactivity could not be established. In contrast, bioassays with *Medicago* saponins against human pathogenic fungi and bacteria have highlighted that the presence of sugar residues in the molecule is not determinant for the antibiotic activity (Avato et al., 2006). It is worth noting that the majority of the reports currently available on *Medicago* saponins bioactivity is focused on the chemical structure and composition of these compounds, while only few studies have been published on the biological effects. Thus, a specific correlation must be established between saponins and their cellular or subcellular target. For instance, Thakur et al. (2011), have recently deduced some structural features essential for cytotoxic activity, by carrying out *in vitro* and preclinical studies carried out with novel synthetic saponins.

In animal cells, saponins target the plasma membrane, binding cholesterol and forming micelle-like aggregates that disrupt membrane functionality and cause lysis. Other cellular targets of saponins in animals are microtubules, endoplasmic reticulum and voltage-dependent anion channels (Thakur et al., 2011). By affecting these cellular components, saponins alter cell cycle progression, with the consequent inhibition of cell proliferation and induction of apoptosis. Balestrazzi et al. (2011a,b, in press) have evidenced that animal and plant cells share common elements in their response to saponins, suggesting for the possible use of plant systems for preliminary large-scale cytotoxicity tests performed on wide collections of natural extracts and/or purified compounds.

The biological activity of alfalfa root saponin extracts on white poplar (*Populus alba* L.) cell suspension cultures has been investigated by Balestrazzi et al. (2011a), who described dose-dependent cell death, as well as reactive oxygen species (ROS) and nitric oxide (NO) production. Furthermore, the up-regulation of the *VFMT2* gene, encoding a type 2 metallothionein, was evidenced in white poplar cells exposed to root saponins and this finding demonstrated that, similarly to animal cells, also plant metallothioneins play a role in the response to saponins (Balestrazzi et al., 2011b). In recent years, several investigations have been conducted in order to unravel saponin biosynthesis in model plants and optimize their production for industrial applications (Confalonieri et al., 2009; Tava et al., 2011a,b; Carelli et al., 2011). The large-scale preliminary screening of compounds relevant for pharmaco-industrial applications, includes the assessment of potential genotoxic effects. There are reports dealing with the effects of saponins on genome integrity in animal cells, while no information is currently available on saponin toxicity in plant cells. Liu et al. (2011) demonstrated that saponins extracted from *Nauclea* spp. were able to induce DSBs (double strand breaks) in CHO (Chinese Hamster Ovary) cells, as evidenced by micronucleus and SCGE (Single Cell Gel Electrophoresis) assays. A direct link between DNA repair and saponins activity was evidenced by Cai et al. (2009) who found that the treatment of human keratinocytes with steroidal saponin Rb1 from *Ginseng* enhanced NER (Nucleotide Excision Repair) pathway response.

In the present work, four saponins having different structural features (aglycone moieties and sugar chains) were selected out of a preliminary screening of fourteen molecules purified from *M. sativa* leaves and root extracts. The purified saponins were tested for their cytotoxicity against highly proliferating white poplar cell suspension cultures to assess the effects they have on plant cells. Transmission Electron Microscopy (TEM) imaging provided for the first time evidence of the effects exerted by saponins on plant cell wall integrity. Based on the reported data, a working hypothesis is presented, which includes the overall physiological changes triggered by saponin treatment and highlights some key molecular aspects of the plant cell response that still remain an open question.

2. Results

2.1. Purification and structural elucidation of M. sativa saponins/sapogenins and preliminary screening for cytotoxic activity against white poplar cells

The chemical structure of the purified compounds from alfalfa (*M. sativa* L.) used in this investigation is reported in Fig. 1. The detailed structural elucidation of the purified saponins (**1**–**11**) and sapogenins (**12**–**14**) was obtained by combining Nuclear Magnetic Resonance (NMR), Electrospray Ionization Mass Spectrometry/Mass Spectrometry (ESI-MS/MS), and Gas Chromatography/Mass Spectrometry (GC/MS) analyses (Bialy et al., 1999; Tava et al., 2005, 2009, 2011a). The saponins/sapogenins listed in Fig. 1 were previously described as the most abundant and representative compounds in alfalfa plant material (Tava and Avato, 2006). A preliminary screening of the cytotoxic effects caused by the purified alfalfa saponins (**1**–**11**) and sapogenins (**12**–**14**) on white poplar cell suspension cultures was carried out using Evans Blue staining assay. Loss of plasma membrane integrity was used as hallmark of saponin cytotoxicity in the cell suspension cultures stained with Evans Blue dye. For each saponin/sapogenin, different concentrations (in the range 0–100 nmol mL^{-1}) were added to exponentially growing four-day-old white poplar cultures. For each *M. sativa* saponin/sapogenin, the concentration able to induce 50% of plasma membrane damage of the total cell population at 24 h following treatment was established (Fig. 1). Based on results of these preliminary experiments, the alfalfa purified saponins were grouped in different classes according to their different cytotoxic activity. As shown in Fig. 2, high amounts of saponins **1** and **3** (106.1 nmol mL^{-1}, and 94.3 nmol mL^{-1}, respectively) were required to cause damage at the plasma membrane level in 50% of the white poplar cell population. Both these compounds are bidesmosidic saponin of hederagenin (Fig. 1) and had low cytotoxic activity towards white poplar cells. Almost half of the dose (54.6 and 51.0 nmol mL^{-1}, respectively) was able to cause the expected cellular damage in the case of saponins **2** and **7**, which consequently revealed higher cytotoxic activity, compared to saponins **1** and **3** (Fig. 2). Compound **2** is an hederagenin monodesmoside, while saponin **7** is a medicagenic acid bidesmoside with a glucuronic acid unit (Fig. 1). The group including saponins **4**–**6** and **9**–**11** was characterized by enhanced cytotoxicity. Indeed the doses required to induce damage at the plasma membrane in 50% of the white poplar cell population progressively decreased: saponin **10**, 12.9 nmol mL^{-1}; saponin **11**, 9.7 nmol mL^{-1}; saponin **5**, 12.1 nmol mL^{-1}; saponin **4**, 7.5 nmol mL^{-1}; saponin **6**, 6.3 nmol mL^{-1}; saponin **9**, 5.8 nmol mL^{-1} and saponin **8**, 3.6 nmol mL^{-1} (Fig. 2). This was observed for saponins showing different structures, such as zanhic acid bidesmosides (compounds **9** and **10**), soyasaponin I (**11**), and for mono- and bidesmoside derivatives of medicagenic acid, namely saponins **4**, **5**, **6** and **8**, respectively (Fig. 1). A range of cytotoxicity was also observed for the tested sapogenins: the lowest cytotoxic effect was registered for zanhic acid (compound **14**) for which the 34.6 nmol mL^{-1} concentration was requested, while medicagenic acid (compound **13**) showed the