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ORIGINAL PAPER

Increased cardenolides production by elicitation of *Digitalis lanata* shoots cultured in temporary immersion systems

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Abstract *Digitalis lanata* is an important source of cardenolides such as digoxin and lanatoside C, which have been widely applied in the treatment of cardiac insufficiencies. Elicitation is one of the most effective methods to enhance the biosynthesis of several secondary metabolites in medicinal plants. We studied the effect of elicitation with Chitoplant®, Silioplant® and methyl jasmonate on biomass and cardenolides accumulation in shoots of *D. lanata* cultivated in temporary immersion systems. Morphological response of the shoots was influenced by elicitors. A reduction in length and number of shoots was evident with all MJ concentrations. Regarding biomass production, Chitoplant® (0.1 g l^{-1}) was found to impact significantly on fresh and dry weight of the shoots. HPLC analysis revealed a higher content of lanatoside C compared to digoxin in all treatments. The highest accumulation of lanatoside C was achieved with Chitoplant® (0.1 g l^{-1}), which resulted in 316 μg g-DW^{-1} and with Silioplant® (0.01 g l^{-1}; 310 μg g-DW^{-1}), which accounted for a 2.2-fold increase in lanatoside C content compared to non-elicited shoot cultures. Additionally, elicitation of *D. lanata* shoots in temporary immersion systems resulted in an oxidative stress characterized by hydrogen peroxide and malondialdehyde accumulation. These observations point to a connection between hydrogen peroxide generation, lipid peroxidation and cardenolide accumulation. The optimization of elicitor treatment and culture conditions for cardenolide production as well as the advantages of TIS for this purpose are discussed.

Keywords Cardiotonic glycosides · Chitoplant · Silioplant · Methyl jasmonate · Temporary immersion systems · Oxidative stress

Abbreviations

6-BAP	6-Benzylaminopurine
ChP	Chitoplant®
DW	Dry weight
FW	Fresh weight
IAA	Indole-3-acetic acid
MDA	Malondialdehyde
MJ	Methyl jasmonate
MS	Murashige and Skoog medium
ROS	Reactive oxygen species
SiP	Silioplant®
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid
TIS	Temporary immersion systems

Introduction

In vitro culture of *Digitalis* species is a useful alternative for the production of therapeutically interesting cardenolides. Isolated cardenolides show similar pharmacodynamic properties but only a few, such as digitoxin, digoxin and lanatosides are used in humans for the treatment of cardiac insufficiency (Hornberger et al. 2000). Plants still are the sole source for their acquisition (Kuate et al. 2008).

Many biotechnological strategies have been developed to enhance the production of valuable cardenolides from *Digitalis*. Some of these include media modification

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(Hagimori et al. 1983; Gavidia and Pérez-Bermúdez 1997) and organ culture in temporary immersion systems (TIS; Pérez-Alonso et al. 2009). Also, specialized techniques as genetic transformation (Saito et al. 1990; Sales et al. 2007), metabolic engineering to modify the biosynthetic pathway (Gärtner et al. 1990; Kreis et al. 1998; Gavidia et al. 2002; Gavidia et al. 2007; Herl et al. 2008; Pérez-Bermúdez et al. 2010) and elicitation (Cacho et al. 1999) have been reported.

Biomass production in bioreactors is a key step towards commercial production of secondary metabolites by plant biotechnology (Karuppusamy 2009). Several valuable medicinal plants have been cultivated using different bioreactor configurations such as temporary immersion systems (Wilken et al. 2005; Quiala et al. 2006; Georgiev et al. 2008). TIS is a cheap technology for automation of in vitro plant propagation and the production of plant secondary metabolites. Previously, Pérez-Alonso et al. (2009) described the development of a TIS based shoot culture system as a reliable alternative for steady production of biomass in *Digitalis purpurea*. On the other hand, elicitation proved to be an effective method to enhance the production of several secondary metabolites from medicinal plants (Namdeo 2007). The combination of TIS and elicitation could open an opportunity to improve the production of plant secondary metabolites.

Most research on elicitation for secondary metabolites production has been carried out on cell cultures. In contrast, comparatively little research has been undertaken to investigate the elicitation on shoot cultures. Interestingly, Kim et al. (2004) reported the effect of different elicitors on asiaticoside accumulation in whole plants of *Centella asiatica* cultivated in bioreactor (air lift type). Similarly, Orlita et al. (2008) studied the effects of abiotic and biotic elicitors on the biosynthesis of coumarins and alkaloids in shoots of *Ruta graveolens* concluding that it is a useful biotechnological source of these valuable metabolites. Recently, Coste et al. (2011) investigated the effects of elicitors on plant growth and production of hypericins and hyperforin in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum* cultivated on agitated liquid medium.

Although several biotic and abiotic elicitors have been used in plant culture applications, only a few examples such as chitosan, silicon and methyl jasmonate (MJ) will be mentioned here. Oligosaccharides as chitosan have shown strong elicitation effects in cell cultures for the production of secondary metabolites (Jeong and Park 2005; Prakash and Srivastava 2008). Chitosan is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (e.g. crabs, shrimp) and cell walls of fungi (Korsangruang et al. 2010). Silicon is a bioactive element associated with effects on mechanical and physiological properties of plants, mainly with plant defense mechanisms (Hammerschmidt 2011). On the other hand, the potential benefits of silicon in plants include the enhancement of growth and yield (Fauteux et al. 2005). Elicitors like MJ have been widely employed to induce secondary metabolite biosynthesis in cell and tissue cultures of various plants (Ketchum et al. 1999; Zhao et al. 2004; Kim et al. 2009; Roat and Ramawat 2009; Sakunphueak and Panichayupakaranant 2010; Bonfill et al. 2011; Qu et al. 2011; Krzyzanowska et al. 2012; Veerashree et al. 2012). However, to our knowledge, elicitors as Chitoplant®, Silioplant® or MJ have not yet been applied to increase the production of cardiotonic glycosides in *Digitalis* species.

The understanding of plant stress-related responses will contribute to the rational design of new technologies for secondary metabolite production. Elicitation in plants can induce the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and products of lipid peroxidation as malondialdehyde (MDA; Hancock et al. 2002). Few studies have provided detailed information concerning oxidative stress and biosynthesis of secondary metabolites (Yu et al. 2002; Chong et al. 2004). ROS may serve as signaling molecules upon induction of some defense responses in plants like production of secondary metabolites (Yu et al. 2002).

The objective of this research was to study the effect of elicitors on biomass accumulation, cardenolide biosynthesis and oxidative stress generation on *D. lanata* shoots cultivated in TIS. Results derived from this study will be useful in improving biomass and glycosides production in shoots of *Digitalis* species and will also contribute to increase the knowledge on the biosynthetic pathway of cardenolides. The effect of elicitors on cardenolide biosynthesis, to our knowledge, has not been described previously for shoot cultures in TIS.

Materials and methods

Plant material and culture conditions

Digitalis lanata shoot cultures were initiated from in vitro germinated seeds. Seeds were provided by Pharmasaat GmbH (Artern, Germany). Seeds were disinfected and cultured as previously described (Pérez-Alonso et al. 2009). The plantlets were subcultured twice every 28 days on semisolid medium before culture in TIS.

Temporary immersion culture

TIS cultures were performed as described by Pérez-Alonso et al. (2009). Briefly, each TIS contained 250 ml of MS

medium (Murashige and Skoog 1962) supplemented with 1.0 mg l^{-1} thiamine HCl, 1.0 mg l^{-1} 6-BAP, 0.1 mg l^{-1} IAA, 100 mg l^{-1} myo-inositol and 30 g l^{-1} sucrose (*M*ultiplication *M*edium: MM). The pH was adjusted to 5.8 with 0.5 N KOH or 0.5 N HCl before autoclaving at 1.1 kg cm^{-2} and 121 °C for 20 min. Twelve individual shoots were inoculated per TIS (weighing about 1.5–3.0 g fresh weight (FW) per TIS). Shoots were immersed for 2 min every 4 h. All cultures were incubated for a 16 h photoperiod under cool white fluorescent lamps at a photosynthetic photon flux density of 125–150 µmol m^{-2} s^{-1} at 27 ± 2 °C.

Elicitor treatment

Silioplant® (SiO_2, 35 % v/v) and Chitoplant® were provided by Chipro GmbH, Germany. MJ (Duchefa) was dissolved in 95 % ethanol and sterilized by membrane filtration (0.22 µm) before addition to the culture medium. Elicitor concentrations were chosen based on previous studies (data not shown). Elicitors were added to culture medium before shoot inoculation. The concentrations used were: ChP (0.001; 0.01; 0.1 g l^{-1}), SiP (0.01; 0.1; 1.0 g l^{-1}) and MJ (60, 80 and 100 µM). A set of control cultures without elicitors was included.

Four TIS were inoculated per treatment and the experiment was repeated twice. Plantlets were collected at the end of culture period (28 days) and biomass accumulation was expressed and determined as fresh and dry weights (g) produced per TIS. For dry weight, the biomass was dried at 60 °C until a constant weight was obtained. Also, the shoot length (cm) and number of shoots produced per TIS were recorded.

Content of cardiotonic glycosides

Shoots were collected, rinsed with distilled water and freeze-dried in a lyophilizer. Lyophilized shoots were finely ground in a mortar. Samples of 1.5 g powdered plant material were extracted with 15 ml ethanol (70 %) in an ultrasonic bath at 70 °C for 15 min using a method previously described by Pérez-Alonso et al. (2009). The residue obtained after extraction and rotaevaporation was dissolved in 1 ml ethanol for HPLC analysis. Ten microliters of this solution were injected in an Agilent 1100 HPLC system equipped with a diode array detector and an Inertsil ODS-3 column (150 × 4.6 mm; 5 µm). A mixture of acetonitrile/water (25/75; v/v) was used as eluent at a flow rate of 1.5 ml min^{-1}. All measurements were carried out at 40 °C and glycosides were detected at a wavelength of 220 nm. Digitoxin, digoxin and lanatoside C were identified on the basis of their retention time and the comparison of their UV spectra with those of authentic standards obtained from a commercial source (Sigma-Aldrich).

Measurement of hydrogen peroxide and lipid peroxidation

Hydrogen peroxide levels were determined according to Sergiev et al. (1997). Samples were homogenized with 2 ml 0.1 % (w/v) Trichloroacetic acid (TCA). The homogenate was centrifuged at 12.000 g for 15 min. A 0.5 ml aliquot of supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1 M potassium iodide. The blank was prepared in the same manner except that 1 ml 10 mM potassium phosphate buffer (pH 7.0) was used instead of the sample. Absorbance was read at 390 nm (UV 1800, Shimadzu, Japan). The amount of H_2O_2 content was calculated using a standard curve prepared with known concentrations of H_2O_2.

Lipid peroxidation of leaf tissue was estimated by the level of malondialdehyde (MDA) production using thiobarbituric acid (TBA, Sigma-Aldrich) method. The crude extract was prepared as described by Heath and Packer (1968). Absorbance at 532 nm was recorded and corrected for nonspecific absorbance at 600 nm (ε, 155 mM^{-1} cm^{-1}) using UV-Visible Spectrophotometer (Shimadzu). Total MDA equivalents were calculated according to Heath and Packer (1968) and expressed as nmol g^{-1} FW.

Data were analyzed by non-parametric Kruskal–Wallis test. In order to distinguish between comparisons a post-hoc Mann–Whitney test was performed. Differences were considered significant at $p < 0.05$. A correlation analysis (Spearman′s correlation) was performed with data from cardenolides content (lanatoside C and digoxin) and oxidative stress markers (H_2O_2 and MDA). Statistical analyses were performed using computer software SPSS package for Windows ver. 18.

Results and discussion

Effect of elicitors on biomass accumulation in TIS

Morphological response of the shoots was influenced by elicitors. The effect of tested elicitors on the length and number of shoots is shown in Table 1. No differences were recorded in shoot length between ChP (0.1 g l^{-1}) treatment and control cultures. The highest concentration of ChP (0.1 g l^{-1}) resulted in a slightly increased number of shoots compared to 0.01 g l^{-1} and showed a significant difference with respect to the control. Positive responses of shoot cultivated in TIS to ChP elicitation are possibly associated with the fact that oligosaccharides have been reported as potent signalling molecules that regulate growth and

Table 1 Effect of elicitors on biomass production of *Digitalis lanata* shoot cultured in temporary immersion systems

Treatment	Shoot length (cm)	No of shoots/explants	Fresh weight/TIS	Dry weight/TIS
Control	7.62ab	3.25b	21.9b	2.35c
ChP 0.001 g l^{-1}	7.07b	3.33b	23.7b	2.55b
ChP 0.01 g l^{-1}	7.33b	3.92ab	24.5b	2.05d
ChP 0.1 g l^{-1}	7.99a	4.33a	33.7a	3.38a
Control	7.44 b	3.83a	21.5ab	2.25b
SiP 0.01 g l^{-1}	8.59a	3.50a	23.3a	2.55a
SiP 0.1 g l^{-1}	7.43b	3.42a	20.5b	2.32b
SiP 1.0 g l^{-1}	7.28b	2.83b	18.5c	1.79c
Control	7.70a	3.67a	22.0a	2.45a
MJ 60 μM	6.34b	2.17b	18.0b	1.88b
MJ 80 μM	6.28b	2.25b	16.5b	1.91b
MJ 100 μM	6.23b	2.33b	16.7b	1.77b

Data are means from two independent experiments, each with four replicates

Values followed by different letters in the same column between same section are significantly different ($p < 0.05$) based on Kruskal–Wallis/Mann Whitney Test

Control MMb without elicitor, *ChP* Chitoplant®, *SiP* Silioplant®, *MJ* methyl jasmonate

development in plants (Sudha and Ravishankar 2002). SiP also influenced shoot growth; at 0.01 g l^{-1} promoted the highest shoot length while the higher concentration (1.0 g l^{-1}) significantly suppressed the number of shoots. The potential benefits of Si nutrition in plants have been extensively reviewed (Epstein 2001). Fauteux et al. (2005) reported several properties of silicon, such as the enhancement of growth, improvement of mechanical properties, reduction of transpiration and increased resistance to pathogens. A reduction in length and number of shoots was evident with all MJ concentrations. Crozier et al. (2000) reported that MJ was detected as senescence-promoting or growth-retarding substance in many plant species, including *Artemisia absinthium*, *Vicia faba* and *P. vulgaris*.

A large number of biotic and abiotic elicitors have been tested for the stimulation of biomass production. Table 1 shows the effect of biotic and abiotic elicitors on biomass production in shoots of *D. lanata* cultured in TIS. Our results demonstrated that ChP was the most effective elicitor tested. ChP (0.1 g l^{-1}) was found to impact significantly on the biomass accumulation (FW and DW per TIS) compared to the other elicitor treatments and control cultures. No difference in the relation DW/FW was evident (DW of the samples accounted for approximately 10 % of their respective FW for all treatments), suggesting that elicitors application had no effect on the water content and on biomass quality, which is supported by the fact that no hyperhydric shoots were observed.

MJ concentrations resulted in a marked reduction of biomass accumulation, contrary to Korsangruang et al. (2010) who reported that MJ did not significantly affect the growth of *Pueraria candollei*. This elicitor is a plant-specific signalling molecule which mediates and steers a diverse set of physiological and developmental processes (Kim et al. 2009).

In a previous work, we demonstrated that TIS is a simple but very effective alternative to increase *D. purpurea* shoot growth and biomass accumulation. Shoots cultures in TIS were regarded as a good system to study cardenolides production due to several advantages avoiding problems as hyperhydricity, which resulted in increased quality and homogeneous biomass production (Pérez-Alonso et al. 2009). TIS combine ventilation of the plant tissues and intermittent contact between the entire surface of the tissue and the liquid medium, allowing more efficient nutrient uptake. In addition, toxic substances or growth inhibitors exuded by plant tissues are dispersed by liquid (Berthouly and Etienne 2005). Liquid culture systems with elicitor addition are increasingly being investigated to improve secondary metabolite production and to reduce process cost in several plant cell/hairy root cultivation systems (Prakash and Srivastava 2008).

Contents of cardiotonic glycosides, hydrogen peroxide and malondialdehyde

HPLC analysis revealed the presence of bioactive compounds in shoots of *Digitalis lanata* cultivated in TIS even without the application of elicitor. Significant changes in secondary metabolite production were induced by elicitor treatment with respect to control cultures. The accumulation of secondary metabolites in plants is part of the defense response against pathogenic attack, which is

triggered and activated by elicitors, the signal compounds of plant defense responses (Namdeo 2007; Pawar et al. 2011).

The main cardenolides of *D. lanata* are the lanatosides, i.e. lanatoside C (Kandzia et al. 1998). This lanatoside was found in higher content than digoxin in all treatments (Fig. 1) and no digitoxin was detected. A connection between cardenolides content and oxidative stress was observed. Elicitor treatment has been effective for enhancing secondary metabolites biosynthesis in several plant species using different culture systems e.g. cell suspensions (Lu et al. 2001; Bonfill et al. 2011; Korsangruang et al. 2010; Pawar et al. 2011; Qu et al. 2011; Veerashree et al. 2012); roots (Zhang et al. 2009; Sakunphueak and Panichayupakaranant 2010) shoots and whole plants (Kim et al. 2004; Orlita et al. 2008; Coste et al. 2011).

Lanatoside C and digoxin contents are elicitor-concentration dependent. Several authors confirmed this observation in other plants (Zhao et al. 2001; Kim et al. 2004). For instance, Chong et al. (2005) reported that different elicitors depending on concentration exerted different effects on cell growth and anthraquinone production in *Morinda elliptica*. Similarly, Bonfill et al. (2011) reported the dose-dependency of MJ on centellosides and phytosterol production in *C. asiatica* suspension cultures. Responses of shoots to each elicitor were observed and are discussed below.

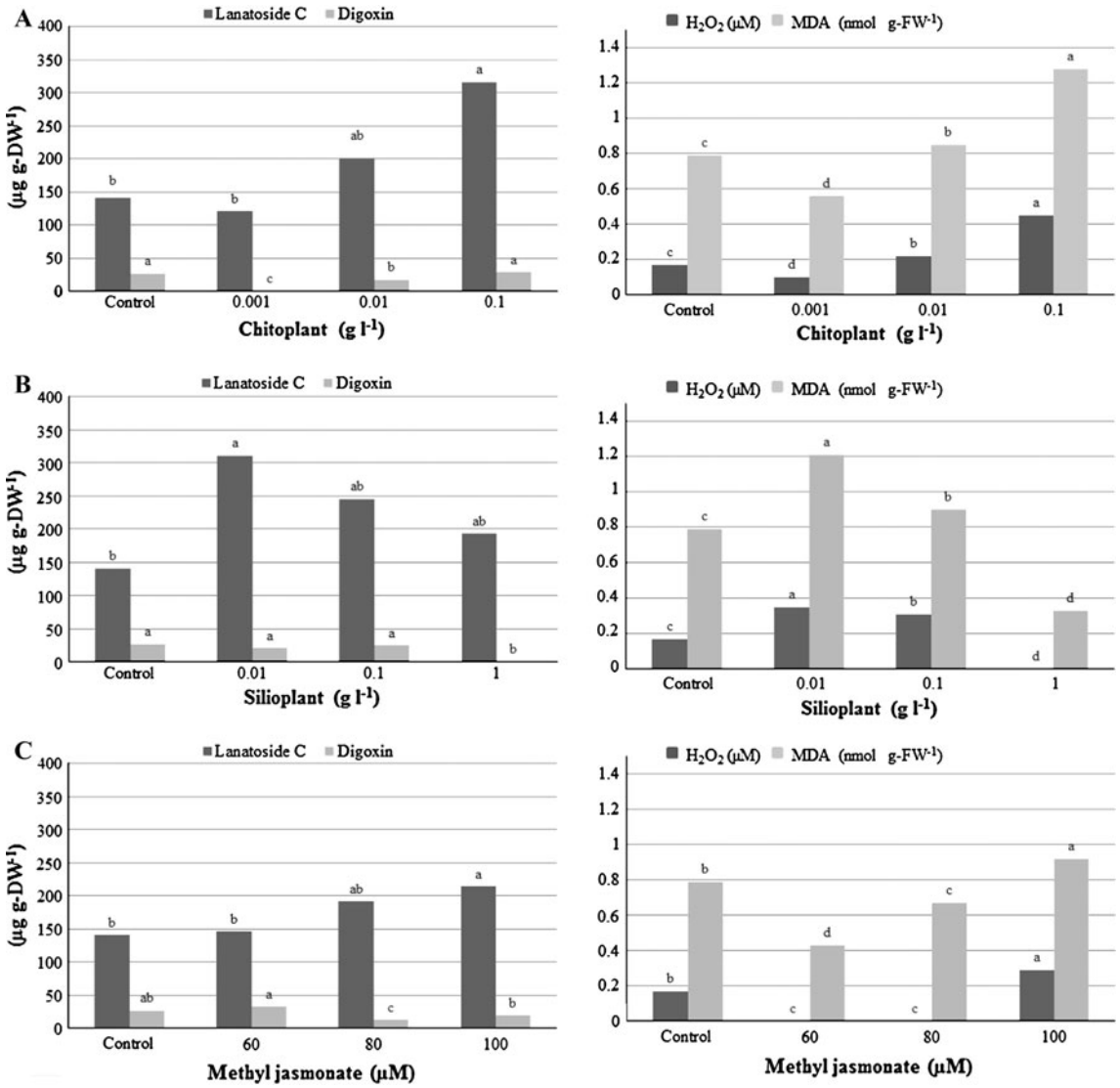

Fig. 1 Effect of elicitors on cardenolides, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents in *Digitalis lanata* shoots cultured in temporary immersion systems, Control (MM without elicitor), **a** Chitoplant®, **b** Silioplant®, **c** methyl jasmonate. *Bars* with *different letters* for each parameter in the same figure section are significantly different ($p < 0.05$) based on Kruskal–Wallis/Mann Whitney Test

Chitoplant®

The effect of ChP on cardenolides accumulation was not the same as for cardenolides detected (lanatoside C and digoxin). However, lanatoside C and digoxin contents were increased relatively by increasing ChP concentrations (Fig. 1A). A high concentration of ChP (0.1 g l^{-1}) significantly increased lanatoside C accumulation compared to control cultures, which resulted in 316 μg g-DW^{-1} without significant differences with 0.01 g l^{-1} (201 μg g-DW^{-1}). On the other hand, the highest concentration of ChP resulted in only slightly increased production of digoxin, without statistically significant differences to control cultures. Nevertheless, this was the best treatment compared to the rest of the concentrations evaluated. No digoxin was detected in the shoots treated with ChP 0.001 g l^{-1}.

ChP produced the same effect on H_2O_2 and MDA concentrations as on cardenolides contents. An increase in oxidative stress was observed by increasing ChP concentrations. ChP 0.1 g l^{-1} increased the H_2O_2 concentration and lipid peroxidation product (i.e. MDA) compared to all elicitors and concentrations studied. This means that ChP provoked higher stress and can effectively induce the biosynthesis of cardiotonic glycosides in shoots of *D. lanata*. Chitosan is known as a potential elicitor of plant defense responses, which form a semi-permeable film around plant tissues (El Ghaouth et al. 1994). The effectiveness of chitosan to increase flavonoid glycoside production in *Onosis arvensis* by about 500 % after elicitation for 24 h was reported by Tumová and Baĉkovská (1999). Linden and Phisalaphong (2000) induced paclitaxel biosynthesis in plant cell suspension cultures of *Taxus canadensis* by chitosan elicitation.

Silioplant®

There were no significant differences on lanatoside C content between SiP concentrations (Fig. 1B). However, SiP at 0.01 g l^{-1} resulted in a twofold higher content than in untreated shoot cultures with statistical differences. An inhibitory effect on lanatoside C accumulation was observed when SiP concentration exceeded 0.01 g l^{-1} (lowest concentration). SiP did not increase digoxin content with respect to the control and no digoxin was detected in the higher concentration (1.0 g l^{-1}). On the other hand, SiP application at 0.01 and 0.1 l^{-1} increased oxidative stress in shoots, except in the highest concentration (1.0 l^{-1}) were MDA content is reduced and no H_2O_2 was detected. The exact nature of silicon interaction with biochemical pathways of the plants leading to disease resistance remains unknown. Fauteux et al. (2005) reported that silicon acts on mechanisms shared by all plant species, such as those leading to the expression of plant stress genes (signaling cascades). Our data suggests that, SiO_2 could be associated with the accumulation of secondary metabolites related to plant defense mechanisms such as cardiotonic glycosides, if we consider the positive effect on lanatoside C content. In previous studies, it has been shown to enhance the accumulation of phytoalexins in *Cucumis sativus* (Fawe et al. 1998).

Methyl jasmonate

Lanatoside C content was slightly increased by MJ, significant differences were only observed between the highest concentration (100 μM) and the control. However, incremented MJ concentrations affected digoxin content (Fig. 1C). Increased content of H_2O_2 and MDA was only found with the highest MJ concentration (100 μM). Many researchers have widely used MJ at concentrations up to 100 μM to improve secondary metabolite production (Korsangruang et al. 2010). It has been shown in other plants that metabolites production in response to MJ could be markedly distinguished from non-treated controls (Chong et al. 2005).

MJ is one of the most frequently used elicitors. For instance, Gundlach et al. (1992) demonstrated the integral role of jasmonic acid and its derivatives in the intracellular signal cascade that begins with the interaction of an elicitor molecule with the plant surface and results, ultimately, in the accumulation of secondary compounds. They found that induction by jasmonate does not appear to be specific to any type of secondary metabolite but rather general to a wide spectrum of low molecular weight substances ranging from flavonoids, guaianolides and anthraquinones to various classes of alkaloids. Lu et al. (2001), Palazón et al. (2003), Choi et al. (2005) and Bae et al. (2006) showed its positive effect on ginsenoside biosynthesis. In previous studies, the total triterpene saponin content in *Panax notoginseng* cell suspension cultures elicited by MJ is known to increase (Hu and Zhong 2008). Veerashree et al. (2012) showed the effect of MJ on secondary metabolite production in cell suspension cultures of *Gymnema sylvestre*. The highest gymnemia acid content was obtained after 15 days of elicitor treatment (50 μM). In spite of several studies, which reported the positive effect of MJ on secondary metabolites production, we found that MJ was less effective than the rest of the elicitors tested for cardenolides production from *D. Lanata* in TIS, although higher MJ concentrations should be tested to confirm these results. Veerashree et al. (2012) showed a negative influence of MJ at 200 μM concentration on both biomass and gymnemic acid accumulation.

Cardenolides are involved in plant defense by acting as deterrents to herbivores (Malcolm and Zalucki 1996), thus, improved cardenolides biosynthesis can be achieved by

certain stress factors. Progesterone 5-β reductase (P5βR) is considered a key enzyme in cardenolides biosynthesis (Gavidia et al. 2007). Recently, Pérez-Bermúdez et al. (2010) demonstrated the existence of a second gene encoding for a protein with P5βR activity. This new gene was named *P5βR2* and they demonstrated that *P5βR2* is a defense-related gene involved in cardenolide biosynthesis. Contrary to *P5βR*, that shows a constitutive expression in leaves of *D. purpurea*, *P5βR2* is highly inducible in response to heat shock, cold shock, increased NaCl and wounding. The higher expression of *P5βR2* in wounded plants was correlated to increased biosynthesis of cardenolides. Its expression seems to be regulated via ethylene signaling, because of the highest transcripts accumulation in plants treated with 1-aminocyclopropane-1-carboxylic acid, while *P5βR2* expression was independent of MJ.

Control of plant defense is interconnected by a complex network of cross-communicating hormone signaling pathways (Pieterse et al. 2009). Several authors suggest that both ethylene and jasmonates significantly contribute to resistance against herbivores. Our results on the effects of MJ on cardenolide content agree with those of Perez-Bermúdez et al. (2010), however the slight increase in lanatoside C content by the highest MJ concentration could be explained by synergistic interaction between ethylene and jasmonate. Onkokesung et al. (2010) revealed that nicotine accumulation after challenging the leaves of *Nicotiana attenuata* with stimulated herbivory suggested a direct role of ethylene and jasmonic acid in alkaloid accumulation.

As shown here, elicitors in the concentrations tested induced different levels of lipid peroxidation and H_2O_2 production, indicating that oxidative stress was evident in shoots of *D. lanata.* Highly significant correlation coefficient ($p < 0.01$, $r = 0.893$) was found between lanatoside C-digoxin content and MDA-H_2O_2 levels. This suggests an interesting connection between H_2O_2, lipid peroxidation and cardenolide contents. However, their relationship and involvement in elicitor-induced cardenolide production is not known.

Studies have been conducted to provide information about the relationship between oxidative stress and secondary metabolite biosynthesis. Some authors accepted that compound biosynthesis may be related to the stress levels of the cell cultures, including the effectiveness of each elicitor (Vasconsuelo and Boland 2007). Plant cells generate H_2O_2 after receiving a signal from elicitors and many reports have focused on the role of H_2O_2 in plant resistance to pathogen infection or elicitor stimulation (Xiaojie et al. 2005). The occurrence of MDA, as one of the final products of peroxidation of polyunsaturated fatty acids, has been considered a useful index of lipid peroxidation (Yu et al. 2002). Guo et al. (1998) reported that ROS induced phytoalexin accumulation in *Glycine max* cells, while Yu et al. (2002) reported that oxidative stress had a deleterious effect on taxol production in *Taxus chinensis* cell suspension cultures.

Although many hypotheses have been considered regarding the mechanisms of action of biotic and abiotic elicitors, extensive fundamental studies are still required. Moreover, since little is known about the biosynthetic pathways of secondary metabolites, the effect of an elicitor on a plant cell or tissue culture is not easily predictable (Orlita et al. 2008). For example, Bhagwath and Hjortso (2000) reported elicitation strategies for secondary metabolites in hairy root cultures of *Ambrosia artemissifolia* and noted that most elicitation approaches are empirical.

There is limited information about cardenolides accumulation in *Digitalis* species by elicitors. The influence of mineral nutrition on cardiac glycoside production was reported by Hagimori et al. (1983) and Gavidia and Pérez-Bermúdez (1997). On the other hand, Gavidia et al. (2002) described the effects of several stress conditions, such as heat-shock, mechanical wounding and salt stress on the expression of a cardenolide biosynthesis related gene in *D. purpurea* greenhouse plants. Gurel et al. (2010) obtained higher digoxin content when shoots of *D. davisiana* were regenerated on Linsmaier and Skoog (1965) medium, producing 12 μg g-DW^{-1} digoxin. Ohlsson et al. (1983) and Cacho et al. (1999) described the effect of continuous light on cardenolide accumulation in *Digitalis* cell cultures.

Large amount of secondary metabolites produced are often stored in the vacuole, nevertheless, sequestration of these compounds is a critical feature that must be taken into consideration (Peters and Croteau 2004). Secondary metabolism seems most often to be regulated at the level of transcription and crucial roles thus exist for the corresponding transcriptional regulators and glucosylation mechanism seems to be very important on secondary metabolites accumulation in vacuoles such as anthocyanins (Matsuba et al. 2010). In particular, important roles are played by cardenolide-transforming enzymes such as acetyltransferases and glucosyltransferases in cardenolide biosynthetic pathways, but only a few results have been reported (Kreis et al. 1986; Kreis and May 1990; Kandzia et al. 1998). In this context, it is possible that Chitoplant® and Silioplant® promote glucosylation and acetylation in order to increase cardenolide accumulation in vacuoles, although further experimental approaches are needed to prove that hypothesis.

Enhancement of secondary metabolites by elicitation is one of the few strategies recently finding commercial application (Savitha et al. 2006). Besides, the advantages of TIS are obvious for this purpose, elicitors can be easily supplied with the culture medium and the culture conditions changed to induce the accumulation of secondary metabolites in the biomass produced.

The simultaneous use of some strategies could be very attractive in enhancing yield of plant secondary metabolites. For instance, Zhao et al. (2000) described a two-stage process developed in bioreactor for enhanced ajmalicine production in elicited *Catharanthus roseus* cell cultures. This strategy seems to be a practical approach to produce indole alkaloids. In the same way, the combination of metabolic engineering and elicitation was an effective strategy for increasing flavonoid production in hairy roots of *Glycyrrhiza uralensis* (Zhang et al. 2009). Arora et al. (2010) demonstrated the elicitation effect on growth and stilbene accumulation in cell cultures of *Cayratia trifolia* in combination with sucrose feeding.

Further studies should be undertaken to obtain data on the effectiveness of elicitation over time. The optimization of elicitor treatment and other culture conditions may improve TIS performance. Our results suggest that elicitation of shoots cultivated in TIS could influence the competition between biomass production, secondary metabolite content and oxidative stress. The combination of elicitor and TIS could be useful in developing rational strategies for enhancing the production of cardenolides in *D. lanata* shoots and an effective alternative instead or combined with genetic modification. To our knowledge, this is the first report on stimulation of cardenolides production in *Digitalis* species shoots cultivated in TIS by elicitation.

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