

SECONDARY METABOLITE PRODUCTION IN *HYPERICUM PERFORATUM* L. CELL SUSPENSIONS UPON ELICITATION WITH FUNGAL MYCELIA FROM *ASPERGILLUS FLAVUS*

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Abstract – We investigated the production of phenylpropanoids (phenolic compounds, flavanols, flavonols and anthocyanins) and naphthodianthrone (hypericin) in elicited *Hypericum perforatum* L. cell suspensions. To determine whether secondary metabolite production could be enhanced, *Hypericum* cell suspensions were exposed to mycelia extract from the fungus *Aspergillus flavus*. Elicited *Hypericum* cell suspension cultures displayed reduced growth and viability and a modification of secondary metabolites production. Anthocyanins were only stimulated in fungal-elicited cell suspensions. Secondary metabolite production in elicited *Hypericum* cells revealed an antagonism between the flavonoid/naphthodianthrone and anthocyanin pathways. The data suggest a modification of the channeling of the phenylpropanoid compounds. Together, these results represent useful data for monitoring the channeling in different secondary metabolite pathways during the scaled-up production of naphthodianthrone for medicinal uses.

Key words: Anthocyanins, *Aspergillus flavus*, elicitation, flavanols, flavonols, hypericin, *Hypericum perforatum* L., naphthodianthrone, phenolics, phenylpropanoids.

INTRODUCTION

Hypericum perforatum L. is a well-known medicinal plant that has been in use for a decade. Studies of the pharmacological effects of the constitutive compounds are still in progress. Recently, the antiviral and antidepressant properties of *Hypericum* have become widely demonstrated (Di Carlo et al. 2001). Extracts from *H. perforatum* are known to contain compounds from six major natural product groups: naphthodianthrone, acylphloroglucinols, flavonol

glycosides, biflavones, proanthocyanidins, and phenylpropanes (Nahrstedt and Butterweck, 1997). The importance of *H. perforatum* as a medicinal plant is mainly due to the presence of naphthodianthrone such as hypericin and pseudohypericin and their precursors: protohypericin, protopseudohypericin and cyclopseudohypericin (Falk, 1999). Hypericin is described as a polyketide-derived quinone named naphthodianthrone, alluding to the configuration of the compound constructed from two tricyclic anthrones called emodin anthrone (Katz and Donadio,

1993). Little is known about the biosynthesis of hypericin other than it lies on the polyketide pathway (Zobayed et al., 2006). The gene *hyp-1* encoding for a hypericin biosynthetic enzyme was cloned and characterized from *H. perforatum* cell cultures (Bais et al., 2003). However, the coordination of the production of naphthodianthrone with other secondary metabolites is still unknown. The levels of hypericin and pseudohypericin in *Hypericum* plants can vary from 0.03 to 0.3% dry weight under different environmental parameters, but the precise environmental factors which influence the biosynthesis of these naphthodianthrone and the partitioning with other secondary metabolites in *Hypericum* are not well understood.

Elicitation of secondary metabolite production in plant cell cultures could be induced either by biotic or abiotic molecules. Elicitors are now considered as signal molecules that activate the signal-transduction cascade and lead to the activation and expression of genes related with the biosynthesis of secondary metabolites (Zhao et al., 2005). Furthermore, elicitors stimulated the antioxidant defense systems of plant cells (De Gara et al., 2003). Treatments of cultured cells with fungal elicitors have also been shown to induce the phenylpropanoid/flavonoid biosynthetic pathways (Dixon et al., 2002; Tan et al., 2004). The fact that fungal elicitors stimulate strongly and rapidly plant secondary metabolite accumulation has recently attracted considerable attention (Dixon et al., 2002; Zhao et al., 2005).

Cell suspension cultures from *H. perforatum* have already been established to study the overproduction of naphthodianthrone using phytohormones (Gadzovska et al., 2005) or various elicitors such as mannan, β -1,3-glucan, pectin (Kirakosyan et al., 2000), methyl jasmonate (Sirvent and Gibson, 2002), jasmonic acid (Walker et al., 2002; Gadzovska et al., 2007), salicylic acid (Sirvent and Gibson, 2002; Walker et al., 2002) or fungal elicitors from *Colletotrichum gloeosporioides* (Conceição et al., 2006) and *Phytophthora cinnamomi* (Walker et al., 2002). *Hypericum in vitro* culture systems have been shown to respond to elicitors by increasing naphthodianthrone, which have been considered to be the chemical defence

arsenal of plants against pathogens and herbivores (Zhao et al., 2005). Nevertheless, the coordination of the production of naphthodianthrone compounds with other secondary metabolites (flavonoids and phenolic compounds) remains unknown.

In this study, *Hypericum* cell suspensions were treated with mycelia extract from the pathogenic fungus *Aspergillus flavus*. *A. flavus* is a common plant pathogen fungus which causes diseases of agronomically important crops, such as corn and peanuts (St. Leger et al., 2000). *A. flavus* is responsible for the aflatoxin contamination of crops prior to harvest or during storage (Yu et al., 2004). Investigations in this study focused on two areas: (1) the consequences of fungal elicitor treatments on cell biomass production, and (2) the partitioning between secondary metabolites as naphthodianthrone (hypericins) and phenylpropanoids (phenolic compounds, flavanols, flavonols and anthocyanins) in elicited cells. Activation of the non-enzymatic antioxidant system was assessed by measuring the non-enzymatic antioxidant properties (NEAOP) in elicited cell suspensions.

MATERIALS AND METHODS

Plant material

H. perforatum seeds were collected from field-grown plants on the Mt. Bistra (Mavrovo), Republic of Macedonia, at the altitude of 1200-1500 m. The seeds were washed overnight, air dried, surface sterilized with 1% NaOCl for 10 min, rinsed 3 times in sterile deionized water and cultured on MS macro and oligoelements (Murashige and Skoog, 1962), B₅ vitamin solution (Gambrog et al., 1968), supplemented with 30 g·L⁻¹ sucrose, and solidified with 7 g·L⁻¹ Difco Bacto agar. No growth regulator was added. The medium was adjusted to pH 5.6 before autoclaving (20 min at 120°C). The seeds were maintained in a growth chamber at 22°C under a 16 h photoperiod (700 μ M/m²/s). The first pair of leaves were excised from 2 week-old *in vitro* plants and used as explants to establish callus cultures. They were cultured in Petri dishes on MS/B₅ medium supplemented with 1.0 mg·L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-

D), 0.5 mg·L⁻¹ N⁶-benzyladenine (BA), 0.1 mg·L⁻¹ α-naphthaleneacetic acid (NAA), 30 g·L⁻¹ sucrose and solidified with 7 g·L⁻¹ Difco Bacto agar. The callus cultures were initiated on the leaf surface after 2 weeks. Subcultures were carried out every 14 days.

Cell suspension cultures were established from 28 day-old callus cultures. Green calli (2 g) were introduced in Erlenmeyer flasks containing 100 mL of MS/B₅ medium, supplemented with 1.0 mg·L⁻¹ 2,4-D, 0.5 mg·L⁻¹ BA, 0.1 mg·L⁻¹ NAA and 30 g·L⁻¹ sucrose. The cultures were maintained on a rotary shaker at 100 rpm in the growth chamber at 25±1°C, under a 16 h photoperiod (700 μM/m²/s). After two weeks, the cells released from the calli were transferred to 4 volumes of fresh liquid medium and subcultured, before treatment, every two weeks.

Fungal mycelium from *Aspergillus flavus* was grown in 250 ml flasks containing Sabouraud-Dextrose broth (Oxoid). One-month-old mycelia were homogenized and centrifuged for 5 min. The fungal mycelia supernatant was re-suspended with sterile distilled water to a final concentration of 50 mg·mL⁻¹ and then autoclaved for 10 min at 120°C. The *A. flavus* fungal mycelia extracts were stored at 4°C, until treatment.

Treatments of the cell suspensions with the fungal mycelia extract were performed 7 days after subculture when the cells were in the log phase of growth. Cell suspensions cultivated on MS/B₅ medium without elicitor were used as a control. Treated and control cell suspensions were then harvested by vacuum filtration on days 1, 4, 7, 14, and 21 post-elicitation, weighed for growth analysis, frozen in liquid nitrogen or lyophilized and stored at -80°C, until analysis.

Extraction and quantification of secondary metabolites

Phenolic quantification was performed as described by Gadzovska et al. (2007). Phenolic compounds were extracted from freeze-dried lyophilized and powdered plant material (0.2-0.5 g) with 80% (v/v)

methanol in an ultrasonic bath for 30 min at 4°C. The total phenol content was determined when the methanolic extracts were mixed with the Folin-Ciocalteu reagent (Carlo Erba Reagenti, Rodano, Italy) and 0.7 M Na₂CO₃. Samples were incubated for 5 min at 50°C and then cooled for 5 min at room temperature. Absorbance was measured spectrophotometrically at 765 nm. The concentration of total phenolic compounds was calculated using (+)-catechin (0-10 mg·ml⁻¹) as a standard.

The flavanol contents were determined in the methanolic extracts with 4-(Dimethylamino)cinnamaldehyde (DMACA) reagent (Gadzovska et al., 2007). The DMACA reagent was added to the diluted (1:10-1:100, v/v) extracts. The mixtures were incubated for 1 h at room temperature. Absorbance was measured at 637 nm. The content of flavanols was calculated using (+)-catechin (0-20 μg·ml⁻¹) as a standard.

The flavonol contents were determined in the methanolic extracts by the method described by Gadzovska et al. (2007). The flavonols were quantified in the methanolic extracts filtered through Sep-pack C₁₈ cartridges (Waters) to exclude chlorophyll and carotenoid pigments (solid-phase extraction). Spectrophotometric measurements of the absorbance were made at 360 nm. Molar extinction coefficient of quercetin (ε₃₆₀=13.6 mM⁻¹·cm⁻¹) was used to determine total flavonol contents.

Anthocyanin determination was performed as described by Gadzovska et al. (2007). Anthocyanins were extracted from freeze-dried lyophilized and powdered plant material (0.2-0.5 g) with 2 mL solution of 1% HCl/CH₃OH (15/85, v/v), ultrasonicated for 60 min at 4°C and then centrifuged at 20,000 g for 30 min. The absorbance of supernatant was measured at 530 nm. The anthocyanin content was calculated using the molar extinction coefficient of cyanidin-3-glucoside (ε₅₃₀=34300 M⁻¹·cm⁻¹) in acidic methanol.

Hypericin extraction was performed with the method described by Gadzovska et al. (2005). Spectrophotometric measurements of the absorbance

Table 1. Correlations between secondary metabolite contents and non-enzymatic antioxidant properties (NEAOP) in *H. perforatum* cell suspensions elicited with fungal mycelia extract from *Aspergillus flavus*.

Pearson coefficient of correlation (r)	Phenolics	Flavanols	Flavonols	Anthocyanins	Hypericin
Flavanols	0.91 ***				
Flavonols	0.92 ***	0.85 ***			
Anthocyanins	0.12 ns	0.18 ns	0.31 ns		
Hypericin	0.72 ***	0.67 **	0.83 ***	0.46 ns	
NEAOP	0.13 ns	0.20 ns	0.21 ns	0.54 **	0.21 ns

* Denoted values are significantly different (* $p < 0.05$; * $p < 0.01$; *** $p < 0.001$). Values represent $n = 36 \pm SE$. Letters ns marked non significant changes.

were made at 590 nm. Standard solutions of hypericin ($0-100 \mu\text{g}\cdot\text{mL}^{-1}$) were prepared from pure commercially available standard of hypericin (Sigma, Germany).

Non enzymatic antioxidant properties (NEAOP) assay

The antioxidant properties of soluble methanolic extracts were estimated by the linoleic acid- β -carotene oxidation method adapted from Gadzovska-Simic, (2010). A linoleic acid- β -carotene emulsion was prepared by mixing 10 mg of linoleic acid with 750 μL of $0.2 \text{ mg}\cdot\text{mL}^{-1}$ chloroformic β -carotene solution and 100 mg of Tween 40 (polyoxyethylene sorbitan monopalmitate). Chloroform was evaporated under nitrogen flow for 10 min. The resulting mixture was adjusted to 25 mL with distilled water and shaken for 10 seconds. The reaction mixture was prepared as follows: 10 μL of extract were adjusted with 15 μL 80% (v/v) methanol and 225 μL of linoleic acid- β -carotene emulsion was added. The mixture was heated to 50°C . The control consisted of 25 μL 80% (v/v) methanol and 225 μL of linoleic acid- β -carotene emulsion. Absorbance was measured at 470 nm every 15 min for 45 min. Results were computed as the ratio of β -carotene protection of the extract to the control (80 % methanol).

Statistical analyses

The experiments were independently repeated twice under the same conditions and all analyses

were performed in triplicate. Error bars of graphs show the standard error of mean value ($\pm SE$). The statistical analyses were performed with the SPSS statistical software program (SPSS version 11.0.1 PC, USA, IL). Means were expressed with their standard error and compared by one-way ANOVA (GML procedure). All statistical tests were considered significant at $p \leq 0.05$.

RESULTS

Growth and viability of elicited H. perforatum L. cell suspensions

Optimization of cell proliferation was the first step toward establishing elicited *H. perforatum* cell suspensions. The factors considered were the time of exposure to the treatment and cell biomass production. From 14 days after elicitation, browning and cell aggregation appeared in the elicited cells (Fig. 1A). Outgoing results from this study showed that long-term treatment with the fungal elicitor caused a brown color and cell aggregation in the elicited cells. *Hypericum*-elicited cells tend to combine and form aggregates, resulting in heterogeneous populations of cell aggregates, ranging from a few cells to thousands of cells, and often reaching a few centimeters in diameter. Localization of anthocyanins in the cell suspensions is shown in Fig. 1B (arrows). The fresh weight of the elicited cells was always below or equal to the control values throughout the time of culture maintenance (Fig. 1C). The effect of the fungal elicitor was not significantly different compared to the

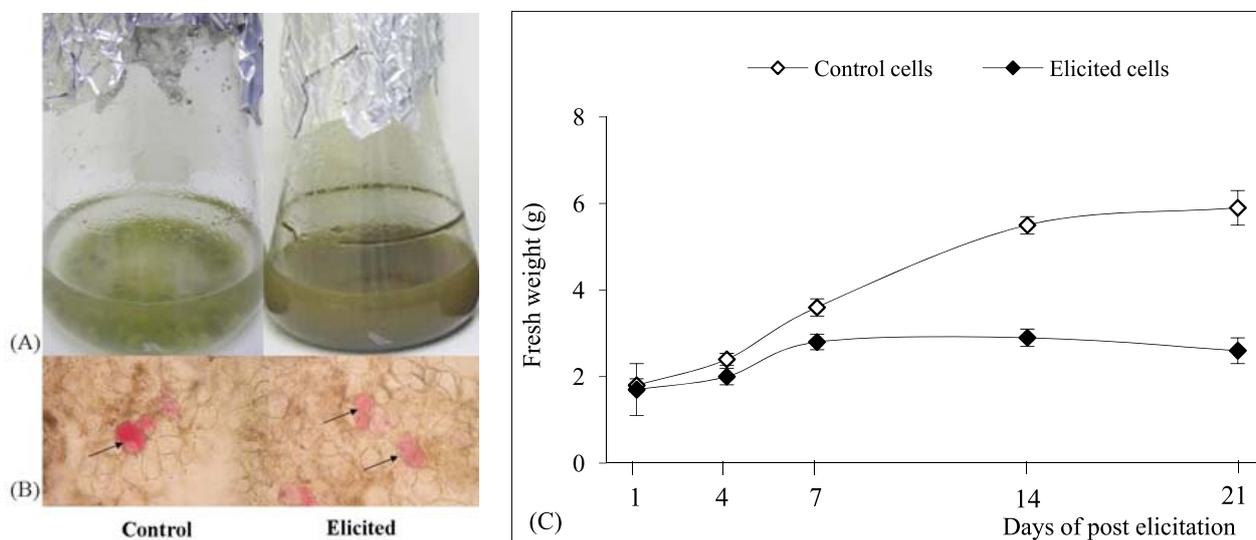


Fig. 1. (A) Morphology of control and elicited *H. perforatum* cell suspensions at day 14. (B) Localization of anthocyanins in cell suspensions (arrows). (C) Growth curves of control and elicited *H. perforatum* cell suspensions.

control until day 7 of post-elicitation. From day 14, it was clear that biomass production in elicited cells was lower (2- to 3-fold in elicited cells) than in the control. Exogenously applied fungal elicitor mediates certain types of stress responses in *Hypericum* cells and its action results in a negative regulation of growth and development.

Secondary metabolite production in elicited H. perforatum cell suspensions

The influence of the *Aspergillus* mycelium extract (50 mg·mL⁻¹) on the secondary metabolite contents and non-enzymatic activity (NEAOP) in the cell suspensions was tested. The fungal mycelia extract caused a 2-fold decrease of phenolic compounds after 4 to 21 days of elicitation compared to the control (Fig. 2A). *Hypericum*-elicited cells had higher flavanol (Fig. 2B) and flavonol contents (Fig. 2C) at day 7; thereafter flavonoid accumulation decreased compared to the control cells. Anthocyanins were only stimulated in the elicited cell suspensions (Fig. 2D). Transient accumulations with maxima at day 14 of elicitation were found for anthocyanins. Namely, a 2- to 4-fold increase of anthocyanins from day 1 to day 14 was observed in elicited cells compared to the control

(Fig. 2D). Hypericins remained unchanged (Fig. 2E) in the corresponding samples of elicited cells compared. Induction of non-enzymatic antioxidant systems was appraised by measuring the NEAOP in the elicited suspensions and an increase was monitored during the time of post-elicitation. NEAOP were higher (Fig. 2F) from day 1 to day 14 in elicited cells compared to the control.

Statistical analysis

Statistically analyses (Pearson's correlation coefficient) showed a positive correlation between the hypericin and phenylpropanoid contents in the cell suspensions upon elicitor treatment (Table 1). The production of phenolic compounds in the elicited cells was in positive correlations with the amounts of flavanols ($r=0.91$, $p<0.001$), flavonols ($r=0.92$, $p<0.001$) and hypericin ($r=0.72$, $p<0.001$). These results provide biochemical proof of a connection between hypericin biosynthesis and flavonoid metabolism. NEAOP were only in positive correlation with anthocyanins ($r=0.54$, $p<0.05$). These results provide evidence that anthocyanins exhibit high antioxidant properties in *Hypericum*-elicited cells.

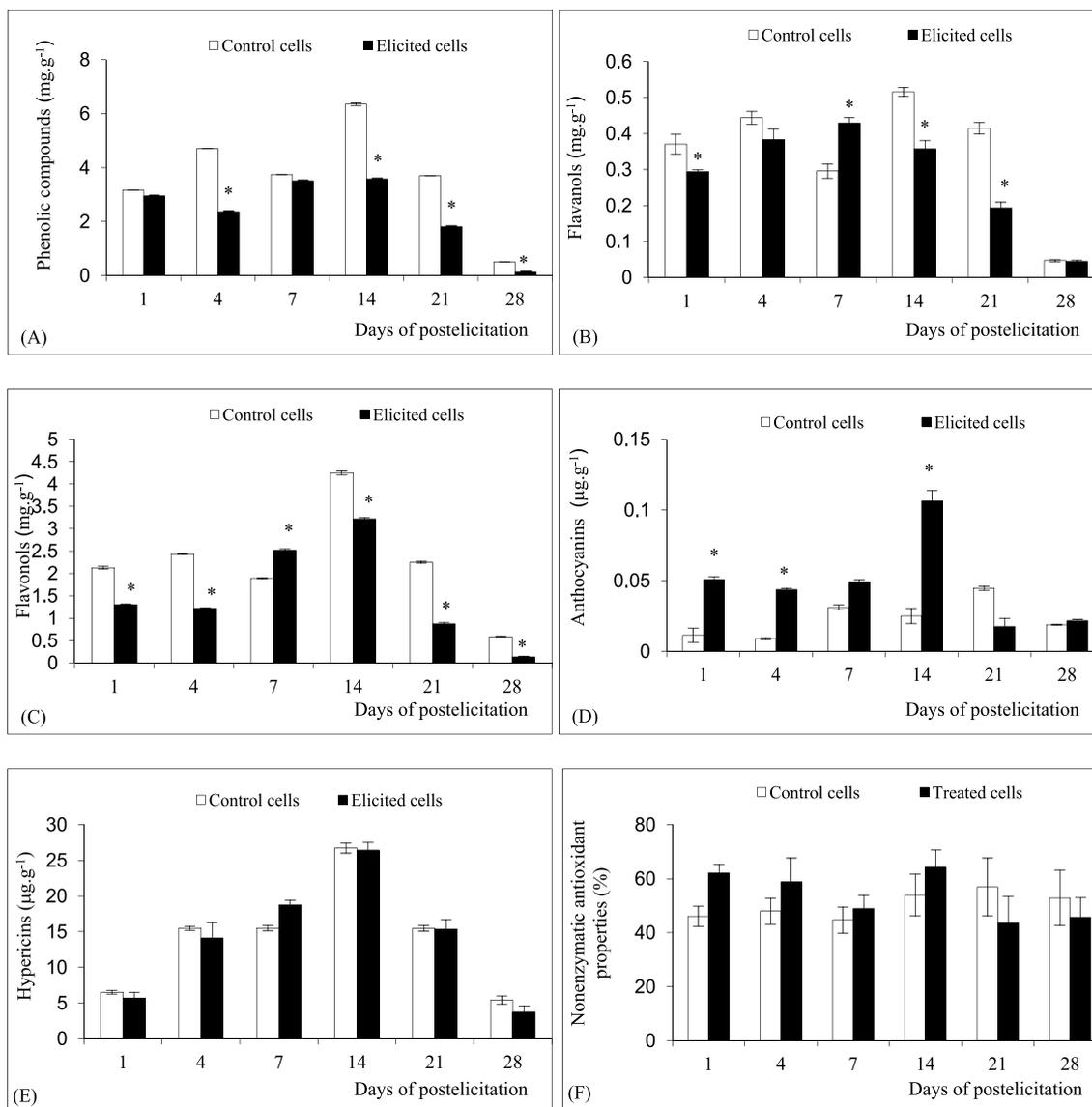


Fig. 2. Production of secondary metabolites of control and elicited *H. perforatum* cell suspensions. (A) Phenolic compounds; (B) flavanols; (C) flavonols; (D) anthocyanins; (E) hypericins; (F) non-enzymatic antioxidant properties of plant extracts (NEAOP). * indicates a significant difference relative to the control.

DISCUSSION

Effects of fungal elicitor on growth and viability in H. perforatum cell suspensions

Hypericum cell suspension cultures treated with the *Aspergillus* mycelia extract were negatively affected

for growth and turned brown in coloration. Such a result has already been reported in *H. perforatum* cell suspensions treated with jasmonic acid (Gadzovska et al., 2007), salicylic acid and fungal cell-wall elicitors from *Phytophthora cinnamomi* (Walker et al., 2002). It has also been reported that the reduction in biomass might be due to membrane lipoxidation

induced by elicitor treatment (Radman et al., 2003). Therefore, elicited plant cells show increased oxygen uptake that can manifest itself in the transient production of reactive oxygen species (ROS). The oxidative burst is a fast response of cells to elicitor treatment (Ortmann et al., 2004). Plant cells are usually protected against the detrimental effects of oxygen species using non-enzymatic antioxidant and/or enzymatic scavenging systems (Blokchina et al., 2003; De Gara et al., 2003). In this study, exogenously applied *Aspergillus flavus* mycelia extract stimulated the non-enzymatic antioxidant system in *Hypericum*-elicited cells. Thus, the NEAOP were found to be higher in elicited cells than in control ones. All these results indicated an efficient elicitation of *Hypericum* cell suspensions.

The relationships between subsequent suspension browning and the formation of cell aggregates with a strong induction of secondary metabolite pathways have already been reported (Zhao et al., 2005). Thus, it has also been proposed that cells on the surface of cell aggregates may be strongly elicited, leading to the formation of a large number of apoptotic cells (Yuan et al., 2002). Accordingly, *Hypericum* cell suspensions elicited with fungal elicitor from *Aspergillus* turned brown, formed cell aggregates and accumulated the highest amounts of anthocyanins. This result is in agreement with relatively high NEAOP in *Hypericum*-elicited cells which positively correlated with the anthocyanins.

Effects of fungal elicitor on secondary metabolite production in H. perforatum cell suspensions

One of the problems encountered in the analysis of the metabolites produced by *Hypericum* is that anthocyanins may have been misidentified as hypericins; this probably occurred because both are red in color and because studies on hypericins do not take into consideration the presence of anthocyanins (Mulinacci et al., 2008). According to this study, hypericin only accumulates in the glandular structures of the leaf (Onelli et al., 2002), whereas anthocyanins accumulate in the vacuole of parenchymatic cells of the leaf mesophyll (Pasqua et al., 2006). In contrast with this,

the results from our study suggested that one reason for the accumulation of both anthocyanins and hypericins in *Hypericum*-elicited cells might be the existence of differentiation and compartmentalization of cell aggregates in suspension cultures. Bais et al. (2003) reported that both cell growth and hypericin production were greatly affected by cell aggregate size and dark growth conditions in *H. perforatum* L. cell suspensions. According to these authors, the size of cell aggregates has major implications in secondary metabolite production, including hypericin content. A smaller size of cell aggregates has been preferred from the standpoint of process engineering, whereas a certain degree of cell-cell contact and cell differentiation was required for hypericin biosynthesis (Walker et al., 2002). These authors reported that the production and localization of hypericin in cell suspensions is entirely different from intact plants. This could be because compounds like hypericins are accumulated in specialized cells (dark glands) in plants (Zdunek and Alfermann, 1992).

Hypericins have been identified in the callus with vegetative buds and plantlets without roots but not in suspended cells and undifferentiated calli (Pasqua et al., 2003). Actually, *Hypericum* cells and undifferentiated calli produced only anthocyanins and xanthenes (Mulinacci et al., 2008). This study indicated that the total absence of hypericins in suspended cells and calli seems to be in contrast with their ability to synthesize xanthenes, which partially share the same pathway as hypericins. The authors suggest that the enzymes involved in anthranol synthesis and dimerization to hypericins are missing. It had been considered for a long time that undifferentiated cells, such a callus or cell suspensions, were not able to produce secondary metabolites, unlike differentiated cells and specialized organs (Krikorian and Steward, 1969). Zenk (1991) showed that this theory was wrong. Results from our previous study (Gadzovska et al., 2007) confirm the view that *Hypericum* cells grown with jasmonic acid as elicitor were able to acquire a certain degree of differentiation and the capability for the production of secondary metabolites such as hypericin and pseudohypericin, as well anthocyanins.

Few studies have reported the effects of fungal elicitors on secondary metabolite production in *Hypericum* cells. *Aspergillus* mycelia extract strongly and usually early activated the biosynthesis of anthocyanins analyzed in this study. The statistical analysis demonstrated positive correlations between the amounts of phenolics, naphthodianthrones and flavonoids. However, an antagonism between flavonoid/naphthodianthrone and anthocyanin pathways in *Hypericum* cells was shown. All these data suggest a partitioning of the secondary compounds during elicitation. Thus, the *Aspergillus* fungal elicitor was the most efficient in anthocyanin production. The effect of the biotic elicitor *Colletotrichum gloeosporioides* on secondary metabolite production in *H. perforatum* cells has been examined (Conceição et al., 2006). In this study, elicited *Hypericum* cells showed a significant increase in xanthone accumulation. The fungal cell-wall elicitor from *Phytophthora cinnamomi* induced the production of hypericin in cell suspension cultures of *H. perforatum* (Walker et al., 2002). Little is known about the biosynthesis of naphthodianthrones; the unknown participating enzymes have to be characterized before enlarging the study to the effects of elicitation in *Hypericum* cell suspensions. It is still unclear whether distinct elicitors acted on the same or on distinct signaling pathways (Zhao et al., 2005). Therefore, more work was needed to better understand the effects of various elicitors on the partitioning between the secondary metabolites in *Hypericum* cell suspensions. Such a result could be explained by the distinct potential signaling pathways and could be a useful tool to monitor the scaled-up production of secondary metabolites for medicinal uses. Thus, the mycelia extracts seem to be the most promising elicitors for such an application.

REFERENCES

- Bais, H.P., Vepachedu, R., Lawrence, C. B., Stermitz, F. R., and J. M. Vivanco (2003). Molecular and biochemical characterization of an enzyme responsible for the formation of hypericin in St. John's Wort (*Hypericum perforatum* L.). *J. Biol. Chem.* **34**, 32413-32422.
- Blokhina, O., Virolainen, E., and K. V. Fagerstedt (2003). Antioxidants, oxidative damage and oxygen deprivation stress. *Ann. Bot.* **91**, 179-194.
- Conceição, L., Ferrares, F., Tavares, R., and A. Dias (2006). Induction of phenolic compounds in *Hypericum perforatum* L. cells by *Colletotrichum gloeosporioides* elicitation. *Phytochem.* **67**, 149-155.
- De Gara, L., De Pinto, M., and F. Tommasi (2003). The antioxidant systems vis-à-vis reactive oxygen species during plant-pathogen interaction. *Plant Physiol. Biochem.* **41**, 863-870.
- Di Carlo, G., Borrelli, F., Ernst, E., and A. Izzo (2001). St. John's wort: Prozac from plant kingdom. *Trends Pharm. Sci.* **22**, 292-297.
- Dixon, R. A., Achnine, L., Kota, P., Liu, C. J., Reddy, M. K. S., and L. Wang (2002). The phenylpropanoid pathway and plant defence - a genomics perspective. *Mol. Plant Pathol.* **3**, 371-390.
- Falk, H. (1999). From the photosensitizer hypericin to the photoreceptor stentorin-The chemistry of phenanthroperylene quinines. *Angew. Chem. Int.* **38**, 3116-3136.
- Gadzovska, S., Maury, S., Ounnar, S., Righazza, M., Kascakova, S., Refregiers, M., Spasenoski, M., Joseph, C., and D. Hagège (2005). Identification and quantification of hypericin and pseudohypericin in different *Hypericum perforatum* L. *in vitro* cultures. *Plant Physiol. Biochem.* **43**, 591-601.
- Gadzovska, S., Maury, S., Delaunay, A., Spasenoski, M., Joseph, C., and D. Hagège (2007). Jasmonic acid elicitation of *Hypericum perforatum* L. cell suspensions and effects on the production of phenylpropanoids and naphthodianthrones. *Plant Cell Tiss Organ Cult.* **89**, 1-13.
- Gadzovska-Simic, S. (2010). Secondary metabolite production in cultures *in vitro* of *Hypericum perforatum* L. Effects of different exogenous factors. LAP Lambert Academic Publishing, Saarbrücken, Germany, 1-192.
- Gamborg, O. L., Miller, R. A., and K. Ojima (1968). Nutrient requirements of suspension cultures soybean root cells. *Exp. Cell Res.* **50**, 148-151.
- Katz, L., and S. Donadio (1993). Polyketide synthesis: Prospects for hybrid antibiotics. *Ann. Rev. Microbiol.* **47**, 875-912.
- Kirakosyan, A., Hayashi, H., Inoue, K., Charchoglyan, A., and H. Vardapetyan (2000). Stimulation of the production of hypericins by mannan in *Hypericum perforatum* shoot cultures. *Phytochem.* **53**, 345-348.
- Krikorian, A. D., and F. C. Steward (1969). Biochemical differentiation, the biosynthetic potentialities of growing and quiescent tissue, In: *Plant Physiology*, (Ed. F. C. Steward), Academic Press, London, 227-326.
- Mulinacci, N., Giaccherini, C., Santamaria, A., Caniato, R., Ferrari, F., Valletta, A., Vincieri, F., and G. Pasqua (2008). Anthocyanins and xanthenes in the calli and regenerated

- shoots of *Hypericum perforatum* var. *angustifolium* (sin. Fröhlich) Borkh. *Plant Physiol. Biochem.* **46**, 414-420.
- Murashige T., and F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-497.
- Nahrstedt, A., and V. Butterweck (1997). Biologically active and other chemical constituents of the herb of *Hypericum perforatum* L. *Pharmacopsych.* **30**, 129-134.
- Onelli, E., Rivetta, A., Giorgi, A., Bignami, M., Cocucci, M., and G. Patrignani (2002). Ultrastructural studies on the developing secretory nodules of *Hypericum perforatum*. *Flora*, **197**, 92-102.
- Ortmann, I., Sumowski, G., Bauknecht, H., and B. M. Moerschbacher (2004). Establishment of a reliable protocol for the quantification of an oxidative burst in suspension-cultured wheat cells upon elicitation. *Physiol. Mol. Plant Pathol.* **64**, 227-232.
- Pasqua, G., Avato, P., Monacelli, B., Santamaria, A., and M. Argentieri (2003). Metabolites in cell suspension cultures, calli, and in vitro regenerated organs of *Hypericum perforatum* cv. Topas. *Plant Sci.* **165**, 977-982.
- Pasqua, G., Avato, P., and N. Mulinacci (2006). High-value metabolites from *Hypericum perforatum*: a comparison between the plant and *in vitro* systems, In: *Ornamental and Plant Biotechnology: Advances and Topical Tissue*, (Ed. J. A. Teixeira da Silva), vol. II, Floriculture, Global Science Books, Japan, 507-513.
- Radman, R., Saez, T., Bucke, C., and T. Keshavrz (2003). Elicitation of plants and microbial cell systems. *Biotechnol. Appl. Biochem.* **37**, 91-102.
- Sirvent, T., and D. Gibson (2002). Induction of hypericins and hyperforin in *Hypericum perforatum* L. in response to biotic and chemical elicitors. *Physiol. Mol. Plant Pathol.* **60**, 311-320.
- St. Leger, R.J., Screen, S.E., and B. Shams-Pirzadeh (2000). Lack of host specialization in *Aspergillus flavus*. *App. Environ. Microb.* **66**, 320-324.
- Tan, J., Bednarek, P., Liu, J., Schneider, B., Svatos, A., and K. Hahlbrock (2004). Universally occurring phenylpropanoid and species-specific indolic metabolites in infected and uninfected *Arabidopsis thaliana* roots and leaves. *Phytochem.* **21**, 691-699.
- Walker, S. T., Bais, H., and M. J. Vivanco (2002). Jasmonic acid - induced hypericin production in cell suspension cultures of *Hypericum perforatum* L (St. John's wort). *Phytochem.* **60**, 289-293.
- Yu, J., Whitelaw, C. A., Nierman, W. C., Bhatnagar, D., and T. E. Cleveland (2004). *Aspergillus flavus* expressed sequence tags for identification of genes with putative roles in aflatoxin contamination of crops. *FEMS Microbiol Lett.* **237**, 333-340.
- Yuan, Y. J., Li, J. C., Hu, Z. D., Wu, J. C., and A. P. Zeng (2002). Fungal elicitor-induced cell apoptosis in suspension cultures of *Taxus chinensis* var. *mairei* for taxol production. *Proc. Biochem.* **38**, 193-198.
- Zdunek, K., and W. Alfermann (1992). Initiation of shoot organ cultures of *Hypericum perforatum* and formation of hypericin derivatives. *Planta Med.* **58**, 621-625.
- Zenk, M. H. (1991). Chasing the enzymes of secondary metabolism: plant cultures as a pot of gold. *Phytochem.* **30**, 3831-3863.
- Zhao, J., Davis, L., and R. Verpoorte (2005). Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol. Adv.* **23**, 283-333.
- Zobayed, S. M. A., Afreen, F., Goto, E., and T. Kozai (2006). Plant-Environment Interactions: Accumulation of hypericin in dark glands of *Hypericum perforatum*. *Ann. of Botany* **98**, 793-804.

