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Jasmonic acid-induced hypericin production in cell suspension cultures of *Hypericum perforatum* L. (St. John's wort)

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Abstract

Hypericum perforatum L. (St. John's wort) is an herbal remedy widely used in the treatment of mild to moderate depression. Hypericin, a photosensitive napthodianthrone, is believed to be the compound responsible for reversing the depression symptoms. In this study, novel in vitro cell culture systems of *H. perforatum* were used to monitor the effect of elicitation on cell growth and production of hypericin. A dramatic increase in cell growth and hypericin production was observed after exposure to jasmonic acid (JA). However, other elicitors such as salicylic acid (SA) and fungal cell wall elicitors failed to show any stimulatory effect on either cell growth or hypericin production. Cell cultures treated with JA and incubated in the dark showed increased growth and hypericin production as compared to the cultures grown under light conditions. Jasmonate induction in dark conditions played an important role in growth and hypericin production in cell suspension cultures, to our knowledge an undocumented observation.

Keywords: *Hypericum perforatum*; Guttiferae; St. John's wort; Callus cultures; Suspension cultures; Hypericin; Jasmonic acid; Elicitation

1. Introduction

Hypericum perforatum L. (St. John's wort), a perennial flowering plant native to Europe, has nearly a 200-year history of use in traditional folk medicine for the treatment of various ailments (Deltito and Beyer, 1998; Dias et al., 1998). Currently, St. John's wort is widely used as an herbal remedy for the treatment of mild to moderate depression (DeSmet and Nolen, 1996). Research on St. John's wort has focused primarily on hypericin and pseudohypericin as the major constituents responsible for the antidepressant activity (Thiede and Walper, 1994). Although the exact mechanism of action of hypericin remains unknown, some evidence suggests that it functions as an inhibitor of enzymes such as monoamine oxidase and catechol-*O*-methyl transferase, which permits the development of newly formed neurotransmitters in the central nervous system (Thiede and Walper, 1994). In addition to its medicinal properties, hypericin and other dianthrone pigments have several known biological and physiological activities within the plant (Diwu, 1995).

Since many of the aforementioned plant pigments are known photodynamic agents (Diwu, 1995; Kirakosyan et al., 2000), these natural products, including hypericin, exhibit light-mediated and photodynamic biological activity against insects, microorganisms and viruses (Towers et al., 1997). Due to hypericin's insecticidal activity, it has been observed that insects feeding on leaves of *H. perforatum* adjust not only their feeding pattern by avoiding the part of the leaf lacking the dark red colored organelles containing hypericin, but they also exhibit negative phototaxis to circumvent the light-mediated phototoxic effects of hypericin (Guillet et al., 2000).

Plant cell culture is often an effective system to study the biological significance of bioactive metabolites under in vitro conditions, as well as for producing natural products for bioprocessing applications (Yanpaisan et al., 1999). For instance, cell suspension cultures of *Lithospermum erythrorhizon* have been widely used for increased production of shikonin (Fukui et al., 1990; Kim and Chang, 1990), a pigmented naphthoquinone with broad range antimicrobial activity (Brigham et al.,

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1999). Given the potential for deriving biologically active compounds from *H. perforatum,* hypericin-producing cell cultures of *H. perforatum* were established to study the overproduction of hypericin using chemical and fungal cell wall elicitors. Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MJ), have been proposed to be key signaling compounds in the process of elicitation leading to the accumulation of various secondary metabolites (Szabo et al., 1999). The jasmonates have been shown to induce rosmarinic acid and shikonin production in cell cultures of *Coleus blumei* and *Lithospermum erythrorhizon,* respectively (Mizukami et al., 1993; Szabo et al., 1999). They have also been reported to play an important role in signal transduction processes that regulate defense genes in plants during assaults such as insect feeding (Farmer and Ryan, 1990; Walling, 2000).

In this communication, we report for the first time the induction by jasmonic acid treatment and dark growth conditions of both cell growth and hypericin production in suspension cultures of *Hypericum perforatum* L.

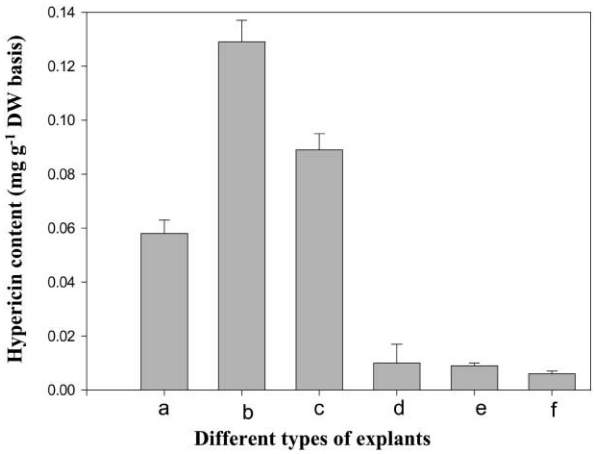

Fig. 1. Hypericin variation in cell cultures and multiple plant parts of *Hypericum perforatum*. Hypericin content was reduced in cell cultures grown in light (a), compared to cell cultures grown in dark (b). Variation of hypericin content in leaves (c), stem (d), roots (e), and media exudates (f). Plant parts were removed from 1-month-old plants cultured in MS media. Hypericin content of cell cultures and plant parts was determined and quantified by HPLC analysis (values are mean ±s.d., $n=5$).

2. Results and discussion

Optimization of cellular proliferation in suspension cultures is the first step toward establishing large scale-up production of biologically active compounds from *H. perforatum*. In an earlier report, we showed that growth and production of hypericin in suspension cultures is strictly regulated by dark conditions (Bais et al., 2002). It has also been reported that the biosynthesis of hypericin is connected with the morphogenesis and formation of dark red colored oil glands on leaves of intact plants (Zdunek and Alfermann, 1992). Accordingly, our in vitro plant cultures displayed similar glands on newly developing leaves, while red-pigmented areas were visible on the majority of callus cultures. The presence of hypericin in our cell culture extracts of *H. perforatum* was previously established by comparing the HPLC retention time to that of an authentic hypericin standard and by HPLC-MS analysis (Bais et al., 2002).

We compared the hypericin content in various parts of in vitro, light-grown plants to that of cell suspension cultures grown under light and dark conditions. To date, leaves of *H. perforatum* have been the organ of choice for hypericin extraction (DeSmet and Nolen, 1996). As shown in Fig. 1, we observed a higher content of hypericin in cell cultures grown in the dark compared to that of leaves removed from a one-month-old in vitro grown plant culture. It has previously been reported that both light conditions and growth regulator treatment influence the growth of cell cultures of *H. perforatum* in static media (Pretto and Santarem, 2000). In our cell suspension cultures of *H. perforatum*, similar sigmoidal curves for biomass accumulation and hypericin production were observed, indicating growth-associated hypericin accumulation (Bais et al., 2002).

Elicitors are defined as molecules that stimulate defense or stress-induced responses in plants (VanEtten et al., 1994). The exogenous application of elicitors to in vitro cultures is useful for studying plant responses to potential microbe/insect attack as well as for enhanced biotechnological production of value-added secondary metabolites in fermentation systems. Elicitors including JA and its derivatives are known to stimulate production of secondary metabolites in plants (Mizukami et al., 1993; Sanz et al., 2000). Salicylic acid (SA) is an important plant-signaling compound that activates defense-related genes, and when used as an elicitor, SA is useful for studying the accumulation of pathogenesis-related compounds (Jirage et al., 1999). Fungal elicitors, mainly derived from the cell walls of fungal pathogens, are known to induce the *de novo* synthesis of antimicrobial phytoalexins (Szabo et al., 1999). Therefore, we examined the effects of JA, SA and fungal cell wall elicitors from *Phytophthora cinnamoni* on growth and production of hypericin in cell suspension cultures of *H. perforatum*. Supplementation of 250 μM JA to *H. perforatum* cell suspension cultures grown under light conditions showed increased biomass production (2090± 156.5 mg l^{-1} dry wt (DW) basis). This was a 1.9-fold increase over the untreated light-grown control (1098.8± 81.75 mg l^{-1} DW basis) (Fig. 2). We observed that treatment with 250 μM JA under dark conditions triggered even greater cell growth (2598.6±195.8 mg l^{-1} DW basis) as compared to the dark-grown control (1679.8± 126.7 mg l^{-1} DW basis) and JA-elicited cultures under light conditions (Fig. 2). Other elicitors such

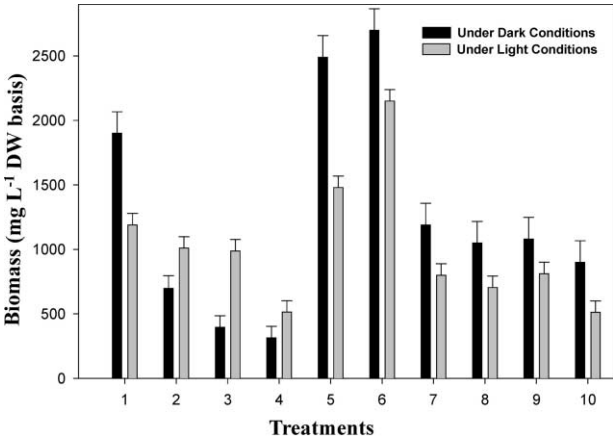

Fig. 2. Effect of different elicitors on biomass accumulation in cell cultures of *Hypericum perforatum* on day 28. Total biomass of *H. perforatum* elicited cell cultures grown under light and dark conditions. Treatments refers to: 1: Control; 2: SA (50 μM); 3: SA (100 μM); 4: SA (200 μM); 5: JA (100 μM); 6: JA (250 μM); 7: JA (500 μM); 8: *P. cinnamoni* CWE (0.1% v/v); 9: *P. cinnamoni* CWE (0.3% v/v); 10: *P. cinnamoni* CWE (0.5% v/v) (values are mean ±s.d., $n=5$).

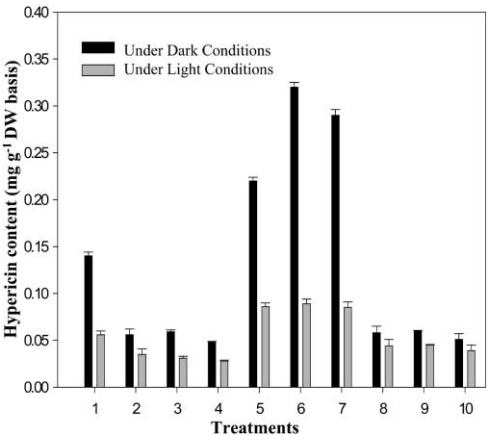

Fig. 3. Influence of different elicitors on hypericin content in *H. perforatum* in suspension cultures on day 28. Total hypericin content in elicited cell cultures of *H. perforatum* grown under light and dark conditions. Treatments refers to: 1: Control; 2: SA (50 μM); 3: SA (100 μM); 4: SA (200 μM); 5: JA (100 μM); 6: JA (250 μM); 7: JA (500 μM); 8: *P. cinnamoni* CWE (0.1% v/v); 9: *P. cinnamoni* CWE (0.3% v/v); 10: *P. cinnamoni* CWE (0.5% v/v) (values are mean ±s.d., $n=5$).

as SA (50–200 μM) and fungal cell wall elicitors from *P. cinnamoni* (0.1–0.3% v v^{-1}) negated induction of growth under both light and dark conditions (Fig. 2).

Hypericin, which belongs to the group of pigmented napthodianthrones, is a well-known photosensitive compound that undergoes transformation under light to its nearest proto-pigments including protopseudohypericin and protohypericin (Torssell, 1997; Kirakosyan et al., 2000; Poutaraud et al., 2001). We have previously shown the production levels and localization of hypericin in cell suspension cultures is entirely different from those of an intact plant (Bais et al., 2002). Therefore, cell suspension cultures of *H. perforatum* should be considered as an entirely different experimental system for elicitation studies. The total hypericin content in untreated cell suspension cultures grown under light conditions was 2.4-fold less than in cell suspension cultures grown under dark conditions (Fig. 3). It was observed that administration of 250 μM JA induced increased accumulation of hypericin in the cultured cells grown under dark conditions (0.318 0.02 mg g^{-1} DW basis) compared to JA-elicited cultures under light conditions (0.089 ± 0.006 mg g^{-1} DW basis) and their respective controls (Fig. 3). Although 250 μM JA induced growth and hypericin production in cell suspension cultures of *H. perforatum*, other tried concentrations of JA (100, 500 μM) also showed a similar effect under both light and dark conditions (Figs. 2 and 3). Elicitors such as SA (50–200 μM) and fungal cell wall elicitors from *P. cinnamoni* (0.1–0.5% v v^{-1}) failed to induce growth and hypericin production under both light and dark incubation (Figs. 2 and 3). This result implies that at the concentrations we tested, SA and fungal cell wall elicitors from *P. cinnamoni* do not channel hypericin elicitation. The transformation of hypericin to the proto-pigments was evident by the lower content of hypericin in leaves and cell cultures grown under light compared to the elevated hypericin content in cell cultures grown under dark conditions (Fig. 1). Increased hypericin content in cell cultures grown under dark conditions may be attributed to stressed cell cultures, which would activate the synthesis of secondary metabolites. In our earlier studies, we established that in dark-grown cultures hypericin was stored in special organelles, rather than in vacuolar oil glands on the leaf surface (Bais et al., 2002). Some investigators have observed under continuous light conditions the transformation efficiency of hypericin increases, resulting in less accumulation of pigments in *H. perforatum* (Poutaraud et al., 2001). It is likely that secondary metabolites, in particular phenolic compounds, can constitute a photo-block, resulting in hindered photo-conversion under continuous light conditions (Hahlbrock and Scheel, 1989). Our results suggest that JA is an effective elicitor for the production of hypericin in cell suspension cultures of *H. perforatum*. Our results also show that incubating JA- elicited cultures under dark conditions can modify the production of hypericin in cell suspension cultures of *H. perforatum*.

The present study shows that the signaling compound JA induces the production of hypericin. It is interesting to note the production of hypericin, a light-mediated insecticide (Towers et al., 1997; Guillet et al., 2000), is stimulated upon JA treatment, since JA is known to be a key signaling compound activated upon insect feeding (Walling, 2000). JA has also been implicated as the signal molecule responsible for increased synthesis of nicotine, an insecticidal compound produced by *Nicotiana sylvestris* upon leaf wounding (Zheng et al., 1997). A biological explanation for the observed overproduction of hypericin in the dark upon JA treatment

may potentially be attributed to the nocturnal feeding patterns of many destructive insect herbivores (De Moraes et al., 2001). In response to nightly insect herbivory on *H. perforatum*, an immediate cascade of events involving JA signal transduction would result in the overproduction of an insecticide, hypericin, for the nocturnal insect to consume.

Although the data presented in this study demonstrate that JA and dark growth conditions increase the production of hypericin in cell suspension cultures of *H. perforatum*, it is not known whether these factors induce different biosynthetic enzymes responsible for the overproduction of hypericin.

3. Experimental

3.1. Plant material

Seeds of *Hypericum perforatum* L. (S5195) were obtained from Richters, Ontario, Canada.

3.2. Chemicals

Plant growth regulators were obtained from Sigma Co (St. Louis, USA); all other chemicals were of analytical and HPLC grade.

3.3. Callus and suspension culture initiation

Seeds were washed in running tap water and were surface sterilized using sodium hypochlorite (0.3% v/v) for 10–15 min, followed by 3–4 washes in sterile distilled water. Surface sterilized seeds were placed on static MS (Murashige and Skoog, 1962) basal media for germination. Callus cultures were initiated using leaves from a 10-day old plant grown in vitro and were subsequently cultured in media containing MS salts and vitamins supplemented with 2, 4-dichlorophenoxyacetic acid (2,4-D) (0.90 μM), kinetin (0.11 μM) and sucrose (30 g l^{-1}). Cultures were maintained under dark and light conditions at 25±2 °C. The light intensity was 4.4117 J m^{-2} s^{-1} under the continuous mode.

Cell suspension cultures were established from the callus cultures, and were maintained in 125 ml Erlenmeyer flasks with 50 ml of nutrient medium containing 2,4-D (0.90 μM), kinetin (0.11 μM) and sucrose (30 g l^{-1}) by biweekly sub-culturing on a rotary shaker at 90 rpm maintained at 25±2 °C.

3.4. Preparation and addition of elicitor

An initial inoculum of 200 mg (fresh weight basis) of callus mass was used for all the growth and elicitation experiments. The cell suspension cultures of *H. perforatum* were analyzed periodically for growth and hypericin content during the culture period of 28 days. Cell suspension cultures of *H. perforatum* grown in 40 ml MS flasks were elicited with jasmonic acid (JA), salicylic acid (SA) and fungal cell wall preparations. Fungal cell wall extracts (CWE) from fungi such as *Phytophthora cinnamoni* were used. The fungal cell wall elicitors were prepared and used according to McKinley et al. (1993). Fungal elicitors were administered at various concentrations (0.1–0.5% v/v), into 125 ml flasks containing 40 ml of MS basal media. Solutions of SA and JA were prepared in ethanol and were added individually to cells grown in 40 ml MS flasks at final concentrations of 50–200 μM and 100–500 μM respectively. Cell cultures were harvested at 7-day intervals for 4 weeks to monitor the influence of various elicitors on biomass and hypericin production. A non-elicited, positive control was also harvested during the same period for biomass and hypericin content.

3.5. Estimation of hypericin using liquid chromatography mass spectrometry (HPLC–MS)

The fresh cells (200 mg) were extracted using 5 ml absolute ethanol (Fisher Co USA) and were sonicated (Branson Co USA, ~60 Hz) for 10 min. The extracts were centrifuged at 10,000 rpm for 10 min. The supernatants were further concentrated by freeze-drying (Model 25LL, Vir Tis, Genesis) and the powders were dissolved in 1 ml of methanol (Fisher Co USA). The samples were prepared for LC analysis after filtration through a 0.2-μm filter (Gelman Sciences). Samples were subjected to gradient elution on a reverse phase HPLC system. (5 μm, C_{18} column, 25 cm×4.6 mm) (Supelco Co USA); the chromatographic system consisted of P580 pumps (Dionex Co, USA) connected to an ASI-100 Automated Sample Injector (Dionex Co, USA). The visible absorbance at 590 nm was measured by a PDA-100 Photodiode array variable UV/VIS detector (Dionex Co, USA). Mobile phase Solution A consisted of a 70% solution of 1% ammonium phosphate (adjusted to 7.0 pH with NaOH) and 30% acetonitrile (Fisher Co USA); solution B was 70% acetonitrile-30% water. A multistep gradient was set up for hypericin estimation as described by Liebes et al. (1991), with an initial injection volume of 15 μl and a flow rate of 1 ml min^{-1}. Hypericin was quantified at 590 nm. Authentic hypericin was purchased from Sigma Co (USA) and samples were compared with the hypericin standard on the basis of retention time and peak area. Chromelon software (Dionex Co, USA) was used for peak integration analyses. Both standard hypericin and the extracted sample eluted at 22.71 min. The LC eluant was injected into an API-2000 mass spectrometer (Dionex Co USA) at a flow rate of 0.25 ml min^{-1}. The MS parameters were optimized using hypericin as the reference standard compound. Subsequent high gas temperature (200 °C)

and gas flow (50 psi) were applied to the HPLC-MS parameters. Hypericin [$(M–H)^-$ 504.5)] (minus one proton after proton-proton splitting, to produce a negatively charged molecule), was used for negative mode monitoring. Scan ranges of 100–750 amu (milli absorbance units) were used for negative ions. A step size of 1 amu and dwell time of 1 ms was used during the analysis.

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