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Stimulation of the production of hypericins by mannan in *Hypericum perforatum* shoot cultures

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Abstract

Shoot organ cultures were established from callus derived from anthers of *Hypericum perforatum* flowers and the effect of elicitors on hypericin and pseudohypericin production in shoot organ cultures was investigated. Mannan stimulated pseudohypericin production up to four fold (0.82 mg/g dry wt) and hypericin production up to two fold (0.04 mg/g dry wt). β-1,3-glucan and pectin slightly stimulated pseudohypericin production (ca. two fold), but had no effect on hypericin production. On the other hand, yeast extract showed no stimulatory effect, on either hypericin or pseudohypericin production. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Hypericum perforatum*; Guttiferae; Shoot organ culture; Hypericin; Pseudohypericin; Yeast extract; Mannan; β-1,3-Glucan; Pectin; Elicitor

1. Introduction

Hypericum perforatum L. (St. John's Wort) is a traditional medicinal plant with wound healing and antidepressive activities (Schulz, 1995). Although it is still unclear which constituents in *H. perforatum* are responsible for its antidepressive activity, hypericin and pseudohypericin, dianthrone pigments in *H. perforatum*, have several known physiological activities (Diwu, 1995; Butterweck, Petereit, Winterhoff & Nahrstedt, 1998; Bork, Bacher, Schmitz, Kaspers & Heinrich, 1999). These activities are presumed to be due to their photodynamic properties, since these plant pigments are known to be photodynamic agents (Diwu, 1995).

Since hypericin and pseudohypericin are important as novel therapeutic agents, the production of these constituents by plant tissue cultures has been examined (Zdunek & Alfermann, 1992; Kartnig, Gobel & Heydel, 1996). However, our understanding of the regulation of the biosynthesis of hypericins is rather limited. In the present study, we established shoot organ cultures of *H. perforatum*, and examined the effect of elicitors on the production of hypericins.

2. Results and discussion

Deep-green calli, derived from anthers of *H. perforatum* (Kirakosyan, Vardapetyan & Charchoglyan, 2000), were cultured separately on a modified Murashige Skoog (MS) agar medium (Murashige & Skoog, 1962) under permanent light (3000 lux) to induce shoot morphogenesis. Regenerated shoots were transferred to a modified MS liquid medium and subcultured at intervals of 15 days.

Production of hypericin and pseudohypericin by plant tissue cultures of various *Hypericum* species has

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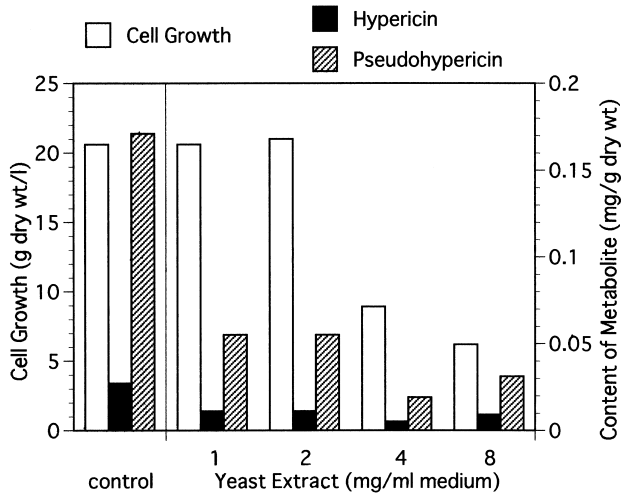

Fig. 1. Effect of yeast extract on the production of hypericin and pseudohypericin by *Hypericum perforatum* shoot organ cultures (Mean of two replicates).

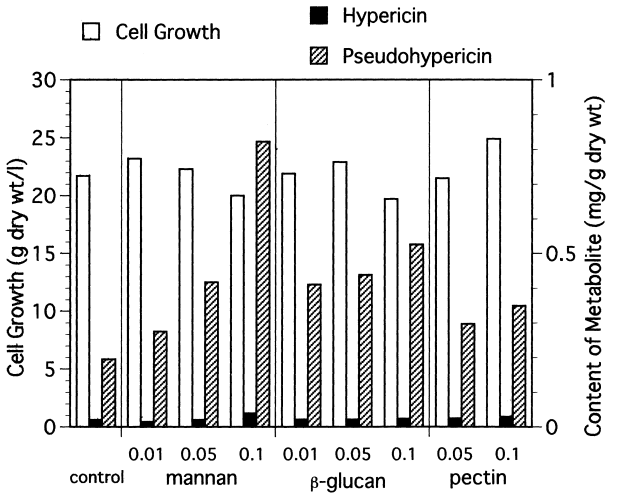

Fig. 2. Effect of yeast mannan, β-1,3-glucan (curdlan) and pectin (from citrus) on the production of hypericin and pseudohypericin by *Hypericum perforatum* shoot organ cultures (Mean of two replicates).

been reported (Zdunek & Alfermann, 1992; Kartnig et al., 1996). It was also reported that biosynthesis of hypericins is connected with the morphogenesis and formation of dark red colored oil glands on leaves of the intact plant (Zdunek & Alfermann, 1992). Our shoot culture also showed similar glands on the newly forming leaves, and both hypericin and pseudohypericin were detected in the extracts by HPLC analysis. In our shoot cultures, pseudohypericin content was much higher than that of hypericin, as reported in cell suspension cultures of *H. perforatum* (Kartnig et al., 1996).

Yeast extract stimulates production of secondary metabolites in plant cell and tissue cultures (Yamamoto, Ichimura & Inoue, 1995). Thus the effect of yeast extract on production of hypericins by *H. perforatum* shoot organ cultures was examined (Fig. 1). Both hypericin and pseudohypericin production was inhibited by yeast extract at concentrations of 1–8 mg/ml. Shoot growth was not inhibited by yeast extract at 1–2 mg/ml, but 4–8 mg/ml yeast extract reduced shoot growth.

Some polysaccharides are also known to act as elicitors of secondary metabolism in higher plants (Yamamoto et al., 1995). Therefore, we investigated the effect of commercial yeast mannan, β-1,3-glucan (curdlan) and pectin (from citrus) on production of hypericins in *H. perforatum* shoot organ cultures (Fig. 2).

Unlike yeast extract, mannan (from yeast) stimulated both hypericin and pseudohypericin production at 0.05–0.1 mg/ml. Pseudohypericin production (0.82 mg/g dry wt) at 0.1 mg/ml of mannan was almost four times higher than that of control shoot cultures (0.19 mg/g dry wt). In addition, hypericin production (0.04 mg/g dry wt) at the same concentration of mannan was two times higher than that of control (0.02 mg/g dry wt). Pseudohypericin production at 0.5 mg/ml of mannan was three times higher than that of control with no inhibition of shoot growth observed (data not shown). These results suggest that commercial mannan is an effective elicitor of the production of hypericins in *H. perforatum* shoot organ cultures. β-1,3-Glucan stimulated pseudohypericin production about 2.5 times at 0.1 mg/ml, an effect lower than that of mannan; no effect of β-1,3-glucan on hypericin production was observed. Pectin stimulated pseudohypericin production to a lesser degree (1.8 times stimulation), while not affecting hypericin production. It is noteworthy that no inhibitory effect of mannan, β-1,3-glucan and pectin (0.05–0.1 mg/ml) on shoot growth was observed in the present study. In all experiments, large amounts of shoots (about 20–22 g dry wt/l) were harvested after a 15-day cultivation.

It was reported that production of hypericin and pseudohypericin by *H.perforatum* plants is strongly dependent on genetic and environmental factors (Büter, Orlacchio, Soldati & Berger, 1998). Our results also indicate that production of hypericins by *H. perforatum* shoot organ cultures can be modified by elicitors such as mannan. This is the first report showing the stimulatory effects of elicitors on the production of hypericins. It is known that hypericins are photodynamic pigments, originating from emodin anthrone, which are dimerized (presumably via phenol oxidation) and further oxidized to form hypericins (Torssell, 1997). Whether these elicitors induce different biosynthetic enzymes responsible for the production of hypericins is not known. Further experiments on the effect of elicitors on the biosynthesis of hypericins in shoot organ cultures are in progress.

3. Experimental

3.1. Chemicals

Hypericin and mannan (from *Saccharomyces cerevisiae*) were obtained from Sigma. β-1,3-Glucan (curdlan) and pectin (from citrus) were obtained from Wako Pure Chemical, Japan. Yeast extract was obtained from Difco Laboratories.

3.2. In vitro culture

Callus cultures were derived from anthers of *H. perforatum* flowers, collected in the National Park of Idjevan, Armenia, in July, 1995 using a modified MS agar medium (Murashige & Skoog, 1962) containing 30 g/l sucrose, 7 g/l agar, 0.1 mg/l thiamine HCl, 250 mg/l *myo*-inositol, 1 mg/l nicotinic acid, 1 mg/l pyridoxine HCl, 1 mg/l 2,4-dichlorophenoxyacetic acid and 1 mg/l kinetin (Kirakosyan et al., 2000). Callus cultures were incubated at 24°C under continuous light (3000 lux) and subcultured at intervals of 5–7 weeks. Another modified MS medium containing 30 g/l sucrose, 8 g/l agar, 0.1 mg/l thiamine HCl, 100 mg/l *myo*-inositol, 1 mg/l nicotinic acid, 1 mg/l pyridoxine HCl, 0.05 mg/l 1-naphthaleneacetic acid and 0.5 mg/l 6-benzyladenine was used for initiation of shoot organ cultures. Shoot organs (2 g fresh wt) derived from the calli were transferred to 50 ml of the modified MS medium without agar. Shoot cultures were incubated at 24°C under continuous light (3000 lux) on a rotary shaker at 100 rpm and subcultured at 15 day intervals. For elicitation experiments, yeast extract (1–8 mg/ml) or polysaccharide (0.01–0.1 mg/ml) was dissolved in medium and autoclaved. The pH of the medium was adjusted to 5.8 before autoclaving. Shoots (2 g fresh wt) were cultured in 50 ml of medium in 300 ml Erlenmeyer flasks for 15 days with or without elicitors under the same conditions. All experiments were means of two replicates.

3.3. Extraction and HPLC analysis of hypericins

Freeze-dried shoot organ cultures (0.2 g dry wt) were extracted with 10 ml of CH_2Cl_2 for 24 h, and the whole was then filtered. The remaining shoot was extracted with 10 ml of 80% MeOH for 10 min, and then 10 ml of Me_2CO for 2 h. The MeOH and Me_2CO solubles were combined and evaporated in vacuo. The residue was dissolved in 2 ml of 80% MeOH and an aliquot (20 μl) was subjected to HPLC (with photodiode array detection) to determine hypericin and pseudohypericin contents. Conditions of HPLC were, column, Cosmosil 5C18-MS (4.6 mm i.d. × 250 mm, Nacalai Tesque, Japan); solvent A: 0.5% TFA in water, solvent B: 70% MeCN–29.5% MeOH–0.5% TFA, A/B gradient of 50% B to 100% B in 15 min, then 100% B for 25 min; flow rate, 1 ml/min; column temperature 40°C; detector, Shimadzu photodiode array SPDM-6A system (Shimadzu, Japan).

Two peaks were observed on the chromatogram by UV absorption at 588 nm. The compound with the retention time of 27.0 min was identified as hypericin by comparison of the retention time and UV spectrum with those of the authentic sample. The major peak (R_t = 16.9 min), whose UV spectrum was identical with that of hypericin, was deduced to be that of pseudohypericin on the following basis: (1) pseudohypericin has the same dianthrone structure as hypericin except for the substitution of a methyl group in hypericin with a hydroxymethyl group in pseudohypericin, indicative of the same UV spectrum as that of hypericin; (2) pseudohypericin was reported to be a major constituent in the intact plants and cell suspension cultures of *H. perforatum* (Kartnig et al., 1996; Büter et al., 1998). Content of hypericins was determined on the basis of the peak area of UV absorption at 588 nm.

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