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ORIGINAL PAPER

Regulation of metabolite production by precursors and elicitors in liquid cultures of *Hypericum perforatum*

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Abstract Four precursors (L-phenylalanine, L-tryptophan, cinnamic acid and emodin) and one signal elicitor (methyl jasmonate, MeJA) were added to liquid cultures of *Hypericum perforatum* L. to study their effect on production of hyperforin and hypericins (pseudohypericin and hypericin). The addition of L-phenylalanine (75 to 100 mg l^{-1}) enhanced production of hypericins, but hyperforin levels were decreased. Hypericin, pseudohypericin and hyperforin concentrations were all decreased when L-tryptophan (25 to 100 mg l^{-1}) was added to the medium. However, addition of L-tryptophan (50 mg l^{-1}) with MeJA (100 μM) stimulated hyperforin production significantly (1.81-fold) and resulted in an increased biomass. Cinnamic acid (25, 50 mg l^{-1}) and emodin (1.0 to 10.0 mg l^{-1}) each enhanced hyperforin accumulation in *H. perforatum*, but did not affect accumulation of hypericins.

Keywords Cinnamic acid · Emodin · Hyperforin · Hypericins · L-Phenylalanine · L-Tryptophan

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Abbreviations

BA	6-Benzylaminopurine
DMF	*N,N*-dimethylformamide
DW	Dry weight
IAA	Indoleacetic acid
IBA	3-Indolylbutyie
MeJA	Methyl jasmonate

Introduction

Extracts of St. John's wort (*Hypericum perforatum* L.) have become a valuable commodity in the United States and Germany, where they are used as a dietary supplement and an anti-depressive phyto-medicine (Di Carlo et al. 2001). Functional metabolites in *H. perforatum* include hyperforin, hypericins (hypericin and pseudohypericin) and other compounds. The quality of these phyto-chemicals in field-grown plants may be affected by physical and chemical factors, as well as biological processes and environmental factors (Mosaleeyanon et al. 2005; Zobayed et al. 2005; Couceiro et al. 2006; Sirvent and Gibson 2002).

The use of bioreactors to grow liquid cultures provides several advantages for plant propagation, such as controlling and enhancing bioactive compound production, avoiding intensive manual handling, and increasing propagation speed and efficiency (Ziv 2005). Zobayed et al. (2004)

established a protocol for large-scale propagation of *H. perforatum* organogenesis in bioreactors and showed that total fresh mass production in a balloon-bubble bioreactor was 2.5-fold greater than that of plantlets grown on gelled medium. However, hyperforin and hypericin production in the bioreactors was only one-third the production in plantlets grown on gelled medium. It may be possible to utilize regulators such as precursors and elicitors, to stimulate metabolite production in bioreactors.

Plant secondary metabolites were synthesized by the shikimate and phenypropanoid pathways in vivo, and several middle chemicals, including L-phenylalanine, L-tryptophan and cinnamic acid were produced (Yu and Tang 1998). Phenylalanine, cinnamic acid and tryptophan were shown to function as precursors in taxoid, shikonin, phenylethanoid glycosides, melatonin, and serotonin synthesis, as well as in the biosynthesis of other compounds in various plants (Liu et al. 2007; Mei et al 2001; Okamoto et al. 1995; Lu et al. 2001; Murch et al. 2000; Gill 2001). In addition, Bais et al. (2003) suggested that hypericin and its naphthodianthrone analogs resulted from dimerization of emodin anthrone. Abe et al. (2004, 2005) reported that type III polyketide synthases (PKSs), which belong to the chalcone synthase (CHS) superfamily, catalyzed the formation of flavonoids, pentaketide, heptaketide and other polyketide chromones. Based on the molecular structures of the hypericins and hyperforin, we hypothesized that small molecule chemicals, including L-phenylalanine, L-tryptophan, cinnamic acid, emodin and others, might also be precursors for biosynthesis of metabolites with type III PKSs catalysis. Further, these metabolites might be regulated by the addition of precursors.

The objective of this study was to regulate metabolite production in *H. perforatum* through organogenesis in a bioreactor. Four putative precursors and one elicitor were selected for the study. Each was added to Murashige and Skoog (MS) liquid medium and biomass production and hyperforin and hypericin levels were compared in each culture. Identification of precursors or elicitors capable of regulating metabolite production will be useful for enhancing the effectiveness and economic value of *H. perforatum*.

Material and methods

Plant materials, treatments and growing conditions

The seed surfaces of *H. perforatum* (obtained from the Chongqing Academy of Traditional Chinese Medicine, Chongqing, China) were sterilized by immersion in a 75% ethanol solution for 30 s, followed by immersion in a 4.5% (v/v) solution of sodium hypochlorite with 0.1% Tween-80 (10 min), and then rinsed five times with sterile distilled water. Sterile seeds were germinated on hormone free MS medium (Murashige and Skoog 1962) in Petri dishes containing 0.6% agar (Qingdao Haiyang Chemical Co., Ltd, Qingdao, China) and 3% sucrose. The pH of the medium was adjusted from 5.7 to 5.8 prior to autoclaving (121°C, 0.105 MPa, 15 min). The seeds were incubated for 25 d and new shoots were transferred to MS medium supplemented with 0.44 μM 6-benzylaminopurine (BA) (Gentel Co., Ltd, Beijing, China) and 0.049 μM 3-indolylbutyie (IBA) (Gentel). Plantlet shoots were cultured for 25 d, under dark and light condition (8 h/16 h) at 25 ± 2°C in 72.35 ± 2.96 μmol m^{-2} s^{-1} light intensity. Elongated shoots were used as experimental material for subsequent experiments.

Plantlet shoots were cut into pieces 20 to 25 mm long (each with 3 to 4 nodes). Ten of the explants were incubated in 50 ml of MS liquid medium (0.88 μM BA + 0.049 μM IBA, agar free) in 250-ml conical flasks. L-phenylalanine (Amerisco, USA) concentrations were 25, 50, 75, 100 and 150 mg l^{-1}, and L-tryptophan (Amerisco, USA) concentrations were 25, 50 and 100 mg l^{-1} in medium, respectively, with amino acid free medium as control. Methyl jasmonate (>95%, Sigma-Aldrich, Inc., St. Louis, MO, USA) was dissolved in *N*, *N*-dimethylformamide (DMF, Sigma, USA) and sterilized by filtering through 0.22-μm nylon syringe filters (Shenghe & Sincerity Membrane Technical Development Centre, Beijing, China). After plantlet shoots were cultured for 12 d in MS liquid medium containing L-tryptophan (50 mg l^{-1}), MeJA (100 μM) was added to the cultures in 0.5 μl of DMF per 1 ml of culture medium for subsequent culture. Emodin solution (dissolved in DMF, 10 mg ml^{-1}) (National Institute for the Control of Pharmaceutical and Biological Products

[NICPBP]) was sterilized by filtering through 0.22-μm nylon syringe filters. The stock emodin solution was diluted in DMF and added to the medium at 1.0, 5.0, 10.0, and 20.0 mg l^{-1}. The emodin control was the culture plus 2 μl of DMF per 1 ml medium. Cinnamic acid (NICPBP, dissolved in ethanol) was added to the medium in 1 μl of ethanol per 1 ml of culture medium and sterilized by filtering through 0.22-μm nylon syringe filters. The final media concentrations were 25 mg l^{-1} and 50 mg l^{-1}. The cinnamic acid control consisted of culture plus 1 μl of ethanol per 1 ml medium.

Extractions and determination of pseudohypericin, hypericin and hyperforin

Extraction methods were those previously described by Ang et al. (2002) with all sample preparation steps performed under dimmed light and at room temperature. Briefly, plant materials cultured for 25 d were collected in 50-ml Eppendorf tubes and then frozen in liquid N_2 and stored at −40°C until needed. Fresh weights were obtained for each sample prior to freezing. Frozen tissues were freeze-dried for 24 h using a freeze dry system (FD-1D-50, <20 Pa, −50°C) (Boyikang Laboratory Instrument Co., Ltd., Beijing, China). Dry samples were weighed (dry weight) and then pulverized using a mortar. Pulverized sample powder (0.2 g) was placed into amber-colored 50-ml vials for extraction with 9.8 ml of methanol containing 200 μl of pyridine for 20 min in an ultrasonic sonicator (JY92-II, Science Biotechnology Co., Ltd. Ningbo, China) in an ice bath. Samples were centrifuged (8,000 *g*, 15 min, GL-20B, Anke Instruments Co., Ltd., Shanghai, China) and filtered through a 0.45-μm nylon syringe filter prior to high performance liquid chromatography (HPLC) analysis. A 20-μl sample of the extract was injected into a Dikma Diamonsil C_{18} column (5.0 μm; 4.6 mm × 150 mm) with a C_{18} guard column (10 mm × 4.6 mm) (Shimadzu, Japan) in a Shimadzu HPLC system consisting of an SCL-10A system controller and a CTO-10AS column oven. The mobile phase was acetonitrile and 0.05 M triethylammonium acetate buffer (80:20) at 1.0 ml min^{-1} flow rate. Hyperforin ($\geq$97.0%, Alexis Co., Lausen, Switzerland) was quantified at 290 nm, and hypericin ($\geq$95.0%, Fluka Co., Buchs, Switzerland) and pseudohypericin ($\geq$98.0%, Alexis Co., Lausen, Switzerland) were quantified at 588 nm in an SPD-M10AV photodiode array detector. Standard curves were obtained by plotting the peak areas of standard concentrations of hypericin (1.0, 2.0, 4.0, 6.0, and 10.0 μg ml^{-1}), pseudohypericin (5.0, 7.5, 10.0, 25.0, 50.0, and 75.0 μg ml^{-1}) and hyperforin (2.5, 5.0, 10.0, 25.0, and 50.0 μg ml^{-1}). Three linear regression equations ($R^2 > 0.99$) were obtained. Quantification of pseudohypericin, hypericin and hyperforin was based on peak area (RT, retention time of 6.4, 9.5, and 19.3 min, respectively) in comparison with the standard curves.

Statistical analysis

Each treatment consisted of eight replications and 80 shoots ($n = 3$ independent experiments). The dry weight of each plantlet was measured on day 25 to establish biomass production. Statistical significance was determined by one-way analysis of variance (ANOVA) (V11.5, SPSS for Windows). Differences between means were assessed with the Student-Newman-Keuls test.

Results

The amounts of pseudohypericin and hypericin produced increased significantly with the addition of L-phenylalanine (75 mg l^{-1}) to the MS liquid medium. Contents increased 1.32-fold and 1.20-fold, respectively, compared with the control (Fig. 1a, b). In contrast, hyperforin content was not changed significantly (Fig. 1c) by the addition of L-phenylalanine at a concentration of 75 mg l^{-1}. However, higher concentrations of L-phenylalanine (150 mg l^{-1}) did cause a significant increase in hyperforin production, while hypericin production decreased. Additionally, biomass appeared to increase with added L-phenylalanine, but the increase was not significant (Fig. 1d).

Biomass production increased significantly in *H. perforatum* when L-tryptophan (25 to 100 mg l^{-1}) was added to the medium (approximately 1.25-fold compared to the control) (Fig. 2d). However, the biomass did not increase when higher concentrations of L-tryptophan were added. The production of

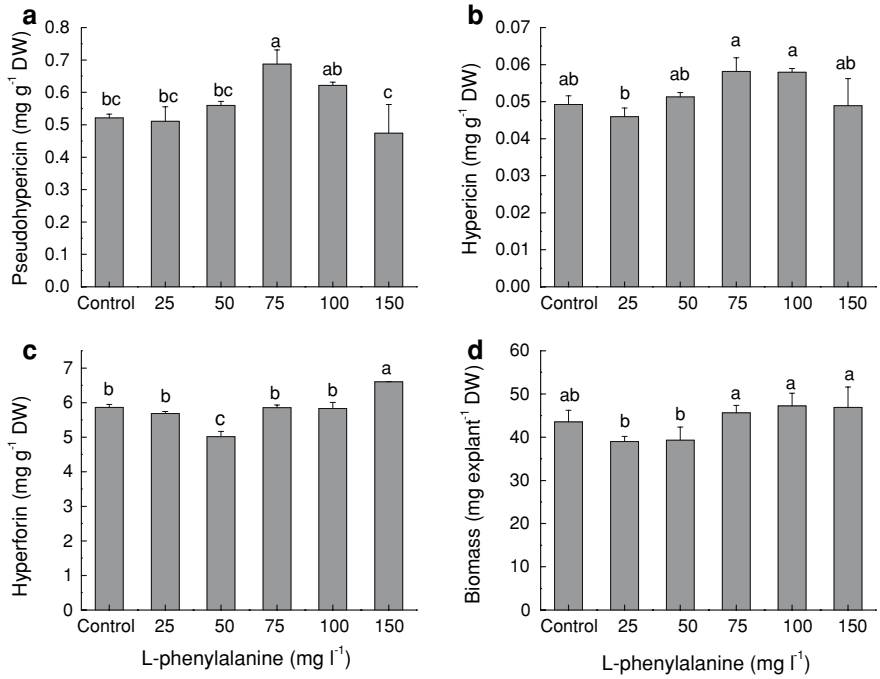

Fig. 1 Effects of L-phenylalanine supplementation on growth and metabolite production in *H. perforatum*. Shoots were transferred to MS liquid medium supplemented with BA (0.88 μM) and IBA (0.049 μM), containing L-phenylalanine (25–150 mg l^{-1}). Plantlets cultured in the same hormone medium (L-phenylalanine free) were used as controls. Mean ± SD (vertical bars, $n = 3$). Different letters indicate significant differences at $P \leq 0.05$

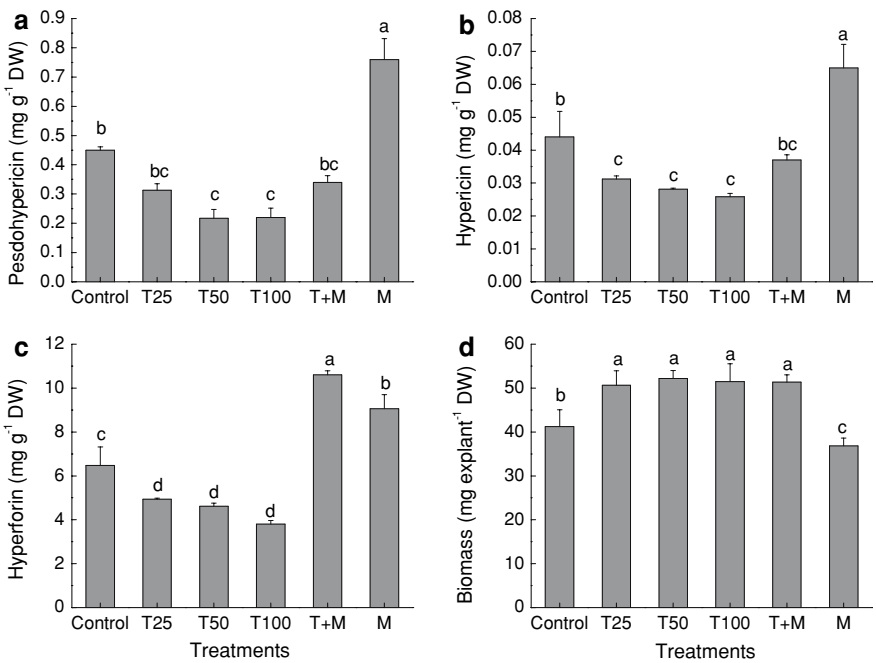

Fig. 2 Effects of L-tryptophan and MeJA supplementation on growth and metabolite production in *H. perforatum*. Shoots were transferred to MS liquid medium supplemented with BA (0.88 μM) and IBA (0.049 μM), containing L-tryptophan (25, 50, 100 mg l^{-1}; T25, T50, T100). MeJA (100 μM) was added to the media containing L-tryptophan 50 mg l^{-1} (T + M) and L-tryptophan free (M) after plantlets were cultured for 12 d. Plantlets cultured in the same hormone medium (both L-tryptophan and MeJA free) were used as controls. Mean ± SD (vertical bars, $n = 3$). Different letters indicate significant differences at $P \leq 0.05$

hyperforin and the hypericins were both decreased significantly by L-tryptophan supplementation. At higher concentrations of L-tryptophan, lower production of the metabolites was linearly observed (Fig. 2a–c). However, hyperforin production was stimulated approximately 1.81-fold compared to the control when 100 μM of MeJA was added to the culture medium containing 50 mg l^{-1} L-tryptophan on the 12th day of culturing (Fig. 2c). Hypericin and pseudohypericin production remained lower than the control when MeJA was added to the medium. When MeJA (100 μM) was added to medium alone, hyperforin, hypericin and pseudohypericin contents were significantly higher than the control, however, plantlet growth was retarded and biomass production was decreased significantly (Fig. 2d).

Addition of emodin (10 mg l^{-1}) stimulated hypericin and pseudohypericin production (Fig. 3), but the increase was not significant. In contrast, hyperforin increased significantly, and was 1.56-fold greater than the control. Both hyperforin and the hypericins were decreased significantly when 20 mg l^{-1} emodin was added to the medium. Additionally, emodin did not have a positive effect on biomass production.

Hyperforin production was significantly increased (1.3-fold) with cinnamic acid supplementation (Fig. 3c), while the hypericins content decreased significantly. As shown in Figs. 1 and 3, when ethanol (1 μl ml^{-1}) was added to the medium, biomass production was decreased. In contrast, DMF (2 μl ml^{-1}) added to the medium had a positive affect on plantlet growth.

Discussion

Recently, Zobayed et al. (2007) found that hyperforin accumulates in plants under water stress. In this study, we found that the combination of L-tryptophan with MeJA abolished hyperforin metabolism retardation and decreased biomass caused by L-tryptophan or MeJA alone in liquid cultures of *H. perforatum*. L-tryptophan is the precursor of indoleacetic acid (IAA) (Wang et al. 2002). It is possible that the increase in biomass is the result of IAA accumulation

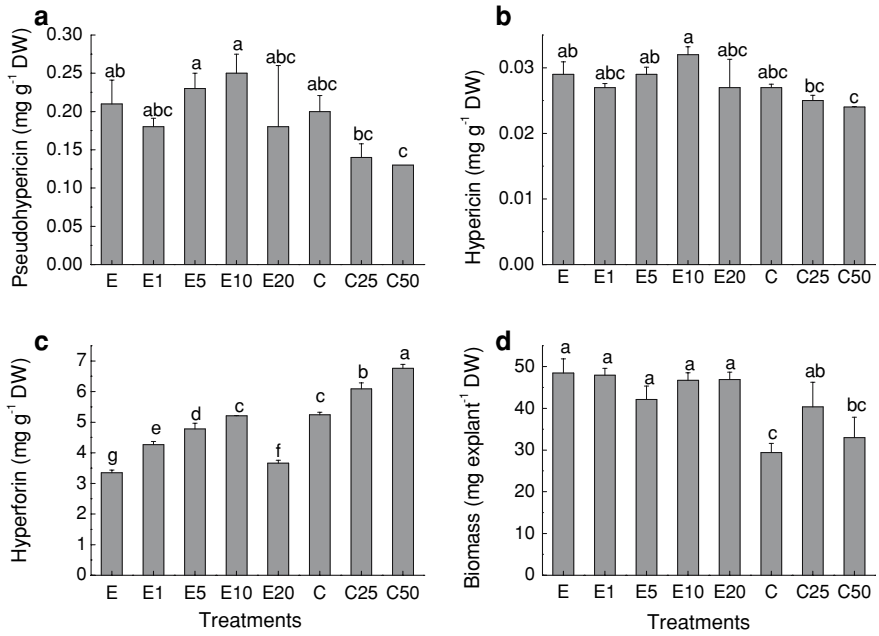

Fig. 3 Effects of emodin and cinnamic acid supplementation on growth and metabolite production in *H. perforatum*. Shoots were transferred to MS liquid medium supplemented with BA (0.88 μM) and IBA (0.049 μM), containing emodin (1.0, 5.0, 10.0, 20.0 mg l^{-1}; E1, E5, E10, E20) and cinnamic acid (25, 50 mg l^{-1}; C25, C50). Plantlets cultured in the same hormone medium containing DMF (2 μl ml^{-1}) and ethanol (1 μl ml^{-1}) were used as the emodin control (E) and cinnamic acid control (C). Mean ± SD (vertical bars, $n = 3$). Different letters indicate significant differences at $P \leq 0.05$

induced by L-tryptophan supplementation. Additionally, L-tryptophan may attenuate the key transcription enzymes for hyperforin or hypericins biosynthesis, suggesting the attenuator may be in the L-tryptophan operon (Wang et al. 2002). MeJA was an elicitor involved in plant defense response pathways, which could cause plant secondary metabolite biosynthesis (Zhao et al. 2005). From this, we infer that L-tryptophan inhibits production of the hypericins and hyperforin via one pathway in plants, while MeJA stimulates hyperforin biosynthesis via another pathway. Different biosynthetic pathways may exist in *H. perforatum* plantlets.

Couceiro et al. (2006) proposed that hypericin and hyperforin biosynthetic pathways might be different, based on the low correlation coefficient obtained for the total hypericins and hyperforin contents. Our research showed a high linear relationship between the hypericins and total flavonoids contents in *H. perforatum* (data not shown). Therefore, we hypothesized that hypericin biosynthesis could be stimulated by supplementing the medium with low concentrations of L-phenylalanine via the phenylalanine ammonia-lyase (PAL) pathway. Then, flavones, anthrones, dianthrones and other polyketides chemicals might be stimulated to synthesize by reactions catalyzed by PKSs. Gill (2001) reported that emodin was the precursor of octaketides via some biosynthetic steps in fungus. In this study, we found that the addition of emodin increased both hypericins and hyperforin formation in *H. perforatum*. Emodin might serve as the precursor of the hypericin and hyperforin, but both may be regulated in different pathways by step-wise PKSs catalysis. Additionally, hyperforin synthesis could be activated with high concentrations of L-phenylalanine. It is possible that high concentrations of L-phenylalanine promote PAL activity to catalyze cinnamic acid synthesis. Meanwhile, cinnamic acid, as a possible precursor could increase hyperforin accumulation. Further research is needed to test this hypothesis.

Hyperforin and its analogs exhibit anti-depressive activity and other neurological effects, as well as effects on inflammation, and antibacterial, anticancer and anti-angiogenic effects (Medina et al. 2006). As a result, increased pharmacological interest has led to more research on these metabolites. Recently, it was suggested the skeleton of hyperforin is formed by isobutyrophenone synthase from isobutyryl-CoA and three molecules of malonyl-CoA. It appears dimethylallyl diphosphate (DMAPP) and geranyl diphosphate (GPP) catalyzed the whole molecular formation (Beerhues 2006; Klingauf et al. 2005). In our study, we also presumed a possible pathway for hyperforin synthesis, as well as for the hypericins. However, the biosynthesis process still remains unclear (Nicolaou et al. 2005). Additionally, cross-talk multiple signaling pathways are important for plant secondary metabolite production, but this operation process is also unclear (Zhao et al. 2005).

In conclusion, hyperforin biosynthesis was stimulated in *H. perforatum* by the presence of high concentrations of L-phenylalanine (150 mg l^{-1}), cinnamic acid (50 mg l^{-1}) and emodin (10 mg l^{-1}). Additionally, L-tryptophan (50 mg l^{-1}) and MeJA (100 mg l^{-1}) in combination also stimulated hyperforin biosynthesis. Two different biosynthetic pathways were assumed for biosynthesis of hyperforin and hypericins based on different chemical signals in liquid culture of *H. perforatum*. Our results show specific plant metabolites could be modulated by exogenous signaling chemicals supplemented in the bioreactor, according to their specific metabolite pathway.

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