Nitric Oxide Mediates the Fungal Elicitor-Induced Hypericin Production of *Hypericum perforatum* Cell Suspension Cultures through a Jasmonic-Acid-Dependent Signal Pathway

Mao-Jun Xu*, Ju-Fang Dong, and Mu-Yuan Zhu

State Key Lab of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou 310012, People’s Republic of China (M.-J.X., M.-Y.Z.); and Department of Biotechnology, Zhejiang Gongshang University, Hangzhou 310035, People’s Republic of China (M.-J.X., J.-F.D.)

Fungal elicitor prepared from the cell walls of *Aspergillum niger* induces multiple responses of *Hypericum perforatum* cells, including nitric oxide (NO) generation, jasmonic acid (JA) biosynthesis, and hypericin production. To determine the role of NO and JA in elicitor-induced hypericin production, we study the effects of NO scavenger 2- to 4-carboxyphenyl-1,4, 5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPITO), nitric oxide synthase inhibitor S,S’-1,3-phenylene-bis(1,2-ethanediyl)-bis-isothiourea, and inhibitors of the octadecanoid pathway on elicitor-induced NO generation, JA biosynthesis, and hypericin production. Pretreatment of the cells with cPITO and JA biosynthesis inhibitors suppresses not only the elicitor-induced NO generation and JA accumulation but also the elicitor-induced hypericin production, which suggests that both NO and JA are involved in elicitor-induced hypericin biosynthesis. S,S’-1,3-phenylene-bis(1,2-ethanediyl)-bis-isothiourea and cPITO inhibit both elicitor-induced NO generation and JA biosynthesis, while JA biosynthesis inhibitors do not affect the elicitor-induced NO generation, indicating that JA acts downstream of NO generation and that its biosynthesis is regulated by NO. External application of NO via its donor sodium nitroprusside induces hypericin production in the absence of fungal elicitor. Sodium-nitroprusside-induced hypericin production is blocked by JA biosynthesis inhibitors, showing that JA biosynthesis is essential for NO-induced hypericin production. The results demonstrate a causal relationship between elicitor-induced NO generation, JA biosynthesis, and hypericin production in *H. perforatum* cells and indicate a sequence of signaling events from NO to hypericin production, within which NO mediates the elicitor-induced hypericin biosynthesis at least partially via a JA-dependent signaling pathway.

Production of secondary metabolites with distinct and complex structures in plants by cell cultures has been one of the most extensively explored areas in recent years owing to the enormous commercial value of those compounds, the scarcity of the plants in the world, and the extremely low levels of such compounds in plants. Application of plant cell culture for the production of useful secondary metabolites, however, is still limited due to the low yield of the desired compounds.

The synthesis of many secondary metabolites in plants is widely believed to be part of the responses of plants to pathogenic attack. The use of elicitors from microorganisms has been one of the most effective strategies for improving the productivity of useful secondary metabolites in plant cell cultures (Roberts and Shuler, 1997). Plant cells respond to fungal elicitor treatment by activating a wide variety of reactions, such as ion fluxes across the plasma membrane, synthesis of reactive oxygen species, and phosphorylation and dephosphorylation of proteins, which have frequently been discussed as putative components of signal transduction chain(s) leading to the elicitor-induced defense responses, such as the activation of defense genes and hypersensitive cell death (Dietrich et al., 1990; Nürnberger et al., 1994; Baker and Orlandi, 1995). However, the molecular basis of elicitor signaling cascades leading to the stimulation of secondary metabolite production is largely unknown.

Nitric oxide (NO) is a bioactive molecule that exerts a number of diverse signal functions in phylogenetically distant species (Beligni and Lamattina, 2000). NO has emerged as a key signaling molecule in plants recently (Neill et al., 2003; Romero-Puertas et al., 2004). Studies have shown that NO generation is a hallmark of plant defense responses to fungal elicitors (Delledonne et al., 1998; Durner et al., 1998). NO is believed to have multiple functions in plants, such as the stimulation of seed germination and root growth, induction of plant defense responses, and defense gene activation (Beligni and Lamattina, 2000; Delledonne et al., 2001; Morot-Gaudry-Talarmain et al., 2002). Recently, NO has been reported to induce the expression of genes related to phytoalexin biosynthesis in...
soybean (*Glycine max*) and tobacco (*Nicotiana tabacum*) cells in culture (Durner et al., 1998; Modolo et al., 2002). In a previous study, we reported that external application of NO via its donor sodium nitroprusside (SNP) induced catharanthine production of *Catharanthus roseus* cells (Xu et al., 2005) and that NO-specific scavenger 2- to 4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPITO) not only suppressed the elicitor-induced NO burst but also blocked the elicitor-induced secondary metabolite production in *C. roseus* and *Taxus chinensis* suspension cells (Xu et al., 2004; Xu and Dong, 2005). These observations suggest the existence of a NO-mediated signaling pathway in elicitor-induced secondary metabolite biosynthesis in plant cells. However, the components of this signal chain and the relationship between NO and other known signal molecules (pathways) are not well characterized.

In addition to NO, jasmonic acid (JA) and its derivatives, such as methyl jasmonate (MeJA), have been recognized as another class of elicitor signal transducers in plant cells (Creelman and Mullet, 1997). JA is derived from the octadecanoid pathway, which involves the peroxidation of linolenic acid by lipoxygenase (LOX). It has been reported that JA and MeJA accumulate rapidly in tobacco and other plant cells after exposure to fungal elicitor (Creelman and Mullet, 1997; Mei, 2003). JA and its octadecanoid precursors have also been implicated as intermediate signals in elicitor-induced secondary metabolite accumulation in plants (Gundlach et al., 1992; Mueller et al., 1993; Ellard-Ivey and Douglas, 1996; Nojiri et al., 1996). In parsley (*Petroselinum crispum*) cells, phenylpropanoid biosynthetic genes are induced by octadecanoids, and the elicitor-induced gene expression is blocked by a LOX inhibitor (Ellard-Ivey and Douglas, 1996). A correlation between elicitor-induced accumulation of endogenous JA and secondary metabolite accumulation has been shown in cells of California poppy (*Eschscholzia californica*; Mueller et al., 1993) and rice (*Oryza sativa*; Nojiri et al., 1996). In *C. roseus* cells, both JA biosynthesis and the expression of terpenoid indole alkaloid biosynthetic genes are induced by fungal elicitor (Menke et al., 1999). External application of the precursor of jasmonate precursor α-linolenic acid or MeJA alone induces the expression of terpenoid indole alkaloid biosynthetic genes, and the fungal elicitor-induced gene expression is blocked by JA biosynthesis inhibitors (Menke et al., 1999). These results suggest that the jasmonate biosynthetic pathway is an integral part of the elicitor-triggered signal transduction pathway leading to the biosynthesis of secondary metabolites in plant cells.

Although both JA biosynthesis and NO accumulation are the best-characterized early responses of plants to elicitor and pathogen infection, their relationship in mediating elicitor-induced useful secondary metabolite biosynthesis in plant cells is still not well characterized. This information is essential for elucidating the elicitor signaling mechanism in plant cells. Here, we report that the elicitors prepared from the cell walls of *Aspergillum niger* stimulated hypericin production of *Hypericum perforatum* cell cultures and that both NO and JA were involved in mediating the elicitor-induced hypericin production in the cells. Furthermore, our results demonstrated that NO and JA triggered the elicitor-induced hypericin biosynthesis at least partially through the same signal transduction pathway within which JA acted downstream of NO.
RESULTS

Elicitor-Induced Hypericin Production

Figure 1 shows the time courses of hypericin production of normal (control) H. perforatum suspension cells and cells treated with 50 μg mL⁻¹ elicitor on day 5. The results showed that the production of hypericin by H. perforatum cells was significantly increased after elicitor treatment, achieving 4.2-fold higher over the control after 25 d of elicitor treatment. This is in agreement with the previous observation, which showed that the elicitor derived from the cell walls of A. niger stimulated secondary metabolite biosynthesis of plant cells (Zhao et al., 2001).

As shown in Figure 2, elicitor-induced hypericin production of the cells is dose dependent. Hypericin production increased relatively with the addition of elicitor over the concentration ranged of 0 to 60 μg mL⁻¹, but once the concentrations of the elicitor exceeded 80 μg mL⁻¹, addition of elicitor actually had a light inhibitory effect on hypericin production.

Elicitor-Induced NO Generation

Greiss reagent and the hemoglobin assay are the most frequently used methods for the determination of NO released by cells (Delledonne et al., 2001; Hu et al., 2003). The elicitor-induced NO generation of H. perforatum cells was measured by both Greiss reagent method (Fig. 3A) and the hemoglobin assay (Fig. 3B) in this work. As shown in Figure 3, A and B, NO in the culture medium increased immediately after elicitor treatment, reaching a first maximum of about 2 μM at 1 h and a second, more sustained one at 9 h, and then fell back to the level of untreated (control) cells at about 21 h. Maximum concentration of NO in the culture medium of elicitor-treated cells reflected about a 5-fold increase above the level of untreated cells.

Nitric oxide synthase (NOS) is instrumental in the generation of NO in the animal systems. As shown in Figure 3B, the activities of NOS were also significantly increased after elicitor treatment, which suggested that NOS or NOS-like enzymes in H. perforatum cells were strongly induced by the fungal elicitor.

Elicitor-Induced JA Accumulation

JA accumulation is one of the common responses of plant cells to fungal elicitors (Creelman and Mullet, 1997; Mei, 2003). As shown in Figure 4, JA contents and LOX activities significantly increased after 6 h of
elicitor treatment, which indicated that the elicitor derived from A. niger triggered JA biosynthesis in H. perforatum cells.

Dependence of the Elicitor-Induced Hypericin Production on NO Generation and JA Biosynthesis

The results of our work indicated that NO generation and JA biosynthesis were two early reactions of H. perforatum cells to the fungal elicitor. However, their relationship and involvement in elicitor-induced hypericin production is not known. To investigate the role of NO and JA in elicitor-induced hypericin production, we determined the effects of NO and JA inhibitors on elicitor-induced hypericin production. As shown in Figure 5, the elicitor-induced hypericin production was blocked by NO-specific scavenger cPITO (Fig. 5C), NOS inhibitor S,S'-1,3-phenylene-bis(1,2-ethanediyl)-bis-isothiourea (PBITU; Fig. 5D), and LOX inhibitors ibuprofen (IBU; Fig. 5E) and nordihydroguaiaretic (NDGA; Fig. 5F), showing that the elicitor-induced hypericin production was dependent on NO generation and JA biosynthesis, or the synthesis of NO and JA were two upstream signaling events essential for the elicitor-induced hypericin production. This conclusion gains further support from the finding that external application of MeJA and NO via its donor SNP reverses the inhibition of JA and NO biosynthesis inhibitors on elicitor-induced hypericin production (Fig. 5, G–I).

Dependence of Elicitor-Induced JA Biosynthesis on NO

To investigate the relationship between elicitor-induced JA biosynthesis and NO accumulation, we studied the effects of PBITU and cPITO on elicitor-induced JA biosynthesis and NDGA and IBU on elicitor-induced NO accumulation. As shown in Figure 6, the elicitor-induced JA biosynthesis was strongly inhibited by NOS inhibitor PBITU and cPITO (Fig. 6B), while JA biosynthesis inhibitors did not significantly affect the elicitor-induced NO generation (Fig. 6A). The results suggested that the elicitor-induced JA production was dependent on NO generation and JA biosynthesis, or the synthesis of NO and JA were two upstream signaling events essential for the elicitor-induced hypericin production.
biosynthesis was dependent on NO generation, or the elicitor-induced NO generation was localized upstream of JA and controlled the biosynthesis of JA.

Dependence of NO-Induced Hypericin Production on JA Biosynthesis

SNP is utilized as the donor of NO to investigate the effect of exogenous NO on hypericin production of *H. perforatum* cells. As shown in Figure 5, both SNP and MeJA induce hypericin production of *H. perforatum* cells in the absence of fungal elicitor (Fig. 5, J and P). SNP-induced hypericin production is blocked by IBU and NDGA (Fig. 5, L and M), while cPITO and PBITU do not inhibit MeJA-induced hypericin biosynthesis (Fig. 5, Q and R). The results indicate that NO triggers hypericin production dependent on JA existence. This conclusion gains further support from the finding that the inhibition of IBU and NDGA on SNP-induced hypericin production is abolished by external application of MeJA (Fig. 5, N and O).

DISCUSSION

This study shows that elicitors prepared from the cell walls of *A. niger* induce NO generation, JA accumulation, and hypericin production of *H. perforatum* cells. The elicitor-induced hypericin production is blocked by NO and JA inhibitors, showing that NO generation and JA biosynthesis are essential for elicitor-induced hypericin production. Pretreatment of cells with NO inhibitors not only suppresses the elicitor-induced NO generation but also inhibits the elicitor-induced JA biosynthesis, which implies that NO and JA act at least partially in the same signaling pathway within which NO is localized upstream of JA. NO-induced hypericin production is blocked by JA biosynthesis inhibitors, indicating that JA is involved in transducing the signal form NO to hypericin production. Thus, our results suggest that NO mediates the elicitor-induced hypericin production through a JA-dependent signaling pathway in *H. perforatum* cells.

So far, the source of NO in plants is far from fully understood. In animals, biosynthesis of NO is primarily catalyzed by the enzyme NOS that oxidizes L-Arg to L-citrulline and NO. In plants, NO can be synthesized either by enzymatic catalysis or by an inorganic nitrogen pathway. During the last few years, several groups have provided evidence for the existence of NOS-like activity in several plant species such as tobacco, soybean, maize (*Zea mays*), pea (*Pisum sativum*), *Lupinus albus*, and *Mucuna hassjoo* (Neill et al., 2003). Mammalian NOS inhibitors have been reported to suppress elicitor-induced NO generation in soybean, tobacco, and *T. chinensis* (Wendehenne et al., 2001; Xu et al., 2004). Recently, a gene encoding a protein with sequence similarity to another protein that has been implicated in NO synthesis in snail *Helix pomatia* was cloned from *Arabidopsis* (*Arabidopsis thaliana*; Guo et al., 2003). These results confirmed the existence of NOS-like activity in plants. NOS-like activities have been identified in several plant species such as tobacco, soybean, maize (*Zea mays*), pea (*Pisum sativum*), *Lupinus albus*, and *Mucuna hassjoo* (Neill et al., 2003). Mammalian NOS inhibitors have been reported to suppress elicitor-induced NO generation in soybean, tobacco, and *T. chinensis* (Wendehenne et al., 2001; Xu et al., 2004). Recently, a gene encoding a protein with sequence similarity to another protein that has been implicated in NO synthesis in snail *Helix pomatia* was cloned from *Arabidopsis* (*Arabidopsis thaliana*; Guo et al., 2003). These results confirmed the existence of NOS in plants. However, other enzymes, such as nitrate reductase, were also found to be implicated in NO synthesis in plants (Yamasaki, 2000; Yamamoto et al., 2003). Furthermore, the nonenzymatic production of NO has recently been demonstrated in seeds (Bertke et al., 2004). In our experiments, NOS-like activity was significantly increased after elicitor treatment, which suggested that NOS or NOS-like enzymes in...
*H. perforatum* cells were strongly induced by the fungal elicitor. However, the NOS-like activities and NO generation in *H. perforatum* cells did not match kinetically, and the elicitor-induced NOS-like activity (production of citrulline) was much lower than NO production (Fig. 3). Therefore, our results suggested that the elicitor-induced NO in *H. perforatum* cells was not entirely dependent on NOS or NOS-like enzymes, other NO-generating systems might exist in the cells, and was mainly responsible for elicitor-induced NO generation.

JA accumulation is another common response of plants to biotic and abiotic stresses. The accumulation of JA relatives occurred rapidly in plant tissues and cells after wounding and exposure to fungal elicitor (Mei, 2003). JA accumulation was induced by fungal elicitor in *C. roseus* cells and was demonstrated to be involved in elicitor-induced expression of the key genes of terpenoid indole alkaloid biosynthesis (Menke et al., 1999). In our study, elicitor prepared from *A. niger* induced LOX activation, JA biosynthesis, and hypericin production in *H. perforatum* cells, and LOX inhibitor IBU and NDGA suppressed not only JA biosynthesis but also hypericin production, which indicated that JA, produced via octadecanoid pathway, was essential for the elicitor-induced hypericin production.

Although both JA accumulation and NO generation are two best-characterized reactions of plant cells to fungal elicitors and are demonstrated to be involved in elicitor-induced secondary metabolite biosynthesis (Modolo et al., 2002; Wu and Ge, 2004), their relationship in elicitor-induced secondary metabolite biosynthesis is still not well established. The previous work has suggested that a signal transduction pathway inducing the production of secondary metabolites mediated by NO might exist in higher plant cells (Modolo et al., 2002; Xu et al., 2004). The results of this work demonstrated that NO and JA mediated the elicitor-induced hypericin production through the same signal pathway within which JA is localized downstream of NO. NO signaling often operates in mammalian cells through cGMP (cGMP)- and cADP Rib (cADPR)-dependent pathways (Wendehenne et al., 2001), and similar mechanisms also appear to be active in plants (Klessig et al., 2000). The involvement of cGMP-dependent components in NO-dependent defense gene activation is suggested by accumulation of phenylalanine ammonia-lyase (PAL) and defense-related protein PR-1 transcripts in tobacco cell suspensions treated with a membrane-permeable analog of cGMP and by suppression of NO-mediated induction of PAL by several inhibitors of mammalian guanylate cyclase (Durner et al., 1998). cADPR has also been reported to induce expression of PAL and PR-1 in tobacco, whereas cADPR antagonists suppress the induction of PR-1 by NO (Durner et al., 1998). Other intracellular targets for NO in mammalian cells are mitogen-activated protein kinases (Huwyler and Pfeilschifter, 1999). Recently, a mitogen-activated protein kinase has been found to be activated by NO in Arabidopsis (Clarke et al., 2000) and in cucumber (*Cucumis sativus*) explants (Pagnussat et al., 2004).

However, most of the information on the biochemical mechanisms by which NO affects intracellular signaling in plants has arisen from studies of plant defense responses, such as the activation of defense genes and hypersensitive cell death (Wendehenne et al., 2002; Neill et al., 2003; Romero-Puertas et al., 2004). Whether these downstream molecules participate in NO signal transduction leading to secondary metabolite biosynthesis in plant cells and their relationships with JA still remain unrevealed. Therefore, it is apparent that we are only at the early stage in understanding the biochemical mechanisms of the NO-signaling pathway leading to secondary metabolite production in plant cells.

**MATERIALS AND METHODS**

**Cell Line and Culture Conditions**

The plant cell line for the study was induced from the young stems of *Hypericum perforatum* growing in Zhejiang Province in China with Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1.0 mg L$^{-1}$ of α-naphthalenacetic acid, 2.0 mg L$^{-1}$ of indole-3-acetic acid, 0.2 mg L$^{-1}$ of kinetin, 20 g L$^{-1}$ of Suc, and 8 g L$^{-1}$ of agar. The callus line had been in culture for 14 months by the time of this study. The suspension culture of the cell line was initiated from the callus culture on a liquid medium similar to that for the callus culture but with 30 g L$^{-1}$ Suc and excluding the agar. The medium was adjusted to pH 5.8 and then sterilized at 121°C for 20 min. The suspension culture was maintained in 250-ml Erlenmeyer flasks with a liquid volume of 100 ml in each flask capped with Magenta B-Caps (Sigma-Aldrich 38648) and incubated on an orbital shaker incubator in the dark at 120 rpm and 25°C. The suspension culture was subcultured every 2 weeks.

**Elicitor Preparation**

The elicitor was prepared from the liquid culture of the isolate of *Aspergillus niger*. Liquid cultures were initiated from 7-d potato dextrose agar by inoculating 2-cm² squares of agar block to 150 ml of potato dextrose agar liquid medium. The flasks were incubated in the dark at 120 runs/min and 25°C for 5 d. The mycelia were collected by filtration, and the elicitor was prepared according to method described by Zhang et al. (2000). The elicitor dose was measured by the total carbohydrate content of the fungal homogenate, which was determined by the phenol/sulfuric acid method using Glc as the standard.

**Hypericin Determination**

Hypericin extraction and determination was carried out as the method reported by Micali et al. (1996). Hypericin was identified by thin-layer chromatography, coelution, and HPLC diode array detection (Hewlett-Packard 1100 LC-DAD HP) compared with standards.

**Determination of Elicitor-Induced NO Generation**

Greiss reagent method and the hemoglobin assay are used to determine NO released by the cells (Delledonne et al., 1998; Hu et al., 2003). Briefly, 1 ml of filtrate of *H. perforatum* suspension cells from various treatments were obtained via passage through a 0.22-μm microsieve, 1 ml of Greiss reagent [1% (w/v) sulfanilamide/0.1% (w/v) N-(1-naphthyl)-ethylenediamines dihydrochloride in 5% (v/v) phosphoric acid] was added for 30 min at room temperature, and the absorbance was quantified spectrophotometrically at 550 nm. Different concentrations of NaNO$_3$ were used to prepare a standard curve. NO released to the culture medium was also determined by monitoring the conversion of HbO$_2$ to metHb as described by Delledonne et al. (1998). HbO$_2$ was added to the filtrate to a final concentration of 10 μM. After 2 min, the changes in the absorbance of the medium at 421 and 401 nm were measured, and NO levels were calculated by using extinction coefficient of 77 μm$^{-1}$ cm$^{-1}$ [A$_{421}$(metHb)-A$_{421}$(HbO$_2$)].
Determination of Intracellular JA

JA was extracted from the cells and quantified following the methods reported by Alami et al. (1999). Fresh mass of cells from the suspension culture was extracted three times with pure methanol (1 mL g⁻¹ fresh weight) using a pestle and mortar in ice. The liquid extract was separated from the homogenate by centrifuging at 12,000g for 20 min at 4°C and then evaporated to dryness in vacuo. The residue was dissolved in 100 μL of methanol and analyzed of the JA content by gas chromatography on an HP59890GC unit with an HP-5 fused silica capillary column of 30 m × 0.25 mm inner diameter and 0.25-μm film thickness (Agilent Technology). The column temperature was initially held at 95°C for 0.5 min, then shifted to 240°C at 4°C/min, and the injector and detector temperatures were set at 240°C and 250°C, respectively. Hydrogen was used as the mobile phase, flowing at a rate of 1 mL min⁻¹.

Extraction and Assay of LOX Activity

LOX was extracted from fresh mass of cells with 50 mM potassium phosphate buffer (pH 7.0) containing 1% (w/v) polyvinylpolypyrrolidone and 10 mM mercaptoethanol. The cell mass was homogenized in the buffer with a pestle and mortar on ice and then centrifuged at 12,000g for 30 min at 4°C to retain a cell-free extract for assay. LOX activity was determined by measuring the formation of 13(S)-hydroperoxylinolenic acid at 25°C using linolenic acid as the substrate (Fourmier et al., 1993). The extract (50 μL) was mixed with quartz cuvette with 1.95 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 50 μM substrate. The change of absorbance at 234 nm, the absorbance peak of 13(S)-hydroperoxylinolenic acid, was measured at 25°C over 2 min after the addition of extract. One unit of LOX activity is equivalent to an increase of 0.01 absorbance units per minute. The protein content of enzyme extract was determined by the method of Bradford (1976) with bovine serum albumin as a standard. The activity of LOX was shown as units mg⁻¹ protein.

Determination of NOs Activity

The activity of H. perforatum cells with various treatments was determined by the citrulline assay method modified from Rees et al. (1995). In brief, 1.0 g of the cell, together with 50 mg of polystyrene-polypyrrolidone, was homogenized in 1.0 mL of cooled extraction buffer (50 mM Tris, pH 7.4, containing 320 mM Suc, 10 mM mercaptoethanol, and 10 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 10,000g for 10 min at 4°C. NOs activity was determined by measuring the formation of L-[U-¹⁴C]citrulline using L-[U-¹⁴C]Arg as the substrate. Forty microliters of the supernatant was added to 100 μL of scintillation liquid and was counted in counter (LS 6000; Beckman).

Test of Elicitor, Inhibitors, SNP, and MeJA on Hypericin Production

Chemicals used in the experiment were mainly obtained from Sigma-Aldrich (cPITO, PBITU, IBU, NDGA, and SNP) and other chemical companies. For the experiments of inhibitors on elicitor- or SNP-induced hypericin production of H. perforatum cells, PBITU, cPITO, IBU, and NDGA dissolved in water or 0.2% dimethyl sulfoxide solution and filtered through 0.22-μm sterile filters (Millipore) were added to 5-d-old cell cultures 20 min before addition of the fungal elicitor or SNP. The cells were filtered and washed with 50 mL of fresh medium to remove the inhibitors after 24 h at which the baseline levels of NO, NOs, and LOX were restored (Figs. 3 and 4) and then resuspended at 0.05 g fresh weight mL⁻¹ in fresh culture medium. The cells were harvested for determination of hypericin after 20 d on which the contents of hypericin reached the highest levels (Fig. 1). For the experiments of external NO and JA on hypericin biosynthesis of H. perforatum cells, 5-d-old cell cultures were treated with NO donor SNP and MeJA. After 24 h, the cells were filtered and washed with 50 mL of fresh medium to remove SNP and MeJA and then resuspended at 0.05 g fresh weight mL⁻¹ in fresh medium. The cells were then cultured for 20 d and harvested for determination of hypericin. Controls received equivalent volumes of vehicle solvent to ensure that they did not interfere with the experiments.

Received May 31, 2005; revised July 8, 2005; accepted July 8, 2005; published September 16, 2005.

LITERATURE CITED


Mei XG (2003) Production of Taxol by Taxus chinensis Cell Culture. East China University Press, Wuhau, China


in phytotherapeutic vegetable extracts and alcoholic beverages.
J Chromatogr A 731: 336–339


