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# Methyl Jasmonate Stimulates Jaceosidin and Hispidulin Production in Cell Cultures of *Saussurea medusa*

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## Abstract

Cell cultures of *Saussurea medusa* produce valuable secondary metabolites, and jaceosidin and hispidulin are the major bioactive compounds. In the present study, the cultures were challenged by methyl jasmonate (MJ). The highest jaceosidin and hispidulin concentrations (65.2 ± 3.67 mg/L and 12.3 ± 0.47 mg/L) were achieved with 5 μ*M* MJ added to 9-d-old subcultures, being 2.2-fold and 4.2-fold, respectively, higher than those from controls. The elicitor had little influence on cell growth, indicating that the changed biological processes did not include alterations in cell division. Furthermore, we observed that the activities of phenylalanine ammonia lyase were transiently increased after treatment with MJ, which suggests that this elicitor modifies jaceosidin and hispidulin production by regulating the phenylpropanoid pathway.

**Index Entries:** Jaceosidin; hispidulin; methyl jasmonate; phenylalanine ammonia lyase; *Saussurea medusa*.

## Introduction

*Saussurea medusa* Maxim. (Asteraceae) is a valuable medicinal plant in China, which has been used to treat arthritis, high-altitude diseases, and

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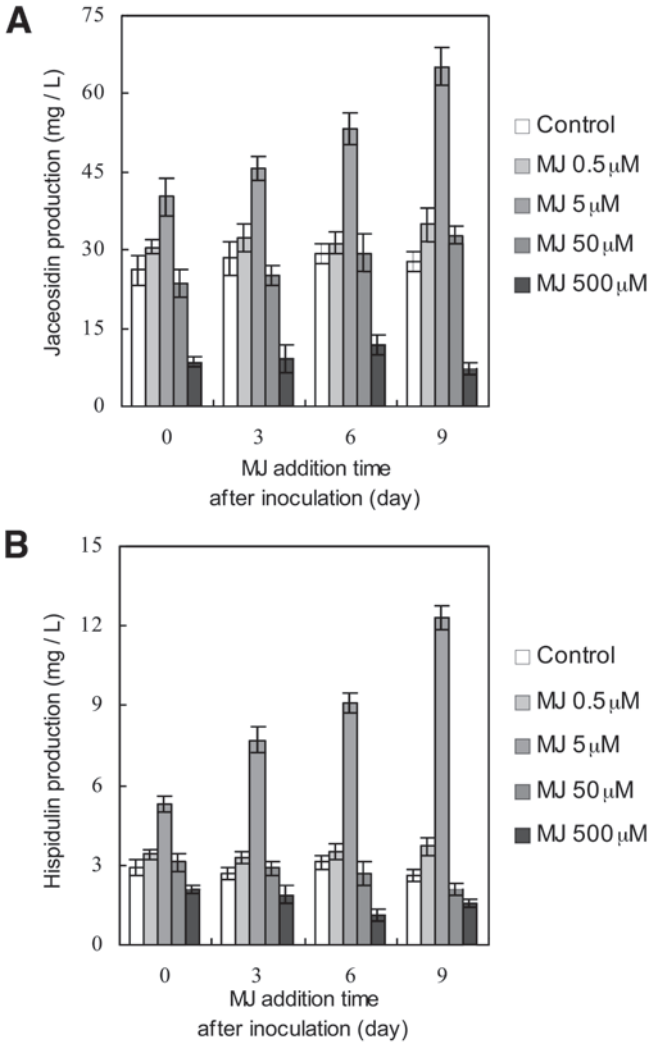


Fig. 1. Effects of MJ on jaceosidin and hispidulin production in suspension culture of *S. medusa.* All cultures were harvested at 12 DAI. Nine shake flasks were used per experimental point and data are means of triplicate determinations. Vertical bars represent standard errors.

gynecological diseases. This is apparently attributable to the many biologically active phenols that it contains *(1)*. Among these compounds, 4′, 5, 7-trihydroxy-3′, 6-dimethoxy flavone (jaceosidin), and 4′, 5, 7-trihydroxy-3′ methoxy flavone (hispidulin), which are derived from the phenylpropanoid pathway, are the major bioactive components *(2)*. Recently, several pharmaceutical experiments on these two compounds have demonstrated anti-cancer, anti-ageing, and anti-inflammatory activities *(3,4)*.

Besides direct extraction from wild *S. medusa* plants that represent a very limited natural supply, cell culture has been developed as a promising

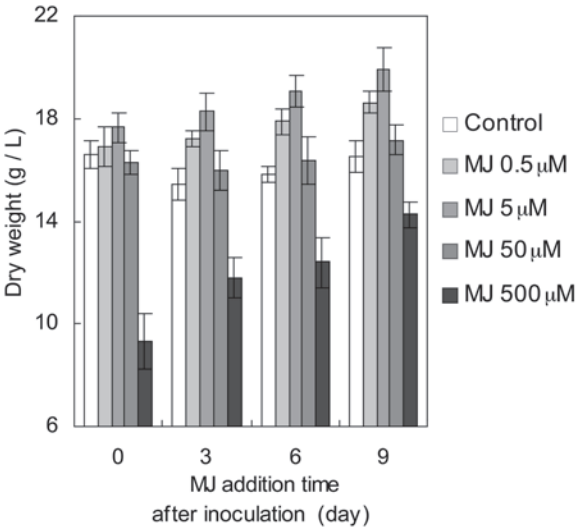


Fig. 2. Effects of dosage and addition time of MJ on cell growth rate. Both elicitor doses and addition times were the same as in Fig. 1. All cultures were harvested at 12 DAI.

alternative for producing the bioactive compounds mentioned previously *(5)*. For commercial exploitation, enhancement of jaceosidin and hispidulin synthesis by *S. medusa* cell cultures has been the focus of our recent research efforts. We have screened for cell lines with high capacity of flavonoids synthesis *(5)*. In a previous study, we challenged the culture system with silver nitrate and glutathion elicitors, and the yields of jaceosidin and hispidulin reached about 1.7-fold and 1.9-fold, respectively, higher than those of the control *(6)*. In the present study, we investigated the effects of Methyl jasmonate (MJ) as an elicitor for jaceosidin formation in the same cell culture system, yet with the hope of an even better outcome. MJ is the methyl ester of jasmonic acid (JA) and both compounds are important hormones regulating many aspects of plants' lives, most notably the defense response to pathogen attack *(7,8)*. Indeed, exogenously applied MJ was able to induce some secondary metabolite production *(9)*. Interestingly, similar responses have been observed in cell cultures *(10,11)*.

Phenylalanine ammonialyase (PAL), which is regarded as the primary enzyme that leads to the phenylpropanoid pathway, and consequently may protect plants against various biotic (infection by viruses, bacteria, fungi, and the like) and abiotic (exogenous chemicals, UV-B light, wounding, and the like) stresses *(12)*. Usually, increased PAL activities and accumulation of many phenolics are observed *(13)*. In our previous studies, the increase of PAL activities was also observed as an early event in the defense response leading to accumulation of flavonoids *(6)*. In the present study, the effect of MJ on PAL activity was investigated.

## Materials and Methods

### Cell Culture and Methyl Jasmonate Treatment

Cell suspension cultures of *S. medusa* were previously established in our laboratory *(5)* and the red cell line has been maintained since then. This line, named according to its color, was found to perform satisfactorily in flavonoids production under optimum culture conditions. Callus was maintained in MS medium supplemented with 30 g/L sucrose, 100 mg/L myoinositol, 2 mg/L α-naphthalene acetic acid, and 0.5 mg/L 6-benzyladenine, at pH 5.8–6.0, 60 μmol/$m^2$ · s light, 25 ± 2°C, 7 g/L agar, and subcultured every 14 or 15 d. Suspension cultures were grown in 100-mL batches on a rotary shaker (110 rpm).

After 12 d in subculture, when the cultures had reached a suitable cell density, large clumps were removed, and single cells and small clumps of about 1.0 g (fresh weight) were inoculated to a 100-mL shake flask containing 25 mL of the same medium without agar (as stated previously). At 0, 3, 6, or 9 d after inoculation (DAI), MJ (Sigma) was added to the cultures, at a final concentration of 0.5, 5, 50, or 500 μ*M* (delivered in 0.25 mL ethanol). As a control, MJ was omitted and only 0.25 mL ethanol was added. For all treatments, cell cultures were harvested on the 12th d after the initial inoculation. Cells were collected by centrifugation, washed with distilled water, filtered using filter paper, and weighed. They were then heated at 60°C to a constant weight and the dry weight was recorded.

### Preparation of Standards

Stock solutions of jaceosidin and hispidulin standards (a generous gift from Prof. Zhong-jian Jia) were prepared in MeOH (0.1 mg/mL). Five different solutions, containing respectively 2, 10, 20, 40, and 50 μg/mL of jaceosidin or hispidulin standard were prepared in MeOH and used for the quantitative study of flavonoids.

### Extraction and Analyses of Flavonoids

Jaceosidin and hispidulin were extracted and determined according to the procedure of Zhao et al. *(6)*, with some modifications. In brief, dried cells were ground using a mortar and pestle, and then 1.0 g sample was extracted using 20 mL of 70% (v/v) ethanol at 50°C for 48 h, with agitation. The extract was vacuum-dried. The residue was then dissolved in 20 mL MeOH and filtered through a 0.2-μm nylon filter.

Jaceosidin and hispidulin were analyzed with an Agilent 1100 preparative HPLC, equipped with a Agilent Zorbax Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm, Agilent Co.). The mobile phase was made up from 90% solvent A (methanol) and 10% solvent B (HPLC grade water acidified to pH 3.0 with orthophosphoric acid). The flow rate was 0.8 mL/min and the injection volume was 10 μL. The chromatogram was monitored at 365 nm with photodiode array detection (DAD). Standards were run the

same way. Limit of detection (LOD) was determined as analyte contents giving signal-to-noise ratios of 3. The compounds were identified by comparison with the authentic samples on the UV spectra. Quantification was based on the peak area of the original sample injection.

### *PAL Activity Assay*

The specific activities of PAL were determined using the method of Zhao et al. *(6)*. Cinnamic acid formed by PAL was measured spectrophotometrically at 290 nm. PAL activity was expressed as nanomoles of cinnamic acid per gram dry weight per hour, which was further standardized by using a relative value: relative PAL activity = $U_{MJ}/U_{Control} \times 100\%$, where $U_{MJ}$ is the PAL activity after treatment with MJ, and $U_{Control}$ is the PAL activity in control culture (i.e., treated with 0.25 mL ethanol only).

### *Statistical Analysis*

Data for dry weight, flavonoids production and PAL activity were collected from triplicate determinations and nine shake flasks were used per experimental point, upon which. A Student's *t*-test was conducted at the 0.05-significance level.

## Results

After inoculation, single cells had grown into various sizes of callus and small calli had grown to larger ones. After MJ addition, cells continued to grow, but at slower rates. At the time of harvest, callus sizes ranged from 2 to 4 mm.

### *Effects of Methyl Jasmonate on Flavonoids Synthesis*

With 5 µ*M* MJ treatment, jaceosidin and hispidulin production were higher than those of control cultures at all time points examined. Addition of 5 µ*M* MJ at 9 DAI resulted great levels of jaceosidin and hispidulin (being 65.2 ± 3.67 and 12.3 ± 0.47 mg/L, respectively), which were about 2.2 and 4.2 times, respectively, higher than those of the control (Fig. 1A,B). Either lower or higher MJ concentrations did not lead to significant changes in jaceosidin and hispidulin production. For example, compared with control cells, jaceosidin or hispidulin production was only slightly different after exposure to 0.5 or 50 µ*M* MJ. In fact, too high MJ concentrations, such as 500 µ*M*, significantly reduced jaceosidin or hispidulin production ($p < 0.05$).

Cell growth was also influenced by MJ, but to less extent (Fig. 2). For example, with 5 µ*M*, the cell growth increased by 20% over the control. At higher concentrations of MJ, cell growth rate was significantly decreased ($p < 0.05$).

The optimum age of culture for elicitation was approx 9 d after subculture, when jaceosidin and hispidulin were produced during the exponential phase of cell growth (Fig. 3). This result shows that jaceosidin and hispidulin accumulation is growth-associated as reported previously *(5)*.

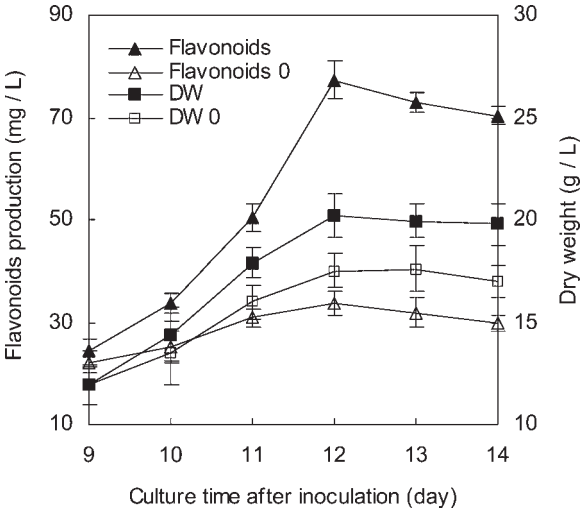


Fig. 3. Time course of flavonoids production and cell growth rate after addition of 5 µ*M* MJ at 9 DAI. The cell cultures were harvested 9, 10, 11, 12, 13, or 14 d after initial inoculation. DW0: dry weight of control cells; Flavonoids 0: jaceosidin and hispidulin in control cells; DW: dry weight of cells in MJ-treated culture; Flavonoids: jaceosidin and hispidulin in MJ-treated culture.

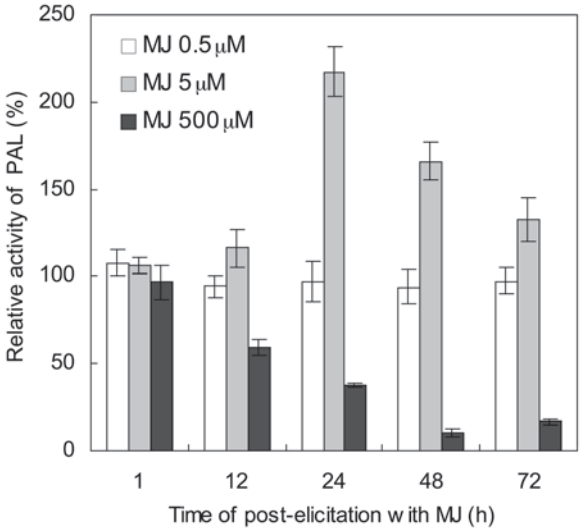


Fig. 4. Time course of specific activities of PAL in suspension cultures of *S. medusa* after treatment with 5 or 500 µ*M* MJ initiated at 9 DAI. Cultures were harvested after 1, 12, 24, 48, or 72 h of incubation with MJ.

## *Specific Activities of PAL After Addition of MJ*

Treatment with 0.5 µ*M* MJ slightly elevated PAL activities (Fig. 4). With 5 µ*M* MJ added on the 9th d of culture, the PAL activity increased from 12 h and reached maximum at 24 h after treatment and then decreased after

MJ treatment ($p < 0.05$). By contrast, 500 μ*M* MJ resulted in a significantly decreased PAL activity ($p < 0.05$).

## Discussion

MJ has been widely used to enhance secondary metabolite synthesis in plant cell culture. For example, MJ is able to increase flavonoids production in transformed *Scutellaria baicalensis* roots *(15)*. Our results suggested that MJ is an effective chemical for increasing the production of jaceosidin and hispidulin in *S. medusa* cell cultures. It has been firmly established that MJ, works as a biosignal to boost the plants' defense system against pathogens *(7)*. In the context of cell culture, it functions most probably as an elicitor or a signal transducer in target cells *(16)*.

A great number of secondary metabolites are derived from the phenylpropanoid pathway. Flavonoids are one group of these metabolites. Rosmarinic acid is another major compound derived from this pathway. Among the enzymes involved is PAL. Accumulation of rosmarinic acid was reported for *Coleus blumei* suspension cultures, which was related to elevated PAL activities *(2)*. Another important enzyme is chalcone synthase (CHS). In suspension cultures of *Petunia*, MJ was able to induce "flavonoids-synthesizing" gene expression, including PAL and CHS, leading to enhanced production of corollasin *(17)*. Our investigations suggest that MJ-enhanced jaceosidin and hispidulin production is brought about, at least in part, through the elevated activity of PAL. These results are similar to those reported in studies on elicitation effects from several other plants *(18–20)*.

The concentration of the elicitor is an important factor to consider. For the MJ concentration series tested, 5 μ*M* was found to be the best for maximum jaceosidin and hispidulin production. In suspension cell culture, the maximum saponin accumulation was achieved using 500 μ*M* MJ *(23)*. The difference is huge.

To optimize secondary metabolism in cell culture systems, the time to add elicitors or other enhancing agents to cell cultures is also critical but this, again, is a variable according to the system. Lu et al. *(21)* reported that for *Panax ginseng* cell culture the most suitable time for elicitor addition was the day of callus inoculation. In the present study, the best time to add MJ is 9 DAI. This result is in line with observations on several other systems *(18–20)*. In some systems, the best time to add the elicitor was around 10 d after inoculation, in terms of enhancement of secondary metabolite production; but once the elicitor was added, cells stopped to grow *(21,22)*. Furthermore, cell growth was not almost related to time for adding the elicitor. Our results showed a different scenario: cells continued to grow with the addition of MJ, only at lower rates.

Why cell cultures from different plant species respond so differently to elicitor treatment? Little information is available for this question. We speculate that there is a trade-off between cell growth and secondary metabolism as affected by elicitors, but this balance is species-specific.

Use of elicitors is one of our strategies to boost jaceosidin and hispidulin production from *S. medusa* cell cultures. Earlier, we challenged the culture system with silver nitrate and glutathion elicitors. The yields of jaceosidin and hispidulin in cell cultures were about 1.7-fold and 1.9-fold (32.01 to 51.25 mg/L and 3.11 to 5.13 mg/L), respectively, higher than those of the control *(6)*. The results of the present work were much better, with the highest jaceosidin and hispidulin production of 65.2 ± 3.67 and 12.3 ± 0.47 mg/L, respectively. The findings from the present study, along with information generated from our previous studies *(5,6)*, will allow us to take an integrative approach toward a successful system for jaceosidin and hispidulin production for commercial scales.

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