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EFFECTS OF METHYL JASMONATE WITH INDOLE-3-ACETIC ACID AND 6-BENZYLAMINOPURINE ON THE SECONDARY METABOLISM OF CULTURED *ONOSMA PANICULATUM* CELLS

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Summary

Methyl jasmonate (MeJA) interacted significantly with both indole-3-acetic acid (IAA) and 6-benzylaminopurine (BA) to influence cell growth of cultured *Onosma paniculatum* cells. Cell growth decreased with increasing concentrations of MeJA from 0.004–4.45 μ*M* with or without IAA and BA. The same concentrations of MeJA (0–4.45 μ*M*) increased the cell growth with IAA and BA, when administered to the cultured cells in M_9 medium. This was found to enhance the production of shikonin. The optimum time for MeJA addition for enhanced shikonin formation was 4 d after cell inoculation in M_9 medium. Furthermore, shikonin formation was affected significantly by both MeJA/IAA and MeJA/BA combinations. Shikonin content was enhanced by increasing MeJA concentrations with IAA concentrations in the range of 0–28 μ*M* and with BA concentrations in the range of 0–44.38 μ*M* in MeJA/IAA and MeJA/BA experiments, respectively. The optimal combination of MeJA and IAA was 4.45 μ*M* and 0.28 μ*M*, while MeJA and BA concentrations of 4.45 μ*M* and 2.22 μ*M* were optimal for shikonin formation. The result also showed that MeJA increased phenylalanine ammonia-lyase (PAL) and *p*-hydroxybenzoic acid-geranyltransferase (PHB-geranyltransferase) activities during the course of shikonin formation, but decreased the activity of PHB-*O*-glucosyltransferase within 9 d after inoculation. These results suggest that enhanced shikonin formation in cultured *Onosma paniculatum* cells induced by MeJA involves regulation of the key enzyme activities.

Key words: Boraginaceae; enzyme activity; *in vitro* culture; medicinal plant; phytohormonal interaction; shikonin.

Introduction

It is well known that jasmonic acid (JA) and methyl jasmonate (MeJA) are signal molecules in biotic and abiotic stresses (van der Fits et al., 2000; Clarke et al., 2001). Exogenous jasmonates applied to plants have been shown to have various morphological and physiological effects, either inhibiting or promoting change (Ji et al., 1995; Suehara et al., 1996; Ketchum et al., 1997; El-Sayed and Verpoorte, 2002). Thus, jasmonates induce gene expression leading to synthesis of many proteins, some of which are probably associated with the defense responses of plants (Reinbothe et al., 1994a, b). Jasmonates have now been associated with the accumulation of some secondary metabolites, which are also part of the defense response (Singh et al., 1998; van der Fits and Memelink, 2000). Thus, while endogenous JA and MeJA accumulate after treatment of cell cultures with a fungal elicitor (Gundlach et al., 1992; Blechert et al., 1995), conversely, treatment with JA or MeJA can elicit the accumulation of several classes of alkaloids (Aerts et al., 1994, 1996; Zabetakis et al., 1999), phenolics (Lee et al., 1997; Sharan et al., 1998), or coumarins (Miksch and Boland, 1996; Sharan et al., 1998) in a wide variety of plant species.

It has also been reported that jasmonate enhanced shikonin or alkannin formation in cell cultures of boraginaceous plants such as *Lithospermum erythrorhizon* in M_9 medium (Gaisser and Heide, 1996) and *Alkanna tictoria* in M_9 medium (Urbanek et al., 1996). Experiments with cultured cells showed that MeJA caused a rapid increase in the activities of enzymes involved in the biosynthesis of shikonin (Yazaki et al., 1997). However, there has been no report of effects of MeJA on growth and shikonin formation in cultured cells of *Onosma paniculatum* (another species for shikonin production), and no attempt has been made to investigate the interaction between exogenous MeJA, indole-3-acetic acid (IAA), and 6-benzylaminopurine (BA) in shikonin production. In the present study, we report on the interactions of MeJA with either IAA or BA in the control of shikonin formation and biochemical mechanisms of MeJA-enhanced secondary metabolism in *Onosma paniculatum* cultured cells.

Materials and Methods

Material preparation and culture methods. The callus used is a somatic line YN121, derived from young shoots of *Onosma paniculatum* (Yang et al.,

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1999). We adopted a two-stage culture system with a growth stage in B_5 medium (solid) and a production stage in M_9 medium (liquid) as described previously (Yang et al., 2003). IAA at 0.28 μ*M* and BA at 4.44 μ*M* were added to the growth medium (B_5) as a basic growth regulator combination, while IAA at 0.57 μ*M* and BA at 4.44 μ*M* were added to the production medium (M_9 liquid). Varying concentrations of MeJA (0, 0.004, 0.04, 0.45, 4.45 μ*M*), IAA (0, 0.28, 0.57, 28 μ*M*), and BA (0, 2.22, 4.44, 44.38 μ*M*) were used to study the interactions of MeJA with IAA (MeJA/IAA) and MeJA with BA (MeJA/BA) on secondary metabolism. In order to exclude the effect of MeJA residual on the production stage, the calluses transferred from the growth stage into the shikonin production stage were fresh, and subcultured in the growth medium without MeJA added.

The method and conditions of callus subculture and harvest for shikonin were the same as previously reported (Yang et al., 2003).

Measurement of cell growth and shikonin content. During the growth and production stages, subculture was carried out every 18 d at 25°C under 80 μmol m^{-2} s^{-1} 8 h light before the cells entered the stationary phase. The cells growing in the media were weighed at the beginning and the end of the culture. The cell increase ratio for fresh weight (CIRFW) was defined as the difference of these two weights divided by the beginning weight (Yang et al., 1999). The content of shikonin and its derivatives (red naphthoquinonone compounds) in the cells were determined as described by Heide and Tabata (1987).

Assay of enzyme activity. Preparation of cell-free extracts and assay for the enzymes involved in shikonin production, including phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), *p*-hydroxybenzoic acid (PHB)-geranyltransferase, and PHB-glucosyltransferase, were conducted as described previously (Yang et al., 2003).

Statistical analysis. All treatments were replicated three times. Statistical analyses were conducted using SAS software (SAS Institute, Cary, NC, USA). One-way ANOVA was used for the time for MeJA addition experiment, separate two-way ANOVAs were used for statistical analyses of MeJA/IAA + BA, MeJA/IAA, and MeJA/BA experiments, and least significant difference (LSD) tests were used for multiple comparisons among treatments.

Results

Interaction of MeJA/IAA + BA on cell growth. MeJA added to the B_5 medium at the first day was shown to have a significant interaction with IAA and BA on cell growth of cultured *O. paniculatum* cells ($P < 0.05$, unpublished data). Without the addition of IAA and BA, MeJA addition at 0.004–4.45 μ*M* significantly increased the CIRFW by 2–8-fold compared with a control (−MeJA/−IAA − BA) (Fig. 1). When IAA and BA were added, MeJA addition at the same concentrations decreased the CIRFW by 9.5–32.2% compared with a control (−MeJA/+IAA + BA). The same concentrations of MeJA tested at 0–4.45 μ*M* increased the cell growth with +IAA/+BA significantly more than that of −IAA/−BA. However, cell growth decreased with increasing concentrations of MeJA from 0.004 to 4.45 μ*M* with IAA and BA (+MeJA/+IAA + BA) or without IAA and BA (+MeJA/−IAA − BA) in B_5 medium (Fig. 1).

Effect of time for MeJA addition on shikonin formation. To determine the optimum time of administration of MeJA for inducing the production of shikonin, 4.45 μ*M* MeJA was administered to cell cultures at intervals of 4 d after their initiation in M_9 medium. The results showed that MeJA administered 4 d after inoculation was the most effective for increasing the yield of shikonin (Fig. 2).

Interactions of MeJA/IAA and MeJA/BA on shikonin formation. In M_9 medium, MeJA interacted significantly with IAA on shikonin formation ($P < 0.01$). In the absence of IAA, compared with a control (0 μ*M* IAA, 0 μ*M* MeJA), MeJA addition at high concentrations (0.45–4.45 μ*M*) increased the content of shikonin and its derivatives by 22.6% and 67.7%, respectively;

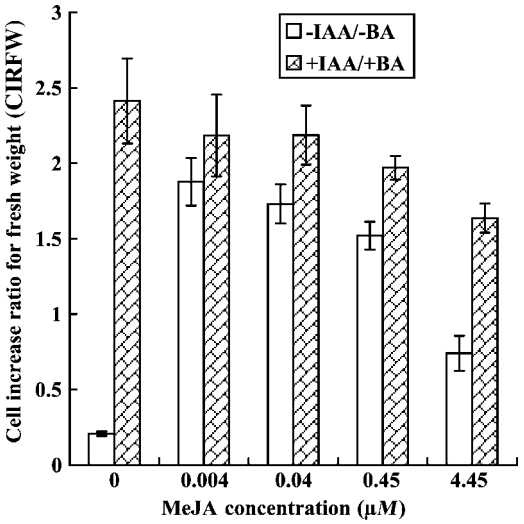

FIG. 1. Effects of MeJA addition on the growth of *Onosma paniculatum* cells in B_5 medium with or without IAA and BA added. IAA and BA were added at 0.28 μ*M* and 4.44 μ*M* in treatments, respectively. *Vertical bars* represent standard deviation (SD) of the means with three replications. LSD 5% = 0.3119.

0.004 μ*M* MeJA inhibited shikonin formation (Fig. 3). When IAA concentration was fixed at 0.28 μ*M*, MeJA addition at 0.04–4.45 μ*M* increased shikonin content by 25.8–80.0% compared with the control (0.28 μ*M* IAA, 0 μ*M* MeJA). Similar effects of MeJA addition on shikonin formation were also found when IAA was fixed at 0.57 or 28 μ*M*. Further, for a given concentration of IAA, increasing concentrations of MeJA resulted in increased shikonin content. In addition, at the same concentration of MeJA, IAA addition at low concentrations produced a greater increase in shikonin than that at higher concentrations. For example, at 4.45 μ*M* MeJA, IAA addition at 0.28, 0.57, and 28 μ*M* increased shikonin content by 38.5%, 21.8%, and 5.1%, respectively, compared with the control (0 μ*M* IAA, 4.45 μ*M* MeJA). Among all MeJA/IAA combinations, 4.45 μ*M* MeJA with 0.28 μ*M* IAA was optimal for shikonin formation (Fig. 3).

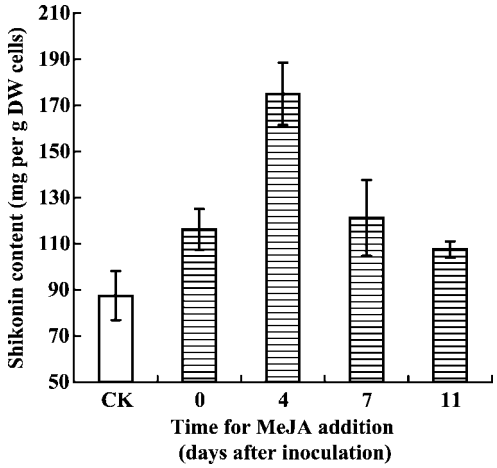

FIG. 2. Effect of time for MeJA addition on shikonin formation of *Onosma paniculatum* cells in M_9 medium. IAA and BA were added at 0.28 μ*M* and 2.22 μ*M* in treatments, respectively. *Vertical bars* represent SD of the means with three replications. LSD 5% = 24.85.

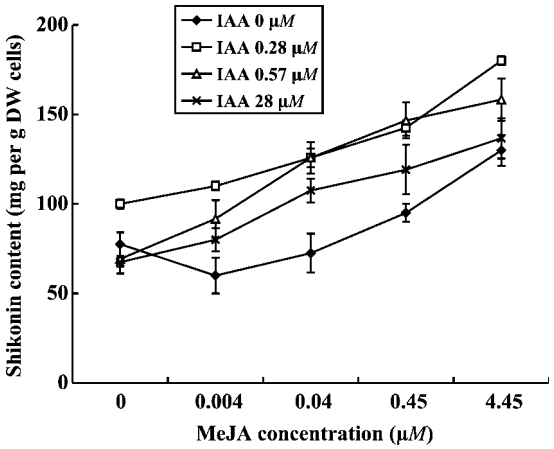

FIG. 3. Effect of MeJA and IAA combinations on shikonin formation of *Onosma paniculatum* cells in M_9 medium. BA was added at 2.22 μ*M* in all treatments. *Vertical bars* represent SD of the means with three replications. LSD 5% = 13.45.

MeJA interacted significantly with BA in controlling shikonin formation ($P < 0.05$). In the absence of BA, MeJA at concentrations in the range of 0.45–4.45 μ*M* significantly promoted shikonin formation by 95.7–143.5% compared with a control (0 μ*M* BA, 0 μ*M* MeJA) (Fig. 4). When the concentration of BA was fixed at 2.22 μ*M*, compared with a control (2.22 μ*M* BA, 0 μ*M* MeJA), MeJA addition at concentrations of 0.004–4.45 μ*M* increased the content of shikonin by 33.3–78.3%. Similar effects of MeJA addition on shikonin formation were also found when BA was fixed at 4.44 or 44.38 μ*M*. For a given concentration of BA added in the range of 2.22–44.38 μ*M*, MeJA also increased shikonin content with increasing concentrations, similar to the effect observed for IAA addition. However, BA at 44.38 μ*M* with MeJA at 0.45–4.45 μ*M* significantly decreased shikonin by 25.6–26.7% compared with the BA-free treatment. Among all MeJA/BA combinations, 4.45 μ*M* MeJA with 2.22 μ*M* BA was optimal for shikonin formation (Fig. 4).

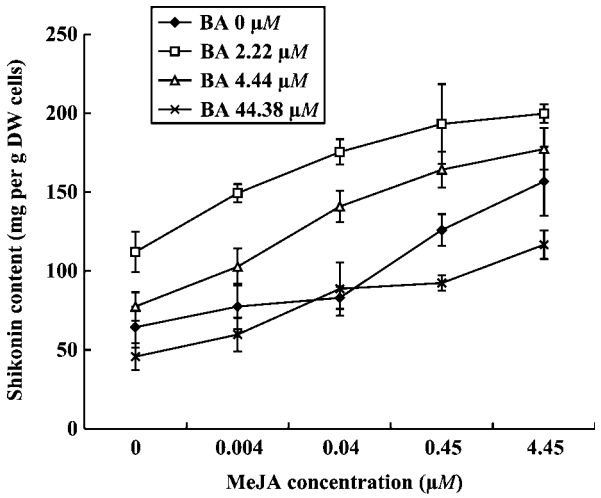

FIG. 4. Effect of MeJA and BA combinations on shikonin formation of *Onosma paniculatum* cells in M_9 medium. IAA was added at 0.28 μ*M* in all treatments. *Vertical bars* represent SD of the means with three replications. LSD 5% = 20.63.

MeJA effect on the activities of enzymes related to shikonin formation. The activities of three enzymes involved in shikonin biosynthesis were measured during the production stage. PAL activity in the cells treated with MeJA was significantly higher than that observed in cells not treated with MeJA during the course of the study (6–18 d after inoculation) (Fig. 5). This same effect was observed for PHB-geranyltransferase activity in the MeJA-treated cells during the same time (6–18 d after inoculation). MeJA addition at 4.45 μ*M* increased the activity of PHB-geranyltransferase by 51.8%, 36.7%, 42.8%, and 37.1%, at the 6th, 9th, 13th, and 18th day after inoculation, compared with controls, respectively (Fig. 6). However, the addition of MeJA significantly decreased PHB-*O*-glucosyltransferase activity from 6 to 9 d after inoculation.

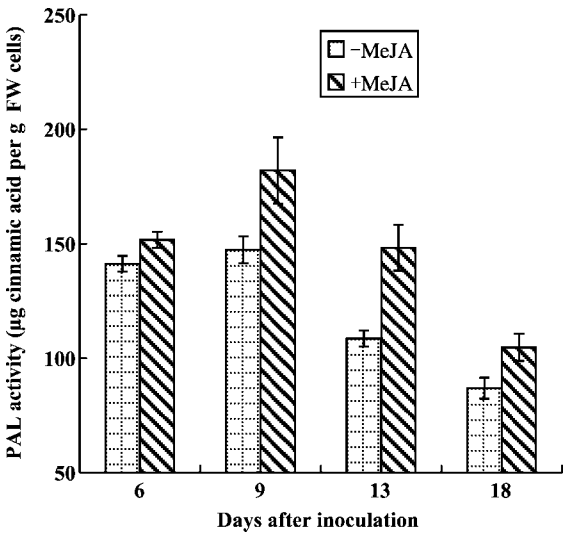

FIG. 5. PAL activity of cultured *Onosma paniculatum* cells with or without MeJA at 4.45 μ*M* added in M_9 medium. IAA at 0.28 μ*M* and BA at 2.22 μ*M* were added in all treatments. MeJA was added on day 4 after inoculation. *Vertical bars* represent SD of the means with three replications.

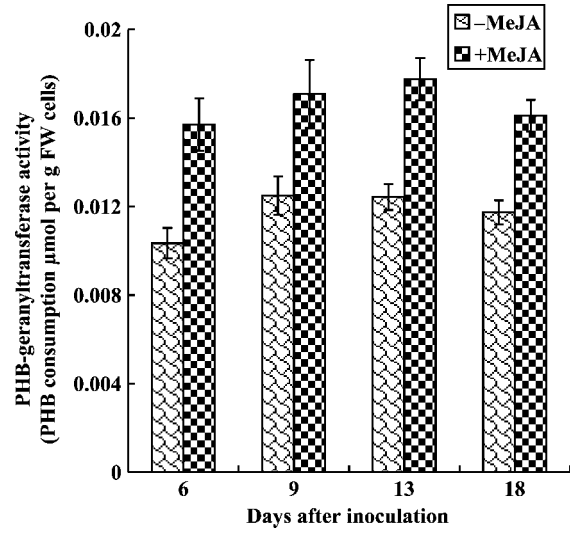

FIG. 6. PHB-geranyltransferase activity of cultured *Onosma paniculatum* cells with or without MeJA at 4.45 μ*M* added in M_9 medium. IAA at 0.28 μ*M* and BA at 2.22 μ*M* were added in all treatments. MeJA was added on day 4 after inoculation. *Vertical bars* represent SD of the means with three replications.

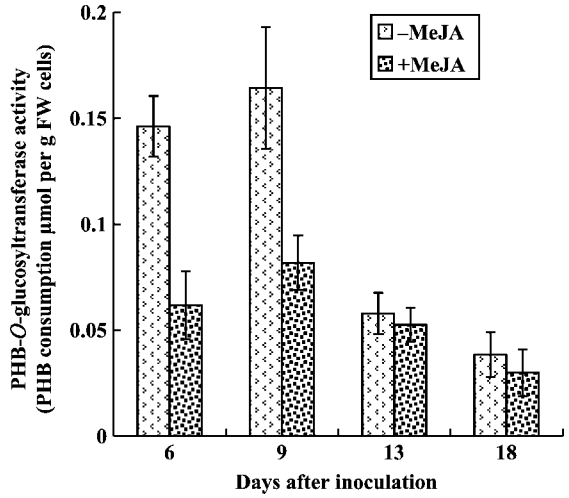

FIG. 7. PHB-*O*-glucosyltransferase activity of cultured *Onosma paniculatum* cells with or without MeJA at 4.45 μ*M* added in M_9 medium. IAA at 0.28 μ*M* and BA at 2.22 μ*M* were added in all treatments. MeJA was added on day 4 after inoculation. *Vertical bars* represent SD of the means with three replications.

MeJA addition decreased the activity of PHB-glucosyltransferase by 57.7% and 50.2%, at the 6th and 9th day after inoculation, compared with controls, respectively (Fig. 7).

DISCUSSION

It is known that JA or MeJA have been associated with the accumulation of some secondary metabolites (van der Fits and Memelink, 2000). Our experiments showed that MeJA increased the content of shikonin and its derivatives (red naphthoquinonone compounds) in *Onosma paniculatum* cultured cells, which corresponds with previous results that MeJA addition can elicit the accumulation of alkaloids (Aerts et al., 1994; Zabetakis et al., 1999), phenolics (Lee et al., 1997), or coumarins (Sharan et al., 1998) in plants. The effects of MeJA on promoting plant secondary metabolism may be associated with the defense responses of plants, in which MeJA plays the important role of second messenger in signal transduction.

In addition, it has been reported that at a certain concentration, MeJA can promote the biosynthesis of endogenous IAA in plants (Grsic et al., 1999; Li and Zhou, 2002). Significant interaction between MeJA and IAA for shikonin formation was also found in cultured *O. paniculatum* cells in the present study. However, at the same concentration of MeJA, the addition of IAA had a greater effect on shikonin content at low concentrations than that at higher concentrations. Furthermore, MeJA at 0.004 μ*M* without the addition of IAA actually inhibited shikonin formation compared with the control (−MeJA/−IAA), suggesting that the addition of IAA at low concentrations is useful for MeJA to enhance shikonin formation. The interactive effects of MeJA and IAA found in the present study seem to support other reports where MeJA and IAA appeared to be additive for the promotion of the percentage of shoots forming roots and number of roots per shoot or the decrease of root length (Dolcet-Sanjuan and Elisabet, 1995) and for ethylene production (Kong et al., 1994).

A significant effect from the interaction between MeJA and BA on shikonin formation also was found in our experiments. It has been reported that MeJA had a significant interaction with BA in the senescence of cotyledons of *Helianthus annuus* (Naik et al., 2002). MeJA promoted senescence parameters (chlorophyll loss, electrolyte leakage, ethylene production, and 1-aminocyclopropane-1-carboxylase oxidase activity) while the effects of MeJA were partially blocked by BA pretreatment (Naik et al., 2002). BA addition at low concentrations (2.22–4.44 μ*M*) showed a combined effect with MeJA on enhanced shikonin formation; however, BA at a high concentration (44.38 μ*M*) inhibited the content of shikonin. This extends our understanding of the known interactive effects between MeJA and BA in plant physiological and metabolic processes.

Our experiments showed that the addition of MeJA can change the activities of some key enzymes involved in shikonin formation. MeJA increased the activity of PHB-geranyltransferase and decreased the activity of PHB-glucosyltransferase, which indicated the involvement of MeJA in the enhanced shikonin formation through the regulation of key enzyme activities.

This MeJA effect on shikonin formation supports other findings that MeJA induced shikonin production in cells of *Lithospermum erythrorhizon* (another species for shikonin formation) through increasing the activities of enzymes involved in the biosynthesis of shikonin, such as PHB-geranyltransferase (Yazaki et al., 1997). The induction patterns observed were similar to those elicited by oligogalacturonides in *Lithospermum* cells, suggesting that jasmonic acid or its derivative may act as a signaling molecule in the elicitation of shikonin biosynthesis (Yazaki et al., 1997).

It has been reported that fungal elicitors can induce the biosynthesis of endogenous MeJA (Blechert et al., 1995), and that a fungal elicitor can promote shikonin formation in *O. paniculatum* cell culture (Ning et al., 1993, 1998). Therefore, our experimental results, demonstrating that MeJA addition promoted shikonin formation in *O. paniculatum* cells, suggest that it was through the regulation of biosynthesis of endogenous MeJA that fungal elicitors enhanced shikonin formation. The molecular and genetic mechanisms for the involvement of MeJA in regulation of shikonin biosynthesis in *O. paniculatum* need to be addressed further.

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