

## Selective Enhancement of Scopadulcic Acid B Production in the Cultured Tissues of *Scoparia dulcis* by Methyl Jasmonate

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**The effects of methyl jasmonate (MeJA) on isoprenoid production were evaluated in cultured tissues of *Scoparia dulcis*. It was found that MeJA suppressed the accumulation of chlorophylls, carotenoids, phytol and  $\beta$ -sitosterol in the tissues. MeJA, however, remarkably enhanced the production of scopadulcic acid B (SDB), with 10  $\mu\text{M}$  being optimal observed concentration for stimulation of SDB production. The maximum concentration of SDB was observed 6 d after MeJA treatment.**

**Key words** scopadulcic acid B; *Scoparia dulcis*; methyl jasmonate; diterpene; biosynthesis

Scopadulcic acid B (SDB) is a tetracyclic diterpene produced by the tropical medicinal plant *Scoparia dulcis*.<sup>1)</sup> It displayed a broad range of biological activities including inhibitory effects on replication of herpes simplex virus type-1 (HSV-1),<sup>2)</sup> gastric proton pump activity<sup>3)</sup> and bone resorption stimulated by parathyroid hormone<sup>4)</sup> as well as antitumor and antitumor promoting activities.<sup>5,6)</sup> The reduced derivative of SDB, known as scopadulciol (SDC), which was also isolated from the plant, was found to be a potentiator of acyclovir and ganciclovir, clinically used as antiherpes drugs. Its synergistic activity was due to the activation of viral thymidine kinase.<sup>7,8)</sup> Because of their unique chemical structures and various biological activities, SDB and SDC were selected as molecular targets for chemical synthesis and the total syntheses of their racemates were accomplished.<sup>9,10)</sup>

Our previous studies on diterpenes of *S. dulcis* revealed that there are three chemotypes based on the major components, *i.e.*, SDB type, SA type producing scoparic acid A and SDX type producing SDC together with scopadiol.<sup>11,12)</sup> These diterpenes were found to be mainly accumulated in younger leaves.<sup>11)</sup> In addition, their production is closely related to the differentiation level of leaves.<sup>13)</sup> Accordingly, we designed a leaf-organ culture system of *S. dulcis* using Murashige–Skoog's culture medium containing 0.1  $\mu\text{M}$  4-PU

(*N*-phenyl-*N'*-(4-pyridyl) urea).<sup>14)</sup>

Recently, the production of secondary metabolites in various plant cell cultures was shown to be highly sensitive to jasmonate. Treatment with methyl jasmonate (MeJA) enhanced the production of taxol and its biosynthetic intermediates in cell suspension cultures of *Taxus*,<sup>15)</sup> and of phenylpropanoid in tobacco cell cultures.<sup>16)</sup> In view of the potential regulatory effect of jasmonate on the production of secondary metabolites, we investigated the effect of MeJA on the production of SDB in the cultured tissues of *S. dulcis*. In order to obtain a comparison, the effects of MeJA on the production of phytol,  $\beta$ -sitosterol and photosynthetic pigments such as chlorophylls and carotenoids in cultured tissues were also examined.

MeJA was reported to suppress growth of tissues and cells.<sup>17–19)</sup> Thus, we examined the dose-dependent effect of MeJA on the growth of the cultured tissues. The indicated concentration of MeJA was added to the medium at day 6 and cultured for 4 d. Although the growth of the tissues was not noticeably inhibited in response to 50  $\mu\text{M}$  or less MeJA, a significant inhibition in growth ( $p < 0.05$ ) was observed when the tissues were treated with 100  $\mu\text{M}$  MeJA (Table 1). This result confirms the anti-cytokinin effect of jasmonate which is known to inhibit cytokinin-induced soybean callus growth and to nullify the senescence-retarding effect of kinetin on an oat leaf segment culture.<sup>20)</sup>

Treatment with MeJA induced a color change in the cultured leaves (from green to yellowish green) suggesting a degradation of the photosynthetic pigments. The decrease in the total content of chlorophylls and carotenoids in the cultured tissues supports this finding (Table 1). The content of chlorophylls in the cultured tissues declined to 53 and 44% of the control tissues when cultured in the presence of 50 and

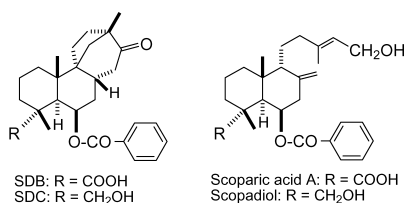


Chart 1

Table 1. Effects of MeJA on the Tissue Growth and the Content of Photosynthetic Pigments in the Cultured Tissues of *S. dulcis*

MeJA ( $\mu\text{M}$ )	Growth (mg F.W.)	Chlorophylls ( $\mu\text{g/g}$ F.W.)	Carotenoids ( $\mu\text{g/g}$ F.W.)
0	845.0 $\pm$ 95.6	562.5 $\pm$ 49.4	97.9 $\pm$ 11.4
10	815.0 $\pm$ 108.5	394.5 $\pm$ 20.2*	73.0 $\pm$ 6.5*
25	802.5 $\pm$ 98.8	387.4 $\pm$ 20.2*	69.1 $\pm$ 2.7*
50	785.0 $\pm$ 131.8	299.2 $\pm$ 11.9**	50.8 $\pm$ 3.6**
100	677.5 $\pm$ 86.5*	252.5 $\pm$ 23.1**	49.6 $\pm$ 8.3**

The tissues were treated for 4 d with different concentrations of MeJA 6 d after inoculation. F.W. means fresh weight. Data represent the mean $\pm$ S.D.,  $n=4$ , \* $p < 0.05$ , \*\* $p < 0.01$ .

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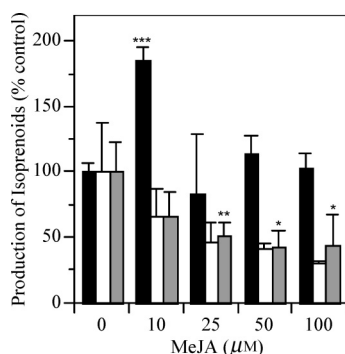


Fig. 1. Effect of MeJA on Isoprenoids Production

The tissues were treated for 4 d with different concentrations of MeJA 6 d after inoculation. After 4 d of MeJA addition, SDB (closed bar), phytol (open bar), and  $\beta$ -sitosterol (gray bar) were quantified. Data represent the mean  $\pm$  S.D,  $n=4$ , \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .

100  $\mu$ M MeJA, respectively. A similar result was observed for the content of carotenoids. A significant reduction of chlorophyll content and carotenoids was observed even at 10  $\mu$ M MeJA. This rapid breakdown of chlorophylls and carotenoids in the tissues treated with MeJA was consistent with the results of experiments using *Arabidopsis thaliana*,<sup>21</sup> and it could be explained by the chlorophyllase gene *AtCLH1* inductive effect, which is known to promote senescence and chlorophyll degradation in plants.<sup>22</sup>

An SDB-type plants of *S. dulcis* produce SDB as a major diterpene together with  $\beta$ -sitosterol.<sup>13</sup> Since exogenously supplied MeJA has been reported to elicit accumulation of secondary metabolites,<sup>15,23</sup> the effects of MeJA on the production of SDB, phytol and  $\beta$ -sitosterol were evaluated. A dose-dependent decrease in the total content of phytol and  $\beta$ -sitosterol was observed in the tissues treated with MeJA, while SDB content in the tissues increased solely in the presence of 10  $\mu$ M MeJA. No significant change was induced at other concentrations. Only this condition significantly enhanced the SDB production ( $p<0.01$ ) when compared with the control or other conditions. It was shown that wounding of plant tissues induced *hmg1* gene that encodes HMGR enzymes involved in the biosynthesis of phytosterols and its induction was suppressed by elicitors in a potato<sup>24</sup> and methyl jasmonate in *Camptotheca acuminata*.<sup>25</sup> These findings might explain the decrease in the content of  $\beta$ -sitosterol in the tissues treated with MeJA. In our previous communication, the involvement of 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway, so called mevalonate-independent pathway, in the biosynthesis of SDB and phytol was suggested, while  $\beta$ -sitosterol was indicated to be biosynthesized *via* the mevalonate pathway.<sup>26</sup> Despite the fact that both SDB and phytol are biosynthesized from geranylgeranyl diphosphate (GGDP) *via* the DOXP pathway, the present results indicate the occurrence of different regulatory mechanisms in diterpene biosynthesis. This might be explained by a flux of GGDP toward SDB production due to MeJA treatment, although the actual mechanism involved in the process remains unclear.

The time course of tissue growth and production of SDB by tissues was followed over a 10 d after treatment with 10  $\mu$ M MeJA. The control tissues without MeJA increased steadily in growth during culture period, while MeJA suppressed tissue growth significantly 6 d after treatment

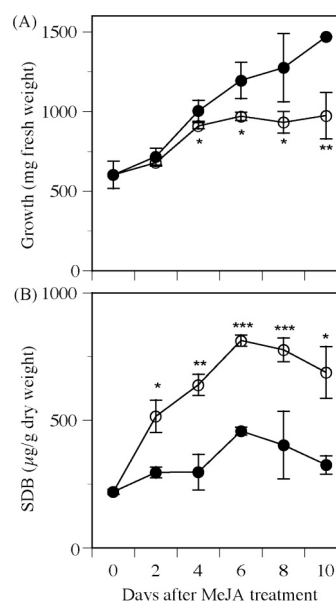


Fig. 2. Time Course of Tissue Growth (A) and SDB Production (B) in the Tissues after Treatment with MeJA (10  $\mu$ M)

The data of control (closed circles) and the treated tissues with MeJA (open circles) are expressed as the mean  $\pm$  S.D,  $n=4$ , \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .

( $p<0.05$ ) (Fig. 2A). On the other hand, SDB production was remarkably enhanced in the presence of MeJA. SDB production increased rapidly over 2 d, peaking at day 6 before falling slightly, although the amount of SDB remained at a significantly greater levels (*ca.* 2-fold) than that of controls ( $p<0.01$ ) (Fig. 2B). It has been reported that MeJA induces production of taxol and other taxoids with a concomitant increase in GGDP synthase and other enzymes involved in taxol biosynthesis.<sup>19</sup> In addition, Martin *et al.* showed that the increased accumulation of resin terpenoids (monoterpenoids and diterpenoids) in Norway spruce was parallel to the increase in prenyltransferase or/and terpenoid synthases.<sup>27</sup> Therefore, MeJA might selectively stimulate biosynthetic enzymes such as GGDP synthase and/or GGDP cyclase and other enzymes involved in SDB production in *S. dulcis*.

#### Experimental

**General Experimental Procedures** GC was performed using a GC-353 (GL Sciences Inc.) gas chromatograph equipped with a SPB-1 fused silica capillary column (30 m  $\times$  0.32 mm  $\times$  0.25 mm film thickness, Supelco Inc.), and N<sub>2</sub> carrier gas with a flow rate of 1.4 ml/min, split ratio 10:1. HPLC was performed on an LC-6A HPLC system equipped with an SPD-6A UV spectrometric detector (Shimadzu Corp.). A MIR-153 incubator (Sanyo Electrical Biomedical Co., Ltd.) and a LX-2100 biophotometer (Taitec Corp.) were used for seed germination and leaf organ cultures, respectively. MeJA was purchased from Wako Pure Chemical Industries Ltd. and the sterile 0.20  $\mu$ m Millipore-LCR filters used in the experiment. All chemicals were of the highest purity commercially available.

**Plant Materials and Culture Conditions** Seeds of *S. dulcis* were obtained from SDB-type plants grown in the herbal garden of Toyama Medical and Pharmaceutical University. Seed germination, culture of seedlings and leaf organ cultures were performed as described previously.<sup>14</sup> The cultured leaf organ tissues were subcultured every two weeks. In all quantitative experiments, about 20 leaf segments were transferred into 100 ml culture flasks containing 50 ml MS medium and incubated on a rotary shaker at 140 rpm at 26  $^{\circ}$ C under continuous illumination (6000 lux). After 6 d, a known volume of filter-sterilized solution of MeJA in DMSO or DMSO as control was added to the medium, and the tissues were harvested 4 d later for quantitative analyses of photosynthetic pigments and isoprenoids.

**Analysis of Chlorophylls and Carotenoids** The content of chlorophylls and carotenoids were determined using the method reported by Lichtenthaler.<sup>28)</sup> Briefly, the cultured tissues were homogenized with 80% acetone and the pigments were extracted with an equal volume of *n*-hexane. The organic pool was dried and the residue was dissolved in 80% acetone. All steps were carried out under diminished light. The optical density of the solution was examined spectrophotometrically at 663.2, 646.8 and 470 nm. The total chlorophylls (Chl *a*+Chl *b*) and carotenoids (Cx+c) were determined on the basis of the following equations:

$$\text{Chl } a = 12.21 \times A_{663.2} + 2.81 \times A_{646.8}$$

$$\text{Chl } b = 20.13 \times A_{646.8} + 5.03 \times A_{663.2}$$

$$\text{Cx+c} = 1000 \times A_{470} - 3.27 \times \text{Chl } a - 104 \times \text{Chl } b / 229$$

**Analysis of Isoprenoids** The harvested tissues were freeze-dried and extracted three times with CHCl<sub>3</sub>-MeOH (3:1) with sonication for 20 min. The combined extracts were evaporated and the residue was dissolved in CHCl<sub>3</sub> (2 ml). The soln. was subjected to a solid phase extraction using a Bondesil-SI Cartridge (50 mg, Varian Inc.). The column was eluted with CHCl<sub>3</sub> (5 ml) and then with MeOH (5 ml). The CHCl<sub>3</sub> eluate containing phytol and  $\beta$ -sitosterol was treated with 6% KOH in MeOH at room temp. overnight and extracted with *n*-hexane. The *n*-hexane fraction was washed three times with an equal volume of H<sub>2</sub>O and then dried under a vacuum. The residue was dissolved in *n*-hexane and then put through a solid phase extraction. The column (Bondesil-SI) was washed successively with *n*-hexane and *n*-hexane-CHCl<sub>3</sub> (1:1) and then eluted with CHCl<sub>3</sub>-MeOH (1:1). After evaporating the eluate, the residue was dissolved in *n*-hexane containing cholesterol (1 mg/ml) as an internal standard and analyzed using GC. Samples (1  $\mu$ l) were injected *via* a split-injector set up to 280 °C onto a column. The oven temp. was maintained at 180 °C for 5 min and then ramped at 10 °C/min to 280 °C and held constant for 24 min, and peaks were detected by FID (300 °C). The content of SDB present in the MeOH eluate was analyzed as described elsewhere.<sup>13)</sup>

**Statistical Analysis** Four samples were analyzed in each experiment and the data represented the mean  $\pm$  S.D. Data were analyzed by the Dunnett's multiple comparisons or by the Student's *t*-test.

**Acknowledgement** The authors wish to thank the staffs of the Herbal Garden of Toyama Medical and Pharmaceutical University for taking care of the plants.

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