Establishment of *Croton stellatopilosus* Suspension Culture for Geranylgeraniol Production and Diterpenoid Biosynthesis

Juraithip Wungsintaweekul[a,*], Chutima Sriyapai[a], Sanlaya Kaewkerd[a], Supinya Tewtrakul[a], Damrong Kongduang[b], and Wanchai De-Eknamkul[b]

[a] Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand. Fax: +66 74 42 82 20. E-mail: juraithip.w@psu.ac.th

[b] Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

* Author for correspondence and reprint requests

Z. Naturforsch. **62 c**, 389–396 (2007); received December 18, 2006/January 19, 2007

Diterpenoids in higher plants are biosynthesized from isoprene units obtained from two distinct pathways: the mevalonate pathway and the deoxyxylulose phosphate pathway. The metabolic partitioning of both pathways in plant species is dependent upon the type of culture. In order to study the diterpenoid biosynthesis in *Croton stellatopilosus* cell culture, callus culture was firstly induced from *C. stellatopilosus* young leaves in Murashige and Skoog (MS) medium in the presence of 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 1.0 mg/l benzyladenine (BA), 3% (w/v) sucrose and 0.8% (w/v) agar. The suspension culture was further induced from its callus in the same medium without gelling agent. Detection of diterpenoid accumulation by gas chromatography-mass spectrometry revealed that a cell culture could accumulate a low amount of geranylgeraniol (GGOH) and a high content of fatty acids and phytosterols. To improve the GGOH production, the culture conditions were optimized by medium manipulation in terms of hormonal factors. The growth rates of cell cultures were similar in all kinds of media. The GGOH production curve indicated that GGOH plays an important role as a primary metabolite in the cell culture. The optimum medium for GGOH production was MS medium supplemented with 2.0 mg/l 2,4-D and 2 mg/l BA that could produce GGOH with a yield of 1.14 mg/g FW.

Key words: Croton stellatopilosus, Geranylgeraniol, Suspension Culture

Introduction

Croton stellatopilosus Ohba (Plau-noi) belongs to the Euphorbiaceae family and is widely distributed in the Southeast Asian region. It was known previously as *C. sublyratus* Kurz. Because of the discovery of stellate-dendritic trichome, the name has been changed to *C. stellatopilosus* Ohba (Esser and Chamarit, 2001). Diterpenelactones, furanoid diterpenes, diterpene alcohols such as plaunotol (18-hydroxygeranylgeraniol), ent-13α-hydroxy-13-epimanol, ent-16β-,17-dihydroxykaurane and an ester of 18-hydroxygeranylgeraniol have been reported from *C. stellatopilosus* (Ogiso *et al.*, 1978; Kitazawa *et al.*, 1979, 1980, 1982; Kitazawa and Ogiso, 1981; Takahashi *et al.*, 1983). Among these compounds, plaunotol is a pharmacologically active component responsible for the eradication of *Helicobacter pylori*, which is highly associated with gastritis and peptic ulcers and accelerates ulcer healing effects (Ogiso *et al.*, 1985; Koga *et al.*, 2002). Furthermore geranylgeraniol (GGOH), a precursor of plaunotol, was shown to induce apoptosis in various lines of cancer cells (Takeda *et al.*, 2001).

Because of the pharmacological potential of plaunotol and GGOH, attempts in searching for an alternative source of these have been made. Types of cultures, kinds of media, hormones and gelling agents have been investigated to increase the plaunotol production in tissue cultures (Morimoto and Murai, 1989; Shibata *et al.*, 1996). However, yields of plaunotol were low because of the slow growth rate of the cells. It can be noted that a complexity of cells is associated with the plaunotol biosynthesis, which has not been observed in unorganized cells (Morimoto and Murai, 1989).

As part of our interest in diterpenoid biosynthesis, we aimed to establish a model culture by induction of *C. stellatopilosus* callus and suspension cultures. This study reports a culture condition

that was optimized to improve the GGOH production. This model culture will be useful for further studies of the diterpenoid biosynthesis.

Experimental

Chemicals

Authentic geranylgeraniol (GGOH) was purchased from Sigma-Aldrich Pte., Ltd. (Singapore). 2,4-Dichlorophenoxyacetic acid (2,4-D) and plant agar were from Duchefa Biochemie (Haarlem, The Netherlands). *N*-Benzyladenine (BA), 1-naphthylacetic acid (NAA) (95% GC), and 6-furfurylamino-purine (kinetin) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Phytagel™ was from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals were standard commercial products of analytical grade.

Plant material

Young leaves of *Croton stellatopilosus* Ohba used for callus induction were collected from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. A voucher specimen of this plant was deposited in the Herbarium, Royal Forest Department in Bangkok, Thailand, under the number 21867.

Callus culture

Young leaves of *C. stellatopilosus* were soaked in 70% (v/v) ethanol (Merck KGaA) for 3 min, sterilized in 0.5% (w/v) sodium hypochlorite (Clorox®) for 5 min and washed thoroughly with sterile distilled water. The sterile leaves were excised and placed on Murashige and Skoog (MS) (Murashige and Skoog, 1962) agar medium containing 2.0 mg/l 2,4-D, 1.0 mg/l kinetin and 3% (w/v) sucrose solidified with 0.8% (w/v) plant agar. The leaf explants were maintained under a 16-h photoperiod (2000 lux) at (25 ± 2) °C. Under these conditions, calli were formed and rapid proliferation occurred within 2 weeks. After 4 weeks, the calli formed were maintained by subculturing every 3 weeks on MS agar medium containing 1.0 mg/l 2,4-D, 0.1 mg/l BA, 0.1 mg/l NAA, 3% (w/v) sucrose and 0.8% (w/v) plant agar. The callus culture appeared to be friable with the greenish colour of the tissues.

Suspension culture

The maintained callus culture was used for preparation of the suspension cultures. Firstly, maintained calli were subcultured on MS agar media supplemented with different compositions of sugar, hormones and gelling agents as follows: 1) MS1: 1.0 mg/l 2,4-D, 0.1 mg/l NAA, 0.1 mg/l BA, 3% (w/v) sucrose, and 0.8% (w/v) plant agar; 2) MS2: 1.0 mg/l 2,4-D, 1.0 mg/l BA, 3% (w/v) sucrose, and 0.8% (w/v) plant agar; 3) MS3: 2.0 mg/l NAA, 2.0 mg/l BA, 1% (w/v) sucrose, and 0.2% (w/v) Phytagel™, kept at (25 ± 2) °C under a 16-h photoperiod. After 3 weeks, suspension cultures were further induced from callus cultures in the media, containing the same composition but without gelling agent. The suspension cultures were incubated at (25 ± 2) °C and a 16-h photoperiod with shaking at 120 rpm. The suspension cultures were maintained in each medium and subcultured every 3 weeks.

Screening of geranylgeraniol by gas chromatography

Suspension cultures (2 g) were harvested and extracted with 10 ml of absolute ethanol under reflux for 1 h, and filtered. The filtrate was evaporated under reduced pressure (Speedvac Plus SC210A, Savant, Holbrook, NY, USA). The residue was re-dissolved in 50% (v/v) methanol. Solution of 10% (w/v) NaOH was added to a final content of 1% (v/v) and the mixture was heated at 70 °C for 30 min. The solution was then partitioned with *n*-hexane three times. Pooled *n*-hexane fractions were dried under reduced pressure. For GC analysis, the residue was further dissolved in 500 μl of *n*-hexane, centrifuged and 2 μl of the solution was analyzed by a GC Agilent 6850 instrument equipped with a flame ionization detector (FID). The GC conditions for GGOH determination were: HP1 methylsiloxane column (Hewlett Packard, CA, USA), 30 m × 0.32 mm i. d., 0.25 μm film thickness, flow 1.0 ml/min helium; splitless injection; temperature program from 220 °C (hold 3 min) to 280 °C (rate 10 °C/min) and hold of 2 min; injector temperature 300 °C; detector temperature 280 °C; sample size 1.0 μl. GGOH was eluted at 6.2 min.

Isolation of geranylgeraniol, fatty acids and phytosterols

Cells in MS2 were harvested, washed thoroughly with distilled water and dried in a hot air oven at 55 °C for 12 h. The dried cells (50 g) were ground in a mortar and extracted with 300 ml of

methanol. A solution of 10% (w/v) NaOH was added to give a final content of 1% (v/v) and the mixture refluxed for 1 h, filtered and evaporated. The residue was dissolved in 100 ml of 80% (v/v) methanol and partitioned with 100 ml of *n*-hexane three times. The *n*-hexane fractions were pooled, evaporated to dryness (1.3 g) and loaded on the top of a silica gel (silica gel 60, Scharlau GE0048, La Jota, Barcelona, Spain) column (7 × 20 cm). The column was consecutively eluted with CH_2Cl_2/MeOH 95:5, 90:10, 80:20, and 70:30. The components of fatty acids and geranylgeraniol were analyzed by GC-MS. The other fraction was purified on a silica gel column and eluted with CH_2Cl_2/*n*-hexane 6:4. Two compounds were obtained and their structures were elucidated by ^{1}H NMR, ^{13}C NMR and MS and by comparison with authentic stearic acid and phytosterols.

Medium manipulation

The hormonal factors in MS media were manipulated in terms of different concentrations of 2,4-D and BA. The cells were harvested every 2 d of the culture period (18 d). The cell growth rate and GGOH production rate of the investigated suspension cultures from different media were evaluated.

Determination of fresh weight (FW): Cells were harvested, washed thoroughly with an excess of distilled water, dried on filter paper, and weighed.

Determination of GGOH: The harvested cells were extracted by the same procedure as the sample used for screening the GGOH content in the cells. Determination of GGOH was performed by using gas chromatography. All sample analyses were carried out in triplicate.

Quantification of geranylgeraniol content

Extraction of GGOH from cell cultures was performed as described. Each of the resulting extracts was determined for its GGOH content by gas chromatography. GC analysis was carried out using a GC Agilent 6850 instrument equipped with a flame ionization detector (FID) under the same conditions as described above. The peak areas were converted to concentration by using a calibration curve. The calibration curve of GGOH was established. The linearity of the GGOH calibration curve was observed in the range 0.005–0.125 mg/ml with r^2 more than 0.9999. Each calibration point was carried out in triplicate. For inter-day analysis, the calibration curve was checked for consistency prior GC analyses.

Gas chromatography-mass spectrometry

GC-MS was performed on a HP5890 gas chromatograph-HP5972 mass selective detector (Hewlett Packard), equipped with electron ionization (inlet temperature 200 °C; injection split mode; transfer line temperature 300 °C; threshold 100; mass range 20–550 amu). The mixture was analyzed on a HP-1 methylsiloxane (30 m × 0.25 mm i. d.; film thickness 0.25 μm) capillary column (Hewlett Packard). The helium carrier gas had a delivery rate of 1 ml/min (controlled constant flow) and a column temperature programming as follows: 70 °C initial temperature, then the temperature was increased to 300 °C for 15 min at the rate of 7 °C/min, sample size 1.0 μl. Compounds were identified using mass spectral fragmentation patterns. The WILEY275 database was used for automatic identification of the GC peaks.

Spectroscopy

^{1}H and ^{13}C NMR spectra were measured with a JEOL NMR spectrometer (^{1}H–270 MHz; ^{13}C–67.5 MHz; JEOL, Co., Tokyo); the chemical shifts are represented as δ (ppm) with tetramethylsilane (TMS) as the internal standard. Samples were dissolved in $CDCl_3$ prior to determination by the NMR spectrometer. An electron-impact mass spectrometer (EI-MS) as high-resolution mass spectrometer, JEOL JMS-GC MATE, was used to detect mass spectra of samples.

Results

Callus and suspension cultures of Croton stellatopilosus

Young leaves containing a high yield of plaunotol were used to induce a callus culture according to a previous report (Vongchareonsathit, 1994). In an attempt to establish a model culture for the diterpenoid biosynthetic study, callus cultures were pre-cultured in three kinds of media differing in sugar content, hormone types and gelling agents. The characteristics of the callus cultures were differences between calli in MS1, MS2 and MS3. Cells in MS1 and MS2 were soft, friable and yellow-green in colour, whereas cells in MS3 were hard, friable and green. This evidence

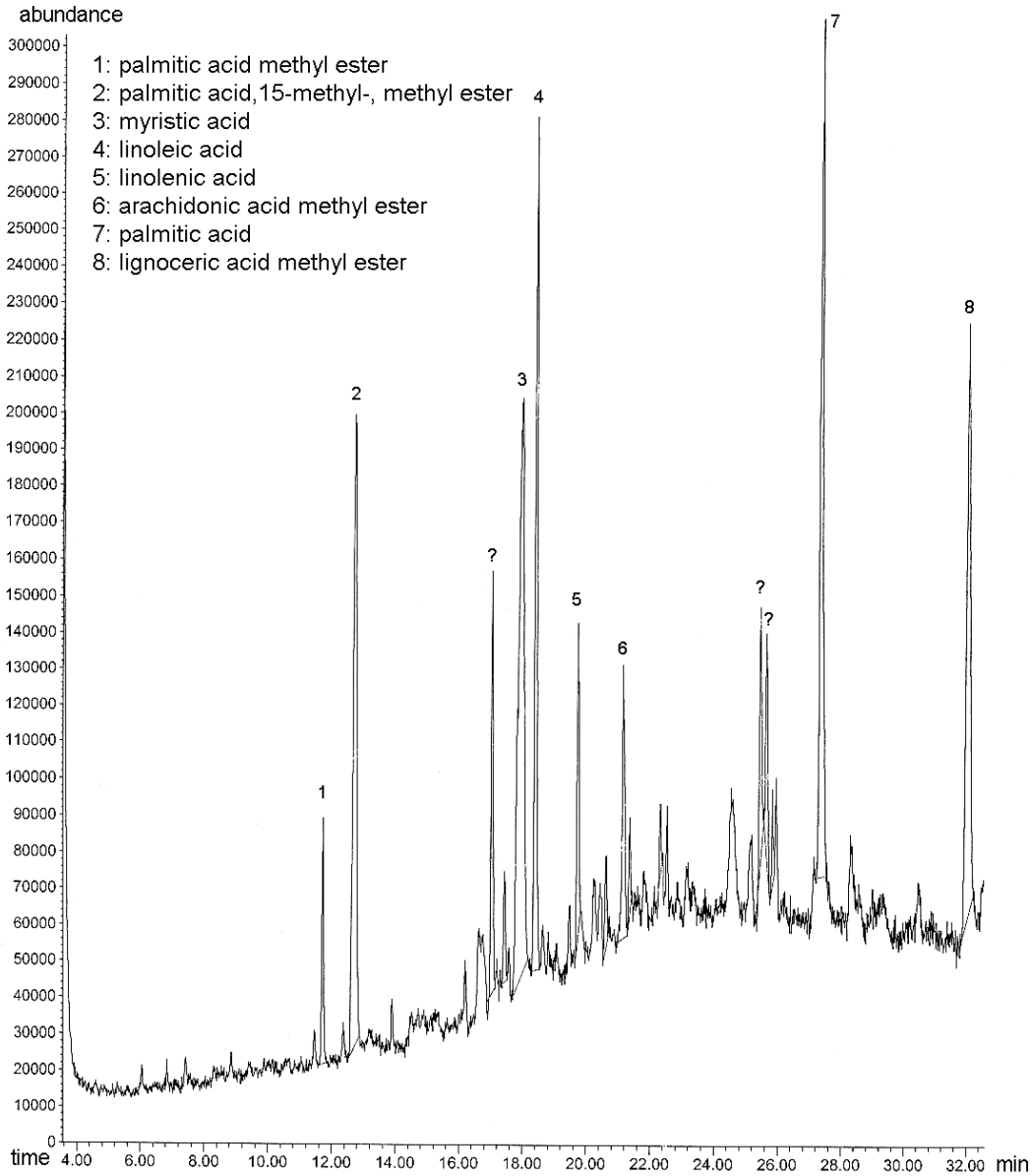

Fig. 1. Gas chromatogram of fatty acid fraction isolated from *C. stellatopilosus* suspension culture.

showed that the amount of sucrose, hormones and gelling agent affected the callus characteristics. Establishment of suspension cultures was performed from callus cultures in the same media. The appearance of suspension cultures was observed after the 2nd passage of subculture. Suspension cultures appeared to be homogeneous cells with pale-yellow colour. However, the cells in MS2 were more aggregated and grew faster than cells in MS1 and MS3, respectively.

Accumulation of geranylgeraniol, fatty acids and phytosterols

Preliminary screenings of plaunotol in *C. stellatopilosus* calli and suspension cultures were performed using the gas chromatography technique. None of the cultures could produce plaunotol. However, the *n*-hexane extracts of suspension cultures from MS1, MS2 and MS3 were analyzed in comparison with the authentic GGOH. The abili-

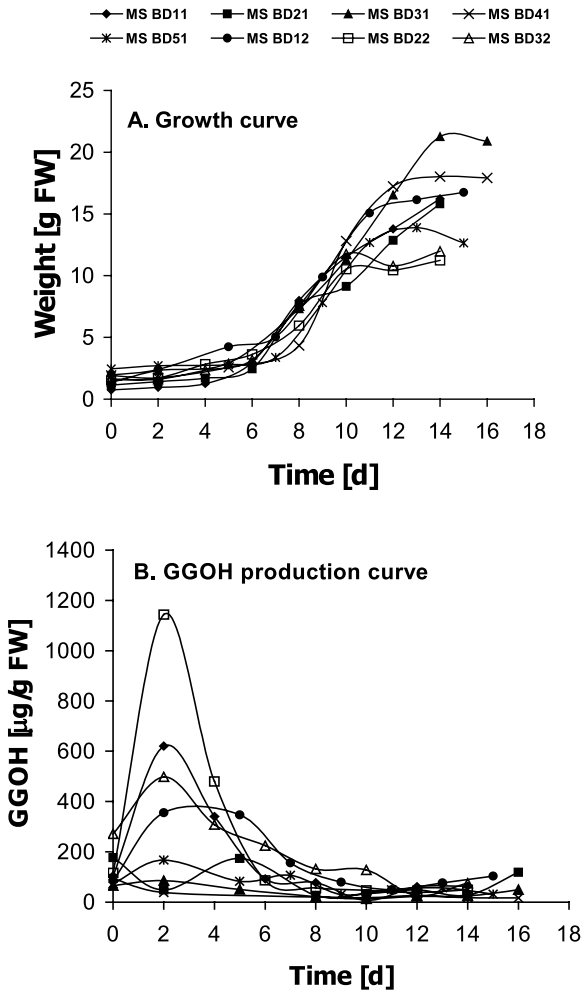

Fig. 2. Medium manipulation of *C. stellatopilosus* suspension culture: (A) growth curve; (B) geranylgeraniol (GGOH) production curve.

ties of GGOH production in each medium were evaluated. The result showed that only cells grown in MS2 could produce and accumulate GGOH. Mass spectral data of the peak at 6.2 min confirmed it to be GGOH, as similar fragmentation compared with the authentic GGOH was found.

Additionally, secondary metabolites, obtained from MS2, were purified using silica gel column chromatography. Isolation of pure GGOH failed because of the small amount and losses during the process of isolation. After one column of *n*-hexane extract, two fractions were obtained. These fatty acid fractions were analyzed by GC-MS. The resulting data in comparison with the WILEY275 database indicated that the fraction contained a mixture of fatty acids, saturated and unsaturated, as shown in Fig. 1. Another fraction was further purified, and two fractions were obtained. One fraction produced needle crystals whose structure was elucidated using ^{1}H and ^{13}C NMR spectroscopy and identified as phytosterols. The fraction that contained pure compound was analyzed by ^{1}H and ^{13}C NMR. NMR data indicated that this compound was a fatty acid as it contained a carboxy group and was a long chain hydrocarbon. Mass spectral data showed, a molecular mass of 284 and fragmentations at *m*/*z* 73 [base peak], 59, 87, 115, 143, 171, 199, 227, confirming the NMR data that this compound was stearic acid.

Improvement of geranylgeraniol production

As shown above, cells in MS2 could produce and accumulate a low amount of GGOH. In this study, the medium manipulation was investigated in order to improve the GGOH production. The concentration of 2,4-D and BA was varied and added to MS2 medium. Cell growth and GGOH content were evaluated. Cells from MS2 were harvested and analyzed every 2 d up to day 18. In eight kinds of media, cells were grown with a similar pattern. The growth cycle was 15 d: 5 d for the lag phase, 2 d for the exponential phase, 3 d for the linear phase and then gradual entering into the stationary phase (Fig. 2A). From the GGOH production curve it can be seen that the GGOH content increased dramatically in the lag phase and gradually decreased in the stage of the exponential phase (Fig. 2B). From this study, it is indi-

Table I. The GGOH content of cells in MS2 medium at day 2 after subculture.

Medium	Contents of BA:2,4-D[a] [mg/l]	GGOH content [mg/g FW]
MS BD11[b]	1:1	0.62 ± 0.023
MS BD21	2:1	0.05 ± 0.009
MS BD31	3:1	0.08 ± 0.002
MS BD41	4:1	0.04 ± 0.005
MS BD51	5:1	0.17 ± 0.008
MS BD21	1:2	0.36 ± 0.023
MS BD22	2:2	1.14 ± 0.006
MS BD32	3:2	0.50 ± 0.003

[a] BA, benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid.
[b] Numbers indicate the concentration of BA and 2,4-D, respectively.

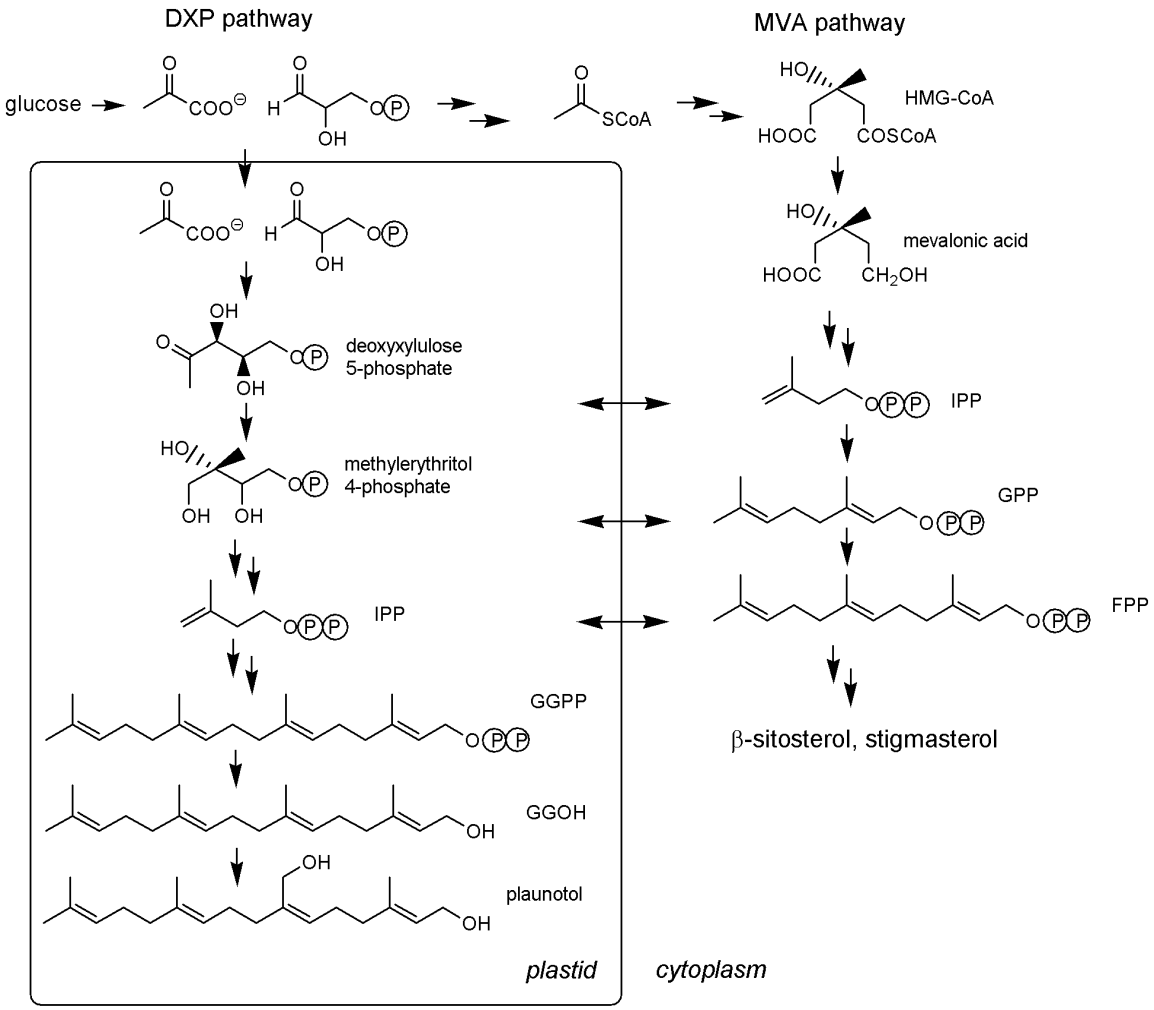

Fig. 3. Cross-talk between the cytosolic mevalonate (MVA) pathway and plastidic deoxyxylulose phosphate (DXP) pathway in isoprenoid biosynthesis.

cated that GGOH played a role as the primary metabolite. The amount of 2,4-D and BA affected the GGOH production in the cells. As shown in Table I, cells grown in MS2 that was supplemented with 2.0 mg/l 2,4-D and 2.0 mg/l BA could produce GGOH with a yield of 1.14 mg/g FW, which is two times higher than that of the original medium.

Discussion

In this study we attempted to establish a model plant culture for diterpenoid biosynthesis. A suspension culture of *C. stellatopilosus* was manipulated in terms of hormonal factors in the basal medium in order to augment the geranylgeraniol production. Growth and production curves of investigated suspension cultures suggested that geranylgeraniol was produced in the lag phase and gradually decreased in the later stages. Therefore, geranylgeraniol acts as primary metabolite in the cells and is further utilized to produce terpenoid compounds such as derivatives of geranylgeraniol (Kitazawa *et al.*, 1982). Lack of essential enzymes to convert geranylgeraniol into diterpenoid compounds in suspension culture can be suggested, probably caused by the absence of organelles. High content of phytosterols and fatty acids, accumulated in the cells, lead to the conclusion that

the cells have mostly produced the fundamental elements for survival.

Nowadays, a building block of terpenoid compounds in higher plants, the isoprene unit, can be supplied from two distinct pathways: the classical mevalonate (MVA) pathway and the deoxyxylulose phosphate (DXP) or non-mevalonate pathway as described in Fig. 3. Monoterpenoids, diterpenoids, and carotenoids are produced from the plastidic DXP pathway, whereas sesquiterpenoids and steroids arise from the cytosolic MVA pathway. Cross-talk could occur in between two pathways by transference of IPP or metabolite downstream from IPP such as GPP, FPP (Sponsel, 2002) (Fig. 3). Studies of diterpenoid biosynthesis in *C. stellatopilosus* have been reported recently. Feeding experiments of labeled glucoses into the shoot and green callus of *C. stellatopilosus* suggested that the isoprene units can be supplied from different sources according to plant maturation. In genuine plants, the isoprene units, incorporated in the skeleton of plaunotol, originate from the DXP pathway (Wungsintaweekul and De-Eknamkul, 2005). In contrast, observation of the labeling pattern of the β-sitosterol skeleton after [1-^{13}C]glucose feeding experiment into the green callus revealed the mixed origin of isoprene units (De-Eknamkul and Potduang, 2003). It can be noted that the metabolic partitioning of both pathways in plant compartments is dependent upon the type of plant source. Interestingly, it has been reported that the effect of the gelling agent on plaunotol production is correlated to chlorophyll formation (Morimoto and Murai, 1989). Therefore, the ability to produce plaunotol is absolutely dependent upon the plastid compartment as it is in line with the finding of localization of plaunotol in the plastid (Sitthithaworn *et al.*, 2006). A study of the origin of the isoprene unit in geranylgeraniol accumulated in the cells may be useful for understanding the diterpenoid biosynthesis, which is currently under investigation in our lab. The high-production of cells of geranylgeraniol can be used as a model for diterpenoid biosynthetic studies.

Acknowledgement

This work was supported by the National Center for Genetic Engineering and Biotechnology (BIOTEC) and Prince of Songkla University grants. The authors thank Dr. Mazayuki Yoshikawa, Kyoto Pharmaceutical University for NMR and MS measurement.

De-Eknamkul W. and Potduang B. (2003), Biosynthesis of β-sitosterol and stigmasterol in *Croton sublyratus* proceeds via a mixed origin of isoprene unit. Phytochemistry **62**, 389–398.

Esser J. and Chamarit K. (2001), Two new species and a new name in Thai *Croton* (Euphorbiaceae). Thai For. Bull. **29**, 51–57.

Kitazawa E. and Ogiso A. (1981), Two diterpene alcohols from *Croton sublyratus.* Phytochemistry **20**, 287–289.

Kitazawa E., Ogiso A., Takahashi S., Sato A., Kurabayashi M., Kuwano H., Hata T., and Tamura C. (1979), Plaunol A and B, new anti-ulcer diterpene lactones from *Croton sublyratus.* Tetrahedron Lett. **13**, 1117–1120.

Kitazawa E., Sato A., Takahashi S., Kuwano H., and Ogiso A. (1980), Novel diterpene lactones with antipeptic ulcer activity from *Croton sublyratus.* Chem. Pharm. Bull. **28**, 227–234.

Kitazawa E., Kurabayashi M., Kasuga S., Oda O., and Ogiso A. (1982), New esters of a diterpene alcohol from *Croton sublyratus.* Ann. Rep. Sankyo Res. Lab. **34**, 39–41.

Koga T., Inoue H., Ishii C., Okazaki Y., Domon H., and Utsui Y. (2002), Effect of plaunotol in combination with clarithromycin or amoxicillin on *Helicobacter pylori in vitro* and *in vivo*. J. Antimicrob. Chemother. **50**, 133–136.

Morimoto H. and Murai F. (1989), The effect of gelling agents on plaunotol accumulation in callus cultures of *Croton sublyratus* Kurz. Plant Cell Rep. **8**, 210–213.

Murashige T. and Skoog F. (1962), A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant **15**, 473–497.

Ogiso A., Kitazawa E., Kurabayashi M., Sato A., Takahashi S., Noguchi H., Kuwano H., Kobayashi S., and Mishima H. (1978), Isolation and structure of antipeptic ulcer diterpene from a Thai medicinal plant. Chem. Pharm. Bull. **26**, 3117–3123.

Ogiso A., Kitazawa E., Kobayashi S., Komai T., Matsunuma N., and Kataumi S. (1985), Plaunotol (CS-684), A new anti-ulcer agent. Annu. Rep. Sankyp Res. Lab. **37**, 1–39.

Shibata W., Murai F., Akiyama T., Siriphol M., Matsunaga E., and Morimoto H. (1996), Micropropagation of *Croton sublyratus* Kurz – a tropical tree of medicinal importance. Plant Cell Rep. **16**, 147–152.

Sitthithaworn W., Potduang B., and De-Eknamkul W. (2006), Localization of plaunotol in the leaf of *Croton stellatopilosus* Ohba. Science Asia **32**, 17–20.

Sponsel V. M. (2002), The deoxyxylulose phosphate pathway for the biosynthesis of plastidic isoprenoids: early days in our understanding of the early stages of gibberellin biosynthesis. J. Plant Growth Regul. **20**, 332–345.

Takahashi S., Kurabayashi M., Kitazawa E., Haruyama H., and Ogiso A. (1983), Plaunolide, a furanoid diterpene from *Croton sublyratus*. Phytochemistry **22**, 302–303.

Takeda Y., Nakao K., Nakata K., Kawakami A., Ida H., Ichikawa T., Shigeno M., Kajiya Y., Hamasaki K., Kato Y., and Eguchi K. (2001), Geranylgeraniol, an intermediate product in mevalonate pathway, induces apoptotic cell death in human hepatoma cells: death receptor-independent activation of caspase-8 with down-regulation of Bcl-xL expression. Jpn. J. Cancer Res. **92**, 918–925.

Vongchareonsathit A. (1994), Shoot regeneration from tissue cultures of *Croton sublyratus* Kurz. Master Thesis, Chulalongkorn University, Bangkok, Thailand.

Wungsintaweekul J. and De-Eknamkul W. (2005), Biosynthesis of plaunotol in *Croton stellatopilosus* proceeds via the deoxyxylulose phosphate pathway. Tetrahedron Lett. **46**, 2125–2128.