รายงานวิจัยฉบับสมบูรณ์

โครงการ

การเหนี่ยวนำและการศึกษาทางพฤกษเคมีของรากขนอ่อนที่ชักนำ
ด้วย *Agrobacterium rhizogenes* ของต้นกระท่อม

Induction and phytochemical studies of the *Agrobacterium rhizogenes*-transformed hairy root cultures of *Mitragyna speciosa* (Roxb.) Korth.

โดย

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โครงการ: การเหนี่ยวนำและการศึกษาทางพฤกษเคมีของรากขนอ่อนที่ชักนำด้วย *Agrobacterium rhizogenes* ของต้นกระท่อม

บทคัดย่อ

รากขนอ่อนของต้นกระท่อม ได้จากการเหนี่ยวนำด้วยเชื้ออะโกรแบคทีเรียมไรโซจิเนส ATCC 15834 จากชิ้นส่วนบริเวณเส้นกลางใบ รากขนอ่อนที่ได้ถูกนำมาวิเคราะห์หายีนที่ก่อให้เกิดรากได้แก่ *rol*A และ *rol*B ด้วยวิธี polymerase chain reaction ผลการทดลองพบว่าประสิทธิภาพของการถ่ายยีนจาก เชื้ออะโกรแบคทีเรียมไรโซจิเนส มีค่าประมาณ 60% ลักษณะรากขนอ่อนที่ได้มีลักษณะแตกแขนงและมีขนอ่อนจำนวนมาก เมื่อศึกษาผลของอาหารเพาะเลี้ยงต่อการเจริญเติบโตของรากขนอ่อน จากอาหารเพาะเลี้ยงรวม 9 สูตรอาหาร พบว่าสูตรอาหาร WPM ที่เสริมด้วย 0.5 mg/l naphthaleneacetic acid เป็นสูตรอาหารที่เหมาะสมที่สุดต่อการเจริญเติบโตของรากขนอ่อน เมื่อศึกษาถึงการสร้างสารทุติยภูมิในรากขนอ่อน โดยการเตรียมเป็นสารสกัดเฮกเซนและสารถูกแยกด้วยเทคนิคคอลัมภ์โครมาโตกราฟี ผลการทดลองได้สาร MSF1 และ MSF2 ในปริมาณ 0.016% (w/w) และ 0.027% (w/w) ตามลำดับ ทำการวิเคราะห์โครงสร้างของสารทั้งสองด้วยเทคนิค ^{1}H- และ ^{13}C-NMR spectroscopy พบว่าสาร MSF1 เป็นสารผสมระหว่าง β-sitosterol และ stigmasterol (อัตราส่วน 1:1) และสาร MSF2 เป็นสารผสมระหว่าง ursolic acid และ oleanolic acid (อัตราส่วน 5:1) จากนั้นได้สร้างกราฟเจริญเติบโตและกราฟการสร้างสาร ursolic acid ในรากขนอ่อน กราฟการเจริญเติบโตแสดงให้เห็นว่ารากขนอ่อนมีวงจรการเจริญเติบโตประมาณ 40 วัน และจากกราฟการสร้างสาร ursolic acid แสดงให้เห็นว่ารากขนอ่อนนั้นสามารถสร้างสาร ursolic acid และมีปริมาณ ursolic acid สะสมสูงสุดคือ 3.47±0.03 มก. ต่อน้ำหนักแห้ง ในช่วงปลายของระยะ linear phase

สัญญาเลขที่ PHA5122020041S

Title: Induction and phytochemical studies of the *Agrobacterium rhizogenes*-transformed hairy root cultures of *Mitragyna speciosa* (Roxb.) Korth.

ABSTRACT

The hairy root culture of *Mitragyna speciosa* was induced with the wild type *Agrobacterium rhizogenes* ATCC 15834 from leaf vein explants. The hairy roots were identified for the rooting genes loci of *rol*A and *rol*B by the polymerase chain reaction. The results showed that the efficiency of *A. rhizogenes* genes transformation was about 60%. The transformed hairy roots appeared branching with pubescent hairs. Study on the appropriate medium for growth of the hairy roots suggested that among 9 different kinds of media, WPM supplemented with 0.5 mg/l naphthaleneacetic acid was suitable medium for promoting the growth. Study on secondary metabolite production, the *n*-hexane extract of the hairy roots was prepared and the compounds were isolated by silica gel column chromatography, affording the MSF1 and MSF2 with the yield of 0.016% (w/w) and 0.027% (w/w), respectively. Elucidation of their structures using ^{1}H- and ^{13}C-NMR spectroscopic methods, indicated that MSF1 was a mixture of β-sitosterol and stigmasterol (ratio 1:1) and MSF2 was a mixture of ursolic acid and oleanolic acid (ratio 5:1). The growth curve and the ursolic acid production curve of the hairy root culture were plotted. The growth cycle of the hairy root culture was realized within 40 days. The production curve of the hairy roots indicated that *M. speciosa* hairy roots contained ursolic acid and reached the maximum yield of 3.47±0.03 mg/g dry wt at a late linear phase.

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Juraithip Wungsintaweekul

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LIST OF ABBREVIATIONS AND SYMBOLS

µg	=	microgram
µl	=	microliter
B5	=	Gamborg's B5 medium
BA	=	benzyladenine
bp	=	base pair
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleoside triphosphate
HPLC	=	high performance liquid chromatography
MS	=	Murashige and Skoog (1962)
NAA	=	1-naphthalene acetic acid
OD	=	optical density
PCR	=	polymerase chain reaction
rpm	=	round per minute
TDZ	=	thidiazuron
TLC	=	thin layer chromatography
T_m	=	melting temperature
UV	=	ultraviolet
WPM	=	Lloyd and McCown (1981) Woody plant medium
YEB	=	yeast extract broth

CHAPTER I

1.1 Introduction and rationale

Mitragyna speciosa (Roxb.) Korth. (Rubiaceae) is an endemic plant found in tropical Southeast Asia. It is of particular medicinal importance known as "Kratom" in Thailand, "Biak-Biak" in Malaysia (เต็ม สมิตินันท์, 2544). For folklore medicine, Kratom has been used as an opium-substitute for pain relief and treatment of diarrhea. The pharmacological activities of mitragynine and its derivatives have been reported from studies in tested animals such as analgesic activity, antinociceptive activity (Watanabe *et al.*, 1997), anti-stress activity, muscle relaxant activity (Aji *et al.*, 2001) and inhibition of gastric acid secretion (Tsuchiya *et al.*, 2002). The mechanism of action of mitragynine for analgesic activity is binding to the opioid receptors, similar to morphine. Interestingly, mitragynine has a characteristic of less addiction than morphine. Therefore, it highlights the relevance to be an alternative to opioid analgesic drug (Thongpraditchote *et al.* 1998).

Contrary to the potential of *M. speciosa*, it is recognized as an illegal plant and growing of this plant is prohibited in Thailand. As such the basic knowledge of mitragynine biosynthesis is very little. From the biosynthesis point of view, the steps of mitragynine formation are still unknown. As part of our interest on plant biosynthesis, the plant cell cultures, such as callus and suspension cultures are established in order to use as a model instead of the intact plant. For those reasons the model plant is controlled under specific conditions. Therefore, the chemicals produced by the plant cell culture do not change by seasons. However, the disorganized cells, sometimes, do not produce the secondary metabolites as found in the intact plant. Techniques of medium manipulation and elicitation then are used to stimulate the disorganized cells growing, which are able to produce the secondary metabolites. On the other hand, establishing the organ culture is probably making cells complex enough for production of secondary metabolites. One of the techniques widely used for inducing the organ culture is the genetic transformation of *Agrobacterium* to plant cells. For instance, *Agrobacterium rhizogenes*, the causative agent of hairy root disease in plants, was transformed into plant cells and later forming the hairy root culture. This technique has emerged as an important method for secondary

metabolic production. An advantage of using transformed hairy root culture is practically scaling up in the bioreactor for commercial purposes. For the sources of indole alkaloid production, hairy roots of *Catharanthus roseus, Rauwolfia serpentina* and *R. micranta* have been reported to accumulate medicinal compounds such as ajmaline, ajmalicine and serpentine (Sim *et al.*, 1994, Benjamin *et al.*, 1994, Sudha *et al.*, 2003). These cultures have been currently used in the biosynthetic studies of indole alkaloid.

In this study, we aim to induce the hairy root culture of *M. speciosa* with a wild type *Agrobacterium rhizogenes* ATCC 15834. The potential of secondary metabolites production was evaluated. Then, secondary metabolites accumulated in the *M. speciosa* hairy roots are isolated and elucidated for their structures by NMR spectroscopy.

1.2 Review of literature

1.2.1 Botanical aspects of *Mitragyna speciosa* (Roxb.) Korth.

Mitragyna speciosa (Roxb.) Korth. (Fig. 1.1) belongs to the Rubiaceae family, found in the regions of Africa and Asia. The genus, named *Mitragyna* was given by Korthals due to the shape of the stigmas in the species. However the nomenclature has frequently been confused; the genuses have been variously named and are consistently recognized as *Naucleeae*, *Sarcocephalus*, *Stephegyne* and *Uncaria*. In Thailand, there are four species of *Mitragyna,* which are *M. speciosa, M. hirtusa, M. diversifolia* and *M. rotundifolia* (เต็ม สมิตินันท์, 2544). Taxonomically, plant species can be categorized by types of indole alkaloids (Keawpradub, 1990). Recently, these species were authenticated by molecular approach, based on rDNA ITS sequence (Sukrong *et al.*, 2007).

For botanical aspects, *M. speciosa* is characterized by the globular flowering head each containing up to 120 florets (Fig. 1.1 B). Each floret is surrounded by many overlapping bracteoles which completely cover the developing florets during the flower-bud stage. The inflorescence is a dichasial cyme. The fruit is a capsule containing numerous small flat seeds. The young woody shoots bear 10-12 leaves arranged in opposite and decussate each pair of leaves being

accompanied by two interpetiolar stipules (Fig. 1.1 A) which initially are closely oppressed and protect the apical bud.

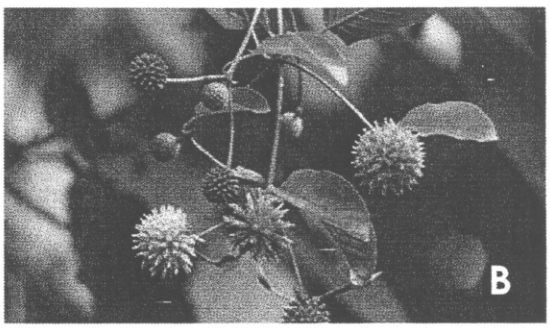


Figure 1.1 *Mitragyna speciosa* (Roxb.) Korth. (Rubiaceae) (A); Globular flowering head (B).

1.2.2 Chemical constituents of *M. speciosa*

Leaves of *M. speciosa*, found in Thailand and Malaysia, contain common indole alkaloids. Takayama (2004) reviewed the abundance of mitragynine in crude alkaloidal extract of Thai and Malay strains. It has been reported that the *M. speciosa* of Thai contained mitragynine up to 66% of the total alkaloid content, whereas only 12% was found in the *M. speciosa* of Malay sample. Reviews of the distribution of indole alkaloids in leaves, twigs, stem barks and root barks of *M. speciosa* are summarized in Table 1.1.

Table 1.1 Chemical constituents of *M. speciosa* (Roxb.) Korth.

Plant part	Category	Chemical substance	Reference
leaves	alkaloid	ajmalicine, akuammigine, angustine, corynantheidine, corynantheidaline, corynantheidalinic acid, corynoxeine, corynoxine, corynoxine B, hirsutine, hirsuteine, isocorynoxeine, isomitraphylline, isorhynchophylline, isocorynantheidine, javaphylline, mitraciliatine, mitragynine oxindole B, mitrajavine, mitraphylline, mitrasulgynine, mitragynaline, mitragynalinic acid, mitralactonal, paynantheine, mitragynine, pinoresinol, speciociliatine, speciogynine, 3-isoajmalicine, 3,4,5,6-tetradehydromitragynine, 7α-hydroxy-7*H*-mitragynine	Phillipson *et al.*, 1973; Phillipson *et al.*, 1973; Shellard *et al.*, 1966; Shellard *et al.*, 1978a; Shellard *et al.*, 1978b Takayama, 2004
	flavones	apigenin, apigenin-7-O-rhamnoglucoside, cosmosiin	Hinou and Harvala, 1988
	flavonol	astragalin, hyperoside, kaempferol, quercetin, quercitrin, quercetin-3-galactoside-7-rhamnoside, quercitrin, rutin	Harvala and Hinou, 1988
leaves	phenylpropanoid	caffeic acid, chlorogenic acid	Hinou and Harvala, 1988
	flavonoid	(-)-epicatechin	Houghton and Said, 1986
	lignin	(+)-pinoresinol	Takayama *et al.*, 1998
	triterpene	ursolic acid	Said *et al.*, 1991
young twigs, stem bark	alkaloid	ciliaphylline, rhynchociline, ciliaphylline, isomitraphylline, isorhynchophylline, isospecionoxeine, javaphylline, mitraciliatine, mitragynine oxindole A, mitragynine oxindole B, mitraphylline, rhynchociline, rhynchophylline, speciogynine, speciociliatine, specionoxeine	Shellard *et al.*, 1978a Shellard *et al.*, 1978b

root bark	alkaloid	ciliaphylline, corynoxeine, isocorynoxeine, isomitraphylline, isorhynchophylline, isospecionoxeine, mitraciliatine, mitraphylline, rhynchociline, rhynchophylline, speciociliatine, speciogynine, specionoxeine	Shellard *et al.*, 1978b; Houghton and Shellard, 197

1.2.3 Biological activities

1.2.3.1 Indole alkaloids

As mentioned earlier, *M. speciosa* is a major source of indole alkaloids. Mitragynine is the most abundant component that is present in the leaves of *M. speciosa*. For biological activities of alkaloids obtained from *M. speciosa*, the crude extract and pure alkaloid-mitragynine were used as materials *in vitro* and *in vivo* experiments. In 1996, a Japanese group investigated the antinociception of mitragynine in mice and the results showed that mitragynine acted as opioid receptors agonist in the brain of mice (Matsumoto *et al.*, 1996a). Later, they found that the mechanisms for antinociceptive effects differed from those of morphine in mice (Matsumoto *et al.*, 1996b). Mitragynine preferred to bind μ and δ opioid receptors, unlike morphine that binds only to μ opioid receptor specifically. Therefore, mitragynine exhibits 10 fold less potent than morphine (Thongpraditchote *et al.*, 1998). Caused by the affinity to opioid receptor, mitragynine also acts as morphine-like action on inhibition of gastric secretion (Tsuchiya *et al.*, 2002). Studies on the synthesis of mitragynine-related indole alkaloids discovered mitragynine pseudoindoxyl and 7-hydroxymitragynine, which exhibited opioid agonistic activity with higher potency than morphine (Takayama *et al.*, 2002). With regards to the psychological effects, *M. speciosa* extract was known to have a stimulatory effect on the dorsal raphe nucleus and an antidepressant-like activity. Stimulation of this brain area has been known to cause antinociception (Kumarnsit *et al.*, 2007).

1.2.3.2 Triterpenoids : ursolic acid

Ursolic acid was found in *M. speciosa* leaves (Said *et al.*, 1991). To date, there is no report about the biological activity of ursolic acid from *M. speciosa*. However, ursolic acid normally exists in plants, especially the Lamiaceae family. Contemporary scientific researches revealed and confirmed that ursolic acid exhibited several pharmacological effects, such as antitumour, hepatoprotective, anti-inflammatory, anti-ulcer, antimicrobial, anti-hyperlipidemic and antiviral activity (Liu, 1995). Ursolic acid was also identified as one of the active principles in *Rosmarinus officinalis* (Lamiaceae) to inhibit the growth of some food borne pathogens (Kamatou *et al.*, 2007). Somova *et al.*, 2003 found that African wild olives contain oleuafricein (mixture of oleanolic and ursolic acid) exhibiting the antihypertensive, diuretic/natriuretic, antihyperlipidemic, hypoglycemic and antioxidant activities. Ursolic acid from *Bouchea fluminensis* has anti-inflammatory activity inhibiting significantly the edema induced by either carrageenan or serotonin (Costa *et al.*, 2003). The anti-inflammatory effects of ursolic acid have been attributed to various mechanisms including inhibition of lipoxygenase and cyclooxygenase activities, inhibition of elastase and inhibition of C3-convertase (Costa *et al.*, 2003).

1.2.4 Genetic transformation and plant tissue culture of Rubiaceous plant

The most popular bacteria, used for genetic transformations, are *Agrobacterium tumefaciens* and *A. rhizogenes*. *A. tumefaciens* contains tumor-inducing plasmid (Ti-plasmid), therefore, causing the crown-gall tumor to plant cells, whereas *A. rhizogenes* contains root-inducing (Ri-plasmid), causing the hairy roots (Oksman-Caldentey and Hiltunen, 1996). In this study, we used *A. rhizogenes* for genetic transformation to *M. speciosa* cells in order to induce the hairy root cultures.

A. rhizogenes is a gram negative soil bacterium and is supplied in many varieties such as wild-type strain-ATCC 15834, A4 (Sevón and Oksman-Caldentey, 2002). *A. rhizogenes* carries the Ri plasmid, its size about 200 to greater than 800 kb. It contains T-DNA and a virulence (*vir*) region, responsible for induction of hairy root formation (Oksman-Caldentey and Hiltunen, 1996) The Ri-plasmid carries the borders of T_L and T_R (or LB and RB), which are expressed after

stimulation of virulence (*vir*) region. The *vir* region is about 35 kb and encodes six transcriptionals: *vir* A, B, C, D, E and G, which have important functions in gene transfer.

Fig. 1.4 illustrates the pathogenic responses of the interaction between *A. rhizogenes* and plant cells. After plant cells are wounded, they then release the phenolic compounds e.g. acetosyringone, which activates the genes of *vir*-region of the bacterial plasmid. Finally, the T-DNA is cleaved (at the region of LB and RB), transferred to plant cells and later incorporated into the nuclear DNA of host plant (Sevón and Oksman-Caldentey, 2002). The resulting genetic-transformed plant cells synthesize novel metabolites e.g. opine, which are essential nutrients for auxin production (Oksman-Caldentey and Hiltunen, 1996).

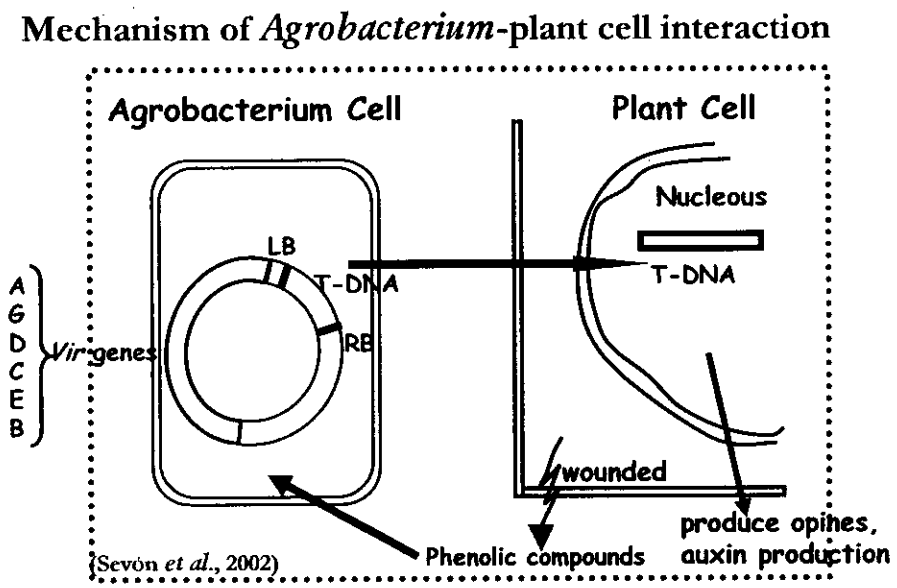


Figure 1.4 Interaction of *Agrobacterium rhizogenes* to plant cell.

In conclusion, the plasmid itself has three regions, those are important for the gene transfer, which are 1) the T-DNA, which is integrated into the genome of the plant cells; 2) the border sequences of the T-DNA and 3) the virulence area (*vir*), which does not enter the plant cell but causes the transfer of the T-DNA to the plant cells (Oksman-Caldentey and Hiltunen, 1996).

A. rhizogenes Ri-plasmid transformed to plant cells causes the hairy roots and the transformed cells are more sensitive to extracellular supplied auxins than the normal roots (Sevón *et al.*, 1998). Susceptibility of plant species to *Agrobacterium* strains varies greatly. It is dependent upon factors, which are 1) transformation ability of different strains of *Agrobacterium*; 2) age and differentiation status of plant tissues; 3) presence of phenolic compounds e.g. addition of acetosyringone. For identification of gene-loci of Ri-plasmid, the genetic transformation can be confirmed by assays: 1) detection of opine production by paper electrophoresis; 2) detection of T-DNA by Southern blot hybridization and 3) detection of genes in *A. rhizogenes* by polymerase chain reaction (PCR) (Oksman-Caldentey and Hiltunen, 1996). The hairy roots are morphologically characterized by laterally highly branched roots, fast growing, and are able to grow in hormone-free medium. They are genetically stable and produce high contents of secondary metabolites. The ability of secondary metabolite production of hairy roots is highly linked to cell differentiation.

For the studies on plant tissue culture of the Rubiaceous plants, the establishment of callus, cell suspension and organ cultures under specific conditions have been reported. Table 1.2 summarized the plant tissue cultures of the Rubiaceous plants. It can be noted that they were able to produce the alkaloids and triterpenoids. An example of the hairy roots of *Ophiorrhiza pumila* convinced the utility of hairy root culture for camptothecin production in the industrial scale (Saito *et al.*, 2007). Suspension culture of *Uncaria tomantosa* could produce ursolic acid and oleanolic acid but no alkaloid was present in the culture (Feria Romero *et al.* 2005).

Table 1.2 Plant tissue cultures of the Rubiaceous plants

Plant source	Chemicals	Culture conditions	Reference
Cinchona ledgerina	cinchonine, quinidine, quinine	suspension culture : Gamborg B5 basal medium containing 2,4-dichlorophexoyacetic 1 mg/l acid and saccharose 20 mg/l	Hamill *et al.*, 1989
Ophiorrhiza pumila	Camptothecin	hairy root culture : Gamborg B5 medium containing sucrose 2%(w/v)	Saito *et al.*, 2007
Psychotria umbellata	umbellatine	embryogenic regeneration : Murashige and Skoog medium containing 1-naphthaleneacetic acid 1 mg/l and kinetin 1 mg/l	Paranhos *et al.*, 2005
Rudgea jasminoides	-	callus cell culture : Murashige and Skoog medium containing kinetin, 2.22 μM and picloram 2.07 μM	Stella *et al.*, 2002
Uncaria rhynchopylla	hirsuteine, hirsutine,3α-dihydrocadambine, ursolic acid	callus culture : Gamborg B5 containing indole acetic acid 10^{-4} M and benzyladenine 3 x 10^{-5} M and sucrose 2% (w/v)	Kohda *et al.*, 1996
U. tomentosa	ursolic acid, oleanolic acid	suspension culture : Nitsch & Nitsch medium containing 2,4-dichlorophenoxyacetic acid 2 mg/l, kinetin 2 mg/l and sucrose 20 g/l	Feria Romero *et al.*, 2005

CHAPTER II

Materials and Methods

2.1 Materials and equipments

2.1.1 Plant material

Mature plants of *M. speciosa* (6 years-old), grown in the open field in Hat Yai District, Songkhla Province, Thailand, were used in this study. A voucher specimen of this plant was deposited in the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai campus, Songkhla, Thailand. Seeds were collected from the globular fruit (Fig. 2.1A) and flat seeds (Fig. 2.1B) were separated. These are used for plantlet preparation and further for genetic transformation with *A. rhizogenes* ATCC 15834.

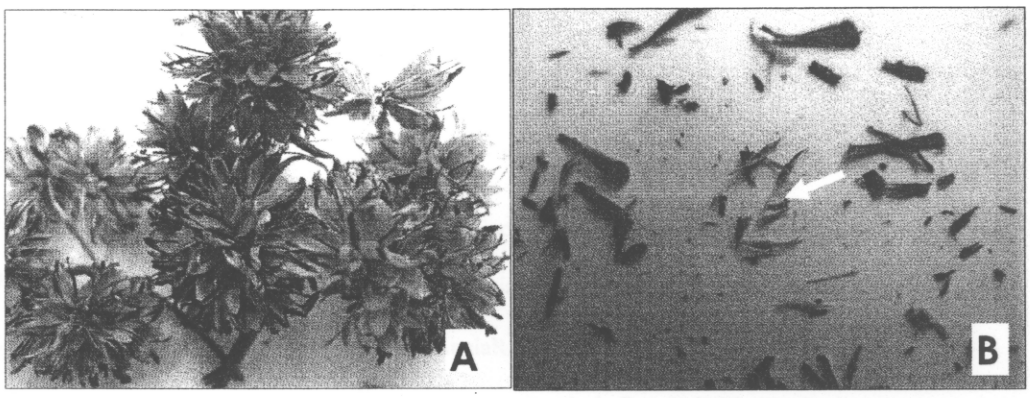


Figure 2.1 *M. speciosa* (Roxb.) Korth.: the globular fruits (A); flat seeds (B)

2.1.2 Chemicals

The authentic mitragynine was kindly provided by Assist. Prof. Dr. Niwat Keawpradub, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. Chemicals and equipments used in this study are listed below.

Chemicals	*Company, Country*
acetic acid, glacial	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
agarose gel	Research Organics, USA
agar (Bacto)	Himedia laboratories, Ltd., India
amberlite XAD-4	Sigma, USA
cefotaxime disodium	M&H Manufacturing, Thailand
chloroform	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
clorox®	Clorox, USA
DNA markers	Sib-enzyme, Russia 2-Log DNA Ladder (0.1-10.0 kb) NEB (New England Biolabs), UK
ethanol (95%v/v)	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
ethidium bromide	Bio Basic INC, Canada
ethyl acetate	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Gamborge's B5 medium	Duchefa Biochemie , The Netherlands
n-hexane	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
McCown Woody plant (WPM) medium	Duchefa Biochemie, The Netherlands
Murashige-Skoog (MS) medium	Duchefa Biochemie, The Netherlands
methanol	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
NH_3 25%(v/v)	BDH AnalaR®, England
petroleum ether	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
plant agar	Duchefa Biochemie, The Netherlands
sucrose	MITR PHOL, Thailand
thidiazuron	Sigma, USA
TLC-plate silica gel GF_{254}	Merck, Germany
ursolic acid	Sigma, USA
yeast extract	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
6-benzyladenine (BA)	Fluka, Switzerland
1-naphthaleneacetic acid (NAA)	Fluka, Switzerland
1-propanol	Lab-scan Asia Co., Ltd., Bangkok, Thailand.

2.1.3 Equipments

Equipments	*Company*
autoclave	Model HA-3D, Hirayama, Japan
balances	Explorer, Ohaus, USA; Avery Berkel, USA
centrifuge	Hermle Z 323 K, Germany
electrophoresis chamber	Mupidα Mini electrophoresis system, Japan
gel documentation	Gel Doc model 1000, BIO-RAD, USA
	Molecular Analyst® Software, Windows Software for BioRad's Image Analysis Systems Version 1.4
gene amplifier	GeneAmp, PCR system 9600, Perkin Elmer, USA
hot air oven	Memmert, Germany
hot plate and stirrer	Model PC-101,CORNING; Fisher Scientific, USA
incubating block	Thermomixer comfort, Eppendorf, Germany
laminar air flow cabinet	Holten, Denmark
micropipette	Socorex, Switzerland
microwave oven	LG, Thailand
pH meter	pH meter Model 710A, ORION Research, USA
platform shaker	Innova 2300, USA
refrigerator	4°C: Sanden Intercool, Thailand; -20°C: Whirlpool, Thailand; -80°C, Forma Scientific, USA
rotary evaporator	aspirator A-3S, EYELA, Japan
rotary shaking	orbital Shaker OS-20
UV-VIS spectrophotometer	Labomed, USA
TLC-scanner	TLC SCANNER 3 and software cats version 4.01, CAMAG, Switzerland
UV-CABINET II for 254, 366 nm	CAMAG, USA
vortex	Vortex-Genie 2™, USA
water bath	Digital water bath SB-1000, EYELA, Japan

2.1.4 Media preparations and solutions

Anisaldehyde spray reagent	Anisaldehyde (5 ml) was mixed with 10 ml of acetic acid and 5ml of HCl (37%), and then adjusted the volume to 100 ml with distilled water.
Cefotaxime stock solution (1g/5 ml)	Cefotaxime Na (1 g) was dissolved in 5 ml of sterile distilled water. The solution was sterilized using 0.22 μm membrane.
Dragendorff's spray reagent	Solution A: bismuth subnitrate (850 mg) was dissolved in 40 ml of distilled water and 10 ml of glacial acetic acid. Solution B: potassium iodide (8 g) was dissolved in 20 ml of distilled water. The mixture was prepared mixing by 5 ml of solution A and 5 ml of solution B. Then 20 ml of glacial acetic acid was added and the volume was adjusted to 100 ml with distilled water.
Ethidium bromide solution	Ethidium bromide (10 μl) was mixed in 100 ml of distilled water.
Loading buffer	Loading buffer contains glycerol (30% v/v) and bromophenol blue (0.025% w/v).
TAE buffer, 50x	The concentrated TAE (Tris-acetic acid-EDTA) buffer composed of Tris base (121 g), EDTA.3Na (19.7 g), glacial acetic acid (35ml), which were dissolved in distilled water, pH was adjusted with HCl (37%) to 8.0 and the volume was adjusted to 500 ml with distilled water.
YEB medium	The mixture contained beef extract (5 g), yeast extract (1 g), peptone (5 g), sucrose (5 g) and 10% w/v $MgSO_4$ (5 ml) and distilled water. The volume was adjusted to 1 L with distilled water. For YEB solid medium, 1.5 g Bacto-agar

was added to 100 ml of mixture. The culture medium was sterilized by autoclaving at 121°C, 15 Ib/in^2, for 20 minutes.

2.1.5 Plasmid isolation kits and enzyme kits

GFX Micro Plasmid Prep Kit (GE Healthcare, USA)

DNeasy Plant Mini Kit (Qiagen, Germany)

ThermoPol *taq* polymerase (NEB (New England Biolabs), USA)

2.2 Methods

2.2.1 Preparation of *M. speciosa* plantlets

Flat seeds (2.1.1) of *M. speciosa* were surface sterilized by rinsing with 70% (v/v) ethanol for 5 min, then sterilizing with 20% (v/v) Clorox® for 5 min and finally rinsing thoroughly with sterile distilled water (x3). Sterilized seeds were placed on WPM solid medium supplemented with BA (1.0 mg/l) for seed germination. After incubating at 25°C and under light for 16 h/day for 1 week, the seedlings germinated. The 2-month-old plantlets were used for bacterial infection.

2.2.2 Bacterial strain and preparation

Agrobacterium rhizogenes wild-type strain ATCC 15834 (TISTR 1450) was obtained from the Microbiological Resources Centre, Pathumthani, Thailand. *A. rhizogenes* ATCC 15834 was grown and maintained in YEB medium. Before bacterial infection, *A. rhizogenes* was activated by culturing on YEB solid medium and incubated at 28°C overnight (16 h). A single bacterial colony was obtained and used for inoculation into 5 ml YEB liquid medium. The culture was then placed on rotary shaker (218 rpm) and incubated at 28°C overnight. The bacterial suspension was harvested by centrifugation at 3,500 rpm for 10 min. The pellet was then re-suspended. The bacterial concentration was adjusted with sterile WPM liquid medium to obtain an OD_{600} about 0.5-0.6. Finally, the bacterial suspension was further used to infect the explants using needle injection method (Dhakulkar *et al.*, 2005).

2.2.3 Plant tissue culture media preparations

The composition of the MS, WPM and B5 media (Duchefa Biochemie) are shown in table A-1, table A-2 and table A-3, respectively (see Appendix). For MS medium preparation, the MS powder (4.4 g) and sucrose (30 g) were dissolved in distilled water. The pH of the solution was adjusted with 1 N NaOH or 1 N HCl to 5.8. For WPM medium preparation, the McCown WPM powder (2.5 g) and sucrose (20 g) were dissolved in distilled water in the final volume of 1 L. The pH of WPM medium was adjusted to 5.7. For B5 medium preparation, the B5 powder (3.2 g) and sucrose (20 g) were dissolved in distilled water; volume was adjusted to 1 L. The pH of B5 medium was adjusted to 5.5. Plant growth regulator was added as needed. For solid medium preparation, plant agar (Duchefa, The Netherland) was added to the final concentration of 0.8% (w/v), and the mixture was heated gently with continuous stirring until a clear solution was obtained. All culture media were sterilized by autoclaving at 121°C, 15 lb/in^2, for 15 minutes.

2.2.4 Induction of hairy roots

For hairy root induction, explants (young stems and leaves) were infected with *A. rhizogenes* (2.2.2). Firstly, the explants were wounded with needle and then the wounded explants were submerged in bacterial suspension for 30 min. The excess bacterial suspension was washed thoroughly with sterile distilled water for 3 times. After removing the water with sterile filtered paper, the infected explants were transferred to hormone-free WPM solid medium and incubated at 25 °C in darkness. To kill the residual *A. rhizogenes*, those explants were transferred to WPM solid containing 500 mg/l of cefotaxime after infection for 3 days. The cultures were further incubated for 1 week. Then, the cefotaxime concentration was reduced each week from 500, 250, 100 mg/l, respectively. Finally the cultures, free from *A. rhizogenes*, were transferred to WPM solid medium without plant growth regulators. For the controlled root culture, the untransformed root culture was induced from the adventitious roots of plantlets. Adventitious roots were excised from 2-month old plantlets and cultured in WPM liquid medium. All cultures were incubated at 25°C, on rotary shaker (80 rpm) in dark condition. The hairy root culture and

untransformed root culture were sub-cultured into fresh medium every 2 months. For large scale production, the hairy roots and untransformed roots were sub-cultured to 200 ml of WPM medium in 1 L Erlenmeyer flask.

2.2.5 Identification of transformed hairy roots

The hairy root culture was identified for the genetic transformation from *A. rhizogenes*. The presence of the virulence genes of *rol*A and *rol*B were detected using the specific primers (Table 2.1), which were designed from the nucleotide sequence of T-DNA region of *A. rhizogenes* (GenBank) (Oksman-Caldentey and Hiltunen, 1996). The DNA fragment was amplified using polymerase chain reaction (PCR). The genomic DNA of the hairy roots was isolated using the DNeasy Plant Mini Kit (Qiagen), which was used as the DNA template in the PCR reaction. For negative control reaction, the DNA template was the genomic DNA of untransformed roots, which were isolated using the same procedure. For positive controlled reaction, the DNA template was the *A. rhizogenes* plasmid, which was isolated from the *A. rhizogenes* cells using the GFX Micro Plasmid Prep Kit (GE Bioscience). The protocols of plant genomic DNA and bacterial plasmid isolation were summarized in the Appendix. PCR reactions were performed for the detection of a 300 base pairs fragment of the *rol*A gene and a 780 base pairs fragment of the *rol*B gene amplifications. The PCR reaction was performed in 50 µl of mixture (Table 2.2) and the PCR conditions were summarized as shown below.

Table 2.1 Primers used in this study

Name of sequence	*Nucleotide sequence (5' to 3')*	*Tm (℃)*
*Rol*A-1	CAGAATGGAATTAGCCGGACTA	60.8
*Rol*A-2	CGTATTAATCCCGTAGGTTTGTTT	59.4
*Rol*B-1	ATGGATCCCAAATTGCTATTCCTTCCACGA	66.0
*Rol*B-2	TTAGGCTTCTTTCTTCAGGTTTACTGCAGC	66.0

Table 2.2 Standard procedure for PCR

Reagents	*Volume/reaction (μl)*	*Final concentration*
DNA Template	5	*ca.* 6 pg
dNTP mix, 10 mM each	1	200 μM of each dNTP
10 μM primer 1	2.5	0.5 μM
10 μM primer 2	2.5	0.5 μM
10X ThermoPol Buffer	5	1x
Taq DNA polymerase (NEB)	0.5	2.5 units/reaction
Sterilize distilled water	33.5	
Total volume	50	

PCR conditions for *rol*A amplification

Segment	*Step*	*Temperature (°C)*	*Time (min)*	Number of cycles
1	Denaturing	94	2	1
2	Denaturing	94	2	1
	Annealing	58	2	
	Extension	72	2	
3	Denaturing	94	1	24
	Annealing	58	1	
	Extension	72	1	
4	Extension	72	10	1
	Holding	4	∞	

PCR conditions for *rol*B amplification

Segment	*Step*	*Temperature* (°C)	*Time* (min)	Number of cycles
1	Denaturing	94	3	1
2	Denaturing	94	1	30
	Annealing	55	1	
	Extension	72	3	
3	Extension	72	10	1
	Holding	4	∞	

2.2.6 Agarose gel electrophoresis

To determine the size of the DNA fragments obtained from the PCR reactions, agarose gel (1.2% (w/v)), was prepared. Agarose (0.48 g) and TAE buffer (50x) were mixed with 40 ml of distilled water. The mixture was heated using the microwave oven until a clear solution was obtained. Then, the mixture was poured on a plastic tray, and a comb was placed to mold the wells for sample applications. After the agarose gel was completely set (30-45 minutes at room temperature), the comb was carefully removed and the gel was installed on the platform in the electrophoresis tank containing 1x TAE buffer (dilution from TAE buffer (50x) with distilled water). The PCR products (10 µl) including loading buffer (1 µl) were loaded into the wells of 1.2% w/v agarose gel using micropipette. The gel was run at 100 volts. The electrophoresis process was stopped when the loading dye had migrated to two third of the agarose gel (about 30 min). After that, the agarose gel was stained with ethidium bromide solution for 10 minutes and the DNA patterns were observed under UV 312 nm (a UV light box equipped with Gel Doc model 1000, BIO-RAD, USA) and a photograph was taken.

2.2.7 Medium manipulation

After obtaining the positive clones, the hairy root cultures (clone no.4) were grown in different liquid media in order to optimize the growth media for the hairy root cultures.

In order to increase the growth of the hairy root cultures, the culture media were manipulated as well as the plant growth regulator-NAA was added in the final concentration of 0.5 mg/l. The manipulated culture media were WPM, MS, ½WPM, ½MS, ½B5, WPM plus NAA, MS plus NAA, B5 plus NAA, and ½B5 plus NAA. The control cultures were the untransformed roots in WPM and WPM plus NAA. The culture conditions were at 25°C on rotary shaker (80 rpm) and under dark condition. The morphology of cultures was observed after sub-culture for 1 month. The growth of cultures was evaluated by weighing the dry weight. The dry weight was obtained from weight of the hairy roots, which were dried at 50°C overnight in hot air oven. The time course of growth was also established for the period of 40 days of culture. The samples (n=3) were consecutively taken every five days of culture. Only the hairy roots cultures grown in WPM, WPM plus NAA, MS plus NAA, B5 plus NAA and ½B5 plus NAA were used to construct the growth curves. To decrease the error of the inoculation size, the growth curve of the hairy roots cultures was constructed by plotting the relative growth (in comparison to day 5^{th} of culture) against the age of culture.

2.2.8 Isolation of secondary metabolites from transformed hairy root culture

The potential of secondary metabolites production by the hairy roots culture was evaluated. Mass preparation of hairy roots was prepared (2.2.4). The hairy roots were harvested when the age of culture reached 1 month. The hairy roots were washed thoroughly with distilled water to get rid of excess culture medium. Then, the hairy roots were dried at 50°C overnight in the hot air oven and ground in mortar. The dried power (37.4 g) was further macerated with methanol (100 ml, 3 times), filtered and evaporated. Crude methanol extract (8.5 g) was obtained and then re-dissolved in methanol (100 ml). The methanol fraction was partitioned with *n*-hexane (100 ml, 3 times) in the 1 L separatory funnel. The *n*-hexane extract was pooled and evaporated. The crude *n*-hexane (403 mg) was obtained and further purified by silica gel column chromatography. Silica gel column (size 3x18 cm) was prepared. Then, the crude *n*-hexane extract was loaded on the top column and eluted with sequential solvent of *n*-hexane: ethyl

acetate; 9:1, 8:2, 7:3 and 5:5. The column was then eluted with polar solvent of ethyl acetate, ethyl acetate: methanol; 8:2 and 5:5. Finally, the column was washed with methanol. After following the elution fraction by TLC analysis, the 11 fractions were obtained. The scheme of isolation of secondary metabolites from the *M. speciosa* hairy roots was shown in Fig. 2.2.

For further purification, fractions of F6 (10 mg) and F10 (17.05 mg) were purified for re-crystallization. The fraction F6 was re-crystallized with the ratio of solvent of $CHCl_3$ and MeOH; 7:3. The white needle crystal was obtained, resulting in MSF1 (6.1 mg). The fraction F10 was washed with *n*-hexane. The white amorphous solid was obtained, resulting in MSF2 (10.11 mg). The resulting MSF1 and MSF2 were elucidated for chemical structures by ^{1}H- and ^{13}C-NMR spectroscopy.

2.2.9 NMR spectroscopy

^{1}H and ^{13}C-Nuclear magnetic resonance (^{1}H-125 MHz and ^{13}C NMR-500 MHz), DEPT, COSY, HMQC, HMBC spectra were recorded on Fourier transformed NMR (FTNMR), Varian Unity Innova (USA), measured at the Scientific Equipment center, Prince of Songkla university. $CDCl_3$ and CD_3OH were used as solvents and tetramethylsilane (TMS) was used as the reference.

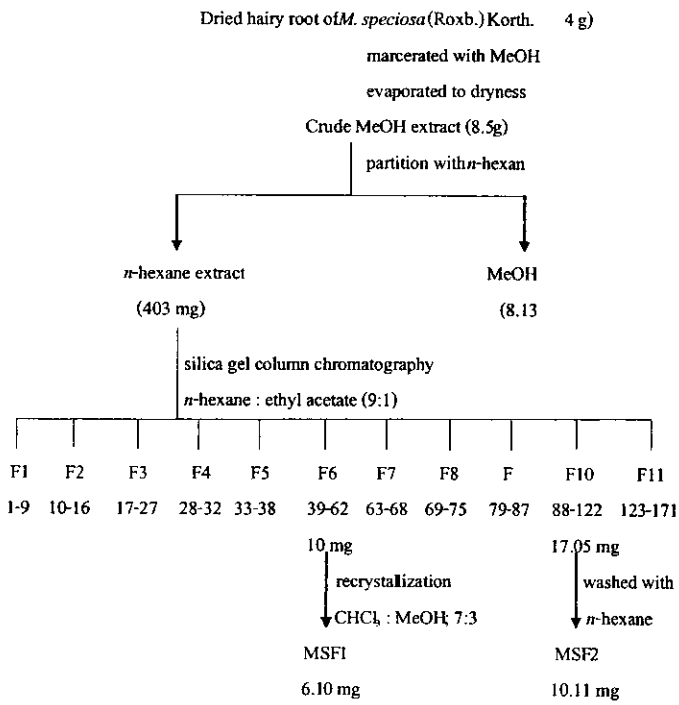


Figure 2.2 Scheme of isolation of compounds from the *n*-hexane extract of *M. speciosa* hairy roots

2.2.10 Establishment of the growth and production curve of *M. speciosa* hairy roots

The growth curve and production curve of the hairy roots, cultured in WPM medium supplemented with 0.5 mg/l of NAA, were constructed. Time course study was performed. The samples (n=3) were taken every 5 days of culture, for a period of 40 days. The dry weight of each culture was determined and the data were plotted against the age of culture. For constructing the production curve, the ursolic acid content was determined from each sample (total of 24 samples) using the HPLC method. The production curve showed the relationship between the ursolic acid content and the age of culture. The ability of *M. speciosa* hairy roots for ursolic acid production was compared to the untransformed roots. In this study, the growth curve and production curve of the untransformed roots culture were also determined in the same procedure.

Determination of dry weight The hairy roots cultures and the untransformed roots cultures were harvested and washed with distilled water. The excess water was removed by

vacuum. Then, the cultures were dried at 50 °C in a hot air oven overnight and ground into powder. The cultures were weighed for the dry weight (g).

Determination of the ursolic acid content The ursolic acid content was determined using HPLC method as described in Chen *et al.* (2003) with slight modification. The samples were prepared for *n*-hexane extract. After the samples were weighed for dried weight, the dried powder (100 mg) was extracted with *n*-hexane (50 ml, 3 x) under reflux for an hour and filtered. After that the filtrates were evaporated to dryness and were dissolved in acetonitrile. The volume was adjusted in a 5 ml-volumetric flask. The solution was further filtered through a 0.45 µm membrane filter prior to subjection to the HPLC system. Identification of the peak was performed from the peak retention time and UV absorption spectra of the corresponding peak, in comparison with the authentic ursolic acid (Sigma). The calibration curve of ursolic acid was constructed from the authentic compound. Standard ursolic acid 4.68 mg was dissolved in of acetonitrile and adjusted the volume to 10 ml. It was diluted with acetonitrile to concentration of 117, 58.5, 29.25, 14.625, 7.3125 µg/ml. The standard solutions were injected (10 µl) and run for calibration curves.

To calculate the ursolic acid content, the areas under the peaks were converted to concentrations by using the calibration curve. The HPLC analysis of each sample was performed in triplicate. The HPLC condition for separation of the ursolic acid was described below. The ursolic acid content was reported as mg per g dry weight.

HPLC conditions:

HPLC Column	: WATERS® USA, µ-BONDAPAK C18, 5 µm particle size column size 3.9 x300 mm
Mobile phase	: acetonitrile : 0.1% H_3PO_4 in H_2O (70:30), isocratic elution
Flow rate	: 1.0 ml/min
Detector	: UV 206 nm (photodiode array)
Injection volume	: 10 µl

CHAPTER 3

Results

3.1 Establishment of transformed hairy root cultures of *M. speciosa*

3.1.1 Induction of hairy roots

Sterile seedlings were prepared from flat seeds of *M. speciosa*, *in vitro* culture. They were germinated within 1 week, and then maintained under culture condition for 2 months. Fig. 3.1 shows the sterile plantlets and parts of the plant including leaves, stems and adventitious roots, all used in this study.

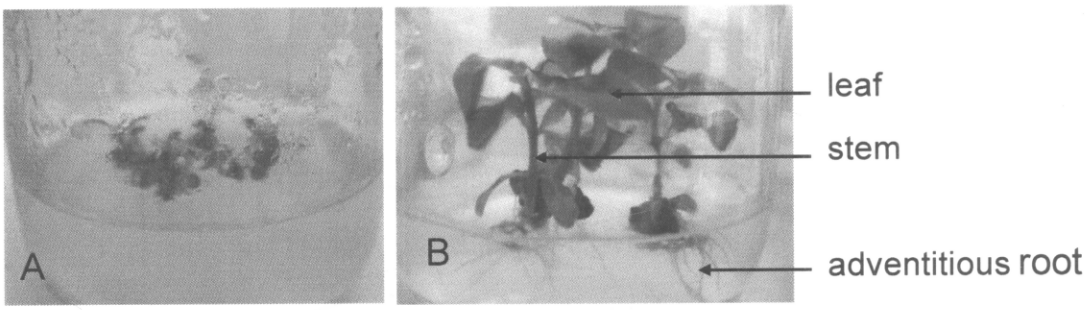


Figure 3.1 *M. speciosa* grown in solid WPM medium supplemented with 1.0 mg/l BA
A: 10 days old, B: plantlets after growing for 2 months.

According to Dhakulkar *et al.* (2005), the transformed hairy root culture of *M. speciosa* was induced with *Agrobacterium rhizogenes* ATCC 15834 with slight modification. Firstly, the hairy roots were induced by wounding at sites of the whole plantlet, including leaf veins and stems. Steps of hairy roots induction are summarized as shown in Fig. 3.2. From the results, the rootlets were initiated from injured sites and observed within 10 days. It can be noted that the frequency of hairy root induction was different in between leaf veins and stems.

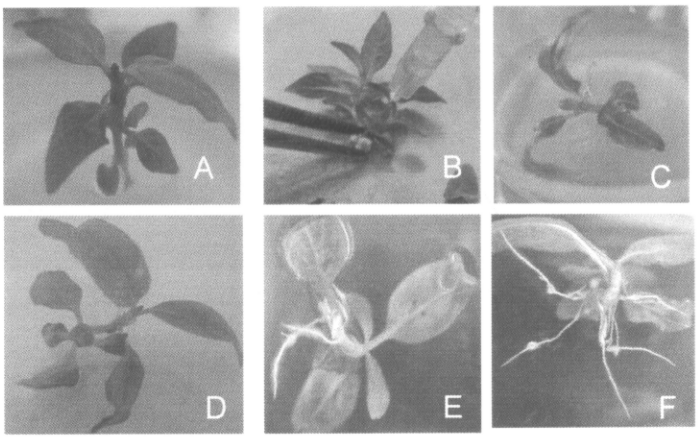


Figure 3.2 Steps of the hairy roots induction from *M. speciosa* plantlet

A. 2-month-old plantlets

B. infection with *A. rhizogenes* ATCC 15834

C. incubated the infected plantlet for 3 days

D. culture in WPM plus cefotaxime 500 mg/l for 7 days

E. culture in WPM plus cefotaxime 250 mg/l for 7 days

F. culture in WPM plus cefotaxime 100 mg/l for 7 days

Therefore, a further experiment was done to determine an appropriate explant for the hairy roots induction. The leaves and stems of the isolated explants were then excised and infected with *A. rhizogenes* ATCC 15834. The results of hairy roots induction were recorded on the 28th day after infection, as shown in Fig. 3.3.

Table 3.1 Induction of hairy roots from different explants

Explants	*Number of cultures used for genetic transformation*	*Number of cultures showing hairy root induction*	*% Hairy root induction*
Stem	189	128	67.72
Leaf vein	339	289	85.25

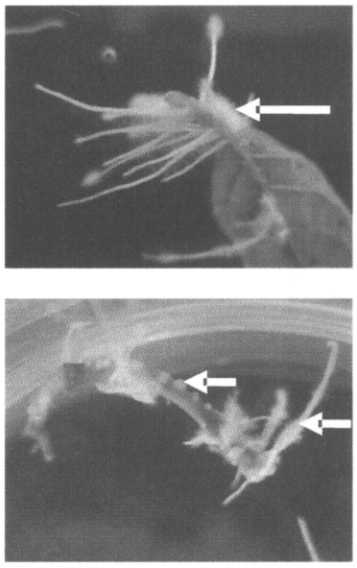


Figure 3.3 Characteristics of hairy roots induced from leaf veins (A) and stems (B)

Fig. 3.3 illustrates the hairy roots characteristics, which were induced from the leaf vein and the stem. From Fig. 3.3, infection sites of the leaf vein gave a number of hairy roots ranging from 2-25 hairy roots per leaf vein, whereas the stem could only produce 3-8 hairy roots. Notably, the hairy roots obtained from the leaf vein appeared as thin and long and contained small pubescence. In contrast, the hairy roots obtained from the stem contained nodules (undeveloped hairy roots) and thick roots. To determine the potential of hairy root formation in these explants, the percentages of hairy roots induction were calculated from the number of cultures used for *A. rhizogenes* infection and the number of cultures showing the hairy roots. As shown in Table 3.1, explants of leaf veins gave better number of hairy roots formation of 85%. Therefore, the explant of leaf vein was used for further genetic transformation with *A. rhizogenes* ATCC 15834 in order to get more clones for gene identification.

After the hairy roots grew in cefotaxime-free WPM medium, they were excised from the explant and placed on the surface of WPM solid medium when its length was about 3-4 cm. The hairy root culture was maintained under culture condition for 1 month (Fig. 3.4). After

that, the hairy roots were transferred to WPM liquid medium and incubated at 25 °C, on a rotary shaker (80 rpm) under dark condition.

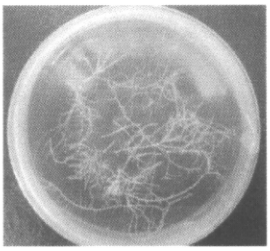


Figure 3.4 The characteristics of hairy root culture in WPM solid medium

For the untransformed root culture, the adventitious roots (Fig. 3.1) were excised and cultured in WPM liquid medium. Fig. 3.5 shows the characteristics of the transformed and untransformed roots after transferring to WPM liquid medium. Morphologically, transformed hairy roots induced by *A. rhizogenes* ATCC 15834 were thin and branching (Fig. 3.5A), whereas untransformed roots were thick, branching and shorter (Fig. 3.5B). From Fig. 3.5, young roots appeared white and gradually turned to slightly yellow-brown, when the cultures were aging. Under eye observation, both cultures were relatively the same under similar conditions.

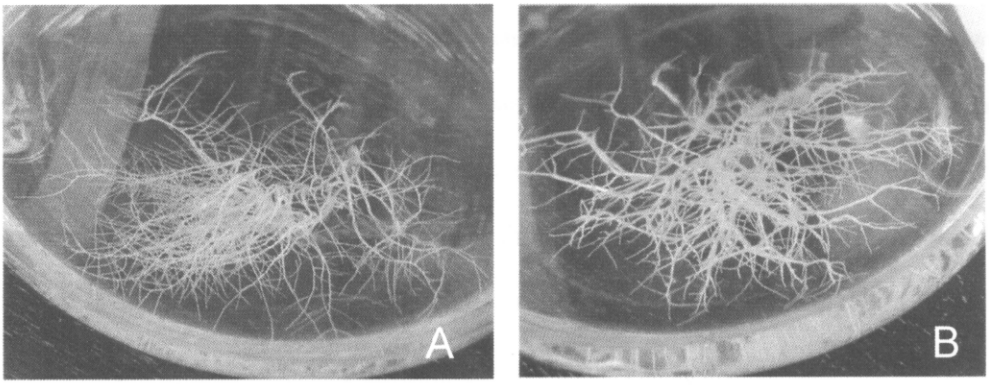


Figure 3.5 Characteristics of transformed hairy roots (A) and untransformed root (B) cultures in WPM liquid medium for 22 days of culture.

3.1.2 Identification of *rol*A and *rol*B genes

The hairy roots (10 clones) were obtained from *A. rhizogenes* infections and used as materials for *rol*A and *rol*B identifications. The genomic DNAs from 10 clones of hairy roots (1 month old) were separately isolated and used as template in the PCR reactions. The genomic DNA of untransformed roots and plasmid DNA of *A. rhizogenes* were used as templates in the PCR reactions for the negative control and positive control, respectively. After performing the genomic DNA isolations, the concentrations of the DNA were determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. The total DNA content was calculated according to the equation. To check the quality of isolated DNA, the absorbance at 280 nm (A_{280}) was also measured. The A_{280} indicated the amount of proteins, contaminated in the DNA fraction. Therefore, the ratio of A_{260}/A_{280} exhibited the purity of the DNA. Theoretically, the purity of total DNA should have an A_{260}/A_{280} ratio of 1.7-1.9.

Table 3.2 shows the concentrations of isolated DNAs and their purities. Data indicated that the total DNA concentrations were about 0.05-0.09 μg/μl. Calculation of A_{260}/A_{280} ratio of DNAs revealed that the isolated DNA contaminated with protein, meaning less quality of DNA was obtained (A_{260}/A_{280} about 0.2-0.6). Nevertheless, the total DNAs were further used as templates in the PCR reactions. After that the PCR reactions were performed, aliquots of reaction (5 μl) were analyzed for the DNA fragment in 1.2% agarose gel electrophoresis as shown in Fig. 3.6. For positive control (lane 2), DNA fragments, corresponding to *rol*A and *rol*B, were observed at the sizes of *ca.* 300 base pair and 780 base pair, respectively. None of DNA fragment appeared in the negative control (lane 3). Clones no. 4, 5, 6, 8, 9 and 10 (lanes 7, 8, 9, 11, 12, 13) were shown to carry the *A. rhizogenes* genes since the DNA fragments of 300 base pair and 780 base pair have been observed (Fig. 3.6). From these results, the genetic transformation rate of *A. rhizogenes* to *M. speciosa* leaf vein explant was accounted for 6/10 clones or 60%.

Table 3.2 Concentrations and purities of total DNA, extracted from the hairy root cultures

Clone no.	A_{260}	A_{280}	*Concentration of total DNA* ($\mu g/\mu l$)	*Purity* (A_{260}/A_{280})
1	0.028	0.071	0.056	0.394
2	0.023	0.103	0.046	0.223
3	0.032	0.040	0.064	0.800
4	0.043	0.089	0.086	0.483
5	0.027	0.076	0.054	0.355
6	0.027	0.045	0.054	0.600
7	0.026	0.102	0.052	0.254
8	0.040	0.058	0.080	0.689
9	0.044	0.081	0.088	0.543
10	0.014	0.023	0.028	0.608

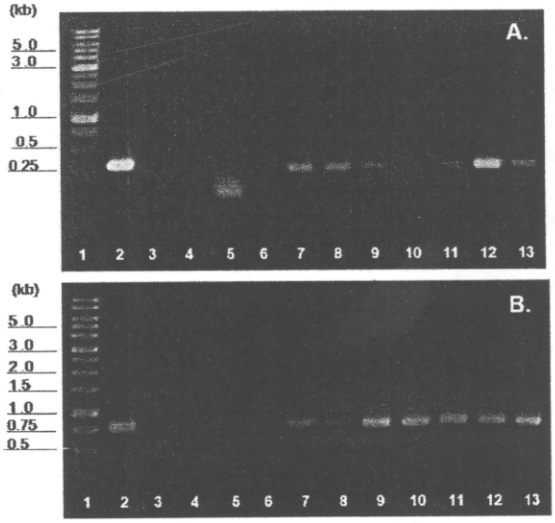


Figure 3.6 1.2% Agarose gel electrophoresis. PCR was performed with primers for the rooting locus genes *rol*A (A, 300 bp) and *rol*B (B, 780 bp). Lane 1 marker (10 kb DNA ladder), lane 2 *A. rhizogenes* ATCC 15834, lane 3 untransformed roots, lane 4-13 transformed hairy root (lines 1-10)

3.2 Medium manipulation

The hairy roots, in the WPM medium, grew slowly. The doubling time was about 2 months. In order to reduce the doubling time of the hairy root culture, the type of medium and the addition of plant growth regulator were manipulated. As described in 2.2.7 (Materials and methods), 9 types of media were investigated in the hairy roots (clone no.4). The appearances of the culture were observed after 1 month (Table 3.3). The untransformed roots in WPM medium and WPM plus NAA (0.5 mg/l) were used as control cultures. For time course determination, dry weights of hairy roots were determined from the hairy roots, which grew in WPM, WPM plus NAA, MS plus NAA and B5 plus NAA. The results were plotted and constructed for the growth curve (Fig. 3.7) and the relative growth plot (Fig. 3.8).

Table 3.3 shows the appearances of the hairy roots cultures of *M. speciosa* in different media. In the case of the untransformed root culture in WPM plus NAA (0.5 mg/l), the roots were transformed to callus-like tissue. This culture was unlike the hairy roots, which cultured in media: WPM, MS or B5, supplemented with NAA. The hairy roots in ½WPM, MS and ½MS media grew badly. In contrast, the hairy roots could grow faster in the media, with the addition of NAA (0.5 mg/l). Interestingly, the appearances of the hairy roots in MS plus NAA and B5 plus NAA were slightly white-green. The time course of growths of the hairy roots were established in order to determine an appropriate medium for the hairy root culture.

Table 3.4 summarizes the dry weights of the hairy roots, which grew in WPM, WPM plus NAA, MS plus NAA and B5 plus NAA media, in comparison with the untransformed roots in WPM medium. The hairy roots were sampled every 5 days for over 40 days. The results were used to construct the growth curves (Fig. 3.7). The results revealed that the manipulated media could promote growth of the hairy roots as reduction of doubling time from 2 months to 1 month. Growth curves were re-plotted in terms of the relative growth in order to eliminate the error of inoculation size. The relative growth plots indicated that additional 0.5 mg/l NAA in WPM, MS and B5 gave similar results. With the omission of NAA, hairy roots significantly grew slowly (Fig. 3.8). Therefore, the WPM plus 0.5 mg/l NAA was selected as an appropriate medium

for biomass production, and the hairy roots were further evaluated for secondary metabolites production.

Table 3.3 The appearances of the hairy roots cultures in different media

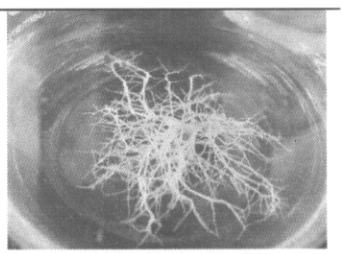
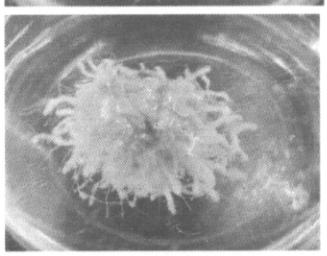
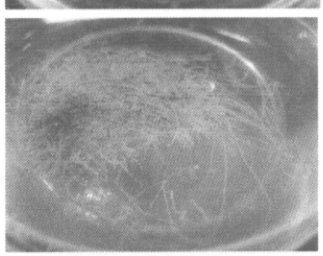
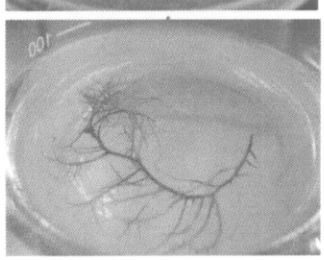
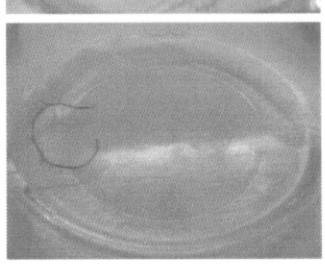
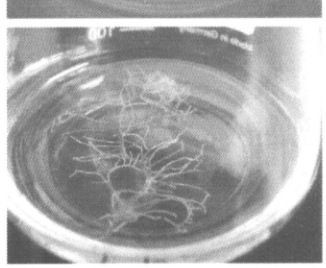
Type	*Medium*	*Characteristics*	*After for 30 days in culture*
Untransformed root	WPM	thick and short	
Untransformed root	WPM medium supplemented with plus 0.5 mg/l NAA	callus, brown color	
Hairy root	WPM	brown color and white color	
	MS	brown color	
	1/2 B5	dark brown and not grown	
	1/2 MS	brown color	

Table 3.3 (continued)

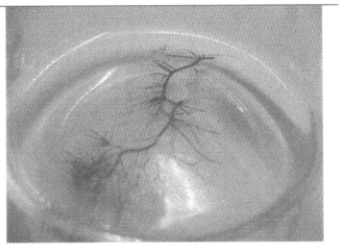
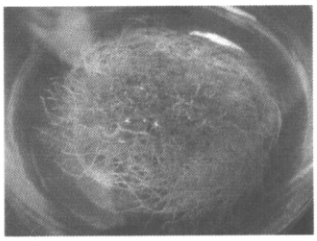
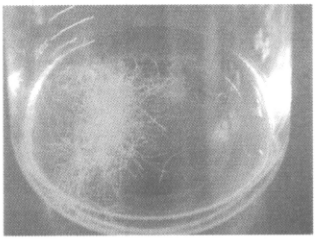
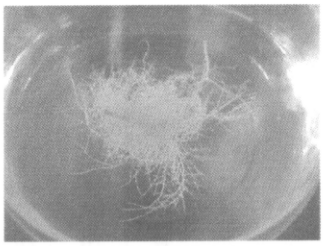
Type	*Medium*	*Characteristics*	*After 30 days in culture*
	1/2WPM	dark brown and not grown	
	WPM medium plus 0.5 mg/l NAA	dark brown and white color	
	B5 plus 0.5 mg/l NAA	green color	
	MS plus 0.5 mg/l NAA	green color	

Table 3.4 The dry weights of the hairy roots in different media (clone no.4). (n=4)

Day of culture	Dry weight (mg ± S.D.)				
	Untransformed roots biomass	Hairy roots biomass			
	WPM	WPM	WPM plus NAA*	B5 plus NAA*	MS plus NAA*
5	2.2 ± 0.2	26.6 ± 2.2	27.9 ± 4.6	8.8 ± 0.3	7.1 ± 0.4
10	2.2 ± 0.4	36.0 ± 0.2	25.3 ± 1.6	11.4 ± 0.6	9.1 ± 0.6
15	8.9 ± 2.5	40.8 ± 0.9	38.0 ± 6.2	15.8 ± 0.1	12.4 ± 0.4
20	13.5 ± 0.8	43.1 ± 0.6	59.4 ± 1.6	18.3 ± 0.1	14.0 ± 0.8
25	14.8 ± 1.9	46.4 ± 2.3	66.0 ± 11.5	20.3 ± 0.5	17.3 ± 0.3
30	25.0 ± 4.0	53.7 ± 2.3	72.6 ± 9.1	24.9 ± 0.3	20.7 ± 0.5
35	33.6 ± 2.0	60.7 ± 3.3	72.1 ± 10.6	26.5 ± 0.8	22.6 ± 0.7
40	31.9 ± 2.6	54.3 ± 0.3	72.4 ± 4.1	27.8 ± 1.5	26.6 ± 1.2

*The culture medium was supplemented with NAA at the concentration of 0.5 mg/l.

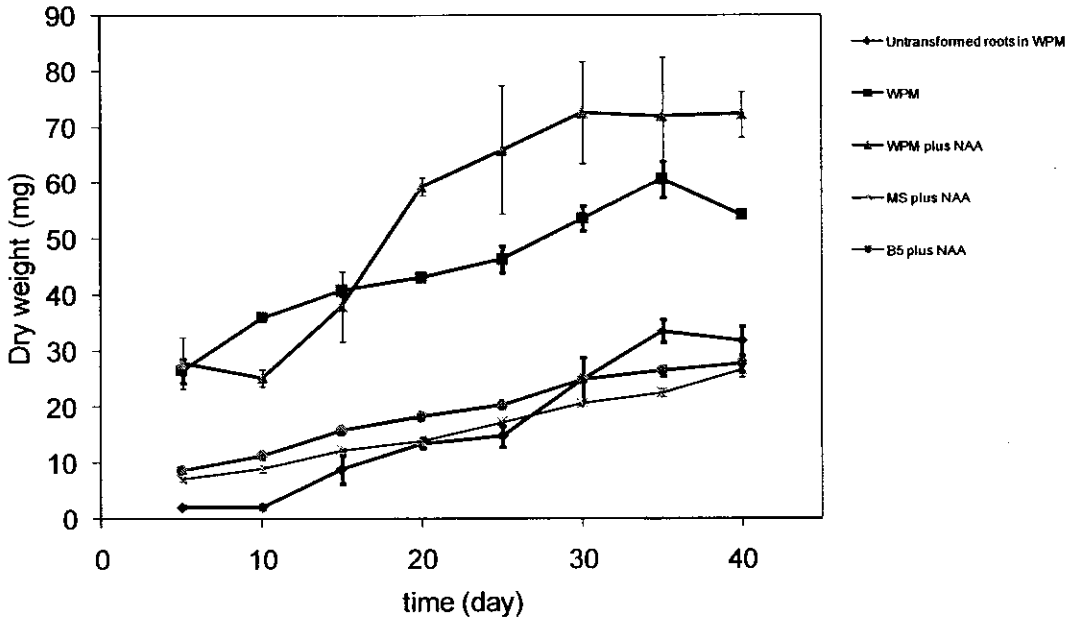


Figure 3.7 Growth curves of hairy roots in selected media. Error bars represent standard deviations. (n=4)

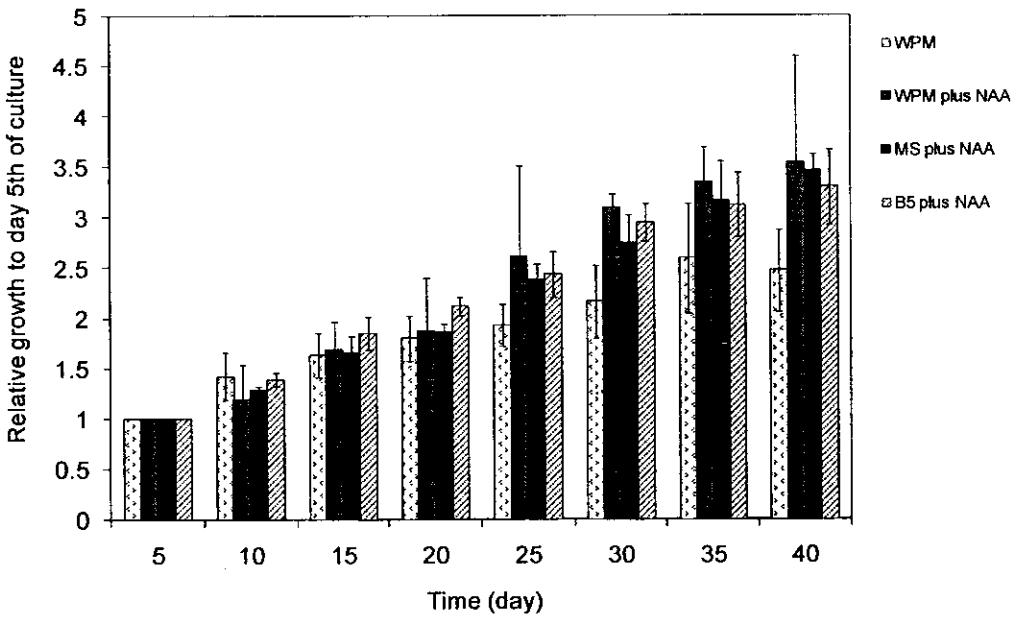


Figure 3.8 Relative growth of hairy roots in selected media. Error bars represent standard deviations. (n=4)

3.3 Structure elucidation of MSF1 & MSF2 from *M. speciosa* hairy roots culture

For preliminary screening, the hairy roots culture of *M. speciosa*, grown in WPM medium supplemented with 0.5 mg/l NAA was extracted and subjected to TLC plate. After developing, the TLC plate was then sprayed with Dragendroff's reagent to evaluate the potential of alkaloid formation. In the beginning, 2 spots of alkaloids were detected and they were positive to the Dragendroff's reagent. However, those spots were absent when the hairy roots were sub-cultured for 2-3 passages. This could probably have been due to the unstable compounds or because the alkaloids were from parts of the explant. Nevertheless, our attempts were to try to isolate the compound in order to evaluate the secondary metabolites. Therefore, *M. speciosa* hairy root culture was scaled up as described earlier (2.2.4). The compounds were isolated (2.2.8) and the structures of MSF1 and MSF2 were elucidated (2.2.9). MSF1 and MSF2 were obtained in percent yields of 0.016% and 0.027%, respectively.

3.3.1 MSF1

MSF1 was isolated from the *n*-hexane extract of transformed hairy root of *M. speciosa*, obtaining white needle crystal solids. In comparison with authentic β-sitosterol, the TLC analysis suggested that MSF1 was sterols. Analyses of ^{1}H- , ^{13}C-NMR spectra revealed that MSF1 was a mixture of β-sitosterol ($C_{29}H_{50}O$) and stigmasterol ($C_{29}H_{48}O$) (6.1 mg, 0.016 %w/w).

β-sitosterol, ^{1}H NMR ($CDCl_3$, 500 MHz): δH3.52 (1H, *m*, H-3), 5.35 (1H, *dd*, H-6), 0.68 (3H, *s*, Me-18), 0.98 (3H, *s*, Me-19), 0.91(3H, *d*, J = 6.4 Hz, Me-21), 0.83 (3H, *d*, J = 6.8 Hz, Me-26), 0.81 (3H, *d*, J = 6.9 Hz, Me-27), 0.85 (3H, *t*, J = 7.8 Hz, Me-29)

Stigmasterol, ^{1}H NMR ($CDCl_3$, 500 MHz): δH3.53, (1H, *d*, H-3), 5.35 (1H, *d*, H-6), 0.66 (3H, *s*, Me-18), 0.98 (3H, *s*, Me-19), 0.90 (3H, *d*, J = 6.4 Hz, Me-21), 0.81 (3H, *d*, J = 6.8 Hz, Me-26), 0.79 (3H, *d*, J = 6.9 Hz, Me-27), 0.81 (3H, *t*, J = 7.8 Hz, Me-29)

β-sitosterol, ^{13}C NMR ($CDCl_3$, 125 MHz): δC37.21 (C-1), 31.61 (C-2), 71.80 (C-3), 42.17 (C-4), 140.71 (C-5), 121.72 (C-6), 31.86 (C-7), 31.86 (C-8), 50.3 (C-9), 36.47 (C-10), 21.04 (C-11), 39.72 (C-12), 42.25 (C-13), 56.72 (C-14), 24.28 (C-15), 28.23 (C-16), 55.98 (C-17), 11.83 (C-18), 19.38 (C-19), 36.12 (C-20), 18.75 (C-21), 33.89 (C-22), 26.3 (C-23), 45.77 (C-24), 29.07 (C-25), 19.81 (C-26), 18.89 (C-27), 23.01 (C-28), 11.96 (C-29)

Stigmasterol, ^{13}C NMR ($CDCl_3$, 125 MHz): δC37.21 (C-1), 31.61 (C-2), 71.80 (C-3), 42.25 (C-4), 140.71 (C-5), 121.72 (C-6), 31.86 (C-7), 31.86 (C-8), 50.07 (C-9), 36.47 (C-10), 21.04 (C-11), 39.63 (C-12), 42.25 (C-13), 56.82 (C-14), 24.34 (C-15), 28.92 (C-16), 55.92 (C-17), 11.96 (C-18), 19.38 (C-19), 40.51 (C-20), 21.04 (C-21), 138.32 (C-22), 129.21 (C-23), 51.21 (C-24), 31.86 (C-25), 21.09 (C-26), 18.95 (C-27), 25.4 (C-28), 12.02 (C-29)

Analysis of chemical shifts, integration and spin coupling patterns from ^1H NMR data were in agreement with NMR data of the published report (Subhadhirasakul and Pechpongs, 2005). From ^{13}C-spectrum, δ at 138.32 (C-22) and at 129.21 (C-23) were observed. Both signals corresponded to the double bond in the structure of stigmasterol. Therefore, signals of H-22 and H-23 in ^1H-NMR were integrated and calculated for the molarities. The results suggested that MSF1 was a mixture of β-sitosterol and stigmasterol, present in the ratio of 1:1. The structures of both compounds are shown in Fig. 3.9. \

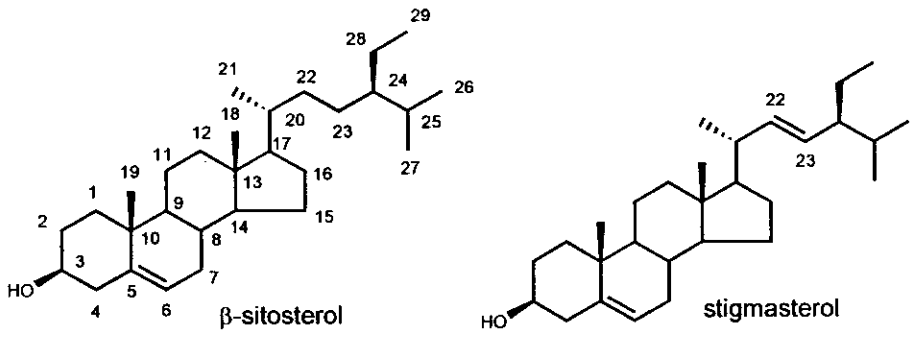


Figure 3.9 Structures of β-sitosterol and stigmasterol

3.3.2 MSF2

MSF2 was isolated from the *n*-hexane extract of the transformed hairy root of *M. speciosa*, obtaining an amorphous solid. The ^{1}H spectrum of MSF2 was suggested that MSF2 exhibited the structure as typical as the structure of triterpene compound. Later, MSF2 was sent to perform for the ^{13}C-NMR, DEPT, 2D-NMR spectra. Analyses of all spectra indicated that MSF2 was ursolic acid ($C_{30}H_{48}O_3$), co-existing with oleanolic acid ($C_{30}H_{48}O_3$) (10.11 mg, 0.027 %w/w).

Ursolic acid, ^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD): 3.20 (t, 1H, J = 7.5 Hz, H-3), 5.24 (m, J = 3.5 Hz 1H, H-12), 2.20 (d, J = 11.0 Hz, 1H, H-18), 2.02-1.15 (m, H-22), 0.98 (s, 3H, Me-23), 0.78 (s, 3H, Me-24), 0.93 (s, 3H, Me-25), 0.82 (s, 3H, Me-26), 1.10 (s, 3H, Me-27), 0.86 (d, J = 6.5 Hz, 3H, Me-29), 0.95 (d, J = 6.5 Hz, 3H, Me-30)

Ursolic acid, ^{13}C-NMR (125 MHz, $CDCl_3$:CD_3OD): 38.4 (C-1), 26.5 (C-2), 78.5 (C-3), 39.2 (C-4), 55.0 (C-5), 18.0 (C-6), 32.8 (C-7), 39.2 (C-8), 47.3 (C-9), 36.7 (C-10), 23.9 (C -11), 125.2 (C-12), 137.9 (C-13), 41.8 (C-14), 23.9 (C-15), 23.0 (C-16), 47.6 (C-17), 52.6 (C-18), 38.8 (C-19), 38.7 (C-20), 30.4 (C-21), 36.6 (C-22), 27.7 (C-23), 15.3 (C-24), 15.1 (C-25), 16.6 (C-26), 23.2 (C-27), 180.5 (C-28), 16.7 (C-29), 20.9 (C-30)

DEPT 90 experiment CH ($CDCl_3$:CD_3OD): 78.5 (C-3), 55.0 (C-5), 47.3 (C-9), 125.2 (C-12), 52.6 (C-18), 38.8 (C-19), 38.7 (C-20)

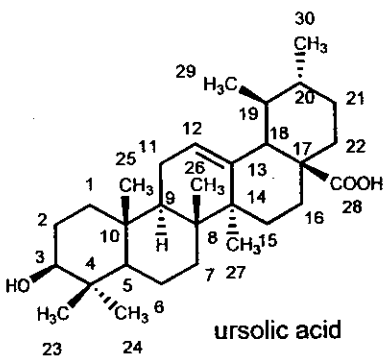
DEPT 135 experiment CH_2 ($CDCl_3$:CD_3OD): 38.4 (C-1), 26.5 (C-2), 18.0 (C-6), 32.8 (C-7), 23.9 (C-11), 23.9 (C-15), 23.0 (C-16), 30.4 (C-21), 36.6 (C-22); CH_3: 27.7 (C-23), 15.3 (C-24), 15.1 (C-25), 16.6 (C-26), 23.2 (C-27), 16.7 (C-29), 20.9 (C-30)

From ^{13}C-NMR spectrum, MSF2 composed of 30 carbons in its structure. DEPT90 and DEPT135 spectra suggested the types of carbons. The presence of δ at 78.5 (C 3; COH), 125.2 (C 12), 137.9 (C 13), 180.5 (C 28; C=C) suggested that MSF2 was ursolic acid. 2D-NMR: COSY

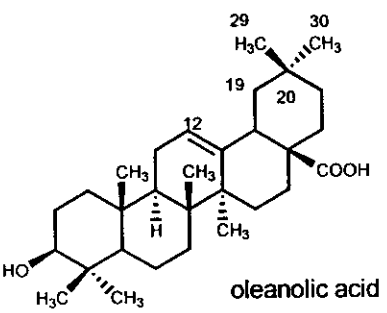
(Fig. B-7), HMQC (Fig. B-8), HMBC (Fig. 3.12 and Fig B-9) indicated the correlation of ^{13}C-^{13}C, ^{1}H-^{13}C and long-length coupling ^{1}H-^{13}C, respectively. Analysis of NMR spectra of MSF2 was in agreement with the previous report (GÜvenalp *et al.*, 2006). HMBC spectrum suggested the correlation of carbon and hydrogen atoms as shown in Table 3.5. The presence of signal at δ 5.28 MHz in ^{1}H-NMR spectra indicated that MSF2 contained not only ursolic acid but also oleanolic acid. This evidence was in line with the presence of signals at δ 122 MHz and 144 MHz in ^{13}C-NMR spectrum (Fig. B-4). Integration of H-12 signal in ^{1}H-NMR indicated that MSF2 was a mixture of ursolic acid and oleanolic acid in the ratio of 5:1. All spectra are shown in Appendix.

Table 3.5 HMBC: ^{1}H and ^{13}C NMR spectroscopic data for ursolic acid

H/C	*δ ^{1}H (J, Hz)*	*δ ^{13}C (ppm)*	*HMBC correlations*
3	3.2 (*t*, 7.5 Hz)	78.5	C-1, C-2, C-23, C-24
12	5.24(*m*, 3.5 Hz)	125.2	C-9, C-14, C-18
18	2.2 (*d*, 11 Hz)	52.6	C-13, C-16, C-28
25	0.93 (*s*)	15.1	C-1, C-5, C-9
26	0.82 (*s*)	16.6	C-7, C-9, C-14
27	1.10 (*s*)	23.2	C-8, C-13, C-14, C-15
29	0.86 (*d*, 6.5 Hz)	16.7	C-18, C-20
30	0.95 (*d*, 6.5 Hz)	20.9	C-19, C-21



ursolic acid



oleanolic acid

Figure 3.10 Structures of ursolic acid and oleanolic acid

3.4 Ursolic acid production in *M. speciosa* hairy roots

Since the hairy roots of *M. speciosa* were maintained in WPM medium and supplemented to 0.5 mg/l NAA, they could produce ursolic acid. Previously it had been reported that ursolic acid exhibits varieties of pharmacological activities (Liu, 1995). In this study, ursolic acid production was, therefore, determined. The time course of ursolic acid production was investigated. Calibration curve of an authentic ursolic acid was constructed. The elution time of ursolic acid was 15.5 min under HPLC condition (2.2.10) (Fig. 3.12). The linearity was ranged from 7.31-117 µg/ml with the linear regression (R^2) of 0.9999 (Fig. 3.11).

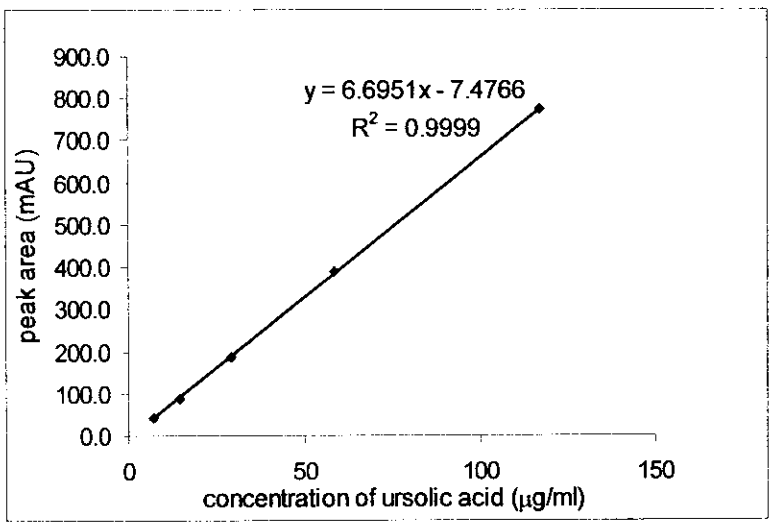


Figure 3.11 Calibration curve of authentic acid ursolic acid.

To construct the growth curve and production curve, the samples (n=3) were taken every 5 days for over 40 days of culture. The ursolic acid content of sample was determined after weighing the dry weight. The untransformed root culture was used as a control culture. The hairy roots and the untransformed hairy roots were extracted and prepared for acetonitrile fractions (2.2.10). The sample (10 µl) was subjected to the HPLC system. The chromatograms of authentic ursolic acid,

the extracts from the untransformed roots and the hairy roots are shown in Fig. 3.12. The identities of ursolic acid were confirmed by UV absorption spectra (200-300 nm) using the diode array detector.

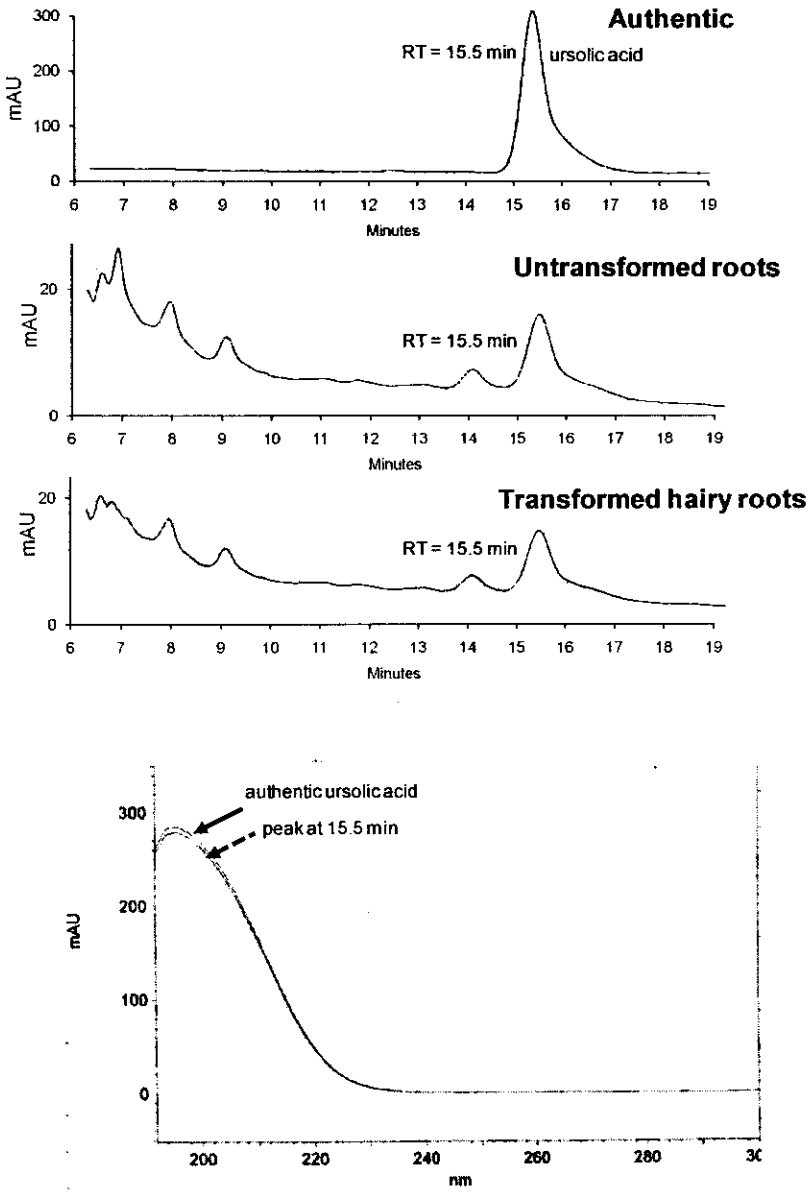


Figure 3.12 HPLC-chromatograms of authentic ursolic acid (RT=15.5 min); the untransformed roots extract; the hairy roots extract and UV absorption spectra of peak at 15.5 min using the HPLC-UV diode array detector.

Table 3.6 Biomass and ursolic acid contents in untransformed roots and hairy roots (n=4)

Day	Untransformed roots		Transformed hairy roots	
	biomass (g dry wt./flask)	ursolic acid content (mg/g dry wt. ± S.D.)	biomass (g dry wt./flask)	ursolic acid content (mg/g dry wt. ± S.D.)
5	0.003 ± 0.001	1.578 ± 0.012	0.025 ± 0.006	1.520 ± 0.004
10	0.004 ± 0.004	1.742 ± 0.008	0.028 ± 0.006	1.902 ± 0.023
15	0.007 ± 0.004	1.891 ± 0.007	0.042 ± 0.009	2.490 ± 0.023
20	0.013 ± 0.001	1.691 ± 0.007	0.058 ± 0.018	1.649 ± 0.012
25	0.016 ± 0.003	2.090 ± 0.009	0.062 ± 0.012	2.004 ± 0.040
30	0.027 ± 0.005	2.410 ± 0.008	0.071 ± 0.010	3.468 ± 0.034
35	0.035 ± 0.004	1.740 ± 0.023	0.085 ± 0.027	1.981 ± 0.027
40	0.035 ± 0.007	2.010 ± 0.004	0.086 ± 0.028	2.398 ± 0.010

The area under peak at 15.5 min was integrated and converted to ursolic acid concentration using the calibration curve. The results are summarized in Table 3.6. Growth curve and production curve of the hairy root culture and the untransformed root culture are shown in Fig. 3.13 and Fig. 3.14, respectively. The results indicated that both types of culture were able to produce ursolic acid. However, at 30 days of culture, the hairy roots accumulated the ursolic acid with the yield of 3.468 ± 0.034 mg/g dry wt, whereas the untransformed roots could produce 2.410 ± 0.008 mg/g dry wt. The results show that the ursolic acid was produced and accumulated at the late linear phase (Fig. 3.13) for both types of culture.

Fig. 3.13 indicates that the cell cycle of the *M. speciosa* hairy roots was within 30 days of culture. It can be seen that the cycle composed of 3 phases: days 1-10 for lag phase, days 10-20 for exponential phase, days 20-30 for linear phase and days 30-40 for stationary phase. The production curve of the hairy roots shows that ursolic acid was usually present in the cells and maintained at a particular level of *ca.* 2 mg/g dry wt. However, the hairy roots were able to

accumulate maximum yield at the late linear phase. Thus, the ursolic acid was utilized by the cells and acted as a secondary metabolite in this *in vitro* culture.

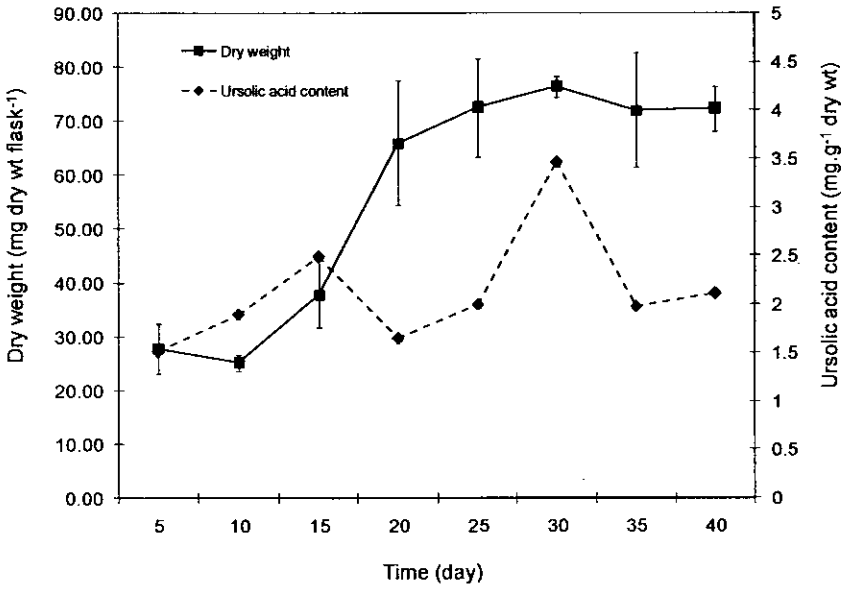


Figure 3.13 Growth curve and production curve of *M. speciosa* hairy root culture. Error bars represent standard deviations.

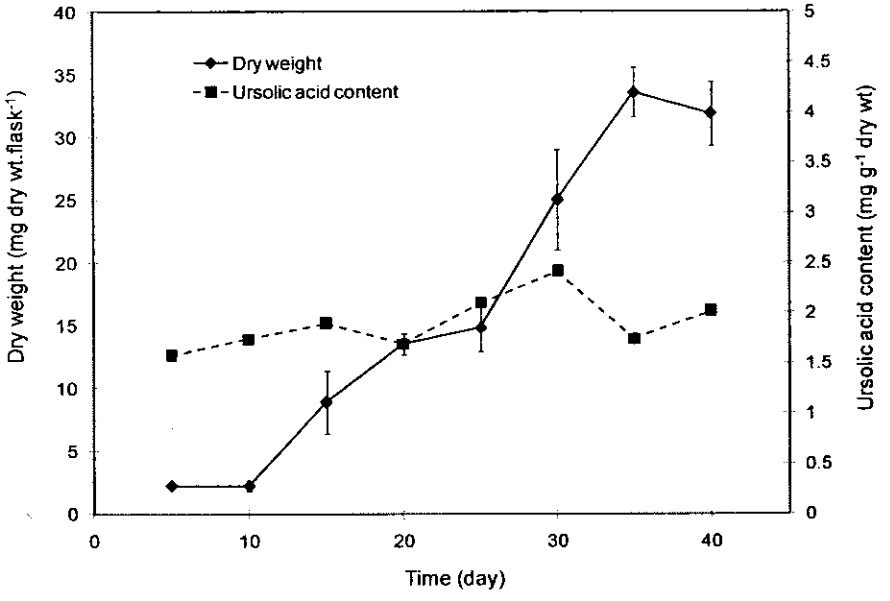


Figure 3.14 Growth curve and production curve of the *M. speciosa* untransformed root culture. Error bars represent standard deviations.

CHAPTER 4

Discussion

Mitragyna speciosa (Roxb.) Korth. (Rubiaceae) is a source of mitragynine, a monoterpenoid indole alkaloid (MIA), which acts as opium-like effect. The main objective of this investigation was to establish hairy roots culture of *M. speciosa* by infection with *Agrobacterium rhizogenes* ATCC 15834. Since the susceptibility of plant cells to *A. rhizogenes* was dependent upon age and differentiation status of plant tissue (Sevón and Oksman-Caldentey, 2002). Therefore, the hairy root culture, in this study, was induced from small plantlets, which were germinated under controlled conditions. The transformed hairy roots were identified for the *rol*A and *rol*B as genetic markers. Six of ten clones were positive to have genes from *A. rhizogenes* ATCC 15834 (transformation rate of 60%), which means low frequency of transformation. Unlike the transformation of *Gmelina arborea*, the herbaceous plants, transformation rate was about 100% (Dhakulkar *et al.*, 2005). Type of woody plant of *M. speciosa* affected the transformation rate which may be discussed. However, the chance of successful transformation may be obtained from infection with different strains of *A. rhizogenes* (Sevón and Oksman-Caldentey, 2002).

Theoretically, the hairy root culture should grow relatively fast and can grow in plant growth regulator free medium (Guillon *et al.*, 2006). But the *M. speciosa* hairy roots obtained from this study grew slowly. For this study, a low concentration of NAA (0.5 mg/l) is required for promoting their growth. This evidence is unusual for normal hairy root culture (Sevón and Oksman-Caldentey, 2002). In the case of other Rubiaceous plants, *Ophiorrhiza pumila* hairy root culture in B5 medium (containing 2% (w/v) sucrose) grew fast and produced the camptothecin, a MIA-anticancer agent (Saito *et al.*, 2007).

The hairy roots of *M. speciosa* could accumulate compounds, which are triterpenoids; ursolic acid and oleanolic acid (with the ratio of 5:1) and phytosterols, β-sitosterol and stigmasterol (with the ratio of 1:1). Nevertheless, many studies reported that the alkaloids such as mitraphylline, rhynchophylline have been found in the root bark of *M. speciosa* (Houghton and

Shellard, 1974; Shellard *et al.* 1978b). But none of the alkaloids were found in this culture. Intermediates of alkaloids were probably unstable and degraded during culture (Fig 4.1). However, the hairy root culture accumulated the ursolic acid, which is pharmacologically important for antibacterial, anti-inflammatory, antiviral activities, etc. (Liu, 1995). It produced in the late linear phase a yield of 3.468±0.034 mg/g dry wt. It can be noted that *M. speciosa* hairy roots could accumulate high yield of ursolic acid when compared to the *Uncaria tomentosa* cell suspension culture (1.680±0.039 mg/g dry wt.) (Feria-Romero *et al.*, 2005). This evidence can be concluded that the differentiated cells such as hairy roots preferred to produce higher amount of secondary metabolites.

For the biosynthetic point of view (Fig. 4.1), *M. speciosa* hairy roots could not produce any alkaloid, especially mitragynine. It may be due to a lack of precursors and enzymes, which is involved in the terpenoid indole alkaloid biosynthesis, since primary metabolites need special cell compartments for storage and degradation (Luckner, 1984). The presence of ursolic acid, coexisting with oleanolic acid, indicated that at least isoprene units have been produced in this culture as shown in Fig. 4.1. These isoprene units are precursors for both triterpenoids and phytosterols (Luckner, 1984), which means their biosynthesis was active in this culture. Therefore, the *M. speciosa* hairy root culture is not only useful for ursolic acid production but also have a potential to be a model plant culture for triterpenoid biosynthesis.

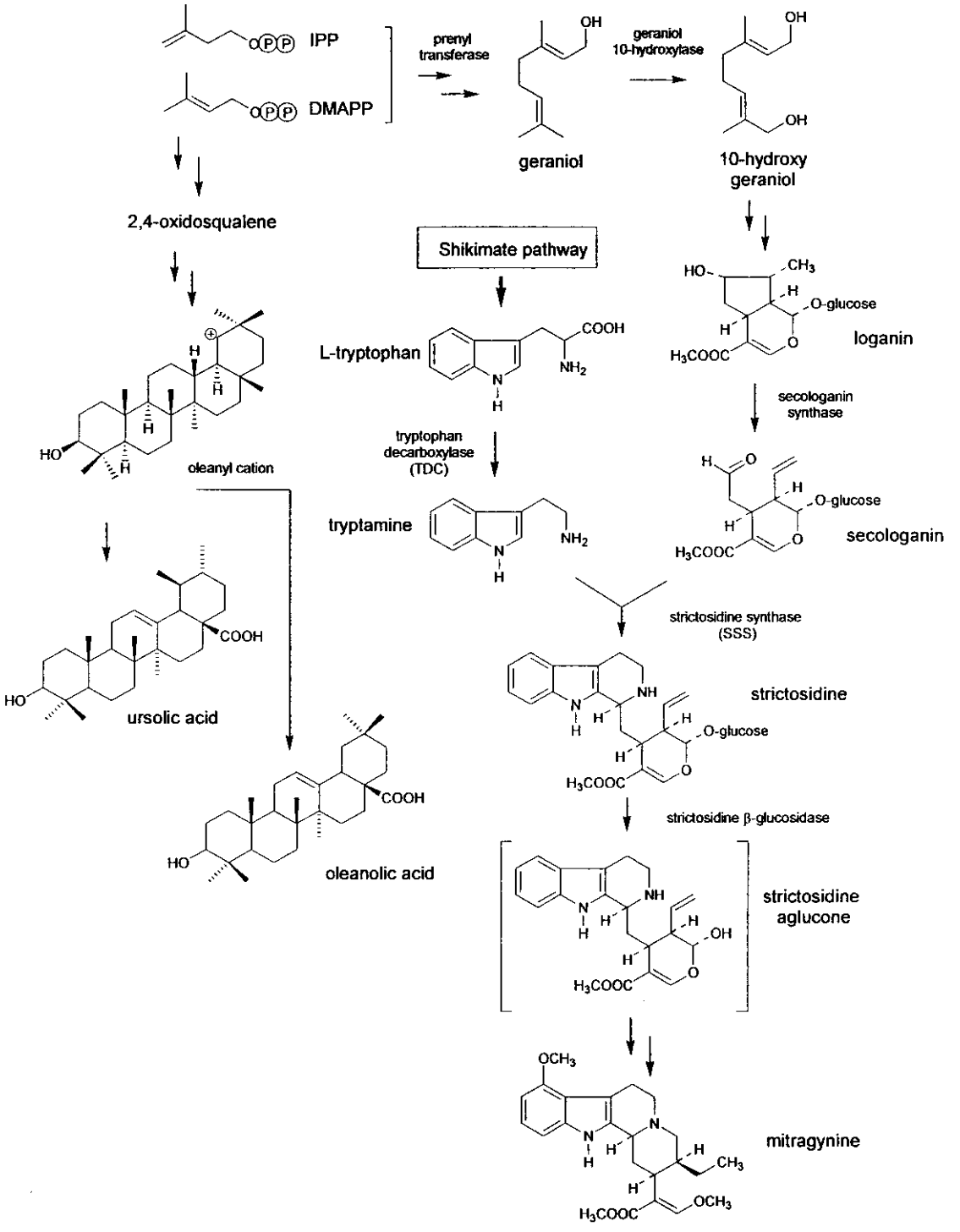


Figure 4.1 Proposed biosynthetic pathway of mitragynine

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