รายงานวิจัยฉบับสมบูรณ์

โครงการ: **การโคลนยีนและการศึกษาคุณสมบัติสตริ๊กโตซิดีนเบต้ากลูโคซิเดสจากพืชกระท่อมและการสร้างพืชต้นแบบเพื่อการศึกษาชีวสังเคราะห์สารมิตรากัยนีน**

[Molecular cloning of strictosidine glucosidase from Kratom and establishment of plant model for mitragynine biosynthetic study]

โดย

รองศาสตราจารย์ ดร.จุไรทิพย์ หวังสินทวีกุล

คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์

กุมภาพันธ์ 2562

สัญญาเลขที่ RSA5880044

รายงานวิจัยฉบับสมบูรณ์

โครงการ: **การโคลนยีนและการศึกษาคุณสมบัติสตริ๊กโตซิดีนเบต้ากลูโคซิเดสจากพืชกระท่อมและการสร้างพืชต้นแบบเพื่อการศึกษาชีวสังเคราะห์สารมิตรากัยนีน**

[Molecular cloning of strictosidine glucosidase from Kratom and establishment of plant model for mitragynine biosynthetic study]

โดย

รองศาสตราจารย์ ดร.จุไรทิพย์ หวังสินทวีกุล

คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยสงขลานครินทร์

บทคัดย่อ

รหัสโครงการ : RSA5880044

ชื่อโครงการ : การโคลนยีนและการศึกษาคุณสมบัติสตริ๊กโตซิดีนเบต้ากลูโคซิเดสจากพืชกระท่อมและการสร้างพืชต้นแบบเพื่อการศึกษาชีวสังเคราะห์สารมิตรากัยนีน

ชื่อนักวิจัย : รศ.ดร.จุไรทิพย์ หวังสินทวีกุล
คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์
E-mail address: juraithip.w@psu.ac.th

ระยะเวลาโครงการ : 3 ปี (มิ.ย. 2558- ก.ค. 2561) (ขอขยายเวลาถึง ธันวาคม 2561)

พืชกระท่อม จัดอยู่ในยาเสพติดให้โทษประเภทที่ ๕ ตามพระราชบัญญัติยาเสพติดให้โทษ พ.ศ. ๒๕๒๒ และปรับปรุงใน พ.ศ. ๒๕๖๒ แต่คุณค่าของพืชกระท่อมในทางยาก็ยังเป็นที่ยอมรับของวงวิชาการในการเป็นยาในกลุ่มแก้ปวดออปิออยด์ วิถีชีวสังเคราะห์ของสารมิตรากัยนีนที่สร้างจากสตริ๊กโตซิดีนยังมีประเด็นที่ต้องศึกษาเนื่องจากยังไม่มีรายงานมาก่อน การศึกษานี้ได้พยายามโคลนยีนสตริ๊กโตซิดีนเบต้า-กลูโคซิเดส เอนไซม์ในชีวสังเคราะห์ของมิตรากัยนีน การโคลนแบ่งเป็นส่วนแกนกลางและส่วนปลายทั้งสองด้านของยีน ขั้นแรกในการโคลนยีนส่วนแกนกลางได้ชิ้นส่วนขนาด 773 นิวคลีโอไทด์ (โค๊ดกรดอะมิโนขนาด 257 หน่วย) เมื่อเปรียบเทียบชิ้นส่วนแกนกลางกับยีนอื่น ๆ ในพืชชั้นสูง พบว่ามีความเหมือนอยู่ในช่วง 55-60% เมื่อวิเคราะห์ด้วยโปรแกรมพบลักษณะสำคัญของยีนในกลุ่มไกลโคซิลไฮโดรเลส จากนั้นนำชิ้นส่วนแกนกลางมาออกแบบไพรเมอร์เพื่อการโคลนส่วนปลาย 5′- และ 3′- ผลพบว่ายังไม่ประสบความสำเร็จในการโคลนยีนส่วนปลาย แม้จะปรับชนิดของไพรเมอร์และสภาวะการทดลองที่เกี่ยวข้องแล้วก็ตาม เกี่ยวกับการสร้างพืชต้นแบบกระท่อมเพื่อศึกษาชีวสังเคราะห์ของสารมิตรากัยนีนและแอลคาลอยด์อื่น ๆ ในการทดลองได้พืชที่เลี้ยงในสภาวะทดลอง, ยอดเพาะเลี้ยง, รากเพาะเลี้ยง, แคลลัส และเซลล์เพาะเลี้ยง โดยพืชต้นแบบทั้ง 5 ชนิดได้ถูกนำมาศึกษาและประเมินการสร้างสารทุติยภูมิ สำหรับพืชเพาะเลี้ยงที่มีลักษณะครบถ้วนเหมือนพืชที่ปลูกในธรรมชาติ (แต่เลี้ยงอยู่ในสภาวะควบคุม) ใช้เทคนิคอิลิซิเตชั่นมากระตุ้นและประเมินการสร้างสาร พบว่าเมื่อกระตุ้นพืชด้วยสารโซเดียม ไนโตรพลัสไซด์ (SNP) สามารถกระตุ้นการสร้างสารเซโคโลกานิน สาร SNP จะปลดปล่อยก๊าซไนตริกออกไซด์ (NO) และกระตุ้นการสร้างสารดังกล่าว ในการทดลองค้นพบว่าผลการสร้างสารเกิดขึ้นผ่านช่องแคลเซียมในระดับเซลล์ และก่อให้เกิดการกระตุ้นยีนที่เกี่ยวข้องในชีวสังเคราะห์เป็นผลให้มีการสร้างสารเซโคโลกานิน สาเหตุที่ได้เห็นความแตกต่างระหว่างการสร้างมิตรากัยนีนของพืชที่ไม่ถูกกระตุ้นและถูกกระตุ้นด้วย SNP คาดว่าเนื่องจากปริมาณกรดอะมิโนทริปตามีนที่อยู่ในเซลล์อาจไม่เพียงพอ และเข้าทำปฏิกิริยาในระดับเซลล์ของการสังเคราะห์มิตรากัยนีน ในการทดลองได้ศึกษาผลของแสงสีแดง สีฟ้า สีขาว และที่มืด ต่อการสร้างแอลคาลอยด์ (มิตรากัยนีน, เพย์แนนเทอีน, สเปซิโอกัยนีน) ในพืชกระท่อม ผลการทดลองพบว่าแสดงสีฟ้ากระตุ้นการเจริญเติบโตของพืชและสร้างสารแอลคาลอยด์ได้ดีที่สุด ในขณะที่แสงสีแดงกระตุ้นการสร้างสารได้ดี และยับยั้งการเจริญเติบโต จากพืชในหลอดทดลองในนำชิ้นส่วนมาเหนี่ยวนำให้เกิดแคลลัสพบว่าส่วนของลำต้นเป็นชิ้นส่วนที่ให้เปอร์เซนต์การเกิดแคลลัสดีที่สุด และเกิดในอาหารชนิด WPM ที่เสริมด้วยฮอร์โมน 2,4-D ขนาด 1 มก./ลิตร หลังจากการเปลี่ยนอาหารหลาย ๆ ครั้ง ทำให้ได้เซลล์ร่วน มีลักษณะเป็นเนื้อเดียวกัน และเจริญเติบโตได้ดี แต่เมื่อประเมินการสร้างสารกลับไม่พบการสร้างแอลคาลอยด์เลย เมื่อออกแบบชนิดของอาหารโดยการใช้ Plackett-Burman Design ก็ไม่พบการสร้างสารมิตรากัยนีน จากนั้นได้พยายามเปลี่ยนชนิดของฮอร์โมน เนื่องจาก 2,4-D อาจมีผลยับยั้งการสร้างสารทุติยภูมิ โดยการปรับให้เป็น NAA และผสมกับ BA หรือ TDZ ในความเข้มข้น 1 มก./ลิตร ผลการทดลองพบว่าเฉพาะแคลลัสที่เลี้ยงใน WPM ที่เสริมด้วย NAA และ TDZ อย่างละ 1 มก/ลิตร แคลลัสที่ได้มีสีเขียว แต่ก็ยังไม่สร้างมิตรากัยนีน แคลลัสทั้งสองสูตร ได้ถูกนำมาเหนี่ยวนำให้เกิดเซลล์เพาะเลี้ยง เมื่อประเมินการสร้างสารในเซลล์เพาะเลี้ยงพบเพียงกรดเออโซลิก (เช่นเดียวกับที่พบในรากเพาะเลี้ยง) การกระตุ้นด้วยเมทิลแจสโมเนท และสารสกัดยีสต์ พบว่าสามารถกระตุ้นการสร้างกรดเออโซลิกในเซลล์เพาะเลี้ยงในอาหารทั้งสองชนิด น่าสนใจที่เมื่อกระตุ้นด้วยสารสกัดยีสต์พบว่าเซลล์มีการหลั่งสารสีน้ำตาลออกมาสู่อาหารเพาะเลี้ยง จากการตรวจสอบเบื้องต้นด้วย LC-MS/MS พบว่ามีแนวโน้มการสร้างสารมิตรากัยนแต่มีปริมาณน้อยมาก ซึ่งต้องปรับสภาวะการทดลองในลำดับต่อไป ส่วนการทดลองในยอดเพาะเลี้ยง มีการกระตุ้นด้วยกรดแจสโมนิก กรดแอบซิสซิก และกรดซาลิไซลิก ออกแบบโดย RSM-CDD พบว่าสารทั้งสามชนิดสามารถกระตุ้นการสร้างสารมิตรากัยนีนได้ นอกจากนี้การกระตุ้นด้วยเมทิลแจสโมแนท กรดซาลิไซลิก และไคโตซาน ก็สามารถกระตุ้นการสร้างมิตรากัยนีนได้เช่นกัน จากผลการทดลองที่กล่าวมา แสดงให้เห็นว่าเนื้อเยื่อและเซลล์เพาะเลี้ยงที่เหนี่ยวนำมีความเหมาะสมในการนำมาใช้ศึกษาชีวสังเคราะห์สารมิตรากัยนีนในระดับโมเลกุลได้

คำหลัก : *Mitragyna speciosa*, mitragynine, strictosidine glucosidase, plant tissue cultures, Kratom

Abstract

Project Code: RSA5880044
Project Title: Molecular cloning of strictosidine glucosidase from kratom and establishment of plant models for mitragynine biosynthetic study
Investigator: Juraithip Wungsintaweekul, Dr.rer.nat., Associate Professor
Faculty of Pharmaceutical Sciences Prince of Songkla University
E-mail address: juraithip.w@psu.ac.th
Project Period: 3 years (June 2015-July 2018) (extended until December 2018)

Medicinal value of *Mitragyna speciosa* (Korth.) Havil or Kratom has been reported for an alternative opioid analgesic. It is classified as a member of narcotic plant level 5 according to the Narcotic Board Control, Ministry of Health of Thailand. From biosynthetic point of view, the distance between strictosidine and mitragynine is still unknown. The present study, we attempted to clone the cDNA encoding strictosidine-β-glucosidase (SGD). The internal sequence of SGD contains 773 nucleotides, encoded for 257 amino acids residues. The alignment of the deduced amino acid to known plant SGD revealed that kratom SGD shared the homology ranging 55-60% identity. Functional analysis of internal sequence found the catalytic motif belonging to glycosyl hydrolases family. In addition, the glutamate catalytic residue also was found in the kratom SGD when compared to *Rauvolfia serpentina* SGD. Unsuccessful cloning of the 5′- and 3′- ends of SGD was tasked. New primers were designed and again cloned using RACE technique as well as optimization of PCR reaction. The resulting fragments of 5′- and 3′- ends of SGD was not obtained.

This study was successful to establish the callus and suspension culture besides of plant, root and shoot cultures. Five different types of tissue cultures were evaluated and manipulated for secondary metabolite production. Plant culture was being a model for complete kratom alkaloid production. The elicited kratom with sodium nitroprusside (SNP) as nitric oxide donor, could stimulate the secologanin production. This result suggested that NO was play an important role in mitragynine biosynthesis. Treatment with 1 mM nifedipine (a calcium channel blocker) together with 1 mM SNP blocked secologanin formation to 2.42±0.44 mg/g dry weight. This result indicated that calcium channel involved in the mitragynine biosynthesis and related to NO response in *M. speciosa* plant culture. Following the mRNA expressions of genes involved in mitragynine biosynthesis revealed that the mRNA levels were increased after treatment with 1 mM SNP for 48 h and reduced when CPTIO or nifedipine was added. Treatment with different wavelength of lights found that blue light enhance growth and kratom alkaloid production while red light stimulated kratom alkaloid production but reduced the growth. In vitro plant was used for inducing the callus culture. Callus culture was obtained from stem and on WPM supplemented with 2,4-D. After several passage, homogenous and friable cells were obtained. Nevertheless, medium manipulation by Plackett-Burman design could not make callus cells to produce mitragynine, albeit phenolic compounds. Changing auxin to NAA and combine with either BA and TDZ was investigated. Callus culture maintained in WPM plus 1 mg/L NAA and 1 mg/L TDZ could survive. Green callus culture was obtained but still no alkaloid was produced. Attempts to use methyl jasmonate and yeast extract as elicitors could only enhance ursolic acid (triterpenoid). We tried to treat the callus culture with yeast extract for 3 days, revealed the production of mitragynine after detection with high-resolution LC-MS/MS, albeit very tiny amount. Shoot culture was established and maintained on WPM plus 2 mg/L TDZ and 1 mg/L BA. Response surface methodology and central composite design suggested the optimal concentration of plant-growth regulator like compounds such as jasmonic acid, abscisic acid and salicylic acid that increased mitragynine production in shoot culture. Point-to-point design also found that chitosan also stimulate mitragynine production. This study illustrated that the differentiate and non-differentiate cell cultures inform us the suitable cultures for mitragynine production. The results obtained from this study would give more insights of an alternative source of mitragynine, molecular regulation in mitragynine biosynthesis.

Keywords: *Mitragyna speciosa*, mitragynine, strictosidine beta-glucosidase, plant tissue cultures, Kratom

Acknowledgments

The author wishes to thank the Thailand Research Fund (TRF) and Prince of Songkla University and the Faculty of Pharmaceutical Sciences for grant support (grant no. RSA5880044).

My sincere thanks also go to my “dream team” who involved in the Mitragyna project. Dr. Supattra Limsuwanchote was responsible for quantification of mitragynine and its derivatives. Ms. Naruemon Sengnon was done a good job on plant tissue cultures and all treatments. All undergraduate students, who involved in the Kratom plant model project are acknowledged.

I would like to thank the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences and the Scientific Equipment Center (SEC), Prince of Songkla University for providing laboratory facilities.

Without their help, this work might be unsuccessful, thank you very much indeed.

Juraithip Wungsintaweekul
Grantee no. RSA5880044
28 February 2019

Executive Summary

1. Background and rationale

Kratom refers to the leaves of *Mitragyna speciosa* (Roxb.) Korth. (Family Rubiaceae). It is an endemic plant found in tropical Southeast Asia, especially in Thailand and Malaysia. Kratom is controlled in Australia, Malaysia, Myanmar, New Zealand, South Korea, and Thailand. Kratom is classified as Narcotic Level 5 in Thailand according to the Royal Decree on Narcotics, 1979. Kratom is traditionally used for diarrhea and as a substitute for opium (Suwanlert 1975). Its leaves have been used in the form of chewed, smoked or brewed as a tea. The medicine man used Kratom's leaves and barks as an ingredient in the decoctions for the treatments of diarrhea, amoebiasis, diabetes, and hypertension.

Scientifically, Kratom is a source of the terpenoid indole alkaloid (TIAs) such as mitragynine, paynantheine, speciogynine and 7-hydroxymitragynine. Kratom's extract and mitragynine have been investigated in several animal models by the pharmacologists. Mitragynine possesses 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). Thongpraditchote and co-workers demonstrated that mitragynine binds to opioid receptors non-specifically and has less addictive than morphine (Thongpraditchote et al. 1998). Therefore, it highlights the relevance of mitragynine and its derivatives to be an alternative opioid analgesic drug. In addition, mitragynine also affects the serotonergic and dopaminergic receptors in the central nervous system (Matsumoto *et al*., 1996a), resulting in the anti-depressant effect. Moreover, 7-hydroxymitragynine has a strong analgesic activity than mitragynine and morphine (Kikura-Hanajiri et al. 2009). This fact also suggested that 7-hydroxymitragynine has the potential to be an oral opioid analgesic.

Looking into Kratom, in the aspect of mitragynine biosynthesis, nowadays, there are reports about the early step genes and enzymes from Kratom. Concerning to mitragynine biosynthesis in *M. speciosa*, Nagakura and co-workers were firstly reported in 1979. By feeding the doubly labeled of [3-^{3}H, 6-^{14}C]strictosidine to *M. speciosa* suggested that radiolabeled substrate was incorporated into a molecule of mitragynine and speciociliatine. Their results indicated that strictosidine play a role as an intermediate in *M. speciosa* (Nagakura et al. 1979). Widely known that strictosidine is a common precursor of the TIAs as shown in *Catharanthus roseus*, *Rauwolfia serpentina*, *Camptotheca accuminata*. Strictosidine is a product of the Mannich reaction of tryptamine and secologanine, catalyzed by the strictosidine synthase (STR) (Rueffer et al. 1978). Tryptamine is the decarboxylation product of an amino acid tryptophan by the tryptophan decarboxylase (TDC) from the shikimate pathway (Dewick 2001). Secologanin, a

secoiridoid moiety, is supplied from the monoterpene biosynthesis. In-depth to manipulate the biosynthetic pathway in higher plant probably affects to the secondary metabolite production. Therefore, gene and enzymes those involved in the TIA biosynthesis are the targets for genetic manipulation. In *M. speciosa* leaves, cDNAs of strictosidine synthase (*STR*) (Jumali et al. 2011), 1-deoxy-D-xylulose 5-phosphate synthase (*DXS*) and 2*C*-methyl-D-erythritol 4-phosphate synthase (*MEPS*) (Wungsintaweekul 2009) were isolated and functionally characterized. An accumulation site of mitragynine is presence dominantly in leaves, less in stem and absence in the root (Charoonratana et al. 2013a). The distance between strictosidine and mitragynine is still unknown. There are proposed to have at least 5 enzymatic steps from the branch point intermediate, strictosidine. Information of genes and enzymes are necessary to understand the regulation of the Kratom's TIAs biosynthesis and have no report so far.

More than 20 enzymatic steps are proposed to involve in the mitragynine biosynthesis. Recently, cDNAs encoding anthranilate synthase alpha subunit (*ASA*) and tryptophan decarboxylase (*TDC*) of Kratom has been reported. The *ASA* of Kratom exists in two isoforms (*ASA1* & *ASA2*). Treatment of Kratom shoot culture with methyl jasmonate resulted in the enhancement of mitragynine (Charoonratana et al. 2013a). The *TDC* gene also cloned and characterized and found that the TDC is a pyridoxal phosphate-dependent protein. Transgenic Kratom hairy roots with *TDC* gene caused the increasing amount of tryptamine (Charoonratana et al. 2013b). As mentioned, five genes of Kratom have been cloned and characterized and most of them involved in the early step of mitragynine biosynthesis. The crucial step next to strictosidine synthase, strictosidine β-glucosidase (SGD), in Kratom is still no information. This enzyme has been shown an important role in producing an intermediate by activating the glucoside, strictosidine, and allowing it to enter the multiple indole alkaloid pathways. The SGDs were cloned, purified and characterized from alkaloid producing plants such as *Tabernaemontana divaricata* (Luijendijk et al. 1996), *Catharanthus roseus* (Luijendijk et al. 1998; Geerling et al. 2000; Zarate et al. 2001), *Strychnos mellodora* (Brandt et al. 2000), *R. serpentina* (Gerasimenko et al. 2002), *Rauvolfia verticillata* (Xu et al. 2012). Crystal structure of SGD from *Rauvolfia serpentina* and site-directed mutagenesis revealed the structural importance of Trp-388 at the catalytic pocket (Barleben et al. 2007). This study, we aim to clone Kratom SGD, heterologous express and characterize its catalytic activity. The SGD reaction product would let us know more information about the step close to mitragynine.

The reason for Kratom is a controlled plant, in this study, we thus established an *in vitro* plant to be our model and grow them under controlled conditions. We successfully produced the Kratom shoot culture. The shoot culture was transferred to a root-inducing medium and further grown in pot soil in the nursery (Phongprueksapattana et al. 2008). For the regulation of

mitragynine biosynthetic study, the shoot culture was also used as a model plant for methyl jasmonate and yeast extract elicitation (Wungsintaweekul et al. 2012). Nevertheless, Kratom shoot culture could produce a small amount of mitragynine. Addition of precursors (tryptophan, tryptamine, and loganin) promoted mitragynine production significantly (Charoonratana et al. 2013a). In contrast, the established hairy root culture of Kratom produced and accumulated only triterpenoids (ursolic acid oleanolic acid) but not alkaloid (Phongprueksapatana et al. 2008). Rare studies of root culture, callus culture, and cell suspension culture were reported. Zuldin et al. (2013) demonstrated that callus and cell suspension culture of Kratom could produce mitragynine albeit low amount. Elicitation cell suspension culture with yeast extract and salicylic acid and additions of tryptophan and loganin enhanced mitragynine in range of 9.3-12.2 mg/L (Zuldin et al. 2013). The task of alkaloid-producing in undifferentiated cells could be explained by subcellular localization of TDC, STR, and SGD. The studies in suspension cultured cells of *C. roseus* and *T. divaricata*. It was found that TDC is an extra-vacuolar enzyme, whereas STR is active inside the vacuole. On the other hand, the localization of SGD is on the outside of the tonoplast. An immediate product after SGD is a result from transporting tryptamine into vacuole where it is condensed with secologanin to form strictosidine and that strictosidine passes the tonoplast and is subsequently hydrolyzed outside the vacuole (Stevens et al. 1993).

Since mitragynine and related alkaloids in Kratom have a potential for medicinal purposes, therefore, arrays of basic knowledge about their biosynthesis are needed and of our interest. We aim to establish plant models including organ cultures (shoot culture, root, and hairy root culture) and cell cultures (callus culture and suspension culture). These are not only for an alternative source of mitragynine but also being used as the artificial models for studying on mitragynine biosynthesis. Using these types of cultures, we can manipulate the regulation of the molecular levels after treatment with elicitors, precursor addition, in situ adsorption, for instance. Statistical experiment design such as Plackett-Burman design and Response Surface Methodology (RSM) are used for design treated experiments. Moreover, transcription profiles of genes involved in mitragynine biosynthesis are measured using quantitative real-time PCR (RT-qPCR). Due to seeking more genes in mitragynine biosynthesis, we also plan to isolate the cDNA encoding strictosidine β-glucosidase as well as its functional characterization. Knowledge obtained from this study will give more insight into mitragynine biosynthesis and can be used for further genetic engineering.

References

Aji, A.M., Effraim, K.D. and Onyeyili, A.P. 2001. Antistress activity of *Mitragyna africanus* (Wild) stembark extract. *Sciences* 1, 105-107.

Barleben, L., Panjikar, S., Ruppert, M., Koeke, J., Stoeckigt, J. 2007. Molecular architecture of strictosidine glucosidase: The gateway to the biosynthesis of the monoterpenoid indole alkaloid family. *Plant Cell*. 19(90, 2886-2897.

Brandt, V., Geerlings, A., Tits, M., Delaude, C., Van der Heijden, R., Verpoorte, R., Angenot, L. 2000. New strictosidine β-glucosidase from *Strychnos mellodora*. *Plant Physiol. Biochem*. 38(3), 187-192.

Charoonratana, T., Wungsintaweekul, J., Pathompak, P., Georgiev, M.I., Choi, Y.H., Verpoorte, R. 2013a. Limitation of mitragynine biosynthesis in *Mitragyna speciosa* (Roxb.)Korth. through tryptamine availability. *Z. Naturforsch*. 68c, 394-405.

Charoonratana, T., Wungsintaweekul, J., Kaewpradub, N., Verpoorte, R. 2013b. Molecular cloning and expression of tryptophan decarboxylase from *Mitragyna speciosa*. *Acta Physiol. Plant* 35, 2611-2621.

Dewick,P.M. 2001. Medicinal natural products. A biosynthetic approach 2nd ed. John Wiley & Sons, Ltd. 350-375.

Geerlings, A., Ibanez, M.M.L., Memelink, J., van der Heijden, R. and Verpoorte, R. 2000. Molecular cloning and analysis of strictosidine beta-D-glucosidase, an enzyme in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. *J. Biol. Chem.* 275, 3051-3056.

Gerasimenko, I., Sheludko, Y., Ma, X., Stoeckigt, J. 2002. Heterologous expression of a *Rauvolfia* cDNA encoding strictosidine glucosidase, a biosynthetic key to over 2000 monoterpenoid indole alkaloids. *Eur. . Biochem/FEBS*. 269(8), 2204-2213.

Jumali, S.S., Said, I.M., Baharum, S.N., Ismail, I., Rahman, Z.A. and Zainal, Z. 2011b. Molecular cloning and characterization of strictosidine synthase, a key gene in biosynthesis of mitragynine from *Mitragyna speciosa*. *Afr. J. Biotechnol.* 10(68), 15238-15244.

Kikura-Hanajiri, R., Kawamura, M., Maruyama, T., Kitajima, M., Takayama, H. and Goda, Y. 2009. Simultaneous analysis of mitragynine, 7-hydroxymitragynine, and other alkaloids in the psychotropic plant “kratom” (*Mitragyna speciosa*) by LC-ESI-MS. *Forensic. Toxicol.* 27, 67-74.

Luijendijk, T.J.C., Nowak, A., Verpoorte, R. 1996. Strictosidine glucosidase from suspension cultured cells of *Tabernaemontana divaricata*. *Phytochemistry*. 41(6), 1451-1456.

Luijendijk, T.J.C., Stevens, L.H., Verpoorte, R. 1998. Purification and characterization of strictosidine β-D-glucosidase from *Catharanthus roseus* cell suspension cultures. *Plant Physiol Biochem*. 36(6), 419-425.

Matsumoto, K., Mizowaki, M., Thongpraditchote, S., Murakami, Y., Takayama, H., Sakai, S-L., Aimi, N. and Watanabe, H. 1996a. Central antinociceptive effects of mitragynine in mice: contribution of descending noradrenergic and serotonergic pathways. *Eur. J. Pharmacol*. 371, 75-81.

Nagakura, N., Rueffer, M. and Zenk M.H. 1979. The biosynthesis of monoterpenoid indole alkaloids from strictosidine. *J. Chem. Soc.* (Perkin I), 2308-2312.

Phongprueksapattana, S., Putalun, W., Keawpradub, N., and Wungsintaweekul, J. 2008. Mitragynine biosynthesis: hairy root culture for triterpenoid production and high yield of mitragynine by regenerated plants. *Z. Naturforch*. 63C: 691-698

Rueffer, M., Nagakura, N. and Zenk, M. 1978. Strictosidine, the common precursor for monoterpenoid indole alkaloids with 3α and 3β configuration. *Tetrahedron Lett*. 18, 1593-1596.

Stevens, L.H., Blom, T.J.M. and Verpoorte, R. 1993. Subcellular localization of tryptophan decarboxylase, strictosidine synthase and strictosidine β-D-glucosidase in suspension cultured cells of *Catharanthus roseus* and *Tabernaemontana divaricata*. *Plant Cell Rep.* 12, 573–576.

Suwanlert, S., 1975. A study of kratom eaters in Thailand. *Bull. Narcotics*. 27, 21-27.

Thongpradichote, S., Matsumoto, K., Tohda, M., Takayama, H., Aimi, N., Sakai, S. and Watanabe, H. 1998. Identification of opioid receptor subtypes in antinociceptive actions of supraspinally-administered mitragynine in mice. *Life Sci*. 62, 1371-1378.

Tsuchiya, S., Miyashita, S., Yamamoto, M., Horie, S., Sakai, S.I., Aimi, N., Takayama, H. and Watanabe, K. 2002. Effect of mitragynine, derived from Thai folk medicine, on gastric acid secretion through opioid receptor in anesthetized rats. *Eur. J. Pharmacol*. 443, 185–188.

Watanabe, K., Yano, S., Horie, S. and Yamamoto, L.T. 1997. Inhibitory effect of mitragynine, an alkaloid with analgesic effect from Thai medical plant *Mitragyna speciosa*, on electrically stimulated contraction of isolated guinea-pig ileum through the opioid receptor. *Life Sci.* 60, 933-942.

Wungsintaweekul, J. 2009. Biosynthesis of terpenoid indole alkaloid: molecular cloning, characterization and expressions of *dxs* and *dxr* genes from *Mitragyna speciosa* (Roxb.)Korth. Final report for the Thailand Research Fund.

Wungsintaweekul, J, Choo-malee, J., Charoonratana, T. and Keawpradub, N. 2012. Methyl jasmonate and yeast extract stimulate mitragynine production in *Mitragyna speciosa* (Roxb.) Korth. shoot culture. Biotech. Lett. Oct; 34(10):1945-1950

Xu, H., Chang, K., Ma, L., Zheng, Y., Liu, X. 2012. Cloning and characterization of the strictosidine-β-D-glucosidase (SGD) from *Rauvolfia verticillata*. *Agricultural Sci. Tech*. 13(7), 1406-1409.

Zarate, R., Bonavia, M., Geerlings, A., Van der Heidijk, R., Verpoorte. R. 2001. Expression of strictosidine β-D-glucosidase cDNA from *Catharanthus roseus*, involved in the monoterpene indole alkaloid pathway, in a transgenic suspension culture of *Nicotiana tabacum*. Plant Physiol. Biochem. 39(9), 763-769.

Zuildin, N.N.M., Said, I.M., Noor, N.M., Zainal, Z., Kiat, C.J., Ismail, I. 2013. Induction and analysis of the alkaloid mitragynine content of a *Mitragyna speciosa* suspension culture system upon elicitation and precursor feeding. *Sci. World.J*. 209434.

2. Objectives

2.1 To isolate and functional characterization the cDNA encoding of strictosidine β-glucosidase (SGD),

2.2 To establish the plant models of Kratom and metabolite profile analysis,

2.3 To improve the mitragynine production in Kratom cultures using manipulation techniques, and study the molecular regulation of genes involved in mitragynine biosynthesis by addition of elicitors.

3. Methodology

Part 1: cDNA isolation and functional characterization of strictosidine β-glucosidase from Kratom leaves.

1.1. Plant material

In vitro culture of Kratom is established according to Phongprueksapattana et al (2008) and is then acclimatized in pot soil for six months. Young leaves are used as materials for total RNA isolation and cDNA preparation.

1.2. Total RNA extraction and cDNA preparation

The plant tissues (300 mg) are collected and immediately frozen in liquid nitrogen. Extraction of total RNA is performed using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany)

under the manufacturer's protocol. The first strand cDNA used for DNA amplification is synthesized using Superscript™ III reverse transcriptase (Invitrogen) in the presence of oligo dT primers. The resulting cDNA is used for the internal fragment cloning.

1.3. Polymerase chain reaction and internal fragment cloning

Pairs of degenerate primers are designed based on the highly conserved region among the plant strictosidine β-glucosidase (SGD) including *Camptotheca acuminata* (GenBank ID: JF508379), *Catharanthus roseus* (GenBank ID: AF112888), *Rauvolfia verticillata* (GenBank ID: JF966733), *Rauvolfia serpentina* (GenBank ID: AJ302044) and *Uncaria tomentosa* (GenBank ID 366085) (http://www.ncbi.nlm.nih.gov). The amino acid residues of the conserved region are selected and converted to degenerate primers (forward and reverse primers). These primers are used to amplify the internal fragment and used the cDNA (from 6.1.2) as a template. Under the optimized condition, the internal fragment is then obtained.

For cloning of the internal fragment, the DNA fragment is separated on agarose gel electrophoresis and purified using the gel extraction kit (Qiagen). The purified internal fragment is then ligated to the cloning vector. The resulting plasmids are chemically transformed into *Escherichia coli* TOP-10 (Invitrogen). Transformants are selected on ampicillin LB-agar plates containing IPTG and X-gal. The recombinant plasmids are extracted using plasmid isolation kit, cut with *EcoR*I and the insertion is analyzed by agarose gel electrophoresis. The nucleotide sequence is obtained from DNA sequencing.

1.4. Cloning of 5'- and 3'-ends of the *SGD* gene

The information of the nucleotide sequence of the internal fragment is used for specific primer design for 5'- and 3'-ends amplifications. For amplification of 5'- and 3'-ends, the rapid amplification of cDNA ends (RACE) technique is used. The cDNA is prepared from SMART™ RACE cDNA Amplification kit (Clontech, USA) according to the manufacturer's instruction, affording the 5'-RACE-ready-cDNA และ 3'-RACE-ready-cDNA. In the presence of a template and specific primers, the 5'- and 3'-ends are amplified under the optimized PCR condition. The PCR products are ligated to the cloning vector and transformed to the host. After checking the insertion, the recombinant plasmids are selected and DNA sequencing is then performed.

1.5. Cloning of the full-length of the *SGD* gene

Information of the internal fragment, 5'- and 3'-ends guide us to design the primers for full-length *SGD* gene amplification. The specific forward and reverse primers are designed, which carry the sites of restriction endonucleases. The full-length *SGD* amplification is performed under the optimized PCR condition. The *SGD* gene is then ligated to the expression vector (pQE30), after treatment with appropriate endonucleases. The recombinant plasmids are then transformed to *E. coli* XL1 BMRF' and selected on ampicillin-containing LB agar. A single white

colony of *E. coli* cells grown on transformation agar was picked up. Cells are cultured and plasmid DNA is isolated using plasmid isolation kit. Identification of the recombinant plasmid harboring *SGD* gene is performed by the treatment the plasmid with restriction enzymes. The DNA fragments are separated on agarose gel electrophoresis. After checking the insertion, the recombinant plasmid is sent for DNA sequencing.

1.6. DNA sequencing and sequencing analysis

The nucleotide sequences are analyzed by the First Base Laboratories (Malaysia). The DNA fragments are sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Comparative analyses of nucleotide sequences and deduced amino acid sequences are performed using BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/) and BioEdit version 7.0.9.0. Multiple alignments are conducted through Clustal W (2.0) (http://www.ebi.ac.uk/clustalw/) using default parameters. The amino acid sequences are subjected to ExPaSy Proteomics Server (http://au.expasy.org/) for prediction of subcellular location, the physio-chemical parameters (http://au.expasy.org/protparam.html) and motif that occur in a sequence (http://au.expasy.org/tools/scanprosite). For the construction of a phylogenetic tree, Clustal W and Treeview computer programs are used.

1.7. Heterologous expression and protein purification

The *E. coli* XL1-BMRF' cells harboring pQE30 vector and SGD gene are inoculated in LB-ampicillin medium and grown overnight. The activated cells are inoculated in 1 L culture and incubated at 37°C until the OD_{600} reaches 0.4. The cell culture is induced by addition of IPTG (2 mM) and further grown for 4 h. Cells are then harvested by centrifugation and washed with buffer. Cells are kept at -20°C until used.

For protein extraction, cells are suspended in the extraction buffer and lysozyme is added. After incubation for 30 min at 37°C, the mixture is ultrasonicated, cell debris is removed by centrifugation. The supernatant is collected. The recombinant protein is purified by loading on the top of the Ni-NTA column. After washing the unbound protein, SGD is eluted from the column by increasing imidazole concentration. The protein fractions are pooled. The purity of protein is estimated from SDS-PAGE. Protein concentration is determined and the SGD activity is investigated.

1.8. Determination of total protein and SDS-PAGE

The amount of protein is determined by the dye-binding method of Bradford assay (Bradford, 1976). The protein sample or standard protein solution and Bradford reagent are mixed. The mixture is measured at 595 nm. BSA is used as a standard protein. Protein amount is estimated from a standard curve.

The purity of protein is determined using discontinuous SDS-PAGE. The gel is prepared with vertical slab gel. The protein sample is mixed with the SDS-sample buffer and heated. The protein sample is then loaded into the wells. After running, the gel is removed, stained and de-stained.

1.9. Assay of SGD activity

SGD activity is performed according to Gerasimenko et al. (2002). The SGD activity is calculated on the basis of strictosidine decrease measured by HPLC. The assay mixture is composed of buffer (pH 5.0), substrate strictosidine, and SGD fraction. After incubation at 30°C for 30 min, the reaction is terminated. The resulting reaction is then subjected to HPLC system and using C-18 column as a stationary phase and acetonitrile/NaH_2PO_4 (pH 2.5) as the mobile phase at a flow rate of 1.5 mL/min. The detector is set at 250 nm. The decrease of strictosidine that utilized by the SGD is measured and calculated for the SGD activity.

It can be noted that substrate strictosidine is not commercially available. In this study, therefore, strictosidine should be enzymatically synthesized and purified according to Hemscheidt and Zenk (1980).

1.10. Properties of SGD

The SGD properties are determined including enzyme kinetic parameters, optimum pH, optimum temperature, native molecular weight. Enzyme kinetic parameters (K_M and V_{max}) are determined in the presence of strictosidine. Temperatures are varied and pH of reaction buffer is investigated. The native molecular weight of SGD is determined using size-exclusion chromatography in compared with the protein standards. To check substrate specificity of the recombinant SGD, various β-D-glucosidase substrates are used.

1.11. Characterization of the catalytic product

The reaction product of SGD enzyme is determined using LC-MS method modified from Gerasimenko et al. (2002). Substrate strictosidine is incubated with the recombinant SGD under the optimal condition. The reaction is subjected to LC separation system and identify by mass spectrophotometer. For the control assay, the enzyme preparation is heated in boiling water.

Part 2: Establishment of plant models for mitragynine biosynthetic study

2.1. Preparation of seedlings

Mature plants of Kratom (6 years-old), grown in the open field in Hat Yai district, Songkhla, Thailand, are 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 are collected from the globular fruit and flat seeds are separated.

Flat seeds of Kratom are 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 are placed on McCown Woody Plant (WPM) (Lloyd and McCown, 1980) 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 1-month old plantlets are used for callus induction. The 2-month old plantlets are used for hairy roots induction.

2.2. Establishment of callus and suspension culture

Young fully expanded leaves of Kratom (2-month old), grown under control condition (6.1), are excised into pieces, 5x5 mm^2, and placed on WPM medium supplemented with appropriate plant growth regulators. The culture is maintained at 25±2^{o}C under 16 h of photoperiods (2000 lux). Optimization of the concentrations of plant growth regulators (auxins-2,4-dichlorophenoxyacetic acid, naphthaleneacetic acid, indolebutyric acid and cytokinins-kinetin, thidiazuron, benzyladenine) is investigated. The percentage of callus formation is recorded. The callus culture is then transferred to a new medium every month. After least 3 times of subculture, the friable and homogenous callus is obtained. The friable cells are transferred to the WPM liquid medium to obtain the cell suspension culture.

2.3. Establishment of shoot culture

For induction of shoot culture, the axillary buds are excised from the 2-month old plantlet and placed on WPM medium supplemented with 2 mg/L thidiazuron (TDZ), 1 mg/L benzyladenine (BA) 2% (w/v) sucrose, 0.8% (w/v) agar, and pH was adjusted to 5.7 (Wungsintaweekul et al. 2012). Plant materials were maintained under control condition at 25 ± 2°C under light for 16 h/day.

2.4. Establishment of root and hairy root cultures

Root culture is initiated by cutting the rootlet from the 2-month old plantlet and placed in liquid WPM medium supplemented with appropriate plant growth regulator. Optimization of the concentrations of plant growth regulators (auxins and cytokinins) is investigated. The growth characteristic is recorded.

For hairy roots induction, leaves were infected with *Agrobacterium rhizogenes* ATCC15834 as described in Phongprueksapattana et al. (2008). Firstly, the explants were wounded with a needle and then the wounded explants were submerged in bacterial suspension for further 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 the culture at 25°C under darkness. To kill the residual *A. rhizogenes*, the explants were transferred to WPM solid

containing cefotaxime. Finally, the cultures, free from *A. rhizogenes*, were transferred to WPM solid medium without plant growth regulators. All cultures were incubated at 25°C, on a rotary shaker (80 rpm) and under dark condition. The hairy root culture and untransformed root culture were sub-cultured into fresh medium every 2 months.

2.5. HPLC analysis for mitragynine content and related alkaloid content in plant tissue cultures

Samples for HPLC analysis are prepared using the method as described in Charoonratana (2012). Freeze-dried cell or tissue cultures are extracted by refluxing with methanol for 1 h. The extract is filtered, and then washed with petroleum ether and concentrated under reduced pressure.

HPLC analysis is carried out. Chromatographic separation is performed on a reverse C18 column with binary gradient mobile phase [1.5 mL/min, acetonitrile: 100 mM H_3PO_4 in water, pH 2.4 (10:90 to 90:10, v/v within 15 min). Identification of metabolites such as tryptamine, secologanin, and alkaloids (mitragynine, speciogynine, paynantheine) is carried out based on the retention time and comparison of the absorption spectra with authentic standards. UV detector is set at 225 nm and 238 nm.

2.6. Enhancing strategies for mitragynine production

Strategies for promoting mitragynine production in callus and cell suspension cultures, shoot culture, root and hairy roots cultures are including medium manipulation (plant growth regulator, nitrogen source, carbon), precursor feedings (tryptophan, tryptamine, loganin, sodium acetate, pyruvate), in situ adsorption (XAD-4, diaion HP-20) and elicitation (biotic elicitors-yeast extract, fungus homogenate, chitosan & abiotic elicitors-methyl jasmonate, nitric oxide, jasmonic acid, abscisic acid, salicylic acid). Statistic experiment designs such as Plackett-Burman design, response surface methodology & central composite design are used to optimize the conditions. Moreover, an integrated process of each manipulation is also investigated.

2.7. Transcription profile analysis

During the addition of elicitors to plant cell cultures, the mRNA levels of genes involved in mitragynine biosynthesis are measured. The reverse transcription quantitative real-time PCR (RT-qPCR) is used as method as described in Charoonratana (2012). The relative expression levels are calculated by the Rest 2009 software v.2.0.13 in comparison with the reference gene. Bootstrapping techniques is used to provide 95% confidence intervals for expression ratio. The relative expression data is presented in Whisker-box plot.

2.8 Statistic analysis

Value are expressed as mean ± S.D. data and analyzed by student *t*-test. The level of statistical significance is taken at $P<0.05$.

4. Research plan

Time frame: June 2015-May 2018 (extended to December 2018)

Plan	Year 1					
	2	4	6	8	10	12
1. Primer design and cDNA synthesis	/					
2. Cloning of internal sequence and 5'- and 3'-ends		/	/	/		
3. Cloning the full-length of SGD gene and DNA sequence analysis				/	/	/
4. Establishment of the root, shoot and hairy root culture		/	/	/		
5. Establishment of callus and cell suspension culture				/	/	/
Plan	Year 2					
	2	4	6	8	10	12
6. HPLC analysis of mitragynine and related alkaloid content in cultures	/	/	/	/	/	/
7. Medium manipulation		/	/			
8. Precursor addition				/		
9. In situ adsorption					/	/
10. Heterologous expression of SGD and determine catalytic activity	/	/	/	/	/	/
Plan	Year 3					
	2	4	6	8	10	12
11. Elicitation	/	/	/	/		
12. Transcription profile analysis			/	/	/	
13. Characterization of a catalytic product of SGD		/	/			
14. Kinetic parameters of SGD				/	/	
15. Data analysis and reports						/

5. ชื่อเรื่องและวารสารที่คาดว่าจะตีพิมพ์ในวารสารนานาชาติ อย่างน้อย 2 เรื่อง พร้อมค่า impact factor

- Title: Precursor addition and in situ adsorption promote mitragynine production in *Mitragyna speciosa* shoot culture. Journal: Biotechnology Letters, impact factor 2013 = 1.736
- Title: cDNA cloning, expression, and characterization of strictosidine β-glucosidase from *Mitragyna speciosa.* Journal: FEBS Journal, impact factor 2014=3.986
- Title: Enhancing mitragynine and related alkaloid production using elicitors. Journal: Plant Cell Reports, impact factor 2014= 2.936

6. งบประมาณโครงการ (ในกรณีที่ได้รับการติดต่อเพื่อปรับงบประมาณจากฝ่ายวิชาการแล้ว กรุณาใช้ข้อมูลที่ได้ปรับแล้วดังกล่าว)

Total budget cannot exceed 1,500,000 Baht for 3 years.

(Not exceeding 500,000 Baht per year)

หมวดงบประมาณ	Expenses			Total
	Year 1	Year 2	Year 3	
1. หมวดค่าตอบแทน	240,000	200,000	280,000	720,000
2. หมวดค่าวัสดุ				
Chemical reagents	230,000	-	135,000	365,000
Glassware & Plastic	10,000	-	36,000	46,000
Office materials	-	-	11,000	11,000
3. หมวดค่าใช้สอย				
DNA sequencing & RT-qPCR	20,000	-	30,000	50,000
HPLC expense	-	-	8,000	8,000
4. หมวดค่าครุภัณฑ์	-	300,000	-	300,000
รวมงบประมาณโครงการ	**500,000**	**500,000**	**500,000**	**1,500,000**

Contents

List of Tables

List of Figures

1. Background and rationale

Kratom refers to the leaves of *Mitragyna speciosa* (Roxb.) Korth. (Family Rubiaceae). It is an endemic plant found in tropical Southeast Asia, especially in Thailand and Malaysia. Kratom is controlled in Australia, Malaysia, Myanmar, New Zealand, South Korea, and Thailand. Kratom is classified as Narcotic Level 5 in Thailand according to the Royal Decree on Narcotics, 1979. Kratom is traditionally used for diarrhea and as a substitute for opium (Suwanlert 1975). Its leaves have been used in the form of chewed, smoked or brewed as a tea. The medicine man used Kratom's leaves and barks as an ingredient in the decoctions for the treatments of diarrhea, amoebiasis, diabetes, and hypertension.

Scientifically, Kratom is a source of the terpenoid indole alkaloid (TIAs) such as mitragynine, paynantheine, speciogynine and 7-hydroxymitragynine. Kratom's extract and mitragynine have been investigated in several animal models by the pharmacologists. Mitragynine possesses 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). Thongpraditchote and co-workers demonstrated that mitragynine binds to opioid receptors non-specifically and has less addiction than morphine (Thongpraditchote et al. 1998). Therefore, it highlights the relevance of mitragynine and its derivatives to be an alternative opioid analgesic drug. In addition, mitragynine also affects the serotonergic and dopaminergic receptors in the central nervous system (Matsumoto et al., 1996a), resulting in the anti-depressant effect. Moreover, 7-hydroxymitragynine has a strong analgesic activity than mitragynine and morphine (Kikura-Hanajiri et al. 2009). This fact also suggested that 7-hydroxymitragynine has the potential to be an oral opioid analgesic.

Looking into Kratom, in the aspect of mitragynine biosynthesis, nowadays, there are reports about the early step genes and enzymes from Kratom. Concerning to mitragynine biosynthesis in M. speciosa, Nagakura and co-workers were firstly reported in 1979. By feeding the doubly labeled of [3-^{3}H, 6-^{14}C]strictosidine to *M. speciosa* suggested that radiolabeled substrate was incorporated into a molecule of mitragynine and speciociliatine. Their results indicated that strictosidine play a role as an intermediate in *M. speciosa* (Nagakura et al. 1979). Widely known that strictosidine is a common precursor of the TIAs as shown in *Catharanthus roseus*, *Rauwolfia serpentina*, *Camptotheca accuminata*. Strictosidine is a product of the Mannich reaction of tryptamine and secologanin, catalyzed by the strictosidine synthase (STR) (Rueffer et al. 1978). Tryptamine is the decarboxylation product of an amino acid tryptophan by the tryptophan decarboxylase (TDC) from the shikimate pathway (Dewick 2001). Secologanin, a secoiridoid moiety, is supplied from the monoterpene biosynthesis. In-depth to manipulate the biosynthetic pathway in higher plant probably affects secondary metabolite production. Therefore, gene and enzymes those involved in the TIA biosynthesis are the targets for genetic manipulation. In *M. speciosa* leaves, cDNAs of strictosidine synthase (STR) (Jumali et al. 2011), 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 2*C*-methyl-D-erythritol 4-phosphate synthase (MEPS) (Wungsintaweekul 2009) were isolated and functionally characterized. An accumulation site of mitragynine is presence dominantly in leaves, less in stem and absence in the root (Charoonratana et al. 2013a). The distance between strictosidine and mitragynine is still unknown. There are proposed to have at

least 5 enzymatic steps from the branch point intermediate, strictosidine. Information of genes and enzymes are necessary to understand the regulation of the Kratom's TIAs biosynthesis and have no report so far.

More than 20 enzymatic steps are proposed to involve in the mitragynine biosynthesis. Recently, cDNAs encoding anthranilate synthase alpha subunit (ASA) and tryptophan decarboxylase (TDC) of Kratom has been reported. The ASA of Kratom exists in two isoforms (ASA1 & ASA2). Treatment of Kratom shoot culture with methyl jasmonate resulted in the enhancement of mitragynine (Charoonratana et al. 2013a). The TDC gene also cloned and characterized and found that the TDC is a pyridoxal phosphate-dependent protein. Transgenic Kratom hairy roots with TDC gene caused the increasing amount of tryptamine (Charoonratana et al. 2013b). As mentioned, five genes of Kratom have been cloned and characterized and most of them involved in the early step of mitragynine biosynthesis. The crucial step next to strictosidine synthase, strictosidine β-glucosidase (SGD), in Kratom is still no information. This enzyme has been shown an important role in producing an intermediate by activating the glucoside, strictosidine, and allowing it to enter the multiple indole alkaloid pathways. The SGDs were cloned, purified and characterized from alkaloid producing plants such as *Tabernaemontana divaricata* (Luijendijk et al. 1996), *Catharanthus roseus* (Luijendijk et al. 1998; Geerling et al. 2000; Zarate et al. 2001), *Strychnos mellodora* (Brandt et al. 2000), *R. serpentina* (Gerasimenko et al. 2002), *Rauvolfia verticillata* (Xu et al. 2012). Crystal structure of SGD from *Rauvolfia serpentina* and site-directed mutagenesis revealed the structural importance of Trp-388 at the catalytic pocket (Barleben et al. 2007). This study, we aim to clone Kratom SGD, heterologous express and characterize its catalytic activity. The SGD reaction product would let us know more information about the step close to mitragynine.

The reason of Kratom is a controlled plant, in this study, we thus established an in vitro plant to be our model and grow them under controlled conditions. We successfully produced the Kratom shoot culture. The shoot culture was transferred to root-inducing medium and further grown in pot soil in the nursery (Phongprueksapattana et al. 2008). For the regulation of mitragynine biosynthetic study, the shoot culture was also used as a model plant for methyl jasmonate and yeast extract elicitation (Wungsintaweekul et al. 2012). Nevertheless, Kratom shoot culture could produce a small amount of mitragynine. Addition of precursors (tryptophan, tryptamine, and loganin) promoted mitragynine production significantly (Charoonratana et al. 2013a). In contrast, the established hairy root culture of Kratom produced and accumulated only triterpenoids (ursolic acid oleanolic acid) but not alkaloid (Phongprueksapatana et al. 2008). Rare studies of root culture, callus culture, and cell suspension culture were reported. Zuldin et al. (2013) demonstrated that callus and cell suspension culture of Kratom could produce mitragynine albeit low amount. Elicitation cell suspension culture with yeast extract and salicylic acid and additions of tryptophan and loganin enhanced mitragynine in range of 9.3-12.2 mg/L (Zuldin et al. 2013). The task of alkaloid-producing in undifferentiated cells could be explained by subcellular localization of TDC, STR, and SGD. The studies in suspension cultured cells of *C. roseus* and *T. divaricata*. It was found that TDC is an extra-vacuolar enzyme, whereas STR is active inside the vacuole. On the other hand, the localization of SGD is on the outside of the tonoplast. An immediate product after SGD is a result from transporting tryptamine into vacuole where it is condensed with secologanin to form strictosidine and that strictosidine passes the tonoplast and is subsequently hydrolyzed outside the vacuole (Stevens et al. 1993).

Since mitragynine and related alkaloids in Kratom have a potential for medicinal purposes, therefore, arrays of basic knowledge about their biosynthesis are needed and of our interest. We aim to establish plant models including organ cultures (shoot culture, root, and hairy root culture) and cell cultures (callus culture and suspension culture). These are not only for an alternative source of mitragynine but also being used as the artificial models for studying on mitragynine biosynthesis. Using these types of cultures, we can manipulate the regulation of the molecular levels after treatment with elicitors, precursor addition, in situ adsorption, for instance. Statistical experiment design such as Plackett-Burman design and Response Surface Methodology (RSM) are used for design treated experiments. Moreover, transcription profiles of genes involved in mitragynine biosynthesis are measured using quantitative real-time PCR (RT-qPCR). Due to seeking more genes in mitragynine biosynthesis, we also plan to isolate the cDNA encoding strictosidine β-glucosidase as well as its functional characterization. Knowledge obtained from this study will give more insight into mitragynine biosynthesis and can be used for further genetic engineering.

2. Literature review

2.1 Botanical aspect of *Mitragyna speciosa* (Roxb.)Korth.

The genus *Mitragyna* belongs to the plant family Rubiaceae. It comprises of ten species of which grow in the tropical and subtropical regions of Asia and Africa (Shellard et al. 1978a). Six species, *M. speciosa* (Roxb.) Korth., *M. tubulosa* (Arn.) Havil., *M. parvifolia* (Roxb.) Korth., *M. hirsuta* Havil., *M. diversifolia* (Wall. Ex G. Don) Havil and *M. rotundifolia* (Roxb.) O.Kuntze, widely distribute in Southeast Asia and the surrounding countries including Thailand, Malaysia, Philippines, Borneo and New Guinea islands. The remaining four species, *M. ciliata* Aubrév&Pellegr., *M .inermis* (Willd.) O.Kuntze., *M. stipulosa* (D.C.) O. Kuntze. and *M. rubrostipulata* (K.Schum.) Havil., is found in West Africa (Shellard 1974). In Thailand, the species of *M. hirsuta*, *M. diversifolia*, *M. rotundifolia*, and *M. speciosa* are commonly found (Smitinand 2001). Taxonomically, plant species can be categorized by types of indole alkaloids (Keawpradub 1990). Recently, these species were authenticated by the molecular approach, based on rDNA internal transcribed sequence (ITS) (Sukrong et al. 2007). There are at least two varieties of *M. speciosa* in Thailand, which are red-veined and green-veined of leaves. Both varieties are believed to accumulate different secondary metabolite profiles.

2.2 Chemical constituents

Kratom is the source of several terpenoid indole alkaloids (TIAs). Over 40 kinds of corynanthe-type alkaloids were isolated from the leaves of Kratom (Adkins et al. 2011). These were indoles and oxindoles with the substitution at C-9 position (Beckett et al. 1966a; 1966b). Mitragynine (MG) is the first isolated TIA and appeared to be exclusive for *M. speciosa* (Shellard 1974). The alkaloid content varies between 0.5-1.5% according to the natural habitat, season and ages (Adkins et al. 2011). The Thai Kratom, the main alkaloid isolated from the young leaves is MG (66.2% based on the crude alkaloid extract) together with its analogs, speciogynine (6.6%), speciociliatine (0.8%), paynantheine (8.6%) and 7-hydroxy-7H-mitragynine (2.0%) (Fig. 1.1) (Ponglux et al. 1994). While Malaysian Kratom contains approximately 12% mitragynine among the total alkaloids (Takayama 2004). Recently, a new indole alkaloid, 7-hydroxyspeciociliatine, has been isolated from the fruits of Malaysian Kratom (Kitajima et al. 2006). Other isolated compounds from this plant which have been reported include corynantheidine,

dihydrocorynantheidine, mitraphylline, isomitraphylline, ajmalicine, 3-isoajmalicine, speciophylline, speciofoline, isospeciofoline, rhynchophylline, isorhynchophylline, 3-dehydromitragynine, corynantheidinalinic acid, mitragynaline, mitragynalinic acid, mitraciliatine, (+)-pinoresinol, 3,4,5,6-dehydromitragynine, mitralactonal, mitrasulgynine, corynantheidaline, 9-methoxymitralactonine, mitralactonine, 7-hydroxyspeciociliatine, mitraciliatine, 3-isopaynantheine, corynoxine, corynoxeine, isocorynoxeine, mitrafoline, specionoxeine, isospecionoxeine, ciliaphylline, mitragynine oxindole B, rhynchociline, mitrajavine, javaphylline, akuammigine, mitragynine oxindole A (Beckett et al. 1966a; 1966b; Shellard et al. 1978a; 1978b; Houghton et al. 1986; 1991; Takayama et al. 1998).

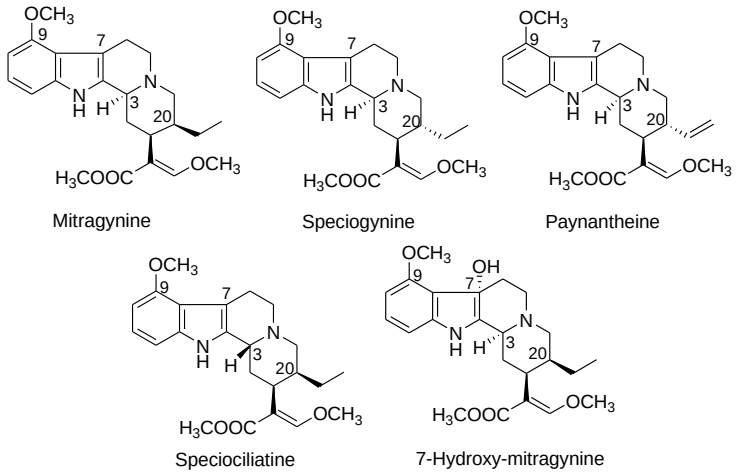


Figure 1.1 Chemical structure of mitragynine and its analogs.

2.3 Pharmacological activities

Mitragynine is the most abundant component that is mostly presented in the leaves. Pharmacological activities of mitragynine, mitragynine derivatives, and Kratom's methanol extract were reported. TIAs in Kratom's leaves have been isolated and elucidated their structures since 1966. Pharmacologically experiments were made in vitro and in vivo models.

In 1996, a Japanese group investigated the antinociception of mitragynine in mice and the results showed that mitragynine acted by binding to opioid receptors 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 an opioid receptor specifically. Thus, mitragynine exhibits 10 fold less potent than morphine (Thongpraditchote et al. 1998). Caused by the affinity to opioid receptors, 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). 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 the antinociceptive effect (Kumarnsit et al. 2007).

Toxicological evaluation of Kratom extract on human cell lines including cHo1, MCL-5 (lymphoblastoid), HEK293 (renal) and SH-SY5Y (neuronal cells) were investigated and found that the extract at a dose > 100 μg/mL inhibited cell proliferation of all cell lines. By flow

cytometry analysis using MCL-5 and SH-SY5Y cell lines suggested that Kratom extract affected a dose-dependent G1 phase arrest at 100 µg/mL and G1/S phase arrest at a concentration > 500 µg/mL in both cell lines (Saidin and Gooderham 2007).

Chittrakarn et al. (2008) reported that methanol extract of Kratom leaves exhibited an antidiarrheal effect on the rat gastrointestinal tract. Their result supported the ethnopharmacological use to treat diarrhea. They suggested that effects on gastrointestinal tract may occur via other pathways besides the binding on opioid receptors. In addition, at the high dose of extract decreased the increment of body weight similar to the effect of morphine (Chittrakarn et al. 2008). Kratom fresh leaves are widely consumed by laborers for stimulating effects that help to improve their tolerance to work and relieves muscle strains. Study of methanol extract of Kratom leaves and mitragynine in rat suggested that the extract and mitragynine had a direct effect on skeletal muscle by decreasing the muscle twitch, thus, produced skeleton muscle relaxation. Its mechanism did not act as a competitive antagonist of acetylcholine. They suggested that there are compounds rather than mitragynine are responsible to effect on the neuromuscular blockade (Chittrakarn et al. 2010). Consuming Kratom leaves does not only the addiction but also altering the working memory. As shown in the study in mice, mitragynine has significantly reduced locomotor activity in the open-field test compared with vehicle. Mice, pretreatment with mitragynine, could not discriminate the change position of objects, therefore, chronic administration of mitragynine affected the cognitive behavioral function (Apryani et al. 2010).

Kratom exhibited the anti-inflammatory effect in animal and cell-based models. The intraperitoneal administration at doses of 100 and 200 mg/kg suppressed the carrageenan-induced paw edema in rats in a dose-dependent manner. This effect was the maximum in the first 3 h after the challenge which indicated that the extract produced the inhibition in the acute phase of inflammation. The authors suggested that it is possible by inhibiting the pro-inflammatory mediator's release and vascular permeability (Shaik Mossadeq et al. 2009). Recently, it has been reported anti-inflammatory activity of mitragynine in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells. It can suppress prostaglandin E_2 (PGE_2) production by inhibiting COX-2 mRNA expression in a dose-dependent manner. (Utar et al. 2011).

2.4 Mitragynine biosynthesis

Based on the similarity of chemical structure of mitragynine to strictosidine, mitragynine, therefore, is proposed to derive from strictosidine, a common intermediate of indole alkaloids. Strictosidine is a condensation product of a nitrogen-containing indole moiety derived from tryptamine and a monoterpenoid component derived from secologanin, catalyzed by strictosidine synthase (STR). Rueffer et al. (1978) was found that the strictosidine plays an important role as a precursor of the TIAs in the higher plant in the family of Apocynaceae (e.g. *Catharanthus roseus*) Loganiaceae (e.g. *Strychnos nux-vomica*), Rubiaceae (e.g. *Mitragyna speciosa*) and Nyssaceae (e.g. *Camptotheca acuminata*) (Rueffler et al. 1978). Later, the same research group demonstrated the experiments that the doubly labeled strictosidine, not vincoside was incorporated into skeletons of mitragynine and speciociliatine, the *Corynanthe* type alkaloid. They also investigated the feeding experiment of [7-^{3}H]secologanin to young shoots of *Rauvolfia canescens* indicated that secologanin, a secoiridoid terpene, was purposed to serve as a precursor of TIA biosynthesis (Nagakura et al. 1979).

Secologanin, a secoiridoid compound, is biosynthesized from a monoterpene, geraniol. Its biosynthetic pathway has been yet fully elucidated. The isoprene unit is supplied either the

classical mevalonate pathway or the recently discovered "deoxyxylulose phosphate pathway". Feeding of [1-^{13}C]glucose into *C. roseus* cell suspension cultures has strongly suggested that secologanin is derived from the deoxyxylulose phosphate pathway rather than the mevalonate pathway (Contin et al. 1998). Feeding studies with the culture of *O. pumila* were also consistent with the utilization of the deoxyxylulose phosphate pathway in secologanin biosynthesis (Yamazaki et al. 2004). In the first committed step of iridoid terpene biosynthesis, geraniol is derived from isopentenyl diphosphate (IPP) and then is hydroxylated by geraniol 10-hydroxylase, providing 10-hydroxygeraniol. Forming of loganin comprises of several steps and iridodial, iridotrial and deoxyloganin are intermediates. Oxidation of the iridotrial to the carboxylic acid is followed by esterification and glucosylation to yield deoxyloganin, subsequent hydroxylation of deoxyloganin yields loganin. Secologanin is then generated by oxidative cleavage by secologanin synthase (O'Conner and Maresh 2006).

Specifically, to mitragynine biosynthesis in *M. speciosa*, a cDNA encoding strictosidine synthase (*STR*) gene has been identified from *M. speciosa* and submitted to the Genbank database under the accession no. EU288197 (Jumali et al. 2011a). The *M. speciosa STR* gene was heterologously expressed in *E. coli* and its gene product catalyzed the condensation of tryptamine and secologanin. Recently, cDNAs encoding 1-deoxy-D-xylulose 5-phosphate synthase (*DXS*) and 2*C*-methyl-D-erythritol 4-phosphate synthase (*MEPS*) were isolated from *M. speciosa* leaves and functionally characterized (Wungsintaweekul, 2009). Moreover, cDNA encoding tryptophan decarboxylase (*TDC*) was cloned and expressed in *E. coli* (Charoonratana et al. 2013a). Findings of those cDNAs suggested that *M. speciosa* performs the TIA biosynthesis via at least the deoxyxylulose phosphate pathway and shikimate pathway. The contribution of the mevalonate pathway and deoxyxylulose phosphate has been proposed to originate from mixed biosynthesis in secologanin biosynthesis (Wungsintaweekul 2009). The late step of mitragynine biosynthesis is unknown up to date.

The localization of TIAs pathway mRNA and enzymes was investigated extensively in *C. roseus* but there is no information from *M. speciosa*. From the *Catharanthus* study, it was found that the enzymes of the TIAs pathway are localized in different parts of the cell and in different tissues of the plant. This compartmentalization can be considered as a kind of regulatory mechanism since the localization requires the transport of different metabolites from one point to another for transformation (Facchini 2001). Localization studies revealed that TDC is located in the cytosol. STR is located within the vacuole. On the other hand, G10H and SGD are located in the endoplasmic reticulum (St-Pierre et al. 1999; Geerling et al. 2000; Guirimand et al. 2009). Moreover, in different organs of the plant, the mRNA for *TDC* and *STR* are located in the epidermal cells of stems, leaves and floral buds (St-Pierre et al. 1999). *DXS*, *DXR*, and *G10H* mRNA were reported to be expressed in the internal phloem parenchyma and were present in roots, flower buds, and leaves (Burlat et al. 2004). Additionally, mRNA for *SLS* and *SGD* were detected in epidermal cells (Murata and De Luca 2005). The genes data of *M. speciosa* and the localization of mRNA and enzymes in *C. roseus* were used in combination in order to propose the biosynthesis and enzyme localization of mitragynine in *M. speciosa* (Fig. 1.2).

2.5 Strictosidine β-glucosidase

Strictosidine β-glucosidase (SGD) was firstly described in *Catharanthus* cell cultures by Hemscheidt and Zenk (1980) and involved in TIA biosynthesis. It removes the glucose moiety of strictosidine yielding an unstable aglycone which in turn opens to form a highly reactive dialdehyde (Treimer and Zenk 1979). This intermediate undergoes further rearrangements and

yield ajmalicine in *Catharanthus* system (Hemscheidt and Zenk 1980). The proposed reaction in mitragynine biosynthesis is shown below (Fig. 1.3).

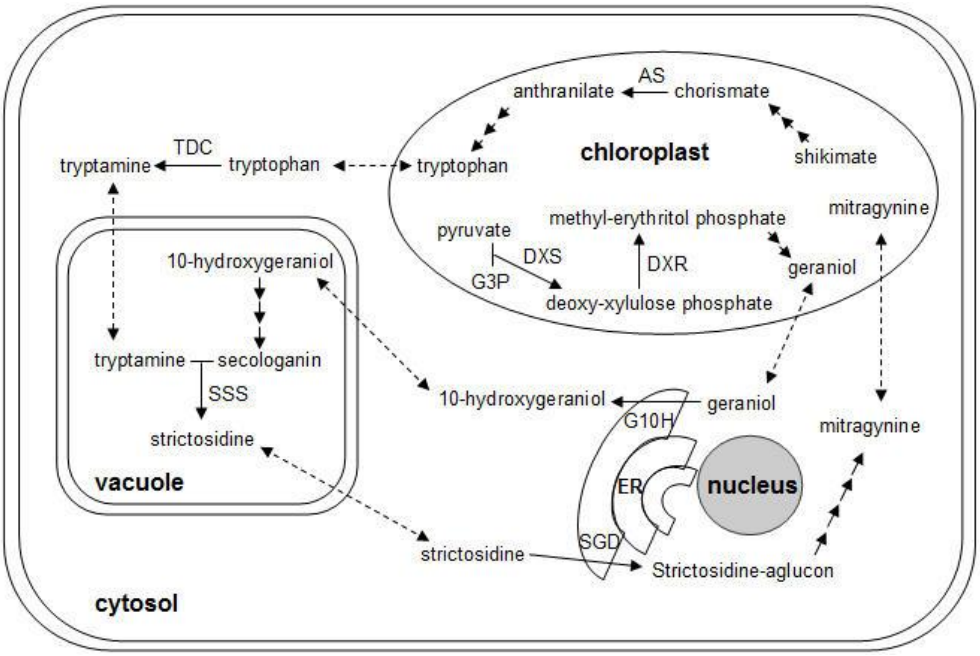


Figure 1.2 Proposed biosynthesis of mitragynine in *M. speciosa*. Dashed arrows indicate multi-step reactions. Two-heads dashed arrows indicate transportation of the metabolites.

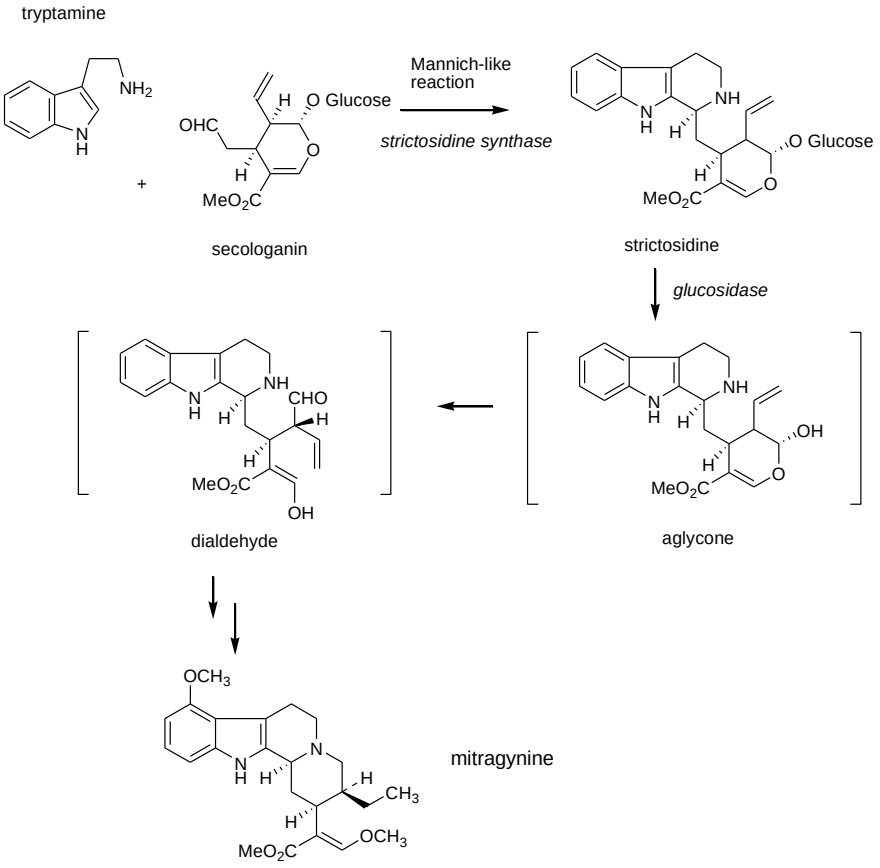


Figure 1.3 Proposed mitragynine biosynthesis via strictosidine synthase and strictosidine β-glucosidase

Later, the SGDs were cloned, purified and characterized from alkaloid producing plants such as *Tabernaemontana divaricata* (Luijendijk et al. 1996), *Catharanthus roseus* (Luijendijk et al. 1998; Geerling et al. 2000; Zarate et al. 2001), *Strychnos mellodora* (Brandt et al. 2000), *R. serpentina* (Gerasimenko et al. 2002), *Rauvolfia verticillata* (Xu et al. 2012). The full-length mRNAs of *SGD* have been submitted to the GenBank (http://ncbi.nlm.nih.gov/) including *Camptotheca acuminata* (accession no. JF508379_1), *Catharanthus roseus* (accession no. AF112888_1), *Rauvolfia serpentina* (accession no. JF966733_1), *R. serpentina* (accession no. AJ302044_1), *Uncaria tomentosa* (accession no. JQ366085_1) and partial fragment of *M. speciosa* (accession no. JF412824_1). The deduced amino acid sequences of those SGDs were aligned and revealed that they shared identity to *M. speciosa* SGD ranging from 41 to 90% as shown in Fig. 1.4.

Figure 1.4 Multiple alignments of alkaloid-producing plant SGDs with a partial amino acid sequence of *M. speciosa*. Shade indicates the similarity. Accession numbers are AFI71457 (*Rauvolfia verticillata*), CAC83098 (*R. serpentina*), AAF28800 (*Catharanthus roseus*), AES93119 (*Camptoptheca acuminata*), AFN69080 (*Uncaria tomentosa*).

Role of SGD in alkaloid biosynthesis has been demonstrated. A transgenic *Saccharomyces cerevisiae* with cDNAs encoding *STR* and *SGD* from *C. roseus*, Feeding of tryptamine and secologanin in the transgenic yeast culture produced high levels of strictosidine and hydrolyzed to cathenamine (Geerlings et al. 2001). In situ hybridization, co-expression of *STR* and *SGD* from *C. roseus* in the epidermal cells revealed that STR was localized to vacuole whereas SGD was

shown to accumulate as highly stable supramolecular aggregates within the nucleus (Guirimand et al. 2010). Elicitation with hydrogen peroxide, buthionine sulfoximine, and jasmonic acid caused oxidative stress in root culture of *U. tomentosa* and stimulated the production of 3α-dihydrocadambine and dolichantoside by increase the activities of STR and SGD in the root culture (Vera-Reyes et al. 2013).

2.6 Elicitation

There are several tissue culture techniques to improve the secondary metabolite production such as transformation methods, selecting high-producing strains and employing precursor feeding (Hussain et al., 2012), two-stage culture, immobilization technique, and elicitation techniques.

Elicitation is defined as plant response processes which the secondary metabolites production (phytoalexin) was attacked with insects, herbivores, and pathogens or to survive from biotic and abiotic stresses (Zhao et. al., 2005). The pathogens or biotic and abiotic stress were called as elicitors. The type of elicitors could be divided into 3 types: 1) biotic elicitors which had biological origin from pathogen or microbe-derived molecules for example polysaccharides, glycoproteins, low-molecular-weight compounds and bacterial and fungi cell walls, 2) abiotic elicitors which had not biological origin such as ultraviolet irradiation, salinity, climate changing, heavy metals and various chemicals, and 3) endogenous elicitors which are produced within the cell itself as methyl jasmonate (secondary messengers) (Tong-Jen et al., 1999). Most of the secondary metabolites caused by elicitation are involved in plant defense response. However, unsuccessful elicitation does not necessarily mean that the metabolic pathway cannot be induced. Thus, the use of elicitors which are not specific to the species (or not general enough) or an inappropriate production medium can cause ineffective elicitation (Tong-Jen et al., 1999).

Addition of biotic elicitor such as microbe homogenate has been reported to increase plant response. Razdan M.K. (2002) was suggested the mode of plant-microbe interaction. These include; first formation, direct release of the elicitor by the micro-organism and its recognition by a receptor site located on the plasma membrane of the plant cell. Second, microbial enzymes responsible for the development of cell-wall components act as elicitors in a respective plant cell. Third, plant enzymes release cell-wall components from the micro-organisms which, in turn, induce phytoalexins then form elicitor-active components from the plant cell wall. Finally, elicitor compounds, endogenous and constitutive in nature are formed in response to various stimulation (Randam, 2002).

In 2007, Vasconsuelo and Poland reported the molecular aspects of the early stages of elicitation on secondary metabolites in a plant cell. They described that the mechanism of elicitor to secondary metabolites production in the plant cell performed as a cascade of stimulation.

Cascade of stimulation can be summarised as below:

1. Elicitors bind to a receptor at specific localized on plasma membrane then activated G-protein.
2. G-protein signals to increase the levels of adenylyl cyclase (AC) and phospholipase C (PLC).
3. The second messengers mediating such as cAMP is stimulated by inositol trisphosphate (IP_3) and diacylglycerol (DAG) is stimulated by phospholipase C.
4. The levels of Ca^{2+}influx in plasma membrane are increasing.
5. The mitogen-activated protein kinases (MAPKs) are stimulated.

6. The regulation gene in nuclease is activated.
7. The secondary metabolite productions are increasing.

Since in 2014, Baenas and research team discovered the elicitor perception in plant cell on the plasma membrane and their defense response after elicited such as ion influx increase, protein phosphorylation, reactive nitrogen species (RNS) and reactive oxygen species (ROS) and hypertensive response. These defense responses were described in Fig. 1.5.

1. Firstly step is elicitor bind to specific receptor on plasma membrane then effect to defense response that involves the systemic acquired response (SAR) and induced systemic resistance (ISR) in the cytoplasm.

2. The effect on defense response (SAR and ISR) includes reactive oxygen species (ROS) and reactive nitrogen species (RNS), protein phosphorylation, ion fluxes increased and hypertensive response.

3. These defense responses cause to the expression of regulatory genes in nuclease and then activation of transcription of enzymes.

4. The secondary metabolites are increased and sometimes activated other signaling molecules such as salicylic acid, jasmonic acid, and ethylene.

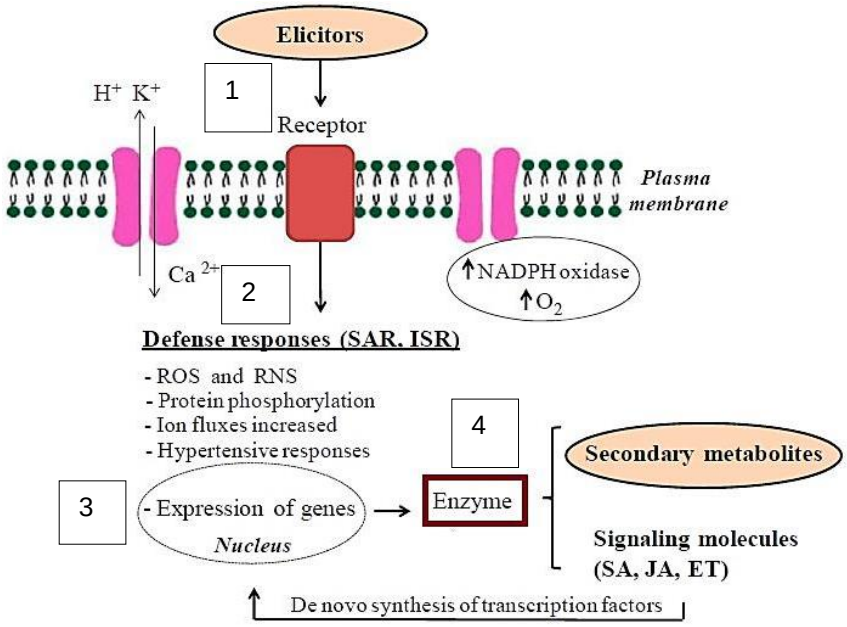


Figure 1.5 General mechanisms after elicitor perception in a plant cell (Baenas *et al.*, 2014)

Abbreviations: ET (ethylene), JA (jasmonic acid), SAR (systemic acquired response), ISR (induced systemic resistance), NADPH (nicotinamide adenine dinucleotide phosphate), ROS (Reactive oxygen species), RNS (reactive nitrogen species) and SA (salicylic acid)

The similar type of elicitors on different cells or organs in the same plant species could have different effects on regulation gene and secondary metabolites production. For example, ethylene elicitor was added in vary concentration in different 2 culture conditions of *C. roseus* (roots and leaves). The results showed that the addition of 15 µM ethylene increased genes expression as *g10h, sgd, as* and enhancing the production of vindoline and catharanthine in root culture (Pan et al., 2014). However, the responsible on genes expression of *str*, *d4h*, *scs*, *tdc*, *sgd* were increased, and the accumulation of vindoline and serpentine were enhanced at 30 µM ethylene elicited in leaves. Clearly, a variety of organ or cell culture in the same plant can be dissimilarly effected on gene expression and secondary metabolites production in the plant cell culture.

2.7 Plant tissue culture of *M. speciosa*

Plant tissue culture technique has been used in many purposes in plant science. The applications in pharmaceutical aspects are including being as alternative sources of secondary metabolite productions, conservation of medicinal plants, the target of gene expression and is a plant model for studying plant sciences. *In vitro* plant has an advantage over nature-plant since it can grow and produce secondary metabolite in-seasonal dependent. It grows under controlled condition; therefore, the contamination of the alien gene/substance to nature can be avoided.

Examples of utilization of plant tissue culture of the Rubiaceous plant to produce the secondary metabolites (Table 1). A wide array of secondary metabolites is produced by the Rubiaceous plants. Well known *Morinda citrifolia* cell culture, a Rubiaceous plant, produces and accumulates abundantly of anthraquinones (Zenk et al. 1975). Focusing the alkaloid-producing plant cell culture such as *Cinchona ledgerina* and *Ophiorrhiza pumila* could be induced in the basal medium of Gamborg B5. *Agrobacterium rhizogenes*-induced hairy root culture from *M. speciosa* has been reported and found that the hairy roots accumulated triterpenoids: ursolic acid and oleanolic acid. TIAs containing plant such as *Ophiorrhiza pumila* has been successful to induce in the form of hairy root culture and could accumulated camptothecin (Asano et al. 2009).

In 2008, Phongprueksapattana and their research group were successfully induced the hairy root by infection with *Agrobacterium rhizogenes* ATCC 15834, which incubation on McCown woody plant medium (WPM) supplemented with 0.5 mg/1 naphthaleneacetic acids. In addition, the triterpenoids (ursolic acid and oleanolic acid) and phytosterols (beta-sitosterol and stigmasterol) from an n-hexane extract of the hairy roots were obtained. The regenerated and differentiated plantlets from the hairy roots which there are accumulated mitragynine about 14.25±0.25 mg/g dry weight higher than in vitro plantlets (4.45 ±0.09 mg/g dry weight) (Phongprueksapattana et al., 2008). Wungsintaweekul and their research group studied the effects of methyl jasmonate and yeast extract on *M. speciosa* shoot culture. Her results indicated that the optimum for methyl jasmonate elicitor was 10 µM and incubation for 24 h which stimulated the highest accumulation of mitragynine content about 0.11± 0.03 mg/g dry weight that higher than control group (0.036 ± 0.005 mg/g dry weight). But, the higher concentration at 500 and 1000 µM of jasmonate decreased mitragynine production. Similarly, the transcription levels of *tdc* and *str* were increased after elicited with 10 µM of methyl jasmonate and incubated on 24 h. And yet, the optimum yeast extract elicitor was 0.1 mg/ml yeast extract incubated for 12 h. *tdc* and *str* transcription levels were enhanced about 15-fold and 7-fold when compared with control, respectively. (Wungsintaweekul et al., 2012). Consideration with the localization of mitragynine production and storage can suggest the hypothesis that mitragynine needs compartment to storage. The preferential storage organ of mitragynine is in leaves and stems but not in roots. This

evidence means that TIA biosynthesis needs essential organelles and enzymes to produce TIAs productions.

In recent year, Malaysian scientists developed *M. speciosa* suspension culture system. They varied hormones including; 2,4-dichlorophenoxyacetic acid (2,4 D), naphthaleneacetic acid (NAA) and benzyladenine (BA) to induce callus culture. Elicitors and precursors feeding to improve mitragynine production has been investigated. Their result showed successfully induced *M. speciosa* callus cell cultures from petiole explants by using 4 mg/L 2, 4 D. The elicitation of 250 mg/L of yeast extract was significantly increased mitragynine production up to 1.5-fold higher than control but salicylic elicitor no affected on mitragynine production. However, 3 µM of tryptophan feeding was achieved 5-fold of mitragynine production higher than control but loganin feeding was unaffected on mitragynine production (Md Zuldin et al., 2013).

2.8 Effect of nitric oxide on secondary metabolite production

In the case of secondary metabolite production, NO has been used as elicitor for more than 10 years. NO elicitor could appear in the cells that cause from biotic elicitor and from NO donor (Table 1.1). For example, Wang and Wu (2004) reported the effect of biotic elicitor on diterpene production of *T. chinensis* cell culture. Fungal elicitor as *Fusarium oxysporu* was used for NO generation and then stimulating diterpene production in cell culture. The NO production detected by a fluorometric assay with 4,5-diaminofluorescein diacetate dye (DAF-2DA) dye. Resulting of the study was shown that treatment of *F. oxysporu* could generate NO production and significantly increased taxol and baccatin III accumulation in *T. chinensis* cell culture (Wang and Wu, 2004). Similarly, 100 µM NO donor SNP treated *Salvia miltiorrhiza* hairy root culture enhanced the tanshinones production, including tanshinone IIA, cryptotanshinone, dihydrotanshinone I and tanshinone I (Du et al., 2015). Moreover, there was reported that SNP treatment affected the regulation gene in a plant cell. Such as, treatment of 5 mM SNP stimulated *str* gene in *C. roseus* leave culture (Dutta et al., 2013). In contrast, treatment of 0.1 mM SNP was completely inhibited the mRNA transcription level such as *as*, *tdc*, *g10h*, *str*, AP2-domain DNA-binding protein (ORCA3), zinc finger DNA-binding protein (*zct1*) and *C. roseus* G-box binding factor (*Crgbf1*) after treatment for 23 days. While, the terpenoid indole alkaloids like serpentine, catharanthine, ajmalicine, lochnericine and tabersonine were increased their accumulation under SNP treatment (Li and Peebles, 2011).

Table 1.1 Effect of NO elicitor on the production of alkaloids in plants

Source of plant	NO source/concentration	Results	References
Catharanthus roseus (suspension)	10 and 20 mmol/L sodium nitroprusside (SNP)	↑ catharanthine	Xu et al., 2005
C. roseus (seedlings)	0.1 mM SNP	↑ serpentine, catharanthine, ajmalicine, lochnericine, and tabersonine but ↓ mRNA transcript levels of *as*, *tdc*, *g10h*, *str*, AP-2 domain DNA-binding protein (ORCA3), zinc finger DNA-binding protein (*zct1*) and C. roseus G-box binding factor (*Crgbf1*)	Lee and Peebles 2011
C. roseus (cell culture)	0-10 mM SNP	↑ catharanthine, ajmalicine	Xu and Dong 2005
C. roseus (leaves)	5 mM SNP	↑ *str* gene expression	Dutta et al., 2013
Lycoris chinensis (seedlings)	100 μM and 5 μM SNP	↑ 1.72-fold galanthamine and ↑ 1.37-fold lycorine but ↓ lycoramine	Mu et al., 2009
Salvia miltiorrhiza (hairy root)	100 μM SNP	↑ tanshinone IIA, cryptotanshinone, dihydrotanshinone I and tanshinone I	Du et al., 2015

2.1 Cloning of the internal sequence of SGD

Plant materials

M. speciosa plantlet was propagated from seeds that germinated on hormone-free McCown Woody Plant medium (WPM) and incubated at 25°C under 16 h daily light. Kratom plant culture was subcultured and maintained under controlled condition.

Total RNA extraction and cDNA preparation

Total RNA was extracted from the leaves (300 mg) using the RNeasy Plant Mini Kit (Qiagen). The quality of total RNA was electrophoresis under the non-denaturing condition on a 1.5% (w/v) agarose. The total RNA was reverse-transcribed to a first-strand cDNA using the Superscript™ III reverse transcriptase (Invitrogen) based on the RNA ligase-mediated and oligo-capping rapid amplification of cDNA (RLM-RACE) (Invitrogen). The GeneRacer RNA oligo dT (5´-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{24}$-3´) was used as a primer. The first-strand cDNA was stored at −20 °C until used for PCR.

Primers design for internal fragment cloning

The amino acids and nucleotide sequences of SGD were obtained from the GenBank database and aligned using the ClustalX software (Thompson et al., 1997). Pairs of degenerate primers were designed based on the highly conserved region (Table 2.1) among the plant strictosidine β-glucosidase (SGD) including *Camptotheca acuminata* (GenBank ID: JF508379), *Catharanthus roseus* (GenBank ID: AF112888), *Rauvolfia verticillata* (GenBank ID: JF966733), *Rauvolfia serpentina* (GenBank ID: AJ302044) and *Uncaria tomentosa* (GenBank ID 366085) (http://www.ncbi.nlm.nih.gov). These primers are used to amplify the internal fragment and used the cDNA as a template.

Table 2.1 Nucleotide sequences of degenerate primers for amplification of SGD.

Degenerate primers	Nucleotide sequence (5´→3´)	Tm (ºC)
SG370F	GGT TYT CAA TYT CAT GGT CMA GA	60
SG1500R	CCY TTY ACA TTY ACW CCA TCR T	58
SG500F	GTA ACT CTC TTC CAC TGG GAT	60
SG850F	TCT TGC TCA CAA AGC TGC TGT	60
SG850R	ACA GCA GCT TTG TGA GCA AG	60

Polymerase chain reaction amplification and cloning of SGD

PCR was performed in a 50 µl reaction system using the KOD plus Taq DNA polymerase. The PCR contained two different conditions: (1) Step-Up (SU); 94°C 3 min, 10 cycles of 94°C 30s, 55°C 30s, 72°C 1 min, 25 cycles of 94°C 30s, 58°C 30s, 72°C 1 min, and 72°C 5 min, and (2) Step-Down (SD); 94°C 3 min, 10 cycles of 94°C 30s, 62°C 30s, 72°C 1 min, 25 cycles of 94°C 30s, 60°C 30s, 72°C 1 min, and 72°C 5 min. The PCR products from were run on 1.5% w/v agarose gel electrophoresis and the DNA bands were separately excised and purified using the QIAquick Gel Extraction Kit (Qiagen). Then, the PCR products were ligated into A-tailed-pGEMT-Easy Vector using the DNA Ligation Kit Ver.2.1 (Takara) in a ratio of 1:1 [PCR products 2.5 ul and vector 2.5 ul, and a solution I from the Kit 5 ul]. The resulting ligation reaction was transformed into *E. coli* competent cells strain DH5α. Recombinant plasmid DNAs were isolated from positive clones followed by PCR and restriction enzyme digestion analysis. Subsequently, the nucleotide sequence was determined using the automated DNA sequencer and searched against the Genbank database using the BLAST program (Altschul *et al.*, 1997).

Results

PCR amplification of SGD cDNA

Combinations of primers have been investigated and by using two-step PCR, the PCR products of about 350 bp and 700 bp were obtained and been chosen for further DNA sequencing. The internal sequence contained 773 nucleotides, encoded for 257 amino acid residues as shown in Fig.2.1. Alignment of the deduced amino acid to the known SGD of alkaloid-producing plant like *Rauvolfia serpentina* revealed that the obtained internal sequence SGD shared the homology to the putative *R. serpentina* SGD for about 61%. In addition, analysis of internal sequence found the catalytic motif belonging to the glycosyl hydrolases family as shown in Fig. 2.2. The glutamate catalytic residue also found in the *M. speciosa* SGD sequence similar to *R. serpentina* SGD.

>JF412824_1_*Mitragyna_speciosa*
AGCATATGGGATACTTTCACCCAGAGAAGACCAGGTATGATTAAGGAAGGAGGCAACGGAAATGTGGCTGTGGATTCAT
ATCATCAGTATAAGGAAGATATCAAGATTTTGAAGAACATGGGGCTGGATGCCTATCGGTTCTCAATATCATGGTCAAG
AGTACTGCCAGGTGGGAATTTAAATGCTGGCGTAAATAAGGAGGGAATCAACTATTACAACAATCTCATTGATGAGCTC
CTAGCCAATGGTATCGAGCCATATGTAACTCTATTTCACTGGGATGTTCCTCAAGCATTGGAAGATAAATATGGTGGCT
TTTTAAGTTCTCAAATTGTGGACGACTTCCGCGAGTACGTAGAGCTTTGCTTTTGGGAGTTTGGAGATCGAGTGAAACA
TTGGATAACACTGAATGAACCATGGAGCTCTAGTGTTGGTGGATATGTAAACGGCACATTTGCACCTGGCCGAGGTGCC
TCTTCATCAGAGCAAGAAAACGATCATCCAGCTCCTGCACTACTGAGCAGATGTTCTCCATGGCAATCACAAAGTATTT
CTAGCAATGGGAATCCAGGGACAGAGCCATATGTGGTGACTCACAATCAGCTTCTTGCTCATGCAGCTGCTGTCGAATT
GTATAAGACCAACTTTCAGAAATCACAAAATGGCAAGATTGGGATTACACTTGTGTCTCAGTGGATGGAACCTTTGGAC
GGAAACAGTAAAGCTGATGTCGAAGCCGCAAAGAGAGCTCTTGATTTCATGCTTGGATGGTT

Figure 2.1 The internal sequence of *M. speciosa* SGD

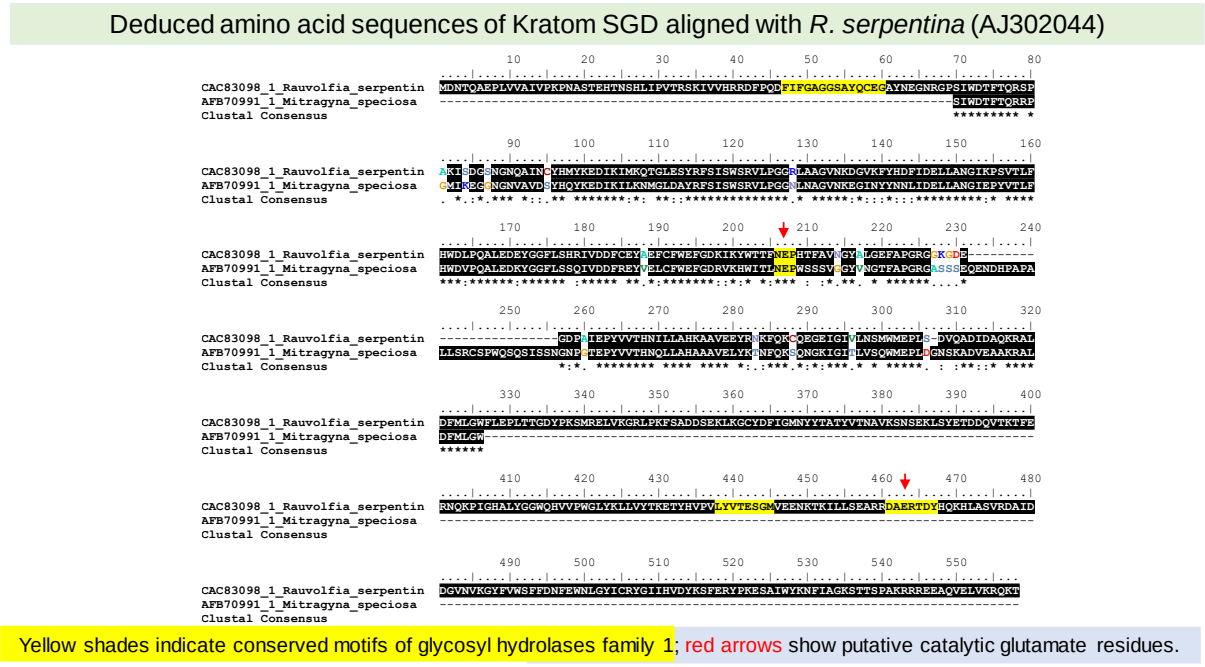


Figure 2.2 Alignment of the amino acid sequence of the internal sequence of *M. speciosa* SGD to *Rauvolfia serpentina* SGD

The deduced amino acid residues of the *M. speciosa* internal sequence of SGD was aligned with known SGD of the alkaloid-producing plant with Clustal W. The result is shown in Fig. 2.3 indicated that *M. speciosa* SGD shared the homology in the range of 55-60% identity. The obtained internal sequence was submitted to the GenBank in accession number of JF412824 and AFB70991.1, respectively. The 3'-end, 5'-end and the full-length SGD cDNA cloning is in progressed.

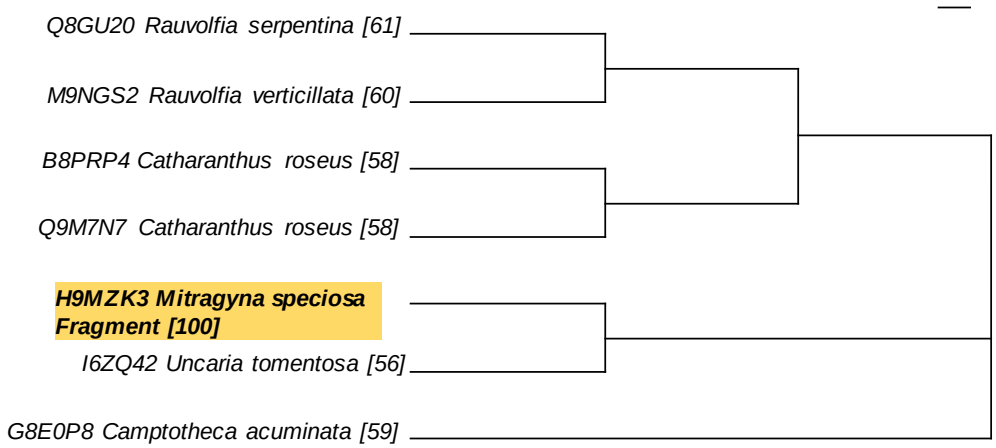


Figure 2.3 The alignment among alkaloid-producing plant SGD with *M. speciosa* SGD by Treeview

Results: Internal sequence of SGD was cloned and its nucleotide sequence is shown to similar to the previous plant SGDs. Primers for 5´- and 3´-ends are designed and is going to clone using SMARTer™ kit (CloneTech)

2. Design primers and cloning of 5´- and 3´-ends

Fragments of 5'- and 3'-ends of the SGD gene were amplified based on the information of SGD internal sequence. An attempt is still unsuccessful. Conditions need to optimize regarding annealing temperature and probably the primer itself. Concerning the *Mitragyna speciosa* tissue culture, such as callus, suspension, shoot, and plant cultures were successfully propagated and maintained. Transcription profiles of target genes such as *DXS*, *DXR*, *AS*, *TDC*, and *STR* were determined in all types of cultures. Interestingly, the abundance of each gene was different, dependent upon types of cultures and organs of plants.

According to the information of internal sequence of SGD (accession number JF412824) and deduced amino acid (accession number AFB70991.1), primers for 5´- and 3´-ends were designed based on the guideline of SMARTER RACE 5'-/3'- kit (Clone Tech, USA) as shown in Table 2.2. Positions of primers are located as shown in Fig.2.4.

Table 2.2 List of primers

Name	Sequence (5'→ 3')
For 5´-end and 3´-end amplification	
RACE5R1	GAGAACCGATAGGCATCCAGCC
RACE5R2	CACATTTCCGTTGCCTCCTTCC
RACE3F1	GTGGATGGAACCTTTGGACGGA
RACE3F2	CTGCACTACTGAGCAGATGTTCTCC
For SGD transcription profile	
F122	GGCTGGATGCCTATCGGTTCTC
R237	GGCTCGATACCATTGGCTAGGA
R371	TTCACTCGATCTCCAAACTCCC

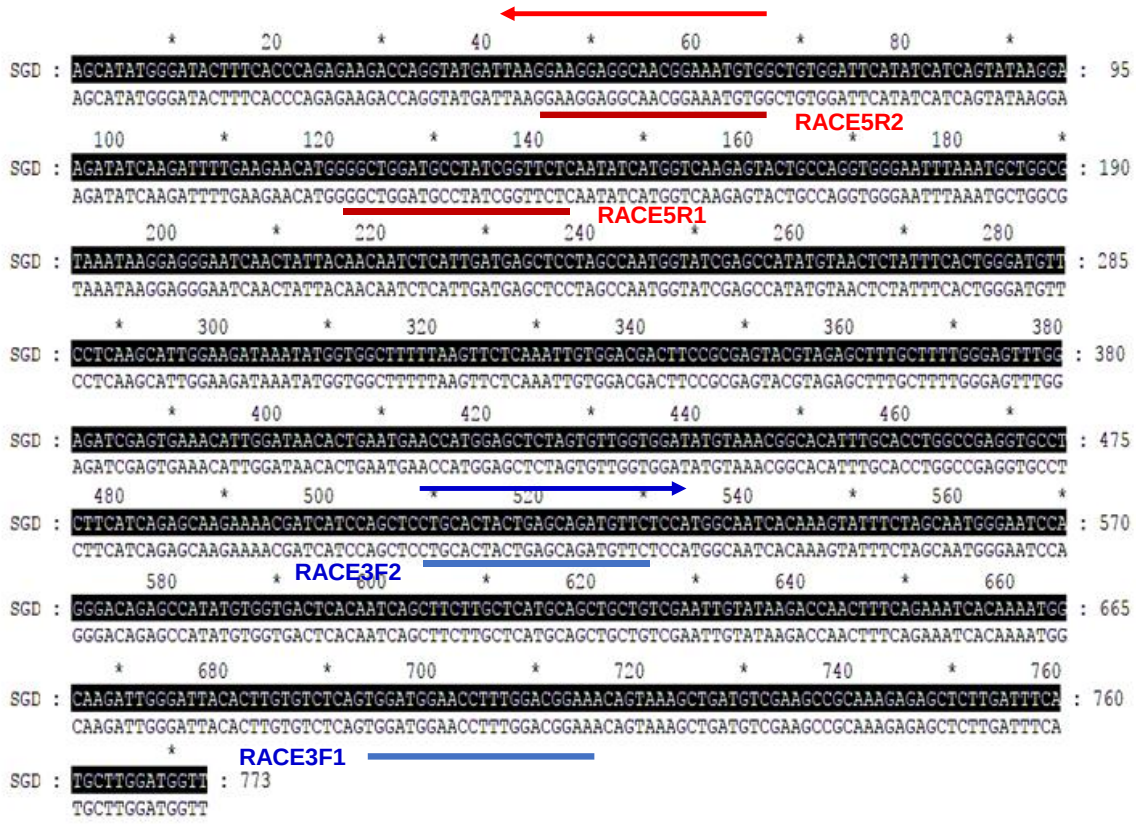


Figure 2.4 SGD internal sequence of *Mitragyna speciosa*. The red arrow indicates the direction of 5'-end amplification and blue arrow indicates the direction of 3'-end amplification. Bars locate the positions of primers.

Isolation of total RNA and cDNA synthesis

Total RNAs were extracted using the Total RNA Mini Kit (Geneaid, Taiwan). *M. speciosa* (kratom) leaves (0.1 g) were ground to a powder with liquid N_2 then transferred to a 1.5 ml microcentrifuge tube. The RB buffer of 500 µl and 5 µl of ß-mercaptoethanol was added. The mixture was incubated at 60ºC for 5 min and transferred to the filter column, centrifuged at 1,000 x g for 1 min. Discard the filter column, the filtrate was transferred to a clean tube. The 0.5 volume of chilled ethanol was added to the filtrate and mixed well with the pipette, and loaded to the RB column, centrifuged at 10,000 rpm for 1 min. Discard flow through, the column was washed with 400 µl of buffer W1 and centrifuged. After drying the column by centrifugation, total RNA was then harvested by adding 50 µl of RNase-free water, incubated for 1 min and centrifuged at 10,000 rpm for 1 min. The purity of total RNA was observed using the ratio between the absorbance at 260 nm and 280 nm. Total RNAs were stored at -80°C until used for RT-PCR.

The isolated total RNA from *M. speciosa* leaves has ratio OD_{260}/OD_{280} of 2.065 with the yield of 16.1 µg (0.322 µg/µl, 50 µl). Total RNA is ready to synthesize the cDNA as appropriate. cDNA synthesis using Superscript III reverse transcriptase (Invitrogen): Briefly, total RNA (3 µg) was mixed with 1 µl of 10 mM dNTP and 1 µl of 10 µM oligo $(dT)_{20}$. The mixture was heated at 65°C for 5 min and then immediately chilled on ice for at least 1 min. After chilling 4 µl of the first-stranded buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOUT (Recombinant RNase Inhibitor) and 1 µl of Superscript III reverse transcriptase were added and incubated at 50°C for 60 min. The reaction was inactivated by heating at 85°C for 15 min. The first-stranded cDNA was kept at -20

°C until used. To check the quality of the obtained cDNA, primers of genes which involved in genes of mitragynine biosynthesis were amplified. Fig. 2 illustrates the utility of cDNA as template for DNAs amplifications and concludes that cDNA is ready for transcription profile analysis.

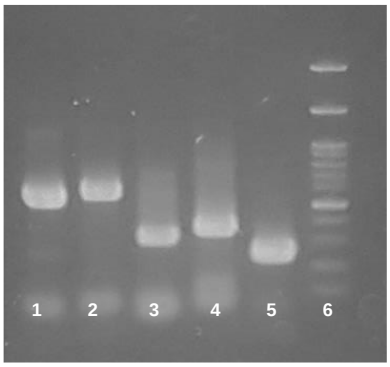


Figure 2.5 1.2% (w/v) Agarose gel electrophoresis of genes involved in mitragynine biosynthesis. 1: *18s rRNA*, 2: deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*), 3: anthranilate synthase alpha-subunit 1 (*AS1*), 4: tryptophan decarboxylase (*TDC*), 5: strictosidine synthase (*STR*), 6: DNA ladder.

cDNA synthesis using Smarter RACE kit (Clontech): This kit is for the 5'- and 3'-RACE-Ready cDNA synthesis. Buffer mix of 4.0 µl 5X first-strand buffer, 0.5 µl DTT (100 mM), 1.0 µl dNTPs (20 mM) for total Volume of 5.5 µl was prepared. For the preparation of 5'-RACE-Ready cDNA, 1.0–10 µl total RNA, 1.0 µl 5'-CDS Primer A were mixed and total volume was adjusted to 11 µl with sterile distilled H_2O. For the preparation of 3'-RACE-Ready cDNA 1.0–10 µl total RNA, 1.0 µl 3'-CDS Primer A were mixed and total volume was adjusted to 12 µl with sterile distilled H_2O. The mixtures were mixed and spun the tubes briefly in a microcentrifuge. The mixture was incubated at 72°C for 3 min and then cooled the tubes to 42°C for 2 min. To collect the content, the reaction tube was centrifuged briefly for 10 s at 14,000 x g.

A master mix of 5'- and 3'-RACE-Ready cDNA synthesis reaction was prepared. The reagents including 5.5 µl buffer Mix, 0.5 µl RNase Inhibitor (40 U/µl) and 2.0 µl SMARTScribe Reverse Transcriptase (100 U) to a total volume of 8.0 µl. Add 8 µl of the Master Mix to the denatured RNA for a total volume of 20 µl per cDNA synthesis reaction. Heat tubes at 70°C for 10 min. Dilute the first-strand cDNA synthesis reaction product with Tricine-EDTA buffer 90 µl. Samples can be stored at –20°C. The cDNA was used as a template for 5'- and 3'-ends amplifications.

Amplifications of SGD fragments
Using RACE cDNA
Fragments of 5'- and 3'-ends were amplified due to complete the SGD full-length. The reaction mixture (50 µl) was composed of 15.5 µl PCR-Grade H_2O, 25.0 µl 2x SeqAmp buffer, 1.0 µl SeqAmp DNA polymerase, 2.5 µl 5'- or 3'-RACE-Ready cDNA, 5 µl 10x UPM, 1 µl 5' or 3' GSP (10 µM). Annealing temperature was optimized from 54-66°C. After 35 cycles of amplification, the PCR product was analyzed in 1.2% (w/v) agarose gel electrophoresis. The results of the PCR products are shown in Fig.2.6. Sizes of the PCR products were not the predicted sizes. Probably, the difficulty of either primer design or the SGD internal sequence made the attempt of amplification unsuccessful.

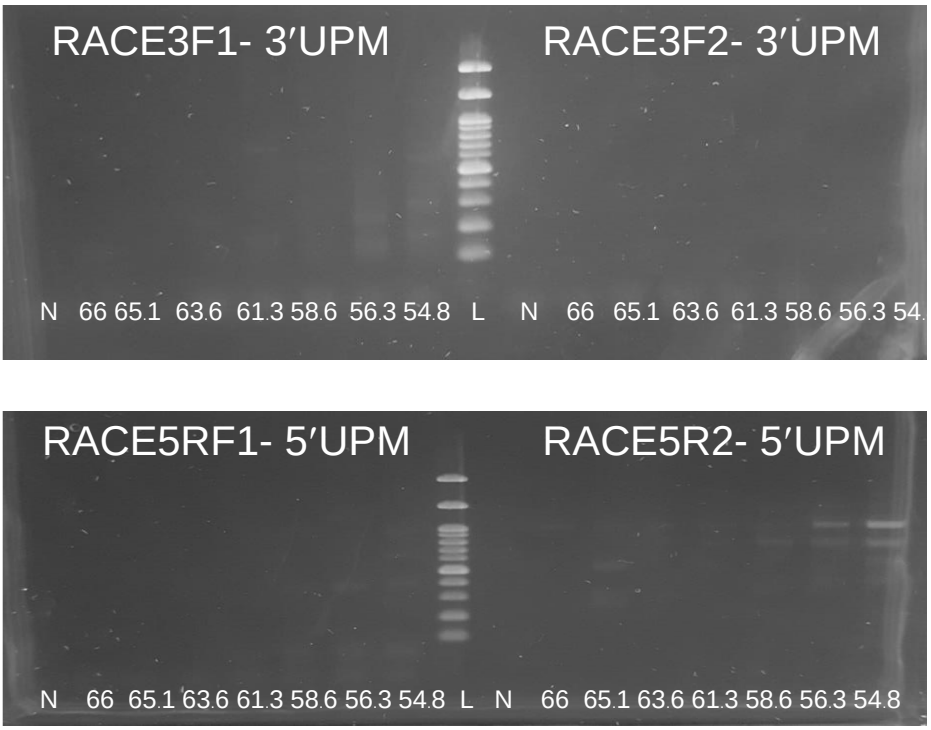


Figure 2.6 Optimization of annealing temperatures of 5'- and 3'-ends amplifications on 1.2% (w/v) agarose gel electrophoresis.

Using Superscript III cDNA

Superscript III cDNA was used as a template for following the transcription profile. As shown in Fig. 2.4, the synthesized template was verified for gene expression. Primers for following the SGD fragment were designed based on the information of SGD internal sequence. However, the sizes of the PCR products were not as predicted (Fig. 2.7).

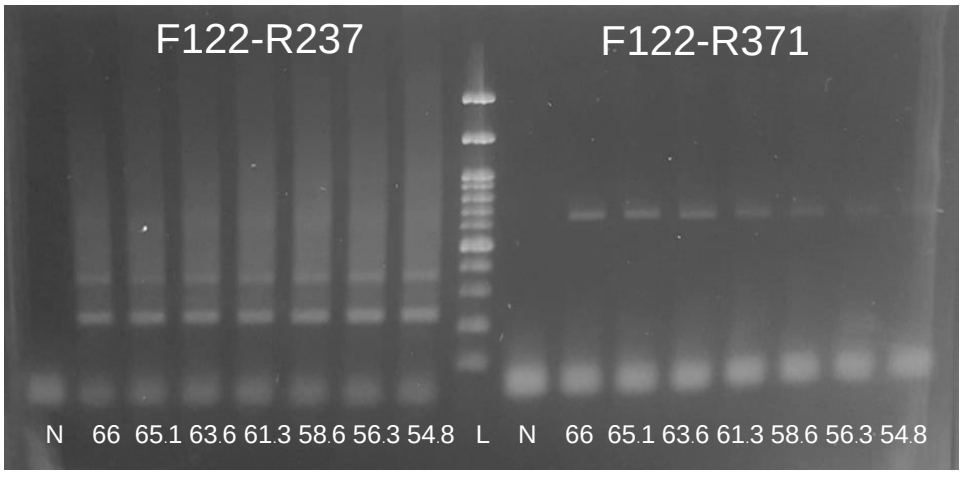


Figure 2.7 Optimization of annealing temperatures of SGD fragment amplification on 1.2% (w/v) agarose gel electrophoresis.

In summary, isolation of the full-length of *SGD* gene from *M. speciosa* was unsuccessful. Consideration of the nucleotide sequences of the full-length SGD from the alkaloid-containing plants which are *Gelsimium semperivirens* (accession no. MF401946.1; 1605 bp), *Rauvolfia verticillata* (accession no. JF966733.1; 1856 bp) and *Catharanthus roseus* (accession no. AF112888.1; 1875 bp) in compare with the internal sequence of *M. speciosa* (773 bp), it shares the identity of 73%, 58%, 57%, respectively. To retrieve the full-length, we need to come back to the origin. The degenerate primers are designed for the internal sequence as well as the primers for 5´- and 3´-ends amplifications. It is illustrated the position of primes as shown in Fig. 2.8 and summarizes in Table 2.3.

Table 2.3 List of designed degenerate primers for SGD amplifications.

Primer	Nucleotide sequences (5´→ 3´)	Tm (°C)	GC content (%)
	For internal sequence amplification		
S280F1 (23 bp)	5´CCY AGT ATA TGG GAT ACT TTC AC 3´	59.2-60.3	39-43
S430F2 (23 bp)	5´TCA TGG TCA AGA RTA YTR CCA GG 3´	59.2-64.6	39-52
S980R1 (15 bp)	5´ARA GGC TCC ATC CAC 3´	46-48.8	53-60
S690R2 (20 bp)	5´TGG TTC ATT CAG HGT HAT CC 3´	55.4-59.5	38-48
	For 5´-end amplification		
5'350R2 (21 bp)	5´AGC CAC ATT TCC GTT GCC TCC 3´	63.2	57
5'550R1 (22 bp)	5´GAG TTA CAT ATG GCT CGA TAC C 3´	60.1	45
	For 3´-end amplification		
3'900F1 (24 bp)	5´ GCT GTC GAA TTG TAT AAG ACC AAC 3´	62	42
3'1010F2 (23 bp)	5´ GCT GAT GTC GAA GCC GCA AAG AG 3´	66	57
	For SGD transcription profile analysis		
Set 1			
SGD Forward	5´ GGC GTA AAT AAG GAG GGA ATC 3´	50.4	47.6
SGD Reverse	5´ TCC AAA CTC CCA AAA GCA AAG 3´	51.3	42.9
	(amplicon: 195 bp; Tm = 73.5°C)		
Set 2			
SGD Forward	5´ TGG ATG CCT ATC GGT TCT C 3´	50.5	52.6
SGD Reverse	5´ TCC CAA AAG CAA AGC TCT AC 3´	45.0	50.2
	(amplicon: 250 bp; Tm = 74.4°C)		

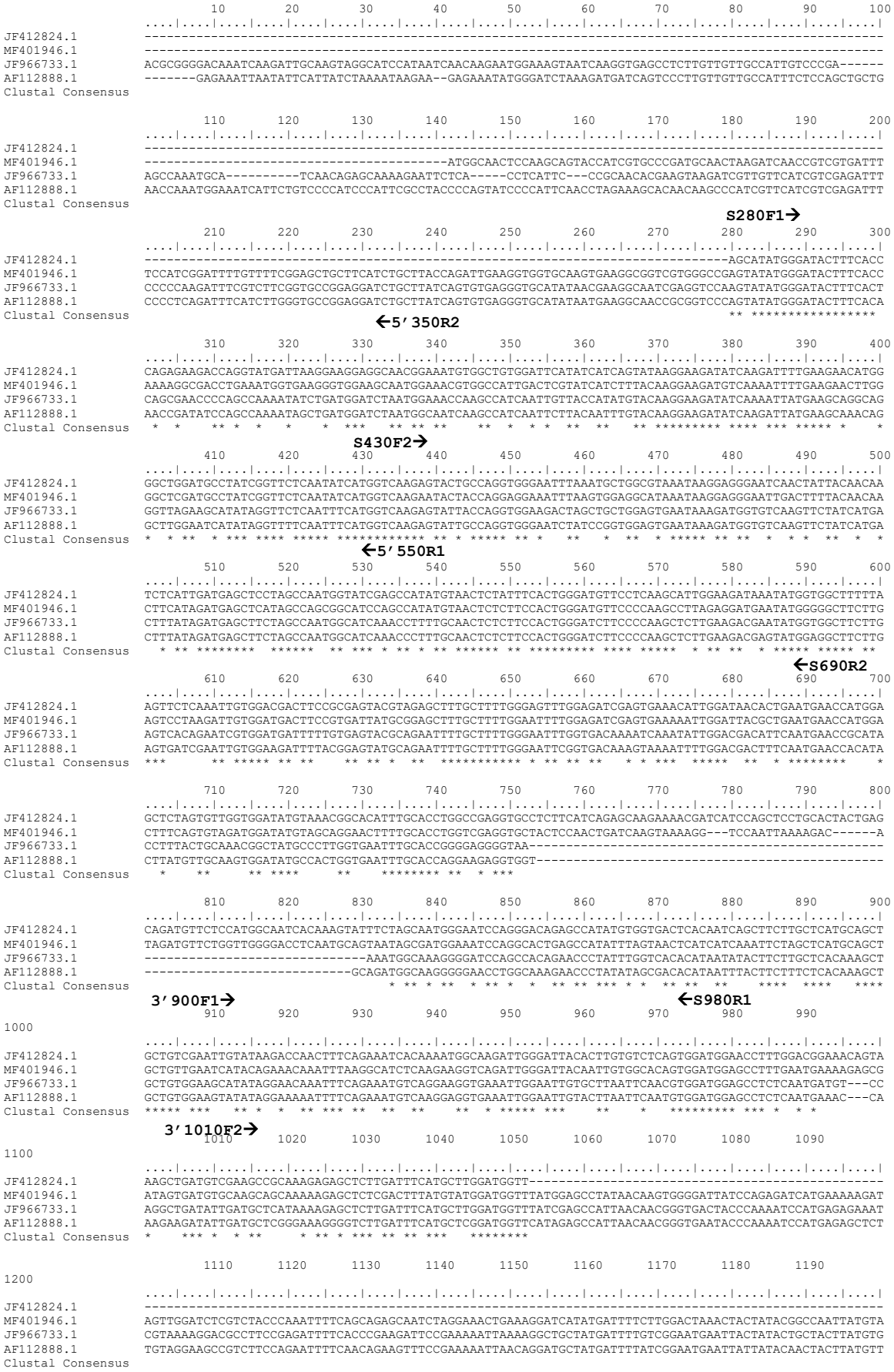


Figure 2.8 The nucleotide sequences of SGD of alkaloid-containing plants.

1. Establishment of kratom tissue cultures

1.1 Preparation of in vitro seedlings

Seeds of kratom were collected from the natural field in South Thailand. The seeds were sterilized in 2% (v/v) of sodium hypochlorite for 5-10 min and washed thoroughly with sterilized distilled water (3 times). The sterilized seeds were washed with 1% (v/v) of hydrogen peroxide (2 times) for 2 min due to get rid of an excess of sodium hypochlorite. After washing with sterilized distilled water (3 times), the sterilized seeds were placed on hormone-free solid WPM medium and incubated at a temperature of 25±2 ºC in dark condition for 2 weeks. Protocol of seed sterilization is illustrated in Fig. 3.1

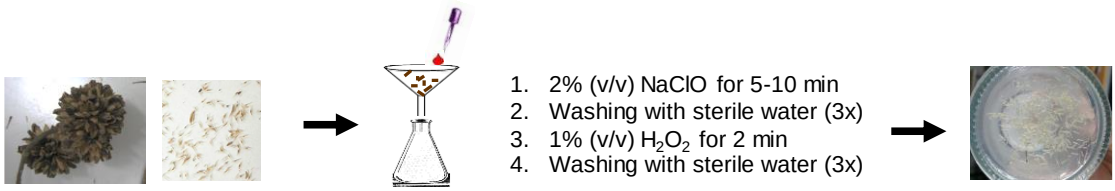


Figure 3.1 Establishment of kratom seedlings

Kratom seeds were placed on WPM hormone-free medium under the sterile condition and germinated. The seedlings were grown for 10 days in the dark condition. Later, the seedlings were exposed to light 16 h/day. After incubating for 1 month, the root of seedling was removed and sub-cultured to the new medium. The 2-month old seedlings were used as starting materials for the induction of several types of tissue cultures (Fig. 3.2).

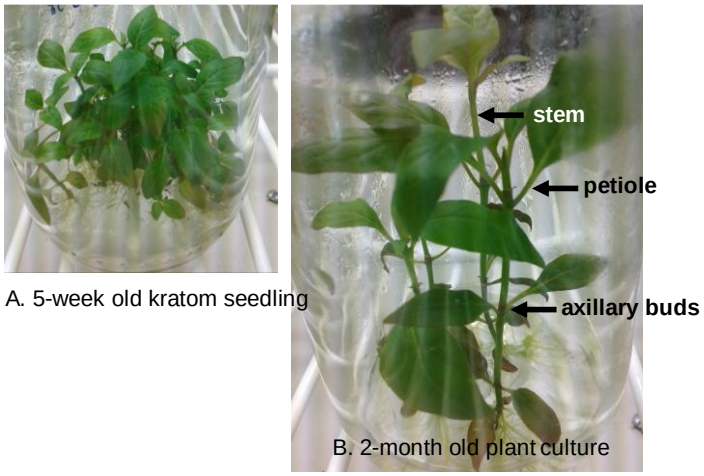


Figure 3.2 A. kratom seedlings and B. kratom plant culture

1.2 Induction of shoot culture

The shoot culture was induced from six-week-old plant culture. The axillary buds were excised and placed on solid WPM medium supplemented with 1 mg/L benzyladenine (BA) and 2 mg/L

thidiazuron (TDZ). Shoot culture was incubated 25 °C under light 16 h/day. After 2 times of passage, shoot culture was transferred into 30 ml liquid WPM medium supplemented with 1 mg/L BA and 2 mg/L TDZ. The shoot culture was kept under 25 °C, 16 h light/day and shaking at 60 rpm. For sub-culturing, the shoot culture, the cluster of the shoot was excised and the dead cells were removed. Five shoot clusters (each cluster contained 3-5 small shoots) were transferred to the liquid medium every month.

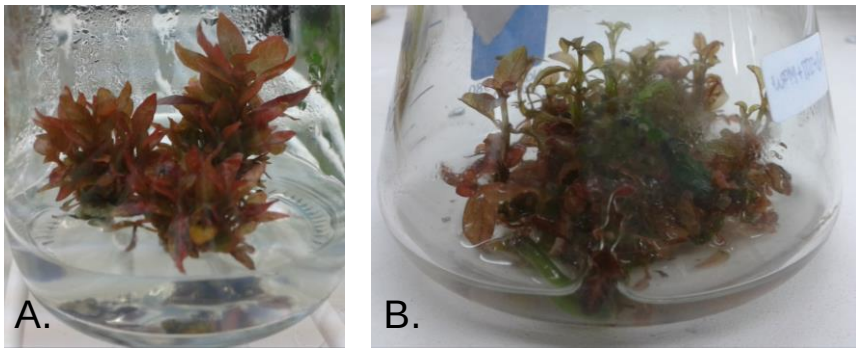


Figure 3.3 Kratom shoot culture A. on solid medium and B. in a liquid medium

1.3 Induction of root culture

An intact root was excised from the 2-month old plant culture and transferred to the liquid medium of WPM supplemented with α-naphthaleneacetic acid (NAA) 0.5 mg/L. The root culture was shaking at 60 rpm under 16 h lights (Fig. 3.4).

1.4 Induction of hairy root culture

Hairy root culture was initiated by infection the *Agrobacterium rhizogenes* ATCC 15834 to petiole of kratom leaves. Suspension of bacterial culture was prepared from the overnight culture. A single bacterial colony was obtained and used for inoculation into 5 ml YEB liquid medium. The culture was then placed on a 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 OD600 about 0.5-0.6. Finally, the bacterial suspension was further used to infect the explants using needle injection method (Dhakulkar et al., 2005).

The hairy root culture, firstly, the explants were wounded with a needle and then the wounded explants were submerged in bacterial suspension for further 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 the culture at 25 °C under 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 (Fig. 3.4).

Figure 3.4 A. kratom root culture in WPM solid medium and liquid medium (supplemented with 1 mg/L NAA) and B. kratom hairy root culture from *A. rhizogenes* infection in the hormone-free WPM medium.

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 a rotary shaker (80 rpm) and under 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.

1.5 Induction of callus and suspension cultures

Callus culture was initiated according to Zuldin et al. 2013. The WPM solid media was prepared supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) in the concentrations of 1, 2.5, 5 and 10 mg/L. The selected explants were petiole and stem (Fig. 3.2). The percentage of callus initiation was recorded. The result is shown in Table 3.1. The WPM supplemented with 2,4-D concentration up from 1 mg/L is suitable for callus induction when petiole and stem were used as explants.

The new cells forming were removed and placed on the new medium. The callus culture was sub-cultured every month until homogenous culture was obtained. Fig. 3.5 exhibits the appearance of 1-month old callus culture. Then suspension culture was induced by transferring the friable callus to the liquid medium, shaking at 120 rpm, light 24 h/day. Fast growing of cells forming in suspension culture was observed. It needs to subculture every 2 weeks.

In overview, we succeed to establish the cell cultures (callus and suspension cultures) and organ cultures (shoot and plant cultures). Due to the required mass culture, the fast-growing culture of root and hairy roots cultures is needed. Nevertheless, those cultures need to be optimized in term of induction condition for better growing culture.

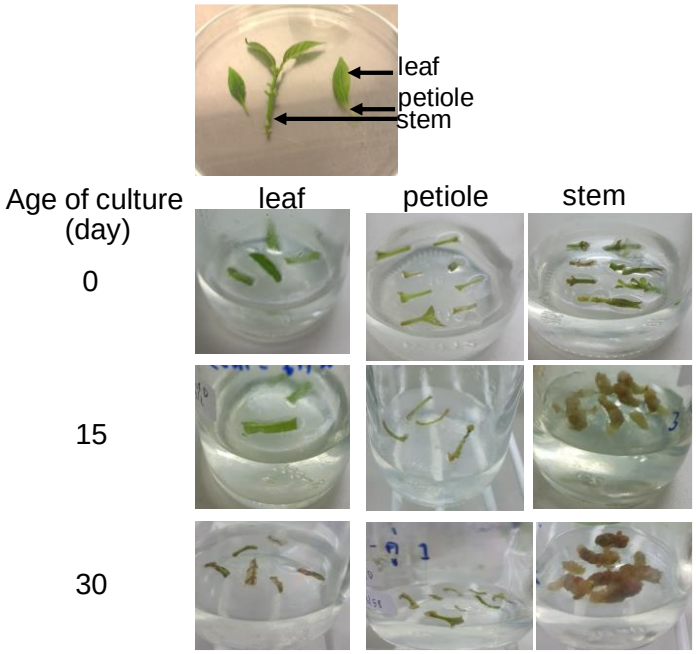


Figure 3.5 Development of callus forming from leaf, petiole, and stem in the WPM supplemented with 1 mg/L 2,4-D

Table 3.1 Percent callus formation on WPM plus 2,4-D.

WPM	**Explant: petiole** Number of explant/Number of callus formed			% Callus formation
	Day 0	Day 9	Day 10	
Hormone-free	79/0	79/0	79/0	0
+ 2,4-D 1 mg/L	91/0	91/0	91/30	33
+ 2,4-D 2.5 mg/L	50/0	50/1	50/50	100
+ 2,4-D 5 mg/L	53/0	53/2	53/47	89
+ 2,4-D 10 mg/L	61/0	61/21	61/43	70
WPM	**Explant: stem** Number of explant/Number of callus formed			% Callus formation
	Day 0	Day 9	Day 10	
Hormone-free	59/0	59/0	59/0	0
+ 2,4-D 1 mg/L	72/0	72/61	72/72	100
+ 2,4-D 2.5 mg/L	46/0	46/42	46/45	98
+ 2,4-D 5 mg/L	50/0	50/45	50/45	90
+ 2,4-D 10 mg/L	49/0	49/45	49/49	100

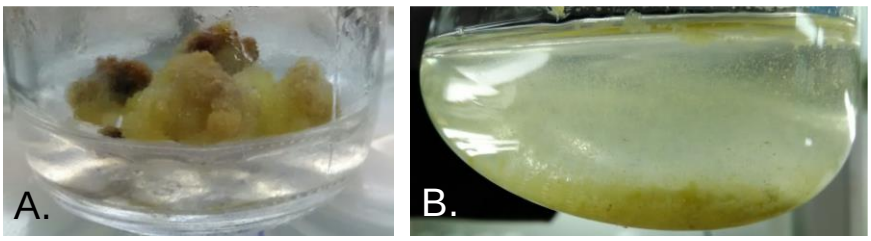


Figure 3.6 A. 1-month old culture and B. 10-day old suspension culture

After several times of subculture (at least 25 times), the appearance of callus culture and suspension culture are shown below. Friable and pale green callus culture was obtained (Fig. 3.7).

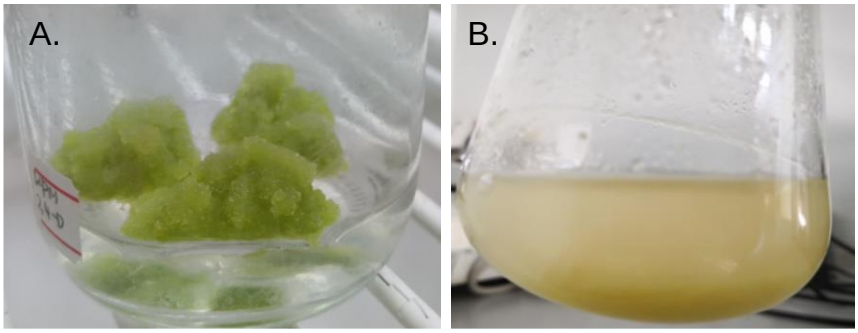


Figure 3.7 Kratom tissue cultures. A. callus culture; B. suspension culture

2. Evaluation of secondary metabolite production

2.1 HPLC analysis

Qualitative and quantitative analyses of mitragynine and related alkaloids and secologanin were performed using HPLC method. The reverse-phase column (C18) was used. The mobile phase and the elution program were optimized as appropriate. At least 3 elution profiles are investigated for the HPLC evaluation as shown below. All HPLC procedures have been validated and reported elsewhere.

System 1: Analysis of mitragynine and secologanin

HPLC analysis was carried out using Agilent 1100 series equipped with Agilent 1100 series Photodiode-arrays detector and a fluorescence detector. Data analysis was performed using Agilent software (USA). Chromatographic separation was performed on a reverse phase VertiSep^{TM} UPS C18 column 250 mm × 4.6 mm i.d., 5 µM particle size (Vertical, Bangkok, Thailand) with binary gradient mobile phase profile [1.5 ml/min, acetonitrile: 100 mM H_3PO_4 in water, pH 2.4 (10:90 to 90:10, v:v within 15 min), injection volume 20 µl]. The identification of secologanin and mitragynine was based on the retention time and comparison of the absorption spectra with authentic standards. The quantitative wavelength was set at 225 nm and 238 nm for mitragynine and secologanin, respectively.

The retention times of mitragynine and secologanin are **9.5 and 6.2** min, respectively. Additional 5 min prior gradient starting was performed when avoid of impurities is needed. Therefore, the retention time of mitragynine and secologanin are shifted to **14.5 and 11** min, respectively.

System 2: Analysis of mitragynine

The HPLC method was modified for mitragynine analysis. Separation of mitragynine and *M. speciosa* metabolites were performed on a C18 reverse column. The preliminary experiment suggested that mitragynine was eluted at 9.60 min when isocratic elution with 5 mM Na_2HPO_4, pH 6.0: acetonitrile; 35:65 at a flow rate of 1 mL/min. By this isocratic elution, mitragynine was eluted at 9.6 min.

System 3: Analysis of mitragynine, paynantheine, speciogynine

HPLC condition was C18 column and mobile phase was acetonitrile: 20 mM CH_3COONH_4 (pH 6) (65: 35), flow rate 1 mL/min, injection volume 20 µL and UV detector 225 nm. The compounds were eluted with an isocratic condition and detected at

225 nm. The retention times of speciogynine, paynantheine and mitragynine are 9.2, 10.3 and 14 min, respectively.

2.2 Alkaloid production in the shoot and plant cultures

Plant culture is a mimic of the intact plant but grown in vitro culture. On the other hand, shoot culture contained stem and leaf, carried the accumulation site of alkaloids. Thus, alkaloid profile accumulation is found only in the shoot and plant cultures (Fig. 3.8). Table 3.2-3.3 summarizes the number of alkaloids and metabolites. Metabolites accumulation profile in plant culture revealed the storage site of secologanin and mitragynine mostly in leaves and following in stem. Secologanin presences in a higher amount than mitragynine in leaves. It has been reported that tryptamine is a limiting factor in mitragynine biosynthesis (Charoonratana et al. 2013). It can be noted that plant and shoot cultures are ready for further manipulations.

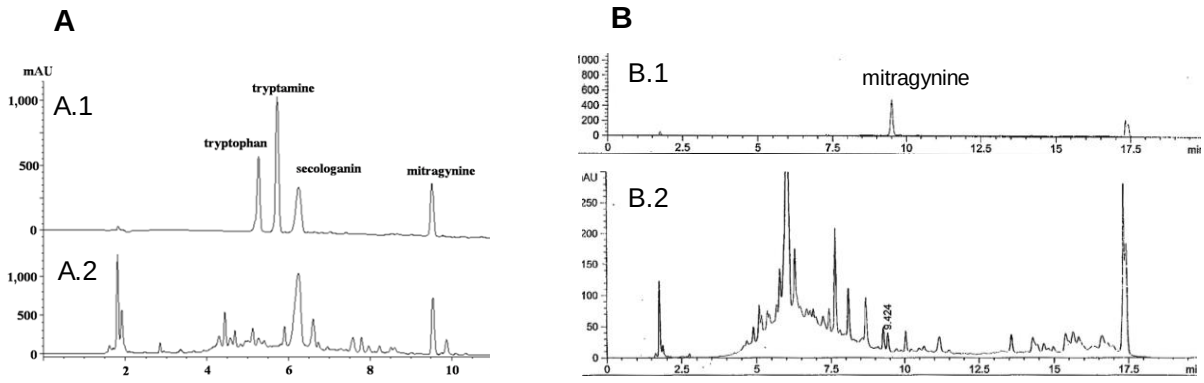


Figure 3.8 HPLC profile of mitragynine and secologanin [system 1].
Lower panel: A. in plant culture B. in shoot culture

Table 3.2 Contents of the metabolites in *M. speciosa* determined by HPLC.

Metabolite	Content (mg/g dry weight)[a]		
	Leaves	Roots	Stems
Plant culture			
Tryptophan	n.d. [b]	n.d.	n.d.
Tryptamine	n.d.	n.d.	n.d.
Secologanin	9.36 ± 0.23	4.11 ± 0.29	4.11 ± 0.24
Mitragynine	3.96 ± 0.21	n.d.	0.78 ± 0.07
Shoot culture	Shoot and stem		
Mitragynine	0.02 ± 0.00		

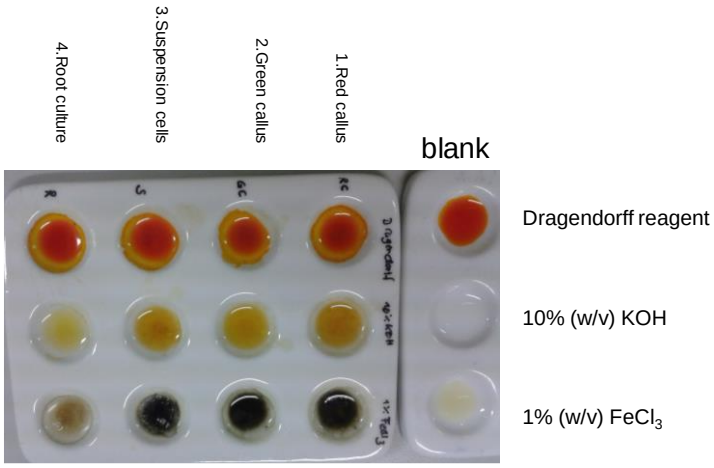
[a] Mean values ± standard deviation; [b] not determined

2.3 Callus and suspension cultures: evaluation of metabolite production

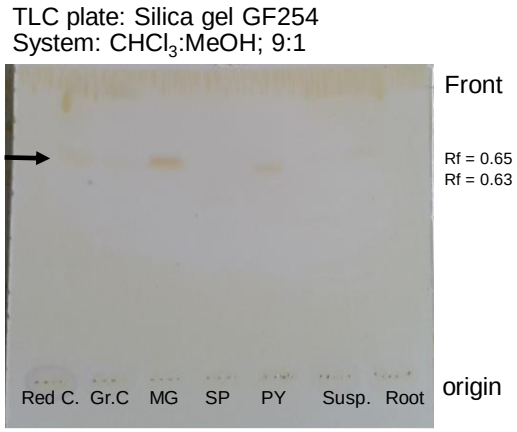
To evaluate the potential of secondary metabolite production by plant cell cultures. Callus (red and green calluses), suspension, root cultures were harvested and extracted with methanol using sonicator bath at 60°C for 30 min. The mixture was centrifuged at 7,000 rpm for 10 min. The supernatant was applied to the separation system. Screening test for chemicals was also investigated.

1. Using chemical test: Since *M. speciosa* has been reported to accumulate several types of compounds such as tannins, flavonoids, alkaloids, etc. We used general reagent to test those mentioned compound.

Preliminary screening indicated that callus (red and green) and suspension culture did not accumulate any alkaloids (negative to Dragendorff reagent). Phenolic compounds were found to the present in all types of cultures (positive to ferric chloride). Attempts of alkaloids producing cell cultures are required.



2. Using TLC: The methanol extracts were applied on silica gel GF254 and eluted with chloroform: methanol; 9:1. The TLC plate was visualized under UV and color was developed after spraying with Dragendorff reagent. Authentics (MG: mitragynine; PY: paynantheine; SP: speciogynine) were used as markers.



3. Using HPLC: The methanol extract (20 µL) was subjected to HPLC system (Shimadzu) equipped with UV-detector (225 nm) and the column was Vertisep^{TM} UPS C18 column (4.6 mm x 250 mm; 5 µm). The column was isocratically eluted with acetonitrile: 20 mM ammonium acetate (pH 6) (65:35) [system 3] at the flow rate of 1 mL/min.

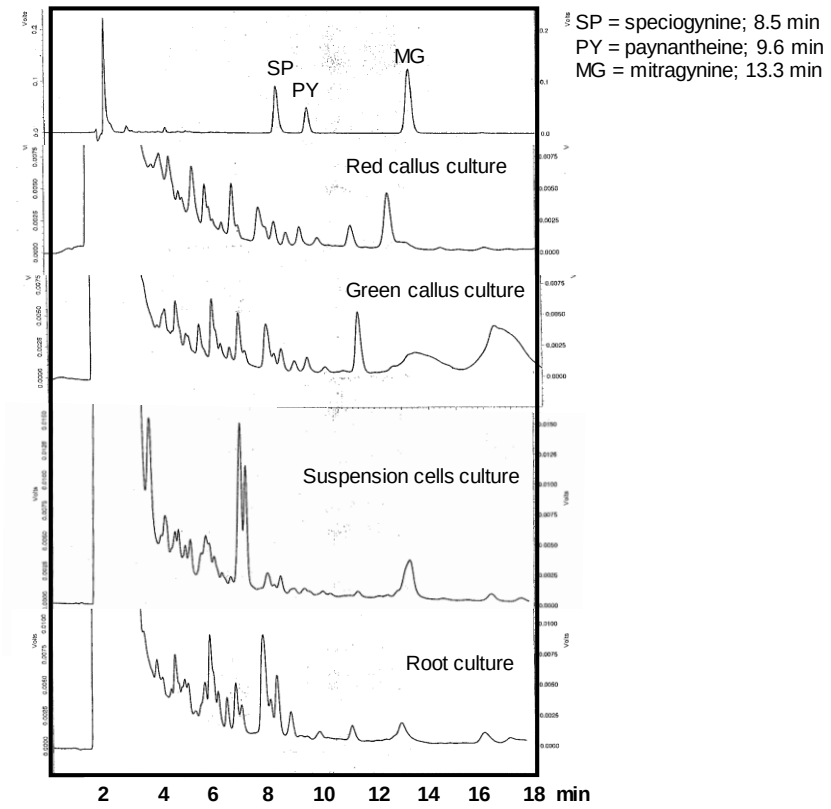


Figure 3.9 The HPLC chromatograms of the extract from plant and tissue cultures of kratom.

3. Medium manipulation in kratom callus culture using Plackett-Burman design

Randomly selected the variable factors including carbon sources (glucose, sucrose), precursors (tryptamine, sodium acetate) and plant growth regulators (2,4-dichlorophenoxyacetic acid-2,4-D, naphthaleneacetic acid-NAA, kinetin, benzyladenine-BA) were investigated. Table 3.3-3.4 summarizes the additional formula in WPM medium. 1-Month old culture (2 g) was inoculated on the WPM supplemented with variable factors. Total experiments were 12 runs. The cultures were incubated for 6 weeks and harvested. After six weeks, callus cultures appeared as dark callus, not healthy and death. The experiment was done in five replicates. Fresh weight was recorded. Callus cells were lyophilized and the crude methanol extract was prepared. Patterns of the chemical profile were evaluated using TLC, HPLC [system 1 and 3].

Table 3.3 Levels of the variables, investigated in the Plackett-Burman (PB) design

Level	Factor	Units	Minimum	Maximum
A	Sucrose	g/L	10	30
B	Glucose	g/L	10	30
C	Tryptamine	mM	0.2	0.6
D	Na acetate	mM	0.2	0.6
E	2,4-D	mg/L	1	5
F	NAA	mg/L	1	5
G	Kinetin	mg/L	1	5
H	BA	mg/L	1	5

Table 3.4 Experimental design and results

variable	unit	Run #1	Run #2	Run #3	Run #4	Run #5	Run #6	Run #7	Run #8	Run #9	Run #10	Run #11	Run #12
sucrose	g/L	10	10	30	10	30	10	30	30	30	10	10	30
glucose	g/L	30	30	10	30	10	10	30	10	30	10	10	30
tryptamine	M	0.6	0.2	0.6	0.6	0.6	0.2	0.6	0.2	0.2	0.6	0.2	0.2
Na acetate	M	0.2	0.6	0.2	0.6	0.6	0.6	0.2	0.2	0.2	0.2	0.2	0.6
2,4-D	mg/L	5	5	1	1	5	1	1	5	1	5	1	5
NAA	mg/L	5	1	5	1	1	5	1	1	5	5	1	5
kinetin	mg/L	5	5	5	1	1	5	5	5	1	1	1	1
BA	mg/L	1	5	5	5	1	1	1	5	1	5	1	1
Fresh weight	g	1.808	1.974	2.090	2.036	1.992	1.966	2.026	1.956	2.072	1.940	3.120	2.106
Peak at 5.12 min (x 10^5)	auc	1.61	0.11	1.99	0.48	0.24	1.33	0.11	0.78	1.65	3.37	0.05	1.27

Results

8 Variables were designed due to check the affluence on the callus growth and the alkaloid production. Fresh weight was recorded. Callus grown in Run 11 medium grew better than callus in other media. However, the fresh weight of callus was still low, only 1.5 x of the starting cells (Fig.3.10A).

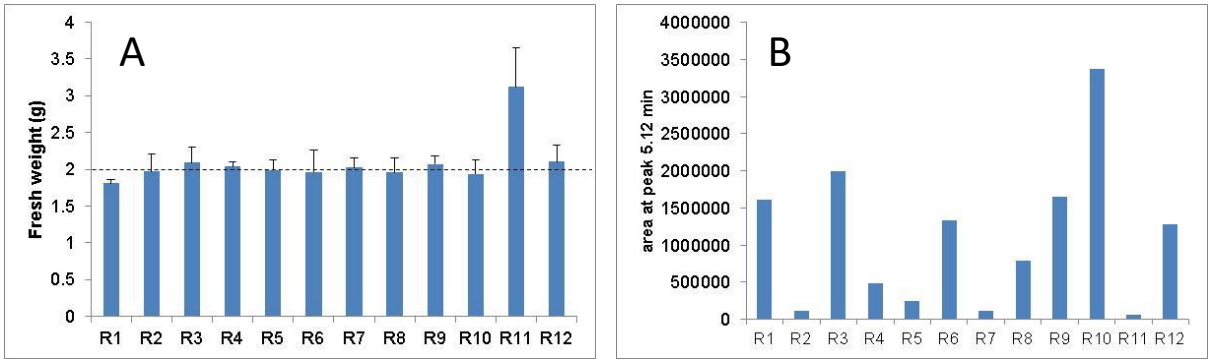


Figure 3.10 Results of experiments.
A: fresh weight and B: Area under the curve (AUC) of the peak at 5.12 min of HPLC [system 3]

Callus cultures under PB design (total 12 runs) were harvested, dried using freeze-dryer and ground. Powder (100 mg) was extracted with methanol (0.5 mL) using sonicator (75 °C for 30 min) and centrifuged (7,000 rpm for 10 min). The supernatant was taken and preliminary screening the presence of mitragynine, secologanin and catechin on TLC was investigated. Under mobile phase elution, none of the tested compounds was a presence in the extract (Fig. 3.11).

Attempt to see the HPLC chromatograms using HPLC [system 3] of the extracts, as shown in Fig. 3.12, comparison with authentic revealed that the extracts of manipulated callus cultures did not accumulate any of them. Nevertheless, the profile of chemicals is not similar especially peaks at 5.12 min and 9 min. The area under the curve of the peak at 5.12 min was integrated and plotted as shown in Fig. 3.8B). No information could be obtained from statistical PB analysis of those data. Changing the elution with mobile phase to system 1, HPLC chromatograms were detected under UV 220 nm for tryptamine, mitragynine and UV 238 nm for secologanin. No tryptamine and (again) mitragynine could be detected. The peak at 10.9 min, which is similar to the retention time of secologanin, was considered. Based on reasons of absorption at 238 nm and UV spectra suggested that peak at 10.9 min was not secologanin. In conclusion, the PB design of eight variables failed to stimulate the callus culture to produce the targets!

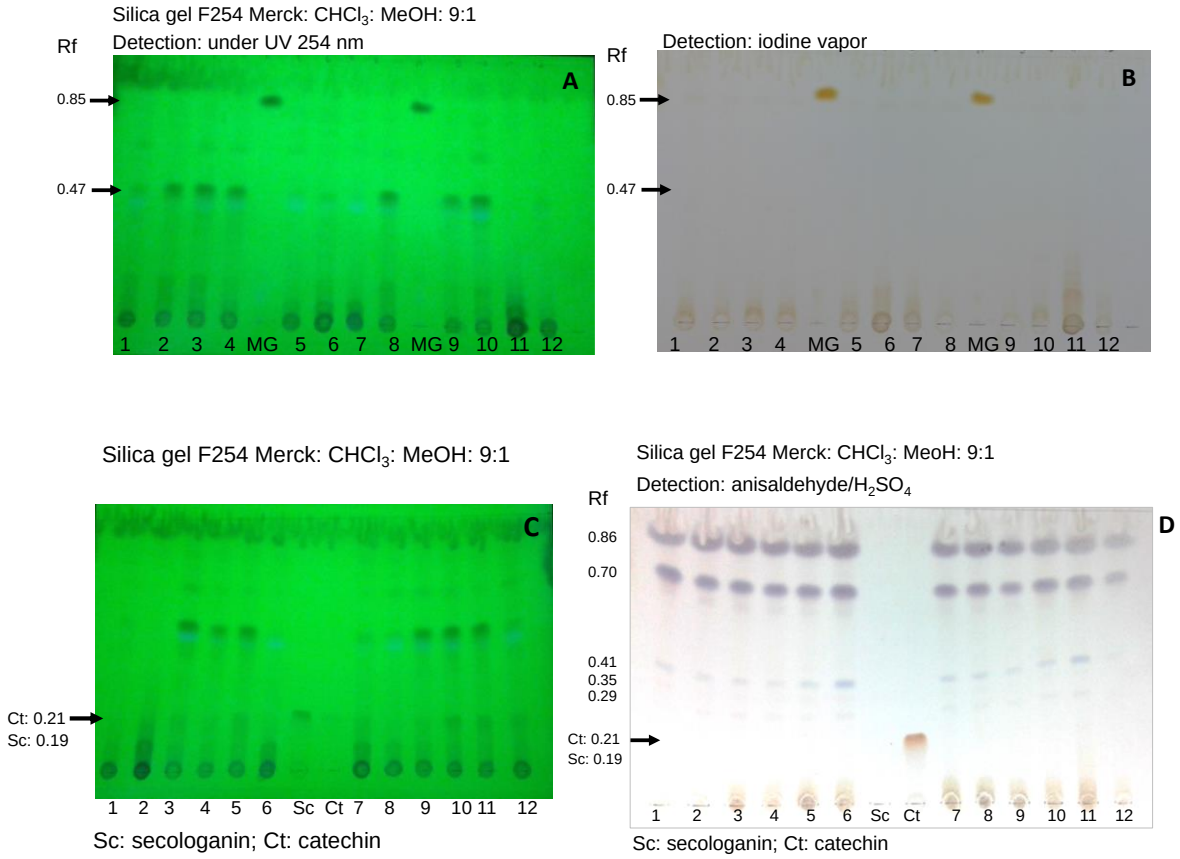


Figure 3.11 TLC fingerprint of methanol extracts from PB designs detected under UV 254 (A & C), iodine vapor (B) and anisaldehyde/H_2SO_4

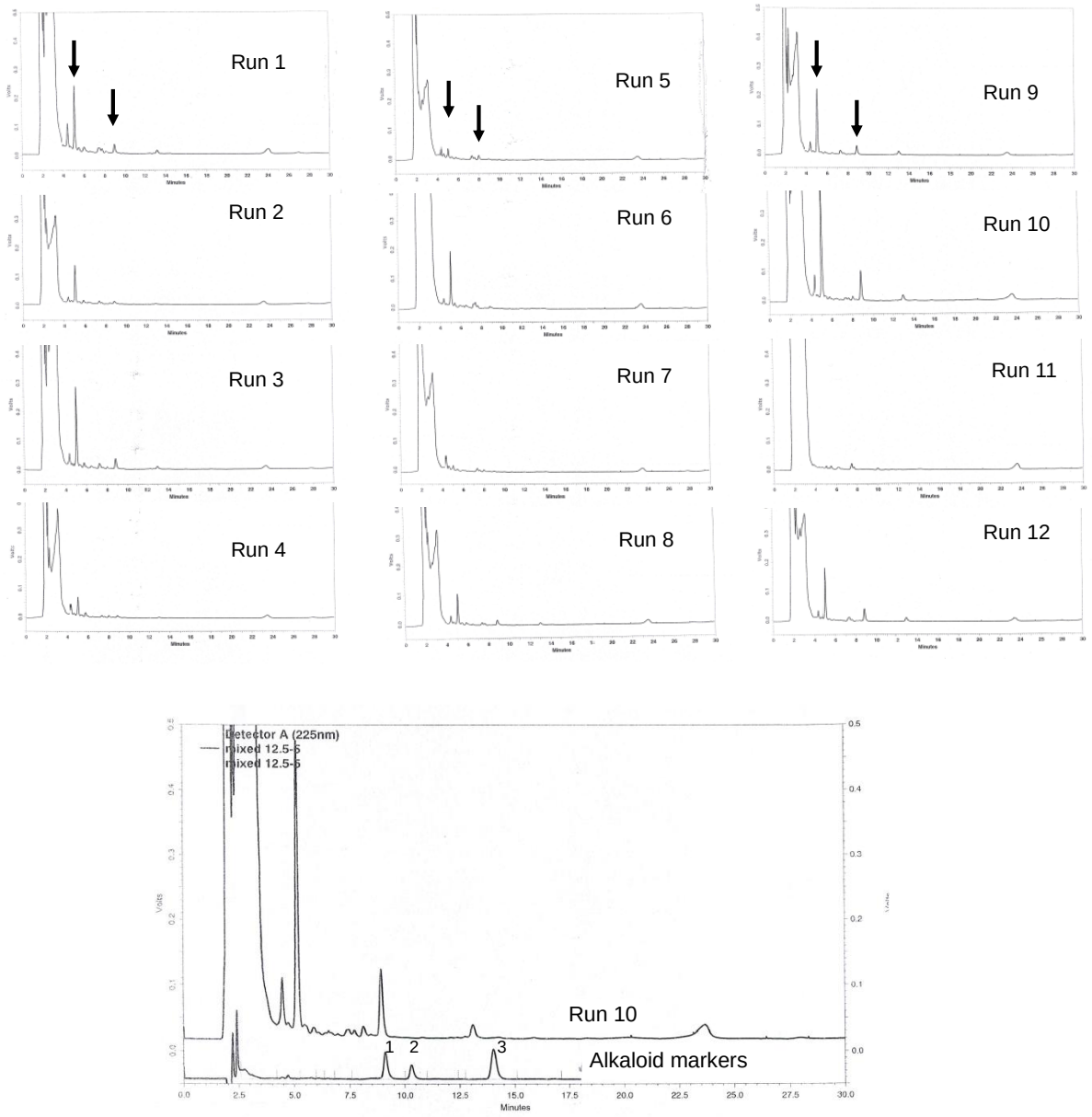


Figure 3.12 HPLC chromatograms of methanol extracts from 12 runs of PB design [system 3] Alkaloid markers: 1= speciogynine at 9.2 min; 2 = paynantheine at 10.3 min; 3 = mitragynine at 14 min

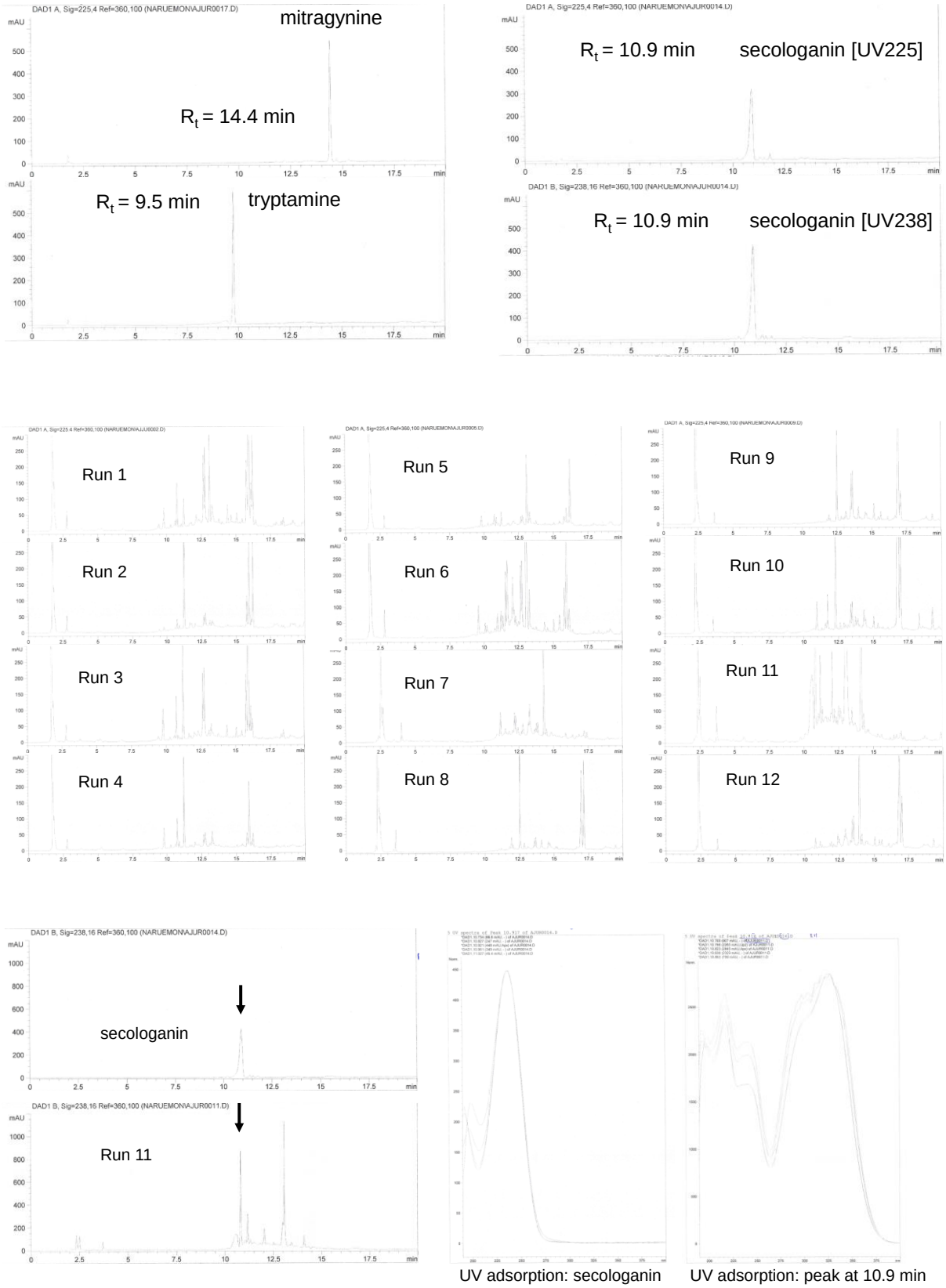


Figure 3.13 HPLC chromatograms of methanol extracts from 12 runs of PB design [system 1]

4. Medium manipulation of kratom callus culture

4.1 Manipulation of plant growth regulators

We could establish the callus and suspension cultures on WPM supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 20 g/L sucrose. Nevertheless, callus and suspension cells did not accumulate any alkaloid (as we expected) although cells growth was very well. The first strategy to enhance the production of secondary metabolite in the callus culture was a manipulation of plant growth regulator. Since the 2,4-D could only proliferate the growth of callus but not for the production. Therefore, we tried to manipulate the plant growth regulators to avoid 2,4-D in the medium. α-Naphthaleneacetic acid (NAA), benzyladenine (BA) and thidiazuron (TDZ) at 1 mg/L of each were selected. Three combinations were including:-

1) 1 mg/L NAA and 1 mg/L TDZ
2) 1 mg/L NAA and 1 mg/L BA
3) 1 mg/L TDZ, 1 mg/L BA and 1 mg/L NAA

One gram of fresh callus was inoculated. The experiment was done in 20-replicates. The cultures were incubated at 16/8 h light/dark cycle.

Fig. 3.14 shows the appearance of callus (1-month old) after three passages. This result indicates that the fastest growth of callus was on WPM medium plus 1 mg/L 2,4-D. Friable callus and pale yellow were obtained. On the other hand, a callus on WPM medium plus 1 mg/L NAA and 1 mg/L TDZ found to grow slower but cells were green and friable. In addition, callus on 1 mg/L NAA and 1 mg/L BA or 1 mg/L TDZ, 1 mg/L BA and 1 mg/L NAA looks not so healthy. From these results, the suitable medium for growing the cells is WPM medium plus 1 mg/L 2,4-D.

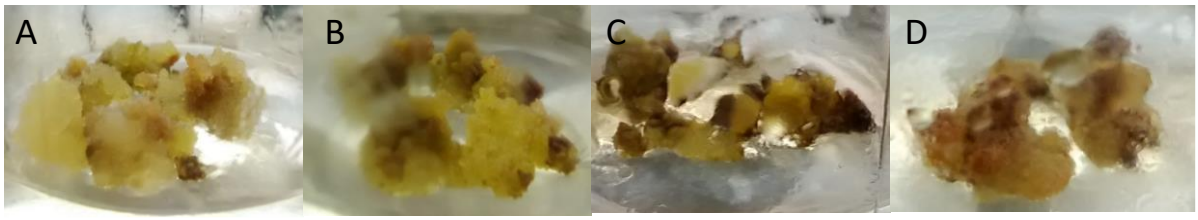


Figure 3.14 One-month-old of *M. speciosa* callus culture.
A. on 1 mg/L 2,4-D; B. on 1 mg/L NAA and 1 mg/L TDZ; C. on 1 mg/L NAA and 1 mg/L BA; D. on 1 mg/L NAA, 1 mg/L TDZ, 1 mg/L BA in the WPM supplemented with 20 g/L sucrose.

The culture cycle of callus culture on the WPM supplemented with 1 mg/L NAA and 1 mg/L TDZ was about 1 month. Callus cells were green and compact (Fig. 3.15). This callus was then evaluated for secondary metabolite production.

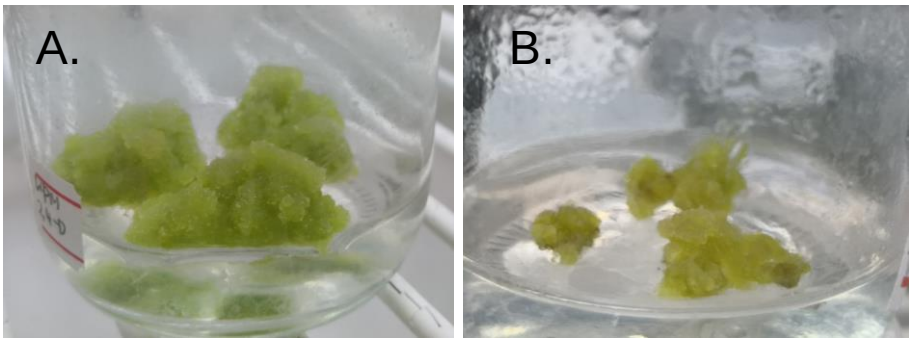


Figure 3.15 The appearance of callus cultures after several passages.
A. on WPM plus 1 mg/L 2,4-D; B. on WPM plus 1 mg/L NAA and 1 mg/L TDZ.

4.2 Screening of metabolites in callus cultures

Callus cultures in 4.1 were evaluated for metabolite production. Cells of one-month-old callus were harvested. Cells were dried at 50°C for overnight and ground. The powder (100 mg) was macerated in methanol for 20 min and sonicated for 10 min. The mixture was refluxed at 70°C for 30 min and filtered. The filtrate was evaporated to dryness. The residue was re-dissolved in 5 mL of methanol. Fig. 3.16 indicates that callus culture, grown in both types of medium, was unable to produce mitragynine. In contrast, both calluses produced ursolic acid either in control culture or elicited with methyl jasmonate (MJ) and/or tryptamine addition.

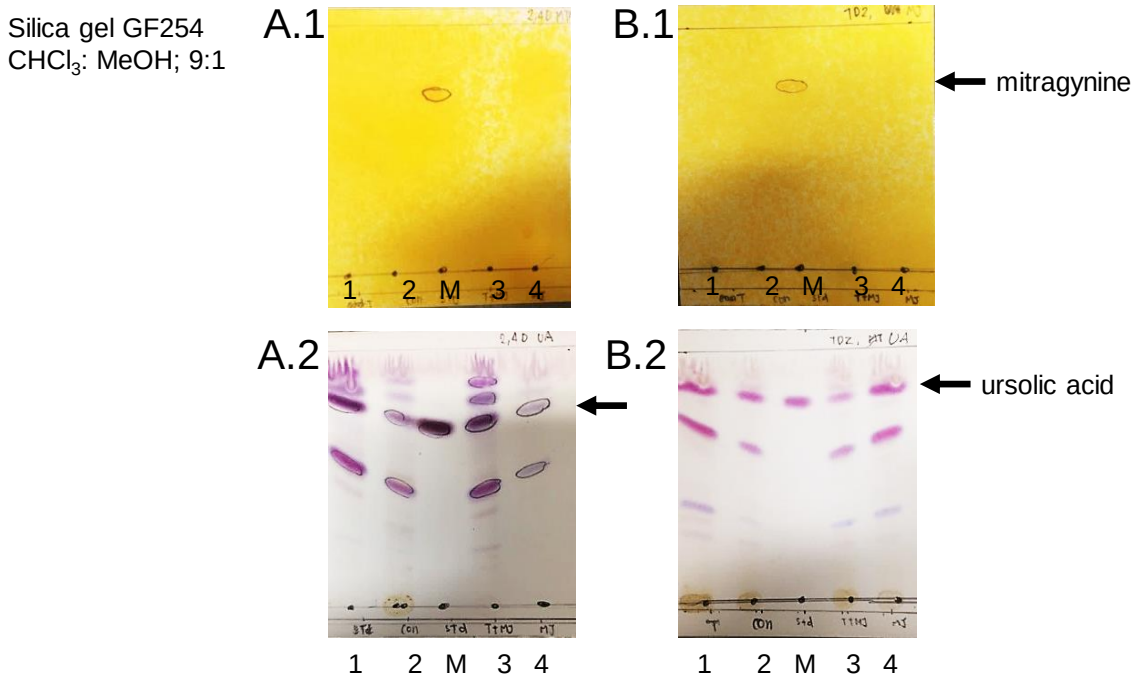


Figure 3.16 TLC chromatograms of the extracts of callus cultures. A. of 1 mg/L 2,4-D and B. of 1 mg/L NAA and 1 mg/L TDZ.
A.1, B.1 screening of mitragynine after spraying with Dragendorff reagent
A.2, B.2 screening of ursolic acid after spraying with anisaldehyde/H_2SO_4 reagent

4.3 Effect of light on the growth of callus culture

Later, we tried to incubate the callus culture under lights (red and blue) is compared with the dark condition. Based on the hypothesis that light with different wavelength probably promoted the cell growth. The result as shown in Fig.3.17 revealed that blue light could promote growth better than red and dark. Nevertheless, it is still slower than incubation under fluorescence.

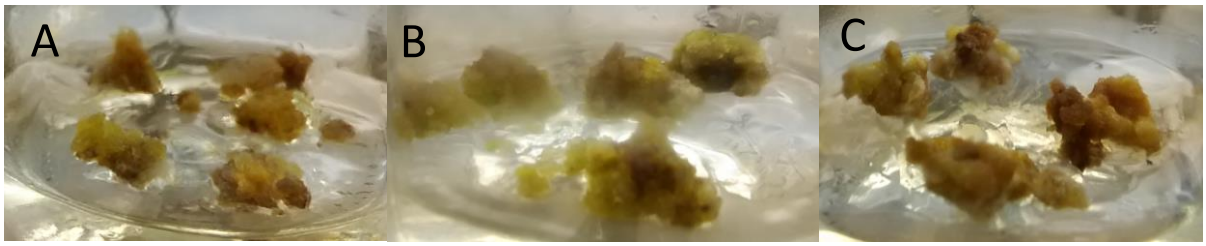


Figure 3.17 1-Month old *M. speciosa* callus culture on 1 mg/L 2,4-D in WPM supplemented with 20 g/L sucrose. A. under dark condition; B. under blue light; C. under red light.

For callus that grown on WPM plus 1 mg/L NAA and 1 mg/L TDZ, lights including white, red and blue and dark were applied to the callus cultures. Calluses were harvested after light treatment for 10 and 15 days. Fig. 3.18 is a summary of the response of callus to light.

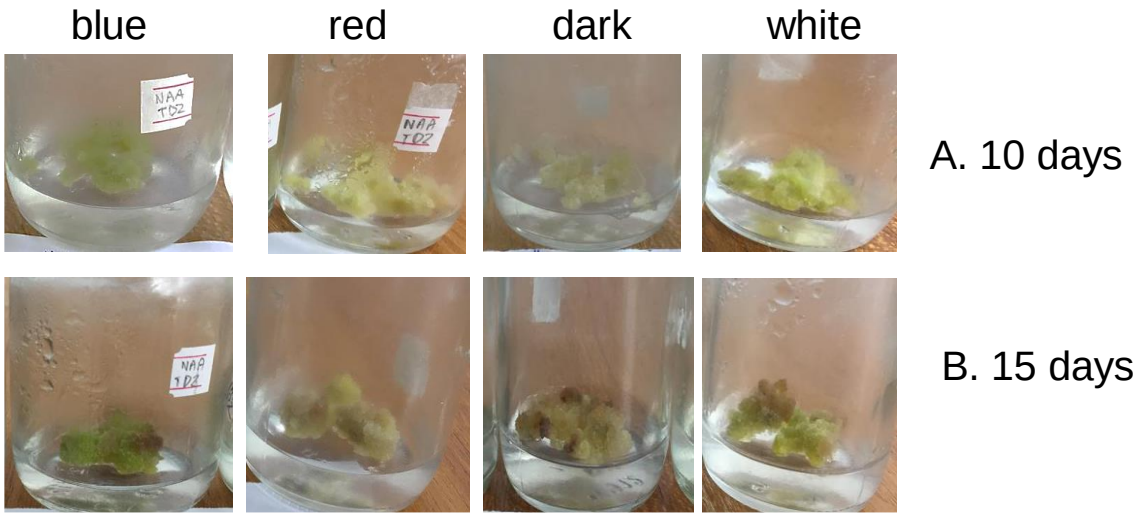


Figure 3.18 The appearance of callus cultures after lights treatment. A for 10 days; B. for 15 days

4.4 Elicitation of kratom suspension cultures

The callus culture on WPM plus 1 mg/L NAA and 1 mg/L TDZ was used as a material for a study on elicitors on ursolic acid production. Firstly, the suspension culture was established. After three passages of subculture, the suspension culture was ready for elicitation. Methyl jasmonate (MJ) and yeast extract (YE) were used as elicitors. Inoculation of fresh cells was 1 g. Experiments were designed to add elicitor on the 12th day of culture and treated for 1, 2, and 3 days. The concentrations of MJ were 50, 100, 200 µM and of YE were 0.5, 1, 2 mg/mL. After treatment, cells were harvested and freeze-dried. Dry weight was recorded. The extract was prepared and analyzed for the amount of ursolic acid.

One hundred mg of powder was refluxed with methanol (10 mL) as mentioned above. Re-dissolved the residue with methanol and centrifuged. The clear supernatant was collected and subjected to HPLC analysis (Phongprueksapattana et al. 2008). An HPLC system (Agilent 1100

Series LC System, Agilent Technologies, Wilmington, USA) was equipped with a C18 reverse phase column (4.6 x 250 nm, 5 µm, Verticep^{TM}) and a UV detector (photodiode array) set at 206 nm. Ursolic acid was eluted isocratically with acetonitrile/0.1% (v/v) H_3PO_4 in water (70: 30, v/v) with a flow rate of 1 ml/min at 14.1 min. A calibration curve of authentic ursolic acid (Sigma-Aldrich) was established. The linearity of the calibration curve was observed in the range 7.5-240 μg/mL with r^2 of 0.9999 (%RSD of 0.09-0.45%). Each calibration point was established in triplicate.

The resulting HPLC chromatograms of ursolic acid is summarized as shown in Fig. 3.19. The chromatographic profile indicated the different chemical accumulation in both types of media. The in the front peak of ursolic acid was expected to be oleanolic acid, an isoform of ursolic acid.

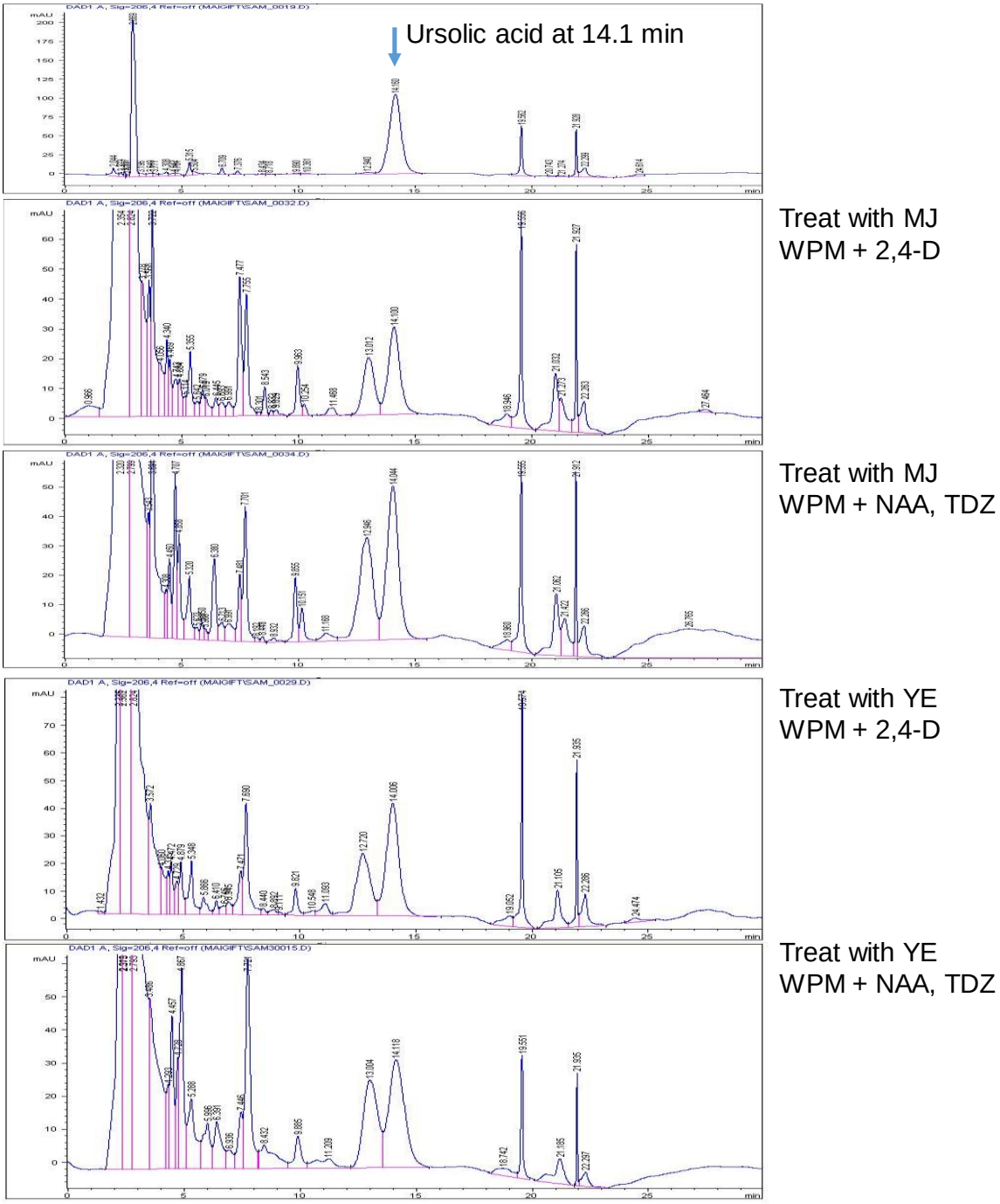


Figure 3.19 The HPLC chromatograms of the extracts from kratom suspension cells.

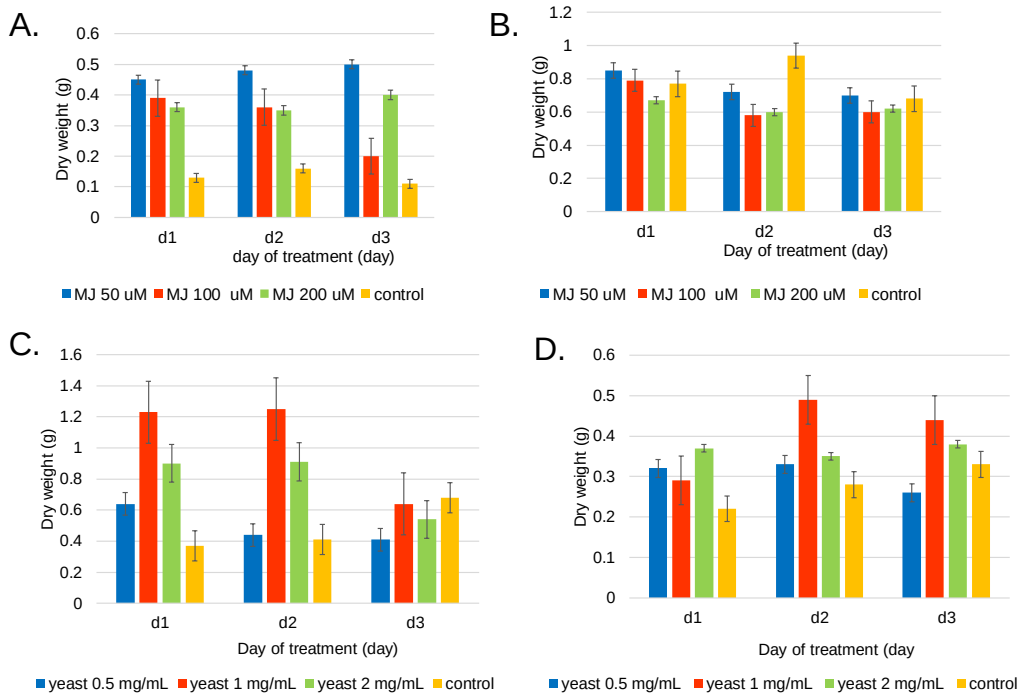


Figure 3.20 Effect of elicitors on the growth of kratom suspension cultures.
A and C: cells grew in the WPM plus 2,4-D and
B and D: cells grew in the WPM plus 1 mg/L NAA and 1 mg/L TDZ.

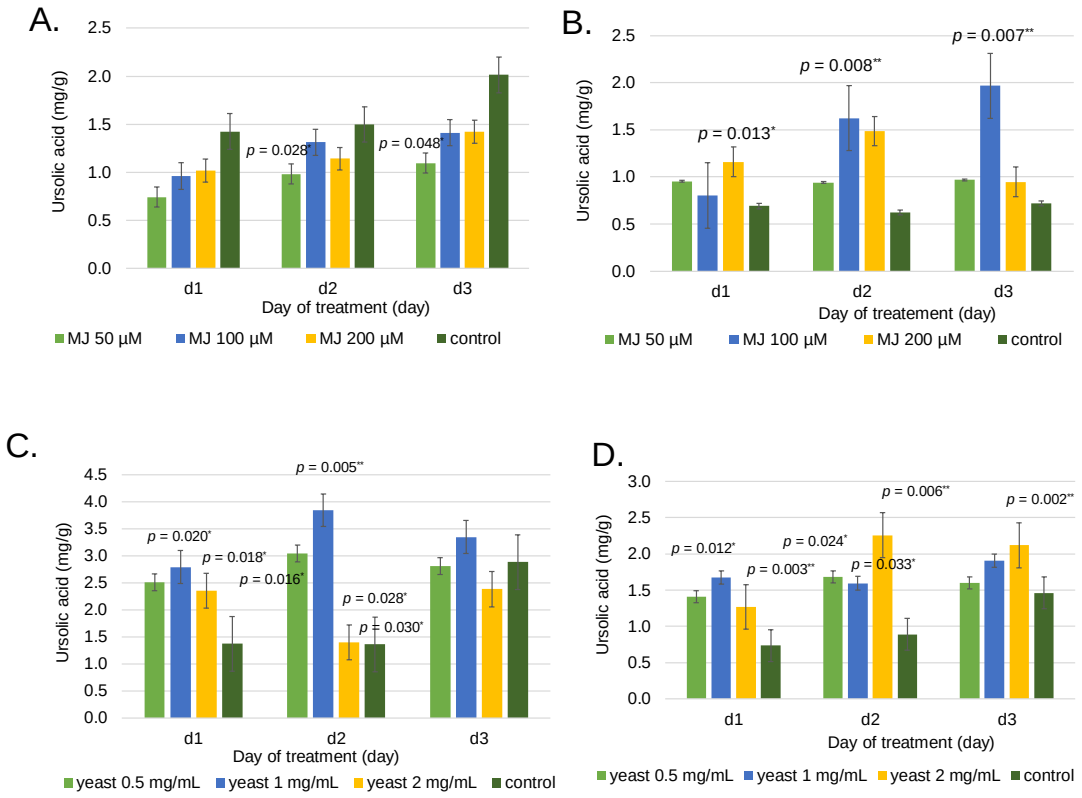


Figure 3.21 Effect of elicitors on ursolic acid production of kratom suspension cultures.
A and C: cells grew on the WPM plus 2,4-D and
B and D: cells grew on the WPM plus 1 mg/L NAA and 1 mg/L TDZ.

Fig. 3.18 summarized the cell suspension growth in two types of medium. Cells still grew very well in 2,4-D supplemented WPM medium.

Elicitation the suspension culture, grown on WPM plus 2,4-D, with YE indicated that YE stimulated ursolic acid production at all treated concentrations. Especially YE 1 mg/mL and treated for 2 days, amount of ursolic acid was 3.846 ± 0.690 mg/g DW when compared to the control of 1.361 ± 0.118 mg/g DW (about 2.8 folds). In contrast, MJ seems not to affect the ursolic production and at test concentrations-, MJ inhibited the production of ursolic acid. Interestingly, suspension cultures, grown on WPM plus 1 mg/L NAA and 1 mg/L TDZ, could enhance the ursolic acid production after YE elicitation. Treated with MJ at 100 µM enhanced the ursolic acid production during elicitation. The amount of ursolic acid increased from 0.719 ± 0.166 mg/g DW in control culture to 1.967 ± 0.199 mg/g DW after 3 days of treatment (about 3.7 folds). On the other hand, elicitation of suspension cell culture with YE promoted ursolic acid production. At day 2 of treatment, ursolic acid production increased from 0.888 ± 0.098 mg/g DW in control culture to 2.257 ± 0.252 mg/g DW at YE 2 mg/ml (about 2.5 folds). In summary, the established callus and suspension cultures in WPM supplemented with 1 mg/L NAA and 1 mg/L TDZ has a potential for further biosynthetic study.

4.5 Elicitation the callus culture with yeast extract

The callus culture that maintained in the WPM supplemented with 1 mg/L NAA and 1 mg/L TDZ was further elicited with YE in order to evaluate the potential of mitragynine production. Inoculation of 0.3 g fresh weight was done during subculture. The callus culture was incubated at 25± 2 °C under white fluorescence for 16 h. After two weeks of subculture, the callus was moved to the solutions containing YE 0.5, 1.0 and 2.0 mg/mL. Cells were elicited for 1, 2 and 3 days, harvested and freeze-dried. During treatment, callus secreted color to the solution and intensify from day 1 to day 3 of treatment. Detection of mitragynine was performed using LC-MS/MS. Cells were extracted with methanol and subjected to the LC system. The HPLC chromatogram and the fragmentation are shown in Fig. 3.23. The preliminary result in YE treatment in callus culture is summary in Fig. 3.22.

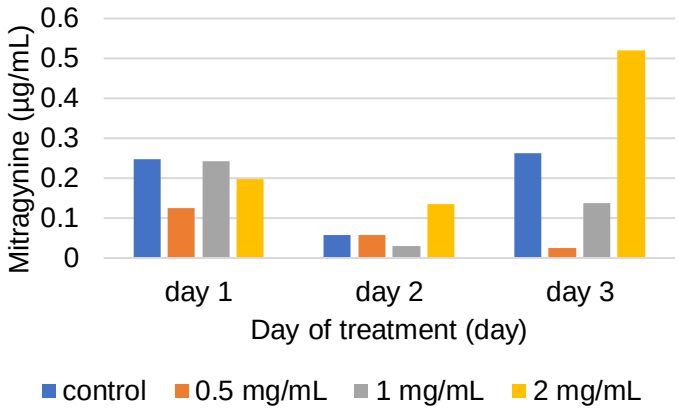
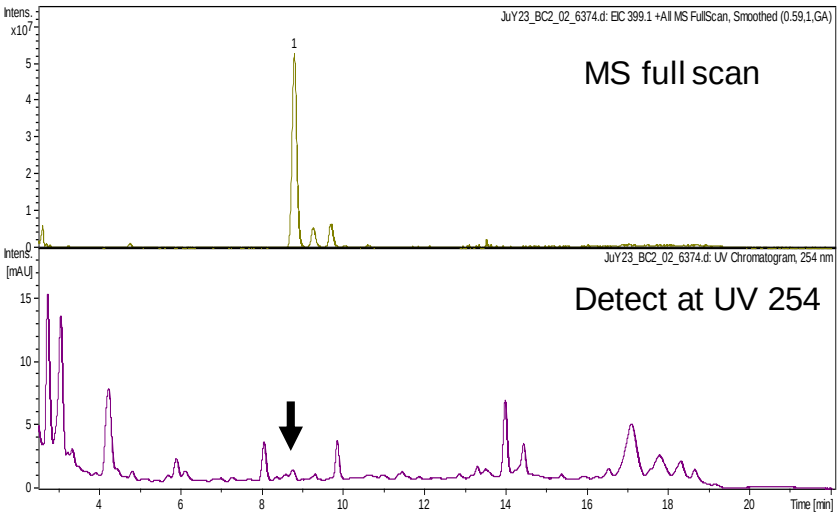


Figure 3.22 Preliminary result of mitragynine accumulation in the callus culture, grown on the WPM medium supplemented with 1 mg/L NAA and 1 mg/L TDZ after elicitation with yeast extract.

A. MS and UV chromatogram (for comparing)



B.

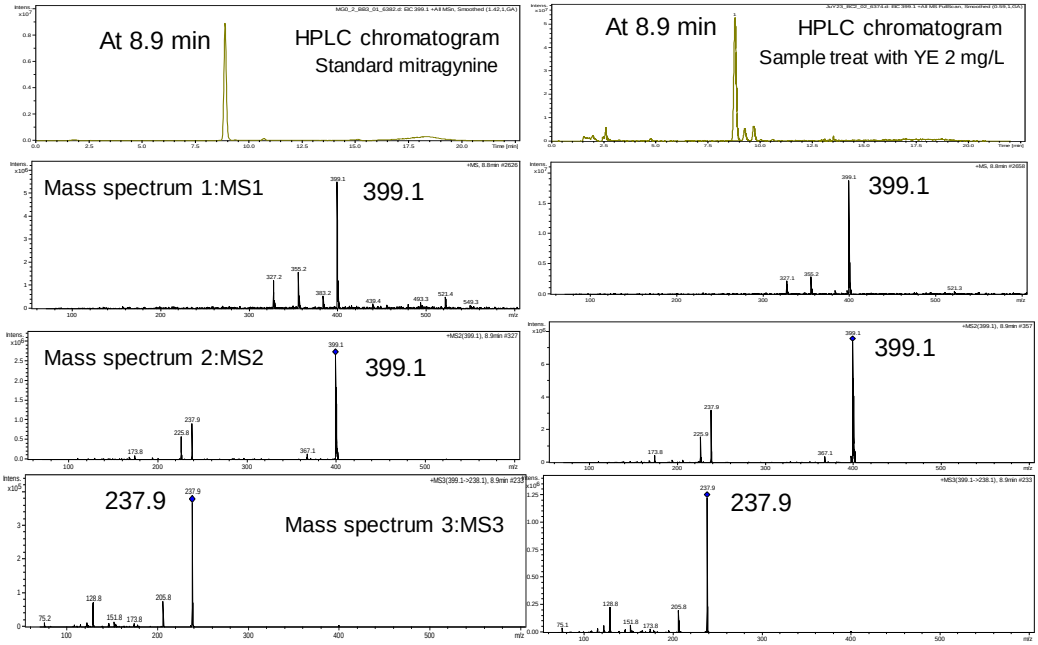


Figure 3.23 LC-MS/MS chromatogram of the extract from the callus culture grown in WPM medium supplemented with 1 mg/L NAA and 1 mg/L TDZ after elicited with yeast extract (2 mg/L) for 3 days. A. comparison HPLC chromatograms of UV detection and MS full scan mode; B. mass fragmentation of peak at 8.9 min of mitragynine standard and from the sample.

5. Kratom shoot culture

In the present study, we established a protocol for shoot multiplication and micropropagation that enables the production of a valuable opioid analgesic from the red-veined *M. speciosa* variety and grown in a completely controlled environment. The plant hormone-like elicitors such as jasmonic acid, abscisic acid, and salicylic acid, were added to *M. speciosa* shoot culture. The concentration and time of exposure of each elicitor were statistically designed using response surface methodology. The ability of the elicited shoot culture in mitragynine production was determined.

5.1 Micropropagation

5.1.1 Plant culture Sterilized *M. speciosa* plants grown *in vitro* were subcultured and maintained in the hormone-free McCown woody plant (WPM) medium according to Phongprueksapattana *et al*. (2008). A two-month-old plant that bore at least three axillary buds used as the starting materials. The axillary buds at the 2^{nd}-3^{rd} position from the top were placed on the hormone-free WPM and incubated at 25±2°C under fluorescence light (3000 lux) for 16 h/day. This protocol served the plantlet for further shoot initiation and proliferation experiments.

5.1.2 Shoot initiation and proliferation Axillary buds were excised from the stems (about 0.5 cm long) and placed vertically on the WPM solid medium supplemented with different thidiazuron (TDZ) concentrations of 0.1, 0.5, 1 or 2 mg/L. The shoot culture was incubated on shelves under a fluorescence light for 16 h/8 h dark cycle period and a temperature of 25±2°C. Using the TDZ optimum concentration of 2 mg/L, various benzyladenine (BA) and kinetin (KN) concentrations of 1, 5 or 10 mg/L was added. After 6 weeks, a number of shoot and shoot length were recorded. Samples were prepared and recorded for at least 12 replicates. Shoot culture that was grown in WPM liquid medium (30 mL) were used for elicitor treatments.

5.1.3 Rooting and acclimatization Regenerated young shoots (about 1 cm in length) were transferred to hormone-free WPM containing 2% (w/v) sucrose and 0.8% (w/v) plant agar. Different types of auxins including 3-indole butyric acid (IBA), 1- naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) at concentrations of 1, 5 or 10 mg/L was added. Shoots, that grew in hormone-free WPM, were used as controls. The samples were performed in 15 replicates. The morphology and characteristic of the roots were photographed and comparison made with the control culture after 6 weeks of culture. The complete plantlets (2-month old) were removed from the culture medium. Roots were gently but thoroughly washed with running tap water to ensure removal of agar. Plantlets were acclimatized by transferring to pots (diameter 20 cm) containing sterilized soils. To control humidity, a plastic bag was used to cover pot-soil containing plant for 1 week. The plastic bag was gradually opened and plants were watered every day. The pots were transferred to a greenhouse. After 2 months of growth in the potting soil, the percentage survival rate during the acclimatization process was calculated.

5.2 Elicitation with plant growth regulators designed with central composite design

5.2.1 Experiment design and statistical analysis *M. speciosa* shoot cultures were elicited with different concentrations and time exposures. The optimization was performed using response surface methodology (RSM) with the central composite design (CCD). The ranges of concentrations of elicitors were 0-100 μM for jasmonic acid (JA) and abscisic acid (ABA) and 0-500 μM for salicylic acid (SA) and the ranges of time exposure were 0-48 h. The experiment design, data analysis, and optimization procedures were conducted using Design-Expert Version 8.0.6 software (Trial version; www.statease.com; Stat-Ease, Inc., Minneapolis, USA). The CCD designed with 2 variables with the quadratic model and 3 center points, afforded 11 experiments in total (Table 3.5). The mitragynine production was chosen as a response. The surface response plots were generated after made data input and analyzed to reveal the effect of elicitor on

mitragynine production. Analysis of variance (ANOVA) for evaluation of second-order response surface model was performed and the regression equations were suggested.

5.2.2 Elicitors treatments Jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) were elicitors in this study. Stock solutions of each elicitor were prepared and add into the medium on day 14th of culture (at exponential phase). For jasmonic acid and abscisic acid, the concentrations were ranging from 0-100 µM. For salicylic acid, the concentrations were ranging from 0-500 µM. Time exposures to elicitor were ranging from 0-48 h. Shoot culture was incubated at 25±2°C on a shaker (60 rpm). After treatments, shoots were harvested, washed with distilled water, and lyophilized.

5.2.3 Mitragynine content Freeze-dried *M. speciosa* (50 mg) were extracted by refluxing with methanol (10 mL) at 70°C for 1h. The extracts were filtered, then washed with petroleum ether twice, and concentrated under reduced pressure. For determination of mitragynine content, HPLC analysis was carried out using Agilent 1100 series equipped with Photo-diode arrays detector. Data analysis was performed using Agilent software (USA). Chromatographic separation was performed on a reverse phase VertiSep^{TM} UPS C18 column × 4.6 mm i.d. 5 µm particle size (Vertical, Bangkok, Thailand) with binary gradient mobile phase profile [1.5 mL/min, acetonitrile: 100mM H_3PO_4 in water, pH 2.4 (10:90 to 90:10, v:v within15 min, injection volume 20 µL]. The identification of mitragynine based on the retention time and comparison of the absorption spectrum with authentic standards. The quantitative wavelength was set at 225 nm. Samples were analyzed in triplicate.

5.2.4 Statistical analysis Data presented as mean value ± standard deviation (SD) and analyzed using SPSS Version 14.0 (SPSS Inc., Chicago, USA). The mean values compared using Duncan's multiple range tests at the 5% level of significance ($P < 0.05$).

Results and discussion

Shoot multiplication and micropropagation *In vitro* propagation strategy began with preparations of plant materials. Seeds of *M. speciosa* red-veined variety were collected from natural sources in Southern Thailand. Sterilized seedlings were prepared, and cultured in hormone-free solid WPM. After two months, the axillary buds were excised and used as explants for shoot proliferation and multiplication experiments. TDZ was tested at 0.1, 0.5, 1 and 2 mg/L in WPM for its effect on shoot formation and 2 mg/L TDZ produced the highest shoot number (Table 3.4).

Table 3.5 Effect of thidiazuron (TDZ), benzyladenine (BA) and kinetin (KN) and in combination on *in vitro* shoot proliferation of *M. speciosa* after 6 weeks of culture

Cytokinin (mg/L)			Number of shoots per explant	Shoot length (cm)
TDZ	BA	KN		
0	0	0	2.54±0.36 f	0.44±0.05 b
0.1	0	0	5.33±0.28 c	0.57±0.05 b
0.5	0	0	6.08±0.26 bc	0.49±0.05 b
1.0	0	0	4.25±0.43 d	0.87±0.18 a
2.0	0	0	6.50±0.26 b	0.48±0.05 b
2.0	1.0	0	9.58±0.63 a	0.48±0.06 b
2.0	5.0	0	4.17±0.40 d	0.33±0.07 b
2.0	5.0	0	3.00±0.52 e	0.22±0.04 bc
2.0	0	1.0	3.67±1.32 de	0.31±0.03 b
2.0	0	5.0	2.17±0.17 f	0.14±0.02 c
2.0	0	10.0	2.33±0.22 f	0.20±0.03 bc

Data represent the mean ± SD of 12 replicates. Values followed by the same letter are not significantly different at $P<0.05$ according to Duncan's multiple range test.

The effects of combinations of BA or Kn at 1, 5 and 10 mg/L with 2 mg/L TDZ on shoot multiplication were compared. TDZ at 2 mg/L and 1 mg/L BA produced the largest number of shoots of 9.58±0.63 per explant. At higher concentrations of BA and any Kn, shoot formation was inhibited (Table 3.5). Only 1 mg/L BA by itself in solid WPM produced only 2.83±1.47 shoot number per explant (Phongprueksapattana 2007) so this emphasizes the importance of TDZ in shoot multiplication. TDZ is a cytokinin-like compound, is widely used for shoot multiplication (for instance, the addition of 5 µM TDZ in Murashige and Skoog (MS) medium increased the frequency of shoot regeneration of *Rauwolfia tetraphylla* (Faisal et al. 2005). However, the best shoot culture of *M. parvifolia* was achieved in MS medium supplemented with 4.44 µM BA (Roy et al. 1998). Manipulation of different basal media such as MS, Gamborg (B5) and WPM for *M. speciosa* root cultures indicated that the WPM was an appropriate medium for hairy root cultures (Phongprueksapattana 2007). For the indole alkaloid-containing plants like *Cinchona ledgeriana* (Hamill et al. 1989) and *Ophiorrhiza pumila* hairy root cultures (Saito et al. 2007), however, required the B5 medium for growing hairy root cultures.

Usually, any rooting procedure is not required for *in vitro* root formation but using an auxin could reduce the time required for root initiation. In this study, three auxins including IBA, NAA and 2,4-D at concentrations of 1, 5, 10 mg/L were studied for inducing roots in comparison with hormone-free WPM. The appearance of roots was recorded after six weeks of culture (data not shown). Root length and root thickness are considered to be the best characteristics of roots. The root characteristic of the plantlets in WPM plus IBA at 5 mg/L produced the healthiest looking roots. Higher IBA concentrations (10 mg/L) caused inhibition of root expansion. NAA (at 1 mg/L) increased the root number but the roots looked unhealthy. NAA at concentrations of 5 mg/L and 10 mg/L inhibited induction of roots but induced callus formation. This effect was also found for 2,4-D. For the control culture on hormone-free WPM, the period of root induction needed was about 10 days of culture (Phongprueksapattana 2007) and fewer rootlets were found when compared to WPM containing IBA (5 mg/L), which took only 5 days for induction of roots.

Plants containing shoots and roots were readied to be transferred to natural conditions by an acclimatization process. Under *in vitro* culture condition, humidity changes and bacterial contamination were avoided. Gradual exposure of the plantlet to the environment was performed

to avoid lower survival rates. Acclimatization began by removing the plantlet from the culture bottle and washing the roots gently but thoroughly with water to remove any gelling agent. Sterilized soil was prepared by autoclaving and packed into the pot (20 cm diameter). The complete plant was then put into pot-soil tenderly and watered every day. Plants in pot-soil were covered with a plastic bag during the first week to prevent loss of humidity. Then the plastic bag was removed for watering. Using this acclimatization protocol the survival rate for *M. speciosa* was 60% and plants reached about 15 cm in length after 2 months (Fig. 3.24). Accumulation of mitragynine was assessed in the regenerated *M. speciosa*. Mature leaves were collected and prepared for the extract prior to HPLC analysis. The regenerated plants produced and accumulated mitragynine, in an age-dependent manner.

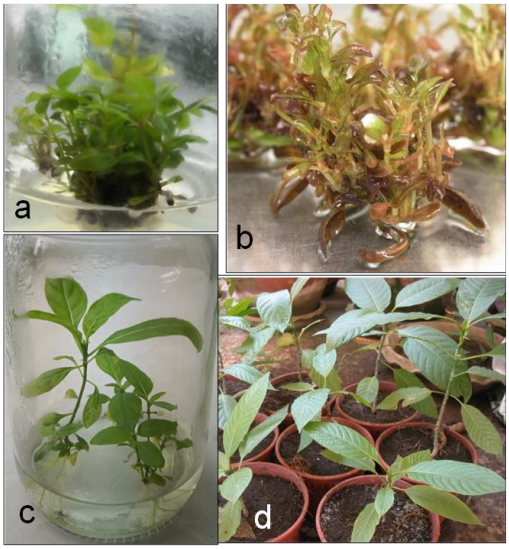


Figure 3.24 Micropropagation of *Mitragyna speciosa* (Korth.) Havil. (*a*) Shoot proliferation on WPM medium with 2 mg/L thidiazuron and 1 mg/L benzyladenine after 2 months, (*b*) Shoot culture in shoot proliferation liquid medium after 2 weeks, (*c*) Plant culture of cutting shoot on hormone-free WPM medium after 4 weeks, and (*d*) Acclimatized plant in pot soil after 3 months.

Elicitation with plant growth regulators

The shoot culture, grown in the liquid medium of WPM supplemented with 2 mg/L TDZ and 1 mg/L BA, was used as plant material for elicitation with plant growth regulators including jasmonic acid (JA), abscisic acid (ABA) and salicylic acid (SA). Using the central composite design, the range of concentration (µM) and time (h) were the variables (Table 3.6). The response of mitragynine production was measured from a shoot that elicited with plant growth regulators (Table 3.7).

The surface response plots were generated after made data input and analyzed to reveal the effect of elicitor on mitragynine production (Fig. 3.25). Analysis of variance (ANOVA) for evaluation of second-order response surface model was performed and the regression equations were suggested (Table 3.8).

Table 3.6 Operating variables and levels of elicitors including jasmonic acid (JA), abscisic acid (ABA) and salicylic acid (SA) using response surface methodology (RSM) and central composite design (CCD)[a]

Treatment	For JA and ABA		For SA	
	Conc. (µM)	Time (h)	Conc. (µM)	Time (h)
1	0	24	427	41
2	50	48	250	24
3	50	24	250	0
4	50	24	0	24
5	15	41	250	24
6	85	41	250	24
7	100	24	250	48
8	85	7	73	41
9	50	24	427	7
10	50	0	500	24
11	15	7	73	7

[a]Design of experiment (DOE) was performed when two variables (concentration and time) and two responses (dry weight and mitragynine content). Central composite design with 2 factors, the model was quadratic: 8 models and 3 center points.

Table 3.7 Experimental values for the responses on mitragynine content after treatment with jasmonic acid (JA), abscisic acid (ABA) and salicylic acid (SA)

Treatment	Response: mitragynine content[a] (mg/g DW)		
	JA	ABA	SA
1	0.176±0.005 a	0.115±0.019 a	0.381±0.031 a
2	0.353±0.019 b	0.244±0.021 c	0.261±0.018 b
3	0.274±0.000 c	0.327±0.023 f	0.208±0.003 c
4	0.269±0.012 c	0.316±0.002 f	0.182±0.007 c
5	0.368±0.019 b	0.205±0.020 b	0.259±0.004 b
6	0.363±0.023 b	0.282±0.005 de	0.282±0.016 b
7	0.307±0.042 c	0.268±0.029 cd	0.282±0.015 b
8	0.357±0.004 b	0.257±0.021 cd	0.212±0.025 c
9	0.276±0.013 c	0.307±0.003 ef	0.269±0.002 b
10	0.199±0.020 a	0.178±0.005 b	0.399±0.033 a
11	0.292±0.029 c	0.197±0.008 b	0.213±0.047 c

[a]Data represent mean ± SD of triplicate experiments. Values followed by the same letter are not significantly different at $P<0.05$ according to Duncan's multiple range test.

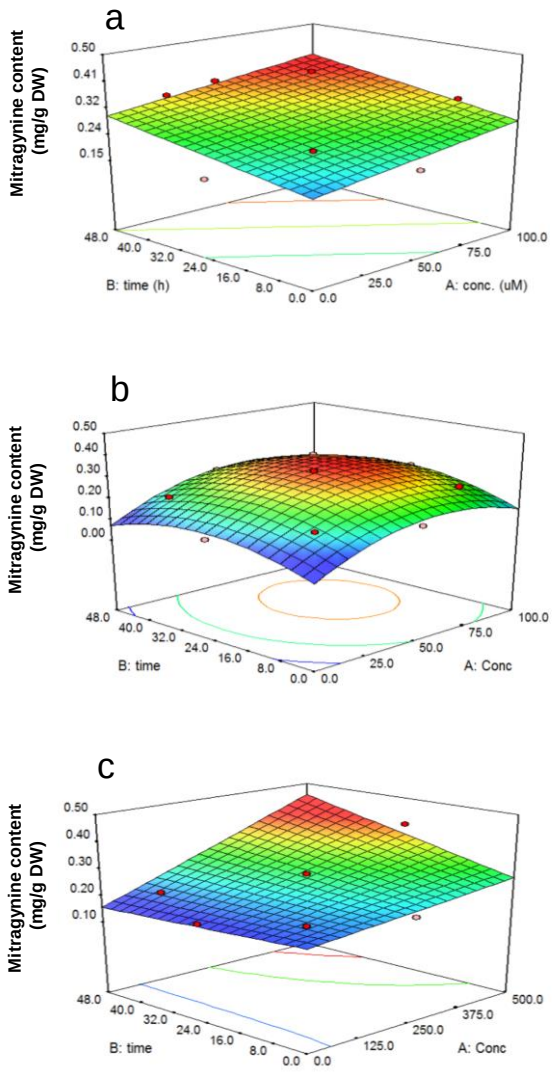


Figure 3.25 Response surface plots between concentration (µM) and time (h) on mitragynine content after elicitation with (*a*) jasmonic acid, (*b*) abscisic acid, and (*c*) salicylic acid

Table 3.8 The regression coefficient of suggested models for the response on mitragynine content

Coefficient	Mitragynine content (mg/g DW)		
	JA	ABA	SA
The model sum of squares	Linear	Quadratic	Two-factor interaction
R squared (R^2)	0.4437	0.9164	0.9302
Coefficient of variation (CV)	18.77	10.94	8.13
P-value[a]	0.0957	0.0100	0.0002

[a]*P*-value < 0.05 = significant; *P*-value > 0.05 = not significant

Table 3.9 Predicted and actual values of mitragynine content after elicitation

Sample (conc.;time)	Mitragynine content (mg/g DW)		*P*-value[a]
	Predicted	Actual	
Control (without elicitor; 24 h)		0.116±0.008	
Control (without elicitor; 48 h)		0.188±0.030	
Jasmonic acid (100 µM; 48 h)	0.346±0.055	0.452±0.021	0.035
Abscisic acid (50 µM; 24 h)	0.317±0.027	0.273±0.007	0.084
Salicylic acid (500 µM; 48 h)	0.456±0.022	0.280±0.017	0.009

[a]The mean values compared between the predicted and actual values using Excel Student *t*-test.

From the response plot, we tried to predict the optimal concentration of JA, ABA and SA and optimal time of elicitation. From the results, elicitors that added to the shoot culture did not affect or inhibit the growth of shoot culture but enhanced mitragynine production. The best combination of elicitor and time of treatment was as followed. Elicited shoot culture with 73 µM SA for 7 h increased mitragynine to 0.409 mg/g DW. Following the treatment with 100 µM, JA for 24 h increased mitragynine production to 0.368 mg/g DW. Elicited shoot culture with 50 µM ABA for 24 h stimulate mitragynine production to 0.305 mg/g DW. Based on the suggested values, the treatments were performed. The resulting response is shown in Table 3.8 and Fig. 3.26. Treatment the shoot culture with 100 µM JA for 24 h, 500 µM SA and 50 µM ABA promoted the mitragynine productions of 3.2-fold, 2-fold and 2-fold when compared to the control cultures, respectively. This experiment indicated that elicitor-like plant growth regulators could stimulate the biosynthesis of mitragynine.

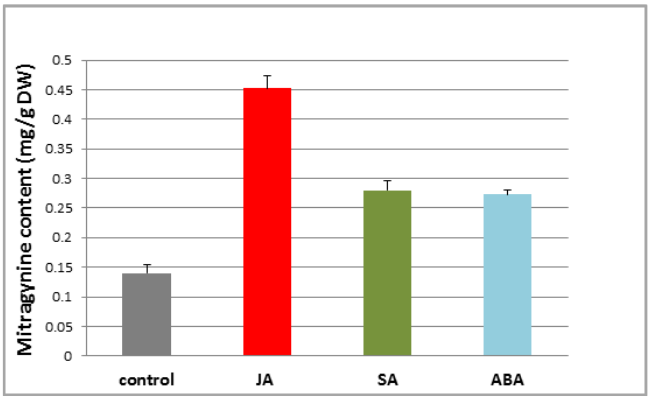


Figure 3.26 The actual value of the response of mitragynine production in the shoot culture

5.3 Elicitation the shoot culture with abiotic elicitor: point-to-point design

In this experiment, we designed to treat the shoot culture with abiotic elicitors including methyl jasmonate (MJ), salicylic acid (SA) and chitosan (CH) by point-to-point design. Mitragynine content was determined by HPLC analysis (as mentioned above).

5.3.1 Stock solution of elicitors

A stock solution of MJ was prepared for the concentration of 45.9 mM in absolute ethanol. A stock solution of SA was prepared for the concentration of 5 M in absolute ethanol. The MJ and SA stock solutions were then diluted with water to the desired amount. For the CH stock solution, CH was prepared to a concentration of 10 g/L. Glacial acetic acid was added to the CH stock

solution to a final concentration of 2% (v/v). The CH stock solution was used by dilution with water. The control of SA and MJ was the final amount of ethanol in water and of CH was water.

5.3.2 Treatment with elicitor

Shoot culture, grown in the shoot induction medium, was subcultured and incubated for 14 days prior to the elicitors treatments. Elicitors were added in the exponential-linear phase of growth. MJ was added to the final concentrations of 0, 50, 100 and 200 µM. SA treatments were 0, 1, 2.5 and 5 mM. CH treatments were 0, 50, 100 and 200 mg/L. The elicited shoot cultures were incubated for 1, 3 and 6 days on the rotary shaker at 60 rpm/min, 25 ± 2 °C and under light 16 h/day. Shoot culture was then harvested, washed with water and freeze-dried. Mitragynine content was determined. The experiment was performed in triplicate.

5.3.3 Effect of MJ in the shoot culture

After treatment shoot culture with MJ, HPLC analysis of mitragynine was performed. The results are shown in Fig. 3.27. MJ did not effect on the growth of shoot culture. Elicited with MJ revealed the shoot culture responded to the elicitor with dose-time dependent manner. Treated with 200 µM for 6 days enhanced mitragynine accumulation to 0.238 ± 0.010 mg/g DW when compared to control culture of 0.040 ± 0.002 mg/g DW (about 6-fold).

5.3.4 Effect of SA in the shoot culture

SA also did not effect on the growth of shoot culture as same as MJ. By the concentration, treatment with SA did not alter the mitragynine production significantly. Fig. 3.28 suggested at 5 mM of SA enhanced mitragynine amount only 0.073 ± 0.001 mg/g DW and the control culture was 0.058 ± 0.001 mg/g DW.

5.3.5 Effect of CH in the shoot culture

Fig. 3.29 suggested at 50 mg/L of CH for 3 days enhanced mitragynine amount from 0.251 ± 0.015 mg/g DW and the control culture was 0.076 ± 0.007 mg/g DW.

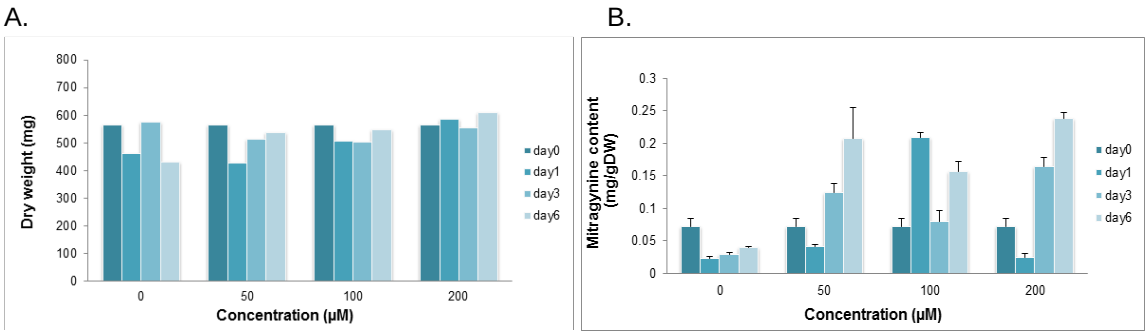


Figure 3.27 The effect of MJ on kratom shoot culture A. on growth; B. on mitragynine production

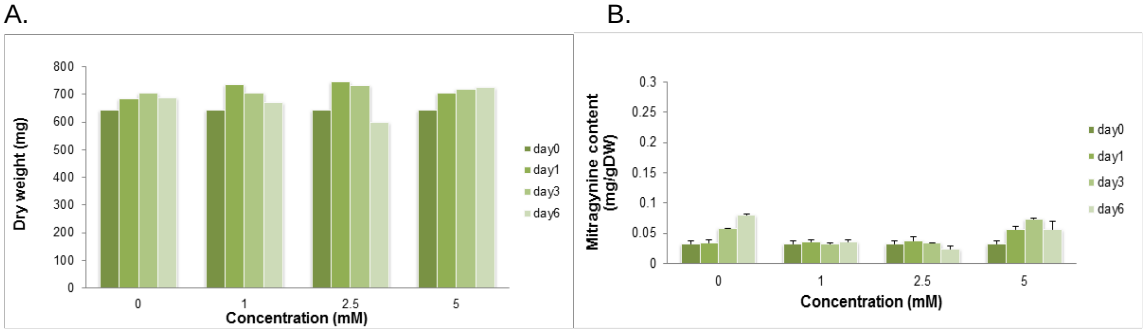


Figure 3.28 The effect of SA on kratom shoot culture A. on growth; B. on mitragynine production

A. B.

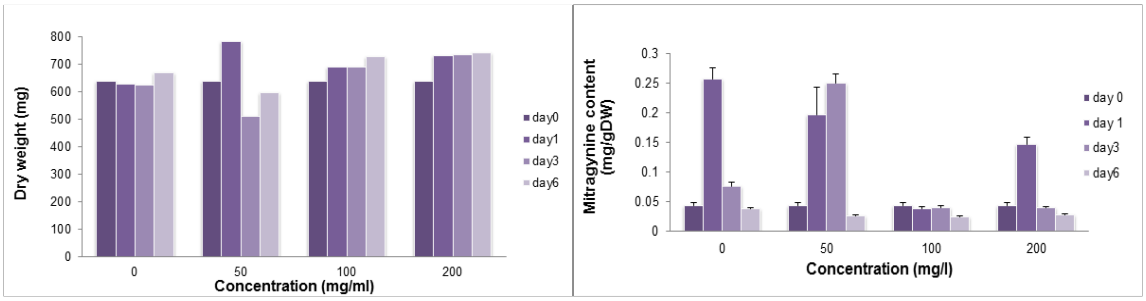


Figure 3.29 The effect of CH on kratom shoot culture A. on growth; B. on mitragynine production

6. Manipulation of kratom plant culture

6.1 Elicitation plant culture with nitric oxide

Plant culture

Kratom plant culture was obtained from micropropagation. From shoot initiation step, the cluster of shoot culture was induced. Then shoots were transferred to the hormone-free WPM. Root is then initiated naturally within 7 days. Plant culture was maintained at the culture condition. Six-week-old plant culture was then used for elicitation.

Treatment with sodium nitroprusside (SNP)

The 6-week old plant culture was removed from the solid medium and placed in SNP solution, nitric oxide donor, at concentrations of 0.25, 0.5, 1, 2.5, 5 and 10 mM. Plant culture placed in distilled water was used as a control. The SNP-treated plant cultures were incubated under the dark condition for 0, 12, 24 and 48 h. The solution was collected and the nitrite content was measured. The plant culture was dried using freeze-dryer and mitragynine and secologanin contents were determined.

Treatment with NO scavenger

The NO scavenger, CPTIO potassium salt (C221, Sigma-Aldrich) was freshly prepared in the concentration of 31.7 mM in distilled water. The 6-week old plant culture was used as plant material. Plant cultures were treated with CPTIO solution by adding into 10 ml medium to the concentration of 1 mM and 2 mM for 30 min. Then, the SNP solution was added to a final concentration of 1 mM. Plant culture that kept in distilled water was used as a control. The plant cultures were incubated under the dark condition for 48 h, harvested and lyophilized. The medium was determined for nitrite content. *M. speciosa* plant was extracted for determination of secologanin and mitragynine contents.

Treatment with calcium channel blocker

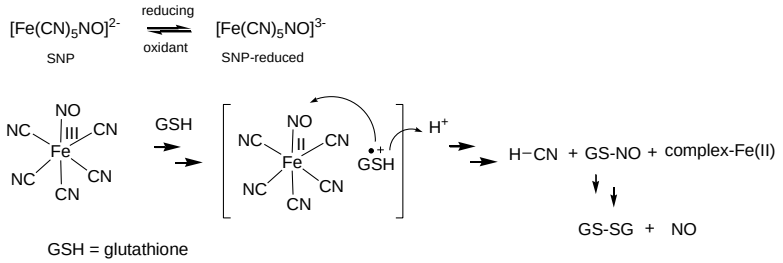
The calcium channel blocker, nifedipine was dissolved in 2% v/v ethanol and added into 6-week old plant culture. The plant culture was treated with nifedipine at a concentration of 0.5 and 1 mM for 30 min before the SNP solution was added to 1 mM. The plant cultures were incubated under the dark condition for 48 h, harvested and lyophilized. The medium was determined for nitrite content. *M. speciosa* plant was extracted for determination of secologanin and mitragynine contents.

Measurement of nitrite content using Greiss assay

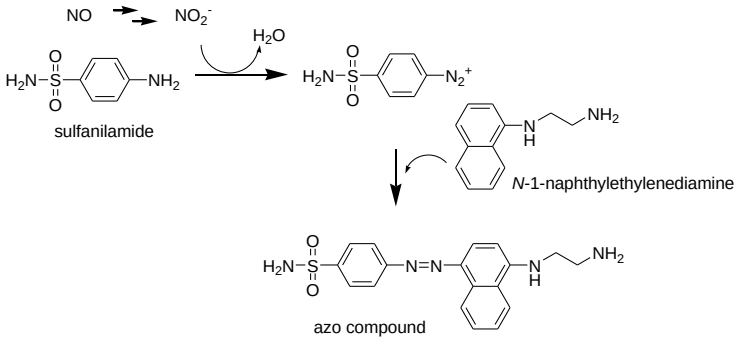
Greiss assay was used for the determination of nitrite content (NO^{2-}), a stable physiological reservoir of nitric oxide (NO). The nitric oxide donor, sodium nitroprusside ($Na_2[Fe^{III}(CN)_5(NO)]$; SNP) is reduced by interacting with sulfhydryl-containing molecule present in vivo such as cysteine, glutathione to paramagnetic $[Fe^{II}(CN)_5(NO)]_3$ as shown in Scheme 1 (Grossi and D'Angelo, 2005). In this study, we used Griess assay as an indirect assay for NO measurement by

determination of nitrite content. Scheme 2 illustrates an interaction of nitrite with sulfanilamide and N-1-naphthyl-ethylenediamine under an acidic condition to produce an azo compound, a pink color that absorbed at 570 nm (Griess, 1879).

Scheme 3.1 Generation of nitric oxide from sodium nitroprusside



Scheme 3.2 Greiss assay



The experiment is performed in a 96-well plate. After treated shoot or plant culture, the medium is separated and immediately measured for nitrite content. The culture medium (50 µL, diluted as appropriate) was reacted with 1% w/v sulfanilamide (50 µL) and incubated at room temperature under the dark condition for 10 min. Then, 0.1% (w/v) N-1-napthylethylenediamine (50 µL) was added into the mixture and incubated further for 10 min under the same condition. The absorbance at 570 nm was measured using a microplate reader. The nitrite content was calculated according to the nitrite standard curve. Distilled water interacted with Griess reagent was used as a blank.

For the construction of the nitrite standard curve, sodium nitrite ($NaNO_2$) was freshly prepared in distilled water at a concentration of 0.1 M. The $NaNO_2$ was diluted to make the concentration of 100 µM. The 100 µM nitrite solution was immediately performed 6 serial twofold dilutions (50 µL/well) to afford the concentrations of 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µM, respectively. The nitrite solution was performed in triplicate. The absorbance at 570 nm was measured. The correlation between nitrite concentration (µM) and OD at 570 nm was plotted. The linear equation was generated and used for nitrite content calculation.

Quantification of secologanin and mitragynine contents

Accumulations of secologanin and mitragynine in shoot culture and plant culture were measured using HPLC analysis as described in Wungsintaweekul et al., 2012. After SNP treatment, shoot and plant cultures were harvested and lyophilized. Dried plant materials were ground and kept at -20°C until used.

The crude extract was prepared. For plant culture, 50 mg of powder was weighed and 10 mL of MeOH was added. The mixture was sonicated at room temperature for 10 min. The mixture

was then refluxed at 80°C for 30 min and filtered. To remove chlorophyll, the filtrate was partitioned with petroleum ether (10 ml, 5 times). The MeOH layer was collected, pooled and evaporated to dryness under vacuum.

HPLC analysis was performed using Agilent 1100 series equipped with a photodiode array detector. The column was a Vertisep™ USP C18 HPLC column (4.6 x 250 mm, diameter 5 µm). The compounds were eluted by gradient elution using a mobile phase: 100 mM H_3PO_4 pH 2.4: acetonitrile, ratio 90:10 for 5 min and gradient to ratio 10:90, within 15 min. Injection volume was 20 µL and the flow rate was 1.5 mL/min. The compounds were detected by diode array detector at UV wavelength 225 nm for mitragynine and at wavelength 238 nm for secologanin. Amounts of secologanin and mitragynine were calculated by extrapolation of the area under the curve. Contents of secologanin and mitragynine were expressed as mg (or µg) per g dry weight.

Transcription profile analysis of genes involved in mitragynine biosynthesis

Plant cultures treated with SNP were harvested and roots were removed. Then, samples were washed with sterile distilled water, immediately frozen in liquid nitrogen and kept at -80°C until used. Total RNA was extracted and isolated using a Total RNA mini kit (plant) (Geneaid). According to the manufacturer's protocol, plant material was ground to powder in the presence of liquid nitrogen and weighed (300 mg) into the sterile microcentrifuge tube. The, RB buffer (500 µl) plus 5 µl of β-mercaptoethanol was added and mixed thoroughly with the vortex. The mixture was then incubated at 60°C for 5 min. After centrifuging at 14,000 g for 1 min, the supernatant was loaded on filter column and centrifuged at 1,000 g for 1 min. The clarified filtrate was collected. The chilled ethanol (½ volume) was added into the filtrate and shake vigorously. The mixture was loaded on the top of RB column and centrifuged at 14,000 g for 1 min, discarded flow-through. To remove DNA, 100 µl of DNase I (2 KU/mL) which was mixed in 100 µl of 2x DNase reaction buffer (20 mM Tris-HCl pH 7.6,5 mM $MnCl_2$, 1 mM $CaCl_2$ at 25 ºC) was loaded onto the RB column and incubated at room temperature for 10 min. For washing step, W1 buffer (400 µl) was loaded into the center of the RB column, centrifuged at 14,000 g for 30 s and discarded flow-through. Wash buffer (600 µl, 2 times) was loaded and centrifuged. To dry the RB column, the RB column was transferred to a new centrifuge tube and centrifuge at 14,000 g for 2 min (2 times). The dried RB-column was transferred to a new centrifuge tube; RNase-free water (50 µl) was loaded to the center of the column matrix and incubated for 2 min, then the total RNA was harvested by centrifuge at 14,000 g for 1 min. The purified RNA was collected.

Transcription profile of genes involved in mitragynine biosynthesis in *M. speciosa* was determined using the qRT-PCR technique. According to manufacturer's protocol, One Step SYBR® PrimeScript™ RT-PCR kit II (Perfect Real Time, Takara, Japan) performs cDNA synthesis from RNA using PrimeScript reverse transcriptase and PCR amplification with Takara Ex Taq HS DNA polymerase within one tube. PCR amplification products are monitored in real time using SYBR® Green I detection. The qPCR solution was prepared in a 96-tube PCR plate. Non-template (no RNA) and non-primers reaction were prepared for checking the unspecific qPCR reaction. The PCR plate was placed into a tray of ABI Prism® 7300 Fast Real-Time PCR system.

After the qPCR is completed, the amplification plot and dissociation curve were recorded and verified. The numbers of the cycle at threshold line (0.2) (CT) were recorded. The house-keeping gene (*18S rRNA*) was used as the endogenous gene. The control group of treatment was used as calibrator. The transcription profile of genes was expressed as relative quantitation (RQ).

Results

Effect of SNP in M. speciosa plant culture

M. speciosa plant culture was maintained and sub-cultured on hormone-free WPM medium. SNP was added into *M. speciosa* plant culture and then follow the secondary metabolites i.e. mitragynine and secologanin productions. The 6 week-old plant cultures were used in this study. The concentrations of SNP were reduced to 0.25, 0.5, 1, 2.5, 5 and 10 mM and the time exposures were within 12, 24 and 48 h. Plant cultures were transferred to SNP solutions and kept under dark condition. After treatment, plants were harvested and prepared for crude extract and later HPLC analysis. And nitrite content analysis was simultaneously determined from the treated solution. The SNP-treated plant culture was lyophilized to dryness, ground and prepared for crude alkaloid extract. After refluxing with methanol, the solution was partitioned with petroleum ether to get rid of impurities such as chlorophylls. The crude extract was evaporated to dryness. After that, the residue was dissolved in 1 ml MeOH (HPLC grade), centrifuged and the supernatant was used for mitragynine and secologanin analyses.

Griess method was again used for NO^{2-} content determination from solutions of SNP-treated plant. For the determination of mitragynine content, the crude extract was diluted for 5 times with methanol prior subjected to HPLC column. The mobile phase used in this study consisted of 100 mM H_3PO_4 pH 2.4 and acetonitrile. Table 3.9 summarizes the amount of mitragynine of SNP-treated plant culture.

Table 3.10 Mitragynine content of SNP treated on 6 week-old plant cultures (n =3)

SNP (mM)	mitragynine (mg/g dry weight)			
	0 h	12 h	24 h	48 h
Control	3.23±0.21			
0.25		2.72±0.72	2.76±0.16*	2.33±0.09*
0.5		2.70±0.29*	2.41±0.07**	1.72±0.02**
1		2.89±0.18	2.86±0.08	2.22±0.28*
2.5		2.41±0.13**	2.68±0.14*	2.08±0.39*
5		2.65±0.13	3.02±0.03	2.75±0.07*
10		2.59±0.17**	2.69±0.07**	2.90±0.12

Data represent mean values ± standard deviation; *indicate $p<0.05$ and **indicate $p<0.01$ when compared with day 0

Results on mitragynine production indicated that SNP did not affect to mitragynine accumulation in *M. speciosa* plant culture. Consideration of mitragynine biosynthesis, secologanin, another precursor of strictosidine beside of tryptamine, may be influenced by SNP treatment. The crude extracts were further subjected to HPLC analysis, observed at 238 nm. Secologanin was eluted at 10.9 min.

Table 3.11 Secologanin content (mg/g dry weight) of SNP treated on 6 week-old plant cultures (n =3)

SNP (mM)	secologanin (mg/g dry weight)			
	0 h	12 h	24 h	48 h
Control	3.05±0.25			
0.25		2.79±1.24	5.59±0.18**	5.57±0.70**
0.5		4.17±0.42	5.98±0.62**	6.24±0.60**
1		6.60±3.01**	6.70±0.60**	8.50±0.55**
2.5		4.94±0.84*	5.82±1.11**	6.75±1.40**
5		0.63±0.13	6.55±0.29**	5.53±0.30**
10		3.07±0.63	4.34±0.47*	3.21±0.55

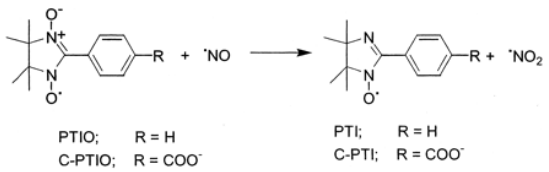
Data represent mean values ± standard deviation; *indicate $p<0.05$ and **indicate $p<0.01$ when compared with day 0

Treatment of various concentrations of SNP at 0.25, 0.5, 1, 2.5, 5 and 10 mM on 6 week-old plant cultures for 12, 24 and 48 h summarizes in Table 3.10. The results are clearly shown that SNP could stimulate secologanin production based on SNP concentration and time exposure to elicitor. Notably, at SNP concentration of 1 mM, secologanin content was increased to 4.87±0.34 mg/g dry weight compared to the control (3.05±0.44 mg/g dry weight). Longer SNP exposure for 24 h and 48 h could induce secologanin production to 6.70±0.60 and 8.50±0.55 mg/g dry weight, respectively. This effect also appears in another group of treatment. The optimal condition of SNP treatment is at 1 mM SNP in hormone-free WPM medium and for 48 h. SNP concentration higher than 5 mM and longer exposure caused inhibit the secologanin production was observed.

Effects of CPTIO and nifedipine treatment in M. speciosa plant culture

M. speciosa plant culture was treated with CPTIO, a nitric oxide scavenger due to confirm that secologanin production was increased in SNP-treated plant caused by NO generation. The optimal condition of 1 mM SNP treatment for 48 h was used in this experiment. The plant cultures were exposed to 1 mM and 2 mM CPTIO solutions for 30 min before SNP treatment. After 48 h, plants were harvested and the solution was filtered. The solutions were determined for nitrite content. The treated plant was dried, ground and prepared for crude extract.

Fig. 3.30 illustrates the nitrite content and secologanin content in SNP- and/or CPTIO-treated plants. There were 5 groups of treatments including SNP, CPTIO, SNP and 1 and 2 mM CPTIO and the control. By Greiss reagent, detecting nitrite species, suggested that SNP could generate NO and in consequence nitrite. However, solutions that contained CPTIO found to accumulate a higher amount of nitrite. Even though CPTIO is NO scavenger, it should theoretically reduce NO and hence nitrite content. Consideration the following equation, CPTIO reacted with NO (endogenous NO and SNP-generated NO) also produce nitrite molecule. Thus, nitrite content in Fig. 3.15 is an accumulation of nitrite from NO and by-product of CPTIO and NO (Goldstein *et al*., 2003).



Determination of secologanin contents in those 8 groups revealed that plant treated with 1 mM and 2 mM CPTIO together with 1 mM SNP have lower amounts of secologanin in compared with

control. This can be proposed that CPTIO scavenged NO that generated from SNP and effect to lowering amount of secologanin. This experiment confirmed that NO elicited *M. speciosa* plant culture and enhancing secologanin production.

Again, plant culture was treated with nifedipine and/or CPTIO prior SNP treatment. Table 3.11 and Fig. 3.30 summarize the effect of nifedipine-treated plant culture. Plant culture that treated with SNP has a considerable amount of secologanin. Secologanin productions were significantly suppressed in the groups those treated with 0.5 mM and 1 mM nifedipine.

Transcription profiles of M. speciosa biosynthetic genes

Mitragynine biosynthesis is nowadays not complete. Mitragynine was proposed to biosynthesize from the indole alkaloid through strictosidine (Rueffer *et al*., 1978). Branch point enzymes in *M. speciosa* including 1-deoxy-D-xylulose 5-phosphate synthase (*dxs1, dxs2*), 1-deoxy-D-xylulose reductoisomerase (*dxr*), anthranilate synthase (*as1, as2*), tryptophan decarboxylase (*tdc*) and strictosidine synthase (*str*) were studied for transcription profile analysis in the SNP-treated plant culture. qRT-PCR using SYBR green as a detecting agent was used to determine the mRNA levels of these genes due to understanding the regulation of secologanin biosynthesis as well as mitragynine biosynthesis.

Four groups of treatments including control, SNP, SNP and CPTIO, SNP and nifedipine were investigated. After treatment for 48 h, plant cultures were harvested, immediately frozen in liquid nitrogen and extracted for total RNA(s). Harvested plants were thoroughly washed with RNase-free distilled water. Plant tissue (300 mg), ground in liquid nitrogen, was used as a material for total RNA extraction (Geneaid).

Takara one-step SYBR® Prime script RT-PCR kit II was used to detect the mRNA levels of genes during PCR reaction. Total RNA of 200 ng was used as template in each PCR reaction. The PCR condition was started in reverse transcription step at 42 ºC for 5 min, to synthesize cDNA single stand. The PCR reaction began at 95 ºC for 5 sec and 60 ºC for 31 sec, 40 cycles. After 40 cycles of PCR, dissociation curve (melting curve) and amplification plot were recorded. Amplification plot of each gene during PCR suggested the specificity of primer and template. Only one band or one product should occur. Number of the cycle at threshold (in exponential phase) of amplification plot was recorded as C_T when *18s rRNA* was used as endogenous gene and the control group was used as calibrator. Relative quantitation (RQ) was calculated by equation $RQ = 2^{(-\Delta\Delta C_T)}$

The specificities of primers to the template of the investigated genes are shown that one band of each gene in the corresponding dissociation curves was found, meaning the C_T value can be used for further RQ analysis. Fig. 3.31 summarizes the values of RQ of each group of genes. The mRNA levels of genes involved in secologanin and mitragynine biosynthesis were shown to accumulate in different levels. After SNP treatment, transcription profiles of all genes were enhanced. On the other hand, treatments with CPTIO and nifedipine suppressed the mRNA levels of all genes.

Table 3.12 Mitragynine and secologanin content (mg/g dry weight) of SNP, CPTIO and nifedipine treatment in *M. speciosa* plant culture (n = 3)

treatment	mitragynine (mg/g dry weight)	secologanin (mg/g dry weight)
control	0.93±0.12	5.44±1.47
2% (v/v) ethanol	1.28±0.08	5.22±1.27
1 mM SNP	1.29±0.07	5.55±0.89
1 mM CPTIO	1.55±0.04	5.04±0.10
1 mM SNP and 1 mM CPTIO	1.58±1.72	4.83±0.43
1 mM SNP and 2 mM CPTIO	1.42±0.02	3.12±0.68*
0.5 mM nifedipine	1.49±0.15	6.69±1.27*
1 mM SNP and 0.5 mM nifedipine	1.26±0.14	4.94±1.34
1 mM SNP and 1 mM nifedipine	1.23±0.17	2.42±0.43*
1 mM SNP, 0.5 mM nifedipine and1 mM CPTIO	1.49±0.38	2.23±0.48*

Data represent mean values ± standard deviation; *indicate $p<0.05$ and **indicate $p<0.01$ when compared with control

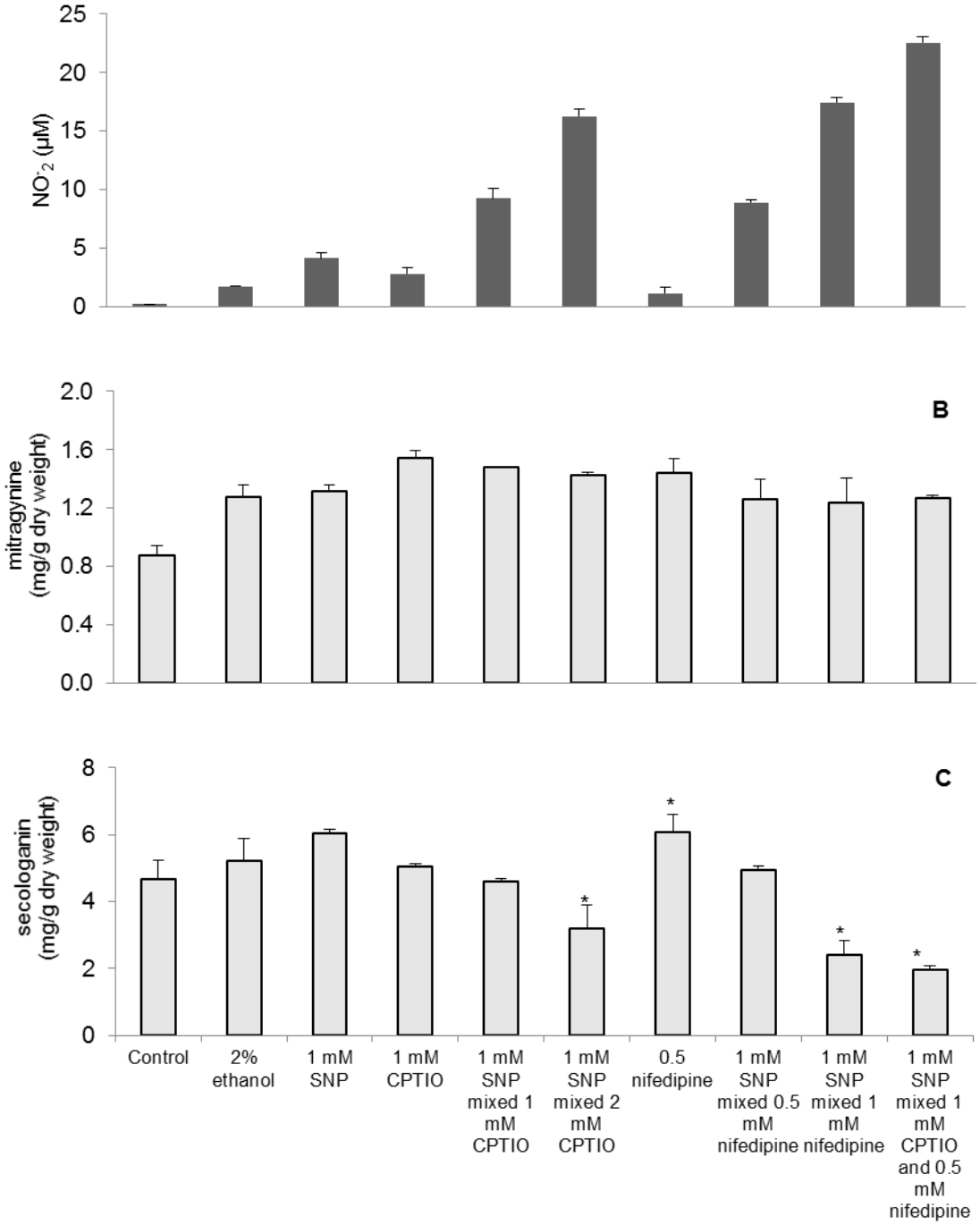


Figure 3.30 Summary of SNP, CPTIO and nifedipine treatment in *M. speciosa* plant culture. A: nitrite content (µM), B: mitragynine content (mg/g dry weight) and C: secologanin content (mg/g dry weight). Data represent mean values ± standard deviation; *indicate $p<0.05$ and **indicate $p<0.01$ when compared with control.

Elicitation is a technique that enhances the biosynthesis of secondary metabolite in the plant. The present study was shown that a nitric oxide (NO) donor like sodium nitroprusside could significantly stimulate the production of secologanin, however, not much effect on mitragynine content. Inhibition NO production by adding a NO scavenger (CPTIO) and a calcium-channel blocker (nifedipine) resulted in the decreasing of secologanin production. These findings

suggested that NO and calcium channel were involved in secologanin biosynthesis. Following the transcription profiles of genes including anthranilate synthase (*as1*, *as2*), tryptophan decarboxylase (*tdc*), strictosidine synthase (*str*) and 1-deoxy-D-xylulose 5-phosphate synthase (*dxs1*, *dxs2*) revealed that the expressions of all genes were increased in plant that treated with 1 mM SNP and decrease in plant that treated with 1 mM CPTIO or 1 mM nifedipine in addition. Our study concluded that SNP (NO donor) enhanced secologanin production in *M. speciosa* plant culture and calcium channel was involved in elicitation cascade. Elicitation with NO increased the transcription profiles of *dxs1*, *dxs2*, *as1*, *as2*, *tdc* and *str* in secologanin/mitragynine biosynthesis. Nevertheless, production of mitragynine did not alter after treatment with NO in plant culture may cause from tryptamine availability.

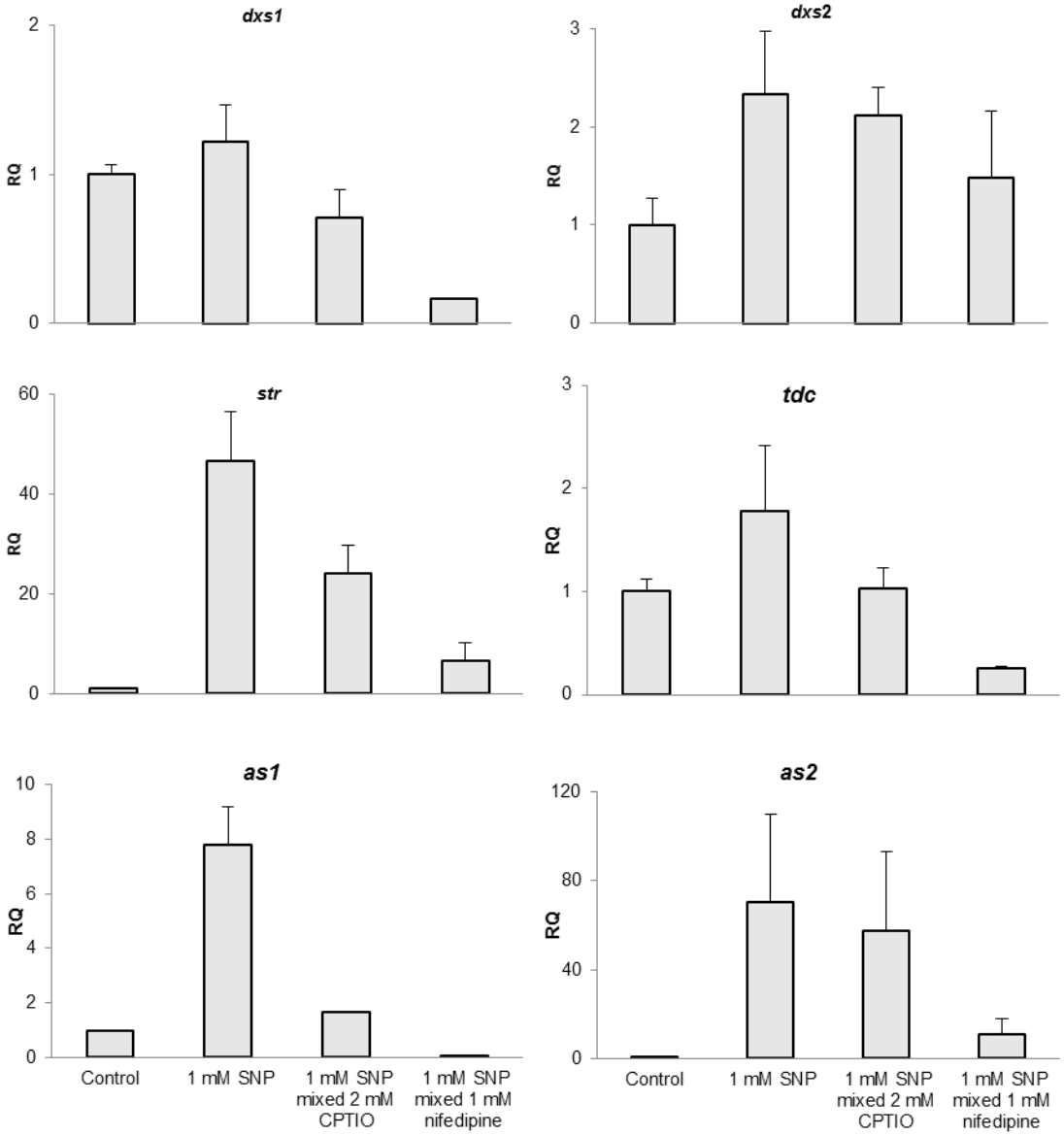


Figure 3.31 The RQ values of genes used in this study.

6.2 Effect of lights on kratom alkaloid production

Establishment of kratom plant culture

An axillary bud of kratom plantlet was excised. The shoot was multiplicated in the shoot induction medium, WPM supplemented with 1 mg/L benzyladenine (BA) and 2 mg/L thidiazuron (TDZ). The shoot culture was incubated at 25°C, under fluorescence light for 16 h/day. The number of shoots could be induced in the liquid shoot induction medium. The shoot was then cut and placed on hormone-free WPM for plant culture induction. After incubation for 7 days, the root appeared. Plant culture was then subcultured to a new medium for three-time (Fig. 3.32). The whole plant was incubated for 2 months and ready to be plant material for the study.

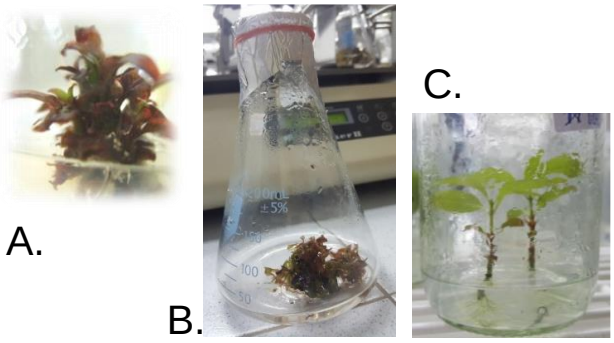


Figure 3.32 Establishment of plant culture from shoot culture. A. shoot culture in the solid medium; B. shoot culture in a liquid medium and C. 2-weeks old plant culture

Light treatment

Lights used in the study were white light (fluorescence; Philips), red light (Toshiba FL18W/T8/DR; wavelength 660 nm), blue light (Toshiba FL18W/T8/DB, wavelength 450 nm).

The treatments were divided into four groups. Group 1 was a plant culture, grown under white light. Group 2 was a plant culture, grown under blue light. Group 3 was a plant culture, grown under red light. All lights treatments were 16 h light/ 8 h dark cycle at 25°C. And group 4 was plant cultures, grown under dark condition. The growth of plant culture was recorded. The appearance of the plant was captured. Plant cultures were harvested at 15, 30 and 45 days of culture and freeze-dried.

Methyl jasmonate elicitation and precursor addition

The suitable light for growth and alkaloid production was chosen. Then treatment with 10 µM methyl jasmonate (MJ) for 12 h was investigated of plant culture that grown for 20 days. For precursor addition, 0.4 mM tryptamine and 0.2 mM secologanin was added on the day of culture and treated for 14 days. Combinations of both MJ elicitation and precursor treatment were done. Plant cultures were harvested and evaluated for alkaloid production. The control plant culture was only light treatment. The experiment was done in triplicate.

Preparation of plant extract

Plant culture was freeze-dried and powdered. Powder (50 mg) was accurately weighed and extracted in 10 mL methanol. The mixture was refluxed at 70°C for 1 h and filtered. The filtrate was transferred to a separatory funnel. To remove the fat material and chlorophyll, 10 mL of petroleum ether was added and partitioned for 2 times. Then methanol fraction was evaporated to dryness. The residue was re-dissolved in methanol and centrifuged. The clear supernatant was subjected to HPLC analysis.

Quantification of total alkaloids: mitragynine, paynantheine, speciogynine

HPLC analysis for quantification of kratom alkaloids including mitragynine, paynantheine, and speciogynine was performed. HPLC system equipped with HPLC SHIMADZU LC2030 3D was used. Column was VertiSepTM USP C18 HPLC Column, 4.6 X 250 mm, 5 µm. The column was isocratically eluted with 20 mM ammonium acetate (pH 6): acetonitrile; 35:65 (% v/v). The flow rate was 1 mL/min. Injection volume was 20 µL and a UV detector was set at 225 nm. Calibration curves were constructed: mitragynine (1.56-25 µg/mL), paynantheine (0.62-10 µg/mL), speciogynine (0.62-10 µg/mL).

Results

Effect of lights on growth and kratom alkaloids production

Kratom plantlet was prepared from shoot culture during micropropagation. Forty-five days old of plant culture appeared as an in vitro plant and has about 7 cm in height. Prior light experiment, plant culture was subcultured by cutting root and trimmed the leaves, only 2 pairs of leaves from the shoot. Then, the explant was placed on the hormone-free medium and was cultured for 15 days under the normal condition of white light. Later, plant culture (12 samples of each group) was moved under blue light, red light and dark as well as white light. Four samples were collected from each group on day 15th, 30^{th,} and 45th (Fig. 3.33).

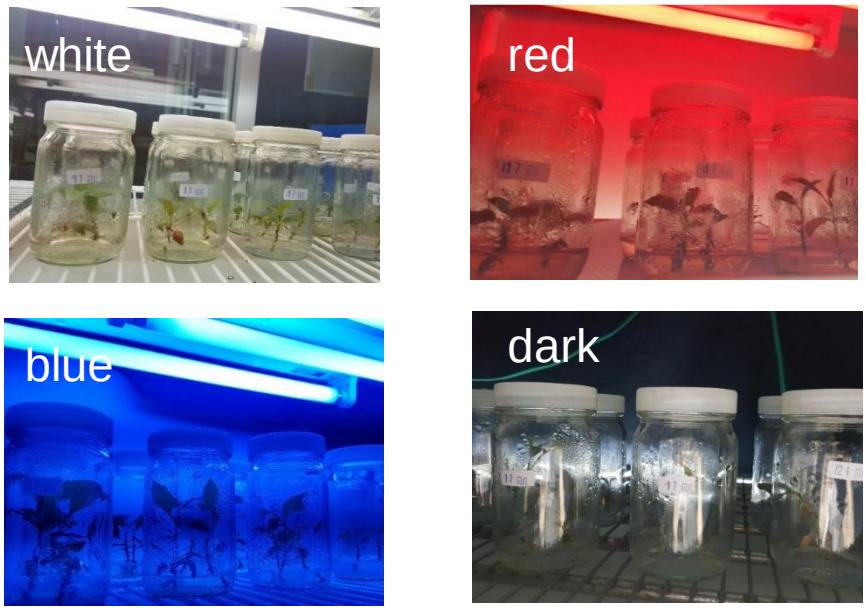


Figure 3.33 Treatment the plant culture with different lights.

Plant cultures had different response under light treatment. As shown in Fig. 3.32, ordinary growth of plant was found in the plant under white light. Interestingly, plant, grown under blue light, has a maximum growth on day 30th of blue light treatment, and then gradually declined on day 45th. Plant cultures, under red light and dark condition, grew slowly and were decline the growth gradually (Fig. 3.34).

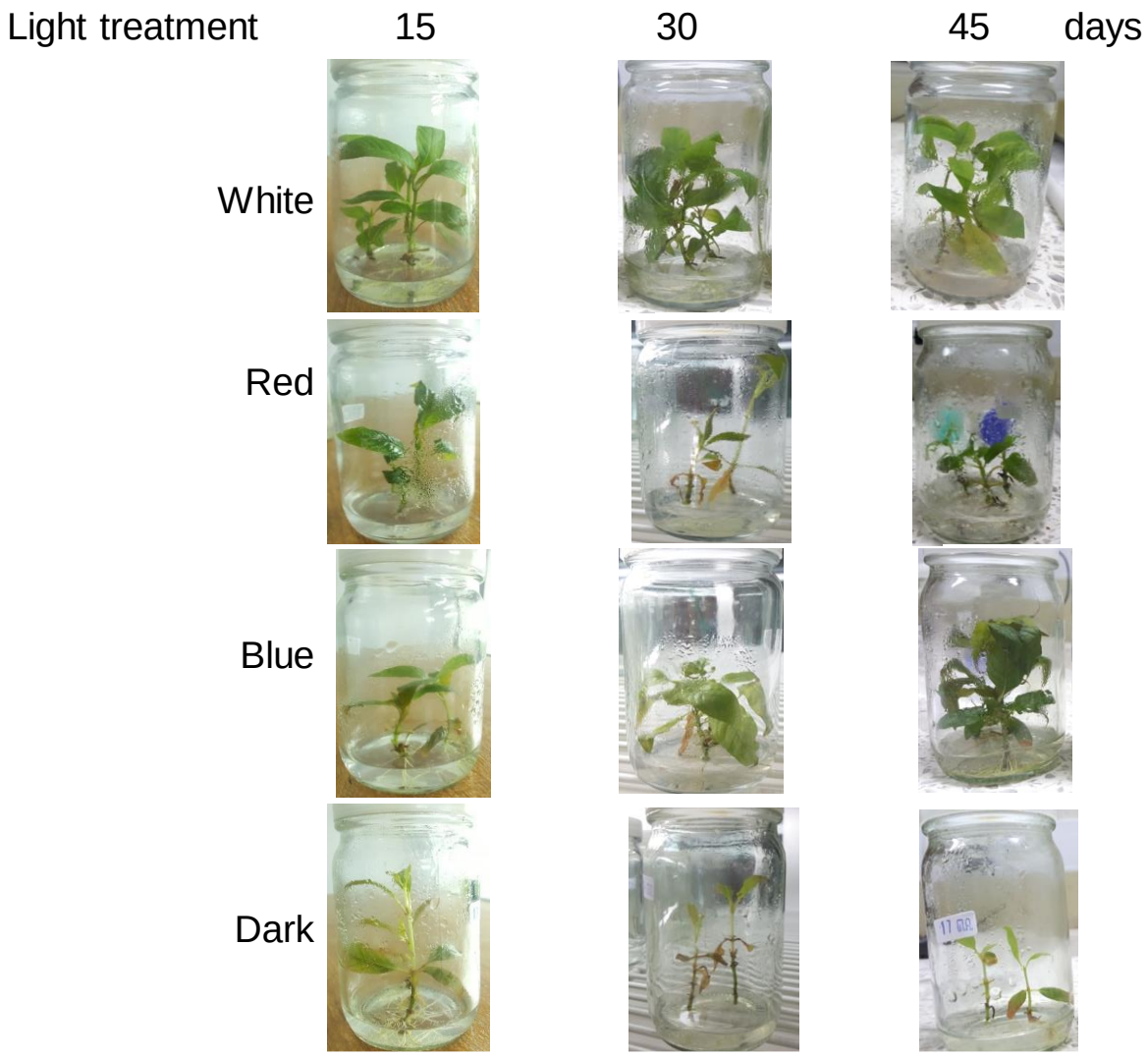


Figure 3.34 The appearances of plant cultures, grown under white, red, blue lights and dark condition after 15, 30 and 45 days of treatments.

To evaluate the production of kratom alkaloids, plants were harvested, dried and extracted. HPLC chromatograms (Fig. 3.35) show peaks of speciogynine, paynantheine, and mitragynine at 8.9, 10.0 and 14.0 min, respectively. The area under the curve was integrated and was calculated for amounts of kratom alkaloids.

The ability of alkaloid production in plant culture was found to produce mitragynine as a major alkaloid, following paynantheine and speciogynine. The rate of alkaloid production was found in the plant culture, grown under red light. The total amount of alkaloid was low, caused by a decline in growth. Based on growth, the amount of alkaloid was found significantly in plant culture, grown under blue light. By this strategy, we decided to elicit plant culture with blue light based on growth and ability of production.

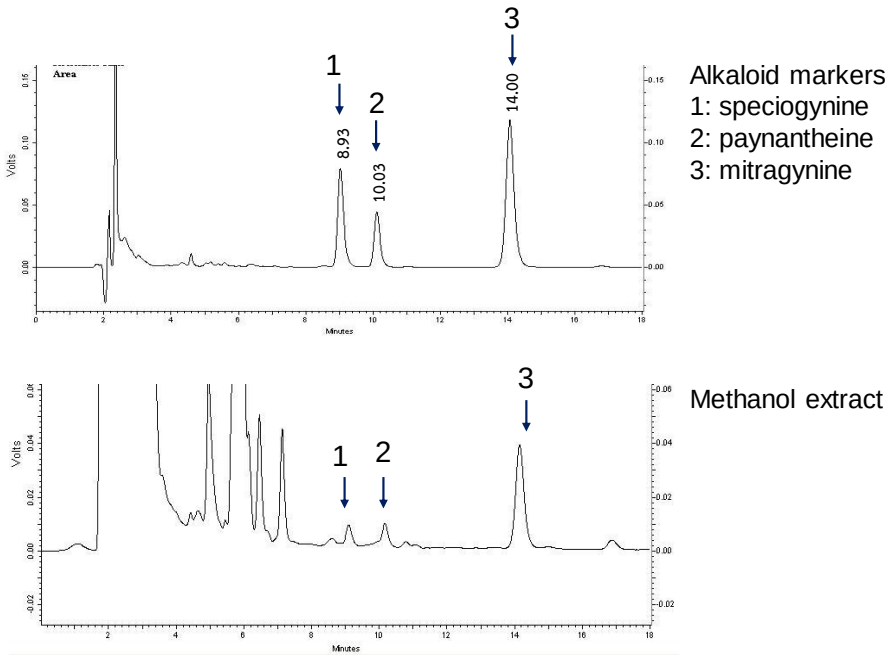


Figure 3.35 HPLC chromatograms of alkaloid markers and of methanol extract.

Table 3.13 Productions of speciogynine in kratom under light treatment.

Speciogynine				
Light	Day	Dry weight (g)	Speciogynine (µg/g DW) ± S.D.	Total Speciogynine (µg) ± S.D.
White	Day 15	0.1975	117.24 ± 20.33	23.16 ± 4.01
	Day 30	0.2318	154.94 ± 86.90	35.92 ± 8.33
	Day 45	0.4645	171.96 ± 21.66	79.87 ± 10.06
Blue	Day 15	0.1395	123.57 ± 13.64	17.24 ± 2.40
	Day 30	0.2582	116.18 ± 5.98	30.00 ± 1.54
	Day 45	0.5246	112.65 ± 19.48	59.10 ± 31.00
Red	Day 15	0.1487	314.29 ± 16.46	46.73 ± 2.45
	Day 30	0.1415	138.27 ± 13.32	19.57 ± 2.77
	Day 45	0.222	205.43 ± 15.07	45.61 ± 3.34
Dark	Day 15	0.1395	229.15 ± 21.44	31.97 ± 4.46
	Day 30	0.1155	199.52 ± 9.70	23.05 ± 1.12
	Day 45	0.0851	304.72 ± 13.94	25.93 ± 2.21

Table 3.14 Productions of paynantheine in kratom under light treatment.

Paynantheine				
Light	Day	Dry weight (g)	Paynantheine (µg/gDW) ± S.D.	Total Paynantheine (µg) ± S.D.
White	Day 15	0.1975	144.57 ± 37.14	28.55 ± 7.34
	Day 30	0.2318	197.36 ± 73.33	45.75 ± 17.00
	Day 45	0.4645	138.88 ± 15.20	64.51 ± 7.06
Blue	Day 15	0.1395	82.18 ± 1.55	11.46 ± 0.22
	Day 30	0.2582	118.25 ± 2.74	30.53 ± 0.71
	Day 45	0.5246	109.61 ± 10.29	57.50 ± 5.40
Red	Day 15	0.1487	112.83 ± 19.83	16.78 ± 2.95
	Day 30	0.1415	302.44 ± 16.20	42.80 ± 2.29
	Day 45	0.222	264.29 ± 10.30	58.67 ± 2.29
Dark	Day 15	0.1395	66.55 ± 1.85	9.28 ± 0.26
	Day 30	0.1155	59.34 ± 3.84	6.85 ± 0.44
	Day 45	0.0851	84.39 ± 0.96	7.18 ± 0.08

Table 3.15 Productions of mitragynine in kratom under light treatment.

Mitragynine				
Light	Day	Dry weight (g)	Mitragynine (µg/g DW) ± S.D.	Total Mitragynine (µg) ± S.D.
White	Day 15	0.1975	350.49 ± 68.32	69.22 ± 13.49
	Day 30	0.2318	368.78 ± 127.31	85.48 ± 29.51
	Day 45	0.4645	298.62 ± 56.59	138.71 ± 26.28
Blue	Day 15	0.1395	243.50 ± 19.12	33.97 ± 2.67
	Day 30	0.2582	542.07 ± 4.92	139.96 ± 1.27
	Day 45	0.5246	486.66 ± 36.31	255.30 ± 19.05
Red	Day 15	0.1487	92.62 ± 6.38	13.77 ± 0.95
	Day 30	0.1415	955.41 ± 35.22	135.19 ± 4.98
	Day 45	0.222	725.09 ± 38.92	160.97 ± 8.64
Dark	Day 15	0.1395	62.26 ± 2.44	8.69 ± 0.34
	Day 30	0.1155	88.30 ± 7.15	10.20 ± 0.83
	Day 45	0.0851	147.90 ± 1.48	12.59 ± 0.13

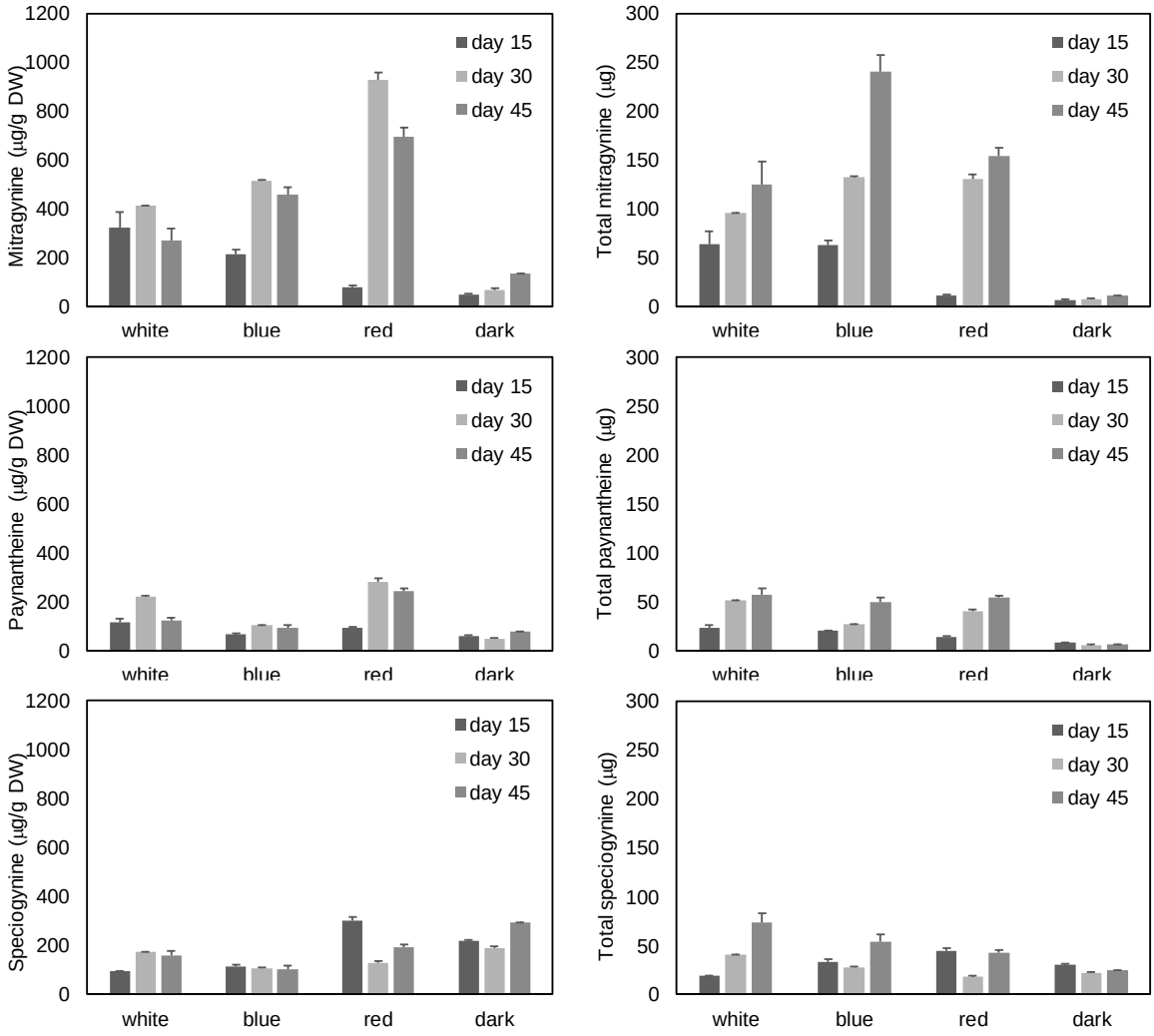


Figure 3.36 Kratom alkaloid production under different light treatment.

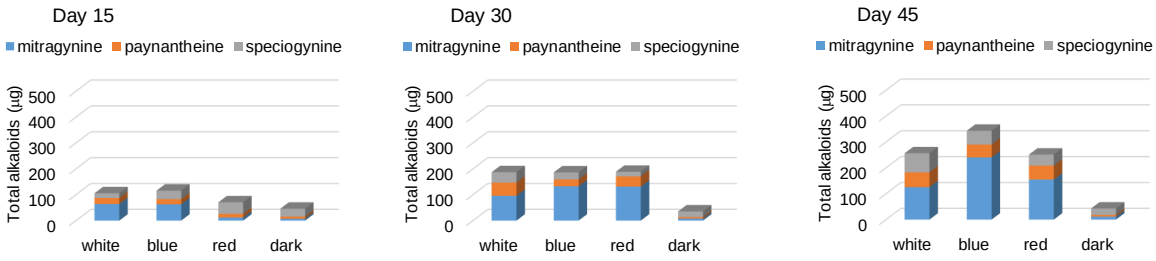


Figure 3.37 Illustration of total alkaloids production after light treatment on day 15, 30 and 45 of culture.

Production of alkaloid after integrated strategy

To improve the alkaloid production in plant culture, we designed the experiment to treat with blue light and either methyl jasmonate or tryptamine/secologanin addition. Plant culture grew under blue light for 30 days. Then, plant culture was removed from the agar and put into the solutions of group 1: 10 µM methyl jasmonate for 12 h; group 2: 0.4 mM tryptamine and 0.2 mM secologanin for 14 days; group 3: treat precursors (0.4 mM tryptamine/0.2 mM secologanin) for 14 days and following 10 µM methyl jasmonate for 12 h. Control culture was set as a blue light treatment only. The result revealed that the alkaloid production in plant culture was increasing in the group of methyl jasmonate elicitation as well as precursor treatment. However, treatment plant culture for 30 days, 10 µM MJ elicitation and precursor addition caused too much stress to the plant. The final amount of alkaloid production was lesser than other groups. This means that plant culture, stimulated with different mechanisms, may cause to death of the plant. The strategy of combination light with either light or precursors was better.

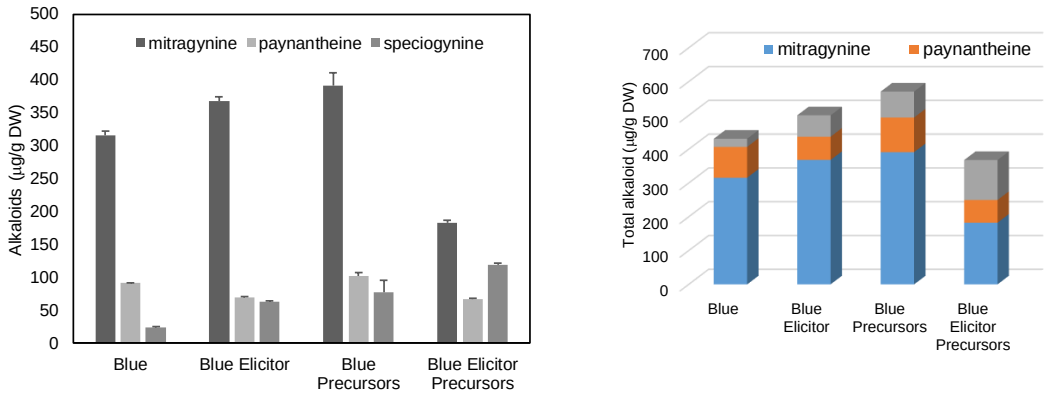


Figure 3.38 Alkaloid production after blue light treatment integrated with methyl jasmonate elicitation and precursors (tryptamine and secologanin)

REFERENCES

Adkins, J.E., Boyer, E.W., McCurdy, C.R., 2011. *Mitragyna speciosa*, A psychoactive tree from Southeast Asia with opiod activity. *Curr. Top. Med. Chem.* 11, 1165-1175.

Aji, A.M., Effraim, K.D. and Onyeyili, A.P. 2001. Antistress activity of *Mitragyna africanus* (Wild) stembark extract. *The Sciences* 1, 105-107.

Apryani, E., Hidayat, M.T., Moklas, M.A.A., Fakurazi, S. and Idayu, N.F. 2010. Effect of mitragynine from *Mitragyna speciosa* Korth. leaves on working memory. *J. Ethnopharmacol.* 129, 357-360.

Arasimowicz, M. and Floryszak-Wieczorek, J. 2007. Nitric oxide as a bioactive signaling molecule in plant stress responses. *Plant Sci.* 172; 876-887.

Barleben, L., Panjikar, S., Ruppert, M., Koeke, J., Stoeckigt, J. 2007. Molecular architecture of strictosidine glucosidase: The gateway to the biosynthesis of the monoterpenoid indole alkaloid family. *Plant Cell*. 19(90, 2886-2897.

Beckett, A.H., Shellard, E.J., Phillipson, J.D. and Lee, C.M. 1966a. The *Mitragyna* species of Asia VI. Oxindole alkaloids from the leaves of *Mitragyna speciosa* Korth. *Planta Med.* 14, 266-276.

Beckett, A.H., Shellard, E.J., Phillipson, J.D. and Lee, C.M. 1966b. The *Mitragyna* species of Asia VII. Indole alkaloids from the leaves of *Mitragyna speciosa* Korth. *Planta Med.* 14, 277-288.

Brandt, V., Geerlings, A., Tits, M., Delaude, C., Van der Heijden, R., Verpoorte, R., Angenot, L. 2000. New strictosidine β-glucosidase from *Strychnos mellodora*. *Plant Physiol. Biochem*. 38(3), 187-192.

Burlat, V., Oudin, A., Courtois, M., Rideau, M. and St-Pierre, B. 2004. Co-expression of three MEP pathway genes and geraniol 10-hydroxylase in internal phloem parenchyma of *Catharanthus roseus* implicates multicellular translocation of intermediates during the biosynthesis of monoterpene indole alkaloids and isoprenoid-derived primary metabolites. *Plant J.* 38, 131-141.

Charoonratana, T., Wungsintaweekul, J., Pathompak, P., Georgier, M.I., Choi, Y.H. and Verpoorte, R. 2013. Limitation of mitragynine biosynthesis in *Mitragyna speciosa* (Roxb.) Korth. through tryptamine availability. *Z Naturforsch C.* 68; 394-405.

Chittrakarn, S., Sawangjaroen, K., Prasettho, S., Janchawee, B. and Keawpradub, N. 2008. Inhibitory effects of kratom leaf extract (*Mitragyna speciosa* Korth.) on the rat gastrointestinal tract. *J. Ethnopharmacol*, 116(1), 173-178.

Chittrakarn, S., Keawpradub, N., Sawangjaroen, K., Kansenalak, S. and Janchawee, B. and 2010. The neuromuscular blockade produced by pure alkaloid, mitragynine and methanol extract of kratom leaves (*Mitragyna speciosa* Korth.). *J. Ethnopharmacol. 129*, 344-349.

Contin, A., van der Heijden, R. Lefeber, A.W.M. and Verpoorte, R. 1998. The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture. *FEBS Lett.* 434; 413-416.

Dewick, P.M. 2001. Medicinal natural product: a biosynthetic approach. 2nd, *John Wiley and Sons*, UK, 167-225.

Du, X., Zhang, C., Guo, W., Jin, W., Liang, Z., Yan, X., Liu, Y. and Yang, D. 2015. Nitric oxide plays a central role in water stress-induced tanshinone production in *Salvia miltiorrhiza* hairy roots. *Molecules*. 20; 7574-7585.

Dutta, A., Sen, J. and Deswal, R. 2013. New evidence about strictosidine synthase (*Str*) regulation by salinity, cold stress and nitric oxide in *Catharanthus roseus*. *J Plant Biochem Biotechnol*. 22; 124-131.

D'Alessandro, S., Posocco, B., Costa, A., Zahariou, G., Schiava, F.L., Carbonera, D. and Zottini, M. 2013.Limits in the use of cPTIO as nitric oxide scavenger and EPR probe in plant cells and seedlings. *Front Plant Sci*. 4; 1-7.

Facchini, P.J. 2001. Alkaloid biosynthesis in plants: Biochemistry, cell biology, molecular regulation, and metabolic engineering applications. *Ann. Rev. Plant Phys.* 52, 29.

Geerlings, A., Ibanez, M.M.L., Memelink, J., van der Heijden, R. and Verpoorte, R. 2000. Molecular cloning and analysis of strictosidine beta-D-glucosidase, an enzyme in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. *J. Biol. Chem.* 275, 3051-3056.

Gerasimenko, I., Sheludko, Y., Ma, X., Stoeckigt, J. 2002. Heterologous expression of a *Rauvolfia* cDNA encoding strictosidine glucosidase, a biosynthetic key to over 2000 monoterpenoid indole alkaloids. *Eur. . Biochem/FEBS*. 269(8), 2204-2213.

Goldstein, S., Russo, A. and Samuni, A. 2003. Reactions of PTIO and Carboxy-PTIO with $^{\bullet}NO$, $^{\bullet}NO_2$ and $O_2^{-\bullet}$. *J Biol Chem.* 278; 50949-50955.

Griess, P. 1879. "Bemerkungen zu der Abhandlung der HH.Weselky und Benedikt Ueber einige Azoverbindungen". *Berichte der Deutschen chemischen Gesellschaft.* 12; 426–428.

Grossi, L. and Angelo, S. 2005. Sodium nitroprusside: mechanism of NO release mediated by sulfhydryl-containing molecules. *J Med Chem.* 48; 2622-2626.

Guirimand, G., Burlat, V., Oudin, A., Lanoue, A., St-Pierre, B. and Courdavault, V. 2009. Optimization of the transient transformation of *Catharanthus roseus* cells by particle bombardment and its application to the subcellular localization of hydroxymethylbutenyl 4-diphosphate synthase and geraniol 10-hydroxylase. *Plant Cell Rep.* 28, 1215-1234.

Hamill, J.D., Robins, R.J. and Rhodes, M.J. 1989. Alkaloid production by transformed root cultures of *Cinchona ledgeriana*. *Planta Med.* 55(4), 354-357.

Houghton, P.J. and Said, I.M. 1986. 3-dehydromitragynine: An alkaloid from *Mitragyna speciosa*. *Phytochemistry* 25(12), 2910-2912.

Houghton, P.J., Latiff, A. and Said, I.M. 1991. Alkaloids from *Mitragyna speciosa*. *Phytochemistry* 30, 347-350.

Hussain, Md.S., Fareed, S., Ansari, S., Rahman, Md.A., Ahmad, I.Z. and Saeed, M. 2012. Current approaches toward production of secondary plant metabolites. *J Pharm Bioallied Sci*. 4; 10-20.

Jumali, S.S., Said, I.M., Ismail, I. and Zainal, Z. 2011a. Genes induced by high concentration of salicylic acid in *Mitragyna speciosa*. *AJCS.* 5(3), 296-303.

Jumali, S.S., Said, I.M., Baharum, S.N., Ismail, I., Rahman, Z.A. and Zainal, Z. 2011b. Molecular cloning and characterization of strictosidine synthase, a key gene in biosynthesis of mitragynine from *Mitragyna speciosa*. *Afr. J. Biotechnol.* 10(68), 15238-15244.

Keawpradub, N. 1990. Alkaloids from the fresh leaves of *Mitragyna speciosa*. Master Thesis, Graduate School, Chulalongkorn University, Bangkok.

Kikura-Hanajiri, R., Kawamura, M., Maruyama, T., Kitajima, M., Takayama, H. and Goda, Y. 2009. Simultaneous analysis of mitragynine, 7-hydroxymitragynine, and other alkaloids in the psychotropic plant "kratom" (*Mitragyna speciosa*) by LC-ESI-MS. *Forensic. Toxicol.* 27, 67-74.

Kitajima, M., Misawa, K., Kogure, N., Said, I.M., Horie, S., Hatori, Y., Murayama, T. and Takayama, H. 2006. A new indole alkaloid, 7-hydroxyspeciociliatine, from the fruit of Malaysian *Mitragyna speciosa* and its opioid agonistic activity. *J. Nat. Med.* 60, 28-35.

Kumarnsit, E., Vongvatcharanon, U., Keawpradub, N. and Intasaro, P. 2007a. Fos-like immunoreactivity in rat dorsal rape nuclei induced by alkaloid extract of *Mitragyna speciosa*. *Neurosci. Lett.* 416, 128-132.

Li, M. and Peebles, C.A.M. 2011. Effect of sodium nitroprusside on growth and terpenoid indole alkaloid production in *Catharanthus roseus* hairy root cultures. *Biotechnol Prog*. 27; 625-630.

Luijendijk, T.J.C., Nowak, A., Verpoorte, R. 1996. Strictosidine glucosidase from suspension cultured cells of *Tabernaemontana divaricata*. *Phytochemistry*. 41(6), 1451-1456.

Luijendijk, T.J.C., Stevens, L.H., Verpoorte, R. 1998. Purification and characterization of strictosidine β-D-glucosidase from *Catharanthus roseus* cell suspension cultures. *Plant Physiol Biochem*. 36(6), 419-425.

Matsumoto, K., Mizowaki, M., Thongpraditchote, S., Murakami, Y., Takayama, H., Sakai, S-L., Aimi, N. and Watanabe, H. 1996a. Central antinociceptive effects of mitragynine in mice: contribution of descending noradrenergic and serotonergic pathways. *Eur. J. Pharmacol*. 371, 75-81.

Matsumoto, K., Mizowaki, M., Thongpraditchote, S., Takayama, H., Sakai, S-L., Aimi, N. and Watanabe, H. 1996b. Antinociceptive action of mitragynine in mice: evidence for the involvement of supraspinal opioid recptors. *Life Sci*. 59, 1149-1155.

Md Zuldin, N., Md. Said, I., Md Noor, N., Zainal, Z., Jin Kiat, C. and Ismail, I. 2013. Induction and analysis of the alkaloid mitragynine content of a *Mitragyna speciosa* suspension culture system upon elicitation and precursor feeding. *Sci World J*. 2013; 1-11.

Nagakura, N., Rueffer, M. and Zenk M.H. 1979. The biosynthesis of monoterpenoid indole alkaloids from strictosidine. *J. Chem. Soc.* (Perkin I), 2308-2312.

Pan, Y.J., Liu, J., Guo, X.R., Zu, Y.G.and Tang, Z.H. 2014. Gene transcript profiles of the TIA biosynthetic pathway in response to ethylene and copper reveal their interactive role in modulation TIA biosynthesis in *Catharanthus roseus*. *Protoplasma*. 252; 813-824.

Phongprueksapattana , S. 2007. Induction of *Agrobacterium rhizogenes* Transformed Hairy Root cultures from *Mitragyna speciosa* (Roxb.) Korth. Ms.C Thesis, Prince of Songkla University, Thailand.

Phongprueksapattana, S., Putalun, W., Keawpradub, N. and Wungsintaweekul, J. 2008. *Mitragyna speciosa*: hairy root culture for triterpenoid production and high yield of mitragynine by regenerated plants.*Z Naturforsch C*.63; 691-8.

Ponglux, D., Wongseripipatana, S., Takayama, H., Kukuchi, M., Kukihara, M., Kitayama, M., Aimi, N. and Sakai, S. 1994. A new indole alkaloid, 7 alpha-hydroxy-7H-mitragynine, from *Mitragyna speciosa* in Thailand. *Planta Med.* 60, 580-581.

Razdan M.K. 2002. Introduction to plant tissue culture (2nd edition).General applications; *Industrial applications; Secondary metabolite production*.Science publisher, Plymouth, U.K.; 263-278.

Rueffer, M., Nagakura, N. and Zenk, M. 1978. Strictosidine, the common precursor for monoterpernoid indole alkaloids with 3 α and 3 β configuration. *Tetrahedron Lett*. 18; 1593-1596.

Shaik Mossadeq, W.M., Sulaiman, M.R., Tengku Muhamad, T.A., Chiong H.S., Zakaria, Z.A., Jabit, M.L., Baharuldin, M.T.H. and Israf, D.A. 2009. Anti-inflammatory and antinociceptive effects of *Mitragyna speciosa* Korth methanolic extract. *Med. Princ. Pract.* 18, 378-384.

Shellard, E.J. 1974. The alkaloids of Mitragyna with special reference to those of *M. speciosa* Korth. *Bull. Narcotics* 26(2), 41-55.

Shellard, E.J., Houghton, P.J. and Resha, M. 1978a. The *Mitragyna* species of Asia part XXXI. The alkaloids of *Mitragyna speciosa* Korth. from Thailand. *Planta Med.* 34, 26-36.

Shellard, E.J., Houghton, P.J. and Resha, M. 1978b. The *Mitragyna* species of Asia part XXXII. The distribution of alkaloids in young plants of *Mitragyna speciosa* Korth. grown from seed obtained from Thailand. *Planta Med.* 34, 253-263.

Smitinand, T. 2001. *Thai plant name.* Forest Herbarium, National Park, Wildlife and Plant Conservation Department, Prachachon Ltd., Bangkok.

Stevens, L.H., Blom, T.J.M. and Verpoorte, R. 1993. Subcellular localization of tryptophan decarboxylase, strictosidine synthase and strictosidine β-D-glucosidase in suspension cultured cells of *Catharanthus roseus* and *Tabernaemontana divaricata*. *Plant Cell Rep.* 12, 573–576.

St-Pierre, B., Vazquez-Flota F.A. and De Luca, V. 1999. Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. *Plant Cell.* 11, 887-900.

Sukrong, S., Zhu, S., Ruangrungsi, N., Phadungcharoen, T., Palanuvej, C. and Komatsu, K. 2007. Molecular analysis of the genus *Mitragyna* existing in Thailand based on rDNA ITS sequences and its application to identify a narcotic species: *Mitragyna speciosa*. *Biol. Pharm. Bull.* 30(7), 1284-1288.

Suwanlert, S. 1975. A study of kratom eaters in Thailand. *Bull. Narcotics* 27, 21-27.

Takayama, H. 2004. Chemistry and pharmacology of analgesic indole alkaloids from the Rubiaceous plant, *Mitragyna speciosa*. *Chem. Pharm. Bull.* 52, 916-928.

Output จากโครงการวิจัย

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

- อยู่ระหว่างการเตรียมต้นฉบับเพื่อตีพิมพ์ในวารสารนานาชาติ จำนวน 2 เรื่อง

Wungsintaweekul J, Perstwong N, Limsuwanchote S. Nitric oxide modulates mitragynine biosynthesis in *Mitragyna speciosa* (Korth.)Havil. Plant Tissue and Organ Culture (in preparation)

Wungsintaweekul J, Choo-Malee J, Nualsri P, Sarata W, Keawpradub N. In vitro shoot multiplication of *Mitragyna speciosa* and plant hormone-like elicitors promote mitragynine production. In Vitro Cellular & Developmental Biology (in preparation).

2. ผลงานอื่นๆ เช่น การไปเสนอผลงาน การได้รับเชิญเป็นวิทยากร

- นำเสนอผลงานในที่ประชุมวิชาการระดับนานาชาติ จำนวน 2 ครั้ง

Wungsintaweekul J, Sarata W., Nualsri P, Chaykul A, Jeoh H. Precursor feeding and elicitation improve mitragynine production in *Mitragyna speciosa* shoot culture. Meeting abstract: 61st International Conference and Annual Meeting of GA 2013, Muenster, Germany. PN120.

Perstwong N, Wungsintaweekul J. Effect of nitric oxide on mitragynine production in *Mitragyna speciosa* shoot culture. **Meeting abstract:** The 3rd Current Drug Development International Conference (CDD2014). PA56: page 266. Aonang, Krabi, Thailand

2 Manuscripts in preparation

Wungsintaweekul J, Perstwong N, Limsuwanchote S. Nitric oxide modulates mitragynine biosynthesis in *Mitragyna speciosa* (Korth.)Havil. Plant Tissue and Organ Culture (in preparation)

Wungsintaweekul J, Choo-Malee J, Nualsri P, Sarata W, Keawpradub N. In vitro shoot multiplication of *Mitragyna speciosa* and plant hormone-like elicitors promote mitragynine production. In Vitro Cellular & Developmental Biology (in preparation).

2 Meeting Abstracts

Wungsintaweekul J, Sarata W., Nualsri P, Chaykul A, Jeoh H. Precursor feeding and elicitation improve mitragynine production in *Mitragyna speciosa* shoot culture. Meeting abstract: 61st International Conference and Annual Meeting of GA 2013, Muenster, Germany. PN120.

Perstwong N, Wungsintaweekul J. Effect of nitric oxide on mitragynine production in *Mitragyna speciosa* shoot culture. **Meeting abstract:** The 3rd Current Drug Development International Conference (CDD2014). PA56: page 266. Aonang, Krabi, Thailand

Title page

Micropropagation

Short communication

In vitro shoot multiplication of *Mitragyna speciosa* and plant hormone-like elicitors promote mitragynine production

Juraithip Wungsintaweekul*, Jutarat Choo-malee, Penjit Nualsri, Wipawee Sarata, Niwat Keawpradub

Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

*Corresponding author: E-mail address: juraithip.w@psu.ac.th (J. Wungsintaweekul); Tel. +6674288887; Fax +6674428220

Running head

In vitro shoot culture of *Mitragyna speciosa*

Abstract

Mitragyna speciosa (Korth.) Havil. [Rubiaceae] or kratom is a unique source of mitragynine, an opioid agonist and possesses an analgesic effect by non-selective binding to opioid receptors. Shoot proliferation of *Mitragyna speciosa* was achieved from axillary buds by culturing on the McCown Woody Plant medium (WPM), supplemented with 2 mg/L thidiazuron, 1 mg/L benzyladenine, 2% (w/v) sucrose and 0.25% (w/v) plant agar, producing a shoot number of 9.58±0.63 shoots/explant and an average shoot length of 0.48±0.06 cm. Plant hormone-like elicitors including jasmonic acid (0-100 µM), abscisic acid (0-100 µM) and salicylic acid (0-500 µM) were added to the shoot culture. The concentration and time exposure of each elicitor were designed using the response surface methodology (RSM) and central composite design (CCD). At optimal conditions, the elicited shoot culture with jasmonic acid (100 µM; 48 h) increased mitragynine production (0.482±0.021 mg/g DW). In addition, abscisic acid (50 µM; 24 h) and salicylic acid (500 µM; 48 h) increased the mitragynine production to 0.273±0.007 mg/g DW and 0.279±0.016 mg/g DW, respectively when compared to the control (0.116±0.008 mg/g DW).

Keywords: Elicitor, *Mitragyna speciosa*, Mitragynine, Shoot culture, Statistical optimization

Introduction

Mitragyna speciosa (Korth.) Havil. (Family Rubiaceae) or Kratom [Thai] is an endemic plant found in tropical Southeast Asia, especially in Thailand and Malaysia. In folklore medicine, its leaf and bark used as ingredients in decoctions for treatment of diarrhea, amoebiasis, diabetes and hypertension (Sirivong Na Ayudhya and Assanangkornchai 2005). Kratom classified as Narcotic Level 5 in Thailand, according to the Royal Decree on Narcotics, 1979. Kratom traditionally used for diarrhea and as a substitute for opium (Suwanlert 1975). Its leaves have used in the form of chewed, smoked or brewed as tea. The medicine man used kratom's leaves and barks as ingredient in the decoctions for the treatments of diarrhea, amoebiasis, diabetes and hypertension. Morphological variation of leaf classifies kratom into 3 variations in Thailand, which are red-veined leaf (Kan Daeng in Thai), green-veined leaf (Tang Gua) and double dentate acuminate leaf (Yak Yai) (Sukrong *et al*. 2007).

Mitragynine, a monoterpenoid indole alkaloid (MIA), is the most abundant component in *M. speciosa* leaves. It possesses antinociception activity by binding to opioid receptors, preferably μ and δ, unlike morphine that binds specifically to μ receptor. Thus, mitragynine is 10-fold less potent and less addictive than morphine (Watanabe *et al*. 1997; Thongpraditchote *et al*. 1998). Because of its affinity for opioid receptors, mitragynine also acts as an inhibitor of gastric secretion (Tsuchiya *et al*. 2002). Studies on the synthesis of mitragynine-related indole alkaloids have discovered that mitragynine pseudoindoxyl and 7-hydroxymitragynine, have a higher opioid activity and potency than morphine (Takayama *et al*. 2002). With regard to the psychological effects, *M. speciosa* extract has known to have a stimulatory effect on the dorsal raphe nucleus together with an antidepressant-like activity (Kumarnsit *et al*. 2007). In addition, mitragynine can act as an antidiarrheal agent; it reduces increments of body weight (Chittrakarn *et al*. 2008), acts as a muscle relaxant (Chittrakarn *et al*. 2010), alters the working memory (Apryan *et al*. 2010) and is an anti-inflammatory agent (Utar *et al*. 2011). These relevant features of mitragynine have encouraged the pursuit of indole alkaloid derivatives, which have an opioid receptor agonistic effect (Horie *et al*. 2012). Therefore, it highlights mitragynine and its derivatives to be an alternative opioid analgesic drug.

Mitragynine accumulated dominantly in leaf, less in stem and absence in the root (Charoonratana *et al*. 2013). It utilizes strictosidine as an immediate precursor similarly to others MIA (Nagakura *et al*. 1979).

Previously, vegetative propagation of *M. speciosa* reported by cutting the nodes and placing them in pot-soil without the rooting hormone. The complete plant obtained after 2 years of propagation (Ajik *et al*. 2010). The established protocol of micropropagation could provide the same clone of plant and increase number of plant efficiently. Since mitragynine accumulated mostly in the leaves and stems, therefore, the shoot culture was an appropriate plant model for studying on the manipulation and production of mitragynine. Previously, we reported the elicited shoot culture with methyl jasmonate and yeast extract increased mitragynine production by promoting the synthesis of mRNA for tryptophan decarboxylase and strictosidine synthase (Wungsintaweekul *et al*. 2012). In MIA biosynthesis, plant stress hormones such as jasmonic acid, methyl jasmonate exhibited to effect on ajmalicine formation in *C. roseus* (Memelink *et al*. 2001; Lee-Parson *et al*. 2004).

In the present study, we established a protocol for shoot multiplication and micropropagation that enables the production of a valuable opioid analgesic from the red-veined *M. speciosa* variety and grown in a completely controlled environment. The plant hormone-like elicitors such as jasmonic acid, abscisic acid and salicylic acid were added to *M. speciosa* shoot culture. The concentration and time of exposure of each elicitor were statistically designed using response surface methodology. Ability of the elicited shoot culture in mitragynine production was determined.

Materials and Methods

Plant culture Sterilized *M. speciosa* plants grown *in vitro* were subcultured and maintained in the hormone free McCown woody plant (WPM) medium according to Phongprueksapattana *et al*. (2008). Two-month old plant that bore at least three axillary buds used as the starting materials. The axillary buds at the 2nd-3rd position from the top were placed on the hormone-free WPM and incubated at 25±2°C under fluorescence light (3000 lux) for 16 h/day. This protocol served the plantlet for further shoot initiation and proliferation experiments.

Shoot initiation and proliferation Axillary buds were excised from the stems (about 0.5 cm long), and placed vertically on the WPM solid medium supplemented with different thidiazuron (TDZ) concentrations of 0.1, 0.5, 1 or 2 mg/L. The shoot culture was incubated on shelves under a fluorescence light for 16 h/8 h dark cycle period and a temperature of 25±2°C.Using the TDZ optimum concentration of 2 mg/L, various benzyladenine (BA) and kinetin (KN) concentrations of 1, 5 or 10 mg/L were added. After 6 weeks, number of shoot and shoot

length were recorded. Samples were prepared and recorded for at least 12 replicates. Shoot culture that grown in WPM liquid medium (30 mL) were used for elicitor treatments.

Rooting and acclimatization Regenerated young shoots (about 1 cm in length) were transferred to hormone-free WPM containing 2% (w/v) sucrose and 0.8% (w/v) plant agar. Different types of auxins including 3-indole butyric acid (IBA), 1- naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) at concentrations of 1, 5 or 10 mg/L were added. Shoots, that grew in hormone free WPM, were used as controls. The samples were performed in 15 replicates. The morphology and characteristic of the roots were photographed and comparison made with the control culture after 6 weeks of culture. The complete plantlets (2-month old) were removed from the culture medium. Roots were gently but thoroughly washed with running tap water to ensure removal of agar. Plantlets were acclimatized by transferring to pots (diameter 20 cm) containing sterilized soils. To control humidity, a plastic bag was used to cover pot-soil containing plant for 1 week. The plastic bag was gradually opened and plants were watered every day. The pots were transferred to a greenhouse. After 2 months of growth in the pot soil, the percentage survival rate during the acclimatization process was calculated.

Experiment design and statistical analysis *M. speciosa* shoot cultures were elicited with different concentrations and time exposures. The optimization was performed using response surface methodology (RSM) with the central composite design (CCD). The ranges of concentrations of elicitors were 0-100 μM for jasmonic acid (JA) and abscisic acid (ABA) and 0-500 μM for salicylic acid (SA) and the ranges of time exposure were 0-48 h. The experiment design, data analysis, and optimization procedures were conducted using Design-Expert Version 8.0.6 software (Trial version; www.statease.com; Stat-Ease, Inc., Minneapolis, USA). The CCD designed with 2 variables with quadratic model and 3 center points, afforded 11 experiments in total (Table 1). The mitragynine production was chosen as response. The surface response plots were generated after made data input and analyzed to reveal the effect of elicitor on mitragynine production. Analysis of variance (ANOVA) for evaluation of second-order response surface model was performed and the regression equations were suggested.

Elicitors treatments Jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) were elicitors in this study. Stock solutions of each elicitor were prepared and add into the medium on day 14th of culture (at

exponential phase). For jasmonic acid and abscisic acid, the concentrations were ranging from 0-100 µM. For salicylic acid, the concentrations were ranging from 0-500 µM. Time exposures to elicitor were ranging from 0-48 h. Shoot culture were incubated at 25±2°C on shaker (60 rpm). After treatments, shoots were harvested, washed with distilled water, and lyophilized.

Mitragynine content Freeze-dried *M. speciosa* (50 mg) were extracted by refluxing with methanol (10 mL) at 70°C for 1h. The extracts were filtered, then washed with petroleum ether twice, and concentrated under reduced pressure. For determination of mitragynine content, HPLC analysis was carried out using Agilent 1100 series equipped with Photo-diode arrays detector. Data analysis was performed using Agilent software (USA). Chromatographic separation was performed on a reverse phase VertiSep^{TM} UPS C18 column × 4.6 mm i.d. 5 µm particle size (Vertical, Bangkok, Thailand) with binary gradient mobile phase profile [1.5 mL/min, acetonitrile: 100mM H_3PO_4 in water, pH 2.4 (10:90 to 90:10, v:v within15 min, injection volume 20 µL]. The identification of mitragynine based on the retention time and comparison of the absorption spectrum with authentic standards. The quantitative wavelength was set at 225 nm. Samples were analyzed in triplicate.

Statistical analysis Data presented as mean value ± standard deviation (SD) and analyzed using SPSS Version 14.0 (SPSS Inc., Chicago, USA). The mean values compared using the Duncan's multiple range tests at the 5% level of significance ($P < 0.05$).

Results and discussion

Shoot multiplication and micropropagation *In vitro* propagation strategy began with preparations of plant materials. Seeds of *M. speciosa* red-veined variety were collected from natural sources in Southern Thailand. Sterilized seedlings were prepared, and cultured in hormone-free solid WPM. After two months, the axillary buds were excised and used as explants for shoot proliferation and multiplication experiments. TDZ was tested at 0.1, 0.5, 1 and 2 mg/L in WPM for its effect on shoot formation and 2 mg/L TDZ produced the highest shoot number (Table 2).

The effects of combinations of BA or Kn at 1, 5 and 10 mg/L with 2 mg/L TDZ on shoot multiplication were compared. TDZ at 2 mg/L and 1 mg/L BA produced the largest number of shoots of 9.58±0.63 per explant. At higher concentrations of BA and any Kn shoot formation was inhibited (Table 2). Only 1 mg/L BA by itself in solid WPM produced only 2.83±1.47 shoot number per explant (Phongprueksapattana 2007) so this

emphasizes the importance of TDZ in shoot multiplication. TDZ is a cytokinin-like compound, is widely used for shoot multiplication (for instance, addition of 5 µM TDZ in Murashige and Skoog (MS) medium increased the frequency of shoot regeneration of *Rauwolfia tetraphylla* (Faisal et al. 2005). However, the best shoot culture of *M. parvifolia* was achieved in MS medium supplemented with 4.44 µM BA (Roy et al. 1998). Manipulation of different basal media such as MS, Gamborg (B5) and WPM for *M. speciosa* root cultures indicated that the WPM was an appropriate medium for hairy root cultures (Phongprueksapattana 2007). For the indole alkaloid containing plants like *Cinchona ledgeriana* (Hamill et al. 1989) and *Ophiorrhiza pumila* hairy root cultures (Saito et al. 2007), however, required the B5 medium for growing hairy root cultures.

Usually, any rooting procedure is not required for *in vitro* root formation but using an auxin could reduce the time required for root initiation. In this study, three auxins including IBA, NAA and 2,4-D at concentrations of 1, 5, 10 mg/L were studied for inducing roots in comparison with hormone-free WPM. The appearance of roots was recorded after six weeks of culture (Fig. 2). Root length and root thickness are considered to be the best characteristics of roots. The root characteristic of the plantlets in WPM plus IBA at 5 mg/L produced the healthiest looking roots (Fig. 2a (ii)). Higher IBA concentrations (10 mg/L) caused inhibition of root expansion. NAA (at 1 mg/L) increased the root number but the roots looked unhealthy. NAA at concentrations of 5 mg/L and 10 mg/L inhibited induction of roots but induced callus formation. This effect was also found for 2,4-D. For the control culture on hormone-free WPM, the period of root induction needed was about 10 days of culture (Phongprueksapattana 2007) and less rootlets were found when compared to WPM containing IBA (5 mg/L), which took only 5 days for induction of roots.

Plants containing shoots and roots were readied to be transferred to natural conditions by an acclimatization process. Under *in vitro* culture condition, humidity changes and bacterial contamination were avoided. Gradual exposure of the plantlet to the environment was performed to avoid lower survival rates. Acclimatization began by removing the plantlet from the culture bottle and washing the roots gently but thoroughly with water to remove any gelling agent. Sterilized soil was prepared by autoclaving and packed into the pot (20 cm diameter). The complete plant was then put into pot-soil tenderly and watered every day. Plants in pot-soil were covered with a plastic bag during the first week to prevent loss of humidity. Then the plastic bag was removed for watering. Using this acclimatization protocol the survival rate for *M. speciosa* was 60% and plants reached about 15 cm in length after 2 months (Fig. 2). Accumulation of mitragynine was assessed in the regenerated *M. speciosa*. Mature leaves were collected and prepared for the extract prior to HPLC analysis. The regenerated plants produced and accumulated mitragynine, in an age dependent manner (Table 2).

Previously, vegetative propagation of *M. speciosa* has been reported by cutting the nodes and placing them in pot-soil without the rooting hormone. The complete plant was obtained after 2 years of propagation (Ajik et al. 2010). The established protocol of micropropagation could provide the same clone of plant and increase number of plant efficiently. In this study, the combination of TDZ (2 mg/L) and BA (1 mg/L) increased the number of shoots, and IBA (5 mg/L) induced root formation. The benefit of using this protocol has been demonstrated in the biosynthetic study of mitragynine. Since mitragynine accumulated mostly in the leaves and stems (Charoonratana 2012), therefore, the shoot culture was appropriate for the study on the regulation of mitragynine production. The elicited shoot culture with methyl jasmonate and yeast extract increased mitragynine production by promoting the synthesis of mRNA for tryptophan decarboxylase and strictosidine synthase (Wungsintaweekul et al. 2012). This is different from the *M. speciosa* hairy root culture, induced by *Agrobacterium rhizogenes* that was unable to produce any alkaloids but only triterpenoids (Phongprueksapattana et al. 2008). The present study reports a micropropagation protocol for *M. speciosa*, a valuable medicinal plant for being a possible source of an analgesic agent. The most important objective was to facilitate studies on mitragynine biosynthesis by using this propagated plant rather than those produced for commercial purposes. Because *M. speciosa* is the only *Mitragyna* spp. in Thailand, that has been designated as a Narcotic Plant Level V. In addition the establishment of callus and suspension cultures could provide alternative methods to investigate mitragynine formation, but this has not been achieved. These sterilized plantlets obtained from this micropropagation are therefore, ideal materials for further investigations.

Acknowledgement

Authors thank the Thailand Research Fund, the Office of Higher Education Commission and Prince of Songkla University (grant nos. RMU5380015; PHA520146S) for financial supports. We also thank Dr. Brian Hodgson for assistance with the English.

References

Ajik M, Kimjus K (2010) Vegetative propagation of Sepat (*Mitragyna speciosa*). Sepilok Bull 12:1-11

Apryani E, Hidayat MT, Moklasa MAA, Fakurazia S, Idayu NF (2010) Effects of *Mitragyna speciosa* Korth. leaves on working memory. J Ethnopharmacol 129(3):357-360

Charoonratana T (2012) cDNA cloning, enzymatic characterization and genetic transformation of tryptophan decarboxylase in *Mitragyna speciosa*. Ph.D. Thesis, Prince of Songkla University, Thailand

Chittrakarn S, Keawpradub N, Sawangjaroen K, Kansenalak P, Janchawee B (2010) The neuromuscular blockade produced by pure alkaloid, mitragynine and methanol extract of kratom leaves (*Mitragyna speciosa* Korth.). J Ethnopharmacol 129(3):344-349

Chittrakarn S, Sawangjaroen K, Prasettho S, Janchawee B, Keawpradub N (2008) Inhibitory effects of kratom leaf extract (*Mitragyna speciosa* Korth.) on the rat gastrointestinal tract. J Ethnopharmacol 116(1):173-178

Faisal M, Ahmad N, Anis M (2005) Shoot multiplication of *Rauvolfia tetraphylla* L. using thidiazuron. Phytochemistry Rev 6:277-305

Gao B, Stiles AR, Liu CA (2012) Thidiazuron enhances shoot organogenesis from leaf explants of *Saussurea involucrata* Kar. Et Kir. In Vitro Cell Dev Biol-Plant 48(6):609-612

Hamill JD, Robins RJ, Rhodes MJC (1989) Alkaloid production by transformed root cultures of *Cinchona ledgeriana*. Planta Med 55:354-357

Horie S, Kitajima M, Matsumoto K, Takayama H (2012) Indole alkaloid derivatives having opioid receptor agonistic effect, and therapeutic compositions and methods relating the same. US patent: US8247428 B2

Kumarnsit E, Vongvatcharanon U, Keawpradub N, Intasaro P (2007) Fos-like immunoreactivity in rat dorsal rape nuclei induced by alkaloid extract of *Mitragyna speciosa*. Neurosci Lett 416:128-132

Phongprueksapattana S (2007) Induction of *Agrobacterium rhizogenes* transformed hairy root culture from *Mitragyna speciosa* (Roxb.) Korth., Master Thesis, Prince of Songkla University, Thailand

Phongprueksapattana S, Putalun W, Keawpradub N, Wungsintaweekul J (2008) *Mitragyna speciosa*: hairy root culture for triterpenoid production and high yield of mitragynine by regenerated plants. Z Naturforsch 63c:691-698.

Roy S, Rahman L, Datta PC (1988) In vitro propagation of *Mitragyna parvifolia* Korth. Plant Cell Tiss Org Cult 12:75-80

Saingam D, Assanangkornchai S, Geater AF, Balthip Q (2012) Pattern and consequences of Karthom (*Mitragyna speciosa* Korth.) use among male villagers in southern Thailand: a qualitative study. Int J Drug Policy, http://dx.doi.org/10.1016/j.drugpo.2012.09.004

Saito K, Sudo H, Yamazaki M, Koseki-Nakamura M, Kitajima M, Takayama H, Aimi N (2007) Feasible production of camptothecin by hairy root culture by hairy root culture of *Ophiorrhiza pumila*. Plant Cell Rep 20:267-271

Sirivong Na Ayudhya A, Assanagkornchai S (Eds) (2005) Kratom plant in Thai society: Culture, behavior, health, science, laws. Ministry of Justice, Bangkok

Takayama H, Ishikawa H, Kurihara M, Kitajima M, Aimi N, Ponglux D, Koyama F, Matsumoto K, Moriyama T, Yamamoto LT, Watanabe K, Murayama T, Horie S (2002) Studies on the synthesis and opioid agonistic activities of mitragynine-related indole alkaloids: discovery of opioid agonists structurally different from other opioid ligands. J Med Chem 45:1949-1956

Thongpraditchote S, Matsumoto K, Tohda M, Takayama H, Aimi N, Sakai S, Watanabe H (1998) Identification of opioid receptor subtypes in antinociceptive actions of supraspinally-administered mitragynine in mice. Life Sci 62:1371-1378

Tikhomiroff C, Jolicoeur M (2002) Screening of *Catharanthus roseus* secondary metabolites by high performance liquid chromatography. J Chromatograph A 443:185-188

Tsuchiya S, Miyashita S, Yamamoto M, Horie S, Sakai S, Aimi N, Takayama H, Watanabe K (2002) Effect of mitragynine, derived from Thai folk medicine, on gastric acid secretion through opioid receptor in anesthetized rats. Eur J Pharmacol 443:185–188

Utar Z, Majid MIA, Adenan MI, Jamil MFA, Lan TM (2011) Mitragynine inhibits the COX-2 mRNA expression and prostaglandin E2 production induced by lipopolysaccharide in RAW264.7 macrophage cells. J Ethnopharmacol 136:75-82

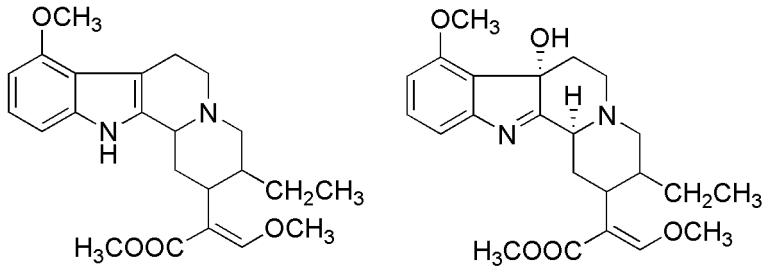
Wungsintaweekul J, Choo-malee J, Charoonratana T, Keawpradub N (2012) Methyl jasmonate and yeast extract stimulate mitragynine production in *Mitragyna speciosa* (Roxb.) Korth. shoot culture. Biotechnol Lett 34:1945-1950

Figure caption

Figure 1. Structures of mitragynine and 7-hydroxymitragynine

Figure 2. Micropropagation of *Mitragyna speciosa* (Korth.) Havil. (*a*) Shoot proliferation on WPM medium with 2 mg/L thidiazuron and 1 mg/L benzyladenine after 2 months, (*b*) Shoot culture in shoot proliferation liquid medium after 2 weeks, (*c*) Plant culture of cutting shoot on hormone-free WPM medium after 4 weeks, and (*d*) Acclimatized plant in pot soil after 3 months.

Figure 3. Response surface plots between concentration (μM) and time (h) on mitragynine content after elicitation with (*a*) jasmonic acid, (*b*) abscisic acid, and (*c*) salicylic acid



mitragynine 7-hydroxymitragynine

Figure 1

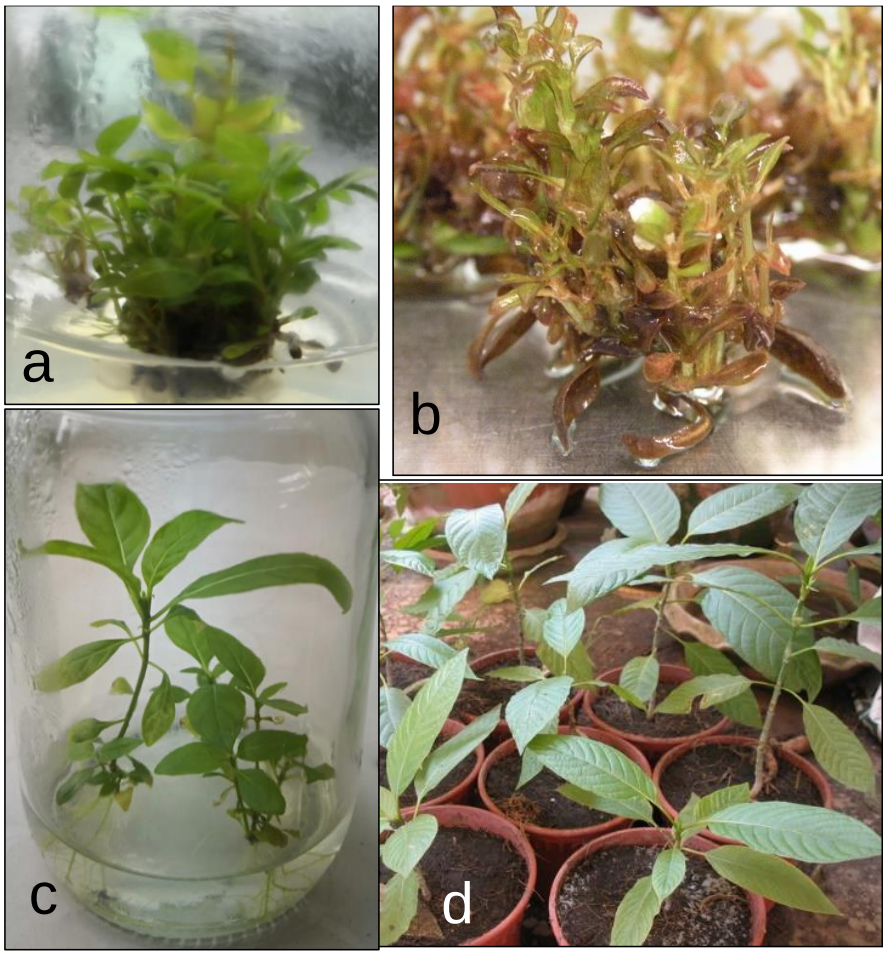


Figure 2

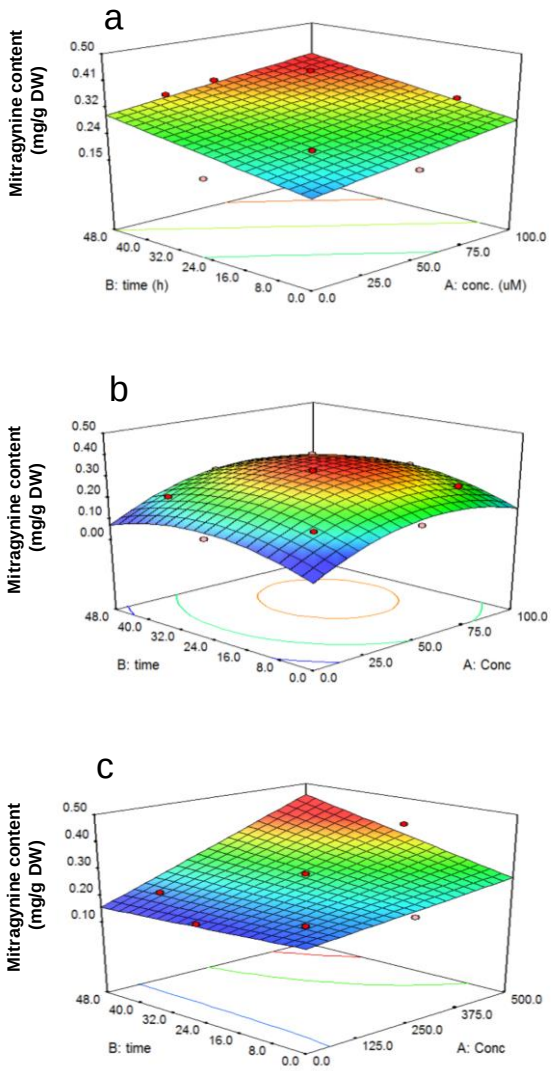

a
Mitragynine content (mg/g DW)
0.50
0.41
0.32
0.24
0.15
48.0
40.0
32.0
24.0
16.0
8.0
0.0
B: time (h)
0.0
25.0
50.0
75.0
100.0
A: conc. (uM)
b
Mitragynine content (mg/g DW)
0.50
0.40
0.30
0.20
0.10
0.00
B: time
A: Conc
c
Mitragynine content (mg/g DW)
0.50
0.40
0.30
0.20
0.10
B: time
A: Conc
125.0
250.0
375.0
500.0

Figure 3

Table 1 Operating variables and levels of elicitors including jasmonic acid (JA), abscisic acid (ABA) and salicylic acid (SA) using response surface methodology (RSM) and central composite design (CCD)[a]

Treatment	For JA and ABA		For SA	
	Conc. (μM)	Time (h)	Conc. (μM)	Time (h)
1	0	24	427	41
2	50	48	250	24
3	50	24	250	0
4	50	24	0	24
5	15	41	250	24
6	85	41	250	24
7	100	24	250	48
8	85	7	73	41
9	50	24	427	7
10	50	0	500	24
11	15	7	73	7

[a]Design of experiment (DOE) was performed when two variables (concentration and time) and two responses (dry weight and mitragynine content). Central composite design with 2 factors, model was quadratic: 8 models and 3 center points.

Table 2 Effect of thidiazuron (TDZ), benzyladenine (BA) and kinetin (KN) and in combination on *in vitro* shoot proliferation of *M. speciosa* after 6 weeks of culture

Cytokinin (mg/L)			Number of shoots per explant	Shoot length (cm)
TDZ	BA	KN		
0	0	0	2.54±0.36 f	0.44±0.05 b
0.1	0	0	5.33±0.28 c	0.57±0.05 b
0.5	0	0	6.08±0.26 bc	0.49±0.05 b
1.0	0	0	4.25±0.43 d	0.87±0.18 a
2.0	0	0	6.50±0.26 b	0.48±0.05 b
2.0	1.0	0	9.58±0.63 a	0.48±0.06 b
2.0	5.0	0	4.17±0.40 d	0.33±0.07 b
2.0	5.0	0	3.00±0.52 e	0.22±0.04 bc
2.0	0	1.0	3.67±1.32 de	0.31±0.03 b
2.0	0	5.0	2.17±0.17 f	0.14±0.02 c
2.0	0	10.0	2.33±0.22 f	0.20±0.03 bc

Data represent mean ± SD of 12 replicates. Values followed by the same letter are not significantly different at $P<0.05$ according to Duncan's multiple range test.

Table 3 Experimental values for the responses on mitragynine content after treatment with jasmonic acid (JA), abscisic acid (ABA) and salicylic acid (SA)

Treatment	Response: mitragynine content[a] (mg/g DW)		
	JA	ABA	SA
1	0.176±0.005 a	0.115±0.019 a	0.381±0.031 a
2	0.353±0.019 b	0.244±0.021 c	0.261±0.018 b
3	0.274±0.000 c	0.327±0.023 f	0.208±0.003 c
4	0.269±0.012 c	0.316±0.002 f	0.182±0.007 c
5	0.368±0.019 b	0.205±0.020 b	0.259±0.004 b
6	0.363±0.023 b	0.282±0.005 de	0.282±0.016 b
7	0.307±0.042 c	0.268±0.029 cd	0.282±0.015 b
8	0.357±0.004 b	0.257±0.021 cd	0.212±0.025 c
9	0.276±0.013 c	0.307±0.003 ef	0.269±0.002 b
10	0.199±0.020 a	0.178±0.005 b	0.399±0.033 a
11	0.292±0.029 c	0.197±0.008 b	0.213±0.047 c

[a] Data represent mean ± SD of triplicate experiments. Values followed by the same letter are not significantly different at $P<0.05$ according to Duncan's multiple range test.

Table 4 The regression coefficient of suggested models for the response on mitragynine content

Coefficient	Mitragynine content (mg/g DW)		
	JA	ABA	SA
Model sum of squares	Linear	Quadratic	Two-factor interaction
R squared (R^2)	0.4437	0.9164	0.9302
Coefficient of variation (CV)	18.77	10.94	8.13
P-value[a]	0.0957	0.0100	0.0002

[a]*P*-value < 0.05 = significant; *P*-value > 0.05 = not significant

Table 5 Predicted and actual values of mitragynine content after elicitation

Sample (conc.;time)	Mitragynine content (mg/g DW)		*P*-value[a]
	Predicted	Actual	
Control (without elicitor; 24 h)		0.116±0.008	
Control (without elicitor; 48 h)		0.188±0.030	
Jasmonic acid (100 µM; 48 h)	0.346±0.055	0.452±0.021	0.035
Abscisic acid (50 µM; 24 h)	0.317±0.027	0.273±0.007	0.084
Salicylic acid (500 µM; 48 h)	0.456±0.022	0.280±0.017	0.009

[a]The mean values compared between the predicted and actual values using Excel Student *t*-test.

Nitric oxide modulates mitragynine biosynthesis in *Mitragyna speciosa* (Korth.)Havil.

Communicated by XX

Key message Sodium nitroprusside (SNP) releases nitric oxide (NO) and enhances the transcription profiles of mitragynine-biosynthetic genes. This is a first report that SNP could trigger the production of secologanin in *Mitragyna speciosa* and thus enhance mitragynine biosynthesis.

Title page

Juraithip Wungsintaweekul[1,*], Naruemon Perstwong[1], Supattra Limsuwanchote[2]

Nitric oxide modulates mitragynine biosynthesis in *Mitragyna speciosa* (Korth.)Havil.

[1]Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, [2]Department of Pharmacology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

*Corresponding author: J. Wungsintaweekul

E-mail address: juraithip.w@psu.ac.th; Telephone and fax: +6674 428220

ORCID number: 0000-0002-4711-3606

Abstract

Mitragynine is an alternative opioid analgesic drug and only accumulated in *Mitragyna speciosa* (Korth.)Havil. Effect of sodium nitroprusside (SNP), a nitric oxide (NO) donor, was studied in *M. speciosa*, on mitragynine and secologanin productions. The results indicated that SNP did not alter the mitragynine content (3.23±0.21 mg g^{-1} DW of control culture), however, *M. speciosa*, elicited with 1 mM SNP for 48 h, produced and accumulated the secologanin content of 8.51±0.55 mg g^{-1} DW (compared with 3.05±0.25 mg g^{-1} DW of the control culture). Treatment with 2 mM of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO, a NO scavenger) significantly suppressed secologanin production from 6.06±0.10 mg/g DW to 3.21±0.68 mg g^{-1} DW. Treatment the culture with 1 mM nifedipine (a calcium channel blocker) blocked secologanin formation to 2.42±0.44 mg g^{-1} DW. This evidence suggested the involvement of calcium channel in the NO response. Transcription profile analysis of genes-associated with mitragynine biosynthesis revealed that plants, treated with 1 mM SNP for 48 h, enhanced the mRNA levels and reduced when CPTIO or nifedipine were added. The present study concluded that NO promote mitragynine biosynthesis by triggering their biosynthetic genes and enhanced the production of secondary metabolite in *M. speciosa*.

Keywords Nitric oxide, Sodium nitroprusside, *Mitragyna speciosa*, Mitragynine, Calcium channel

Introduction

In the fundamental of plant growth process, the oxygen molecule is a by-product of respiration and photosynthesis processes in plant cell. Oxygen can lead to the reactive oxygen species (ROS). Various plant stresses could produce the ROS in the physiological system (Agarwal and Zhu 2005). A bioactive signaling molecule such as nitric oxide (NO) was interestingly used to the study of secondary metabolites production (Zhang et al. 2012) and physiological responses (Domingos et al. 2015). There are two sources of NO in the plant cell including endogenous NO and exogenous NO. Plant cell produces the endogenous NO by enzymatic reactions, similarly in animal cell for instance, nitrite: NO-reductase (Ni-NOR), horseradish peroxidase, cytochrome P450, catalase and haemoglobin (del Río et al. 2004; Arasimowicz and Floryszak-Wieczorek 2007). The exogenous NO was induced from pathogens for instance, yeast extract (Xu et al. 2005), NO donor such as sodium nitroprusside (SNP), *S*-nitroso-N-acetylpenicillamine (SNAP) and *S*-nitrosoglutathione (Arasimowicz and Floryszak-Wieczorek 2007). Role of NO has been extensively studied in higher plant. Recently, many reports demonstrated that NO increased the production of secondary metabolites such as hypericin in *Hypericum perforatum* suspension culture (Xu et al. 2005), indole alkaloids (ajmalicine and catharathine) in *Catharanthus roseus* suspension culture (Xu and Dong 2005), shikonin in *Onosma paniculatum* (Wu et al. 2009), camptothecin in *Camptotheca acuminata* suspension culture (Lu et al. 2011), tanshinone in *Salvia miltiorrhiza* hairy root culture (Liang et al. 2012).

Mitragyna speciosa (Korth.)Havil. or kratom belongs to the Rubiaceae family. *M. speciosa* exhibited numerous pharmacological activities. The psychoactive compounds such as mitragynine and its derivative like 7-hydroxy-7H-mitragynine are opioid agonists (Takayama et al. 2002). Several pharmacological activities of mitragynine have been reported such as antidiarrheal (Chittrakarn et al. 2008), antioxidant and antibacterial (Parthasarathy et al. 2009), muscle relaxant (Chittrakarn et al. 2010), anti-inflammatory (Utar et al. 2011), antidepressant (Idayu et al. 2011) and antidiabetic activities (Purintrapiban et al. 2011). The mitragynine constitutes as indole alkaloid and mostly present in young leaves approximately 66% (w/w) of the total alkaloid content (Takayama 2004).

For this reason, mitragynine is a molecule of interest, nowadays, and being a candidate for oral analgesic drug. Based on the structure of mitragynine, it is similarly to the structure of strictosidine, a common precursor of indole alkaloids. Strictosidine is a condensation product of tryptamine and secologanin catalyzed by strictosidine synthase (*STR*) (Rueffer et al. 1978). Tryptamine is a decarboxylation product of tryptophan catalyzed by tryptophan decarboxylase (*TDC*) (Charoonratana et al. 2013a) Tryptophan is a catalyzed product of

anthranilate in shikimate pathway. On the other hand, loganin converted to secologanin by secologanin synthase. Loganin is an iridoid, a condensation product from the methylerythritol phosphate (MEP) pathway (Contin et al. 1998). Nowadays, the regulation genes in the mitragynine biosynthesis were successfully cloned, and presented in NCBI databases (Fig. 1).

Recently, there are reports about strategies for mitragynine production in the *M. speciosa* tissue cultures. For instance, *Agrobacterium rhizogenes* ATCC 15834-induced hairy root culture, on McCown woody plant (WPM) medium, had no mitragynine production, however, it produced triterpenoids (ursolic acid and oleanolic acid) and phytosterols (β-sitosterol and stigmasterol) (Phongprueksapattana et al. 2008). Shoot culture in WPM supplemented with thidiazuron (TDZ) and 6-benzylaminopurine (BA) enhanced the mitragynine production when elicited with methyl jasmonate and yeast extract, caused by the increasing of *TDC* and *STR* genes expressions (Wungsintaweekul et al. 2012). *M. speciosa* callus culture has been reported from petiole by incubating on 4 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D). Treatment of 250 mg L^{-1} of yeast extract on suspension culture was significantly increased mitragynine production up to 1.5-fold higher than control (Md Zuldin et al. 2013).

In this study, we consequently established an in vitro plant to be our model for enhancing the mitragynine and secologanin productions by using elicitation technique. The chemical elicitors such as nitric oxide donor like SNP, nitric oxide scavenger and Ca^{2+}channel blocker were investigated. Transcription profiles analysis of genes in mitragynine biosynthesis were measured for their expressions by qRT-PCR technique. Regulatory mechanism by NO in mitragynine biosynthesis in *M. speciosa* was discussed.

Materials and methods

Plant materials

Seeds of *M. speciosa* were sterilized in 2% (v/v) of sodium hypochlorite for 10 min and washed thoroughly with sterile water (3 times). The sterilized seeds were washed with 1% (v/v) of hydrogen peroxide (2 times) for 2 min to get rid of an excess of sodium hypochlorite. After washing with sterilized distilled water (3 times), the sterilized seeds were placed on hormone-free solid McCown woody plant medium (WPM) (Phytotechnology Laboratories, KS, USA), and incubated at temperature of 25±2 ºC in dark condition for 2 weeks. Then, the plant cultures were placed under light for 16 h day^{-1}. The 2 month-old plant were subcultured and maintained under the culture condition. The six-week old plant were used as material for elicitation.

Treatment with sodium nitroprusside (SNP)

SNP in crystalline form, Na_2 $[Fe(CN)_5NO]\cdot 2H_2O$ is a chemically NO donor. Stock solution of SNP (20 mM) (CAS; 13755-38-9, Sigma-Aldrich, USA) was freshly prepared in sterile distilled water. The six-week old plant was removed from agar medium. Then placed in SNP solutions at the concentrations of 0.25, 0.5, 1, 2.5, 5 and 10 mM. Control was the plant culture that put in the distilled water. The SNP-treated plant were incubated under dark condition for 1, 12, 24, and 48 h. Plants were harvested and lyophilized.

Treatment with NO scavenger

The NO scavenger, 4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) potassium salt (Sigma Aldrich, USA), was freshly prepared at the concentration of 31.7 mM in sterile distilled water. The six-week old plants were treated with CPTIO solution by adding into 10 mL WPM to final concentration of 1 mM and 2 mM for 30 min, respectively. Later, SNP solution was added at concentration of 1 mM. Plant culture that kept in distilled water was used as control. The plant cultures were incubated under dark condition for 48 h. Plants were harvested and lyophilized.

Treatment with nifedipine

The calcium channel blocker, nifedipine (STADA pharm GmBH, Germany), was dissolved in 2% (v/v) ethanol in water and added into six-week old plant culture. The culture was treated with nifedipine at the concentrations of 0.5 and 1 mM for 30 min before the SNP was added to 1 mM. Plant culture that kept in 2% (v/v) ethanol in water was used as control. The culture was incubated under dark condition for 48 h. Plants were harvested and lyophilized.

Assay for nitrite content

Nitric oxide (NO) was measured in the form of nitrite content (NO_2^-) in the culture medium using Greiss assay (Griess 1879; Mur et al. 2011). The experiment was performed in 96-well plate. After treated plant culture, the medium was separated, and immediately measured for nitrite content. The culture medium (50 µL, diluted as appropriate) was reacted with 1% (w/v) sulfanilamide (50 µL) (Sigma Aldrich, USA) and incubated at room temperature under dark condition for 10 min. Then, 0.1% (w/v) *N*-1-napthylethylenediamine (50 µL) (Sigma Aldrich, USA) was added into the mixture and further incubated for 10 min under the same condition. The

absorbance at 570 nm was measured using microplate reader. The nitrite content was calculated according to the nitrite standard curve. Distilled water, interacted with Greiss reagent, was used as blank.

For construction of nitrite standard curve, sodium nitrite ($NaNO_2$) at 0.1 M was freshly prepared in distilled water. The solution was diluted to make the concentration of 100 µM. The 100 µM nitrite solution was immediately performed 6 serial two-fold dilutions (50 µL/well) to afforded the concentrations of 100, 50, 12.5, 6.25, 3.13 and 1.56 µM, respectively. Nitrite content was determined as mentioned above. The correlation between nitrite concentration and OD at 570 nm was plotted.

Quantification of secologanin and mitragynine contents

Contents of secologanin and mitragynine were determined using HPLC as previously described (Wungsintaweekul et al. 2012). After SNP treatment, plant cultures were harvested, lyophilized and ground. For crude extract preparation, 50 mg of sample was weighed and 10 mL of methanol was added. The mixture was sonicated for 10 min at room temperature. The mixture was then refluxed at 80°C for 30 min and filtered. To remove chlorophyll, the filtrate was partitioned with petroleum ether (10 mL, 5 times). The methanol layer was collected, pooled and evaporated to dryness under vacuum.

HPLC analysis was performed using Agilent 1100 series equipped with photodiode array detector. Column was Vertisep^{TM} USP C18 HPLC column (4.6 x 250 nm, 5 µm). The compounds were eluted by gradient elution using a mobile phase: 100 mM H_3PO_4, pH 2.4: acetonitrile; 90:10 for 5 min and gradient to 10:90 within 15 min. Injection volume was 20 µL and the flow rate was 1.5 mL min^{-1}. Mitragynine was detected at UV wavelength 225 nm and secologanin was at 238 nm. Calibration curves of mitragynine and secologanin were constructed. Contents of secologanin and mitragynine were expressed as mg per g dry weight (DW).

Total RNA isolation

The treated plant was harvested and roots were removed. Then, washed with sterile distilled water, immediately frozen in liquid nitrogen and kept at -80°C until used.

Total RNA was isolated using Total RNA mini kit (plant) (Geneaid, Taiwan). According to manufacturer's protocol, plant material was ground to powder in the presence of liquid nitrogen, weighed (300 mg) and transferred to sterile microcentrifuge tube. The, RB buffer (500 µL) plus 5 µL of β-mercaptoethanol was added and mixed thoroughly with vortex. The mixture was then incubated at 60°C for 5 min. After centrifuge at 17,970 x g for 1 min, supernatant was loaded on filter column and centrifuged at 90 x g for 1 min. The clarified

filtrate was collected. The chilled ethanol (½ volume) was added into the filtrate and shake vigorously. The mixture was loaded on the top of RB column and centrifuged at 17,970 x g for 1 min, discarded flow-through. To remove DNA, 100 µl of DNase I (2 KU mL^{-1}) which was mixed in 100 µL of 2x DNase reaction buffer (20 mM Tris-HCl pH 7.6,5 mM $MnCl_2$, 1 mM $CaCl_2$ at 25°C) was loaded onto the RB column and incubated at room temperature for 10 min. For washing step, W1 buffer (400 µL) was loaded into the center of the RB column, centrifuged at 17,970 x g for 30 s and discarded flow-through. Wash buffer (600 µL, 2 times) was loaded and centrifuged. To dry the RB column, the RB column was transferred to a new centrifuge tube and centrifuge at 17,970 x g for 2 min (2 times). The dried RB-column was transferred to a new centrifuge tube, RNase-free water (50 µL) was loaded to the center of the column matrix and incubated for 2 min, then the total RNA was harvested by centrifuge at 17,970 x g for 1 min. The purified RNA was collected. The concentration and purity of the total RNA were determined using UV spectrophotometer (GENESYS 6, USA).

Quantitative real-time polymerase chain reaction (qPCR)

Transcription profile of genes involved in mitragynine biosynthesis in *M. speciosa* was determined using qRT-PCR technique using the ABI Prism® 7300 Fast Real-Time PCR system. Primers, used in this study, are listed in Table 1 (Charoonratana et al. 2013b).

According to manufacturer's protocol of One Step SYBR® PrimeScript™ RT-PCR kit II (Perfect Real Time, Takara, Japan), the cDNA was synthesized using PrimeScript reverse transcriptase and the PCR amplification with Takara Ex Taq HS DNA polymerase within one tube. PCR amplification products were monitored using SYBR® Green I detection. The qPCR was prepared in 96-tube PCR plate. Non-template (no RNA) and non-primers reaction were prepared for checking unspecific qPCR reaction. The reaction mixture (20 µL) composed of 1x One step RT-PCR buffer IV, 1 unit Primescript 1 step Enzyme Mix II, 0.4 µM of forward primer, 0.4 µM of reverse primer, 1x ROX dye and 200 ng of total RNA. The amplification temperature was 42°C for 5 min, 95°C for 10 s, 40 cycles of 95°C for 5 s, 60°C for 31 s following the 95°C for 15 s, 60°C for 1 min. After the qPCR is completed, the amplification plot and dissociation curve were recorded and verified. The number of cycle at threshold line (0.2) was recorded (C_T). The endogenous gene was *18S rRNA*. The control treatment was used as calibrator. The mRNA level of gene was expressed as relative quantitation (RQ) using the following equation ($RQ = 2^{-\Delta\Delta C_T}$).

Statistical analysis

Set of data was presented in mean ± S.D. Statistical analysis was performed using the student pair *t*-test by Microsoft Excel. *P* values of < 0.05 and < 0.01 were considered as statistically significant of 95% and 99% confidential, respectively.

Results

Effect of sodium nitroprusside (SNP) in M. speciosa plant culture

M. speciosa plant culture was maintained and sub-cultured on hormone-free WPM medium. SNP was added into *M. speciosa* plant culture. NO released from SNP by reacting with glutathione in the cells and trans-eliminated of the cyanide group through the formation of the protonated cyanide. Then the thiol group acted as a reducing agent and carrier of NO. Finally, NO was released to the cells (Grossi and D'Angelo 2005). We followed the effect of NO on the secondary metabolites i.e. mitragynine and secologanin productions.

Firstly, degradation of SNP in the treated vehicles such as water, WPM and WPM plus hormones was studied in light and dark conditions for 1, 2 and 3 days. It suggested that treatment of plant with SNP should be perform in the WPM medium and in dark condition (data not shown). Fig. 2 summarizes the nitrite content, mitragynine and secologanin productions after treatment with SNP. Treatments of various concentrations of SNP at 0.25, 0.5, 1, 2.5, 5 and 10 mM to the six week-old plant for 12, 24 and 48 h were performed. Under dark condition, the kinetic of SNP releasing NO was dependent upon SNP concentrations and time. It can be noted that nitrite content was slightly reduced after 48 h (Fig. 2a). Following the secondary metabolites productions by HPLC, optimal concentration and time exposure of SNP was at 1 mM and for 48 h for secologanin production. Notably, SNP concentration at 1 mM for 12 h, production of secologanin increased to 4.87±0.34 mg g^{-1} DW, when compared to control (3.05±0.44 mg g^{-1} DW). Longer SNP exposure for 24 h and 48 h could induce secologanin production to 6.70±0.60 and 8.50±0.55 mg g^{-1} DW, respectively. SNP concentration higher than 5 mM and longer exposure caused an inhibition of the secologanin production (Fig. 2b). For the case of mitragynine, SNP at tested concentrations seemed to suppress its production in the plant (Fig. 2c). Interference the mitragynine production from the metabolic pooled in plant cells may be postulated.

Effect of CPTIO on mitragynine and secologanin productions

M. speciosa plant-treated with CPTIO, a nitric oxide scavenger, was investigated due to confirm that the modulation of secologanin production caused from NO generation during SNP treatment. Pre-exposed plant with CPTIO for 30 min was performed and SNP was consequently added. After 48 h, plants were harvested and

solution was filtered. Fig. 3 summarizes the nitrite, mitragynine and secologanin contents in SNP- and/or CPTIO-treated plants. By Greiss assay, SNP could generate NO and in consequence nitrite. However, solutions that contained CPTIO found to accumulate higher amount of nitrite. Goldstein et al. (2003) described that the CPTIO reacted with NO (endogenous NO and SNP-generated NO) also produce nitrite molecule. Therefore, nitrite amount resulted from a summation from NO and by product of CPTIO and NO.

Addition of SNP was clearly increase the production of secologanin. However, pre-treatment of 1 mM and 2 mM CPTIO inhibited the synthesis of secologanin significantly. This evidence indicated that adding of CPTIO, a NO scavenger, reduced the level of NO in activating secologanin biosynthesis. On the other hand, it confirmed that NO modulated secologanin production. For mitragynine biosynthesis, SNP slightly enhanced mitragynine production (Fig. 3).

Effect of nifedipine on mitragynine and secologanin productions

Another set of the experiment was treatment with nifedipine, in order to study the involvement of calcium triggering signal during NO elicitation. Because of nifedipine was prepared in 2% (v/v) ethanol, therefore, the plant grown in WPM plus 2% (v/v) ethanol was a control of this study. Addition of SNP and 0.5 mM and 1 mM nifedipine suppressed secologanin production with *P*-value of 0.5239 and 0.0037, respectively. Treatment plant culture with 0.5 mM nifedipine only enhanced secologanin production (*P*-value 0.1516) (Fig. 4b). It may cause from level of calcium to the cell channeling to secologanin production. However, combination of SNP, nifedipine and CPTIO significantly suppressed secologanin content (*P*-value 0.0033). Following mitragynine production, SNP at 1 mM or combined with nifedipine did not alter mitragynine content. However, treatment only nifedipine enhanced amount of mitragynine (*P*-value 0.1038) (Fig. 4c). This evidence was similar to the combination between SNP, nifedipine and CPTIO (*P*-value 0.4004). The present results suggested that NO generated from SNP had effect on calcium channel.

Transcription profiles of M. speciosa biosynthetic genes

Biosynthesis of mitragynine proposed to biosynthesize through strictosidine (Rueffer et al. 1978). Branch point enzymes in *M. speciosa* including 1-deoxy-D-xylulose 5-phosphate synthase (*DXS1*, *DXS2*), 1-deoxy-D-xylulose reductoisomerase (*DXR*), anthranilate synthase (*AS1*, *AS2*), tryptophan decarboxylase (*TDC*) and strictosidine synthase (*STR*) were investigated for transcription profile in the SNP-treated plant culture. qRT-PCR using SYBR green as detecting agent was used to determine the mRNA levels of those genes due to

understand the regulation of secologanin biosynthesis as well as mitragynine biosynthesis. Four groups of treatments including control, SNP, SNP/CPTIO, SNP/nifedipine were performed. After elicited for 48 h, plant were harvested, immediately frozen in liquid nitrogen. Total RNA was extracted and then used as template for one-step qPCR to measure the mRNA level. Validity of amplification plot was evaluated based on the dissociation curve. Then, the relative quantitation (RQ) was calculated when the *18S rRNA* was used as an endogenous gene and the control group was a calibrator as shown above. Consideration of RQ-value of SNP-elicited plant revealed that all genes involved in mitragynine biosynthesis were increased. On the other hand, mRNA expression of all genes was decreased when adding either CPTIO or nifedipine (Fig. 5).

Discussion

The present study, we attempted to increase the production of metabolite in mitragynine biosynthesis by using plant culture under controlled condition. Treatment with abiotic elicitor was our approach. As we interested in the signal molecule, that effect to cell and stimulate the cascade of signaling pathway, nitric oxide (NO) was used in this study. In plant, NO has been introduced also as a signaling molecule in 1998. Since then scientists tried to understand role of NO regarding to primary and secondary metabolisms such as signal in plant defense resistance (Delledonne et al. 1998), defense gene induction (Durner et al. 1998) and regulates potassium and chloride channels in guard cell (Garcia-Mata et al. 2003). Focusing on indole alkaloid biosynthesis, NO has been reported for signaling to catharanthine production in *Catharanthus roseus* suspension cells (Xu et al. 2005). Role of NO in mitragynine biosynthesis has not explored yet.

In plant cell, NO is generated from non-enzymatic and enzymatic reactions. In this study, sodium nitroprusside (SNP) was NO donor. SNP released NO by reacting with sulfhydryl group of the cells. NO is a gaseous molecule and can be indirect measured by Griess reagent and reported as nitrite content. The optimal condition of SNP concentration and time exposure are dependent upon plant species and type of culture (Arasimowicz and Floryszak-Wieczorek 2007). In *C. roseus* suspension culture, SNP at 10 and 20 mM inhibited growth but enhance catharanthine production and low concentration of SNP like 0.1 and 0.2 mM stimulated growth but had no effect on catharanthine production (Xu et al. 2005). In addition, of lower concentration of SNP of 0.5 mM elicited in *Panax ginseng* suspension culture increased 2-fold of total ginsenoside content (Huang et al. 2013). In case of *Lycoris chinensis* seedlings elicited with SNP at concentrations of 0.005, 0.05, 0.1 and 0.5 mM for 3, 10 and 25 days found to increase galanthamine accumulation but inhibit lycorine production (Mu et al. 2009).

Elicited *M. speciosa* plant with 1 mM SNP for 48 h found to enhance secologanin content significantly but has no effect on mitragynine production. High concentration of SNP at 10 mM inhibited secologanin productivity. Our result corresponded to *C. roseus* study, treated with 0.1 mM SNP for 9-30 days in hairy root cultures increased indole alkaloid productions like serpentine, catharantine, ajmalicine, lochnericine and tabersonine (Li et al. 2011). To proof that mitragynine biosynthesis was stimulated, caused from NO (generated from SNP), CPTIO-a NO scavenger was added prior SNP treatment. The result clearly indicated that CPTIO reduced amount of NO in the biological system, resulting lowering amount of secologanin. This evidence is in agreement with *C. roseus* suspension culture. Addition of CPTIO and SNP in the cells found to reduce catharanthine production (Xu et al. 2004). Dutta et al. (2013) demonstrated in *C. roseus* leave that differential regulation of strictosidine synthase (*STR*), a key enzyme of indole alkaloid biosynthesis, was coordinated with oxidative stress, nitric oxide, exogenous calcium as well as protein phosphorylation. They also showed the involvement of NO related to nitric oxide synthase (NOS) after treatment L-NAME (NOS inhibitor) with SNP (5 mM) and suppressed the *STR* expression (Dutta et al. 2013).

To understand the involvement of calcium channel in secologanin/mitragynine biosynthesis, plant cultures were treated with SNP and nifedipine, reduction of secologanin was observed. Intra- and extracellular calcium exhibited an important role to regulate ajmalicine production in methyl jasmonate-induced *C. roseus* cell culture (Lee-Parsons and Ertürk 2005). In case of *Arabidopsis thaliana* suggested that NO increased free cytosolic calcium through a signaling cascade and induced the expression of defense genes (Wendehenne et al. 2004). Hence, we hypothesized that SNP could stimulate calcium channel in *M. speciosa*.

Consideration of mitragynine biosynthesis as shown in Fig. 1, strictosidine is a catalyzing product of strictosidine synthase (*STR*), which condensed tryptamine from shikimate pathway and secologanin from terpenoid pathway. Key enzymes that flux primary metabolite chorismate to tryptamine are anthranilate synthase (*AS1*, *AS2*) and tryptophan decarboxylase (*TDC*) (Charooratana et al. 2012). Secologanin is biosynthesized from methylerythritol phosphate (MEP) pathway, thus, 1-deoxy-D-xylulose 5-phosphate synthase (*DXS1*, *DXS2*) was postulated to involve in mitragynine biosynthesis. To follow the NO effect to the biosynthetic genes of mitragynine biosynthesis, in this study, transcription profile of genes (as mentioned above) were investigated. Our result indicated that treatment with 1 mM SNP stimulated the expression levels of *AS1*, *AS2*, *TDC*, *DXS1*, *DXS2* and *STR*. However, 1 mM SNP in combination with 2 mM CPTIO or 1 mM nifedipine suppressed the genes expressions. Similar to *C. roseus* leaves, *STR* was stimulated by 5 mM SNP treatment. In case of *Artemisia annua* hairy roots culture, NO induced from fungal elicitor and stimulated *DXS* in plastidial

MEP pathway and in cytosolic mevalonate pathway of artemismin biosynthesis (Wang et al. 2009). Our result proposed that abiotic treatment like NO could bind with specific sensor on plasma membrane leading to influx extracellular calcium and initiation of signaling cascade. Activation of mitogen activated protein (MAP) kinase modulated the downstream signaling component, thus enhance *STR* expression and finally alkaloid production (Dutta et al. 2013). From this this study, we found that the mitragynine content did not change or NO has less effect after SNP treatment. In model of *C. roseus* hairy root culture demonstrated the limitation of terpenoid indole alkaloid biosynthesis caused from the presence of tryptamine, secologanin as well as the transcription level of *TDC*, *STR* and strictosidine β-glucosidase (*SGD*) in the cells (Goklany et al. 2009). For mitragynine biosynthesis, Charoonratana et al. (2013b) reported the limitation of mitragynine caused from tryptamine availability. Condensation of tryptamine and secologanin is a crucial step for production strictosidine, an important intermediate of mitragynine. Therefore, enhancing of mitragynine production needs to supply tryptamine to the cells. In conclusion, we demonstrated in the present study that NO could stimulate mitragynine biosynthesis in *M. speciosa*. NO generated from SNP triggered through calcium channel and enhanced mRNA level of genes in mitragynine biosynthesis. Based on our knowledge, our result gave a new insight of NO on mitragynine biosynthesis.

Reference

Aji, A.M., Effraim, K.D. and Onyeyili, A.P. 2001. Antistress activity of *Mitragyna africanus* (Wild) stembark extract. *Sciences* 1, 105-107.

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W. and Lipman D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.* **25**, 3389–3402.

Charoonratana, T., Wungsintaweekul, J., Pathompak, P., Georgiev, M.I., Choi, Y.H., Verpoorte, R. 2013. Limitation of mitragynine biosynthesis in *Mitragyna speciosa* (Roxb.)Korth. through tryptamine availability. *Z. Naturforsch*. 68c, 394-405.

Goldstein, S., Russo, A. and Samuni, A. 2003. Reactions of PTIO and Carboxy-PTIO with $^{\bullet}NO$, $^{\bullet}NO_2$ and $O_2^{-\bullet}$. *J Biol Chem.* 278; 50949-50955.

Griess, P. 1879. "Bemerkungen zu der Abhandlung der HH.Weselky und Benedikt Ueber einige Azoverbindungen".*Berichte der Deutschen chemischen Gesellschaft.* 12; 426–428.

Grossi, L. and Angelo, S. 2005. Sodium nitroprusside: mechanism of NO release mediated by sulfhydryl-containing molecules. *J Med Chem.* 48; 2622-2626.

Jumali, S.S., Said, I.M., Baharum, S.N., Ismail, I., Rahman, Z.A. and Zainal, Z. 2011b. Molecular cloning and characterization of strictosidine synthase, a key gene in biosynthesis of mitragynine from *Mitragyna speciosa*. *Afr. J. Biotechnol.* 10(68), 15238-15244.

Kikura-Hanajiri, R., Kawamura, M., Maruyama, T., Kitajima, M., Takayama, H. and Goda, Y. 2009. Simultaneous analysis of mitragynine, 7-hydroxymitragynine, and other alkaloids in the psychotropic plant "kratom" (*Mitragyna speciosa*) by LC-ESI-MS. *Forensic. Toxicol.* 27, 67-74.

Matsumoto, K., Mizowaki, M., Thongpraditchote, S., Murakami, Y., Takayama, H., Sakai, S-L., Aimi, N. and Watanabe, H. 1996a. Central antinociceptive effects of mitragynine in mice: contribution of descending noradrenergic and serotonergic pathways. *Eur. J. Pharmacol*. 371, 75-81.

Nagakura, N., Rueffer, M. and Zenk M.H. 1979. The biosynthesis of monoterpenoid indole alkaloids from strictosidine. *J. Chem. Soc.* (Perkin I), 2308-2312.

Phongprueksapattana, S., Putalun, W., Keawpradub, N., and Wungsintaweekul, J. 2008. Mitragynine biosynthesis: hairy root culture for triterpenoid production and high yield of mitragynine by regenerated plants. *Z. Naturforch*. 63C: 691-698

Rueffer, M., Nagakura, N. and Zenk, M. 1978. Strictosidine, the common precursor for monoterpenoid indole alkaloids with 3β and 3α configuration. *Tetrahedron Lett*. 18, 1593-1596.

Thongpradichote, S., Matsumoto, K., Tohda, M., Takayama, H., Aimi, N., Sakai, S. and Watanabe, H. 1998. Identification of opioid receptor subtypes in antinociceptive actions of supraspinally-administered mitragynine in mice. *Life Sci*. 62, 1371-1378.

Tsuchiya, S., Miyashita, S., Yamamoto, M., Horie, S., Sakai, S.I., Aimi, N., Takayama, H. and Watanabe, K. 2002. Effect of mitragynine, derived from Thai folk medicine, on gastric acid secretion through opioid receptor in anesthetized rats. *Eur. J. Pharmacol*. 443, 185–188.

Watanabe, K., Yano, S., Horie, S. and Yamamoto, L.T. 1997. Inhibitory effect of mitragynine, an alkaloid with analgesic effect from Thai medical plant *Mitragyna speciosa*, on electrically stimulated contraction of isolated guinea-pig ileum through the opioid receptor. *Life Sci.* 60, 933-942.

Wungsintaweekul, J. 2009. Biosynthesis of terpenoid indole alkaloid: molecular cloning, characterization and expressions of *dxs* and *dxr* genes from *Mitragyna speciosa* (Roxb.)Korth. Final report for the Thailand Research Fund.

Zuildin, N.N.M., Said, I.M., Noor, N.M., Zainal, Z., Kiat, C.J., Ismail, I. 2013. Induction and analysis of the alkaloid mitragynine content of a *Mitragyna speciosa* suspension culture system upon elicitation and precursor feeding. *Sci. World.J*. 209434.

Figure legends

Figure 1 Proposed biosynthesis of mitragynine, a condensation product of shikimate pathway and methylerythritol pathways.

Figure 2 Effect of sodium nitroprusside (SNP) on mitragynine and secologanin productions in *M. speciosa* in 48 h of treatment. A. nitrite content; B. secologanin content and C. mitragynine content. Sample were in triplicate. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively comparison with control treatment.

Figure 3 Summary of SNP and CPTIO compare to water control

Figure 4 Nifedipine treatment in *M. speciosa*. A. nitrite content; B. mitragynine content; C. secologanin content. Data represent mean values ± standard deviation. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively comparison with control treatment (2% ethanol).

Figure 5 Transcription profile analysis of genes involved in mitragynine biosynthesis after treatments.

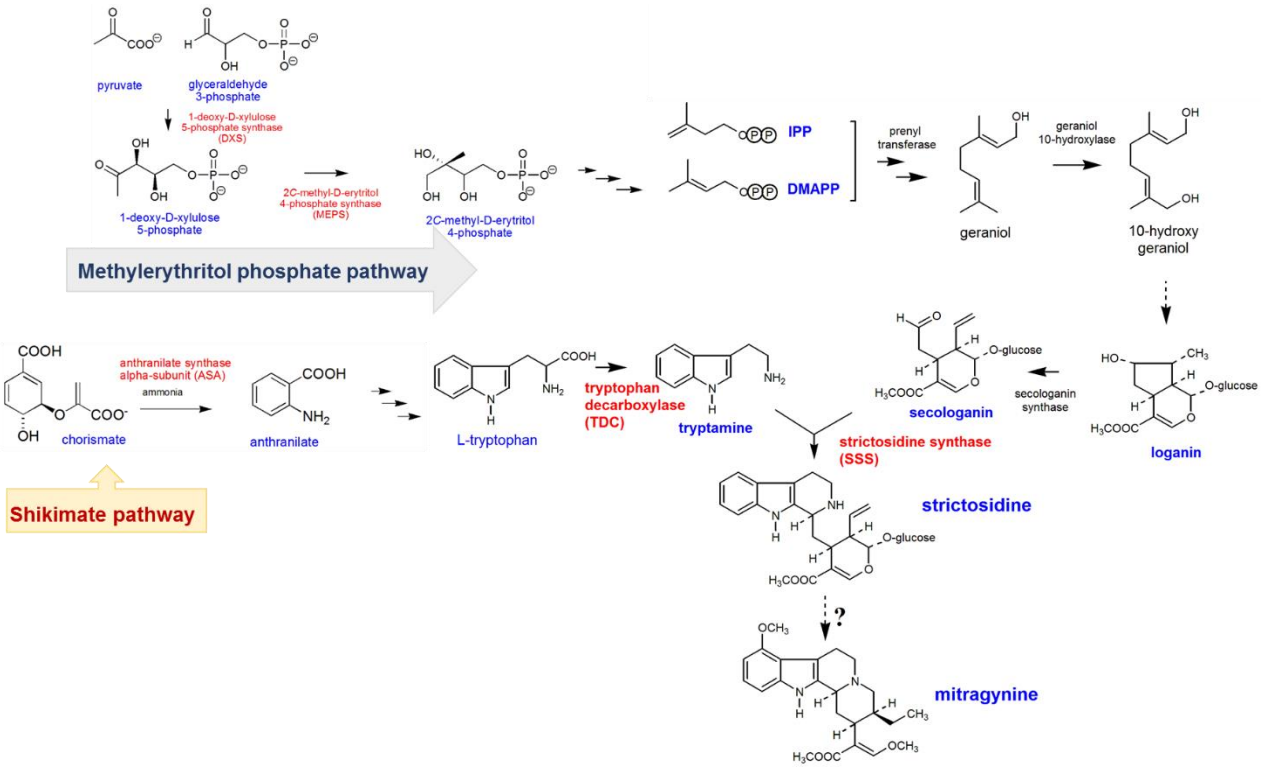

pyruvate
glyceraldehyde 3-phosphate
1-deoxy-D-xylulose 5-phosphate synthase (DXS)
1-deoxy-D-xylulose 5-phosphate
2C-methyl-D-erytritol 4-phosphate synthase (MEPS)
2C-methyl-D-erytritol 4-phosphate
Methylerythritol phosphate pathway
IPP
DMAPP
prenyl transferase
geraniol
geraniol 10-hydroxylase
10-hydroxy geraniol
anthranilate synthase alpha-subunit (ASA)
ammonia
chorismate
anthranilate
L-tryptophan
tryptophan decarboxylase (TDC)
tryptamine
secologanin
secologanin synthase
loganin
strictosidine synthase (SSS)
strictosidine
mitragynine
Shikimate pathway

Figure 1

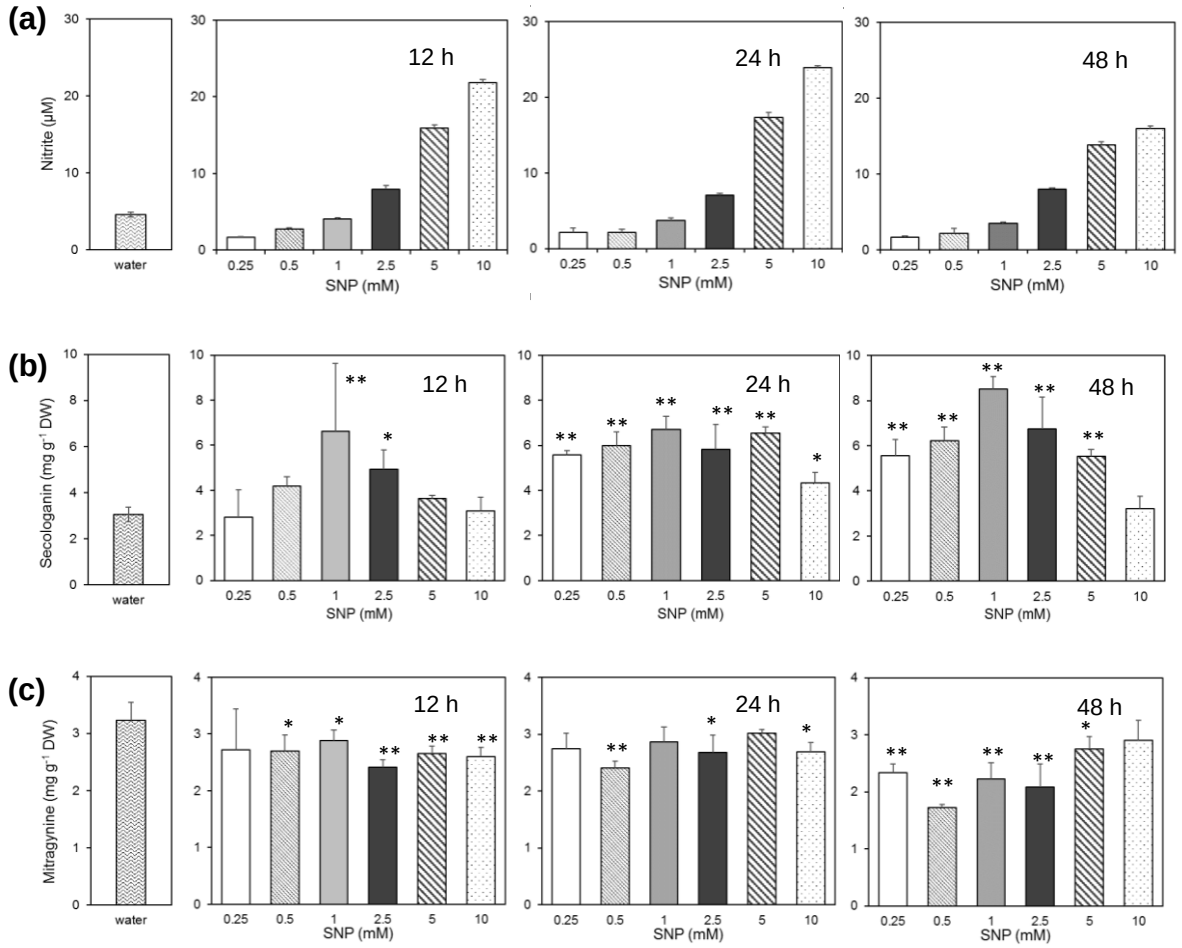

(a)
Nitrite (μM)
water
12 h
24 h
48 h
SNP (mM)
(b)
Secologanin (mg g⁻¹ DW)
(c)
Mitragynine (mg g⁻¹ DW)

Figure 2

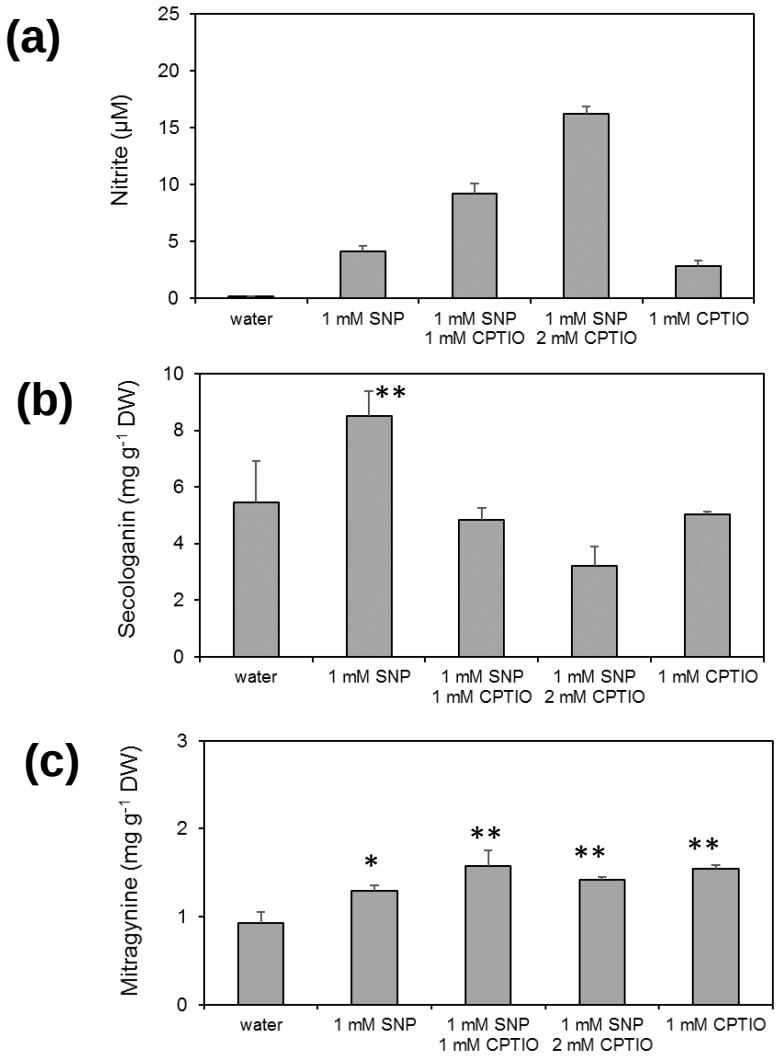

(a)
Nitrite (μM)
25
20
15
10
5
0
water
1 mM SNP
1 mM SNP 1 mM CPTIO
1 mM SNP 2 mM CPTIO
1 mM CPTIO
(b)
Secologanin (mg g-1 DW)
10
8
6
4
2
0
**
water
1 mM SNP
1 mM SNP 1 mM CPTIO
1 mM SNP 2 mM CPTIO
1 mM CPTIO
(c)
Mitragynine (mg g-1 DW)
3
2
1
0
*
**
**
**
water
1 mM SNP
1 mM SNP 1 mM CPTIO
1 mM SNP 2 mM CPTIO
1 mM CPTIO

Figure 3

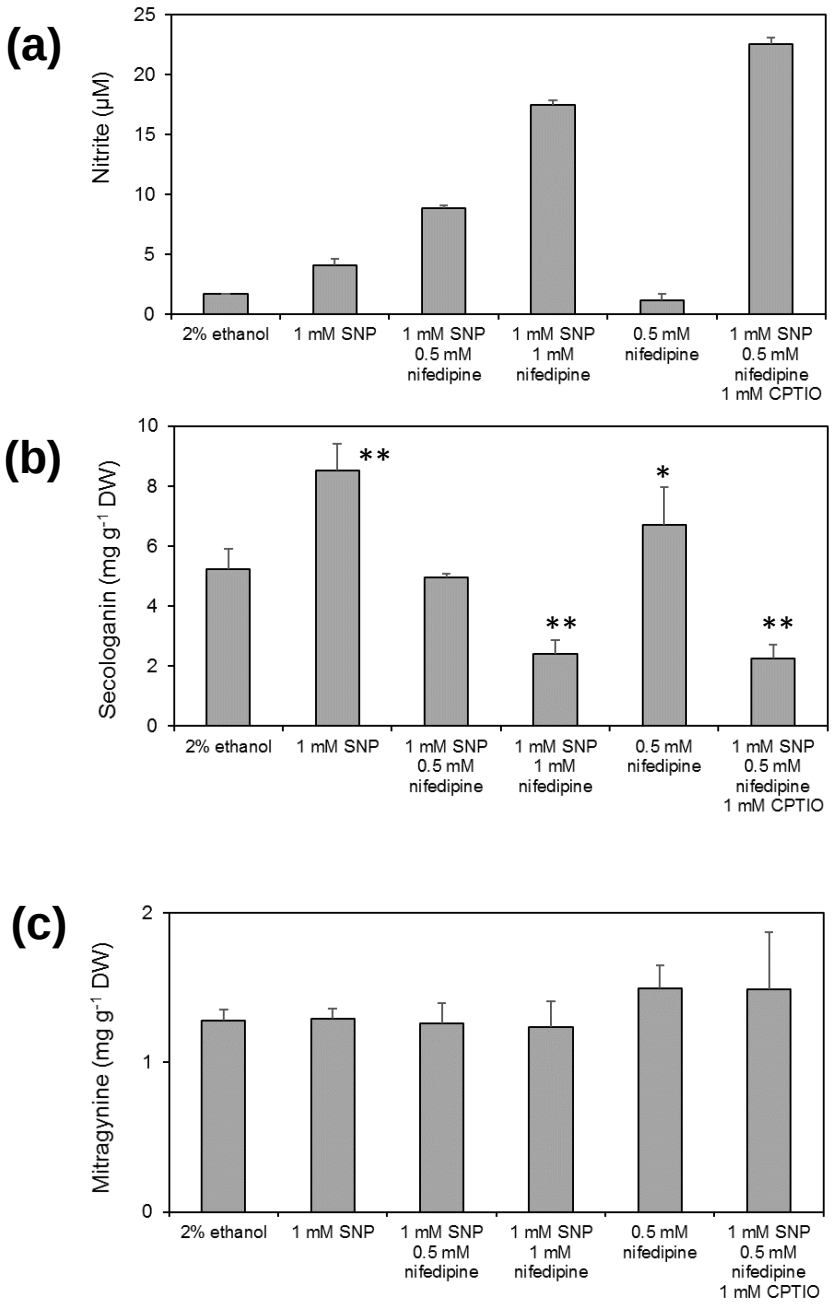

(a)
Nitrite (μM)
25
20
15
10
5
0
2% ethanol
1 mM SNP
1 mM SNP 0.5 mM nifedipine
1 mM SNP 1 mM nifedipine
0.5 mM nifedipine
1 mM SNP 0.5 mM nifedipine 1 mM CPTIO
(b)
Secologanin (mg g-1 DW)
10
8
6
4
2
0
**
**
*
**
2% ethanol
1 mM SNP
1 mM SNP 0.5 mM nifedipine
1 mM SNP 1 mM nifedipine
0.5 mM nifedipine
1 mM SNP 0.5 mM nifedipine 1 mM CPTIO
(c)
Mitragynine (mg g-1 DW)
2
1
0
2% ethanol
1 mM SNP
1 mM SNP 0.5 mM nifedipine
1 mM SNP 1 mM nifedipine
0.5 mM nifedipine
1 mM SNP 0.5 mM nifedipine 1 mM CPTIO

Figure 4

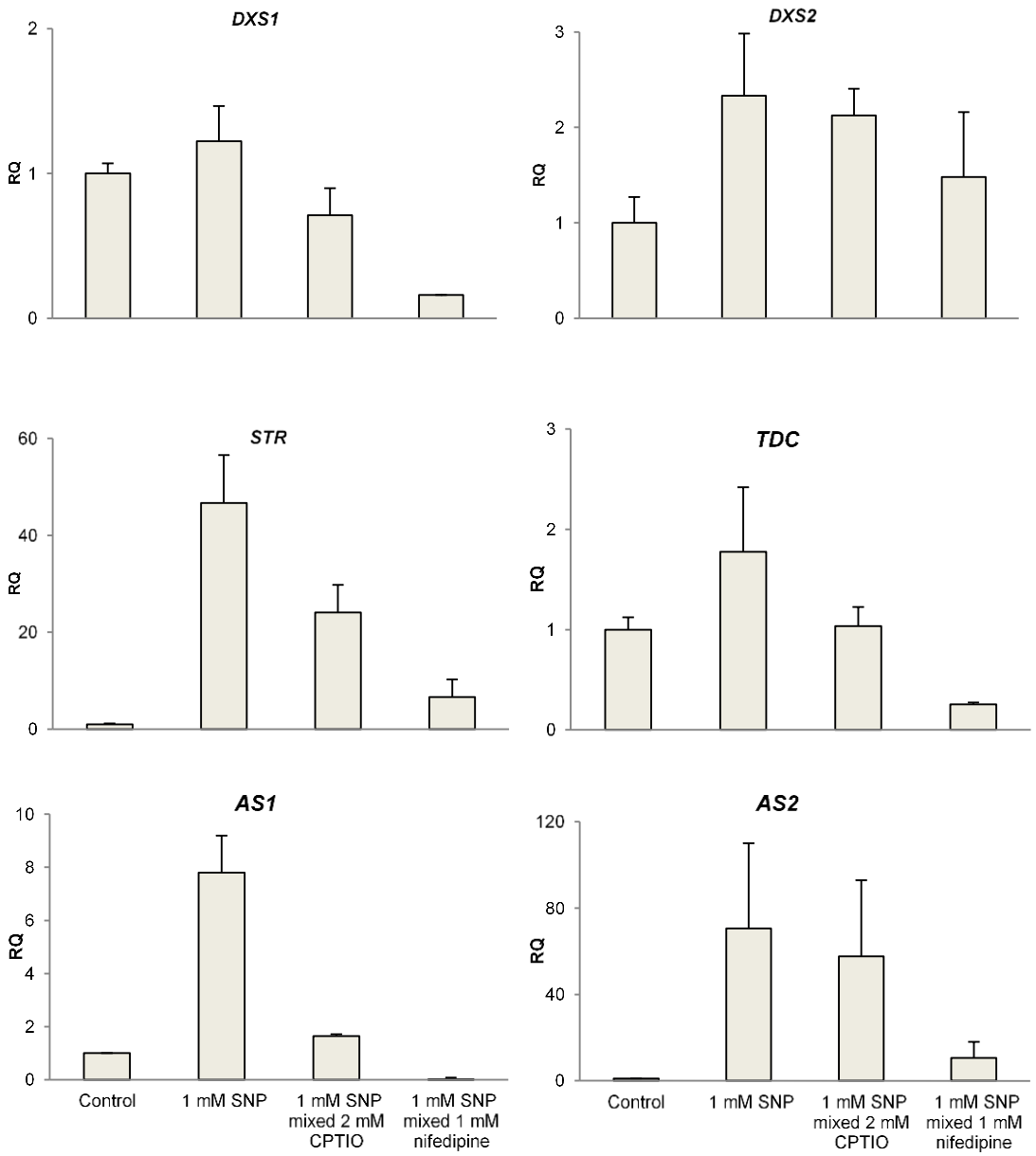

DXS1
DXS2
STR
TDC
AS1
AS2
RQ
Control
1 mM SNP
1 mM SNP mixed 2 mM CPTIO
1 mM SNP mixed 1 mM nifedipine

Figure 5

Table 1 Primers used in this study[a]

Target gene	Accession No.[b]	Primer (5′→3′)	Product size (bp)
18S rRNA F	JF412826	CAAAGCAAGCCTACGCTCTG	530
18S rRNA R		CGCTCCACCAACTAAGAACG	
TDC F	JN643922	GCTTTTTCGTCAAGACTTGCAAATTGGCTG	362
TDC R		GTAACAAAGTAGCCACTTATGAGGGCTC	
AS1 F	JQ775867	CTCTCTCCAGTCTTCATCTCC	316
AS1 R		CACAACATCTCCTTCAACTTCC	
AS2 F	JQ775866	CCACAGTTTCTCCTCCGTCA	311
AS2 R		CTGTTCCACTCTTCGTCCTTG	
STR F	EU288197	GGAATTACTGTTACGCCTAGAGC	246
STR R		AGAAGAAGCCACTCCATTCAAAG	
DXS1 F	JQ038372	GGTGGTTCAGTGCATGAACTTGCAGC	624
DXS1 R		CGGAAGGCATGCCATAAATGCCACATC	
DXS2 F	JQ038373	TGTGACAAAGCAACTAGGAAACCAAGCT	679
DXS2 R		AGGCAGACAAGCCATGTAAGTGGTGTC	

[a]Source: Charoonrattana et al. 2013b; [b]Accession number was retrieved from https://www.ncbi.nlm.nih.gov/