การขยายพันธุ์ด้วยการเพาะเลี้ยงเนื้อเยื่อและการผลิตแคมป์โทเธซิน
ของ *Ophiorrhiza harrisiana* โดยใช้เครื่องไบโอรีแอคเตอร์แบบจมชั่วคราว

นางสาวลัดดา เตชะวิริยะทวีสิน



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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ปีการศึกษา 2557

# MICROPROPAGATION AND CAMPTOTHECIN PRODUCTION OF *OPHIORRHIZA HARRISIANA* BY TEMPORARY IMMERSION BIOREACTOR

Miss Ladda Techawiriyathaweesin



A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacognosy
Department of Pharmacognosy and Pharmaceutical Botany
Faculty of Pharmaceutical Sciences
Chulalongkorn University
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| | |
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| Thesis Title | MICROPROPAGATION AND CAMPTOTHECIN PRODUCTION OF *OPHIORRHIZA HARRISIANA* BY TEMPORARY IMMERSION BIOREACTOR |
| By | Miss Ladda Techawiriyathaweesin |
| Field of Study | Pharmacognosy |
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ลัดดา เตชะวิริยะทวีสิน : การขยายพันธุ์ด้วยการเพาะเลี้ยงเนื้อเยื่อและการผลิตแคมป์โทเธซิน ของ *Ophiorrhiza harrisiana* โดยใช้เครื่องไบโอรีแอคเตอร์แบบจมชั่วคราว (MICROPROPAGATION AND CAMPTOTHECIN PRODUCTION OF *OPHIORRHIZA HARRISIANA* BY TEMPORARY IMMERSION BIOREACTOR) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. ร.ต.อ.หญิง ดร.สุชาดา สุขหร่อง, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ภญ. ดร. สรกนก วิมลมั่งคั่ง, 83 หน้า.

*Ophiorrhiza harrisiana* เป็นพืชที่สามารถผลิตแคมป์โทเธซิน พบได้ทั้งในส่วนรากและใบ ซึ่งแคมป์โทเธซินเป็นสารตั้งต้นในการสังเคราะห์ยาเคมีบำบัด เนื่องจากพืชชนิดนี้เป็นพืชล้มลุก ขึ้นเฉพาะถิ่น และพบได้เพียงบางฤดูกาล ดังนั้นจึงควรอนุรักษ์และขยายพันธุ์พืชชนิดนี้ไว้ การศึกษาในครั้งนี้จึงมีวัตถุประสงค์เพื่อขยายพันธุ์พืชโดยการเพาะเลี้ยงเนื้อเยื่อด้วยสองวิธี ได้แก่ แบบดั้งเดิมโดยเลี้ยงในอาหารแข็งและการใช้เครื่องไบโอรีแอคเตอร์แบบจมชั่วคราว ตลอดจนศึกษาถึงศักยภาพในการผลิตสารแคมป์โทเธซิน การเพาะเลี้ยงเนื้อเยื่อ *O. harrisiana* แบบดั้งเดิมโดยใช้อาหารแข็งสูตร ½ MS ร่วมกับสารควบคุมการเจริญเติบโตไคเนติน 4.6 ไมโครโมลาร์ จะให้ปริมาณยอดทวีคูณสูงที่สุดโดยเฉลี่ย 15.4 ยอดต่อหนึ่งชิ้นส่วนยอด เมื่อทำการเพาะเลี้ยงเนื้อเยื่อโดยใช้เครื่องไบโอรีแอคเตอร์แบบจมชั่วคราวพบว่า สามารถชักนำให้เกิดชิ้นส่วนยอดทวีคูณในอาหารสูตร ½ MS ที่ปราศจากสารควบคุมการเจริญเติบโต เมื่อทำการเปรียบเทียบการเจริญเติบโตในทั้งสองระบบพบว่า จำนวนยอดและความยาวยอดที่ได้จากการเลี้ยงด้วยเครื่องไบโอรีแอคเตอร์มากกว่าการเลี้ยงโดยวิธีดั้งเดิม 4.4 และ 1.4 เท่าตามลำดับ การวิเคราะห์ปริมาณแคมป์โทเธซินของ *O. harrisiana* จากตัวอย่างสี่กลุ่ม ได้แก่ ตัวอย่างจากแหล่งธรรมชาติ จากการเพาะเลี้ยงเนื้อเยื่อแบบดั้งเดิม จากการใช้ไบโอรีแอคเตอร์ และจากพืชที่ออกปลูก พบว่า ปริมาณแคมป์โทเธซินจากพืชที่เลี้ยงด้วยเครื่องไบโอรีแอคเตอร์มีปริมาณสารสูงที่สุดทั้งในส่วนใบและราก คือ 521.79 และ 316.55 ไมโครกรัมต่อกรัมน้ำหนักแห้งตามลำดับ กล่าวโดยสรุปพบว่า การเพาะเลี้ยงเนื้อเยื่อโดยใช้เครื่องไบโอรีแอคเตอร์สามารถผลิตต้นพันธุ์ขนาดเล็กได้มากกว่าการเพาะเลี้ยงแบบดั้งเดิมและสามารถผลิตแคมป์โทเธซินได้ในปริมาณสูงสุดในทุกกลุ่มตัวอย่างพืช ดังนั้นการเพาะเลี้ยงเนื้อเยื่อด้วยเครื่องไบโอรีแอคเตอร์แบบจมชั่วคราวจึงเหมาะที่จะใช้เป็นทางเลือกสำหรับการผลิตสารตั้งต้นของยาเคมีบำบัดในระดับอุตสาหกรรมและการอนุรักษ์พันธุ์พืชชนิดนี้จากการสูญพันธุ์

ภาควิชา เภสัชเวทและเภสัชพฤกษศาสตร์ ลายมือชื่อนิสิต .....

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LADDA TECHAWIRIYATHAWEESIN: MICROPROPAGATION AND CAMPTOTHECIN PRODUCTION OF *OPHIORRHIZA HARRISIANA* BY TEMPORARY IMMERSION BIOREACTOR. ADVISOR: ASSOC. PROF. SUCHADA SUKRONG, Ph.D., CO-ADVISOR: ASST. PROF. SORNKANOK VIMOLMANGKANG, Ph.D., 83 pp.

*Ophiorrhiza harrisiana* is a plant which produces camptothecin (CPT) in its roots and leaves. CPT is a precursor in the synthesis of chemotherapeutic drug. Since this plant is an herb that is found in particular location and season, conservation and propagation of this plant species are needed. The objectives of this study were to propagate the plant in two tissue culture systems including conventional method (CM) and temporary immersion bioreactor (TIB), and to study its potential in CPT production. *O. harrisiana* cultured by CM on semi-solid medium containing 1/2 MS supplemented with kinetin 4.6 μM showed the highest average number of shoots with 15.4 shoots per explant. When the plant was grown by TIB, multiple shoots could be induced on 1/2 MS medium without any plant growth regulator. When comparing between CM and TIB, it was found that the number and length of the shoots derived from TIB were about 4.4 and 1.4 times higher than those from CM, respectively. The CPT content of *O. harrisiana* was analyzed in four plant sources including natural source, CM, TIB, and soil-cultivated plant. The results showed that the highest amount of CPT was observed in both leaf and root of the plant from TIB cultures which were 521.79 and 316.55 μg/g dry weight, respectively. In conclusion, the plant grown in TIB can be propagated to obtain higher number of plantlets than CM and produce the highest amount of CPT among other plant sources. Therefore, TIB can be a suitable system to be used as an alternative method in the production of chemotherapeutic drug in the industrial scale and in the conservation of this plant from extinction.

| | | |
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| Department: | Pharmacognosy and Pharmaceutical Botany | Student's Signature ..... |
| | | Advisor's Signature ..... |
| Field of Study: | Pharmacognosy | Co-Advisor's Signature ..... |
| Academic Year: | 2014 | |

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| α | = alpha |
| BA | = 6-benzylaminopurine or N6-benzyladenine |
| B5 | = Gambrog's medium |
| Cm | = centimeter |
| CM | = conventional method |
| CPT | = camptothecin |
| °C | = degree celsius |
| DW | = dry weight |
| FW | = fresh weight |
| g | = gram |
| hr | = hour |
| HCPT | = 10-hydroxy camptothecin |
| HPLC | = high performance liquid chromatography |
| $H_2O$ | = water |
| IBA | = indole-3-butyric acid |
| L | = liter |
| Kn | = kinetin-6-furfurylaminopurine |
| M | = molar |
| MCPT | = 9-methoxy camptothecin |
| MeOH | = methanol |
| mg | = milligram |
| min | = minute |
| ml | = milliliter |
| mm | = millimeter |
| mM | = millimolar |
| MS | = Murashige and Skoog |
| MW | = molecular weight |
| m/z | = mass to charge ratio |

$[M+H]^+$ = pseudomolecular ion

NAA = $\alpha$-naphthaleneacetic acid

nm = nanometer

PGR = plant growth regulator

rpm = revolution per minute

RT = retention time

SD = standard deviation

sp. = species (singular)

spp. = species (plural)

TIA = monoterpenoid indole-alkaloid

TIB = temporary immersion bioreactor

TIS = temporary immersion system

Top = topoisomerase

Trp = tryptophan

UV = ultraviolet

µg = microgram

µL = microliter

µM = micromolar

1/2 MS = half strength Murashige and Skoog

2, 4-D = 2, 4-dichlorophenoxy acetic acid

# CHAPTER I

# INTRODUCTION

Cancer is a disease caused by the proliferation and growth of abnormal cells. This disease is a public problem. In the year 2002, there were 10.9 million new cancer patients and 6.7 million deaths (Parkin *et al*., 2005). In the year 2008, the World Health Organization (WHO), reported that cancer was the leading cause of worldwide death in about 13 percent. The number of patients increased to 12.7 million in new cases and 7.6 million cancer deaths in 2008 (Jemal *et al*., 2011). In 2012, cancers led causes of mortality worldwide with approximately 14 million new cases and 8.2 million cancer related deaths (World Health Organization, 2014). This information shows increasing trend in the number of cancer patients around the world. In Thailand, according to the information from the Ministry of Public Health, cancer has been the most prominent cause of death for many years (Ministry of Public Health, 2013).

Treatment of cancer involves three main therapies: surgery, radiotherapy and chemotherapy. There have been many containing researches and developments to find new drugs and new methods for cancer treatment. A number of chemotherapeutic agents are derived from natural substances. For example, paclitaxel was discovered from the bark of pacific yew, *Taxus brevifolia*, and used for the treatment of cancers such as breast and ovarian cancer. Vincristine and vinblastine from *Catharanthus roseus* are used in the treatment of leukemia and lymphoma. Camptothecin (CPT) from the bark of *Camptotheca acuminata*, is used as a precursor in the synthesis of topotecan and irinotecan for colorectal and ovarian cancer (Dharmarajan *et al*., 2005). Some chemotherapeutic compounds are difficult to synthesize because of their structural complexity. Therefore, natural products have been interesting extensively in research particularly bioproduction by plant from *in vitro* culture.

CPT was first isolated from *Camptotheca acuminata* in the family Nyssaceae (Wall *et al*., 1966). This substance was later found in several other plant species, for example *Nothapodytes foetida* in the family Icacinaceae and some species of the genus *Ophiorrhiza* in the family Rubiaceae (Dharmarajan *et al*., 2005; Krishnakumar *et al*., 2012). There have been many reports about the isolation and identification of CPT and its derivatives from *Ophiorrhiza* plant such as *O. mungos* (Namdeo *et al*., 2012), *O. pumila* (Sirikantaramas *et al*., 2007), *O. liukiuensis* and *O. kuroiwai* (Asano *et al*., 2004). Many *in vitro* culture techniques have been used to increase CPT production such as hairy root culture (Saito *et al*., 2001; Asano *et al*., 2004), elicitation (Yamazaki *et al*., 2003), and bioreactor (Sudo *et al*., 2002) to increase biomass. Using bioreactor system is one of the most popular methods in commercial scale of secondary metabolite production; however, the system has some problems with oxygen supply. The oxygen supply from agitation in general bioreactor can damage culture cells from bladder leading to reduction in the yield of the product. Temporary immersion system (TIS) is a newly developed system using temporary immersion bioreactor (TIB). TIS allows air to circulate, by air compressor, through the bioreactor can reduce damage to cell culture. This technique has been applied in the micropropagation of many plant species. Moreover, plantlets generated from TIB culture can be regrown in nature. This is a practical approach for the conservation and micropropagation of important plants and production of secondary metabolites at industrial scale.

In our previous work, it was found that *O. harrisiana* could produce CPT in high amount compared with other *Ophiorrhiza* species. Interestingly, CPT was found both in the roots and leaves of *O. harrisiana* while other species of this plant genus produced CPT only in the roots. Because of the limited resources in nature, micropropagation of *O. harrisiana* to increase the production of CPT by tissue culture technique is an interesting alternative. This study aims to compare tissue culture techniques between conventional method (CM) by semi-solid culture and a novel method by TIB for the propagation and production of CPT. The conceptual framework of this study was shown (Figure 1).

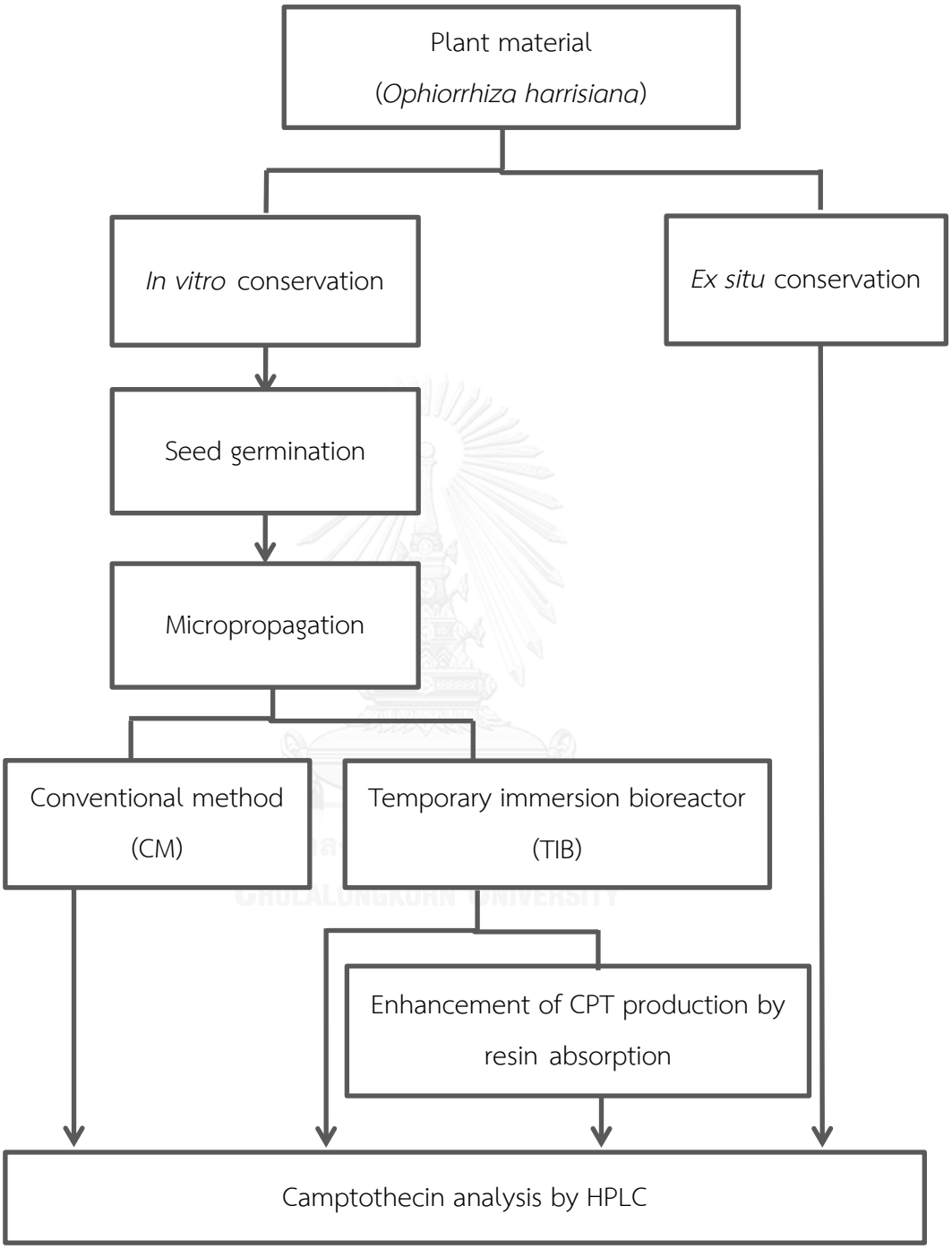



**Figure 1** The conceptual framework of the study.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 *Ophiorrhiza harrisiana*

### 2.1.1 Botanical aspects of the genus *Ophiorrhiza*

The genus *Ophiorrhiza* belongs to the family Rubiaceae. More than 400 species of *Ophiorrhiza* was found (Schanzer, 2005). This predominantly herbaceous genus is distributed from eastern India to the western Pacific and from the south of China to the north of Australia (Puff and Chayamarit, 2007). In Thailand, there are 44 species which can be found throughout the country (Puff and Chayamarit, 2007).

The characteristics of genus *Ophiorrhiza* (Puff *et al*., 2005) are as follows:

"*Ophiorrhiza* L. is distinctly herbaceous plant; prostrate or annual, uncommonly subshrubby; stem sometimes succulent. Leaves are opposite (decussate), rarely slightly anisophyllous, and blades mostly membranous; leaf-like stipules entire or fimbriate. Inflorescence terminal often consist of helicoid or scorpioid cymes, sometimes congested and head-like; bracts well developed or absent. Flowers are 5-merous, hermaphrodite, heterostylous or isostylous, sometimes cleistogamous; calyx lobes often very small; corolla typically narrowly infundibular to hypocrateriform, tube inside glabrous or hairy, base of tube occasionally distinctly bulbous, lobes valvate in bud, ascending to reflexed in open flowers; stamen inserted at different levels in corolla tube, filaments long or short, anthers included or exerted; style filiform, with 2-lobed stigma, included or exerted ovary 2-celled, each locule with numerous ovules on placenta attached to lower half of septum; roof of ovary with conspicuous 2-lobed disk. Fruits are strongly laterally compressed, capsular, loculicidally dehiscent. Seeds are very numerous, small and rhomboid."

Forty-four species of this genus exist in Thailand. Many species were recorded as the endemic and rare plants (Santisuk *et al*., 2006; Puff, 2007). Only some of them

has local name in Thai (Smitinand, 2001; Puff *et al*., 2005). The species of the *Ophiorrhiza* distributed in Thailand were described in Table 1.

*Ophiorrhiza harrisiana* B. Heyne ex Hook. f is an herbaceous plant (Figure 2) This plant was found in Chang Island, Trat province in the east of Thailand (Figure 3). This species was reported in Flora of Koh Chang (Schumann, 1902) and Thai Forest Bulletin (Chamchumroon and Puff, 2003). No Thai name has been recorded for this *Ophiorrhiza* species. This plant is rare and normally grows in shady and moist to wet areas in evergreen forest.

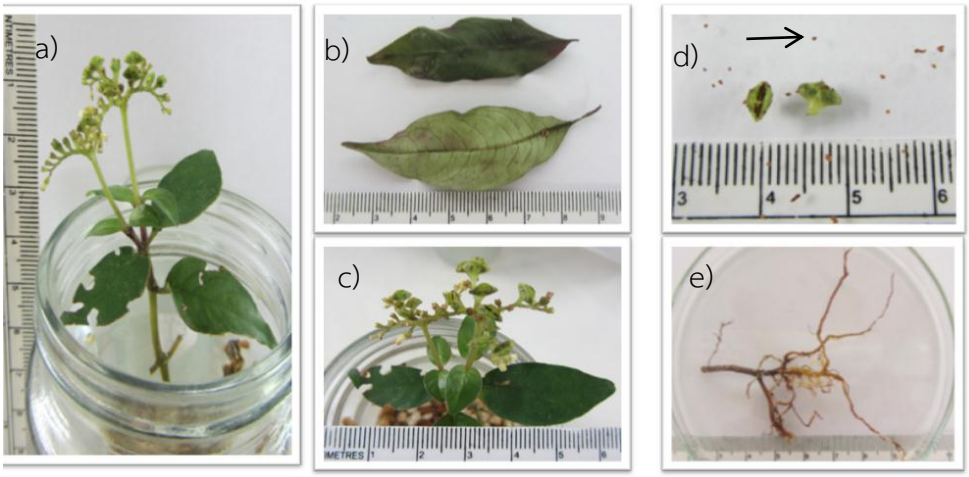



**Figure 2** *Ophiorrhiza harrisiana* plant.

a) *ex situ* three-month plant, b) leaves, c) flowers and fruits, d) fruits and seeds (arrow), and e) roots.



**Figure 3** Habitat of *Ophiorrhiza harrisiana*.

**Table 1** *Ophiorrhiza* spp. distributed in Thailand.

| **Scientific name (Local name)** | **Distribution** | **Reference** |
|---|---|---|
| *O. aggregata* Craib | - | Puff, 2007 |
| *O. alata* Craib (ผักหลอดดอกขาว) | Chantaburi | Smitinand, 2001; Puff, 2007; Ya-ut, Chareonsap, and Sukrong, 2011 |
| *O. angkae* Craib (สร้อยกะจับ) | Chiang Mai, Nan, Tak | Smitinand, 2001; Chamchumroon *et al.*, 2005; Puff, 2007 |
| *O. approximata* Craib | - | Puff, 2007 |
| *O. argentea* Wall. ex G.Don | - | Puff, 2007 |
| *O. bambusetorum* Craib | - | Puff, 2007 |
| *O. bicolor* Craib | - | Puff, 2007 |
| *O. brachycarpa* K.Schum. | Trat | Chamchumroon and Puff, 2003; Puff, 2007 |
| *O. calcarea* Craib | - | Puff, 2007 |
| *O. condensa* Craib | - | Puff, 2007 |
| *O. communis* Ridl. | Yala | Smitinand, 2001; Chamchumroon *et al.*, 2005; Globinmed, 2015 |
| *O. fucosa* Craib | Khao Soi Dao Nua, Chantaburi | Viraporn, 2011 |

**Table 1** (continued)

| **Scientific name (Local name)** | **Distribution** | **Reference** |
|---|---|---|
| *O. harrisiana* B. Heyne ex Hook. f | Koh Chang, Trat | Chamchumroon and Puff, 2003; Puff, 2007 Viraporn, 2011 |
| *O. hispidula* Wall. ex G.Don (หญ้าตีนมือตุ๊ดตู่) | Surat Thani, Doi Chiang Dao, Chiang Mai | Smitinand, 2001; Puff, 2007 |
| *O. kratensis* Craib (กะเสิมหิน) | Trat | Smitinand, 2001; Puff, 2007 |
| *O. lancifolia* Ridl. | - | Puff, 2007 |
| *O. larseniorum* Schanzer | Surat Thani | Schanzer, 2005; Puff, 2007 |
| *O. longifloriformis* Schanzer | Trat | Schanzer, 2005; Puff, 2007 |
| *O. longifolia* Craib | - | Puff, 2007 |
| *O. longipes* Craib | - | Puff, 2007 |
| *O. membranacea* Craib | - | Puff, 2007 |
| *O. nana* Edgew. | Mae Hong Son, Chiang Mai, Nan, Lampang, Tak; Nakhon Si Tamarat | Puff, 2007 |
| *O. oblonga* Craib | - | Puff, 2007 |
| *O. patula* Craib | - | Puff, 2007 |

**Table 1** (continued)

| **Scientific name (Local name)** | **Distribution** | **Reference** |
|---|---|---|
| *O. pedunculata* Schanzer | Mae Hong Son, Chiang Mai, Chiang Rai, Thong Pha Phum, Kanchanaburi | Schanzer, 2004; Puff, 2007 |
| *O. pellucida* Lévl | Mae Hong Son, Chiang Mai, Nan, Lampang, Tak; Nakhon Si Tamarat | Puff, 2007 |
| *O. plumbea* Craib | Bangpae waterfall, Phuket | Puff, 2007; Viraporn, 2011 |
| *O. prostrata* D.Don | - | Puff, 2007 |
| *O. pseudofasciculata* Schanzer | Chang Mai, Nan, Chiang Rai, Lampang | Chamchumroon *et a*l., 2005; Schanzer, 2005; Puff, 2007 |
| *O. ridleyana* Craib | Queen Sirikit Botanical Garden, Chiang Mai | Puff, 2007; Viraporn, 2011 |
| *O. ripicola* Craib (แดงก่อนจาก) | Doi Inthanon National Park, Chiang Mai | Smitinand, 2001; Chamchumroon *et al.*, 2005; Puff, 2007; Puff and Chayamarit, 2007 |
| *O. rosea* Hook.f. | - | Puff, 2007 |

**Table 1** (continued)

| **Scientific name (Local name)** | **Distribution** | **Reference** |
|---|---|---|
| *O. rugosa* Wall | Trat | Chamchumroon and Puff, 2003; Puff, 2007 |
| *O. rugosa* Wall. var. argentea | - | Puff, 2007 |
| *O. scabrella* Ridl. | - | Puff, 2007 |
| *O. schmidtiana* Craib (ผักพรหมมิ) | South eastern | Smitinand, 2001; Puff, 2007 |
| *O. subaequalis* Craib | - | Puff, 2007 |
| *O. subcapitata* Wall. ex Hook.f | - | Puff, 2007 |
| *O. subpunicea* Craib | - | Puff, 2007 |
| *O. trichocarpa* auct., non Blume | - | Puff, 2007 |
| *O. trichocarpon* Blume (ผักสามชาย) | Khao Yai National Park, Northern: Chiang Mai, Chiang Rai, Phayao | Schanzer, 2004; Chamchumroon *et al.*, 2005; Puff and Chayamarit, 2006; Puff, 2007 |
| *O. trichocarpon* Blume | Sa Kaeo | Schanzer, 2004; Puff, 2007 |
| *O. trichocarpos auct.,* non Blume | | Puff, 2007 |
| *O. villosa* Roxb | Doi Chiang Dao, Chiang Mai | Schanzer, 2004; Puff, 2007; Putiyanan and Maxwell, 2007 |

### 2.1.2 Chemical constituents of *Ophiorrhiza* plants

There are reports that *Ophiorrhiza* plants have been used in the treatment of various diseases. For example, the root extract of *O. mungo* is used in Indian folk medicine as anti-venom (Yamazaki *et al*., 2013). In China, *O. japonica* is used to treat skin diseases, leprosy and stomach ulcers (Kai *et al*., 2008).

Phytochemical study of the genus *Ophiorrhiza* showed the presence of several types of compounds, including alkaloids (Yamazaki *et al*., 2003; Yamazaki *et al*., 2013) and anthraquinones (Yamazaki *et al*., 2013). Alkaloids are reported as camptothecin, 9-methoxycamptothecin, 10-hydroxycamptothecin, chaboside and strictosidinic acid (Yamazaki *et al*., 2003). Anthraquinones which was often reported such as 3-hydroxy-2-hydroxymethylanthraquinone, 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone and 2-hydroxymethyl-3-methoxyanthraquinone (Watase *et al*,. 2004). Since CPT is one of the most important drugs in cancer therapy, we will focus on this compound. There are reports that some species of *Ophiorrhiza* can produce CPT such as *O. alata* (Ya-ut *et al*., 2011), *O. pumila* (Yamazaki *et al*., 2003; Roja, 2006), *O. mungos* (Roja, 2006; Krishnakumar *et al*., 2012), *O. prostrata* (Krishnakumar *et al*., 2012), *O. liukiuensis* and *O. kuroiwai* (Asano *et al*., 2004).

*Ophiorrhiza* plants found in Thailand that can produce CPT are *O. fucosa*, *O. harrisiana*, *O. plumbea*, and *O. ridleyana* (Viraporn, 2010; Viraporn *et al*., 2011). CPT was found in the root but not the leaves of *O. fucosa*, *O. plumbea*, and *O. ridleyana.* Interestingly, only *O. harrisiana* has CPT both in its leaves and root, with the amount in the leaves higher than in the root. Moreover, the amount of CPT in the leaves of *O. harrisiana* is the highest compared with the other 3 species (Figure 4). This is the reason that *O. harrisiana* was chosen for this study.

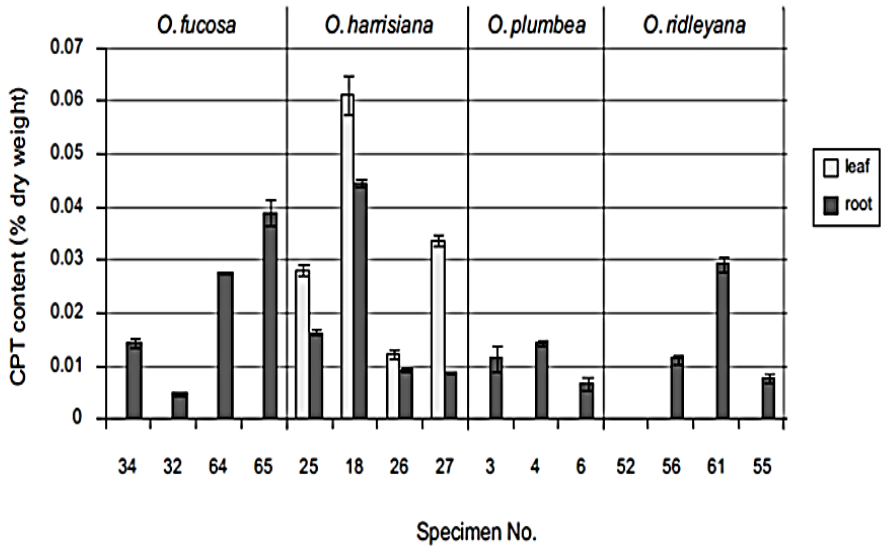
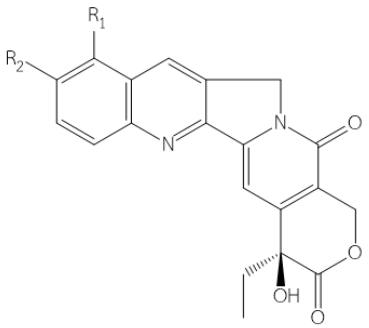



**Figure 4** Camptothecin content in the leaves and root of *O. fucosa*, *O. harrisiana*, *O. plumbea* and *O. ridleyana*.
(source: Viraporn, 2010)

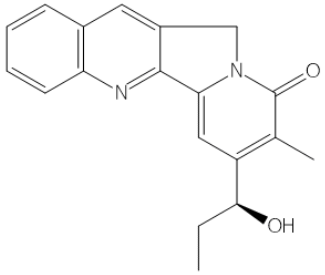
CPT and its derivatives previously isolated from *Ophiorrhiza* plants are shown in Table 2. Structure of CPT and its derivatives are shown in Figure 5.

**Table 2** Camptothecin and its derivatives found in *Ophiorrhiza* plants.

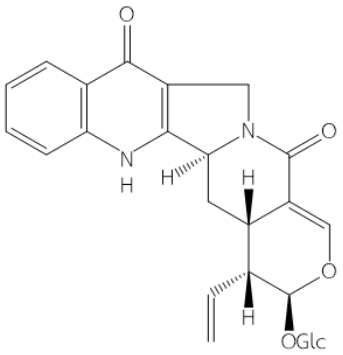
| **Chemical compound** | **MW** | **Plant** | **Reference** |
|---|---|---|---|
| Camptothecin [1] | 348 | *O. kuroiwai,*<br>*O. liukiuensis,*<br>*O. mungos,*<br>*O. pumila,*<br>*O. rugosa,* | Saito *et al*., 2001<br>Yamazaki *et al*., 2003<br>Kitajima *et al*., 2005<br>Roja, 2006<br>Asano *et al*., 2009 |
| 9-Methoxycamptothecin [2] | 378 | *O. liukiuensis,*<br>*O. pumila,*<br>*O. rugosa* | Kitajima et al., 2005<br>Yamazaki et al., 2003<br>Roja, 2006 |
| 10-Hydroxycamptothecin [3] | 364 | *O. pumila* | Yamazaki *et al*., 2003 |
| Chaboside [4] | 556 | *O. pumila* | Yamazaki *et al*., 2003 |
| Mappicine [5] | 306 | *O. pumila* | Yamazaki *et al*., 2003 |
| Pumiloside [6] | 512 | *O. liukiuensis*,<br>*O. pumila* | Kitajima et al., 2005<br>Yamazaki et al., 2003 |
| Deoxypumiloside [7] | 496 | *O. liukiuensis*,<br>*O. pumila* | Kitajima *et al*., 2005<br>Yamazaki *et al*., 2003 |
| Strictosamide [8] | 498 | *O. liukiuensis* | Kitajima *et al*., 2005 |
| Strictosidinic acid [9] | 516 | *O. pumila* | Yamazaki *et al*., 2003 |




| | $R_1$ | $R_2$ |
|---|---|---|
| [1] Camptothecin | H | H |
| [2] 9-Methoxycamptothecin | OMe | H |
| [3] 10-Hydroxycamptothecin | H | OH |
| [4] Chaboside | OMe | OGlc |

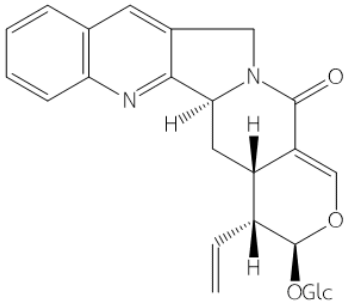



[5] Mappicine

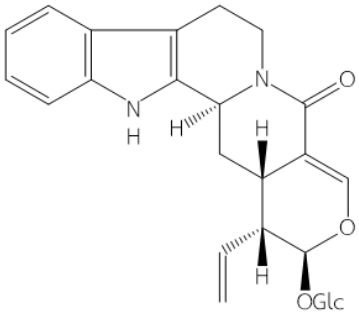



[6] Pumiloside

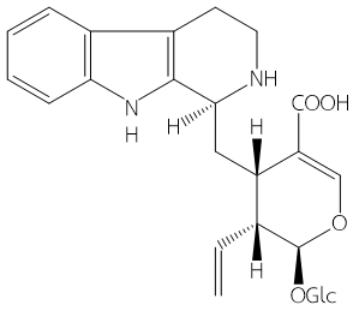
**Figure 5** Structure of camptothecin and its derivatives previously isolated from *Ophiorrhiza* plants.




[7] Deoxypumiloside




[8] Strictosamide




[9] Strictosidinic acid

**Figure 5** Structure of camptothecin and its derivatives previously isolated from *Ophiorrhiza* plants (continued).

### 2.1.3 Camptothecin

Camptothecin (CPT) is a tryptophan-derived indole alkaloid (Figure 6). It has cytotoxic activity by inhibiting the action of enzyme topoisomerase I, which is essential for the proliferation of cancer cell (Dharmarajan *et al*., 2005; Krishnakumar *et al*., 2012).

### Structure and chemistry

CPT is a plant-originated pentacyclic indole alkaloid (Yamazaki *et al*., 2003). The 20(S)-CPT was first isolated from *Camptotheca acuminata.* It was crystallized from methanol-acetonitrile as pale yellow needles. The $M^+$ at m/z is 348.11. The melting point is at 264-267 °C. The λ max of CPT is at 220 mμ, 254, 290 and 370 (Wall *et al*., 1966).

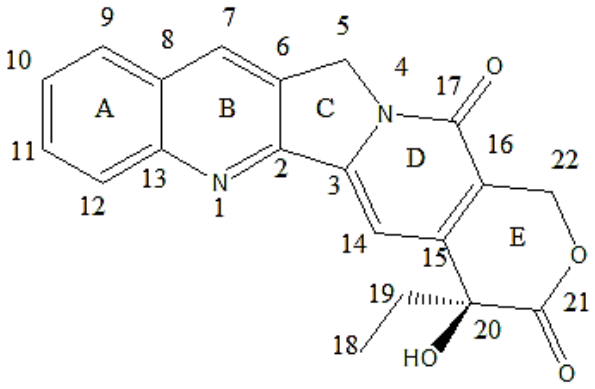



**Figure 6** Chemical structure of camptothecin.

CPT has an asymmetric carbon at position 20. The pentacyclic ring system includes a pyztolo[3,4-b]quinoline (ring A, B and C), a conjugated pyridine (ring D), and six-membered lactone (ring E) with a chiral center at position C-20. The natural 20S CPT is approximately 2-fold more potent than a racemic mixture of synthetic 20S

and 20R CPT. The 20R CPT is inactive as a topoisomerase I inhibitor (Fan *et al*., 1998). The 20(S)-hydroxyl is therefore the structural feature of CPT that is essential for its activity (Wang *et al*., 1999). However, the low water solubility of CPT in the lactone form limited the practical clinical utility of the drug since large volumes of fluid have to be administered to the subject in order to provide an effective dose of the drug (Narkunan *et al*., 2009). The semi-synthetic water-soluble CPT analogs such as topotecan and irinotecan were developed to increase solubility and have been currently used (Figure 7).

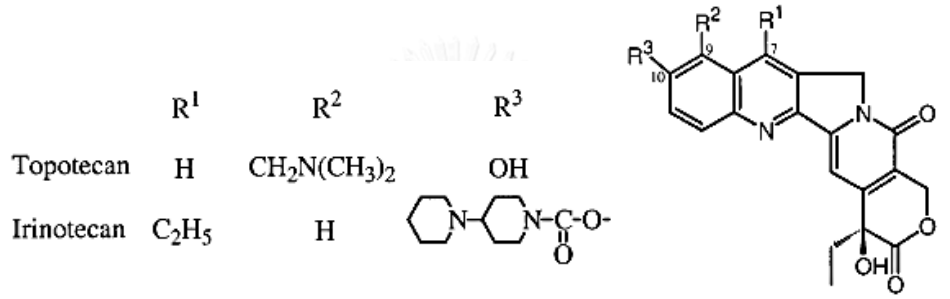



**Figure 7** Clinically used semi-synthetic camptothecin analogs, topotecan and irinotecan.
(source: Yamazaki *et al*., 2003)

**Anticancer camptothecin analogs**

10-Hydroxycamptothecin (10-HCPT) and 9-methoxycamptothecin (9-MCPT) are natural CPT derivatives which have been isolated from many CPT-producing plants (Yamazaki *et al*., 2003). 10-Hydroxycamptothecin was more potent and less toxic than CPT (Zhang *et al*., 1998). The water-soluble CPT analogs, topotecan and irinotecan, were prepared from 10-Hydroxycamptothecin. Topotecan (Hycamtin®) was prepared by oxidation of CPT to 10-Hydroxycamptothecin followed by additional modifications (Kingsbury *et al*., 1991). Irinotecan (Camptosar®) was synthesized by bonding phenolic hydroxyl group of 7-ethyl-10-Hydroxycamptothecin with diamines (Sawada *et al*., 1991). The methoxy analog was found to be more active than CPT

(Tafur *et al*., 1976). 9-Methoxycamptothecin was also reported to have antiviral activity (Das and Madhusudhan, 1999).

**Biosynthetic pathway of camptothecin**

CPT is a product of secondary metabolism from terpenoid indole-alkaloids (TIAs) (Lu *et al*., 2008) derived from amino acid tryptophan (Trp) and terpenoid precursors (Lorence and Nessler, 2004). CPT is formed by the combination of the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway and the shikimate pathway, which involves many distinct enzymatic steps. The first intermediate found from the combination of both pathways is strictosidine. 3(S)-pumiloside and 3(S)-deoxy pumiloside are thought to be biogenetic intermediates in the formation of CPT from strictosamide. Biosynthetic pathway for TIAs in CPT-producing plants was shown in Figure 8 (Yamazaki *et al*., 2003).

**Mechanism of action of camptothecin**

CPT exhibits antitumor activity due to its interaction with the cellular topoisomerase I (TopI) enzyme (Hsiang *et al.*, 1985). This interaction damages the DNA by causing the cancer cell to be destroyed or preventing the growth and reproduction of cancer cell (Pommier *et al*., 1998). CPT is considued to be a topoisomerase poison. CPT not only binds to TopI but also stabilizes a covalent complex between TopI and the nicked DNA (Reid *et al*., 1998). CPT induces TopI-linked DNA breaks by preventing DNA re-ligation, which could be poisonous to the cells (Fan *et al*., 1998).

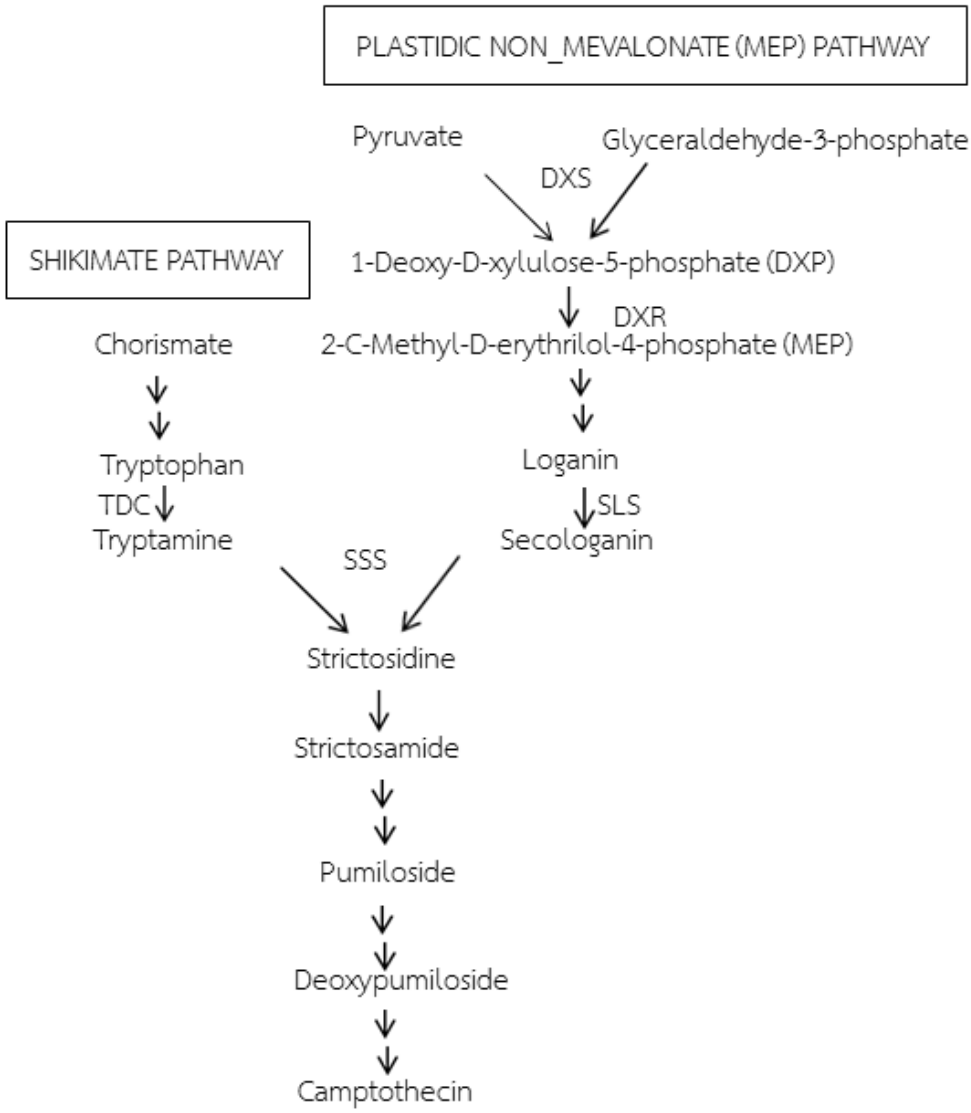



**Figure 8** Biosynthetic pathway for TIAs in camptothecin-producing plants.

The enzymes involve in the pathways: DXP synthase (DXS); DXP reductoisomerase (DXR); secologanin synthase (SLS); tryptophan decarboxylase (TDC); and strictosidine synthase (SSS). Multiple arrows represent multiple steps of intermediates.

**Distribution of camptothecin**

CPT is first isolated from extracts of *Camptotheca acuminata* (Nyssaceae), a deciduous tree native to China and Tibet (Wall *et al*., 1966). Families and orders of plants which have been reported containing CPT belong to the following:

Family Nyssaceae (Order Cornales): *Camptotheca acuminate* (Wall *et al.*, 1966)

Family Icacinaceae (Order Celastrales): *Nothapodytes foetida* (Aiyama *et al.*, 1988) *Pyrenacantha klaineana* (Zhou *et al*., 2000), *Merrilliodendron megacarpum* (Arisawa *et al*., 1981)

Family Rubiaceae (Order Gentianales): some species of the genus *Ophiorrhiza* such as *O. mungos* (Namdeo *et al.*, 2012), *O. pumila* (Sirikantaramas *et al.*, 2007)

Family Apocynaceae: *Ervatamia heyneana* (Gunasekera *et al*., 1979)

Family Gelsemiaceae: *Mostuea brunonis* (Dai *et al*., 1999)

**2.2 Tissue culture techniques**

Plant tissue culture is a general term of *in vitro* manipulations of plant cells, tissues and organs. The dedifferentiation of the parental cells was changed directly into plant organs and whole plants. The small piece of the parent plant, which is used to initiate the culture, is called the explant. Theoretically, every explant has the potential to produce a plant. The final result is the mass multiplication of the parent genotype, known as micropropagation (Watt, 2012).

Currently, plant tissue culture technique has been used as the basis for research. It used to increase the production of secondary metabolites to provide the ability to produce enough to increasing demand. That is including the development and use of new technologies to increase production capacity to be more effective. Many secondary metabolites can be extracted from nature. However, they have often a small amount. The plant may grow slowly in nature. There is also the problem of the uncertainty of the crop caused by the weather, pests and diseases

(Saito *et al*., 2001; วราภรณ์ ภูตะลุน, พ.ศ. 2551). The synthesis of some secondary metabolites is able to do, but the process is difficult and the cost of production is high (Yamazaki *et al*., 2003).

CPT, which is currently used in the manufacture of anticancer drugs, is mainly derived from plant extracts such as *Camptotheca acuminata* (Nyssaceae) and *Nothapodytes foetida* (Icacinaceae) (Asano *et al*., 2004; Saito *et al*., 2001). The possibility of using tissue culture to produce CPT was able to do by conventional method by semi-solid culture and temporary immersion bioreactor.

### 2.1.1 Conventional method by semi-solid culture

The micropropagation is now regularly employed by many research laboratories and commercial agriculture. The advantages of the *in vitro* micropropagation over the conventional propagation methods have been accepted decades ago (Watt, 2012). In pharmaceutical application, there are many advantages to produce a valuable secondary product in plant tissue culture, rather than in the whole crop plant (Karuppusamy, 2009). For example:

a. Mass production can be simpler and predictable

b. The isolation of phytochemical can be more rapid and efficient than the extraction from complex whole plants.

c. Tissue cultures can be parallelly produced compounds which produced from natural plant.

d. Tissue cultures can yield a source of phytochemicals in large quantities.

e. Tissue cultures can avoid interfering compounds which occur in the field-grown plant.

f. Plant can be grown regardless of the limitations of nature such as season and it can produce the product continuously.

g. Tissue cultures can be a potential model to study biosynthetic pathway.

Plant tissue culture also has been used in conjunction with other techniques to increase the amount of secondary metabolites such as elicitation. Elicitation technique was applied by adding elicitor to stimulate the production of secondary metabolites such as methyl jasmonate (Teisson and Alvard, 1995; Asano *et al*., 2004; Rahimi *et al*., 2012), sodium chloride (Rahimi *et al.*, 2012), chitosan (Putalun *et al.*, 2007; Ya-ut, 2008), and yeast extract (Asano *et al.*, 2004; Putalun *et al.*, 2007). Hairy root culture technique was generated by infection of roots with *Agrobacterium rhizogenese* (Ya-ut *et al.*, 2011; Rahimi *et al.*, 2012). Saito's group has reported that hairy root culture of *O. pumila* can produce CPT and CPT can be released into liquid medium in which it can be absorbed by polystyrene resin (Saito *et al.*, 2001). There are many researches that attempt to produce CPT by tissue culture techniques. The accumulation of CPT in natural sources and CPT production by tissue culture techniques were shown in Table 3.

**Table 3** The accumulation of camptothecin in natural sources and CPT production by tissue culture techniques.

| **Species (Family)** | **Plant part** | **CPT content (µg/g dry wt)** | **Reference** |
|---|---|---|---|
| *Camptotheca acuminata* Decaisne (Nyssaceae) | Young leaf<br>Seed<br>Bark<br>Root | 4000–5000<br>3000<br>1800-2000<br>400 | López-Meyer *et al*., 1994 |
| | Young leaf<br>Old leaf<br>Young fruit<br>Old fruit | 2421–3022<br>482<br>842<br>2362 | Li *et al*., 2002 |
| | Hairy root culture | 1000 | Lorence *et al*., 2004 |
| | Callus | 2040–2360 | Wiedenfeld *et al*., 1997 |
| | Cell culture | 2.5-4 | Sakato *et al*., 1974 |
| *Camptotheca lowreyana* Li (Nyssaceae) | Young leaf<br>Old leaf | 3913–5537<br>909–1184 | Li *et al*., 2002 |
| *Camptotheca yunnanensis* Dode (Nyssaceae) | Young leaf<br>Old leaf | 2592–4494<br>590 | Li *et al*., 2002 |
| *Ervatamia heyneana* (Wall) T. Cooke (Apocynaceae) | Wood and stem bark | 1300 | Gunasekera *et al*., 1979 |

**Table 3** (continued)

| **Species (Family)** | **Plant part** | **CPT content (µg/g dry wt)** | **Reference** |
|---|---|---|---|
| *Nothapodytes foetida* (Wight) Sleumer (Icacinaceae) | Stem wood | 1400–2400 | Aiyama *et al*., 1988 |
| | Shoot | 750 | Roja and Heble, 1994 |
| | Callus | 9.5 | Ciddi and Shuler, 2000 |
| | Cell culture | 1.1 | Fulzele *et al*., 2001 |
| *Merrilliodendron megacarpum* (Hemsl.) Sleumer (Icacinaceae) | Leaf and stem | 350 | Arisawa *et al*., 1981 |
| *Ophiorrhiza alata* Craib (Rubiaceae) | Hairy root culture | | Ya-ut *et al*., 2011 |
| *Ophiorrhiza kuroiwae* Makino (Rubiaceae) | Tissue culture | 55 | Asano *et al*., 2009 |
| *Ophiorrhiza liukiuensis* Hayata (Rubiaceae) | Whole plant | 127 | Kitajima *et al*., 2005 |
| *Ophiorrhiza mungos* Linn. (Rubiaceae) | Entire plant | 12 | Tafur *et al*., 1976 |
| | Shoot<br>Root | 96<br>176 | Roja, 2006 |

**Table 3** (continued)

| **Species (Family)** | **Plant part** | **CPT content (µg/g dry wt)** | **Reference** |
|---|---|---|---|
| *Ophiorrhiza pumila* Champ. (Rubiaceae) | Leaf<br>Young root<br>Hairy root culture | 300–400<br>1000<br>1000 | Saito *et al*., 2001 |
| | Entire plant<br>Hairy root culture | 300–510<br>240 | Yamazaki *et al.*, 2003 |
| | Cell culture | None | Kitajima *et al*., 1998 |
| *Ophiorrhiza rugosa* (Rubiaceae) | Shoot<br>Root | 10<br>20 | Roja, 2006 |
| *Ophiorrhiza trichocarpon* Blume (Rubiaceae) | Whole plant | CPT | Klausmeyer *et al*., 2007 |
| *Mostuea brunonis* (Gelsemiaceae:) | Whole plant | 100 | Dai *et al*., 1999 |

However, semi-solid culture has the major limitations of protocols, which are the need for regular subculturing (every 4 weeks), due to nutrient depletion from the small volumes depending on container of media and labor expenditure. Bioreactor is a scaled-up and automated system. This system is designed to increase multiplication rates, reduce the amount of handling time during the steps required for micropropagation and minimize production costs (Watt, 2012).

### 2.1.2 Temporary immersion bioreactor

A bioreactor is a self-contained sterile environment which uses liquid nutrient. It is designed for intensive and scaled-up cultures. Plant tissue culture in the bioreactor is fed to a large volume generally focused on commercial interests. There are many type of laboratory bioreactors, for example, aeration bioreactor, aeration-agitation bioreactor (Gupta and Ibarak, 2008) and temporary immersion bioreactor (McAlister *et al.*, 2005). There are many uses of bioreactor for propagated crop plants. In pharmaceutical study, bioreactor is used to produce secondary metabolites. The CPT production from hairy root cultures of *O. pumila* was evaluated using bioreactor (Saito *et al.*, 2001). This study compared CPT content between hairy root cells cultured in 3 - liters bioreactor and 100 ml - flask. The result showed that cells in bioreactor can produce similar amounts of CPT compared to cells in flask per unit volume. This experiment shows that it is possible to increase the production of large quantities of this material with the bioreactor. However, the weight of the plant tissue grown in 3 liters agitation bioreactor was lower than hairy root culture. Because the roots of this species are very thin and some roots were traumatized by the propeller of bioreactor. Therefore, in our study, we use TIB for micropropagation to reduce tissue damage from agitation. The production of CPT by TIB was also determined.

Temporary immersion system (TIS) is the micropropagation method by using temporary immersion bioreactor (TIB). The name of this system implies that the plant cultures are not constantly immersed in liquid media, which affects abnormal

morphogenesis and plant growth (Watt, 2012). This method is a semi-automated system that combines the advantages of semi-solid culture and liquid culture. Therefore, it has many advantages over traditional plant propagation. For example, the cell culture from TIS will grow faster than semi-solid culture. TIS can reduce hyperhydricity of the plant tissue by avoiding submergence in liquid medium at all times. Hyperhydricity can cause abnormal symptoms of growing plants and plant death. Moreover, this system can feed the sterile air to the plant tissue vessel, which is necessary for plant growth. As a result, when regrowth plantlets, their physical plants are similar to those found in nature (McAlister *et al.*, 2005; Watt, 2012). It also can reduce the labor of subculture (Putalun *et al.*, 2007; Watt, 2012; Georgiev *et al.*, 2009).

The TIB used in this study was the twin flasks type (Escalona *et al.*, 1999). This TIB consists of two main components. There are two glass vessels. First glass is called “culture vessel” for packing plant tissue and another is called “medium vessel” for containing liquid medium. The two vessels are connected by a silicone tube to deliver the liquid medium by pressure from the air compressor through filter. This is a close system with filter 0.2 micron to prevent contamination of bacteria from the atmosphere (McAlister *et al.*, 2005) (Figure 9).

The principle of TIS is a semi-automatic system to control the timing of the medium feeding (Figure 10). When valve (1) is opened and valve (2) is closed, the compressed sterile air is moved through valve (1) and forces the medium in medium vessel into the culture vessel to immerse the plant tissues. This process is called “feeding period” (Figure 10a). The process is reversed when valve (2) is opened and valve (1) is closed. The air pressure forces the medium back into the original medium vessel. This process is called “feeding back period” (Figure 10b). The air pressure is the leading of liquid medium. The frequency of time to feeding medium is called “immersion cycle”. The period of time to immerse plant tissues is called “immersion time”. This system has many advantages over traditional aeration-agitation type bioreactor. Tissues are not destroyed by the centrifugal force of the blades, so TIS will make increases biomass.

**Figure 9** Temporary immersion bioreactor.

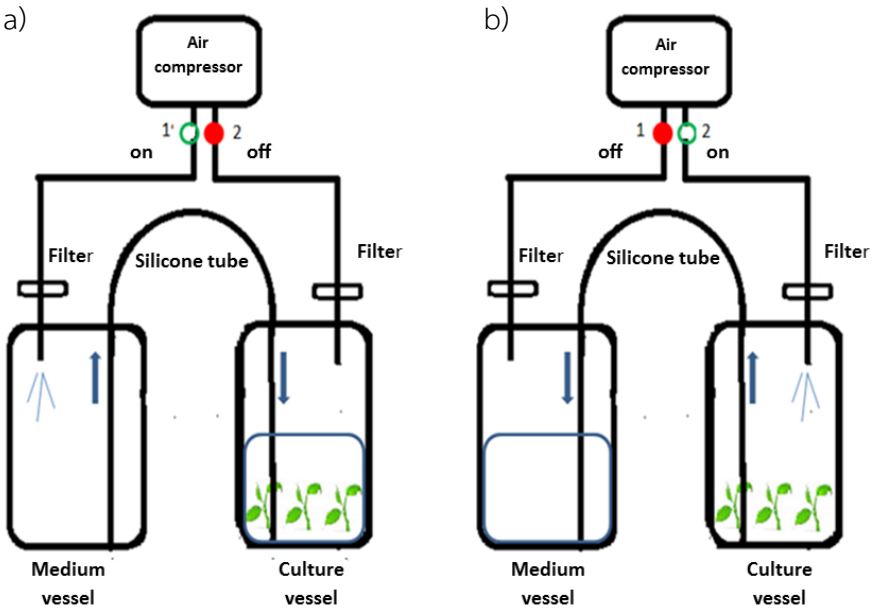



**Figure 10** Principle of temporary immersion bioreactor diagram.
a) feeding period, b) feeding back period.

**Application of temporary immersion bioreactor**

Nowadays, TIB is used in numerous applications for example, micropropagation, regeneration, and phytochemical production. Micropropagation of economic crop plants has been reported in bamboo (*Bambusa vulgaris*) (García-Ramírez *et al.*, 2014), eucalyptus (*Eucalyptus* clones) (McAlister *et al.*, 2005), monk fruit (*Siraitia grosvenorii*) (Yan *et al.*, 2010), new hybrid *Hippeastrum* (Ilczuk *et al.*, 2005), pistachio (*Pistacia vera*) (Akdemir *et al.*, 2014), plantain (*Musa* spp.) (Roels *et al.*, 2005), robusta (*Coffea canephora*) (Ducos *et al.*, 2007) and vanilla (*Vanilla planifolia*) (Ramos-Castellá *et al.*, 2014). Plant regeneration has been reported in cocoa (*Theobroma cacao*) (Niemenak *et al.*, 2008) and hop (*Humulus lupulus*) (Gatica-Arias and Weber, 2013). Improving secondary embryogenesis has been done in oak (*Quercus robur*) (Mallo´n *et al.*, 2012). Some species of medicinal plants have been used to obtain biomass as raw material in the production of secondary metabolites such as North American ginseng (*Panax quinquefolius*) (Uchendu *et al.*, 2011). Many phytochemicals were produced by using this bioreactor such as cardenolides (Perez-Alonso *et al.*, 2012), silymarin, shikonin and ginsenoside (Paek *et al.*, 2005).

**Camptothecin production by resin absorption**

Plant tissue culture has been used in conjunction with other techniques to increase the amount of CPT for example elicitation (Asano *et al*., 2004), hairy root culture (Ya-ut *et al*., 2011) and resin absorption (Saito *et al*., 2001). Absorption technique was applied by using adsorbent for absorb secreted phytochemical compounds in the liquid medium (Yamamoto *et al.*, 1996; Vogel and Todaro, 1996). In addition to increasing the amount of biomass from plant micropropagation, using an adsorbent material can increase the amount of secreted compounds. Absorption technique can apply with some species which is able to secrete phytochemical compounds from cell to environment. There have been reported that in suspension culture, cell culture released some substances into the liquid medium. Some species

of *Ophiorrhiza* can be released CPT into liquid medium such as *O. alata* (Ya-ut *et al*., 2011) and *O. pumila* (Saito *et al*., 2001).

The successful production of CPT by resin absorption was reported in Saito study, which was applied hairy root culture technique in bioreactor with resin absorbent. The CPT production of *O. pumila* was done by 3 liters bioreactor. CPT could be produced from tissue culture and some of CPT could be released from tissue into the liquid medium. The released CPT was harvested from medium by polysterene resin absorption (Saito *et al.*, 2001). The results show that CPT content in absorbed condition is more than a state that is not used as absorbents 5 times. Moreover, it was reported that amount of CPT in hairy root culture of *O. alata* which used same absorbent can increase 7 times (Ya-ut *et al.*, 2011). In addition, hairy root culture of *O. pumila* in agitation type bioreactor can produce CPT which was absorbed by the same resin into liquid medium by 17 percent. The CPT isolation from resin was able to do with methanol, which is the most effective and feasible to be used in the CPT production (Saito *et al.*, 2001).

# CHAPTER III

# MICROPROPAGATION OF *O. HARRISIANA*

*Ophiorrhiza harrisiana* is a rare and endemic plant species. It has a very poor natural regeneration percentage. This plant is an herbaceous plant which is found only in certain seasons and is difficult to breed. *O. harrisiana* is a CPT-producing plant. The harvest of this plant from their natural habitat for CPT extraction can lead to diminution of plant resources.

The aim of this study was to establish *in vitro* micropropagation of *O. harrisiana* for multiple shoot regeneration through shoot explants by conventional tissue culture method (CM) and temporary immersion bioreactor (TIB).

## 3.1 Materials and Methods

### 3.1.1 Plant material

Plant samples were collected from Chang Island, Trat, Thailand (Chamchumroon and Puff, 2003) in May, 2013 and authenticated by Dr. Ivan A. Schanzer of the Herbarium of the Main Botanical Garden, Russia. The voucher specimens were deposited at the herbarium of the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Seeds of *O. harrisiana* were harvested and used for *in vitro* culture technique.

### 3.1.2 Micropropagation by conventional method

#### Preparation of aseptic explants and germination

Seeds of *O. harrisiana* were soaked with running tap water for approximately 1 hour. The optimum condition for sterilization was surface sterilized with 70% ethanol for 1 min, 1% sodium hypochlorite (Clorox®) with 2 additional drops of

Tween-20, shaken for 15 min and then washed 3 times with sterile distilled water for 5 min. All steps were done under aseptic conditions.

After surface sterilization process, seeds were placed on various media, which including half strength MS (1/2 MS) (Murashige and Skoog, 1962), 1/2 MS with 0.3% charcoal (1/2 MSC), MS, and Gamborg's medium (B5) (Gamborg *et al.*, 1968) without plant growth regulator (PGR) for induce seed germination. All media were supplied with 2% sucrose and 0.8% agar. The medium pH was adjusted to 5.7 before autoclaving at 121°C and 1.2 kg $cm^{-2}$ pressure for 20 min. All cultures were kept at 25°C under 16 h light/8 h dark cycle. The germination process was shown in Figure 11. The germinated period was observed for 4 weeks. The plantlets were subcultured to new media with the same formula every 4 weeks for shoot elongation. They were used as explants for the next step. Eight weeks after germination, seedings showing a single shoot with 2-4 true leaves were cut to approximately 1 cm in size and used as shoot explants. Shoot explants were used to study the effects of plant growth regulators on shoot multiplication.

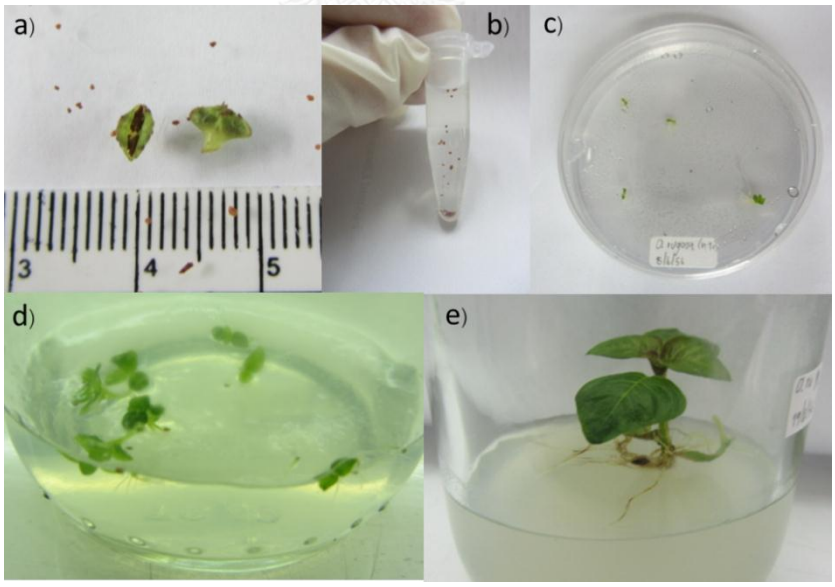



**Figure 11** Germination process.

a) seeds of *O. harrisiana*, b) surface sterilization, c) 4-week old plantlets after germination, d) 6-week old plantlets after germination, and e) 8-week old plantlets on MS medium.

**Preparation of nutrition media**

For semi-solid culture, approximately 800 ml of distilled water were poured into a beaker. The basal media were added and stirred continuously using a magnetic stirrer. Compositions of the culture media were described in Appendix (Table A1). After the media were dissolved, the sucrose and plant growth regulators by the formula were added into the solution. The pH was adjusted to 5.7-5.8 with a few drops of 1M sodium hydroxide and 1M hydrochloric acid solution. The volume of solution was adjusted to 1L with distilled water. Agar was added in the final sequence. The solution was heated until dissolved completely. The media were poured into a container and then autoclaved at 120°C for 20 minutes. The sterile media were stored at room temperature in dark condition. Plant tissue cultures were maintained in a room with 16 hour light period at illumination of 1,500 lux and 8 hour dark period. The incubation temperature was maintained at 25±2°C.

Nutrition media: MS, 1/2 MS, 1/2 MSC and B5 were used as media in this study.

Plant growth regulators: auxins and cytokinins were used as PGRs in different types and concentrations.

Auxins: Naphthaleneacetic acid (NAA), and 2, 4-dichlorophenoxy acetic acid (2, 4-D)

Cytokinins: kinetin-6-furfurylaminopurine (Kn), and benzylaminopurine (BA)

Media for the induction multiple shoots on semi-solid culture were ½ MS media with different types and concentrations of plant growth regulators. They were differently treated as follows:

Group 1: Kn 2.3, 4.6, 6.9, and 9.2 µM (Treatment 1-4)

Group 2: Kn 2.3, 4.6, 6.9, and 9.2 µM +NAA 0.27 µM (Treatment 5-8)

Group 3: Kn 2.3, 4.6, 6.9, and 9.2 µM +NAA 0.54 µM (Treatment 9-12)

Group 4: BA 2.2, 4.4, 8.8, and 13.2 µM (Treatment 13-16)

Group 5: BA 2.2, 4.4, 8.8, and 13.2 µM+NAA 0.27µM (Treatment 17-20)

Group 6: BA 2.2, 4.4, 8.8, and 13.2 µM+NAA 0.54 µM (Treatment 21-24)

Group 7: 2, 4-D 2.3, 4.6, 9.2, and 13.8 µM (Treatment 25-28)

Control: ½ MS without PGRs

**Induction for multiple shoots**

Eight weeks after germination, shoot explants were cut into approximately 1 cm length. Explants were cultured on 1/2 MS medium supplemented with various PGRs for induction of multiple shoots. All cultures were kept at room temperature with 16 hour light period and 8 hour dark period. After 8 weeks, the multiplication rate was recorded in term of the number of shoots, the length of shoot and fresh weight. The data was statistically analyzed by SPSS software.

**Induction of *in vitro* root**

Eight weeks after multiplication process, shoot explants were induced to roots by subculture to 1/2 MS medium, 2% sucrose, 0.8% agar with the supplement of NAA 0.27 µM. Plantlets with rooting were prepared to be grown *ex vitro*.



**Transplanting to greenhouse**

Eight-week old plantlets by *in vitro* rooting were transplant to greenhouse. They were removed agar by thoroughly tap water, transplanted to autoclaved vermiculite about 4 weeks, and then transfered to autoclaved soil (coconut husk: rice ashes (1:1) v/v). All plantlets were covered with a plastic bag in order to maintain humidity.

### 3.1.3 Micropropagation by temporary immersion bioreactor

#### Plant material for temporary immersion bioreactor

Approximately 1 cm of shoot explants was cut from the three-month old of plantlets grown in semi-solid culture. They were tested for microorganism contamination in liquid medium. To perform contamination test, each explants was transferred to 1/2 MS liquid medium without PGR at pH 5.7 and supplemented with 2% sucrose (Figure 12). The tested liquid medium was shaken at 200 rpm for 5 days. The sterile explants, which were not contaminated with microorganisms, were evaluated to pass the test. Sterile shoots were transferred to culture vessel of TIB (Model: National Research Council of Thailand). This equipment was supported by the Plant Genetic Conservation Project under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn.

#### Preparation of nutrition media

Nutrition preparation for growing plantlets in TIB has the same formula and procedure as CM but without agar addition.



#### Optimization condition for TIB

Optimization of immersion time: The volume of liquid medium is 200 ml per vessel. Three immersion times of 1 min, 5 min and 30 min, were used in the experiment. Five shoot explants were used per culture vessel. Each treatment was done in triplicate. The experiment was observed for 3 days. The characteristics of shoots were recorded.

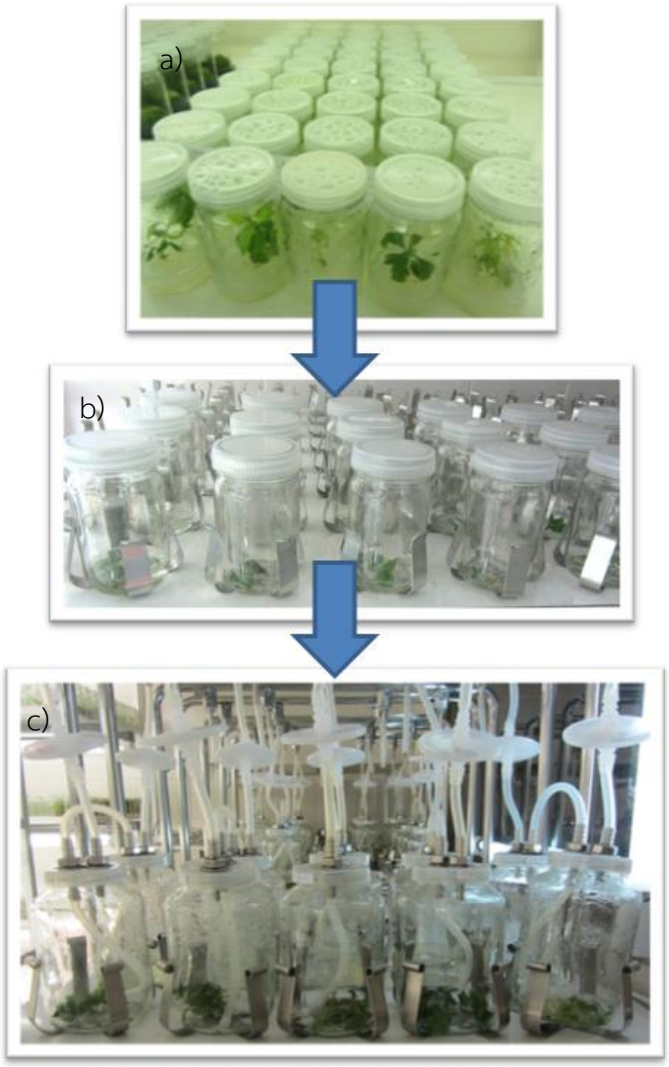



**Figure 12** Contamination test process of explants from conventional method to temporary immersion bioreactor.
a) shoots in conventional method by semi-solid culture, b) contamination test of shoots in liquid medium, and c) sterile shoots in temporary immersion bioreactor.

Optimization of immersion cycle: Three immersion cycles of feeding medium, 6 hours, 8 hours and 12 hours, were used in the experiment. The volume of liquid medium is 200 ml of 1/2 MS medium per vessel without PGR. The length of shoot explants was approximately 1 cm. Five shoot explants were used per culture vessel. Each treatment was done in triplicate. The TIB culture was subcultured every 2 weeks. The growth rate was recorded in term of number and length of shoots every 2 weeks for 8 weeks. The experiment data was collected and statistically analyzed.

The modified TIB used in this study is a twin-flask system that contained with 1.5-cm-diameter size round crystal balls. Approximately 40 balls were used in each culture vessel. This system was adapted to suitable for *in vitro* condition of *O. harrisiana* plant.

The five treatments which were chosen from the three highest multiplication formulas of CM were used as follows:

Treatment 1: 1/2 MS media without PGRs.

Treatment 2: 1/2 MS media with BA 2.2 μM.

Treatment 3: 1/2 MS media with BA 4.4 μM.

Treatment 4: 1/2 MS media with Kn 4.6 μM.

Treatment 5: 1/2 MS media with Kn 2.3 μM and NAA 0.27 μM.

All treatments were supplement with 2% sucrose. The pH was adjusted to 5.7. The cultures were subcultured every 2 weeks. The growth rate was recorded in term of number and length of shoots every week for 4 weeks.

## 3.2 Results

### 3.2.1 Germination of *O. harrisiana* seed

The effect of medium on *O. harrisiana* seed germination was studied. Seed germination was successful on the three media formulas for *in vitro* culture, which

were 1/2 MS, 1/2 MSC and MS (Table 4). Seed germination on 1/2 MS was 85% which was the highest germination rate, followed by 1/2 MSC and MS medium with 80 and 55%, respectively. Time of germination in 1/2 MSC was 21 days which was the fastest. The survival rate 4-week after germination was 100% in all three media (1/2 MS, 1/2 MSC and MS). This experiment found that B5 medium was not suitable for seed germination of this plant. Effect of seeding age on seed germination was studied in both *in vitro* and *ex vitro* conditions. The result showed that 7-day-collected seeds growing on 1/2 MS medium had 85% germination and all seedlings could survive after 4 weeks (Table 5). While *ex vitro* condition, only 25 % of seeds could germinate and none of them could survive after 4 weeks. Seed aged more than a year failed to germinate both *in vitro* and *ex vitro* conditions.

**Table 4** Effect of medium on seed germination.

| Medium | Germination time (days) | % Germination | % Survival |
|---|---|---|---|
| Soil | 60 | 25 | 0 |
| B5 | - | 0 | 0 |
| 1/2 MS | 28 | 85 | 100 |
| 1/2 MSC | 21 | 80 | 100 |
| MS | 35 | 55 | 100 |

n = 10, duplicate experiment.

**Table 5** Effect of seeding age on *O. harrisiana* seed germination.

| Condition | Age of seeds (days) | % Germination | % Survival | | |
|---|---|---|---|---|---|
| | | | 0 week | 2 weeks | 4 weeks |
| *Ex vitro* | 7 | 25 | 100 | 38 | 0 |
| (Soil) | > 365 | - | - | - | - |
| *In vitro* | 7 | 85 | 100 | 100 | 100 |
| (1/2 MS) | > 365 | - | - | - | - |

n = 10, duplicate experiment.

### 3.2.2 Micropropagation of *O. harrisiana* by conventional method

Plantlets were subcultured into two semi-solid media, 1/2 MS and MS, for 4 weeks for shoot proliferation. Plantlets grown in 1/2 MS medium had longer shoots than MS medium in average. Shoot length of plantlets generated from 1/2 MS and MS were 1.35 cm and 1.18 cm, respectively.

**Induction for multiple shoots**

The 1-cm size shoots were used as initial shoot explants to determine the effect of PGRs on shoot multiplication. Each experiment was observed with 1 explant per bottle and repeated 10 times. The effect of PGRs to induce multiple shoots after 8 weeks was shown in Figure 13. The multiplication of shoots in CM was successful on 1/2 MS medium with various concentrations of Kn, BA, and combinations of Kn with NAA. The PGRs which induced the highest number of shoots were 4.6 µM Kn, followed by 6.9 µM Kn and 4.4 µM BA. The number of shoots were 15.4 $\pm$ 4.2, 9.1 $\pm$ 2.18 and 7.8 $\pm$ 1.62 per explant, respectively. The 1/2 MS medium not supplemented with PGRs did not promote the development of shoot multiplication (Figure 14a). The phenotype of multiple shoots was affected by two cytokinins, Kn (Figure 14b) and BA (Figure 14c), on 4-week old *O. harrisiana* was shown different phenotypes in shoots and leaves. The medium supplemented with Kn yielded a number of shoots with small size of leaves while medium supplemented with BA gave less number of shoots, large size of leaves and some abnormal leaves of vitrification. Combinations of Kn and NAA produced low number of shoots and large size of leaves. The length and weight of shoots were shown in Appendix (Table A3, A4).

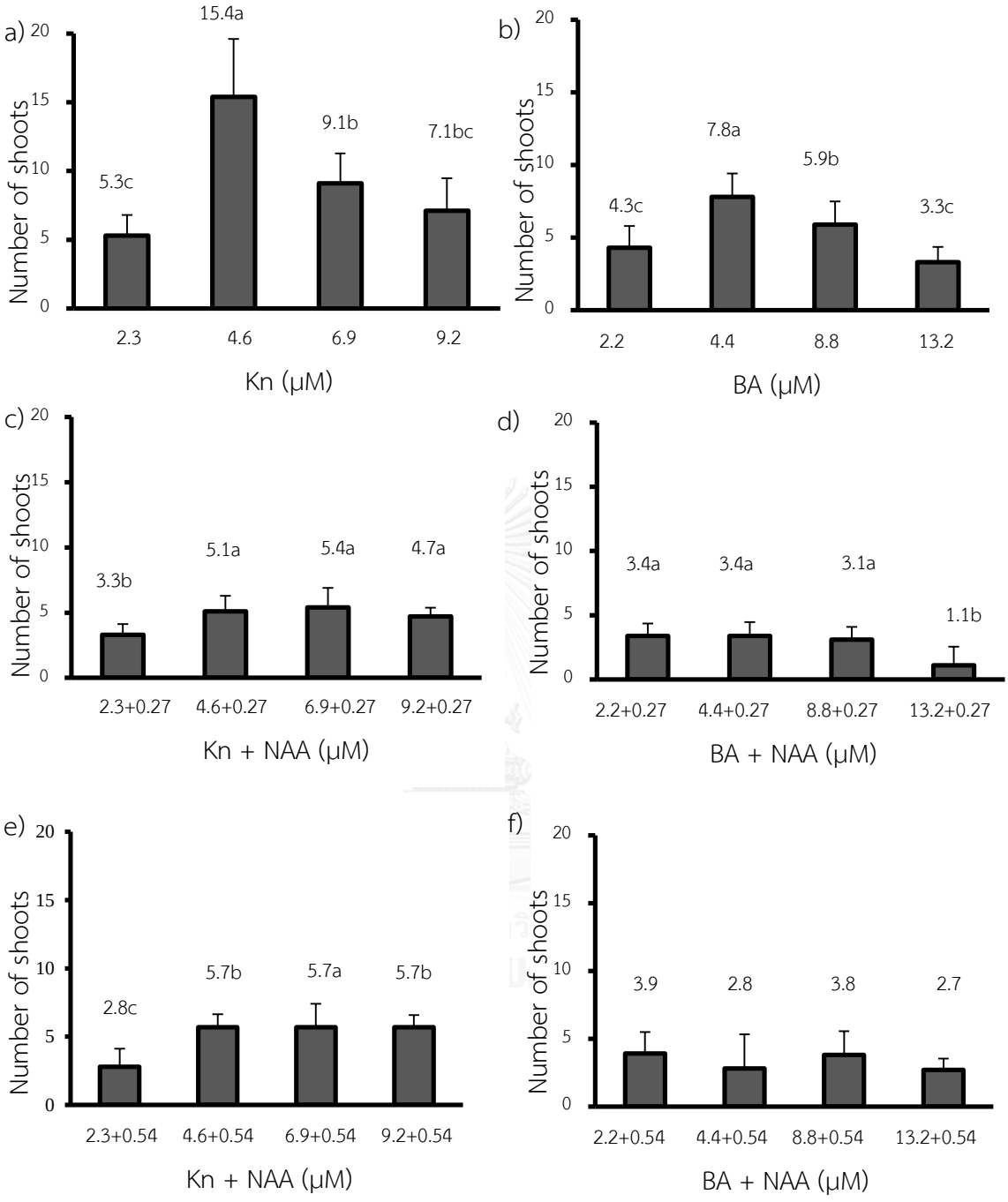



**Figure 13** Effect of plant growth regulators on the number of shoots.

a,b,c,d values followed by a different letter were significantly different at $p < 0.05$.

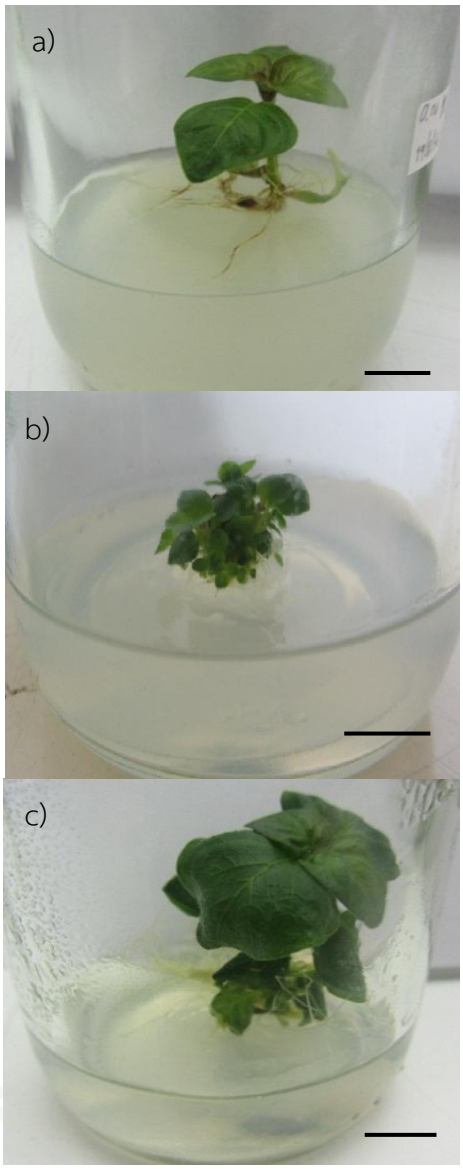



**Figure 14** Effect of plant growth regulators (PGRs) on the phenotype of 4-week old conventional method culture of *O. harrisiana.*
a) 1/2 MS media without PGRs, b) 1/2 MS with 4.6 µM Kn, and c) 1/2 MS with 4.4 µM BA, bar= 10 mm.

**Induction of *in vitro* roots**

Eight weeks after induction of multiple shoots, each shoot explant was subcultured in 1/2 MS medium without PGR to induce the elongation of shoots. After eight weeks of shoot elongation, each shoot was separated from shoot clusters and

used as explant for root induction. Shoots were incubated on 1/2 MS medium supplemented with 0.27 µM NAA to evaluate the induction and proliferation of adventitious roots. The cultures were kept under light and temperature regime as the condition of shoot multiplication cultures. Rooting induction was successful in 4 weeks. The process of transferring from *in vitro* culture to *ex vitro* was done by removing agar, transplanted to autoclaved vermiculite for 4 weeks, and then transferred to soil. All plants were covered with plastic bag in order to maintain humidity (Figure 15). Plants were acclimatized and regrown in green house condition at 25°C.


**Figure 15** The process of transferring tissue cultures of *Ophiorrhiza harrisiana* from *in vitro* culture to *ex vitro*.

a) induced root of *O. harrisiana* from shoot, b) transplanted a plantlet of *O. harrisiana* to autoclaved vermiculite, c) transfer a plant of *O. harrisiana* to autoclaved soil and d) soil - growth plant of *O. harrisiana* with its flowers.

### 3.2.3 Micropropagation of *O. harrisiana* by temporary immersion bioreactor

This is the first report to study suitable condition for micropropagation of *O. harrisiana* in TIB. Optimization of immersiom time, immersion cycle, and PGRs for TIB micropropagation has been done.

**Optimization of immersion cycle and immersion time**

The best time periods of immersion cycle and immersion time were every 8 hour of medium feeding and 1 minute immersion, respectively. The number of shoots was highest (3.2 shoots per explant) in immersion cycle at 8 hour frequency. Six and 12-hour immersion cycle gave 2.2 and 1.8 shoots per explant, respectively (Figure 16). Therefore, the immersion cycle at 8 hour frequency was chosen for the study of micropropagaion and CPT production in the experiment.

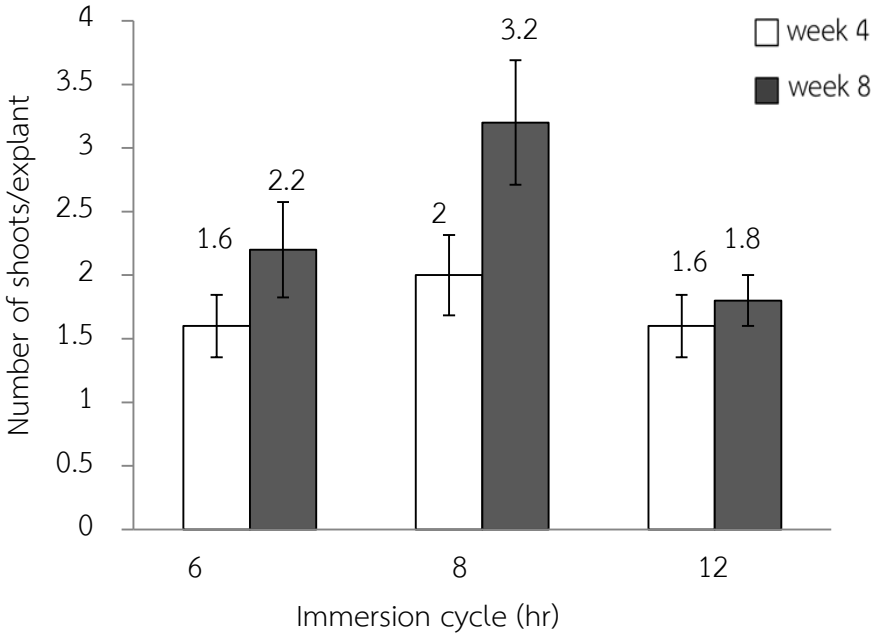



**Figure 16** Effect of immersion cycle on the number of shoots of *Ophiorrhiza harrisiana* by temporary immersion bioreactor culture.

**Optimization of PGRs for micropropagation by TIB**

Comparison of effect of various PGRs on *O harrisiana* shoot length was done in TIB. One-cm shoot explants and 5 explants per one culture vessel were used in the study. The immersion cycle was every 8 hour and 1 min of immersion time. The liquid medium was 200 ml per vessel. After 8 weeks, the result showed that the longest shoots (2.63 cm) was found on 1/2 MS medium supplement with 4.4 µM BA, followed the medium without PGRs (2.26 cm) and medium supplemented with 2.2 µM BA (2.15 cm) (Figure 17). It was observed that shoots on media supplemented with BA showed high degree of leaf hyperhydricity. The highest number of shoots (3.6 per explant) was found on 1/2 MS medium supplemented with 2.3 µM Kn and 0.27 µM NAA, followed the 1/2 MS medium without PGRs (3.4 per explant) and medium supplemented with 4.6 µM Kn (3.2 per explant). It was observed that the length of shoots on media supplemented with Kn was very short. Therefore, the 1/2 MS medium without PGR was the optimum medium for regeneration of *O. harrisiana* by TIB.

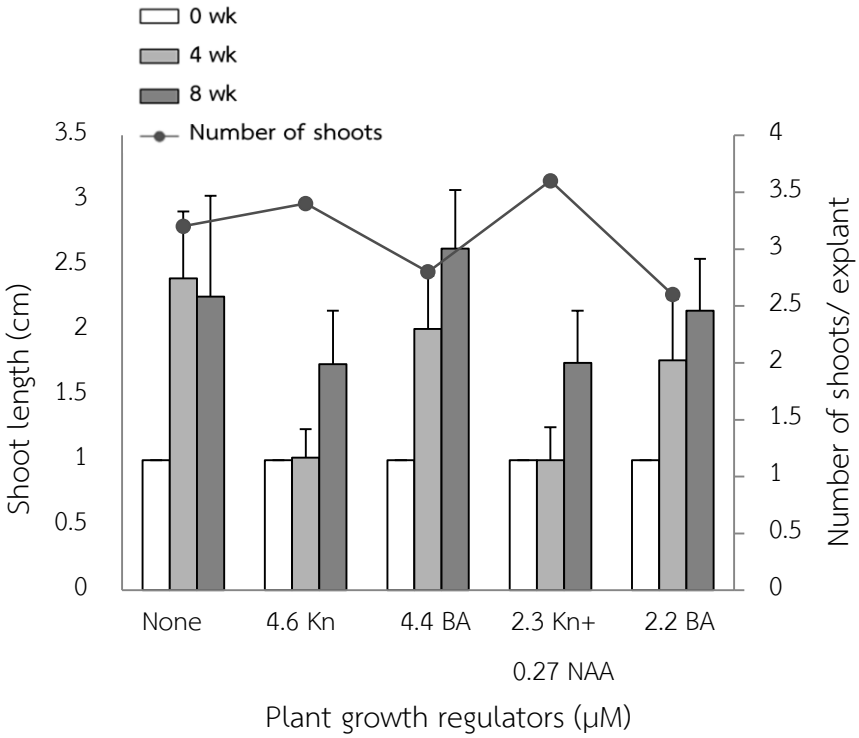



**Figure 17** Effect of PGRs on shoot length and number of shoots of *O. harrisiana* cultured by temporary immersion bioreactor culture in ½ MS medium.

**Comparison of conventional method and temporary immersion bioreactor cultures of *O. harrisiana***

**Effect of culture system on the number of *O. harrisiana* shoots**

At the end of 10 weeks, the number of *O. harrisiana* shoots regenerated from CM and TIB was compared. Two cm shoot explants and 5 explants per culture vessel growing in 1/2 MS medium without PGR were used in the experiment (Figure 18). Three replicates of each treatment were done. The immersion cycle and immersion time of TIB was every 8 hours and 1 min, respectively. The result showed that the number of shoots in TIB and CM was 24.67 and 5.67 shoots per explant at the tenth week, respectively (Figure 19). Shoot generation through TIB was therefore 4.4 times more effective than through CM.

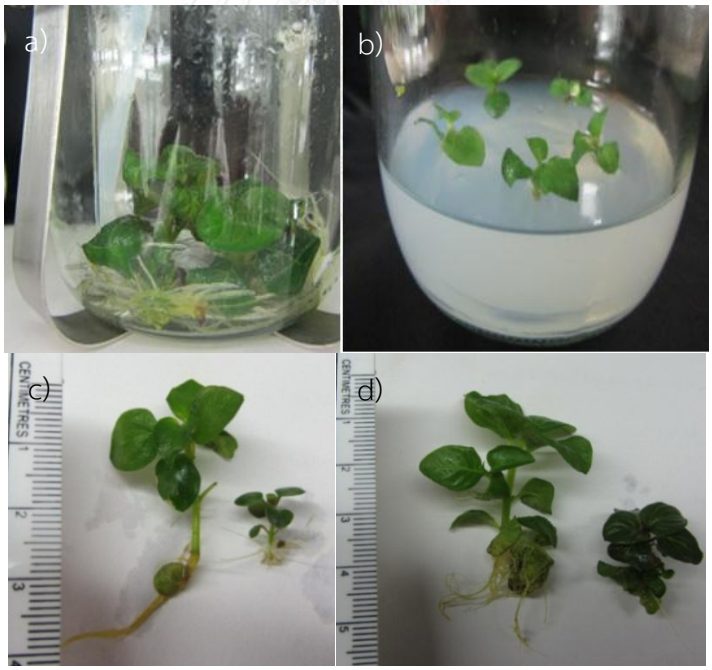



**Figure 18** Comparison of the length of shoot obtained by temporary immersion bioreactor (TIB) and conventional method (CM) culture.
a) 1-week old cultures in 500 ml vessel of TIB, b) 1-week old cultures in 500 ml vessel of CM, c) 2-week old cultures from TIB (left) and CM (right), and d) 4-week old cultures from TIB (left) and CM (right).

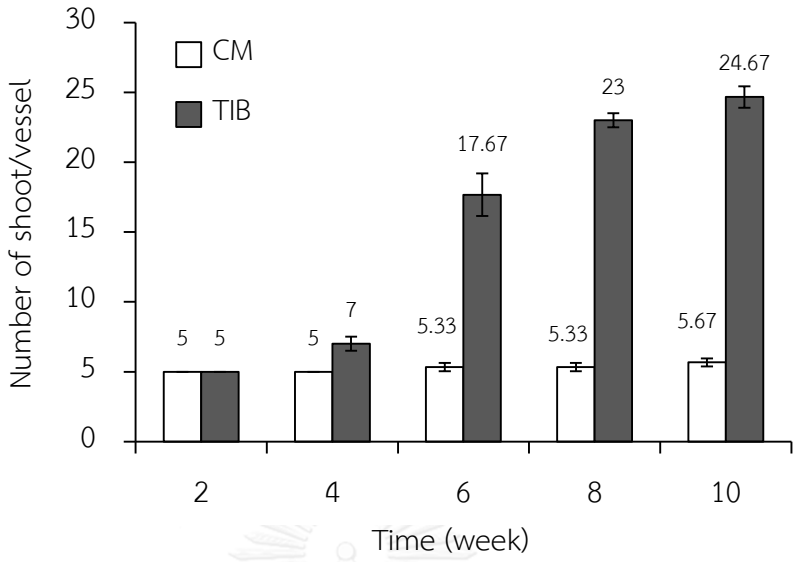



**Figure 19** Effect of culture system on the number of *O. harrisiana* shoots.

**Effect of culture system on *O. harrisiana* shoot length**

Shoot length of *O. harrisiana* generated by CM and TIB culture was compared at the end of the tenth week (Figure 20). Two cm shoot explants with 5 explants per bottle were used. Each treatment was done in triplicate. After 10 weeks, the average shoot length in TIB and CM were 6.83 and 4.87 cm, respectively, with maximum shoot length of 14.8 cm and 6.1 cm, respectively. The average shoot length of plantlets generated by TIB is 1.4 times longer than by CM. The data on average and maximum shoot length were shown in Appendix (Table A4).The phenotypes of CM and TIB culture of *O. harrisiana* at the end of the tenth week were shown in Figure 21.

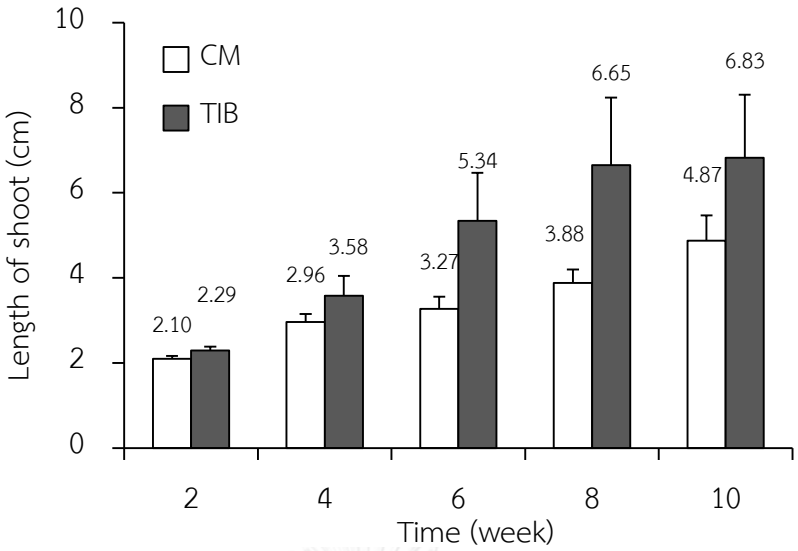



**Figure 20** Effect of culture system on *O. harrisiana* shoot length.


**Figure 21** Comparison between conventional method (CM) and temporary immersion bioreactor (TIB) of *Ophiorrhiza harrisiana* tissue cultures.

a) 10-week old culture in 500 ml vessel of CM, b) 10-week old culture in 500 ml vessel of TIB, and c) the process of micropropagation of *O. harrisiana* by TIB culture, bar = 10 mm.

### 3.3 Discussion

*Ophiorrhiza harrisiana* is an herbaceous plant found in the eastern part of Thailand. Plants in the genus *Ophiorrhiza* can grow only in specifically suitable habitat. *O. harrisiana* is a rare species that normally grows in shady and moist areas in the forest. It appears to make this plant at great risk of extinction. For conservation, micropropagation by plant tissue culture is a popular approach. In this study, the micropropagation of *O. harrisiana* was established using CM and TIB. Seeds of *O. harrisiana* were collected and germinated in *in vitro* and *ex vitro* conditions. The freshly collected seeds germinate well in both conditions. However, after one year storage, these seeds could not germinate at all. It was found that seeds of *O. harrisiana* have short viability for storage. In this study, the ability of *O. harrisiana* seeds to germinate in soil was low. This is similar to a previous report of poor seed germination of *Ophiorrhiza* plants (Sirikantaramas *et al.*, 2013). The *in vitro* propagation by tissue culture techniques could increase the rate of germination and survival of this plant.

The micropropagation by using suitable PGRs combinations has been used to generate many plants (Namdeo *et al.*, 2012). In this study using CM, medium for induction of multiple shoots was ½ MS with different types and concentrations of PGRs (Kn, BA and Kn in combination with NAA). Shoot multiplication on shoot explants was successful on 1/2 MS medium supplemented with different concentrations of Kn and Kn in combination with NAA. Similarly, shoot multiplication from leaf and node explants of *O. alata* was successful when 1/2 MS medium supplemented with different concentrations of Kn and NAA was used (Ya-ut *et al.*, 2011). Maximum multiple shoot induction of *O. harrisiana* was 15.4 shoots per explant in 1/2 MS supplemented with 4.6 µM Kn. This result is in agreement with those from *O. prostrate* (Beegum *et al.*, 2007) and *Ficus carica* (Fra´guas *et al.*, 2004), in which Kn alone gave the best multiplication result. Shoots from *In vitro* culture of *O. harrisiana* on 1/2 MS media with 2.3-9.6 µM of Kn, had normal phenotype. In contrast, shoots in medium supplemented with high concentration of BA (>6.6 µM)

exhibited abnormal character from hyperhydricity. The phenomenon of hyperhydricity induced by BA was found in other *in vitro* plants such as *Aloe polyphylla* (Ivanova and Van Staden, 2011). Treatment with high concentration of BA caused abnormal stomata, broken epidermis layers of guard cells and reduced survival during the acclimatization stage of teak tissue cultures (Quiala *et al.*, 2012).

In this study, temporary immersion of *O. harrisiana* was performed for 1 min every 8 hrs in 1/2 MS medium without PGR. In previous study, supporting net was used as supporting material in large-scale production of *Kalopanax septemlobus* plantlets using bioreactor (Kim *et al.*, 2011). However, in this study crystal balls were used as a supporting system for explants in TIB cultures in order to avoid complete submersion of shoots in the liquid medium. The crystal balls in TIB have 2 main benefits. First, the plantlets were immobilized that can help to promote plant growth. Second, placing plantlets on crystal ball can help to reduce time to contact liquid media. This position of plantlets on crystal ball was similar to *O. harrisiana* position in natural habitat. The induction of multiple shoots by TIB culture could occur in the medium without PGR. However, in CM, the shoot multiplication needed PGRs. At the tenth week of culture, the number of shoots was 24.67 per explant in TIB, while in CM it was 5.67 per explant. Therefore, TIB produced 4.4 times higher number of shoots than CM. The growth rate of *O. harrisiana* growing in TIB was obviously faster than CM. The TIB culture was successful in production of many commercial plant tissues such as african yam (Polzin *et al.*, 2014), eucalyptus (McAlister *et al.*, 2005), pistachio (Gupta and Ibarak, 2008) and vanilla (Ramos-Castellá *et al.*, 2014).

In conclusion, tissue culture by CM and TIB can be done on a continuous year-round basis and not destructive for this plant in nature, thus making it an alternative method for propagation and conservation of the *Ophiorrhiza* plants. We have successfully established the tissue culture of *O. harrisiana* in both CM and TIB. TIB has more advantages than CM. TIB could increase the biomass accumulation of plantlets and reduce cost from using some substance such as gelling agent and PGRs.

# CHAPTER IV

# THE CAMPTOTHECIN PRODUCTION OF *O. HARRISIANA*

CPT was first discovered from the Chinese plant *Camptotheca acuminata* and also in some species of the genus *Ophiorrhiza*. *O. harrisiana* can produce CPT in the highest amount in both root and leaf while other species produce CPT in root only. The aim of this research is to study the potential of *O. harrisiana* for producing CPT by tissue culture techniques and to enhance the CPT production from roots in the presence of a polystyrene resin (Diaion HP-20).

## 4.1 Materials and Methods

### 4.1.1 Plant material

Samples of *O. harrisiana* were collected from Trat, Thailand in 2013. The voucher specimens were deposited at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.



### 4.1.2 Camptothecin determination of *O. harrisiana*

#### Preparation of *O. harrisiana* crude extract

*O. harrisiana* samples from natural source were separated into leaf and root parts for the determination of CPT content. Extraction of CPT was performed according to procedure previously reported (Saito *et al*., 2001) with minor modifications. Each plant part was freeze-dried and ground by mortar and pestle. Ten mg of dried specimens were extracted with methanol (1 mL), then ultrasonicated for 30 min and kept at 4°C overnight. The crude extracts were centrifuged and the supernatants were filtered through syringe filter 0.45-µm filters

(Filtrex, Singapore) and analyzed by HPLC. The tissue culture samples were harvested and kept at -20°C until used.

**The quantitative analysis of camptothecin**

The calibration curve of standard CPT was prepared. CPT (Sigma Chemical Co., USA) was dissolved in methanol to prepare standard solutions. For HPLC analysis, standard CPT was prepared in the concentration range of 5-50 µg/ml. CPT content was calculated with the regression equations obtained from the calibration curve. Triplicate samples of each treatment were measured and the standard deviation was calculated.

CPT and its derivatives in the samples were identified by UV spectroscopy at 254 nm, MS spectra and their retention times. The contents of compounds in each sample were calculated. Average contents and standard deviation (SD) of triplicate analyses were calculated. HPLC analysis of CPT by was performed by comparing the retention time of CPT peak in chromatograms monitored at 254 nm, UV spectra and mass spectra of standard compounds.

Different tissue specimens from *O. hariisiana* were examined for CPT by HPLC model SHIMADZU LC-20AD (Shimadzu, Japan). HPLC analyses were carried out using a ZORBRAX ECLIPSE PLUS C 18 column (4.6 x 250 mm, 5 µm) (Agilent Co., USA) at a flow rate of 0.8 mL/min. Mobile phase is acetonitrile:water (3:7). The CPT determination by HPLC was performed according to previously reported procedure with minor modifications (Yamazaki *et al*., 2003; Wang *et al*., 2009).

### 4.1.3 Camptothecin release into culture medium and its absorption to resin

The polystyrene resin Diaion HP-20 (Mitsubishi Chemical Co., Japan) was used in this study. The diaion HP-20 can adsorb CPT with hydrophobic interaction. This

resin was used to increase the CPT release into the medium by absorption of the released CPT.

**Optimization of suitable quantity of resin for camptothecin absorption**

The *O. harrisiana* explants were cultured in TIB for CPT production. The Diaion HP-20 adsorbent resin was added into the medium vessel to absorb the released CPT. Polystyrene resin was put inside a nylon mesh bag (Saito *et al,* 2001; Ya-ut *et al,* 2011). All resin bags were steriled before putting in liquid medium. The weights of polystyrene resin which used in this study were 100, 500 and 1000 mg per vessel. Later, the resin was collected and analyzed for CPT content. First, the resin was washed with sterile deionized water and then extracted with methanol. The CPT content was analyzed by HPLC. Comparison of CPT production between the presence and absence of resin was done. All treatments were performed in triplicate.

For time-course study of cultures for CPT absorption, the sterilized resin packets added to the liquid culture medium (200 ml per 500 ml vessel) were changed every 2 weeks and extracted with methanol for the determination of CPT retained in the resin. After eight weeks of culture, the cultures were harvested and CPT extracted for analysis.



## 4.2 Results

### 4.2.1 Camptothecin determination of *O. harrisiana*

The calibration curve of standard CPT showed good linearity, with a regression coefficient ($r^2$) of 0.999. The regression equation for CPT was y = 293491x - 232307 (Figure 22). The CPT content was calculated with the equation.

*O. harrisiana* tissue samples were analyzed for the content of CPT and its analogs by HPLC. The UV-visible spectra of the HPLC peak determined by photodiode array detection showed CPT. CPT exhibited $\lambda$max (nm) at 251, 288 and

364 and pseudomolecular mass fragment ($[M+H]^+$) at m/z 349.18. The UV spectrum of CPT was shown in Appendix (Figure A4).

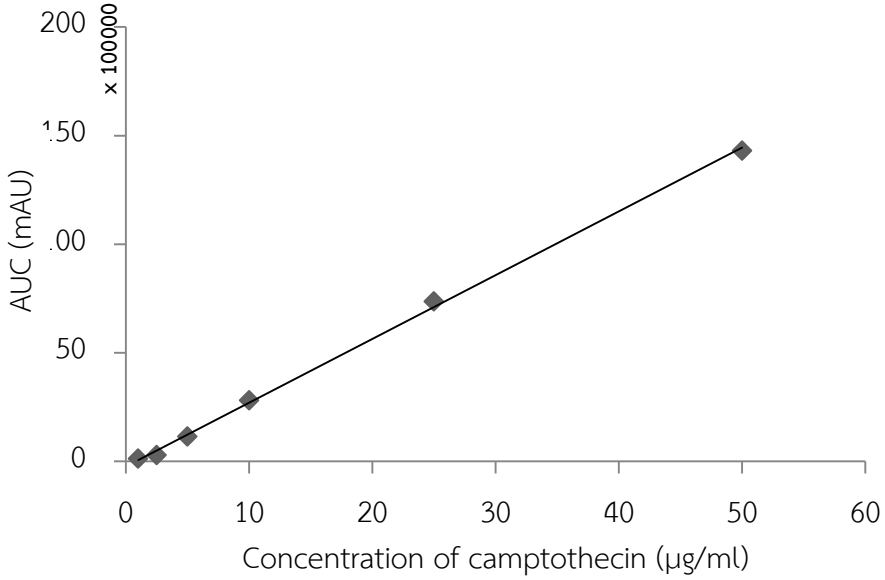



**Figure 22** Calibration curve of standard camptothecin solution with calculated linear regression equation of y = 293491x – 232307, $R^2$ = 0.9999.

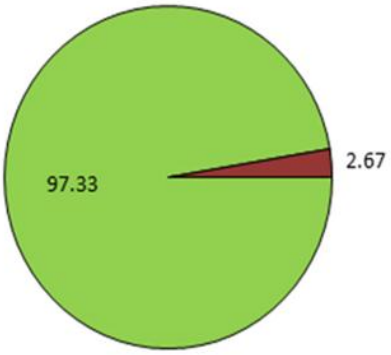
The 4 week old leaves in CM culture could be divided into two main phenotypes. The first phenotype had green color on both adaxial and abaxial surfaces of the leaf (green type), while the other phenotype had red color on abaxial and green color on adaxial surfaces of the leaf (red type) (Figure 23a). By tissue micropropagation, green and red types were 97.33 % and 2.67 %, respectively (Figure 23b). The CPT content in the green type was higher than in red type at 193.465 and 146.82 µg/g dry wt, respectively (Figure 23c).

The phenotypes of *in vitro* and *ex vitro O. harrisiana* roots are shown in Figure 24. The 2-week old *in vitro* root culture was white to light green in color (Figure 24a). The 2-8-week old *in vitro* root culture was light green in color (Figure 24b), whereas the 8-week old *in vitro* root culture was brown (Figure 24c). The 4-week old roots of soil-grown plant and of those from natural source were brown

(Figure 24d-e). The highest amount of CPT content was found in 2-8-week old root culture, followed by the 8-week old root culture and roots from natural source which were 482.76, 217.85 and 714.96 μg/g dry wt, respectively (Figure 25).

a)

b)




c)

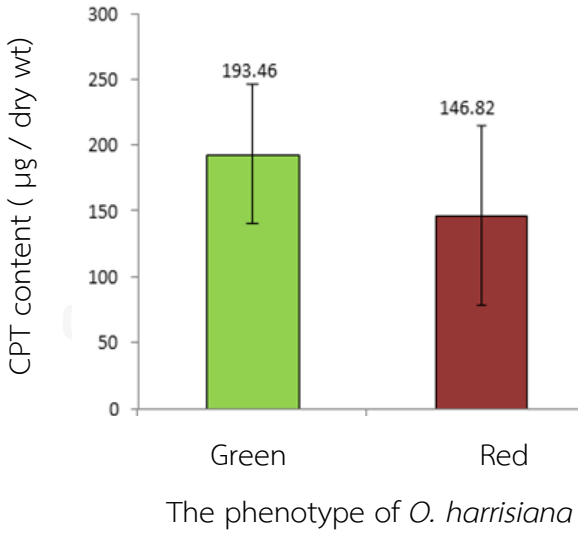



**Figure 23** Different phenotypes of *Ophiorrhiza harrisiana* generated by conventional method and its CPT production.

a) green (left) and red (right) phenotypes of *O. harrisiana* shoot explants, b) pie chart represented percent of the two phenotypes of *O. harrisiana,* and c) CPT contents of green and red leaf phenotypes, bar = 10 mm.

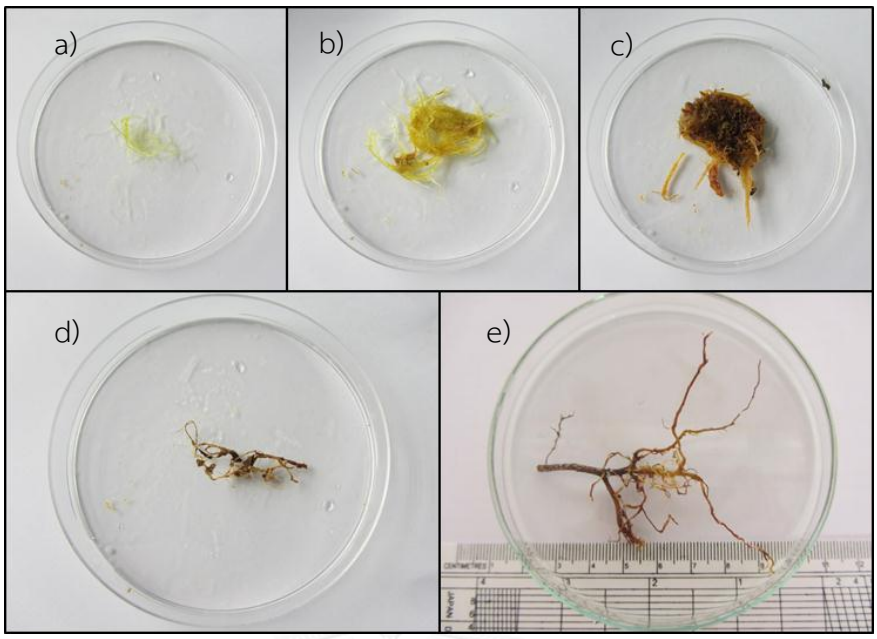



**Figure 24** *In vitro* and *ex vitro* root phenotypes of *Ophiorrhiza harrisiana.*

*In vitro* (a-c) *and ex vitro* (d-e): a) white to light green 2-week old roots, b) light green 2-8-week old roots, c) brown 8-week old roots, d) soil-grown 4-week old roots and e) roots from plants grown in natural source.

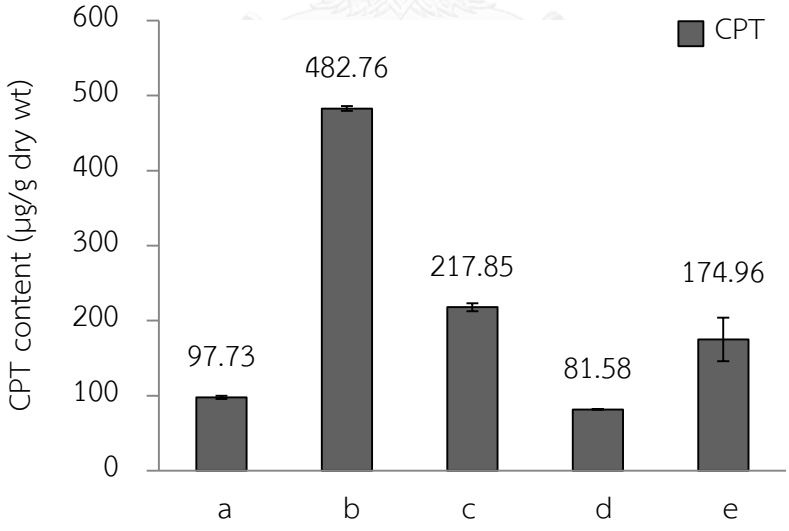



**Figure 25** Camptothecin contents among different phenotypes of roots.

a) white to light green 2-week old roots, b) light green 2-8-week old roots, c) brown 8-week old roots, d) soil-grown 4-week old roots and e) roots from plants grown in natural source.

**Camptothecin content in different parts of natural and tissue cultured *Ophiorrhiza harrisiana* plants**

The contents of CPT in *O. harrisiana* plants from natural source, CM, and TIB were shown in Table 6. The methanol extracts of *in vitro* culture sources (CM and TIB) were analyzed and compared with *ex vitro* sources (natural source and soil-grown plant). The highest amount of CPT content was found in the flower of soil-grown plant, which was 431.27±105.43 µg/g dry wt. The result also showed that CPT amount in the leaf was higher than in the root for every type of sample sources. The highest amount of CPT content in the leaf was found from TIB culture (521.79 ± 150.00) ; followed by from natural source (475.29 ± 40.53) and CM culture (216.81 ± 64.60 µg/g dry wt), respectively. The highest amount of CPT content in the root was found from TIB culture (316.55 ± 117.97), followed by from natural source (186.21 ± 44.11) and by CM culture (178.79 ± 56.01 µg/g dry wt), respectively.

**Table 6** Comparison of the camptothecin contents in different sources of *O. harrisiana.*

| Source | CPT content (µg/g dry wt) | | |
|---|---|---|---|
| | Leaf | Root | Flower |
| Natural source | 475.29±40.53[a] | 186.21±44.11[d] | nd |
| Conventional method | 216.81±64.60[b] | 178.79±56.01[d] | nd |
| Temporary immersion bioreactor | 521.79±150.00[a] | 316.55±117.97[c] | nd |
| Soil-grown plant | 145.86±25.23[b] | 95.43±27.70[e] | 431.27±105.43 |

[a,b,c,d,e] values followed by a different letter were significantly different at $p < 0.05$

Triplicate experiment.

### 4.2.2 Camptothecin release into culture medium and its absorption to resin

Optimization of the amount of resin to absorb CPT release into the liquid medium was done. The amounts of resin used in this study were 100, 500 and 1000 mg per vessel of TIB. The result showed no significant difference between the amounts of resin used to absorb CPT in the medium of two-week old TIB culture (Table 7).

**Table 7** Optimization of resin amount for camptothecin absorption.

| Amount of resin (mg) | CPT content (µg/g ± SD) |
|---|---|
| 100 | 111.58±2.63 |
| 500 | 120.23±5.38 |
| 1000 | 100.71±9.84 |

Tripicate experiment.

**Comparison of the camptothecin production in temporary immersion bioreactor culture in the presence or absence of resin**

**Time–course study of CPT production by TIB**

The measurement of CPT content in liquid medium in the presence or absence of resin was done every 2 weeks for the duration of 8 weeks. The cultures were grown in 500 ml vessel containing 200 ml of 1/2 MS liquid media with and without Diaion HP-20 resin. The polystyrene resin (1000 mg per pack) was added to the culture medium. Resin and liquid medium were collected for measurements of CPT content by HPLC each 2 weeks. In liquid medium without resin, there was no difference in the amount of CPT released from tissue culture into medium during the whole period of experiment. In contrast, the medium with resin showed that the

tissue cultures released more CPT into medium. At the eighth week, the amount of CPT in medium with resin was approximately 3 times higher than control. (Table 8).

**Table 8** Comparison of the camptothecin production in TIB culture in the presence or absence of resin.

| Time | CPT content (µg/ vessel) | |
|---|---|---|
| (week) | Absence of resin | Presence of resin |
| 2 | 97.54±16.00[a] | 198.79±8.72[c] |
| 4 | 81.71±0.65[b] | 184.49±13.34[c] |
| 6 | 84.71±1.78[b] | 232.49±37.64[b] |
| 8 | 95.71±20.31[a] | 285.64±135.86[a] |

[a,b,c,d] values followed by a different letter were significantly different at $p < 0.05$.

Tripicate experiment.

**Effects of polystyrene resin on camptothecin production and excretion**

The polystyrene resin packed with nylon mesh (1000 mg resin per packet) was added to the culture medium (200 ml in a 500-ml vessel). The packets were changed every two weeks and extracted with methanol for the determination of CPT adsorbed to the resin. After 8 week, the tissues were harvested for the determination of CPT retained to the resin. Control was medium no addition of the resin. The result showed that not only did the cultures contain CPT but the liquid medium also accumulated substantial amounts. The amount of CPT from resin HP-20 was higher than medium without resin approximately 3 times (Table 9). The amount of CPT in resin was 2099.24 µg/vessel. The amount of CPT in medium without resin was 770.34 µg/vessel. However, the CPT content between tissue cultures of the medium with resin and the medium without resin was not difference. The amount of CPT from tissue culture with resin was 33.29 mg/vessel. The amount of CPT from tissue culture without resin was 32.62 mg/vessel.

**Table 9** Effects of polystyrene resin on CPT production and excretion.

| Sample | CPT content | |
|---|---|---|
| | Tissue culture (mg/vessel) | Liquid medium (µg/vessel) |
| Presence of resin | 33.29±3.76 | 2099.24±36.44 |
| Absence of resin | 32.62±2.83 | 770.34±14.06 |

Triplicate experiment.

**4.3 Discussion**

*Ophiorrhiza harrisiana* can produce CPT in both root and leaf parts. Since it is an herbaceous, rare and endemic plant, the micropropagation of *O. harrisiana* to increase the production of CPT by tissue culture technique is an interesting solution. Two tissue culture techniques, a conventional method (CM) by semi-solid culture and temporary immersion bioreactor (TIB), were used in this study. CM and TIB also produce CPT in term of the biomass accumulation. The productions of CPT by CM in leaves and roots were 216.81 and 178.79 µg/g dry wt, respectively. For TIB, CPT was found at 521.79 and 316.55 µg/g dry wt in leaves and roots, respectively. The CPT content in leaves and roots of plants grown in natural source was also measured. They were 475.29 and 186.21 µg/g dry wt, respectively. Interestingly, TIB not only gave biomass accumulation, but also produced higher amount of CPT than natural source.

In general, the level of alkaloid production from tissue cultures was 100 - 1000 times lower than from soil-grown plants (Sirikantaramas *et al.*, 2013). Kitajima's group has found that suspension culture, the undifferentiated state, of *O. pumila* failed to produce CPT (Kitajima *et al*., 1998). It is known that the differentiated tissue cultures have better potential to produce secondary metabolites. Therefore, differentiated tissue cultures by CM and TIB were generated for this study of CPT production. Results showed that CPT was produced in the roots and leaves of *O. harrisiana* in both CM and TIB cultures. Moreover, this study showed that the roots

and leaves regenerated from TIB provided higher amount of CPT than natural source. It was similar to previous study (Namdeo *et al*, 2012) in which higher amount of CPT was observed from tissue culture of whole plant than in naturally grown *O. mungos*. In addition, higher amount of some secondary metabolites produced by TIB was also observed. Music and group observed the increase in biomass accumulation and secoiridoid glycosides production from hairy root cultures of *Centaurium maritimum* in TIB (Music *et al*., 2013). This study found that there was a different level of CPT production in different parts of plants. The flower part contains higher amount of CPT than leaf and root. Similar result was supported in that the distribution of CPT content in flower part was higher than leaf and root of *O. pumila* (Sudo *et al.*, 2002).

The large-scale CPT production of *O. harrisiana* by TIB could be to maintain for long period of time. In this case, plantlets have been maintained in the laboratory for more than a year. The plantlets used in the CPT measurement came from three-month–old cultures by TIB. The plantlets retained their ability to produce CPT and be continuously subcultured. Previously successful CPT production by agitation bioreactor was achieved with hairy roots of *O. pumila* (Sudo *et al.*, 2002). However, the culture did not survive after 2 months. It was thus an advantage of *O. harrisiana* culture in TIB that could continuously grow and produce CPT. Many methods are still interesting to explore to get the increase of CPT such as hairy root culture, elicitors and biosynthetic precursors addition, or using adsorbent in liquid medium. In this study, TIB with the presence of adsorbent were used to increase CPT level in *O. harrisiana*.

Addition of adsorbent in tissue culture liquid media is one of several methods to increase CPT production. The polystyrene resin (Diaion HP-20) was used as an absorbent in TIB culture in our study. The presence of Diaion HP-20 increased CPT accumulation in the medium, with the CPT excreted into the medium being absorbed by the resin. Although total CPT production was not enhanced by the presence of resin, the amount of released CPT in medium was approximately three times that of control. This result was similar to the studies of Saito (2001) and Ya-ut (2011). The CPT from hairy root cultures of *Ophiorrhiza* spp., *O. pumila* and *O. alata*, were excreted in the amounts of five to seven times into the medium with the

presence of resin, respectively. This study showed that TIB combination with resin was an effective method, which could enhance the excretion of CPT and continuously produce CPT. The collection of resin and extraction of CPT was simple to do. The presence of absorbent resin could be used in conjunction with the growth of plantlets in TIB.

The research for method development to increase the CPT production by TIB is possible to combine with other methods such as elicitation with biotic and abiotic substrates. The example of biotic substrates which were used to increase CPT production were chitosan and yeast extract (Saito *et al.*, 2001). For abiotic substrates, methyl jasmonate and salicylic acid were used (Saito *et al.*, 2001). The study to increase the CPT production using TIB with the combination of resin absorption and elicitation is feasible. The TIB culture could be continuously realistic CPT production and conjunction with conservation of this threatened plant species.

# CHAPTER V

# CONCLUSION

We have established an efficient system for the in vitro micropropagation of *Ophiorrhiza harrisiana* and the CPT production. The micropropagation of *O. harrisiana* was successful both in conventional method (CM) by semi-solid culture and temporary immersion bioreactor (TIB). Both methods (CM and TIB culture) were successful in the production of CPT.

The plant regeneration by TIB was effective than CM. The number of shoots generated by TIB (24.67 shoots per explants) was 4.4 times higher than CM (5.67 shoots per explants). The shoot length of plants in TIB (6.83 cm) was 1.4 times longer than CM (4.87 cm). Moreover, the experiment of seeds germination showed that the tissue culture technique helped embryo rescued of *O. harrisiana* seeds from low population in natural source. These are many benefits for tissue cultures of this plant, which were micropropagation, conservation and production of CPT.

From this study, the tissue culture of *O. harrisiana* could produce CPT both in the leaf and root. The culture from TIB produced higher levels of CPT than soil-grown and CM culture. The CPT production from TIB (521.79) was 2.4 times higher than CM (216.81 µg/g dry wt) in the leaf. In the root, the CPT production from TIB (316.55) was 1.8 higher times than CM (178.79 µg/g dry wt). In large scale, production of CPT by TIB has more advantages than CM from an increase of rapid mass production, high amount of CPT production, reduction of labor for subculture and improvement of the cost-effectiveness. Moreover, TIB was simply able to combine with other techniques to increase CPT production.

In this study, TIB combination with resin absorption was done for enhanced the excretion of CPT into liquid medium. The CPT accumulation in liquid medium could also be increased three fold by TIB culturing with a polystyrene resin. The isolation of CPT from this combination method could be simple and efficient. This TIB system was a suitable system for CPT production of *O. harrisiana*. The production

of CPT in this plant cell culture can be done on a continuous year-round. Including TIB systems can be the potential factories for secondary metabolites products in biomass in a short time. Therefore, the tissue culture of *O. harrisiana* by TIB has potential for micropropagation and CPT production. The CPT production of this plant by TIB can be the potential source of CPT and reduce exploitation of limited natural source.

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# APPENDIX

**Table A1** Composition of plant culture media

| **Component** | **Chemical formula** | **Concentration (mg/liter)** | | |
|---|---|---|---|---|
| | | **MS** | **1/2 MS** | **B5** |
| **Minerals (mg/L)** | | | | |
| Ammonium nitrate | $NH_4NO_3$ | 1650 | 825 | - |
| Ammonium sulfate | $(NH_4)_2SO_4$ | - | - | 134 |
| Boric acid | $H_3BO_3$ | 6.2 | 6.2 | 3 |
| Calcium chloride anhydrous | $CaCl_2$ | 332.2 | 166.1 | 113.24 |
| Cobalt chloride $6H_2O$ | $CoCl_2 \cdot 6H_2O$ | 0.025 | 0.025 | 0.025 |
| Cupric sulfate $5H_2O$ | $CuSO_4 \cdot 5H_2O$ | 0.025 | 0.025 | 0.025 |
| EDTA disosdium salt $2H_2O$ | $Na_2EDTA$ | 37.3 | 37.3 | 37.3 |
| Ferrous sulfate $7H_2O$ | FeSO4·7H2O | 27.8 | 27.8 | 27.8 |
| Manganese sulfate $7H_2O$ | $MgSO_4 \cdot 7H_2O$ | 370 | 185 | 250 |
| Molybdic acid sodium salt $H_2O$ | $Na_2MoO_4 \cdot 2H_2O$ | 0.25 | 0.25 | 0.25 |
| Potassium iodide | KI | 0.83 | 0.83 | 0.75 |
| Potassium nitrate | $KNO_3$ | 1900 | 950 | 2500 |
| Potassium phosphate monobasic | $KH_2PO_4$ | 170 | 85 | - |
| Zinc sulfate 7H2O | $ZnSO4 \cdot 7H_2O$ | 8.6 | 8.6 | 2 |
| **Organics (mg/L)** | | | | |
| Ascorbic acid | | 5 | 5 | - |
| Glycine (free base) | | 2 | 2 | - |
| Myo-Inositol | | 100 | 100 | 100 |
| Nictotinic acid (free acid) | | 0.5 | 0.5 | 1 |
| Pyridoxine HCl | | 0.5 | 0.5 | 1 |
| Thiamine HCl | | 0.1 | 0.1 | 10 |

**Table A2** Effect of plant growth regulators on multiple shoots by conventional method.

| 1/2 MS with plant growth regulators (μM) | | | | Number of shoots per explant±SD | Length of shoot±SD (cm) | Maximum length (cm) | Leaf color |
|---|---|---|---|---|---|---|---|
| Kn | BA | NAA | 2, 4-D | | | | |
| 0 | 0 | 0 | 0 | 1.00±0.00 | 1.67±0.31 | 2.10 | Light green |
| 2.3 | | | | 5.30±1.49 | 0.55±0.31 | 1.20 | Light green |
| 4.6 | | | | 15.40±4.19 | 0.50±0.27 | 1.40 | Light green |
| 6.9 | | | | 9.10±2.18 | 0.53±0.30 | 1.30 | Green |
| 9.2 | | | | 7.10±2.38 | 0.56±0.31 | 1.40 | Green |
| 2.3 | | 0.27 | | 3.30±0.82 | 0.70±0.41 | 1.30 | Green |
| 4.6 | | 0.27 | | 5.10±1.19 | 0.71±0.34 | 1.40 | Green |
| 6.9 | | 0.27 | | 5.40±1.51 | 0.63±0.38 | 1.40 | Green |
| 9.2 | | 0.27 | | 4.70±0.67 | 0.63±0.36 | 1.30 | Green |
| 2.3 | | 0.54 | | 2.80±1.32 | 0.74±0.36 | 1.30 | Green |
| 4.6 | | 0.54 | | 5.70±0.94 | 0.59±0.34 | 1.30 | Green |
| 6.9 | | 0.54 | | 8.40±1.71 | 0.54±0.29 | 1.20 | Green |
| 9.2 | | 0.54 | | 6.10±0.88 | 0.61±0.37 | 1.40 | Green |
| | 2.2 | | | 4.30±1.49 | 0.73±0.45 | 1.50 | Dark green |
| | 4.4 | | | 7.80±1.62 | 0.51±0.33 | 1.40 | Green |
| | 8.8 | | | 5.90±1.59 | 0.61±0.35 | 1.40 | Green |
| | 13.2 | | | 3.30±1.06 | 0.73±0.37 | 1.30 | Green |
| | 2.2 | 0.27 | | 3.40±0.96 | 0.81± 0.36 | 1.30 | Green |

**Table A2** (continued)

| 1/2 MS with plant growth regulators (μM) | | | | Number of shoots per explant±SD | Length of shoot±SD (cm) | Maximum length (cm) | Leaf color |
|---|---|---|---|---|---|---|---|
| Kn | BA | NAA | 2, 4-D | | | | |
| | 4.4 | 0.27 | | 3.40±1.07 | 0.75±0.41 | 1.40 | Green |
| | 8.8 | 0.27 | | 3.10±0.99 | 0.78±0.42 | 1.40 | Green |
| | 13.2 | 0.27 | | 1.10±1.45 | 1.03±0.34 | 1.40 | Green |
| | 2.2 | 0.54 | | 3.90±1.59 | 0.76±0.41 | 1.40 | Green |
| | 4.4 | 0.54 | | 2.80±2.53 | 0.72±0.35 | 1.30 | Green |
| | 8.8 | 0.54 | | 3.80±1.75 | 0.66±0.43 | 1.40 | Green |
| | 13.2 | 0.54 | | 2.70±0.82 | 0.73±0.39 | 1.30 | Green |
| | | | 2.3 | dead | 0 | 0 | Yellow |
| | | | 4.6 | dead | 0 | 0 | Yellow |
| | | | 9.2 | dead | 0 | 0 | Yellow |
| | | | 13.8 | dead | 0 | 0 | Yellow |

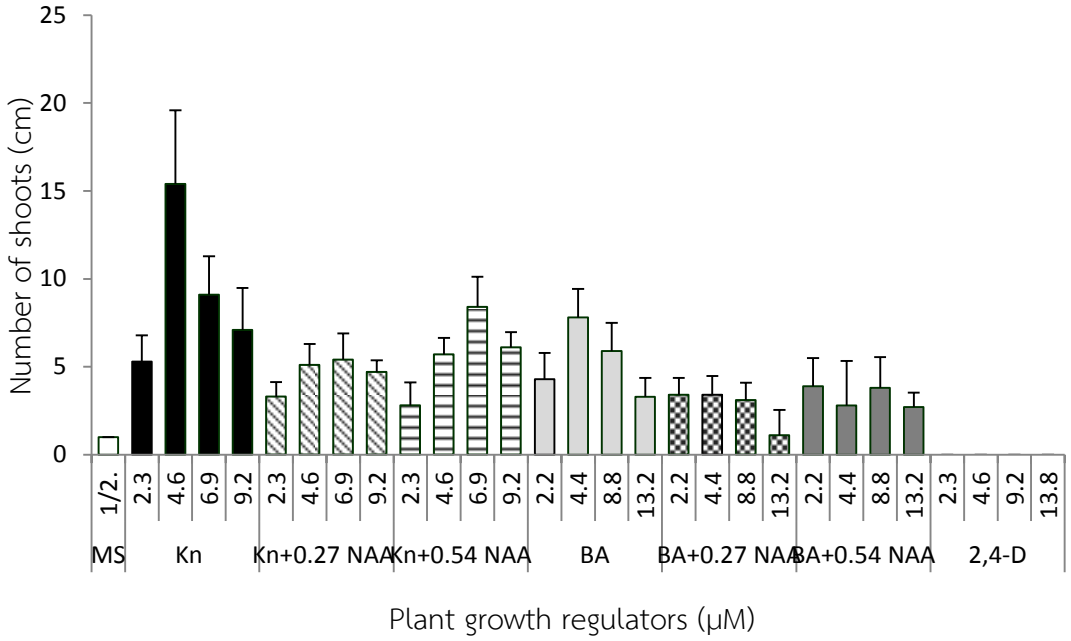


**Figure A1** Effect of plant growth regulators on induced multiple shoots by conventional method culture after 8 weeks of treatments.

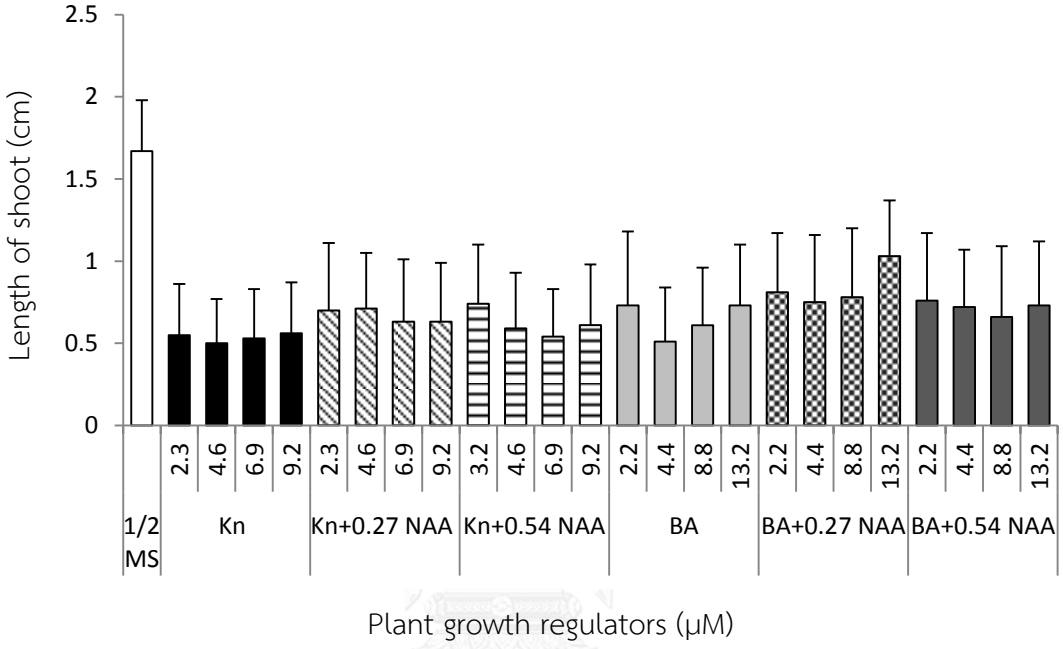


**Figure A2** Effect of plant growth regulators on length of shoot by conventional method culture after 8 weeks of treatments.

**Table A3** Comparison of the length of *O. harrisiana* shoot between conventional method and temporary immersion bioreactor culture.

| Time (weeks) | length of shoots±SD (cm) | | Maximum length (cm) | |
|---|---|---|---|---|
| | CM | TIB | CM | TIB |
| 0 | 2.00±0.00 | 2.00±0.00 | 2.00 | 2.00 |
| 2 | 2.10±0.13 | 2.29±0.18 | 2.40 | 2.60 |
| 4 | 2.96±0.38 | 3.58±0.93 | 3.80 | 5.20 |
| 6 | 3.27±0.57 | 5.34±2.26 | 4.20 | 11.20 |
| 8 | 3.88±0.63 | 6.65±3.17 | 5.10 | 14.10 |
| 10 | 4.87±1.19 | 6.83±2.96 | 6.10 | 14.80 |
| 12 | 5.95±1.41 | 9.50±4.78 | 8.10 | 19.70 |

Triplicate experiment

**Table A4** Effect of plant growth regulators on fresh weight of shoots cultures by conventional method.

| Medium | Fresh weight (mg/bottle) | | |
|---|---|---|---|
| | 0 week | 4 weeks | 8 weeks |
| 1/2 MS | 120.00±0.00 | 186.00±34.82 | 707.10±120.39 |
| 1/2 MS+4.6 μM Kn | 120.00±0.00 | 547.00±158.54 | 1210.40±165.17 |
| 1/2 MS+4.4 μM BA | 120.00±0.00 | 1092.10±367.7 | 2094.50±55.46 |

n = 30

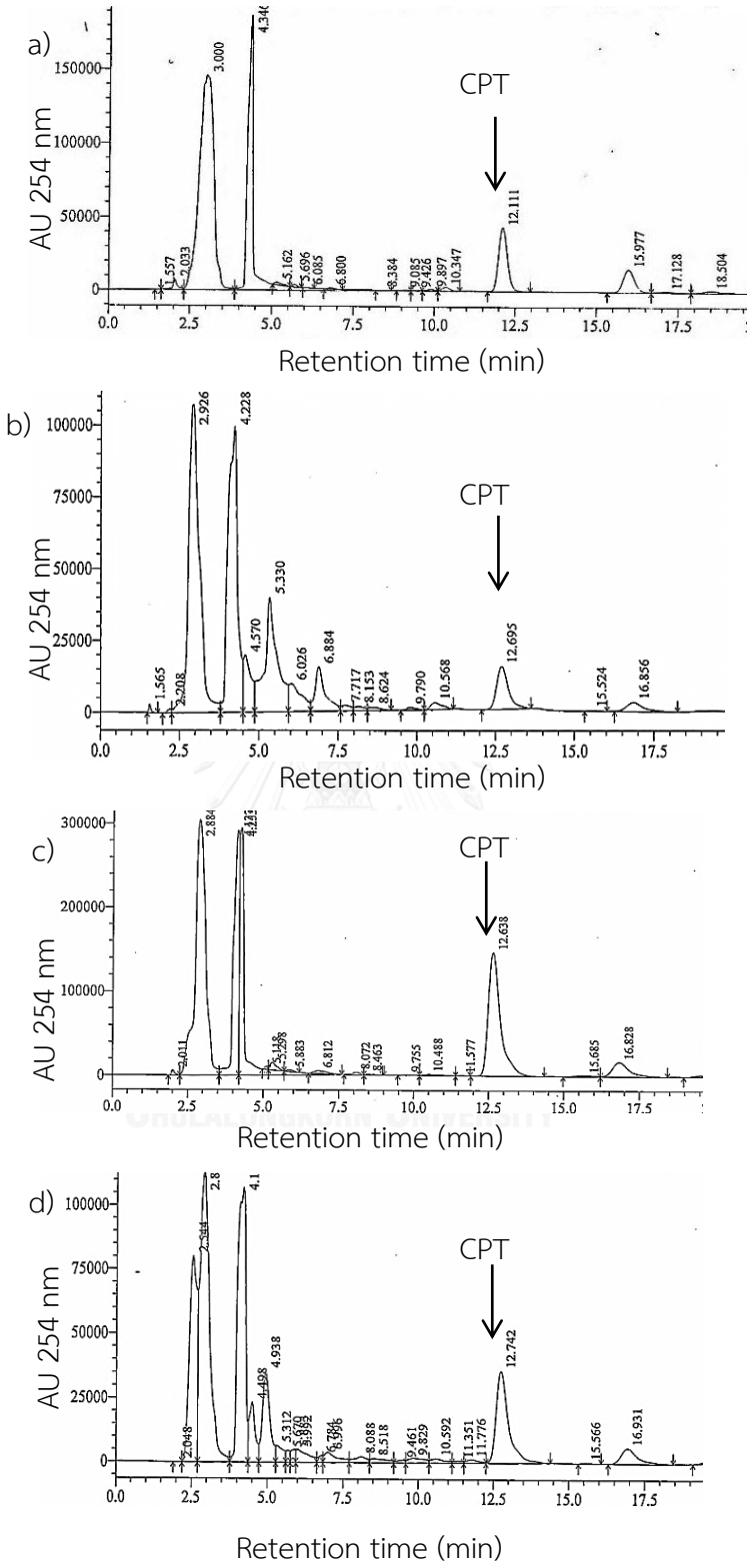


**Figure A3** The HPLC chromatograms of *O. harrisiana* extracts detected at 254 nm. a) leaf extract from CM culture, b) root extract from CM culture, c) leaf extract from TIB culture, d) root extract from TIB culture.

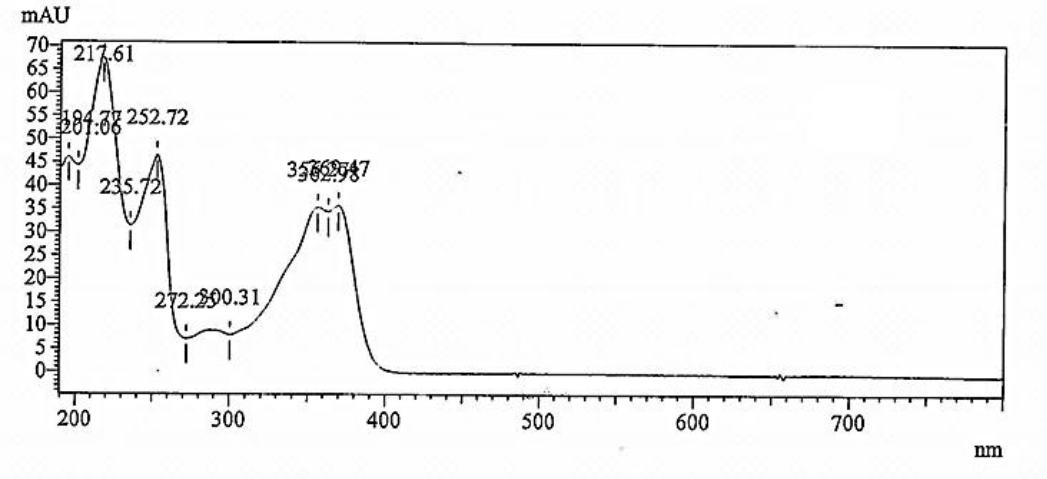


**Figure A4** UV spectra of CPT from *Ophiorrhiza harrisiana* extract.

# VITA

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Poster presentation

Ladda Techawiriyathaweesin, Piyarat Parinyapong Chareonsap, Sornkanok Vimolmangkang, Suchada Sukrong . Tissue culture establishment of Ophiorrhiza harrisiana for camptothecin production. Proceeding of the 34 th National Graduate Research Conference, March 27, 2015. Khon Kaen University, Khon Kaen, Thailand. p.1354-1360.