การเพาะเลี้ยงเนื้อเยื่อและรากขนของผักหลอดดอกขาวเพื่อการผลิตแคมป์โทเธซิน

นางสาว พรวิไล ยะอุตม์

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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MICROPROPAGATION AND HAIRY ROOT CULTURE OF *OPHIORRHIZA ALATA* FOR CAMPTOTHECIN PRODUCTION

Miss Pornwilai Ya-ut

ศูนย์วิทยทรัพยากร
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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
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Thesis Title	MICROPROPAGATION AND HAIRY ROOT CULTURE OF *OPHIORRHIZA ALATA* FOR CAMPTOTHECIN PRODUCTION
By	Miss Pornwilai Ya-ut
Field of Study	Pharmacognosy
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Accepted by the faculty of Phamaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree.

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พรวิไล ยะอุตม์ : การเพาะเลี้ยงเนื้อเยื่อและรากขนของผักหลอดดอกขาวเพื่อการผลิตแคมป์โทเธซิน (MICROPROPAGATION AND HAIRY ROOT CULTURE OF *OPHIORRHIZA ALATA* FOR CAMPTOTHECIN PRODUCTION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ร.ต.อ.หญิง ดร.สุชาดา สุขหร่อง, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร.ปิยรัษฎ์ เจริญทรัพย์, 78 หน้า.

งานวิจัยนี้เป็นการศึกษาถึงศักยภาพการผลิตแคมป์โทเธซินจากการเพาะเลี้ยงรากขนของผักหลอดดอกขาว เริ่มจากการนำชิ้นส่วนใบและข้อที่ได้จากการเพาะเลี้ยงเมล็ดในสภาพปลอดเชื้อที่เลี้ยงบนอาหารความเข้มข้นครึ่งหนึ่งของ Murashige & Skoog (MS) ที่ประกอบด้วยด้วยฮอร์โมน Kinetin (Kn) และ α - naphthaleneacetic acid (NAA) ที่ความเข้มข้นต่างกัน ทำการเพิ่มปริมาณยอดปลอดเชื้อ นำยอดที่เกิดขึ้นมากระตุ้นให้เกิดราก โดยใช้ฮอร์โมน indole-3-butyric acid (IBA) และรากขนโดยใช้เชื้ออะโกรแบคทีเรียมไรโซจีเนส สายพันธุ์ TISTR 1450 เกิดรากขนจำนวนมากขึ้นที่บริเวณบาดแผลของพืช เมื่อเปรียบเทียบอัตราการรอดชีวิตของต้นที่กระตุ้นให้เกิดรากด้วยฮอร์โมนและกระตุ้นให้เกิดรากขนโดยเชื้ออะโกรแบคทีเรียม พบว่าอัตราการรอดชีวิตเป็น 100 และ 80 เปอร์เซ็นต์ตามลำดับ

เมื่อนำผักหลอดดอกขาวที่ได้จากการเพาะปลูกในดิน เพาะเลี้ยงเนื้อเยื่อ เพาะเลี้ยงรากขน และย้ายปลูกในโรงเพาะชำ มาวิเคราะห์และเปรียบเทียบปริมาณแคมป์โทเธซินด้วยเครื่องลิควิดโครมาโตกราฟีสมรรถนะสูง ปริมาณแคมป์โทเธซินพบมากที่สุดในรากที่ได้จากการเพาะปลูกในดินเท่ากับ 39.98 ไมโครกรัมต่อกรัมน้ำหนักแห้ง ใบที่ได้จากการเพาะปลูกในดินมีปริมาณแคมป์โทเธซินใกล้เคียงกับ ราก ใบ และ รากขน ที่ได้จากการเพาะเลี้ยงเนื้อเยื่อและเพาะเลี้ยงรากขนประมาณ 10 ไมโครกรัมต่อกรัมน้ำหนักแห้ง ยกเว้นใบที่ได้จากการเพาะเลี้ยงเนื้อเยื่อมีปริมาณแคมป์โทเธซินเพียง 6.29 ไมโครกรัมต่อกรัมน้ำหนักแห้ง เมื่อมีการย้ายปลูกเป็นระยะเวลา 4 สัปดาห์ ใบและรากผักหลอดดอกขาวที่ได้จากการกระตุ้นรากด้วยฮอร์โมน IBA และ เชื้ออะโกรแบคทีเรียมไรโซจีเนสมีปริมาณแคมป์โทเธซินเพียง 3 ไมโครกรัมต่อกรัมน้ำหนักแห้งเท่านั้น แสดงให้เห็นถึงศักยภาพการผลิตแคมป์โทเธซิน จากการเพาะเลี้ยงเนื้อเยื่อและรากขนของผักหลอดดอกขาว ถึงแม้ว่ารากขนจะผลิตแคมป์โธเทซินได้น้อยกว่าจากรากที่ปลูกเพาะในดิน แต่ก็สามารถผลิตเป็นมวลมากและช่วยลดการทำลายพืชจากธรรมชาติได้

ภาควิชา..เภสัชเวทและเภสัชพฤกษศาสตร์..ลายมือชื่อนิสิต.....

สาขาวิชา.....เภสัชเวท.....ลายมือชื่ออ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2551.....ลายมือชื่ออ. ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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PORNWILAI YA-UT: MICROPROPAGATION AND HAIRY ROOT CULTURE OF *OPHIORRHIZA ALATA* FOR CAMPTOTHECIN PRODUCTION. THESIS ADVISOR: ASSIST. PROF. SUCHADA SUKRONG, Ph.D., THESIS COADVISOR: PIYARAT CHAREONSAP, Ph.D. 78 pp.

In this study, micropropagation and hairy root culture of *Ophiorrhiza alata* for camptothecin production were investigated. Leaf and node explants from germinated seed of *O. alata* were cultured on half strength Murashige & Skoog (MS) medium variously supplemented with Kinetin (Kn) and α- naphthaleneacetic acid (NAA) for shoots multiplication. Multiple shoot explants were induced roots and hairy roots by indole-3-butyric acid (IBA) and *Agrobacterium rhizogenes* TISTR 1450, respectively. Abundant hairy roots were developed at the wound sites. Survival rates between root plantlet induced by plant growth regulator and root plantlet induced by *A. rhizogenes* were 100 and 80 %, respectively when transplanting to greenhouse.

Various *O. alata* plant types from growing in soil, *in vitro* culture, hairy root culture, and transplanting to greenhouse were analyzed and compared the amount of camptothecin content by High Performance Liquid Chromatography. Untransformed root of soil–grown plants had the highest amount of CPT 39.98 µg/g dry wt. Leaf from soil–grown plant had the level of CPT closely to *in vitro* culture including root and hairy root induction with approximately 10 µg/g dry wt. while leaf from *in vitro* culture had CPT content only 6.29 µg/g dry wt. Four week after transplanting to greenhouse, both root and leaf from induced for rooting by IBA plant and composite plant had CPT content approximately 3 µg/g dry wt. These results indicated that *in vitro* culture and hairy root culture of *O. alata* had potential for campothecin production. Even though the camptothecin content in hairy root was lower than roots from soil grown plant, hairy root could produce the biomass accumulation and it is not destruction for nature plant.

Department:.Pharmacognosy and Pharmaceutical Botany..Student's signature.Pornwilai.....
Field of study:..... Pharmacognosy.....Advisor's signature.Suchada Sukrong
Academic year:.....2008.....Co-Advisor's signature.P.Chareonsap

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LIST OF ABBREVIATIONS

%	= percent (part per 100); percentage
µg	= microgram(s)
µl	= microliter(s)
/	= per
ACPT	= O-acetyl-camptothecin
ATCC	= American Type Culture Collection
BA	= 6-benzylaminopurine or N6- Benzyladenine
B5	= Gambrog's medium
cm	= centimeter(s)
CPT	= camptothecin
C-18	= carbon at 18^{th} position
DAD	= diode array detector
dCPT	= (20S)-18,19-dehydro camptothecin
DNA	= Deoxyribonucleic acid
et. al	= et alii
g	= gram(s)
HCPT	= 10-hydroxy camptothecin
HPLC	= high performance liquid chromatography
hr	= hour(s)
H_2O	= Water
IBA	= indole-3-butyric acid
kg	= kilogram(s)
kn	= kinetin-6-furfurylaminopurine
l	= liter(s)
m	= meter(s)
MACPT	= 9-methoxy-20-O-acetyl-camptothecin
$MgCl_2$	= magnesium chloride
MCPT	= 9-methoxy camptothecin

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mg = milligram

MeOH = methanol

min = minute(s)

ml = milliliter

mm = millimeter

MS = Murashige and Skoog

NA = nutrient agar

NB = nutrient broth

NAA = α-naphthaleneacetic acid

nm = nanometer

°C = degree Celsius

OD = optical density

PCR = Polymerase chain reaction

pH = the negative logarithm of the concentration of hydrogen ions

r.p.m. = round per minute

Ri = root inducing

RT = retention time

SD = standard deviation

Top = topoisomerase

TISTR = Thailand Institute of Scientific and Technological Research

UV = ultraviolet light

vir = virulence area

v/v = volume weight/volume (concentration)

wt = weight

w/v = weight/volume (concentration)

w/w = weight/weight (concentration)

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CHAPTER I

INTRODUCTION

Cancer has been a public problem whose estimated worldwide new incidence is about 10.9 million cases per year. There are 6.7 million deaths from the disease. It is estimated that there are 24.6 million people alive who have received a diagnosis of cancer in the last five years (IARC, 2008). In Thailand, new cancer cases for all sites are expected to reach approximately 125,000 by 2008, compared with 81,000 in 1999 (Sriplung *et al.*, 2006; Vatanasapt *et al.*, 2008). Cancer has been a leading cause of death in Thai population for decades. In addition, mortality rates for cancer have substantially increased when compared with heart disease, infectious disease, and accidents, of with mortality rates have decline (Wibulpolprasert, 2005).

Cancer is a disease characterized by unregulated proliferation of cells. However, cancer treatment traditionally involves three model of therapy: surgery, radiotherapy and chemotherapy (Srivastava *et al.*, 2005).

At present, various compounds are used as chemotherapeutic agents. These drugs are antifolates, 5-flouropyrimidines, cystidine analogues, purine metabolism, hydroxyl urea, antimicrotubule agent, alkaylating agents, platinum analogue, antibiotics, anthracyclines, and plant alkaloids (Encyclomedia Britannica, 2009). Drug lead finding, pharmaceutical agents are usually obtained from prepare chemicals and natural products. Some chemically synthetics compounds are difficult to synthesize because of their structural complexity (Wink *et al.*, 2005). Therefore, natural products have been interesting continually in investigation. Secondary metabolites can be applied as starting compounds for further chemical modification. Sustainable bioproduction of the compounds of interest may be achieved by plant *in vitro* cultures.

In recent years, there has been an increasing interest to produce high value natural plant products by tissue culture that can solve many problems associated with industrial production of these natural products by extraction from intact plants (Mulabagal and Tsay, 2004). Among plant tissue culture for secondary metabolite production techniques, researchers reported that undifferentiated callus and suspension cultures very often fail to accumulate the compounds of interest (Lorence *et al.*, 2004).

In contrast, shoot and root cultures as well as hairy roots normally produce the same compounds as in the appropriate organs (saito, 2001; Lorence *et al*., 2004).

Several secondary metabolites of pharmaceutical interest are accumulated in plant roots (Flores *et al*., 1999). However, harvesting roots is destructive for the plants and hence there has been a growing interest to generate hairy roots from medicinal plant species. Hairy roots develop as the consequence of the interaction between *Agrobacterium rhizogenes*, a gram-negative soil bacterium, and the host plant (Tzfira *et al*., 2004). Hairy roots are characterized by high growth rate independent of any source of exogenous plant growth regulators, absence of geotropism and high branching; furthermore, they often produce secondary metabolites for a long period of time, unlike natural roots (Shanks *et al*., 1999). For these reasons, switching from culturing natural plant organs to hairy roots is considered as an attractive alternative for the production of many valuable natural secondary metabolites (Sevon *et al*., 2002)

The productions of anticancer compounds, such as the alkaloids vinblastine, vincristine, paclitaxel, camptothecin (CPT), or the lignan podophyllotoxin, by plant *in vitro* cultures have been reported (Mulabagal and Tsay, 2004). Taxanes can be produced in bioreactors using cell suspensions of various *Taxus* species with good yields. Presently paclitaxel belonging to taxane group is produced on a commercial scale by Phyton Biotech (Germany) (Mulabagal and Tsay, 2004). CPT has low yields in suspension cultures of *Camptotheca acuminata* or *Nothapodytes foetida*, but shows a good production in root and hairy root cultures of *Ophiorrhiza pumila*, *O. mungos* and *C. acuminata*. Podophyllotoxin can be produced in cell suspension and root as well as hairy root cultures of *Podophyllum* and various *Linum* species. The *in vitro* production of dimeric indole alkaloids in *Catharanthus roseus* has failed so far both in undifferentiated and differentiated *in vitro* cultures. In cases where *in vitro* cultures show good yields, they can be employed in biotechnology for the sustainable production of valuable products (Wink *et al*., 2005).

CPT, a tryptophan derived-quinoline alkaloid, is regarded as one of the most promising anticancer drugs of the twenty first century (Wall *et al*., 1966; Nalawade *et al*., 2003). Irinotecan and topotecan, two water soluble derivatives of camptothecin, have been approved by the Food and Drug Administration (FDA) of the United States of

America for treating colorectal and ovarian cancer (Vladu, 2000). They are known for their inhibitory activity on topoisomerase I in eukaryotic cells (Carbonero and Supko, 2002). CPT was first isolated from a Chinese deciduous tree *Camptotheca acuminata* (Wall and Wani, 1968). Later it was isolated from a variety of plant species including *N. foetida* (Govindachari *et al.,* 1972), *O. mungos* (Tafur *et al.,* 1976) and *O. pumila* (Saito *et al*., 2001). The worldwide market size of irinotecan and topotecan in 2002 was estimated at about $750 million and at $1 billion by 2003 (Lorence and Nessler, 2004). In spite of the rapid growth of the market, CPT is still harvested by extraction from bark and seeds of *C. acuminata* and *N. foetida*, respectively (Lorence and Nessler, 2004).

Tafur *et al.* (1976) isolated and identified CPT from the entire plant of *O. mungos* in 1976. Later, a lot of research on isolation of the constituents from the other *Ophirrhiza* spp. has been reported including *O. pumila, O. liukiuensis, O. kuroiwai, O. prostrata* and *O. rogusa* (Kitajima *et al*., 1997; Yamazaki *et al*., 2003; Asano, *et al*., 2004; Aravind *et al*., 2007). In addition, high CPT productions in *O. pumila, O. liukiuensis,* and *O. korowai* hairy root cultures were reported (Saito, 2001; Sudo *et al*. 2002; Asano, *et al*., 2004).

O. alata or known as "ผักหลอดดอกขาว" in Thai is distributed in the southeastern part of Thailand (Smitinand, 1999). There is no any report involved in this species yet. In this study, we aim to establish an *in vitro* culture of *O. alata*. We also want to see the possibility inducing of hairy root culture by infection with *A. rhizogenes* for CPT production. Survival rate for transplanting to greenhouse will be also included.

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CHAPTER II

LITERATURE REVIEW

1. Botanical aspects of *Ophiorrhiza* spp. and *Ophiorrhiza alata*

Ophiorrhiza L. (Rubiaceae) is a predominantly herbaceous genus distributed from eastern India to the western Pacific and from the south of China to the north of Australia (Puff *et al*., 2005).

More than 400 species have been described, though many of them probably superfluously (Schanzer, 2005). However systematic knowledge of this genus is still inadequate. Recent regional revisions are available only for marginal parts of its area: the Pacific, China, and the Indian subcontinent (Darwin, 1976). About 30 species have been recorded from Thailand and adjacent areas of Peninsular Malaysia (Schanzer, 2005).

Ophiorrhiza L. is prostrate or elect perennial (and sometimes also annual) herbs, uncommonly subshrubby; stems sometimes succulent.

Leaves: opposite, rarely slightly anisophyllous, blades mostly membranous; stipules entire or firmbriate. Inflorescence terminal, often consisting of helicoids or scorpioid cymes, sometimes congested and head-like; bracts well developed or absent.

Flowers: 5-merous, hermaphrodite, heterostylous or isostylous, sometimes cleistogamous; calyx lobes often very small; corolla 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; stamens inserted at different levels in corolla tube (usually high up in short-styled, but in the lower part in long-styled morphs), filaments long or short, anthers included or exserted; style filiform, with 2-lobed stigma, included or exserted; style filiform, with 2-lobe stigma, included or exserted (above the level of the anthers in long-styled morphs but not necessarily exserted); ovary 2-celled, each locule with numerous ovuleson placenta attached to lower half of septum; roof of ovary with conspicuous 2-lobed disk.

Fruits: laterally compressed, capsular, loculicidally dehiscent; seeds numerous, small, rhomboild.

An asiatic genus of possibly 150 species. The roughly 30-35 species recorded from Thailand are found in all parts of the country and normally grow in shady, moist to wet areas (often along streams and waterfalls) in both lowland and montane evergreen forest type but also in mixed deciduous and bamboo forests (Puff *et al*., 2005).

Ophiorrhiza alata (Figure 2.1a-e) is distributed in southeast of Thailand (Smitinand, 1999).

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Figure 2.1 *O. alata* collected from Chanthaburi province, Thailand. a) habitat, b) hine-month soil-grown plant, c) and d) flower, and e) fruit

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2. Chemical constituents of *Ophiorrhiza* spp.

In 1976, Tafur *et al.* (1976) isolated and identified CPT from the entire plant of *O. mungos*. Later, publications on isolation of the constituents from the other *Ophirrhiza* spp. have been reported including *O. pumila, O. liukiuensis, O. kuroiwai, O. prostrate* and *O. rogusa* (Kitajima *et al*., 1997; Yamazaki *et al*., 2003; Asano, *et al*., 2004; Aravind *et al*., 2007). The chemical substances isolated from these plants are summerized in Table 2.1.

Table 2.1 Chemical constituents and structures found in *Ophiorrhiza* spp.

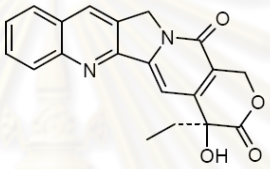
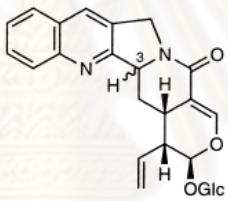
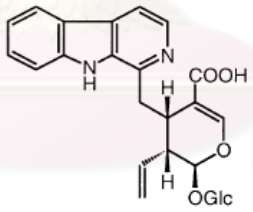
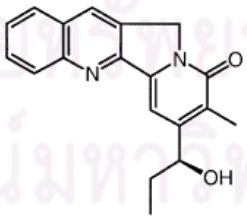
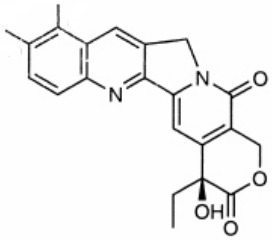
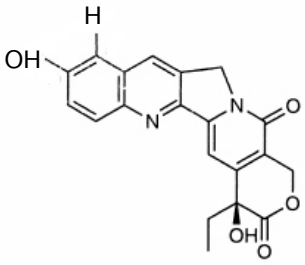
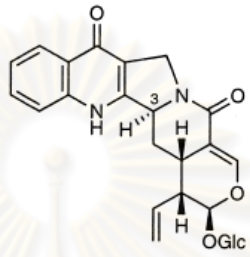
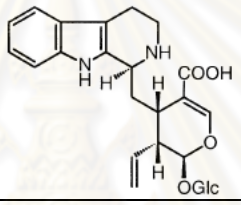
Chemical substance	Chemical structure	Reference
Camptothecin		Kitajima *et al*., 1997 Yamazaki *et al*., 2003 Asano, *et al*., 2004 Aravind *et al*., 2007
Deoxypumiloside		Kitajima *et al*., 1997
Lyalosidic acid		Yamazaki *et al*., 2003
Mappicine		Yamazaki *et al*., 2003
9-Methoxycamptothecin		Kitajima et al., 2002 Yamazaki *et al*., 2003

Table 2.1 (continued)

Chemical substance	Chemical structure	Reference
10-Hydroxycamptothecin		Yamazaki *et al*., 2003
Pumiloside		Kitajima *et al*., 1997 Kitajima *et al*., 2002 Yamazaki *et al*., 2003
Strictosidine		Kitajima *et al*., 1997 Yamazaki *et al*., 2003 Lorence *et al*., 2004

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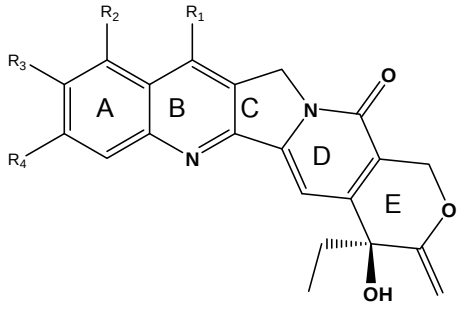
3. Camptothecin (CPT)

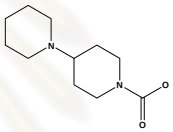
Camptothecin is a cytotoxic tryptophan derived-quinoline alkaloid which inhibits the DNA topoisomerase I (top I) (Nalawade *et al*., 2003). It was discovered in 1966 by Wall and Wani in systematic screening of natural products for anticancer drugs (Wall and Wani, 1966). It was isolated from the bark and stem of *C. acuminata* , a tree native in China and Tibet. CPT showed remarkable anticancer activity in preliminary clinical trials but also low solubility and adverse drug reaction. Because of these disadvantages researchers have made numbers of derivatives to increase the benefits of the chemical, with good results. Two CPT analogues have been approved and are used in cancer chemotherapy today, topotecan and irinotecan (Figure 2.2) (Carbonero and Supko, 2002).

3.1 Structure and Chemistry

The characteristic structural features of the camptothecins include a five-ring backbone comprised of a quinoline subunit fused through two interposed rings to a terminal-hydroxy-lactone ring with a chiral center at position C-20 (Figure 2.2). The naturally occurring 20*S*-isomer of CPT inhibits purified topoisomerase I 10–100 times more potently than the 20*R*-isomer (Wani *et al*., 1987). Potency can be enhanced by appropriate substitutions at positions C-9 and C-10 of the aromatic A ring of the quinoline moiety (Kingsbury *et al.,* 1991).

CPT is a weak acid and therefore the lactone ring highly is susceptible to ring opening by hydrolysis, forming carboxylate. The open ring form is inactive and it must therefore by closed to inhibit top I. The closed form is favored in acidic condition, as it is in many cancer cells microenvironment. CPT is transported into the cell by passive diffusion. Cellular uptake is favored by lipophilicity, which enhances intracellular accumulation. Lipophilicity makes compounds more stable because of improved lactone partitioning into red blood cells and consequently less hydrolysis of the lactone. CPT has affinity for human serum albumin (HSA), especially the carboxylate form of CPT. Because of that, the equilibrium between the lactone ring and the carboxylate form is driven toward the carboxylate. Reduced drug-HSA interactions could result in improved activity (Zunino *et al*., 2002; Adams *et al*., 2005).



Compound	R1	R2	R3	R4
Camptothecin	H	H	H	H
Topotecan	-H	$CH_2N(CH_3)_2$	-OH	H
Irinotecan	CH_2CH_3	H		H

C

Figure 2.2 Chemical structure of CPT and its analogues (Carbonero and Supko, 2005)

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3.2 Biosynthesis pathway of CPT

Camptothecin is found in a number of plant species belonging to the families Apocynaceae, Icacinaceae, Nyssaceae and Rubiaceae (Watase *et al*., 2004). The initial step of CPT biosynthesis involves the strictosidine synthase mediated condensation of tryptamine with secologanin to yield strictosidine (Figure 2.3) (Kutchan, 1995). Strictosidine is then converted to strictosamide via intermolecular cyclization and this compound is a precursor of CPT. The remaining details and precise intermediates between strictosamide and CPT are not completely defined. It has been postulated that CPT could be formed from strictosamide by three trans-formations: (1) oxidation-recyclization of the B- and C-rings, (2) oxidation of the D-ring and removal of the C-21 glucose moiety, and (3) oxidation of ring E (Hutchinson *et al*., 1979). A presumed precursor of CPT was isolated from the polar fraction of a large-scale extraction of *C. acuminata* (Carte *et al*., 1990). 3(*S*)-Pumiloside, 3(*S*)-and 3(*R*)-deoxypumiloside, plausible CPT precursors, have been found in *O. pumila* (Aimi *et al*., 1989; Kitajima *et al*., 1997; Yamazaki *et al*., 2003).

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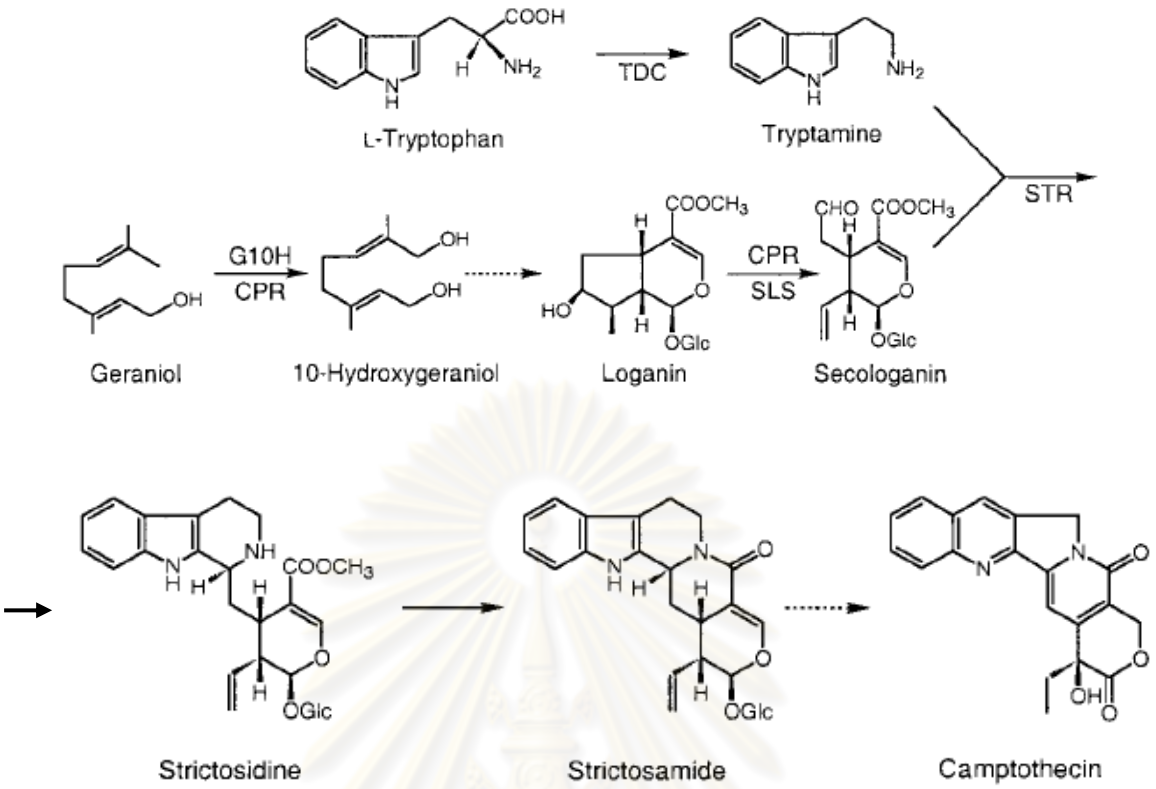

Figure 2.3 The predicted biosynthetic pathway of camptothecin. The enzymes are: TDC, tryptophan decarboxylase; G10H, geraniol 10- hydroxylate; CPR, NADPH: cytochrome P450 reductase; SLS, secologanin synthase; STR, strictosidine synthase. Dashed arrows indicate involvement of multiple enzymatic steps.

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3.3 Distribution of CPT and its metabolites

CPT has been isolated from samples of the following unrelated orders and families of angiosperms: Order *Celastrales* (Icacinaceae): *N. foetida* (Aiyama *et al*., 1988), *Pyrenacantha klaineana* (Zhou *et al*., 2000), and *Merrilliodendron megacarpum* (Arisawa *et al*., 1981); Order *Cornales* (Nyssaceae): *C. acuminata* (Wall *et al*., 1966), *C. lowreyana,* and *C. yunnanensis* (Li *et al*., 2002); Order *Gentianales* (Rubiaceae): *O. mungos* (Tafur *et al*., 1976), *O. pumila, O. filistipula (*Saito *et al*., 2001), Family Apocynaceae: *Ervatamia heyneana* (Gunasekera *et al*., 1979), and Family Gelsemiaceae: *Mostuea brunonis* (Dai *et al*., 1999).

The information generated by multiple research teams regarding the sites of accumulation of CPT and its concentration in multiple natural sources are summarized in Table 2.2.

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Table 2.2 Sites of accumulation of CTP and its derivatives in several natural sources

Species	Tissue analyzed	Sample origin	Camptothecinoids Content (µg/g dry wt)	Reference
Camptotheca acuminata Decaisne	Young leaves	Texas, USA	CPT 4000-5000 HCPT 20-30	Lopez-Meyer *et al.*, 1994
	Seeds		CPT 3000 HCPT 25	
	Bark		CPT 1800-2000 HCPT 2-90	
	Roots		CPT 400 HCPT 13-20	
	Young leaves	Texas, USA	CPT 2421-3022	Li *et al.*, 2002
	Old leaves		CPT 482	
	Young fruit		CPT 842	
	Old leaves		CPT 2362	
	Hairy roots	Texas, USA	CPT 1000 HCPT 150	Lorence *et al.,* 2004
	Callus	Shangai, China	CPT 2040-2360	Wiedenfeld *et al.*, 1997
	Cell cultures		HCPT 80-100 CPT 2.5-4	Sakato *et al.*, 1974 ; Hengel *et al.* 1992
Camptotheca lowreyana Li	Young leaves	Texas, USA	CPT 3913-5537	Li *et al.,* 2002
	Old leaves		CPT 909-1184	
Camptotheca yunnanensis Dode	Young leaves	Texas, USA	CPT 2592-4494	Li *et al.,* 2002
	Old leaves		CPT 590	
Ervatamia heyneana (Wall) T. Cooke	Wood and stem bark	India	CPT 1300 MCPT 400	Gunasekera *et al.,*1979
Nothapodytes foetida (Wight) Sleumer	Steam wood	Okinawa, Japan	CPT 1400-2400 dCPT 19	Aiyama *et al.*,1988
	Steam	Taiwan	ACPT 0.24	Wu *et al.*, 1995;
	Shoot	Mahabaleshwar, India	CPT 750 MCPT 130	Roja and Heble, 1994
	Plantlet culture		MCPT 7	
	Callus		MCPT 1	
	Stem	Godavari, India	MACPT 2.5	Srinivas and Das, 2003; Ciddi and Shuler, 2000
	Callus	Ooty, India	CPT 9.5	
		Satura , India	MCPT traces CPT 1.1 MCPT 0.81	Fulzele *et al.,* 2001
Merriliodendron megacarpum (Hemsl.) Sleumer	Leaves and stem	Guam	CPT 530 MCPT 170	Arisawa *et al.*,1981
Mostuea brunonis Didr.	Entire plant	Lope, Gabon	CPT-20-0-β-glucoside 100 Deoxypumiloside 100 Strictosamide 600	Dai *et al.,* 1999
Ophiorrhiza mungos Linn.	Entire plant	Colombo, Ceylan	CPT 12 MCPT 10.41	Tafur *et al.*, 1976
Ophiorrhiza pumila Champ.	Leaves	Japan	CPT 300-400	Saito *et al.*, 2001
	Young roots		CPT 1000	
	Hairy roots		CPT 1000	
	Entire plant	Kagoshima, Japan	CPT 300-510 MCPT 70-140 Chaboside 300-690	Yamazaki *et al.*, 2003
	Hairy roots		CPT 240	Kitajima *et al.,* 1998
	Cell cultures	Japan	None	
Pyrenacantha klaineana Pierre ex Exell & Mendoca	Stem	Ankasa Game Reserve ,Ghana	CPT 4.8 MCPT 1.6	Zhou *et al.*, 2000

CPT = camptothecin; ACPT= *0*-acetyl-CPT; dCPT = (20*S*)-18,19-dehydro CPT; HCPT = 10-hydroxy CPT; MACPT = 9-methoxy-20-0-acetyl- CPT; MCPT =9-methoxy CPT

3.4 Mechanism of action of CPT

Chemotherapy drugs interfere with cancer cells' ability to grow (multiply) or to survive. There are several types of drugs; each type interferes with the cell's ability to grow or survive in a different way. Many drugs act against cancer cells by interacting with the DNA or RNA of the cancer cell. This interaction damages the DNA, causing the cancer cell to be destroyed or preventing it from growing and reproducing. Four chemotherapy drug types that act directly to impair the DNA in cancer cells are: the DNA-damaging agents (also called antineoplastics); antitumor antibiotics; antimetabolites; and DNA-repair enzyme inhibitors. DNA-repair enzyme inhibitors, such as etoposide or CPT, attack the cancer cell proteins that normally repair any damage to the cell DNA. DNA repairing in a supercoiled state is maintained by combined action of two enzymes. These enzymes are called DNA topoisomerases (Top) (Pommier, 2006).

On the basis of the fundamental differences in their reaction mechanisms, DNA Top are classified in two types, type I and type II (Uluka *et al*., 2002). Top I changes the topological state of DNA via transient enzyme-linked single-strand breaks. In contrast, Top II catalyse the strand passing reaction by transiently breaking both strands of duplex DNA to generate a gap for the passage of second duplex DNA prior to relegation reaction (Hofland *et al*., 2000). Top II is the cellular target of several clinically important drugs such as the epipodophyllotoxin etoposide and the anthracycline doxorubicin, whereas the camptothecins and derivatives are substrates of Top I (Chrencik, 2004).

CPT class of compounds has been demonstrated to be effective against a broad spectrum of tumors. Their molecular target has been firmly established to be human DNA Top I. CPT inhibits top I by blocking the rejoining step of the cleavage/religation reaction of Top I, resulting in accumulation of a covalent reaction intermediate, the cleavable complex. The primary mechanism of cell killing by CPT is S-phase–specific killing through potentially lethal collisions between advancing replication forks and Top-I cleavable complexes. Collisions with the transcription machinery have also been shown to trigger the formation of long-lived covalent Top-I DNA complexes, which contribute to CPT cytotoxicity (Srivastava *et al*, 2005).

3.5 Camptothecin production by plant *in vitro* cultures

As mentioned above, CPT is produced by plants from several unrelated families. Some of them have been established *in vitro* culture for CPT production, especially *C. acuminata, N. foetida, O. mungos*, *O. pumila* and *O. rugosa* (Lorence and Nessler, 2004).

The first tissue culture study of *C. acuminata* was reported by Sakato *et al*. (1974). Those cultures produced 0.002 mg CPT/g dry wt whereas the whole plant contained from 0.2 to almost 5 mg/g dry wt depending on the tissue analyzed (Lopez-Meyer *et al*., 1994). Later reports showed *in vitro* CPT production by *C. acuminata* range from 0.004 mg/g dry wt in cell suspensions (van Hengel *et al*., 1992) to 2 mg/g dry wt in callus cultures (Wiedenfeld *et al*., 1997). Also in cell suspension cultures of *N. foetida*, CPT levels were 100–1000-fold lower than in the intact plant (Ciddi and Shuler, 2000; Fulzele *et al*., 2001; Thengane *et al*., 2003). These data indicate that callus and suspension cultures usually fail on CPT production.

Good results have been obtained from root and hairy root cultures (Saito *et al*., 2001; Lorence *et al*., 2004). First encouraging result was that hairy root cultures of *O. pumila* showed approximately 1 mg/g dry wt of CPT production (Saito *et al*., 2001).

CPT production from *O. pumila* hairy root cultures has been scaled up to 3-litre bioreactors and a final concentration of 0.0085% CPT (fresh weight) was obtained; approximately 17% of CPT was released into the culture medium. It was harvested from medium by binding the alkaloid to a polysterene resin (Diaion HP- 20) (Sudo *et al*., 2002).

CPT production of *O. mungos* on callus, cell suspension, normal root and hairy root cultures have established by Wink *et al*. (2005).

The above mentioned findings indicate that callus and cell suspension cultures have very low yields whereas CPT formation is substantially higher in hairy root cultures.

4. Hairy root and secondary metabolite production

4.1 Hairy root induction

4.1.1 Hairy root

Hairy roots are characterized by a high degree of lateral branching, profusion of root hairs and absence of geotropism (Guillon, 2008). They often grow as fast as or faster than normal (untransformed) roots due to their extensive branching, resulting in the presence of many meristems, and they do not require phytohormones in the medium. The increase in the number of branches is approximately logarithmic during the early stages of growth and thus the overall pattern of growth is similar to cell suspension cultures (Guillon *et al*., 2006).

The name hairy root was first introduced in the literature by Steward *et al.* (1900). Riker *et al.* (1930) later described and named the hairy-root-causing microorganism as *Phytomonas rhizogenes*, which was later renamed *Agrobacterium rhizogenes*. This conclusion was accepted and given wide recognition by many other researchers. The first directed transformation of higher plants using *A. rhizogenes* was made by Ackermann in 1973. A large number of small, fine, hairy roots covered with root hairs originate directly from the explant in response to *A. rhizogenes* infection and hence the term hairy root (Srivastava, 2007).

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4.1.2 Agrobacterium rhizogenes and Ri T-DNA genes

A. rhizogenes (formerly *Phytomonas rhizogenes*) was first identified more than 70 year ago as the causative agent of the plant disease known as hairy root disease or root mat disease (Veena and Taylor, 2007). *A. rhizogenes* is a gram-negative, rod-shaped soil bacterium (Figure 2.4) belong to the genus *Agrobacterium*. *A. rhizogenes* is a close relative of the better known *A. tumefaciens*, which is the causative agent for crown gall disease and the best characterized species among the genus *Agrobacterium* (Giri and Narusu, 2000). *A. rhizogenes* infects wounded plant cells because of the production of phenolic compounds that attract *A. rhizogenes*. Bacteria move to the wound site by chemotaxis. Subsequent infection at wound site followed by integration of *Agrobacterium* derived genetic material into the plant genome results in development of hairy root disease (Giri and Narusu, 2000). Hairy-root disease is characterized by plagiotropic root growth, a high degree of lateral branching, profusion of root hairs, and enhanced growth rates (Guillon *et al*., 2006).

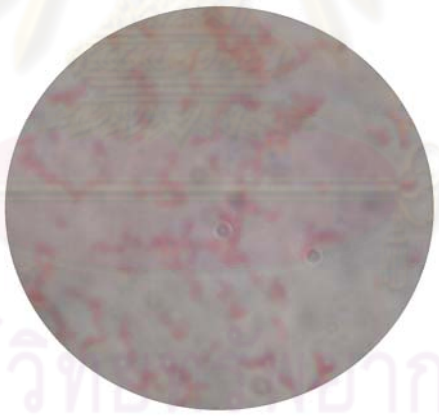

Figure 2.4 *A. rhizogenes* TISTR 1450 gram strain

All *A. rhizogenes* strains are characterized by the presence of a large root inducing (Ri) plasmid. Major groups of genes located on Ri include: the T-DNA (the DNA transferred to the host cell), *vir* (virulence) genes, Ri plasmid conjugation, and opine metabolism (Figure 2.5) (Hansen *et al*. 1997; Veena and Taylor, 2007).

T-DNA is the DNA fragment transferred to the host cell whose expression results in root formation. Ri plasmid has two segments of T-DNA (called TR and TL) with four border sequences. The segments bounded by the border sequences may be transferred individually or as an entire piece carrying both TR and TL. T-DNA carries genes for enzymes for plant growth hormone biosynthesis and for the modification of plant responses to growth hormones (Nilsson and Olsson 1997).

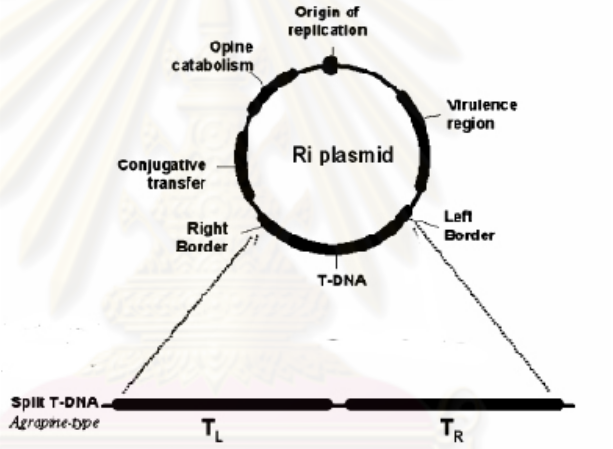

Figure 2.5 Structure of the Ri-plasmid of *A. rhizogenes*. (adapted from Veena and Taylor, 2007)

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4.1.3 Mechanism of *A. rhizogenes*-plant cell interaction.

To date, our basic understanding of the molecular mechanisms of genetic transformation of plants by members of the *Agrobacterium* genus relies on extensive studies using *A. tumefaciens*. The overall process of infection is considered similar in both *A. tumefaciens* and *A. rhizogenese* (Flores *et al*., 1999*:* Veena and Taylor, 2007).

One of the earliest stages in the interaction between *Agrobacterium* and a plant is the attachment of the bacterium to the surface of the plant cell. A plant cell becomes susceptible to *Agrobacterium* when it is wounded. The wounded cells release phenolic compounds, such as acetosyringone, that activate the *vir*-region of the bacterial plasmid. It has been shown that the *Agrobacterium* plasmid carries three genetic components that are required for plant cell transformation (Giri and Narusu, 2000).

The first component, the T-DNA that is integrated into the plant cells, is a mobile DNA element. The second one is the virulence area (*vir*), which contains several *vir* genes. These genes do not enter the plant cell but, cause the transfer of T-DNA. The third component, the so-called border sequences (25 bp), resides in the *Agrobacterium* chromosome (Veena and Taylor, 2007).

The least understood aspect of hairy-root disease is the mechanism of prolific root induction and growth. As above mention, all *A. rhizogenes* strains contain a T-DNA region located on the Ri plasmid that carries genes involved in root initiation and development (*rol*-genes), genes concerned with opine biosynthesis, and genes of unknown function. T-DNA may be present that contains genes involved in auxin biosynthesis along with further genes of unknown function. Whereas both TL-DNA and TR-DNA are known to be transferred and integrated independently into the host plant genome, the transfer of TL-DNA is essential for induction of the hairy-root syndrome (Sevon and Oksman-Caldentey 2002).

4.2 Secondary metabolite production from hairy roots

Various advantages of hairy root culture over cell suspension culture include genotypic and biochemical stability, cytodifferentiation and growth in hormone free medium. These factors play a vital role during secondary metabolite production. Fast growth, low doubling time, ease of maintenance of hairy roots and their ability to synthesize a large range of chemical compounds offer an additional advantage as a continuous source for the production of valuable secondary metabolites (Bourgaud *et al*., 2001).

Hairy roots have also been observed to synthesize novel secondary metabolites, which are not present in the untransformed (control) tissue (Banerjee *et al*., 1995). Even in cases where secondary metabolites accumulate only in the aerial part of an intact plant, hairy root cultures have been shown to accumulate the metabolites. For example, lawsone normally accumulates only in the aerial part of the plant, but hairy roots of *Lawsonia inermis* grown in half- or full strength MS medium (Murashige and Skoog, 1962) can produce lawsone under dark conditions (Bakkali *et al*., 1997). Similarly, artemisinin was thought to accumulate only in the aerial part of the *Artemisia annua* plant (Wallaart *et al*., 1999) but several reports have shown that hairy roots can also produce artemisinin (Weathers *et al*., 1994; Jaziri *et al*., 1995; Liu *et al*., 1999; Giri *et al*., 2000).

Hairy root culture follows a definite growth pattern; however, secondary metabolite production may or may not be growth related. Variables examined for their influence on growth and secondary metabolite production from hairy roots include different basal media (Christen *et al*., 1992), sucrose level (Uozumi *et al*., 1993), exogenous supply of growth hormone (Bais *et al*., 2001), nature of the nitrogen source and their relative amounts (Norton and Towers, 1986), and phosphate concentration (Taya *et al*., 1994).

Physical factors including light (Hirata *et al*., 1991; Yu *et al*., 2005), temperature (Hilton and Rhodes, 1994; Yu *et al*., 2005), presence of chemicals inducing physical stress (Sim *et al*., 1994) have also been reported to affect secondary metabolite production from hairy roots. Betacyanin release from hairy roots of *Beta vulgaris* was achieved by oxygen starvation (Giri and Narasu, 2000).

Permeabilization treatment using Tween-80 (Polyoxy ethylene sorbilane monolaurate) released a high yield of hyoscyamine from roots of *Datura innoxia* without any detrimental effects (Boitel *et al.*, 1995). Treatment with 5 mM hydrogen peroxide induced a transient release of tropane alkaloids from transformed roots without affecting viability (Lee *et al.*, 1998). The fact that individual hairy roots may have different requirements for nutrient conditions suggests that the culture conditions should be optimized separately for each species and for individual clones. Despite such attempts sometimes the efficiency of secondary metabolite production is not as desired.

Hairy roots cultures of many plant species have been widely studied for the *in vitro* production of secondary metabolites. Some secondary metabolites from hairy root culture were shown in Table 2.3.

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Table 2.3 Some secondary metabolite production from hairy root cultures (Giri *et al.*, 2000).

Plant	Secondary metabolite
Aconitum heterophyllum	Aconites
Ajuga replans var. *atropurpurea*	Phytoecdysteroids
Ambrosia spp.	Polyacetylenes, thiophenes
Amsonia elliptica	Indole alkaloids
Anisodus luridus	Tropane alkaloids
Armoracia laphthifolia	Peroxidase, Isoperoxidase, Fusicoccin
Artemisia absinthum	Essential oils
Artemisia annua	Artemisinin
Astragalus mongholicus	Cycloartane saponin
Atropa belladonna	Atropine
Azadirachta indicaA. Juss.	Azadirachtin
Beta vulgaris	Betalain pigments
Bidens sps.	Polyacetylenes, thiophenes
Brugmansia candida	Tropane alkaloids
Calystegia sepium	Cuscohygrine
Campanula medium	Polyacetylenes
Carthamus	Thiophenes
Cassia obtusifolia	Anthraquinone
Catharanthus roseus	Indole alkaloids, Ajmalicine
Catharanthus tricophyllus	Indole alkaloids
Centranthus ruber	Valepotriates
Chaenatis douglasis	Thiarubrins
Cinchona ledgeriana	Quinine
Coleus forskohlii	Forskolin
Coreopsis	Polyacetylene
Datura candida	Scopolamine, Hyoscyamine
Datura stramonium	Hyoscyamine, Sesquiterpene
Daucus carota	Flavonoids, Anthocyanin
Digitalis purpurea	Cardioactive glycosides
Duboisia myoporoides	Scopolamine
Duboisia leichhardtii	Scopolamine

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Table 2.3 (continued)

Plant	Secondary metabolite
Echinacea purpurea	Alkamides
Fagra zanthoxyloids Lam.	Benzophenanthridine
Fagopyrum	Flavanol
Fragaria	Polyphenol
Geranium thubergee	Tannins
Glycyrrhiza glabra	Flavonoids
Gynostemma pentaphyllum	Saponin
Hyoscyamus albus	Tropane alkaloids, Phytoalexins
Hyoscyamus muticus	Tropane alkaloids
	Hyoscyamine, Proline
Hyoscyamus niger	Hyoscyamine
Lactuca virosa	Sesquiterpene lactones
Leontopodium alpinum	Anthocyanins & Essential oil
Linum flavum	Lignans (5-methoxy podophyllotoxins)
Lippia dulcis	Sesquiterpenes, (hernandulcin)
Lithospermum erythrorhizon	Shikonin, Benzoquinone
Lobelia cardinalis	Polyacetylene glucosides
Lobelia inflata	Lobeline, Polyacetylene
Lotus corniculatus	Condensed tannins
Nicotiana hesperis	Nicotine, Anatabine
Nicotiana tabacum	Nicotine, Anatabine
Panax ginseng	Saponins
Panax Hybrid *(P. ginseng X P. quinqifolium)*	Ginsenosides
Papaver somniferum	Codeine
Perezia cuernavcana	Sesquiterpene quinone
Pimpinella anisum	Essential oils
Platycodon grandiflorum	Polyacetylene glucosides
Rauwolfia serpentina	Reserpine
Rubia peregrina	Anthraquinones
Rubia tinctorum	Anthraquinone
Rudbeckia sps.	Polyacetylenes and thiophenes
Salvia miltiorhiza	Diterpenoid Hyoscyamine

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Table 2.3 (continued)

Plant	Secondary metabolite
Serratula tinctoria	Ecdysteroid
Sesamum indicum	Naphthoquinone
Solanum aculeatissi	Steroidal saponins
Solanum lacinialum	Steroidal alkaloids
Solanum aviculare	Steroidal alkaloids
Swainsona galegifolia	Swainsonine
Swertia japonica	Xanthons
Tagetus patula	Thiophenes
Tanacetum parthenium	Sesquiterpene coumarin ether
Tricosanthes kirilowii maxim var japonicum	Defense related proteins
Trigonella foenum graecum	Diosgenin
Valeriana officinalis L.	Valepotriates
Vinca minor	Indole alkaloids (vincamine)
Withania somnifera	Withanoloides

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CHAPTER III

IN VITRO AND HAIRY ROOT CULTURE OF *OPHIORRHIZA ALATA*

O. alata is a rare plant species of Thailand. This plant material has a very low multiplication rate and show very poor natural regeneration percentage. The harvest of plants on a mass scale from their natural habitats for natural compounds extraction is leading to a depletion of plant resources.

The aim of this chapter was to establish *in vitro* multiple shoot regeneration through leaf and node explants. Leaf and node of regenerate shoots were induced hairy root.

1 Materials

1.1 Plant material for *in vitro* culture

Seeds of *O. alata* was collected from Chantaburi Province, Thailand. They were used as initial explant for shoot multiplication.

1.2 *A. rhizogenes* strain TISTR 1450

Wild type *A. rhizogenes* strain TISTR 1450 is equivalent to American Type Culture Collection (ATCC) 15834 (Figure 3.1) were obtained from the Thailand Institute of Scientific and Technological Research (TISTR).

Figure 3.1 Freeze dried of *A. rhizogenes* strain TISTR 1450 in a vacuum sealed ampule

2 Methods

2.2 Preparation of media and cultures condition for plant tissue culture

2.1.1 Nutritional media

Murashige and Skoog media (MS), 1/2 MS and Gambrog's medium (B5) were used as media through out this experiment. The compositions of this culture media were described in Appendix A.

Auxins and cytokinins were used as plant growth regulators at various concentrations. Type of auxins and cytokinins are as follows:

Auxin: α- Napthalenecetic acid (NAA) and Indole-3-butyric acid (IBA)

Cytokinins: 6-benzylaminopurine or N^6- Benzyladenine (BA) and Kinetin-6-furfurylaminopurine (Kinetin, Kn)

For solid media, Gelrite (Sigma, USA) were added to 0.5% (w/v).

2.1.2 General preparation of media

Approximately 800 ml of distilled water was measured and poured into a glass beaker. The basal media were then slowly added and gently continuously stirred using magnetic stirrer. The compositions of this culture media were described in part A of Appendix. After the media were completely dissolved, the required sucrose and plant growth regulator was added to the solution. The pH was adjusted to 5.7-5.8 with a few drops of 1M sodium hydroxide and 1M hydrochloric acid. The solution was made up to 1L with distilled water. In order to get a semi-solid medium, the gelling agent was added at this time. The solution was then heated and stirred to dissolve gelling agent completely. The media were poured into a suitable container. They were then autoclaved at 120 °C for 20 minutes. They were stored at room temperature prior to use.

2.1.3 Plant tissue cultures condition

The *in vitro* cultures of *O. alata* were maintained in a culture room with 16-hour photoperiod at illumination of 1,500 lux and 8-hour dark. The incubation temperature was maintained at 25 ± 2 °C. The pH valve of all media under the study was set to 5.7 before autoclaving.

2.2 Preparation of aseptic explants and surface sterilization

Seeds of *O. alata* were surface sterilized with 70% ethanol for 1 min, 1% sodium hypochlorite containing three drops of Tween 80 for 15 min and then rinsed three times with sterilized water. Seeds were germinated on 1/2 MS medium pH 5.7 supplemented with 1% sucrose and 0.5% Gelrite. Cultures were kept at 25 $\pm$ 2 °C with a 16 h light/8 h dark. Germination started within 2 weeks. Plantlets were subcultured on the same medium every 4 weeks. After 8 weeks shoots with 2 to 4 true leaves were selected to use as explants in the shoots multiplication experiment.

2.3 Preparation of *A. rhizogenes* strain TISTR 1450

2.3.1 Revival *A. rhizogenes* from freeze - dried cultures

Freeze dried *A. rhizogenes* TISTR 1450 (ATCC 15834) (Figure 3.1) obtained from the Thailand Institute of Scientific and Technological Research (TISTR) was revived using the protocol from TISTR. It was maintained on nutrient agar (NA) medium. The petri dishes were kept on 25 °C. After 48 hr cultivation, the colonies appeared. A single colony was transfered from the plate to a 15 ml sterile tube containing 5 ml nutrient broth (NB) medium. The cultures were incubated on a shaker at 150 rpm at 25 °C for 24 hr. For maintenance *Agrobacterium*, 100 µl bacteria culture was put in a sterile eppendorf tube which containing 100 µl sterile 80% (v/v) glycerol solution. Then they were stored at –70°C. The bacteria in glycerol were used as bacterial stocks for hairy root induction.

2.3.4 Growth analysis of *A. rhizogenes*

The bacteria were recovered by streaking *A. rhizogenes* in a glycerol stocks on NA medium. A single bacterial colony was inoculated in 5 ml of nutrient broth medium and the culture was placed on rotary shaker (150 rpm) at 25°C. The optical density (OD) was measured on spectrophotometer at 600 nm during 0 - 72 hr cultivation period. Growth curve of *A. rhizogenes* was plotted overtime. From the growth curve, the appropriate cultivation time of *A. rhizogenes* for using in plant induction was in log phase period.

2.3.5 Preparation of *A. rhizogenes* for plant induction

A. rhizogenes from a glycerol stock was streaked on NA medium. A single bacterial colony was inoculated in 5 ml of nutrient broth medium and the culture was placed on rotary shaker (150 rpm) at 25°C for 24 hr or untill the OD_{600} was about 0.5. The bacterial suspension was used for co-cultivation of explants.

2.7 Plant growth regulators on *in vitro* multiple shoots

. After germination, plantlets were subcultured on the 1/2 MS medium pH 5.7 supplemented with 1% sucrose and 0.5% Gelrite.every 4 weeks. After 8 weeks shoots with 2 to 4 true leaves were selected to use as explants in the shoots multiplication experiment.

After 8 weeks of germination, seedlings were selected for using in multiplication of shoots induction. Leaves and nodes were cut into appropriate size, 1 cm^2 of leaf explant and 1 cm of node, using sterile forceps and a knife. Both explants were cultured on 1/2 MS medium supplement with the combinations of Kn (0, 4.65 and 9.30 µM) and NAA (0, 0.54, and 1.08 µM). Cultures were kept at 25 $\pm$ 2 °C with a 16 h light/8 h dark.

2.8 Hairy root induction

Hairy root was induced according to the previously reported procedure with minor modification (Saito *et al*., 2001; Watase et al.,2004). Leaf and node explants from 8 week-fully grown *in vitro* plantlets were used as explants for hairy root induction. Leaves and nodes were cut into appropriate sizes, 1 cm^2 and 1 cm, respectively. They were wounded with a sterile scalpel and immersed in conical flasks containing the bacteria suspension for 30 min. After dipping, they were co-cultivated with *A. rhizogenese* on 1/2 MS medium pH 5.7 supplemented with 1% sucrose and 0.5% Gelrite in the dark condition for 2 days. After co-cultivation, the explants were subcultured on MS medium with 200 mg/l cefotaxime at one week interval in order to eliminate the *A. rhizogenese*. After 4 weeks, they were subcultured on cefotaxime-free B5 medium solidified with 0.5% Gelrite. All the cultures were maintained in complete darkness at 25 $\pm$ 2 °C. After 4 weeks of incubation, root number, root length, fresh weight, and dry weight were measured. Subsequently, they were harvested for CPT analysis.

2.9 Detection of *rol* B transformation by PCR

DNA from transformed and non-transformed roots were isolated with a Dneasy Plant Mini Kit (Quigen, Germany) using the procedure specified by the manufacturer. Polymerase chain reaction (PCR) primers specific for the amplification of the *rol* B gene were used (Watase,2004.). A 20 µl PCR mix contained 100 ng of genomic DNA, 200 nM of each primer, 200 µM dNTPs, 1 unit of *Taq* DNA polymerase and 1.5 mM $MgCl_2$. The PCR reaction mixture was incubated in a DNA thermal cycle under the following condition; 94°C for 3 min, 40 cycles of 93°C for 1 min, 55°C for 2 min and 72°C for 3 min, and a final extension at 72°C for 10 min. The primers used in the PCR are *rol B* (F) primer: 5'-CCTCTAGAGTAACTATCCAACTCACATCACAAG-3' and *rol B* (R) primer: 5'-TTGAATTCGTG GCTGGCGGTCTTGGATTCATTTC-3' (Minlong *et al*., 2000). Amplified DNA bands were analyzed by 1% agarose gelelectrophoresis at 100 V for 30 min follow by staining with SYBR® safe and visualized under UV light to determine quality and quantity. A Lambda 1 Kb DNA marker was used as standard molecular marker. DNA extract was kept at -20 °C for further use as template in PCR amplification.

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3 Results

3.1 Surface sterilization conditions

The optimum condition for sterilization of *O. alata* seed was surface sterilized with 70% ethanol for 1 min, 1% sodium hypochlorite containing three drops of Tween 80 for 15 min and then rinsed three times with sterilized water.

3.2 *A. rhizogenes* strain TISTR 1450

3.2.1 Revival of *A. rhizogenes*

A. rhizogenes was maintained on nutrient agar (NA) medium. Petri dishes were kept on 25 °C. After 48 hr cultivation, the colonies were noticed (Figure 3.2).

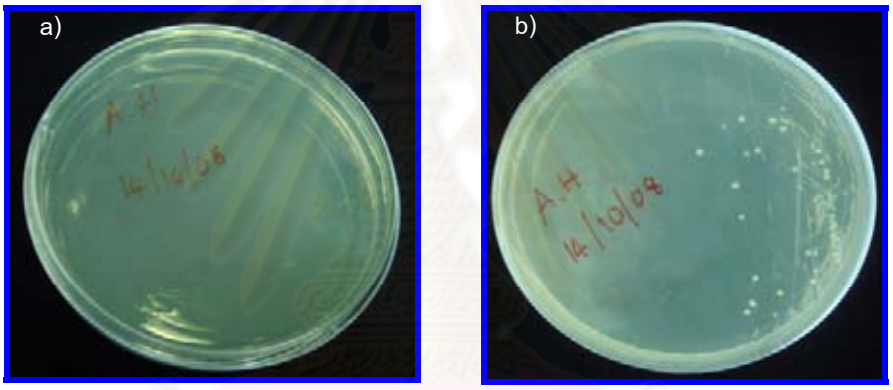

Figure 3.2 *A. rhizogenes* culture on NA medium: a) day 0, b) day 2

3.2.2 Growth of *A. rhizogenes*

The optical density (OD) was measured by spectrophotometer at 600 nm during 0 - 72 hr cultivation period. The growth curve, OD against cultivation time, of *A. rhizogenes* was plotted (Figure 3.3). It showed four stages of bacterial growth including lag phase, log phase, stationary phase, and death phase. The log phase presented a 12 – 48 hr interval of cultivation time. This interval was selected for bacterial suspension preparation.

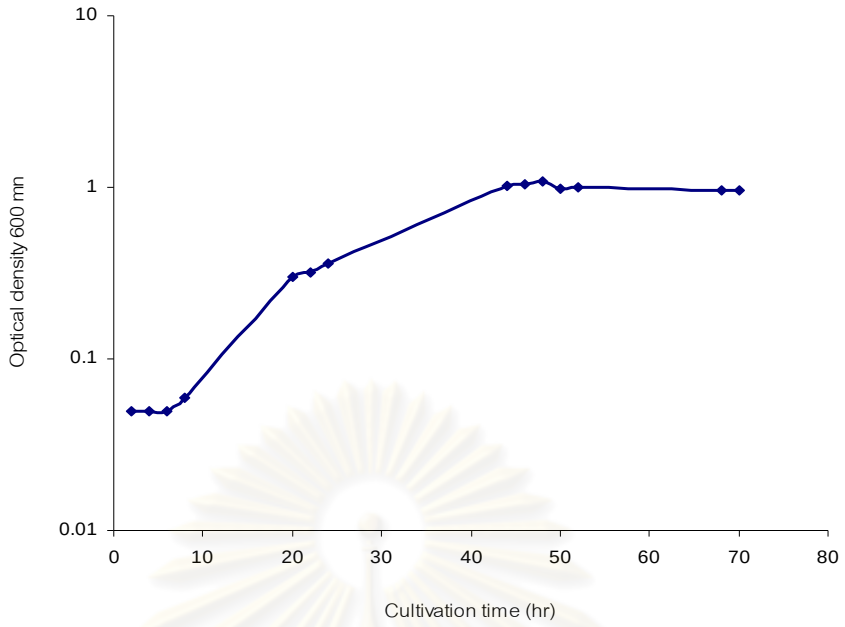

Figure 3.3 The growth curve of *A. rhizogenes* strain TISTR 1450. Cultivation time was plotted against OD_{600}.

3.3 Effect of plant growth regulators on *in vitro* multiple shoots

O. alata seeds were sterilized and germinated on 1/2 MS medium without plant growth regulators. Eight weeks after germination, *O. alata* seedlings showed a single shoot with 2-4 true leaves and were used as explants for shoot multiplication (Figure 3.4).

For the induction of multiple shoots from leaves and nodes of *O. alata*, explants were cultured on 1/2 MS medium supplemented with various concentrations of auxins and cytokinins. The highest percentage of explants forming shoot was 80 % on the medium supplement with combination of 9.30 µM Kn and 0.54 µM NAA (Table 3.1) followed by 60 % on the medium consisting of 4.65 µM Kn and 0.54 µM NAA from leaves explants. On the contrary, the medium supplemented with 4.65 µM Kn alone and free plants growth regulators were not effective on multiple shoots induction. The maximum number of shoots was 1.7 per explants obtained on the medium containing 9.30 µM Kn and 0.54 µM NAA (Table 3.1 and 3.2) followed by 1.3 shoots per explants in the medium having 4.65 µM Kn and 0.54 µM NAA from leaves explants.

In case of node explants, all of treatments could induce 100 % of multiple shoots. The highest number of shoots from node explants was 9.6 per explants which obtained from medium containing 4.65 μM Kn alone (Table 3.1 and 3.3) followed by 7.7 per explants in medium having 9.30 μM Kn. On the other hand, the lowest number of forming shoot from node explants was 1.4 per explant on the medium supplemented with 0.54 μM NAA. Individual obtained shoots were elongated on the same medium without plants growth regulators.

Figure 3.4 Seedling of *O. alata* obtained from seed germination on 1/2 MS medium for 8 weeks

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Table 3.1 Effect of different concentration of Kn and NAA on *in vitro* multiplication of leaf and node explant of *O. alata* .

Plant growth regulator (μM)		Percentage of leaf and node explants forming shoots (%)		Number of shoots per explants ± S.D.	
Kn	NAA	Leaf	node	Leaf	node
0	0	0	100	0.0 ± 0.00^{d}	1.7 ± 0.67^{b}
0	0.54	60	100	1.1 ± 0.39^{abc}	1.4 ± 0.51^{b}
0	1.08	40	100	0.6 ± 0.84^{bcd}	1.5 ± 0.53^{b}
4.65	0	0	100	0.0 ± 0.00^{d}	9.6 ± 4.99^{a}
4.65	0.54	60	100	1.3 ± 1.42^{ab}	3.2 ± 2.39^{b}
4.65	1.08	40	100	0.4 ± 0.52^{cd}	2.3 ± 1.50^{b}
9.30	0	10	100	0.3 ± 0.95^{cd}	7.7 ± 4.45^{a}
9.30	0.54	80	100	1.7 ± 1.41^{a}	3.7 ± 2.11^{b}
9.30	1	30	100	0.3 ± 0.48^{cd}	3.7 ± 1.41^{b}

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

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Table 3.2 Effect of different concentrations of Kn and NAA on *in vitro* multiplication of leaf explant of *O. alata*.

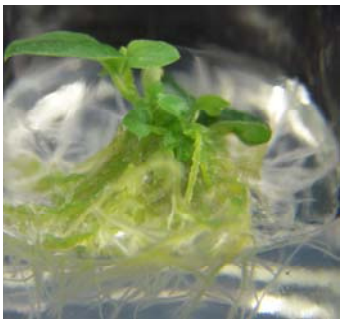
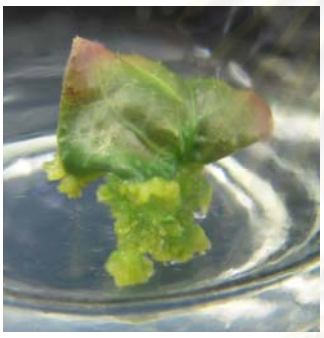
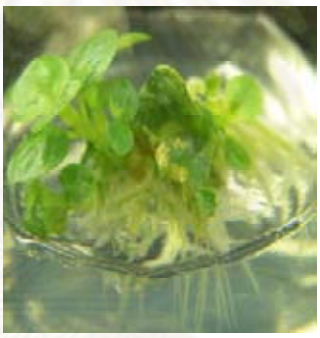
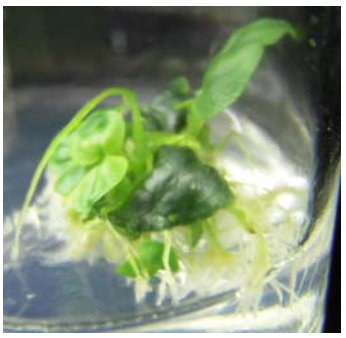
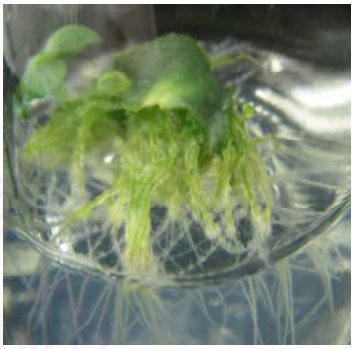
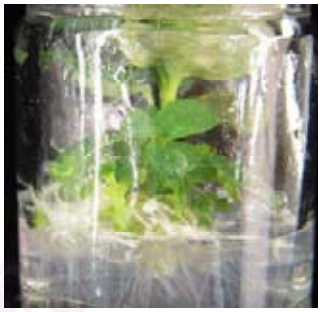
Kn (μM) \ NAA (μM)	0	0.54	1.08
0			
4.65			
9.30			

Table 3.3 Effect of different concentrations of Kn and NAA on *in vitro* multiplication of node explant of *O. alata*.

Kn (μM) \ NAA (μM)	0	0.54	1.08
0			
4.65			
9.30			

3.6 Hairy root induction

Leaf and node of *O. alata* explants were infected with *A. rhizogenes* strain TISTR 1450. After 10 days, hairy roots immerged at wound sites. Percentage of hairy root formation was 100 % and 96.35 % for node and leaf explants, respectively (Table 3.4 and Figure 3.5 – 3.6). The induced hairy roots were grown on fresh 1/2 MS medium containing 200 mg/l cefotaxime to eliminate *A. rhizogenes*. After three passages onto fresh antibiotic containing medium at one week intervals, hairy roots were transferred to B5 free antibiotic medium. Four weeks after inoculation, root number, root length, fresh weight and dry weight of hairy roots were measured. Hairy roots had characteristics of transformed roots such as high lateral branching and lack of geotropism. Root number was 17.65 and 6.32 for node and leaf explants. Root length from leaf and node inductions were 2.10 and 2.37 cm, respectively. Node explants showed higher biomass accumulation of hairy root than leaf explants.

Table 3.4 Growth of hairy root cultures after infection 4 weeks

Type of explants	Percentage of hairy root formation	number of hairy root ± S.D (cm)	Length of hairy root ± S.D (cm)	Fresh wt of hairy root per explant ± S.D (mg)	Hairy root dry wt per explant ± S.D (mg)
Leaf	96.35	6.32 ± 4.52	2.10 ± 1.04	9.90 ± 0.36	1.44 ± 0.33
Node	100	17.65 ± 6.43	2.37 ± 1.13	22.45 ± 0.62	3.22 ± 0.20

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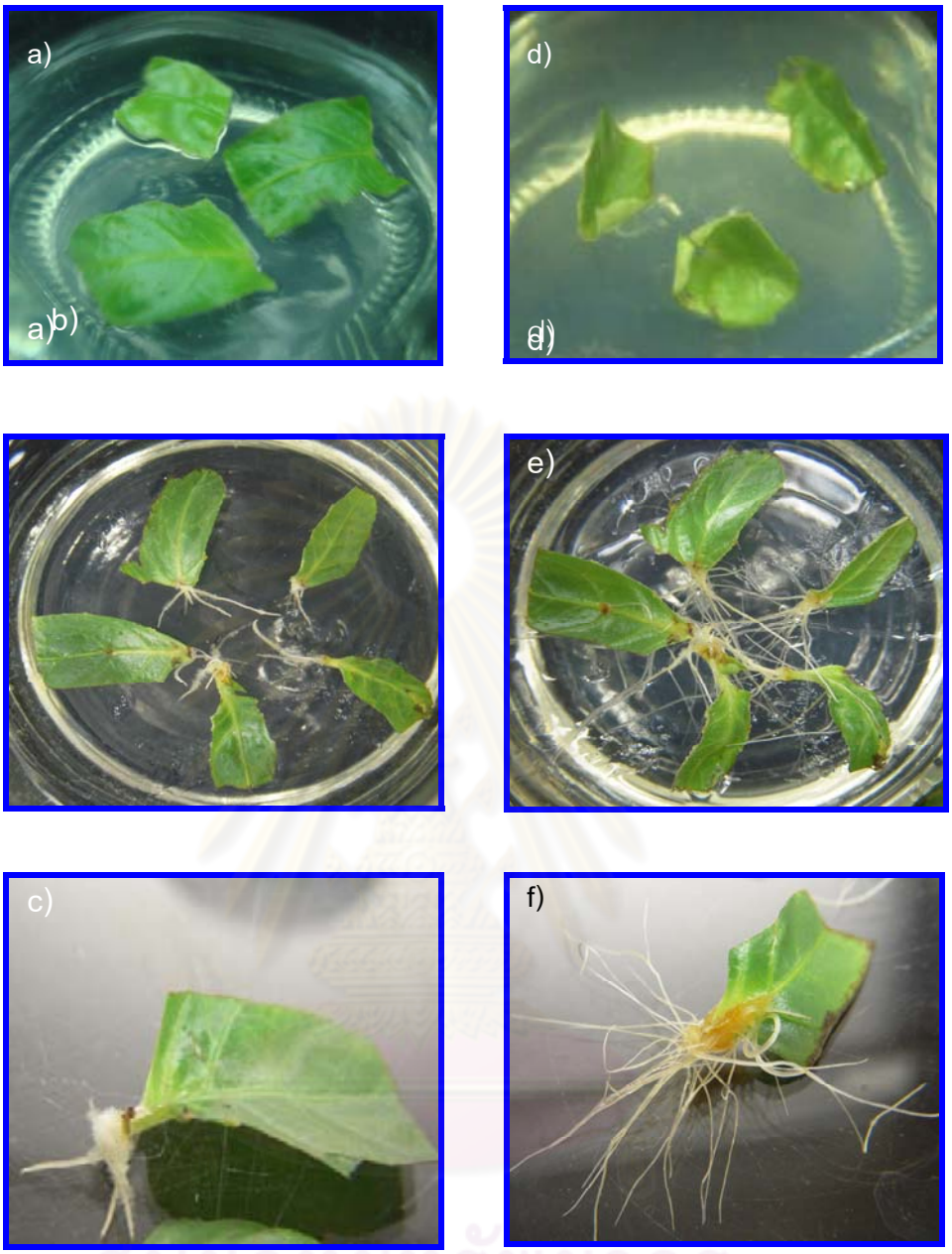

Figure 3.5 Hairy root induction of leaf explants; a) and d) 10 and 30 days after induction with water (negative control), b) and e) 10 and 30 days after induction with *A. rhizogenes*, c) and f) closer views showing hairy root development at day 10 and 30, respectively

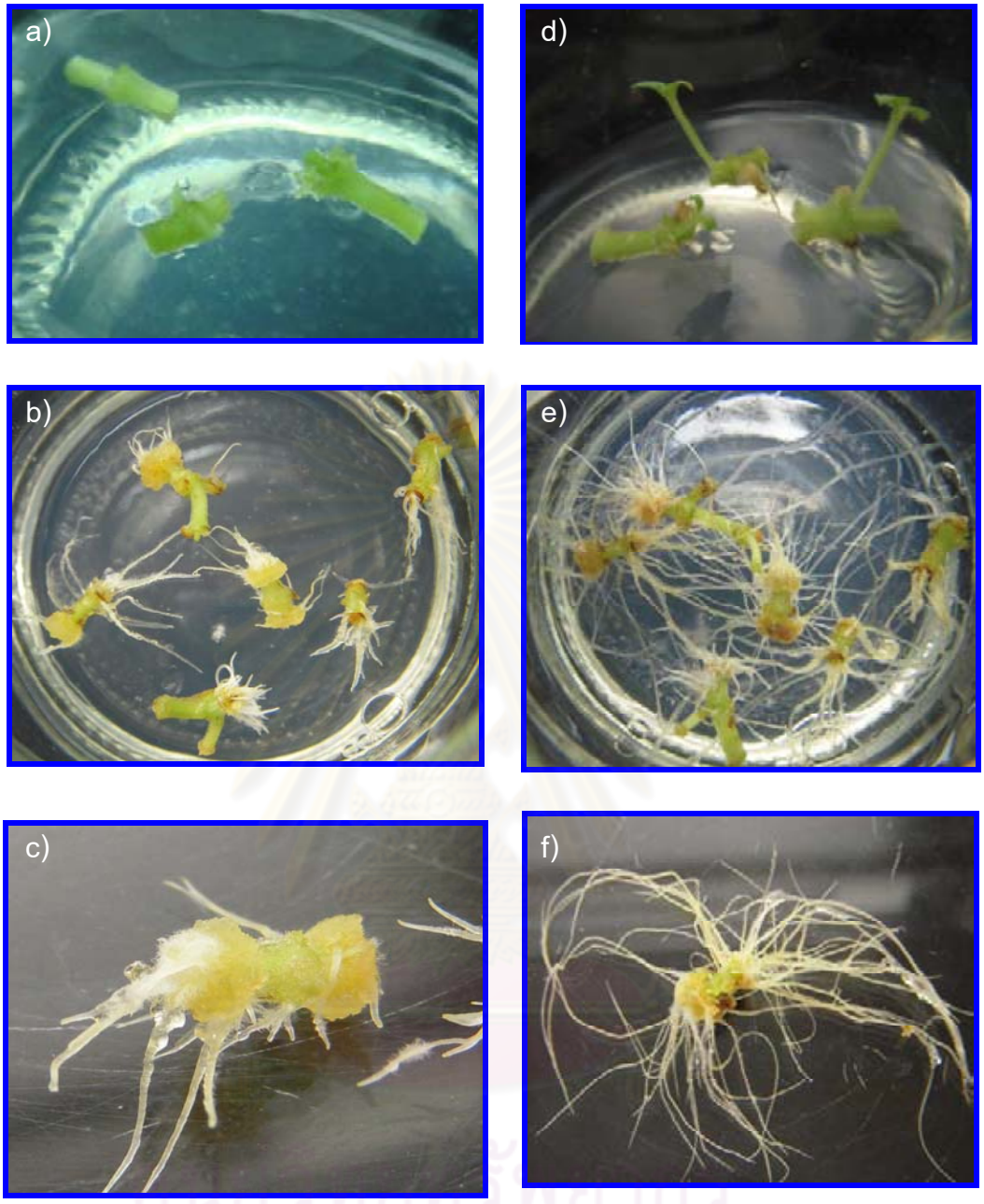

Figure 3.6 Hairy root induction of node explants; a) and d) 10 and 30 days after induction with water (negative control), b) and e) 10 and 30 days after induction with *A. rhizogenes*, c) and f) closer views showing hairy root development at day 10 and 30, respectively

3.7 PCR analysis of hairy root

Hairy root induced from *A. rhizogenes* were confirmed by PCR. The PCR products showed clear bands for *rol* B (approximate 1.3 kb) in transformed root and *A. rhizogenes* strain TISTR 1450 itself. There were no bands in untransformed roots (Figure 3.7). This result indicates that the *rol* B from Ri plasmid of *A. rhizogenes* strain TISTR 1450 were successfully integrated into *O. alata* hairy root.

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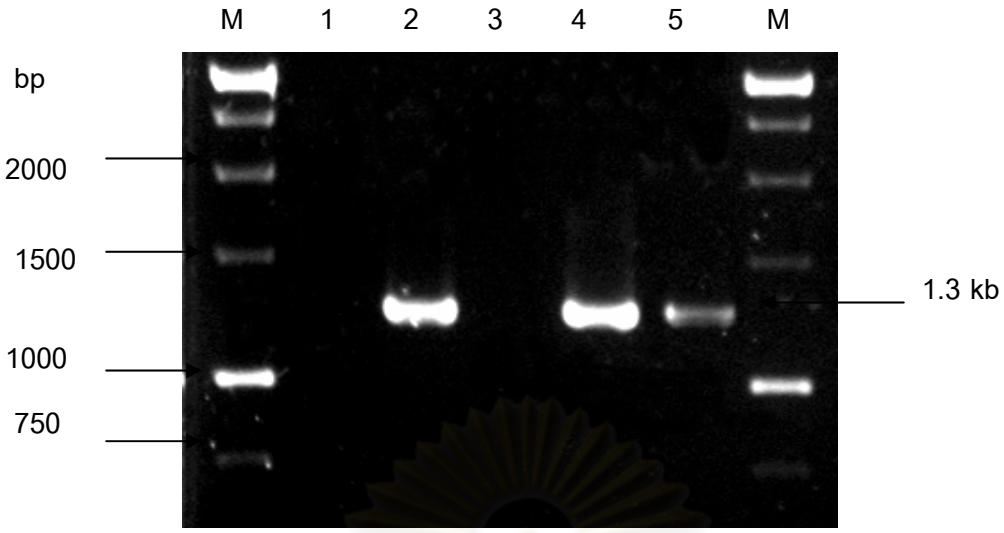

Figure 3.7 Agarose gel electrophoretogram of PCR products amplified with *rol B* (F) and *rol B* (R) primers; Lane M: Lambda 1 Kb DNA marker, Lane 1: non-transformed root obtained from leaf explants, Lane 2: hairy root obtained from leaf explants, Lane 3: non-transformed root obtained from node explants, Lane 4: hairy root obtained from node explants, Lane 5*: A. rhizogenes*

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4. Discussion

Shoot multiplication from leaf explants cultured on 1/2 MS medium containing 9.30 μM Kn and 0.54 μM NAA way found to be the best treatment for maximum multiple shoot induction as well as gave the maximum number of shoots per explants. Similar results were also reported in the other *Ophiorrhiza* spp. such as *O. prostrata* (Beegum *et al*., 2007). In addition, combinations of auxin and cytokinin have been used to induce shoot formation in numerous species (Al-Bahrany, 2002, Adeniyi *et al.,* 2008)

Node explants were found to be superior for maximum multiple shoot induction and gave the maximum number of shoots per explants when cultured on 9.30 and 4.65 μM Kn alone. It has also been reported that cytokinin alone gave successfully multiplication in shoot of *Cissus sicyoides* (Abreu *et al*., 2003), *Salix humboldtiana* (Pereira *et al*., 2000) and *Ficus carica* L. (Fraguas *et al*., 2004).

Therefore, micropropagation of *O. alata* in our study was successfully obtained from node explants cultured on 1/2 MS medium containing 4.65 μM Kn alone.

The main advantage of using hairy root cultures is their ability to grow in defined basal media without supplementation of phytohormones. Due to their differentiated nature, they show genetic stability and tend to produce high levels of secondary metabolites characteristic of the species (Giri *et al*., 2000). The variation in hairy root induction could possibly be attributed to the variation in virulence of different *A. rhizogenes* strains used (Giri *et al.,* 2001). It has been reported that hairy root induction by *A. rhizogenes* strains ATCC 15834 was success in various plant. This strain effected on hairy root formation of *Solanum laciniatum* and *Gmelina arborea* reaching 100 and 32 % (Okrslar, *et al* 2002; Satdive ; *et al*., 2007).

In the present study, induction of *O. alata* hairy roots by *A. rhizogenes* strain TISTR 1450, which is equivalent to ATCC 15834, occurred at a frequency of about 96.35 % in leaf explants and 100 % in node explants. In *Echinacea purpurea*, a high frequency of root induction has also been reported (Wang *et al.,* 2006). Its hairy roots were initiated from leaf discs with a success rate of approximately 80% using *A. rhizogenes* strain A4. There has been reported that *A. rhizogenes* strains ATCC 15834 was successful in providing the most consistant hairy root response (saito et al, 2001; Bolivar *et al*., 2007). In *Eustoma grandiflorum,* hairy roots were initiated from

microshoots with a success rate reaching 100 % using *A. rhizogenes* strains LBA 9402. In contrast, very low root induction rate was obtained with strains NCPPB 2659 (Popa *et al*., 2006).

Besides strain virulence, susceptibility of the explants may also play an important role in determining the frequency of hairy root induction (Satdive *et al.,* 2007). Wang *et al.* (2006) found that leaves were comparatively easier to produce hairy root than leavestalks of *E. purpurea*. Transformed percentage of leaves was 80 %, while the transformed percentage of leavestalks was only 10 %. In our study, similar percentage of hairy root formation from node and leaf explants was observed (Table 3.4).In addition, the percentage of hairy root induction and number of hairy roots per explant varied with infection period (Karthikeyan et al., 2007).

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CHAPTER IV

ROOT AND HAIRY ROOT INDUCTION FOR TRANSPLANTING TO GREENHOUSE

Hairy root is a plant disease caused by *A. rhizogenes*. It shows a numerous and fast growing of roots. The alteration in root growth and development could be important for improvement of root system for propagation. A composite plant was generated by inducing hairy roots by *A. rhizigenes* on untransformed shoots. A normal plant were generated a numerous root with auxin on untransformed shoots.

The goal of this chapter was to compare growth and survival of composite and normal plant after transplanting to greenhouse.

1 Materials

1.1 Plant material for *in vitro* rooting

After multiple shoots induction (CHAPTER III), individual shoot was elongated on 1/2 MS medium without plant growth regulators for 8 weeks. These plantlets were used as explants for *in vitro* rooting assay.

1.2 *A. rhizogenes* strain TISTR 1450

Wild type *A. rhizogenes* strain TISTR 1450 which is equivalent to ATCC 15834 was obtained from the Thailand Institute of Scientific and Technological Research (TISTR). They were grown on nutrient agar medium. A single bacterial colony was inoculated in 5 ml of nutrient broth medium and the culture was placed on rotary shaker (150 rpm) at 28 °C for 48 hr untill the OD_{600} was about 0.5. The bacterial suspension was used for co-cultivation of explants.

2 Methods

2.1 *in vitro* rooting by auxin and *A. rhizogenes*

Shoots were induced into rooting by cultured on 1/2 MS medium supplemented with IBA at 0.49, 0.98 and 1.96 µM and by *Agrobacterium* infection.

For *A. rhizogenes* infection, shoots were wounded with a sterile scalpel and immersed in *A. rhizogenes* suspension for 30 min. Then, the shoots were co-cultivated with *A. rhizogenes* on 1/2 MS medium pH 5.7 supplemented with 1% sucrose and 0.5% Gelrite in the dark condition for 2 days. After co-cultivation, shoots were subcultured on 1/2 MS medium with 200 mg/l cefotaxime at 1 week interval in order to eliminate the *A. rhizogenese* for 4 weeks, and then, subcultured on cefotaxime-free B5 medium. Growth factors such as leaf number, leaf width, leaf length and plant height were measured after incubation for 4 weeks.

For control experiment, shoots were cultured on 1/2 MS medium.

2.2 Detection of *rol* B transformation by PCR

Detection of *rol* B transformation of hairy root was performed according to method 2.3 (CHAPTER III).

2.3 Transplanting to greenhouse

Four-week-old plantlets by *in vitro* rooting with IBA and *A. rhizogenes* were transplanted to greenhouse. They were thoroughly washed under tap water for 2 to 3 min to remove traces of Gelrite medium before transplanting to autoclaved soil (coconut husk: rice ashes (1: 2) v/v). In order to maintain humidity, plantlets were covered with a plastic bag for two weeks. Four weeks after transplanting, growth factors such as plantlets height, leaf number, and leaf length were measured.

3 Results

3.1 Effect of auxin and *A. rhizogenes* on *in vitro* rooting

The formation of roots after *A. rhizogenes* infection and IBA treatment was recorded for 4 weeks. In comparison with *in vitro* root induction by *A. rhizogenes* and IBA treatment, it was shown that no difference in percentage of shoots forming roots. All treatments were 100 % of shoots forming roots (Table 4.1).

The highest number of roots per shoots was 26.1 from the medium supplemented with 0.49 µM IBA. On the contrary, the lowest number of roots per shoot was 8.3 in medium-free plant growth regulators (control). In addition, *A. rhizogenes* treatment plantlets had the number of roots in culture media lower than all IBA treatments but higher than free plant growth regulators.

The highest length of roots per shoots was 3.67 cm from medium with free plants growth regulators. The lowest root length per shoot was 0.81 cm on medium supplemented with 1.96 µM IBA. However, *A. rhizogenes* treatment plantlets had the length of roots longer than 0.98 and 1.96 µM IBA treatments but no significantly difference when were compared with 0.49 µM IBA treatment.

Table 4.1 Growth comparison of *in vitro* rooting by IBA and *A. rhizogenes*

Type of treatment		Percentage of shoots forming roots (%)	Root number per shoot ± S.D.	Root length ± S.D (cm)
IBA (µM)	0 (control)	100	8.3 ± 0.67^{c}	3.67 ± 0.48^{a}
	0.49	100	26.1 ± 5.38^{a}	1.62 ± 0.23^{b}
	0.98	100	22.9 ± 8.46^{ab}	1.17 ± 0.43^{c}
	1.96	100	25.0 ± 8.46^{a}	0.81 ± 0.48^{c}
A. rhizogenes		100	17.8 ± 2.09^{b}	1.76 ± 0.48^{b}

$^{a, b, c}$ values followed by a different letter were significantly different at $p < 0.05$

3.2 PCR analysis of hairy root

The presence of the *rol* B gene in the hairy roots was tested by PCR amplification of the DNA using *rol* B forward and reverse primers. *A. rhizogenes* themselves served as positive control and DNA from the non-transformed roots served as negative control. DNA from hairy root and *A. rhizogenes* showed the presence of the 1.3 kb *rol* B amplified product, while DNA from the non-transformed did not show any amplification at expected sites (Figure 4.1). Thus, These results confirmed the successful transformation.

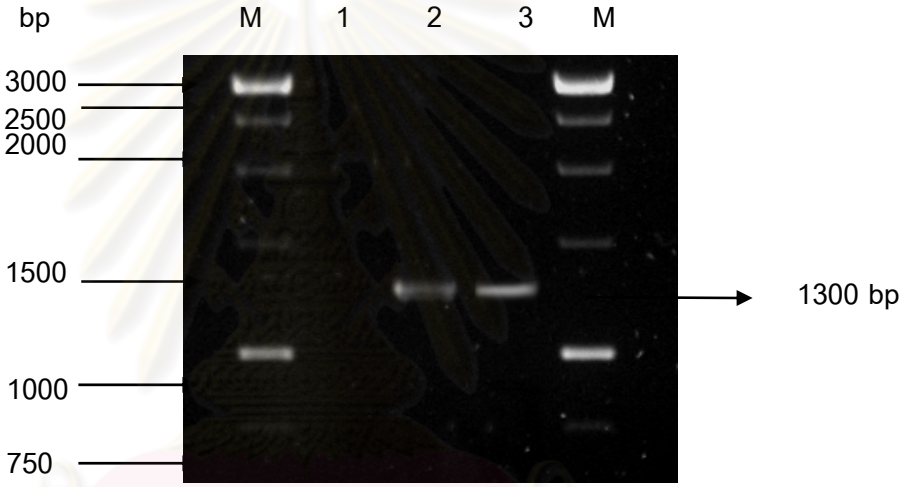

Figure 4.1 Detection of *rol* B gene. Lane M 1 kb DNA Marker, Lane 1: non-transformed root, Lane 2 transformed root, and Lane 3 *A. rhizogenes*

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3.3 Comparison composite plant and normal plant after transplanting to greenhouse

Four weeks after transplanting, survival, plant height, leaf number, and leaf length were measured. The result showed that the highest percentage of survival was 100 % obtained from all IBA treatments. *A. rhizogenes* treatment plantlet showed 80% survival (Figure 4.2a and Table 4.2).

Figure 4.2 Four weeks after root-induction by *A. rhizogenes* and various concentrations of IBA (1-5): 1) *A. rhizogenes*, 2) 0 µM IBA (control), 3) 0.49 µM IBA, 4) 0.98 µM IBA, and 5) 1.96 µM IBA; a) and b) Day 0 of transplanting to greenhouse, and c) four weeks after transplanting to greenhouse

Table 4.2 Survival of rooting plantlets induced by IBA and *A. rhizogenes* after transplanting to greenhouse.

Type of treatment		Percentage of survival (%)
IBA (μM)	0 (control)	100
	0.49	100
	0.98	100
	1.96	100
A. rhizogenes		80

All IBA treatments showed rapid increasing in plant height. *A. rhizogenes* infected plants showed slow increase in height (Figure 4.3). The highest height of plants was 6.45 cm resulted from treatment with 0.49 μM IBA followed by 6 cm from 1.96 μM IBA treatment. The lowest height of plants was 3.31 cm from induction with *A. rhizogenes*.

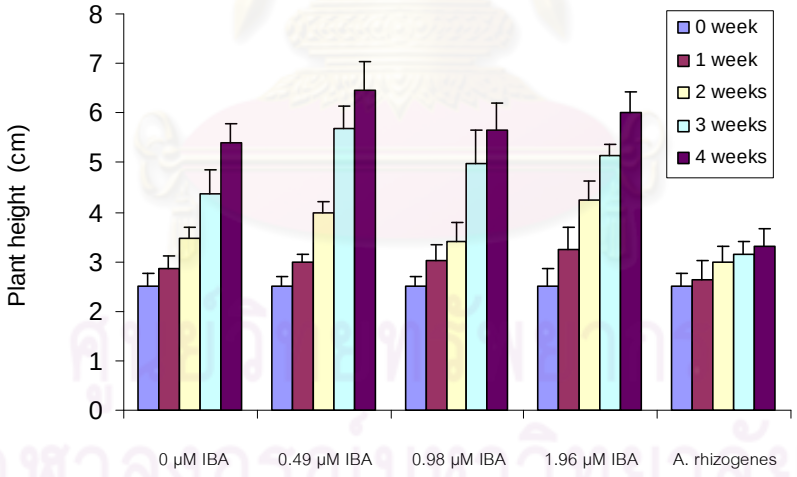

Figure 4.3 Plantlets height of plantlets after transplanting to greenhouse

The increasing in the number of leaf exhibited similar pattern to the increase in height of plants among treatments. They showed rapid increase in the number of leaf in all of IBA concentration and slow increase in *A. rhizogenes* treatment (Figure 4.4). In addition, *A. rhizogenes* treatment also showed the lowest number of leaf with 7.00 per plantlets. The highest number of leaf was 12.4 per plantlets obtained from 0.98 μM IBA treatment.

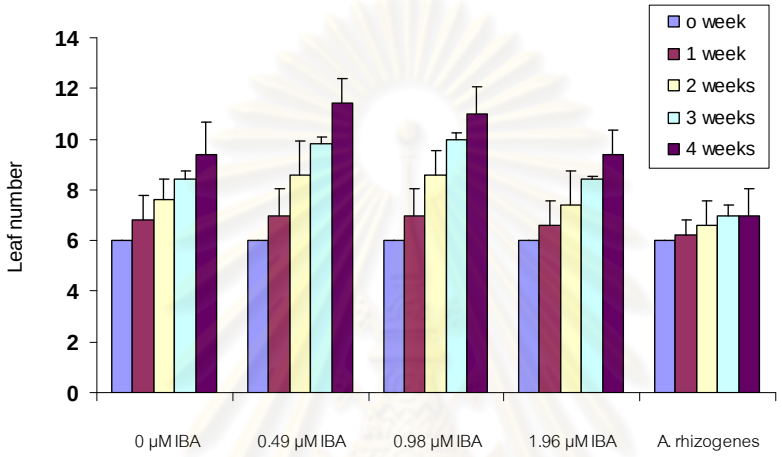

Figure 4.4 Leaf numbers of plantlets after transplanting to greenhouse

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The increase of the length of leaf was similar in both parameters, height of plant and number of leaf (Figure 4.5). The highest length of leaf was 5.45 cm obtained from medium consisting of 0.98 µM IBA. In the other hand, the lowest length of leaf was 2.37 cm obtained from *A. rhizogenes* treatment. And it also showed slow increase in left length.

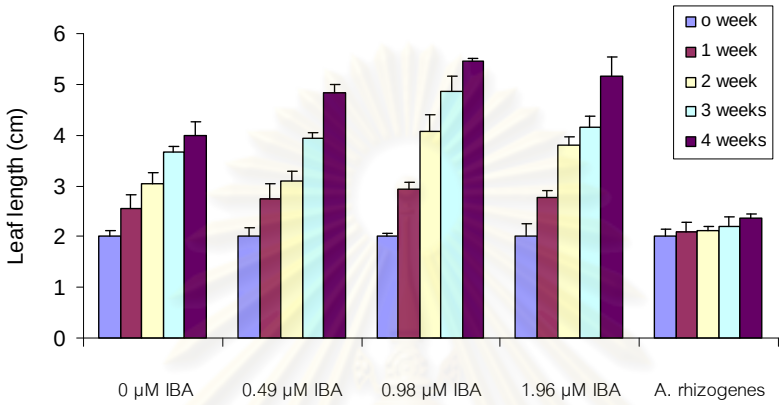

Figure 4.5 Leaf length of plantlets after transplanting to greenhouse

Control and IBA treatment plantlets were healthy and well developed when transferred to soil. On the contrary composite plants with transformed roots exhibited a dwarf and smaller leaves (Figrure 4.2 b-c).

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4. Discussion

We planned to test a hypothesis that a plant with numerous roots have high survival rate and more healthy when transplanted to greenhouse.

In vitro rooting, all of treatments, 0.49, 0.98, and 1.96 μM IBA, and *A. rhizogenes*, were effective for root induction from micropropagated plantlets of *O. alata*. A good result was obtained in IBA treatment with 100 % of explant producing roots. Similar results were also reported in the other plant such as *Tabernaemontana fuchsiaefolia* (Oliveira *et al*., 2003). In this study, root induction with *A. rhizogenes* strain TISTR 1450 gave 100 % rooting efficiency. This high efficiency were also found by using *A. rhizogenes* strain LBA 9402 to induce hairy root of pink and blue genotype of *Eustoma grandiforum* (Popa *et al*., 2006).

In comparison of the root number of *O. alata*, IBA treatment showed higher root number per shoot than *A. rhizogenes* treatment plantlets. Similar results were also reported for *E. grandiforum* (Popa *et al*., 2006). Moreover, there was a report that *in vitro* rooting of *Datura arborea* by *A. rhizogenes*, compositing plants showed number of root higher than untransformed plants. In addition, some of *D. arborea* compositing plant showed shorter length of root than untransformed plants (Giovannini *et al*., 1997).

After transplanting of *O. alata* plantlets, all IBA treatments exhibited 100 % survival rate better than *A. rhizogenes* treatment which showed only 80 %. The high survival rate was also found in *T. fuchsiaefolia* rooting induction by IBA (Oliveira *et al*., 2003). In this experiment, plants with *A. rhizogenes* treatment showed slower growth than all IBA treatment plants. In addition, IBA treatment plants were healthy and well developed when transferred to soil. Composite plants with transformed roots exhibited dwarf and smaller leaves. These phenotype characteristics had been reported for *A. rhizogenes*-mediated transformed plant (Koike. 2003; Crane, 2006). These alternate morphological characters are generally regarded as undesirable, but some of the altered features can have potential applications for the genetic improvement of ornamental plants (Koiki, 2003).

The choice of *in vitro* rooting of *O. alata* was obtained from 1/2 MS medium without plants growth regulators because no different survival rate was shown when compared to IBA treatment but higher than *A. rhizogenes* treatment. Moreover, this

method is easy and has low cost. The results also showed the possibility of applying *A. rhizogenes* infection to *O. alata* shoots for genetic improvement, especially for the development of abundant roots for using as an alternative source for CPT production.

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CHAPTER V

CAMPTOTHECIN PRODUCTION

CPT is a cytotoxic tryptophan derived-quinoline alkaloid which inhibits the DNA topoisomerase I. CPT and its derivatives are at present obtained by extraction from intact plants. Transformed plant tissue cultures may be alternative source of production. *O. mungos* and *O. pumila* were reported to be the source of CPT. *O. alata*, the same genus or known as "ผักหลอดดอกขาว" in Thai is distributed in the southeastern part of Thailand (Smitinand, 1999). It was collected to CPT analysis. Hairy root, in vitro culture plantlet, and transplanting to greenhouse plant were analyzed the amount of CPT content by HPLC. Then, they were compared with soil grown plant

1. Materials

1.1 Plant materials for CPT analysis

As above mentioned, plant types were used for CPT analysis including leaf, root and hairy root. Leaf and root were obtained from 5 month old of soil - grown plant, 4 weeks *in vitro* culture, and 4 week after transplanting to greenhouse (CHAPTER IV). Hairy roots were obtained from 4 weeks after leaf, node, and shoot were induced with *A. rhizogenes* (CHAPTER III – IV). In addition, they were obtained from 4 week after transplanting to greenhouse of composite plant (CHARPTER IV).

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2. Methods

2.1 Phytochemical techniques

2.1.1 Preparation of *O. alata* powder from leaf, root, and hairy root

Leaf and root from soil - grown plants: 5 month old plant, leaves and roots were collected and cleaned with tap water to remove soil. They were dried with freeze drying. Both samples were ground into powder.

Leaf and root from plant induced root by IBA: 4 weeks after cultivation was harvested and cleaned with distilled water for media removal. It was dried by freeze drying. The dried samples were ground into powder.

Leaf and root from plant after transplanting to greenhouse: 4 week old plant, leaves and roots were harvested and dried by freeze drying. The dried samples were ground into powder.

Leaf and root from composite plant after transplanting to greenhouse: 4 week old plant, leaves and hairy roots were harvested and dried by freeze drying. The dried samples were ground into powder.

2.1.2 Extraction

The extraction and detection of camptothecin were performed according to previously reported procedure with minor modification (Saito *et al.* 2001; Yamazaki *et al.* 2003). A 100 g dried wt of sample was then broken by mortar and pestle .The dried powdered material was homogenized with 1 ml of methanol by using ultrasonic bath for 30 min at the room temperature. After sonication, the samples were transferred to polypropylene micro-centrifuge tubes and centrifuged at 10,000 *xg* for 10 min. After centrifugation, the supernatant was collected and filtered through a 0.45 μm filter for high-performance liquid chromatography (HPLC) analysis. The methanol extract was transferred to clean glass vials and applied directly into a HPLC column.

2.1.3 HPLC analysis

Separation was performed with a ZORBAX Eclipse XDB-C18 column (25 cm x 4.6 mm, Agilent) at a flow rate of 0.8 ml/min. Elution gradient program was as follows: 0 –35 min linear gradient from solvent A [H_2O–HOAc–MeOH (79.8:0.2:20)] to solvent B [H_2O–HOAc–MeOH (9.975:0.025:90)], 35–40 min isocratic at 100% of solvent B. CPT were detected at 254 nm by diode array detector (DAD). The identifications were carried out through relative retention time and standard addition. These results were also confirmed after a comparison between the DAD absorption spectra of the peak and that of the standard.

2.2 The quantitative analysis of CPT production

The methanol crude extract of root, leaf, and hairy root of *O. alata* were analyzed by HPLC method. The calibration curve of standard CPT was prepared. Four mg of each standard was dissolved in 1ml methanol. This stock solution was diluted with methanol to the concentration range 5 - 40 µg/ ml for constructing its calibration curve by HPLC analysis. The content of CPT was calculated with the regression equations obtained from the calibration curves.

For each treatment at least triplicate samples were measured independently and the standard deviation calculated.

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3. Results

3.1 HPLC analysis of methanol crude extracts

A characteristic HPLC-DAD chromatogram of CPT standard and methanol crude extracts of *O. alata* monitored at 254 nm and their associated UV-Vis spectra were shown in Figure 5.1-5.3. Retention time of peak obtained with standard CPT was used to identify the corresponding peaks in methanol crude extracts of *O. alata* plant (Figure 5.1). The retention time of CPT standard was 38.7 min.

Beside relative retention time, CPT identification was confirmed by carried out through standard addition. CPT was identified by co-elution after spiking of crude extract with standard CPT (Figure 5.2).

CPT detection confirmed with comparison of the UV-Vis absorption spectra of the peak and that of the standard. UV spectra were signatures produced by each chemical in the detector. Figure 5.3 is the spectrum from the 38.7 minute peak in HPLC/DAD chromatograms of CPT standard and one of methanol crude extract sample. Notice that the spectrum has UV absorbance wavelengths along the x axis. This 38.7 minute peak of methanol crude extract spectrum is very similar to CPT spectrum.

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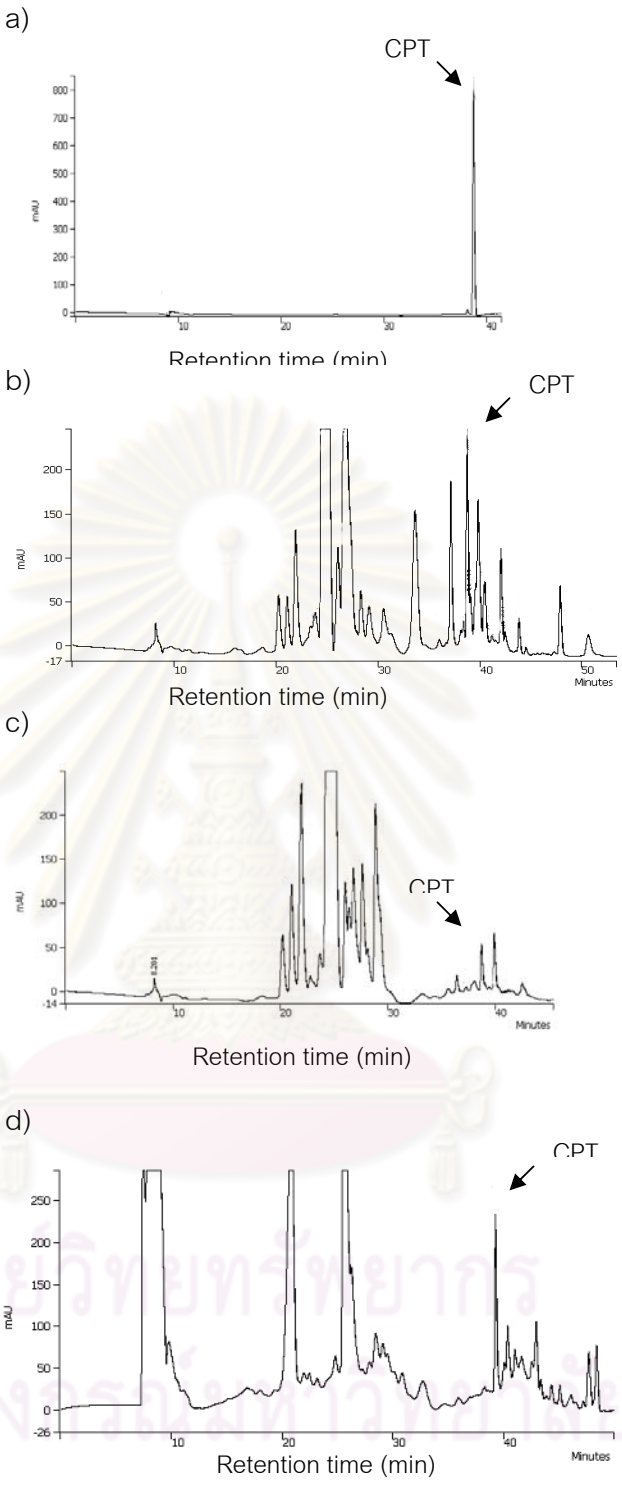
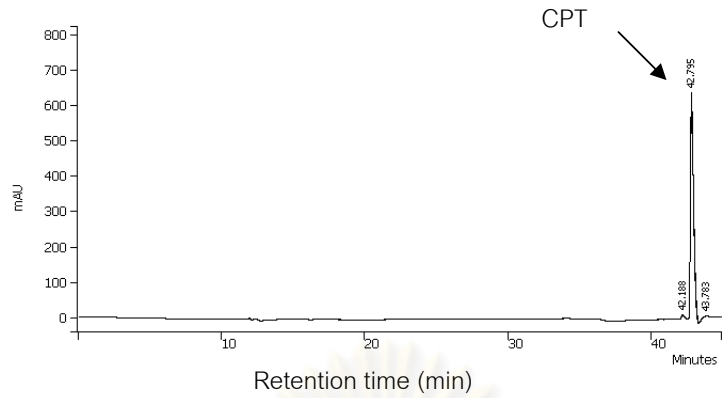
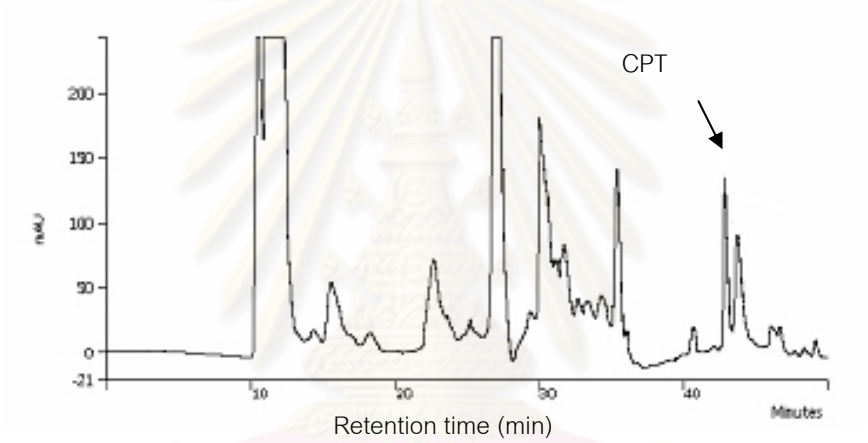


Figure 5.1 HPLC/DAD chromatograms detected at 254 nm of: a) standard CPT, b) untranformed root from soil - grown plant, c) leaf from soil - grown plant, and d) hairy roots of *O. alata*. Mobile phase: 0–35 min linear gradient from solvent A [H_2O–HOAc–MeOH (79.8:0.2:20)] to solvent B [H_2O–HOAc–MeOH (9.975:0.025:90)], 35–40 min isocratic at 100% of solvent B.

a)



b)



c)

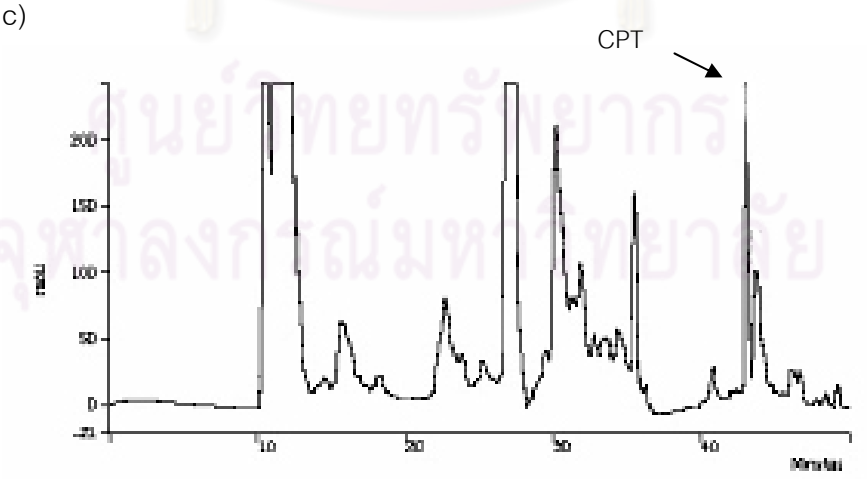


Figure 5.2 HPLC/DAD chromatograms of; a) standard CPT, b) crude extract-hairy root, and c) crude extract -hairy root spiked with internal standard.

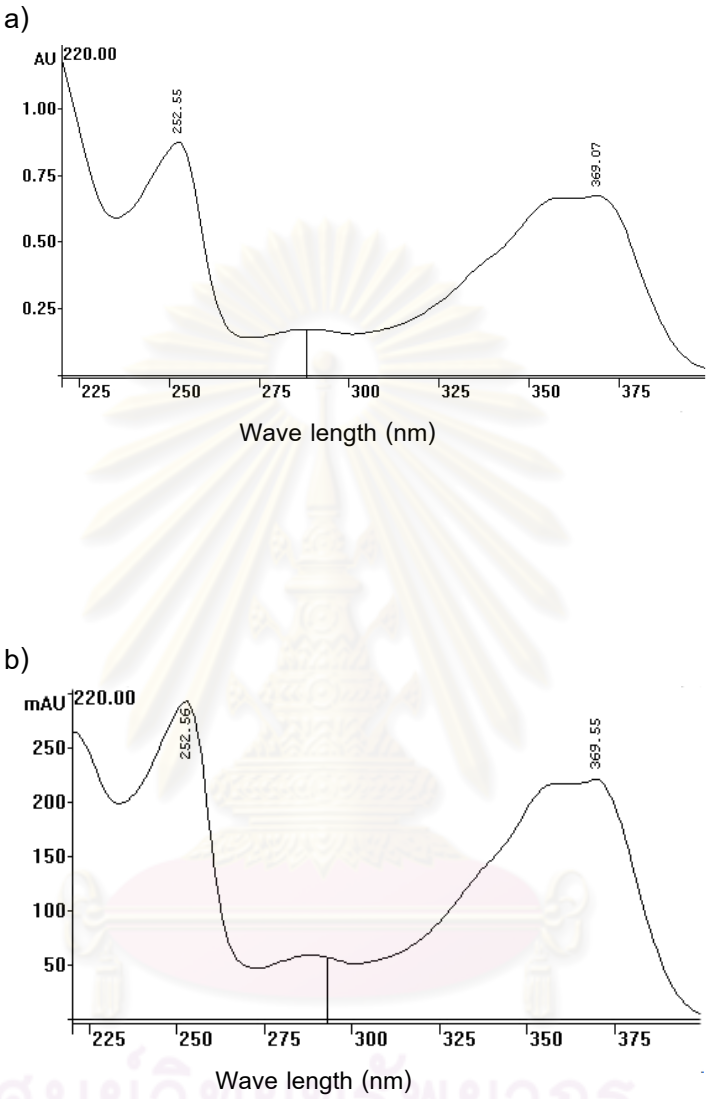


Figure 5.3 UV-visible spectra of the HPLC peak eluting at 38.7 minutes of: a) standard CPT and b) *O. alata* methanol extract determined by photodiode array detection.

3.2 **Camptothecin content** in different parts of plant types

The calibration curve showed good linearity, with a regression coefficient of 0.997. The regression equations for CPT was Y = 8693457X + 4225572 (Figure 5.4). The content of CPT in methanol extracts were calculated with the regression equations obtained from the calibration curves. The results were shown in Table 5.1. Untransformed root of soil–grown plants had the highest amount of CPT 39.98 µg/g dry wt. Leaf from soil–grown plant had the level of CPT closely to *in vitro* culture including root and hairy root induction with approximately 10 µg/g dry wt. except leaf from *in vitro* culture had CPT content only 6.29 µg/g dry wt. After transplanting to greenhouse, CPT content of plant induced rooting by IBA and composite plant were lower than *in vitro* culture. Both transplanted plants had CPT content with approximately 3 µg/g dry wt.

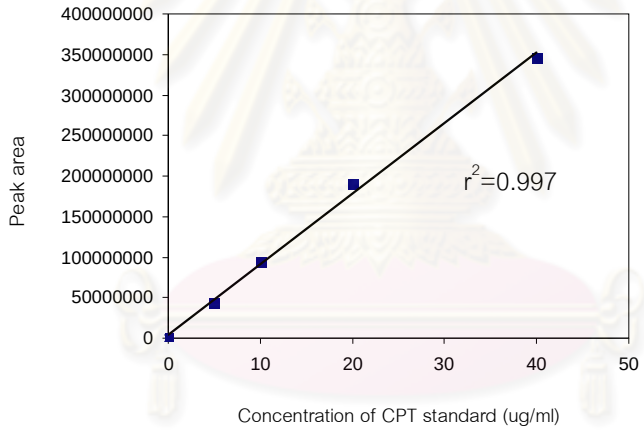


Figure 5.4 Standard curve of CPT, Peak area at 254 nm

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Table 5.1 Comparison of the camptothecin content in different parts of plant types

Source	Plant type	Total CPT contents ± S.D. (μg/g dry wt)
Growing in soil	Leaf	13.13 ± 0.95
	Root	39.98 ± 1.10
In vitro culture	Leaf	6.29 ± 0.83
	Root	13.35 ± 1.32
	Hairy root derived from leaf	10.11 ± 1.49
	Hairy root derived from node	14.18 ± 0.97
	Hairy root derived from shoot	13.76 ± 0.31
	Leaf from shoot induction by *A. rhizogenes*	10.82 ± 0.78
Transplanting to green-house	Leaf of plant induced root by IBA	3.62 ± 0.42
	Root of plant induced root by IBA	3.92 ± 0.52
	Leaf from composite plant	3.71 ± 1.19
	Hairy root	3.63 ± 1.30

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4. Discussion

CPT was detected in 12 samples of *O. alata* plant type from growing in soil, *in vitro* culture, hairy root culture, and transplanting to greenhouse by HPLC. Root of soil–grown plants had the highest amount of CPT which was 39.98 µg/g dry wt. The level of CPT of leaf from soil–grown plant was closely to *in vitro* culture including root and hairy root induction with approximately 10 µg/g dry wt. After transplanting to greenhouse, CPT content of plant induced for rooting by IBA and composite plant were lower than *in vitro* culture. Both transplanted plants had CPT content with approximately 3 µg/g dry wt. The present study indicated that CPT accumulated more in growing in soil, followed by *in vitro* culture, hairy root culture and transplanted plant. This result also suggested that root of soil - grown plant showed highest potential CPT production.

The production of CPT through callus or transformed and untransformed roots by several CPT-producing plants has been documented (Wiedenfeld *et al*., 1997; Saito *et al*., 2001; Fulzele *et al.* 2002). Some of the approaches were inefficient and recovery of CPT was lower than that of the intact plant (Wiedenfeld *et al.* 1997; Ciddi and Shuler 2000). The yield of CPT isolated from cell suspension cultures of C. acuminate was 2.5 µg/g dry wt (Sakato *et al.* 1974). Untransformed root cultures of *N. foetida* incubated on MS medium with 71.36 µM NAA and 8.87 µM BA produced CPT 100 µg/g dry wt (Fulzele et al., 2002). In other *Ophiorrhiza* spp. hairy root cultures exhibited better CPT production than *O. alata*. *O. pumila*, *O. korowai*, and *O. liukieuensis* hairy root cultures produced CPT reaching 1000, 219, and 83 µg/g dry wt, respectively (saito *et al*., 2001; Asano *et al*., 2004).

High-level production of CPT production through hairy roots at equal or higher amounts than the roots in planta has been reported in *O. pumila* (Saito et al. 2001) and *C. acuminata* (Lorence *et al*., 2004). CPT was also obtained from transformed roots of *O. pumila* and plants regenerated from them (Watase *et al*., 2004). Hairy root culture yielded more than 1000 µg/g dry wt and the excretion of relatively large quantities and scaled-up production of it in a bioreactor has been accomplished (Saito et al. 2001; Sudo et al. 2002).

Our result showed that CPT accumulated in growing in soil plant more than transplanted plant. Possibly, development stage of plant may play an important role in

metabolite accumulation. This experiment, plants were harvested for HPLC analysis with age of growing in soil plant more than transplanting to greenhouse plant. Roja (2006) studied on comparison of CPT content in the juvenile plant and mature tree of *N. foetida* intact plant. The juvenile plant had total CPT content lower than in mature tree. An increase in the level of CPT in the mature plant suggests that the accumulation of the CPT may probably be associated with age and maturation of plant. (Roja, 2006)

In the present work, CPT accumulated in *in vitro* culture and hairy root culture more than in transplanting to greenhouse plant. This result suggested that *in vitro* culture optimized to produce contents of CPT within the same time of cultivation in *O. alata*. It has also been found for nicotine alkaloid synthesis in *Nicotiana africana* (Para and Hamill, 1987)

Our result showed that hairy roots of *O. alata* could produce trace CPT content level. Possibly, variation strain of bacteria used may also play an important role in metabolite accumulation. Example for that, Giri et al (2001) reported that transgenic hairy root cultures of *A. annua* with variation of *A. rhizogenes* strain showed significant difference in arteminisinin content. A hairy root line induced by strain 9365 was found to contain highest amount of arteminisinin (0.23 %) (Giri *et al*., 2001).

One possibility that hairy root of *O. alata* produced trace amount of CPT. because substantial CPT content was excreted into the culture medium instead. Saito *et al.* (2001) reported that the amount of CPT excreted into the culture medium increased five fold of *O. pumila* hairy root.

Beside the above – mentioned factors, the type of medium may have possible influence on CPT production of hairy root cultures. *Azadiaracta indica* hairy roots were cultured on three different media: Ohyama and Nitsch (ON), B5 and MS. It was found that hairy roots grown on ON medium accumulated the maximum amount of azadiarachtin content followed by B5 and MS, respectively (Satdive *et al*., 2007).

Accumulation of secondary metabolites could be improved by chemical and physical means in hairy root cultures. Chemical techniques refer to changing nutritious ingredients of culture, adding hormones or precursors. Physical techniques involve changing culture condition, such as light and temperature (Wang *et al,* 2006). As mentioned above, *A. indica* hairy roots grown on ON medium accumulated the

maximum amount of azadiarachtin, followed by B5 and MS. It suggested that ON medium contains higher ionic concentrations of inorganic salts, ascompared to MS and B5 (Satdive *et al*., 2007). Physical techniques have been reported in hairy root cultures of *Panax ginseng* of which biomass accumulation and ginsenoside production was optimal under 20 ˚C/13 ˚C day (12 /8 hr) cycle. Biomass of hairy roots was highest in the cultures grown under dark or red light while ginsenoside accumulation was optimum in the cultures grown under fluorescent light (Yu *et al*., 2005).

Furthermore, biological reactor technologies for hairy root cultures have also been studied; bio-technique, such as transgenic technologies, anti-sense technologies, two-step culture technologies. Addition of precursor compounds or elicitors is also effective in increasing the content of secondary metabolites in hairy roots. For example, sodium acetate (abiotic elicitor) clearly elicited the production and secretion of valued resveratrol and resveratrol derivatives from peanut hairy root cultures (Medina-Bolivar *et al.,* 2003). In addition, hairy root cultures of *Panax ginseng* enhance the biomass accumulation and ginsenoside production in two stages. First, they were cultured under dark, which favors biomass accumulation. In the second stage, the cultures were irradiated with fluorescent light to enhance ginsenoside production (Yu *et al*., 2005).

In this study, we have successfully established a hairy root culture of *O. alata* transformed by *A. rhizogenes* strain TISTS 1450. This hairy root culture produces trace amount of CPT compared with intact roots, however, it is not destructive for the plants in nature. Hairy root biomass accumulation could be as an alternative source for CPT production.

Further work is in progress to investigate methods to enhance CPT production in *O. alata in vitro* culture such as using elicitors and adding biosynthetic precursors, secologanin and tryptamine, and even developing genetic engineered CPT-producing plants.

CHAPTER VI

CONCLUSIONS

Camptothecin (CPT) belongs to a class of anticancer agents which has been rapidly growing in the pharmaceutical market. Despite the increase of its demand, CPT is still supplied exclusively from the intact plants, mainly *C. acuminata* and *N. foetida*. CPT is produced also in some species of the genus *Ophiorrhiza*. Feasible production of CPT by hairy root cultures of *O. pumila*, Japanese species, was also reported. In Thailand, *O. alata* is found in the southern part of the country. We, then, would like to investigate micropropagation and hairy root cultures for CPT production of *O. alata*. In addition, we would like to see the survival rate of root and hairy root-induced plants when transplanting to greenhouse and include theirs CPT production.

In this study, we successfully established shoot multiplication of *O. alata* using seed as initial explants. Leaf explants cultured on 1/2 MS medium containing 9.30 μM Kn and 0.54 μM NAA showed maximum multiple shoot induction. The best shoot multiplication from node explants can be established by culturing on 1/2 MS medium containing 4.65 and 9.30 μM Kn alone. In comparison, node explants showed higher shoot number than leaf explants. Hence, the optimum for micropropagation of *O. alata* in this study was obtained from node explants cultured on 1/2 MS medium containing only 4.65 μM Kn which is minimum concentration.

We have successfully established a hairy root culture of *O. alata* transformed by *A. rhizogenes* strain TISTS 1450. Moreover, we have successfully transformed the *rol* B from Ri plasmid of *A. rhizogenes* into *O. alata*. We found that abundant roots were developed at the wound sites. Hairy root formation was initiated from node better than leaf with a success rate of 100 % and 80 %, respectively.

We successfully established *in vitro* rooting and transplanting to soil. *O. alata* can be induced for *in vitro* rooting from all of treatments. There are 0.49, 0.98, and 1.96 μM of IBA and *A. rhizogenes*. Plantlets were cultured on 1/2 MS medium supplement with 1% glucose and 0.5 % Gelrite for 4 weeks. After transplanting to greenhouse, composite plants with transformed roots showed considerable differences in their

morphology when compared to the corresponding untransformed plants. Survival rate of non-transformed showed no difference when compared to IBA treatment but was higher than *A. rhizogenes* treatment. Therefore, the choice of *in vitro* rooting of *O. alata* for transplanting to greenhouse was obtained from 1/2 MS medium without plants growth regulators.

In HPLC analysis, CPT was detected in 12 samples of *O. alata* plant type from growing in soil, *in vitro* culture, hairy root culture, and transplanting to greenhouse. Untransformed root of soil–grown plants had the highest amount of CPT 39.98 µg/g dry wt. Leaf from soil–grown plant had the level of CPT closely to *in vitro* culture including root and hairy root induction with approximately 10 µg/g dry wt. except leaf from *in vitro* culture had CPT content only 6.29 µg/g dry wt. After transplanting to greenhouse, CPT content of plant induced for rooting by IBA and composite plant were lower than *in vitro* culture with approximately 3 µg/g dry wt. This result suggested that *in vitro* culture optimized to produce contents of CPT within the same time of cultivation. *O. alata* hairy root cultures showed lower potential CPT production than in roots of soil grown-plant. However, it is not destructive for the plants in nature. Hairy root biomass accumulation could be used as an alternative source for CPT production.

Based on the above experimented data, hairy root cultures of *O. alata* would be an alternative experimental system for CPT production and possible resources for developments of anti-cancer drugs originates from plants.

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APPENDICES

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APPENDIX A

Composition of plant culture media

Constituent	Concentration in culture medium (mg/liter)		
	MS	1/2 MS	B5
KNO_3	1900	950	2500
NH_4NO_3	1650	825	-
$NH_4H_2PO_4$	-	-	-
$(NH_4)_2SO_4$	-	-	134
$MgSO_4 \cdot 7H_2O$	370	185	250
$CaCl_2 \cdot 2H_2O$	440	220	150
KH_2PO_4	170	85	-
$NaH_2PO_4 \cdot H_2O$	-	-	150
$MnSO_4 \cdot H_2O$	-	-	10.0
$MnSO_4 \cdot 4H_2O$	22.3	22.3	-
KI	0.83	0.83	0.75
H_3BO_3	6.2	6.2	3.0
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	2.0
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025
$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8
Na_2EDTA	37.3	37.3	37.3
Nicotinic acid	0.5	0.5	1.0
Pyridoxine-HCl	0.5	0.5	1.0
Thiamine-HCl	0.1	0.1	10.0
myo-Inositol	100	100	100
Glycine	2.0	2.0	-

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APPENDIX B

A. rhizogenes media

Nutrient broth formular

Formula per liter

Beef extract	3.0 g
Peptone	5.0 g

Nutrient agar formular

Formula per liter

Beef extract	3.0 g
Peptone	5.0 g
Agar.....	15.0 g

Media preparation

Nutrient broth

1. Weigh out 8.0 grams of nutrient broth powder
2. Add to 1.0 liter of distilled water in a 2.0 liter flask
3. Dissolve the powder completely in the water
4. Dispense into tubes or flasks using a graduated cylinder or a pipette
5. Sterilize at 121°C for 20-25 minutes

Nutrient agar plates:

1. Weigh out 8.0 grams of nutrient broth powder and 15 grams of agar
2. Add to 1.0 liter of distilled water in a 2.0 liter flask
3. Sterilize at 121°C for 20-25 minutes
4. Cool to 50°C
5. Swirl thoroughly to mix agar and nutrients
6. Pour 25-35 ml per plate
7. Yields about 35 plates

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