

**Tissue Culture Establishment of *Ophiorrhiza harrisiana* for Camptothecin Production**

**การเพาะเลี้ยงเนื้อเยื่อ *Ophiorrhiza harrisiana* เพื่อการผลิตแคมป์โทเซซิน**

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**ABSTRACT**

*Ophiorrhiza harrisiana* B. Heyne ex Hook. f (Rubiaceae) is a rare and endemic plant. This plant can produce camptothecin, an anticancer compound. Therefore this research was aimed to establish tissue culture of *O. harrisiana* and to study its potential for the camptothecin production. It was found that 1/2 Murashige and Skoog (MS) medium without plant growth regulator was suitable for seed germination. The 1/2 MS medium with 2 mg/L kinetin and 0.1 mg/L naphthaleneacetic acid was optimum for shoot multiplication. In addition, the tissue culture of *O. harrisiana* can produce camptothecin. It could be a source of camptothecin production in the future.

**บทคัดย่อ**

*Ophiorrhiza harrisiana* B. Heyne ex Hook. f จัดอยู่ในวงศ์ Rubiaceae เป็นพืชหายาก พบเฉพาะถิ่น และสามารถผลิตแคมป์โทเซซินซึ่งเป็นสารต้านมะเร็ง ดังนั้นจึงทำการเพาะเลี้ยงเนื้อเยื่อ *O. harrisiana* ตลอดจนศึกษาศักยภาพในการผลิตแคมป์โทเซซินในเนื้อเยื่อเพาะเลี้ยง จากการศึกษาพบว่าสูตรอาหารชนิด 1/2 Murashige & Skoog (MS) ที่ไม่ผสมสารควบคุมการเจริญเติบโตเป็นสูตรที่เหมาะสมต่อการงอกของเมล็ดมากที่สุด ส่วนสูตรอาหารที่เหมาะสมกับการเพิ่มจำนวนยอดคือ 1/2 MS ที่ผสม kinetin 2 มิลลิกรัมต่อลิตร และ naphthaleneacetic acid 0.1 มิลลิกรัมต่อลิตร นอกจากนี้ยังพบว่าเนื้อเยื่อเพาะเลี้ยงของ *O. harrisiana* สามารถผลิตแคมป์โทเซซินได้ ซึ่งอาจใช้เป็นแหล่งผลิตแคมป์โทเซซินในอนาคต

**Key Words:** Camptothecin, *Ophiorrhiza harrisiana*, Tissue culture

**คำสำคัญ:** แคมป์โทเซซิน พืชสกุลออไฟโอไรซ่า การเพาะเลี้ยงเนื้อเยื่อพืช

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## Introduction

Cancer is a disease caused by the proliferation and growth of abnormal cells. Cancer is the leading cause of worldwide death up to 13% in 2008 according to the World Health Organization (WHO). In Thailand, cancer is the first cause of death in many years. Treatment of cancer involves three main therapies, surgery, radiotherapy and chemotherapy. Chemotherapeutic agents, which are used in current, partly derived from natural substances such as paclitaxel (Miele et al., 2012), vincristine, vinblastine (Mujib et al., 2014) and camptothecin (Zeng et al., 2012).

Camptothecin (CPT) is used as a precursor in the synthesis of anticancer drugs, topotecan and irinotecan, for colorectal cancer and ovarian cancer (Dharmarajan et al., 2005). It was first isolated from a native Chinese tree *Camptotheca acuminate* in the family Nyssaceae (Wall et al., 1966). In Thailand, CPT is found in some species of *Ophiorrhiza* such as *O. alata* (Ya-ut, Chareonsap, and Sukrong 2011), *O. fucosa*, *O. harrisiana*, *O. plumbea* and *O. ridleyana* (Viraporn et al., 2011). Interestingly, *O. harrisiana* can produce CPT in both the leaf and the root while others produce CPT only in the root. Moreover, high amount of CPT was observed in *O. harrisiana*.

*O. harrisiana* is a rare plant species of Thailand (Santisuk et al., 2006). This plant is an herbaceous plant which is found only in certain seasons. It is an endemic plant. The harvest of this plant from their natural habitat for CPT isolation can lead to a diminution of plant resources, so the *in vitro* production of CPT by tissue culture technique is an alternative solution.

## Objective of the study

The aim of this study was to establish the *in vitro* culture of *O. harrisiana*, including its potential for CPT production.

## Methodology

### Plant material

*O. harrisiana* was collected from the southeast of Thailand. It was authenticated by Ivan A. Schanzer, Ph.D. from Herbarium Main Botanical Garden, Russia. The voucher specimens were deposited at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

### Seed germination

Seeds of *O. harrisiana* were surface sterilized by 70% ethanol for 1 min, 1% sodium hypochlorite with 2 drops of tween-20 for 15 min and then washed 3 times with sterile distilled water for 5 min. After surface sterilization process, seeds were placed on various media, which is half strength MS medium (1/2 MS) (Murashige and Skoog 1962), 1/2 MS with 0.3% charcoal (1/2 MSC), MS, and Gamborg's medium (B5) (Gamborg, Miller, and Ojima 1968). All media were supplied with 2% sucrose, 0.8% agar and without plant growth regulator. The pH was adjusted to 5.7. The *ex vitro* seed germination was done on sterile soil. All treatments were incubated at 25°C with 16 h light/8 h dark. Two replicates of 50 seeds were used for all treatments. The germinated rate, duration of germination and survival rate after 4 weeks were recorded.

### Shoot multiplication

The plantlets were subcultured on 1/2 MS medium every 4 weeks after germination. One cm shoot was used as explants in the experiment of multiple shoot induction. The 1/2 MS medium with 0, 1, 2 mg/L kinetin (Kn) combined with 0, 0.05, 0.1 mg/L naphthaleneacetic acid (NAA) was used to study the effect for shoot induction. The 1/2 MS medium without plant growth regulator was used as control. The data was recorded in term of number of shoot per explant and shoot length. Statistical analysis was expressed as the means  $\pm$  SD. The significance of differences was determined by analysis of the variance (one-way ANOVA) and separated the means by LSD ( $P < 0.05$ ) by Duncan's multiple range test.

### Camptothecin analyses

Tissue culture samples were harvested and dissected into the leaf and the root parts for determination of CPT content. A pooled sample of each part was freeze-dried and ground to powder using mortar and pestle. The 10 mg dried specimens were weighed and extracted with 1 mL methanol, and then ultrasonicated for 30 min and kept at 4°C overnight. The crude extracts were centrifuged and the supernatants were filtered through syringe filter 0.45- $\mu$ m filters (Filtrex, Singapore) and analyzed by HPLC (Shimadzu, Japan) (Namdeo, Priya, and Bhosale 2012). HPLC analyses were carried out using a ZORBAX ECLIPSE PLUS C18 column (4.6x250 mm, 5  $\mu$ m) (Agilent Co., USA) at a flow rate of 0.8 mL/min. Mobile phase is acetonitrile:water in the ratio of 3:7. CPT was identified by its UV spectra, mass spectra, and retention time of HPLC monitored at 254 nm compared with that of standard camptothecin (Sigma Chemical Co., USA).

### Results

Seed germination was successful on the three media formula of *in vitro* culture, 1/2 MS, 1/2 MSC and MS (Table 1). Seed germination on 1/2 MS was 85% which was the highest germination rate, followed by 1/2 MSC and MS medium with 80 and 55%, respectively. Time of germination in 1/2 MSC was 21 days which was the fastest among the three medium. The survival rate after 4-week germination was 100% in all media.

**Table 1** Effect of medium for germination

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

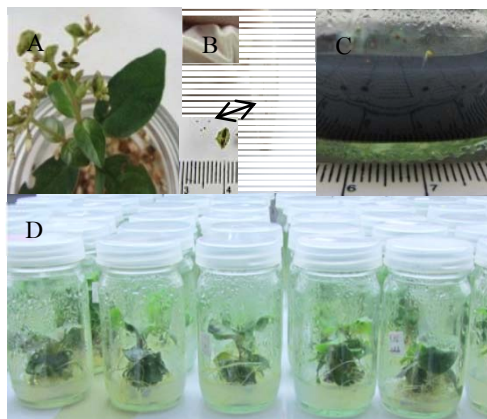
n = 10, duplicate experiment

This experiment found that B5 medium was not optimum for seed germination of *O. harrisiiana*. None of seed in B5 was geminated. In the *ex vitro* condition, 25% of seeds can germinate in 60 days, but none of them can survive after 4 weeks.

After germination, plantlets were subcultured onto the same medium every 4 weeks. Seedlings were selected for use in multiplication of shoots induction after 8 weeks of germination. The germination and multiplication process are shown in Figure 1.

Multiplication process was induced by the combination of plant growth regulators, Kn and NAA (Table 2). The multiplication of shoot was not found

in the 1/2 MS medium without plant growth regulators. It was observed that shoots growing on this medium were longer than others in the average of



**Figure 1** Seed germination and multiplication process of *O. harrisiana*. fruits and seeds (A), surface sterilization of seeds (arrow) (B), 21-day germination on 1/2 MSC medium (C) and multiplication from shoot explants on 1/2 MS media with various Kn and NAA (D).

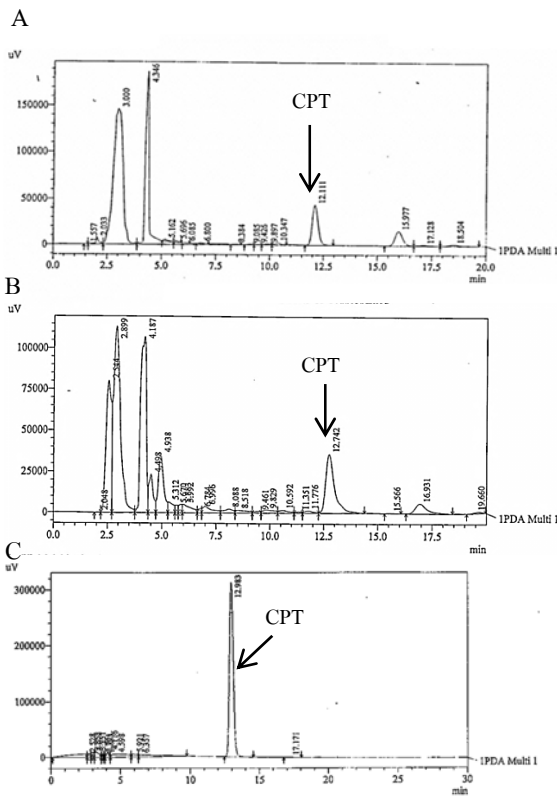
1.67±0.31 cm. The highest number of shoot was observed on 1/2 MS with 2 mg/L Kn and 0.1 mg/L NAA (6.1± 0.88) follow by 1/2 MS with 1 mg/L Kn and 0.1 mg/L NAA (5.7±0.94) and 1/2 MS with 1 mg/L Kn and 0.05 mg/L NAA (5.1±1.19), respectively.

**Table 2** Effect of Kn and NAA on induced multiple shoots

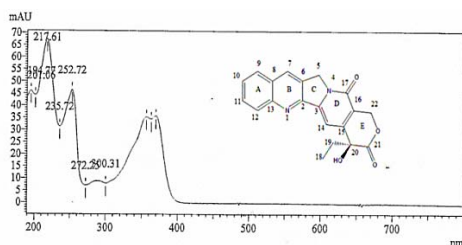
1/2 MS with plant growth regulators (mg/L)		Number of shoot per explant	Shoot length (cm)
Kn	NAA		
0	0	1.00±0.00 <sup>d</sup>	1.67±0.31 <sup>a</sup>
1	0.05	5.10±1.19 <sup>bc</sup>	0.71±0.34 <sup>b</sup>
2	0.05	4.70±0.67 <sup>c</sup>	0.63±0.36 <sup>b</sup>
1	0.1	5.70±0.94 <sup>ab</sup>	0.59±0.34 <sup>b</sup>
2	0.1	6.10±0.88 <sup>a</sup>	0.61±0.37 <sup>b</sup>

Values (mean±SD) followed by different letters are significantly different at P < 0.05, n = 10, triplicate experiment

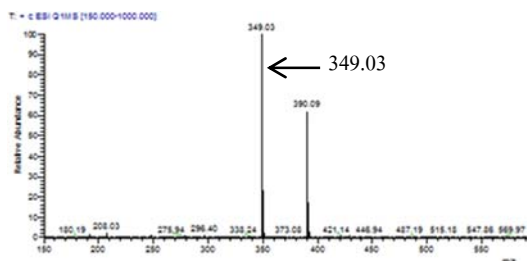
Analysis of CPT was done in the leaf and the root. Plantlets were harvested and divided into two parts and extracted with methanol. From HPLC chromatogram (Figure 2), CPT was found in both the leaf and the root with retention time of approximately 12 min. The identification was confirmed by the retention time of standard camptothecin, UV pattern and mass spectra. The UV spectra pattern of camptothecin (Figure 3) has  $\lambda_{max}$  at 253, 291 and 369 nm, ESI/MS(/MS) (m/z) 349.03 [M+H]<sup>+</sup> as previous study (Yamazaki et al. 2003) (Figure 4).



**Figure 2** HPLC chromatogram of *O. harrisiana* methanolic extract from the leaf (A), the root (B) and standard CPT (C)



**Figure 3** UV spectra pattern and camptothecin structure



**Figure 4** Mass spectra pattern

The amount of CPT was compared between extraction of *O. harrisiana* from natural source and tissue culture (Table 3). The result showed that CPT amount in the leaf was higher amount than the root both from natural source and tissue culture. The leaf from natural source had the highest amount of CPT  $475.29 \pm 40.53 \mu\text{g/g}$  dry wt. The leaf from tissue culture had amount of CPT  $216.81 \pm 64.60 \mu\text{g/g}$  dry wt. While the root from tissue culture had the level of CPT closely to natural source with approximately  $180 \mu\text{g/g}$  dry wt.

**Table 3** Comparison of the CPT content

Source	CPT $\pm$ S.D. ( $\mu\text{g/g}$ dry wt)	
	Leaf	Root
Natural source	$475.29 \pm 40.53^a$	$186.21 \pm 44.11^a$
Tissue culture	$216.81 \pm 64.60^b$	$178.79 \pm 56.01^a$

Values (mean $\pm$ SD) followed by different letters are significantly different at  $P < 0.05$

### Discussion and Conclusions

*Ophiorrhiza* was classified as the endemic and rare plants of Thailand (Santisuk et al., 2006). It is liable to become extinct because of poor seed germination (Sirikantaramas, Yamazaki, and Saito 2013). In natural habitat, *O. harrisiana* is usually found in evergreen forests along the banks or moist soils along water sources. It could not grow in other habitats. The moisture has effect on seed germination (Budelsky and Galatowitsch 1999). From our observation, *O. harrisiana* can germinate and survive in *in vitro* condition (higher moisture) better than in *ex vitro* condition (lower moisture). The low humidity in some seasons may be a cause of low germination, causing near extinction of the plant. This experiment

showed that *in vitro* culture can increase of the germination rate of *O. harrisiana*. The *in vitro* culture on 1/2 MS medium was the most optimum for germination of this plant. It was found that 85% of seeds were germinated and all of them survived. In contrast, only 25% of seeds were germinated in soil and did not survive after 4 weeks. From the experiment with *O. harrisiana*, *in vitro* condition is suitable for seed germination than *ex vitro*.

*In vitro* shoot multiplication of many plants was obtained on specific medium with plant growth regulators (Karuppusamy 2009, Namdeo, Priya, and Bhosale 2012). In this study, the highest number of shoot was approximately 6 shoots per explant, which was found on 1/2 MS with 2 mg/L Kn and 0.1 mg/L NAA. This result was similar to the previous report of *O. alata* in which the combination of Kn and NAA was used successfully in regeneration of node and leaf (Ya-ut, Chareonsap, and Sukrong 2011).

A number of researches were attempted to produce secondary metabolites from *in vitro* plants (Yamazaki et al., 2003, Karuppusamy 2009). This experiment shows the potential of CPT production from organogenesis of *O. harrisiana*. CPT was found both in the leaf and the root of tissue culture. In generally, differentiated cultures produces higher amount of secondary metabolite production than undifferentiated culture (Karuppusamy 2009). These results indicated that tissue culture of *O. harrisiana* had potential for CPT production. Even though the CPT content in tissue culture was lower than natural source, tissue culture could produce the biomass accumulation.

In this study, we have successfully established the tissue culture of *O. harrisiana*. CPT can be produced both from the leaf and the root

culture. In addition, it is not destructive for the plants in nature. Plant cell culture can be conducted on a continuous year-round basis. Tissue culture biomass accumulation could be as an alternative source for CPT production. Further work such as elicitation is in progress to investigate methods to enhanced CPT production in tissue culture of *O. harrisiana*.

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