การเพาะเลี้ยงเนื้อเยื่อเปราะภู (*Caulokaempferia thailandica* Larsen)

An Effective Protocol for Clonal Propagation of "Proh Phu" (*Caulokaempferia thailandica* Larsen)

*สุมนทิพย์ บุนนาค (Sumontip Bunnag)[1]**
ปิยะดา ธีระกุลพิศุทธิ์ (Piyada Theerakulpisut)[1]
ประวีณา มณีรัตนรุ่งโรจน์ (Praweena Maneerattanarungroj)[2]
ดวงกมล ตั้งพงษ์ (Duangkamol Tungpong)[2]
จิรภัทร จันทะพงษ์ (Jirapat Chunthapong)[3]

บทคัดย่อ

ศึกษาวิธีการเพาะเลี้ยงเนื้อเยื่อของเปราะภู (*Caulokaempferia thailandica*) ซึ่งเป็นพืชหายาก โดยขั้นตอนแรกศึกษาวิธีการฟอกฆ่าเชื้อชิ้นส่วนของตาข้าง จากการศึกษาพบว่า การแช่ตาข้างโดยใช้ $HgCl_2$ ความเข้มข้น 0.01% (w/v) เวลา 10 นาที เป็นวิธีที่เหมาะสมที่สุด สำหรับการศึกษาหาสูตรอาหารที่เหมาะสมในการเพิ่มจำนวนยอดและรากของเปราะภู โดยศึกษาความเข้มข้นของธาตุอาหารในสูตร MS (1962) และน้ำตาลซูโครส พบว่าอาหารสูตร MS ที่มีน้ำตาลซูโครส 30 กรัมต่อลิตร โดยไม่เติมน้ำมะพร้าว เป็นสูตรที่เหมาะสมสำหรับการเจริญของเปราะภู และในการศึกษาอิทธิพลของฮอร์โมน BA ต่อการเพิ่มจำนวนยอดจากตาข้าง พบว่าอาหารสูตร MS ที่เติมฮอร์โมน BA 5 มิลลิกรัมต่อลิตร สามารถชักนำให้เกิดยอดจำนวนสูงสุดคือ 15 ยอดต่อต้น และมีอัตราการรอดชีวิต 92 % เมื่อเพาะเลี้ยงเป็นเวลา 4 สัปดาห์ ต้นอ่อนของเปราะภูสามารถย้ายออกปลูกในสภาพแวดล้อมภายนอกและเจริญได้ดี เมื่อเวลา 2-4 เดือน ลักษณะทางสัณฐานวิทยาของต้นอ่อนไม่เปลี่ยนแปลง งานวิจัยนี้เป็นการรายงานครั้งแรกที่ศึกษาเกี่ยวกับการเพาะเลี้ยงเนื้อเยื่อเปราะภู ซึ่งจะเป็นประโยชน์ต่อการขยายพันธุ์ของเปราะภูเพื่อการอนุรักษ์ต่อไป

Abstract

We have established an effective protocol for *in vitro* propagation of young buds of "Proh Phu" (*Caulokaempferia thailandica*), a rare plant species of Thailand. First, we investigated various conditions for surface sterilization and found that 10-min treatment with 0.01% (w/v) mercuric chloride was the most suitable condition for surface sterilization of this plant species. We then studied the effects of MS salt basal strengths and sucrose concentrations on survival rates and number of shoots and roots of explants. The results indicated that full-strength MS (1962) medium with 30 g/l sucrose and without coconut water was the most effective medium for culture initiation. Lastly, we investigated the effect of BA on direct organogenesis of *C. thailandica*. Fifteen shoots per explant and 92% survival rate were recorded after four-week culture on MS medium supplemented with 5 mg/l BA. Plantlets were successfully transferred to soil and acclimatized in a growth chamber. Morphologically normal plants were obtained from 2-4 month potting. To our knowledge, this work represents the first successful *in vitro* clonal propagation of *C. thailandica*, with promising implications in large-scale propagation and *ex situ* conservation of this rare plant.

คำสำคัญ: เปราะภู การขยายพันธุ์ในหลอดแก้ว ตายอด
Keywords: Caulokaempferia thailandica, in vitro propagation, young bud

[1]*Associate Professor, Applied Taxonomic Research Center, Department of Biology, Faculty of Science, Khon Kaen University*
[2]*Research Assistant, Applied Taxonomic Research Center, Khon Kaen University*
[3]*Lecturer, Department of Biology, Faculty of Science, Khon Kaen University*
* *corresponding author, e-mail: sumbun@kku.ac.th*

Introduction

Proh Pru (*Caulokaempferia thailandica* Larsen) is a rare plant species of Thailand. It belongs to the family Zingiberaceae which consists of a dozen tiny plants found in humid environments, mossy rocks along streams, wet walls of rocks, and swampy areas (Larsen, 2002). Members of this genus can be found distributed from the Himalayas to continental Southeast Asia, with a distinct center of diversity in Thailand (Suksathan and Triboun, 2004). This plant can be vegetatively propagated using young buds from the sprouting rhizome. However, these plant materials have a very low multiplication rate and show very poor natural regeneration percentage. In addition, many species of the family Zingiberaceae are known to be susceptible to contamination problems. Hence, *in vitro* culture techniques are required to provide an alternative means fo propagation for this plant. In this report we present, for the first time, a successful *in vitro* clonal propagation of *C. thailandica*, which would be ideal for its large-scale propagation and *ex situ* conservation.

Material and Methods

Plant materials

The plant *C. thailandica* was collected from the northern and northeastern part of Thailand and grown in the greenhouse at the Department of Biology, Faculty of Science, Khon Kaen University, Thailand. Young vegetative buds from sprouting rhizomes of the plant were harvested during May - August and used as a source of explants. These buds were thoroughly washed with tap water to remove mud. They were cleansed further by soaking in soft detergent solution for 5 min and washed with running tap water. The explants were then immersed in 70% (v/v) ethanol for 5 min and surface sterilized with various concentrations of aqueous solution of mercuric chloride or calcium hypochlorite (see Table 1). Explants were then rinsed 3 times with sterile distilled water under aseptic conditions. The sterilized explants were grown in the MS (1962) initiation medium and subcultured every 4 weeks.

Culture conditions and statistical analysis

The MS medium supplemented with 3% (w/v) sucrose, 0.85% (w/v) agar and 0.01% (w/v) myo-inositol was used in all experiments. The pH of the medium was adjusted to 5.5-5.8, prior to autoclaving for 15 min at 1.06 $Kg.cm^{-2}$ (121°C). Cultures were incubated at 25°C under 16 h daily illumination with fluorescent light (approx. 40 $mmol^{-2}s^{-1}$). Each experiment was repeated three times and there were ten replications per treatment. The experiments were laid out according to a completely randomized design (CRD). The datas were statistically analyzed using one-way analysis of variance (ANOVA) and the Least Significant Difference (LSD) test. The mean values were compared using *p* value of 0.01 as the cutpoint.

Effects of basal medium strengths and sucrose concentrations

Three-time subcultured plantlets (size range between 1-1.5 cm) were cultured in a full, half, or a quarter strength of the MS medium with various concentrations of sucrose and with or without 15% (v/v) coconut water. The number of shoots and roots per explant and percentage survival rate were recorded after four weeks of culture.

Effects of BA on shoot proliferations.

Plantlets (size range between 1-1.5 cm) were used as explants which were then cultured on the MS medium supplemented with various concentrations of BA. The number of shoots and roots per explant were recorded after culture for four weeks.

Results and Discussion

Surface sterilization conditions

The optimum condition for sterilization of *C. thailandica* young vegetative buds was first investigated. The best sterilization condition was achieved by immersing the explant in 0.01% (w/v) aqueous solution of mercuric chloride and then rinsing 3 times in sterile distilled water (Table 1). This method is the most effective protocol for reducing the percentage of contamination (12%) while retaining highest survival percentage (88±2.6%). The contamination issue is one of the most serious problems in plant culture initiation. Although our results suggested a good surface sterilization condition, we have not been able to completely eliminate contamination without killing the plant. A possible way to overcome this problem might be by the use of antibiotics. Many groups of researchers were successful in using antibiotics to reduce contamination. For example, Salvi et al. (2000) reported that 10 mg/l of gentamycin sulphate reduced the contamination of immature inflorescence turmeric culture. In addition, Lebowitz (1995) suggested that the failure of plant surface sterilization was decreased by using optimum concentration of rifampicin alone or in combination with other antibiotics. Clearly, further studies are required in order to find the most suitable and effective antibiotics regimen for surface sterilization of *C. thailandica*.

Effects of basal medium strength and sucrose concentrations

Table 2 shows the effects of basal medium strength on shoot and root formation. Analysis of variance showed a highly significant difference among strength of basal medium and sucrose concentrations on plant growth. The full strength of MS medium with 30 g/l sucrose without coconut water was the most effective medium for root and shoot formation (Table 3). On the other hand, young buds cultured on the medium with lower strength of basal media and sucrose concentrations showed lower percentage survival rate and average number of shoots and roots per explant. The effects of basal medium strength on plantlets production observed in this study were similar to those reported by Shirin et al. (2000), who found that 0.75x strength MS medium was the best medium for *Kaempferia galanga* plantlets production. In addition, our results are in agreement with Mamiya and Sakamoto (2000). Although their report showed the effect of sucrose concentrations and strength of basal medium on conversion of somatic embryos to plantlets in *Asparagus officinalis* L., they suggested that sugar concentrations and strengths of basal medium could control shoots and roots growth of somatic embryos.

Effects of BA on direct organogenesis

Since plantlet production was achieved at the initiation of the culture, further investigations were aimed at optimizing the plantlet production. Five treatments with different concentrations of BA were tested. Medium supplemented with 5 mg/l BA produced significantly higher number of shoots and roots per explant (Table 3 and Figure 1). This result is similar to a study on *Curcuma amada* (Prakash et al., 2004), which reported that using BAP alone

gave the maximum number of shoots per explant. However, the most effective plant growth regulators for plant regeneration were the optimum ratio of auxin and cytokinin. Shirin et al. (2000) reported that the optimum media for *Kaempferia galanga* plantlet production was MS supplemented with 12 mM BA and 3 mM NAA. Moreover, Salvi et al. (2000) found that shoots of turmeric (*Curcuma longa*) were directly regenerated from immature inflorescence on MS (1962) containinga suitable ratio of BA (5 or 10 mg/l) in combination with 2,4-D (0.2 mg/l) or NAA (0.1 mg/l) and TDZ (1 or 2 mg/l).

In conclusion, this is the first report describing an effective protocol for clonal propagation of *C. thailandica*. This successful protocol has a strong implication in *ex situ* conservation and large-scale multiplication of this rare plant species. The method may also be beneficial for conserving the genetic resource of *in vitro* germplasm collections as well as cryopreserved materials. Additional studies will provide more complete and rapid clonal propagation systems for this plant species.

Acknowledgements

We wish to acknowledge the Applied Taxonomic Research Center (ATRC), Khon Kaen University, Thailand (grant ATRC_R4808) for funding this study. We are also thankful to Mr. Pramote Triboun for providing plant materials and helping in taxonomic identification of the plant.

References

Larsen, K. 2002. Three new species of Caulokaempferia (Zingiberaceae) from Thailand with a discussion of the generic diversity. Nordic Journal of Botany 22(4): 409-417.

Lebowitz, R.J. 1995. Plant biotechnology laboratory manual. Wm. C. Brown Publisher. Iowa. 115 pp.

Mamiya, K. and Sakamoto, Y. 2000. Effects of sugar concentration and strength of basal medium on conversion of somatic embryos in Asparagus officinalis L. Scientia Horticulturae 84: 15-26.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15: 473-479.

Prakash, S., Elangomathavan, R., Seshadri, S., Kathiravan, K. and Ignacimuthu, S. 2004. Efficient regeneration of Curcuma amada Roxb. Plantlets from rhizome and leaf sheath explants. Plant Cell, Tissue and Organ Culture 78: 159-165.

Salvi, D.N., George, L. and Eapen, S. 2000. Direct regeneration of shoots from immature inflorescence cultures of turmeric. Plant Cell, Tissue and Organ Culture 62: 235-238.

Shirin, F., Kumar, S. and Mishra, Y. 2000. In vitro plantlet production system for Caulokaempferia galanga, a rare Indian medicinal herb. Plant Cell, Tissue and Organ Culture 63: 193-197.

Suksathan, P. and Triboun, P. 2004. A new species of Caulokaempferia (Zingiberaceae) from Thailand. Edinburgh Journal of Botany 60(3): 513-516.

Table 1 Effects of various surface sterilization conditions on % contamination, % survival of explants, and % response of explants.

Treatments	% contamination	% Survival of explants	% Response of explants
1% $Ca(OCl)_2$, 15 min	98	0.00 ± 0.0^{a}	0.3 ± 0.6^{a}
1% $Ca(OCl)_2$, 30 min	64	6.67 ± 6.1^{a}	0.3 ± 0.6^{a}
1% $Ca(OCl)_2$, 10 min and 2% $Ca(OCl)_2$ 5 min	74	10.66 ± 8.9^{a}	4.3 ± 3.0^{ab}
2% $Ca(OCl)_2$, 10 min and 1% $Ca(OCl)_2$ 5 min	53	12.0 ± 2.6^{ab}	7.3 ± 2.0^{b}
0.01% $HgCl_2$, 10 min	12	88.0 ± 2.6^{c}	87.3 ± 4.0^{c}
0.02% $HgCl_2$, 10 min	11	61.0 ± 5.5^{d}	67.3 ± 2.5^{d}

Mean values bearing different letters in the same column are significantly different at p = 0.01 probability according to protected LSD test.

Table 2 Effect of strength of basal medium and concentration of sucrose on shoot and root formation.

Treatments	% Response	Average no. of shoots / explant	Average no. of roots / explant	% survival
MS + 30 g/l sucrose	100	6.3 ± 0.5^{a}	9.7 ± 1.6^{a}	100
MS + 30 g/l sucrose + CW	95	3.3 ± 5.7^{b}	5.6 ± 1.2^{b}	66
½ MS + 15 g/l sucrose	91	3.0 ± 1.0^{bc}	5.3 ± 1.5^{bc}	58
½ MS + 15 g/l sucrose + CW	43	2.0 ± 1.0^{bc}	3.3 ± 0.6^{bc}	50
¼ MS + 7.5 g/l sucrose	23	1.3 ± 0.6^{c}	2.6 ± 0.5^{c}	50
¼ MS + 7.5 g/l sucrose + CW	16	2.0 ± 0.0^{bc}	3.3 ± 0.5^{bc}	40

Mean values bearing different letters in the same column are significantly different at p = 0.01 probability according to protected LSD test.

Table 3 Effect of BA on shoot and root formation.

BA concentrations (mg/l)	% response	Average no. of shoots / explant	Average no. of roots / explant	% survival
0.0	100	5.7 ± 0.6^{a}	9.0 ± 1.0^{a}	100
2.5	100	7.0 ± 1.0^{a}	6.7 ± 1.5^{a}	100
5.0	100	15.0 ± 1.0^{b}	18.7 ± 2.5^{b}	100
7.5	100	10.0 ± 1.0^{c}	14.6 ± 2.5^{ab}	100
10.0	100	13.0 ± 1.0^{b}	18.3 ± 3.5^{b}	100

Mean values bearing different letters in the same column are significantly different at p = 0.01 probability according to protected LSD test.

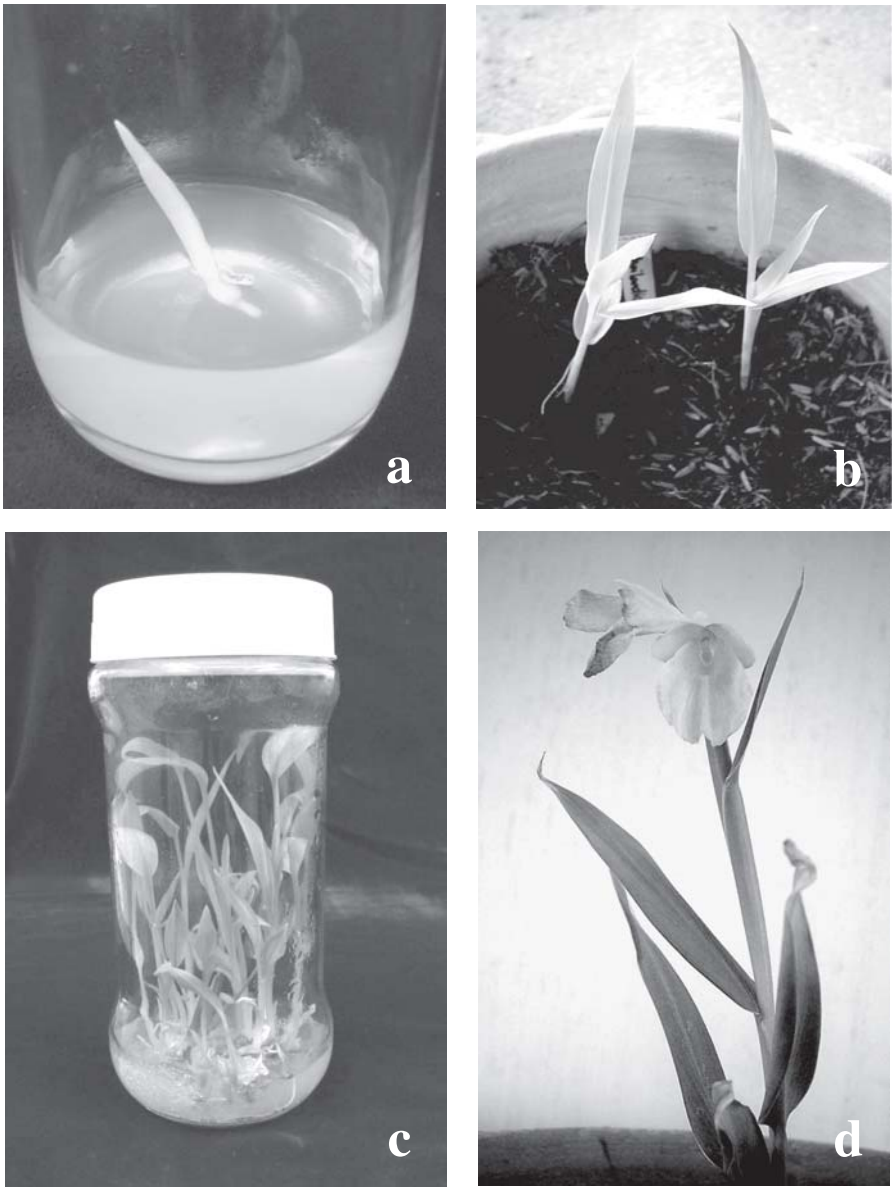

Figure 1 Clonal propagation of C. thailandica (a) initial explant: young vegetative shoot (b) multiple shoot formation in MS (1962) medium containing 5 mg/l BA (c) plantlets transferred to sterile soil (d) flowering stage