Optimization of Explant Surface Sterilization Conditions and Multiple Shoot Induction in Threatened Plant *Phanera sirindhorniae*

Sukanya Sirimat and Arpakorn Sakulsathaporn
School of Natural Resource and Environmental Management, Faculty of Applied Science and Engineering, Khon Kaen University, Nong Khai Campus, Nong Khai 43000
Email: {sukasi, arpasa}@kku.ac.th

***Abstract*—*Phanera sirindhorniae* is a rare species of ornamental and medicinal plants distributed in the upper Northeastern Thailand. It contains many bioactive compounds including tyrosinase inhibitor, which can be used for blocking melanin synthesis of the melasma process. This species has been listed as a threatened species by the Forest Herbarium Department of National Parks, Wildlife and Plant Conservation of Thailand. *In vitro* propagation techniques for *P. sirindhorniae* are currently required to help the utilization and conservation of this species. In this study, sterilization steps of the explants were evaluated. The most effective procedure for sterilization with high survival rates is by using the shoot tips treated with 10% NaOCl for 10 min and then 10% NaOCl for 15 min and the nodal explants treated with 10% NaOCl for 10 min and then 5% NaOCl for 15 min. For propagation, shoot multiplication was studied by using shoot tips, nodal explants and cotyledonary nodes incubated on MS media supplemented with BAP at 1, 2, 3 and 4 mg/L for four weeks. The highest shoots were obtained on the MS medium containing 2 mg/L BAP with 4.00, 3.20 and 3.75 shoots per explants from shoot tips, nodal explants and cotyledonary nodes, respectively.**

***Index Terms*—*Phanera sirindhorniae*, threatened species, NaOCl, shoot multiplication, cotyledonary nodes**

I. Introduction

Phanera sirindhorniae (K. Larsen & S. S. Larse) Mackinder & R. Clark, an endemic plant of *Northeastern* Thailand specifically in Beung-Kan, Nakorn-Panom, Mukdahan and Sakon-Nakhon Provinces. It has been used for ornamental and medicinal purposes for a long time. *P. sirindhorniae* is a synonym of *Bauhinia sirindhorniae* commonly known as Sirindhornwallee, which is a woody climber found in on edges of evergreen forests from altitudes of 150 to 200 meters. The tree can grow up to 10-20 meters tall using means of tendrils. The young leave and nodal explants are covered with a layer of reddish-brown hairs giving leaves as a golden and age to green. The reddish-brown flowers are held on raceame [1]-[3].

P. sirindhorniae are utilized in traditional medicine. Its dried roots help to remove lymphatic waste, getting strong and having more energy [4]. The infusion of nodal explants composes of tyrosinase inhibitor can be used for blocking melanin synthesis of the melasma process. Moreover, many laboratory reports have shown in various parts of *P. sirindhorniae* presented bioactive compounds against with microbial such as (2*S*)-eriodictyol, isoliquiritigenin and isoliquiritigenin 4-methyl ether [5]. However, raising extension of rubber plantations and overexploitation of root plants led to habitat loss and population decrease resulted in *P. sirindhorniae* be listed in the threatened plant by the Forest Herbarium Department of National Parks, Wildlife and Plant Conservation of Thailand.

To protect and conserve *P. sirindhorniae*, it is necessary to ensure plants can be regenerated and survived. Under these circumstances, *in vitro* culture is a useful method to provide mass propagation and plant genetic resource conservation to sustainable prevent this threatened species. Successfully, *in vitro* plant regeneration and multiple shoots induction effected of Cytokinin have been reported in *Phanera variegate*, *Bauhinia cheilantha* and *Bauhinia tomentos* [6]-[8]. The objective of this study is to optimize the sterilization procedure and to investigate the effect of different concentration of BAP at 1, 2, 3 and 4 mg/L on shoot tips, nodal explants and cotyledonary nodes of *in vitro* culture of *P. sirindhorniae.*

II. Methadology

A. Plant Material and Explant Sterilization

The mature shoots and seeds of *P. sirindhorniae* were collected from Seka school at Bueng-Kan province, Thailand during February-April 2017. Shoot tips and nodal explants derived from mature shoots were cut into small pieces ~2.00-2.50 cm long (Fig. 1), before being soaked in a 1% detergent solution for 15 min, washed with running tap water for 30 min and submerged in 70% ethanol for 1 min. Under aseptic conditions, the shoot tips and nodal explants were soaked in a 10% (v/v) sodium hypochlorite (NaOCl) solution (Haiter Bleach, Thailand) containing 3-5 drops of Tween-20 for 10 min and then re-soaked with different concentrations of NaOCl (5, 10 and 15%) for 15 min before rinsing three times with sterilized

Manuscript received June 22, 2019; revised November 14, 2019.

doi: 10.18178/joaat.6.4.263-266

distilled water. Surface sterilized shoot tips and nodal explants were further trimmed to about 1 cm in length. The seeds were sterilized by dipping in 95% ethanol and flame (Table I).

Figure 1. *P. sirindhorniae*; flowers (a), seeds (b), shoot (c) and tips nodal explants (d).

TABLE I. THE SUMMARY OF DISINFECTION TREATMENTS FOR EXPLANT SOURCE MATERIALS

explants	Disinfection Treatment	
shoot tip	10% (v/v) NaOCl	5, 10, 15 % (v/v) NaOCl
nodal		
seed	heating in a flame	

To select disinfected tissues, the explants were placed onto Murashige and Skoog, 1962 (MS) medium [9] containing 30 g/L of sucrose, 7 g/L of agar. The pH was adjusted to 5.7 ± 2 with either 1 N NaOH or 1 N HCl. The medium was melted in microwave, aliquoted into culture glass bottles and autoclaved at 120 ℃ and 1.1 kPa for 15 min. Cultures were incubated under cool fluorescent lamps for 16 h light photoperiod at 25°C.

B. Multiplication Shoot Induction

Uncontaminated shoot tips, nodal explants and cotyledonary nodes were incubated on MS medium supplemented with different concentrations of BAP (0.0, 1.0, 2.0, 3.0 and 4.0 mg/L) and regularly sub-cultured at a 2-week interval in the same fresh medium. The cultures were maintained at 25 ±2 ℃, 16 h photoperiod under white fluorescent lights and. The number of shoot buds per explant were counted after four weeks.

C. Statistical Analysis

Data analysis was performed using SPSS 17 (SPSS Inc., Chicago, IL, USA). ANOVA analysis and subsequently post hoc tests were performed using DMRT at 0.05 level of significance.

III. RESULTS AND DISCUSSION

A. Sterilization of Shoot Tips and Nodal Explants

Surface sterilization of explants is the one of the most important steps for *in vitro* propagations of plant tissues. The NaOCl is the most popular sterilant for eliminating microorganisms from explants. To optimize the sterilization protocol, explants from *P. sirindhorniae* were tested for re-soaking with various concentrations of NaOCl. The effects of NaOCl on sterilization of shoot tips and nodal explants from mature shoots are shown in Table II.

TABLE II. EFFECT OF DIFFERENT CONCENTRATIONS OF NAOCL ON THE PERCENTAGE OF DISINFECTION AND SURVIVAL OF EXPLANTS AFTER 14 DAYS OF CULTURE

10% NaOCl 10 min	Re-soaked NaOCl concentration (%) 15 min	Mean percentage of explants			
		disinfection		survival	
		shoot tips	nodal explants	shoot tips	nodal explants
	0	33.33^{a}	33.33^{a}	0^{a}	33.33^{a}
	5	66.67^{b}	66.67^{ab}	44.44^{b}	51.85^{a}
	10	88.89^{b}	55.56^{ab}	55.56^{b}	44.44^{a}
	15	77.78^{b}	77.78^{b}	37.04^{b}	44.44^{a}

Note: Each treatment consisted of three replications and in each replication three explants were used. Means followed by the same letter in the same column are not significantly different based on DMRT (p=0.05).

After sterilization and cultivation for 14 days, the highest mean percentage of shoot tips disinfection and survival (88.89%, 55.56%) were obtained when using the 10% NaOCl for 10 min and followed by 10% NaOCl for 15 min. The nodal explants were successfully surface sterilization (77.78%) with the 10% NaOCl for 10 min and followed by 15% NaOCl for 15 min. However, an optimum value of nodal survival (51.85%) could be obtained with 10% NaOCl for 10 min and followed with 5% NaOCl for 5 min whereas the lowest disinfection percentage (33.33%) was observed for only soaked one step in NaOCl 10% in both explants (Fig. 2).

NaOCl has widely been accepted to eliminate microorganism since effective rapidly killed vegetative spores, bacteria, fungi, protozoa and viruses by oxidizing sulfhydryl groups of essential enzymes, proteins and damaging of DNA and membrane [10]-[12].

This result indicates that the tendency of increasing concentration of NaOCl in the second time helps to increase the disinfection explants, but will result in lower numbers of survived explants. Therefore, different explants must be surface sterilized, and archive survived by the correct concentration and immersion time in NaOCl. Surface sterilization using repeated NaOCl treatments was previously suggested to be effective for removing bacterial and fungal contamination of explants from field conditions for *in vitro* culture. For example, flower stalks of *Phalaenopsis* hybrids were soaked in 70% ethanol for a few seconds followed by NaOCl solution containing 1% active chlorine with 0.05 % Tween 80 for 10 minutes then re-soaked in sodium NaOCl containing 0.5% active chlorine with 0.05 % Tween 80 [13]. The nodal explants of *Solanecio biafrae* were surface sterilized by immersion in 70% ethanol for 5 min, immersed in 10% NaOCl for 20 min, and then repeated with 5% NaOCl for 5 min [14]. Shoots with buds of *Strobilanthes tonkinensis* washed with tap water and subsequently shaken in 70% ethanol for 1 min and treated with 1.2% NaOCl for 10 min and then 0.6%

NaOCl for 15 min produced 70% good-growing, healthy shoots and sterilized explants [15].

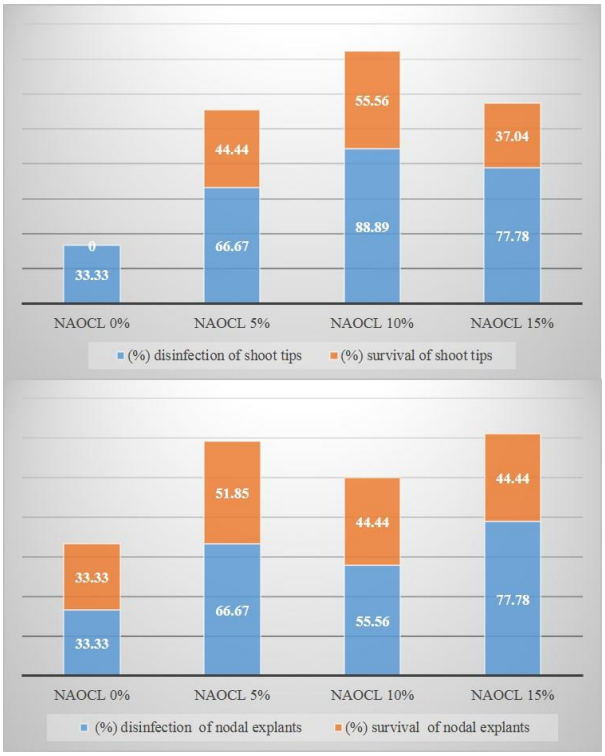

Figure 2. Percentage of disinfection and survival of shoot tips (a) and nodal explants (b) of *P. sirindhorniae* after soaked in 10% NaOCl and re-soaked in 0, 5, 10 and 15 % (v/v) NaOCl.

B. Effects of BAP on Shoot Multiplication

Cotyledonary nodes, shoot tips and nodal explants, which were pre-cultured on the MS medium for 21 days, were transferred onto MS medium supplemented with BAP at 0, 1, 2, 3 and 4 mg/L. After four weeks, from all BAP concentrations, shoots were observed to be directly regenerated from the apical buds of shoot tips, lateral buds of nodal explants and axillary buds of cotyledonary nodes. The shoot tips, nodal explants and cotyledonary nodes produced the best average of 4.00, 3.20 and 3.75 shoots on MS medium containing with 2.0 mg/L of BAP (Table III). Callus induction was observed from cotyledonary nodes on 4.0 mg/L BAP (Fig. 3). Similar results have already been reported in *Bauhinia variegata*, in which multiple shoots were induced on MS medium with 2.5 mg/L BA and callus formed when the BA concentration exceeded 5 mg/L BA [16]. BAP (Benzyl adenine) and BA (6-Benzylaminopurine) are members of Cytokinin and are essential plant growth regulators for plant cell division, organogenesis and lateral bud development in *in vitro* plant culture [17], [18]. Similarly, *Bauhinia holophylla*, calli obtained from leaf segments were induced on MS media supplemented with 4.44-17.75 µM BAP [19]. For *Clitoria ternatea*, the multiple shoot derived from cotyledonary node explants were induced on MS medium containing 1.0 mg/L of BA [20]. In the case of *Baptisia australis*, the most effectively medium for multiple shoot induction from the nodal explants was MS supplemented with 4.4 µM BA [21].

TABLE III. EFFECTS OF BAP IN MS MEDIUM ON SHOOT MULTIPLICATION AT FOUR WEEKS OF CULTURE

BAP (mg/L)	average number of shoots		
	shoot tip	stem	cotyledonary node
0	1.00[a]	0.60[a]	0.75[a]
1	2.40[ab]	2.00[ab]	1.50[a]
2	4.00[b]	3.20[b]	3.75[b]
3	1.00[a]	1.20[a]	1.75[a]
4	0.80[a]	2.20[ab]	1.25[a]

Note: Each treatment consisted of four replications and, in each replication, one explants were used. Means followed by the same letter in the same column are not significantly different based on DMRT (p=0.05)

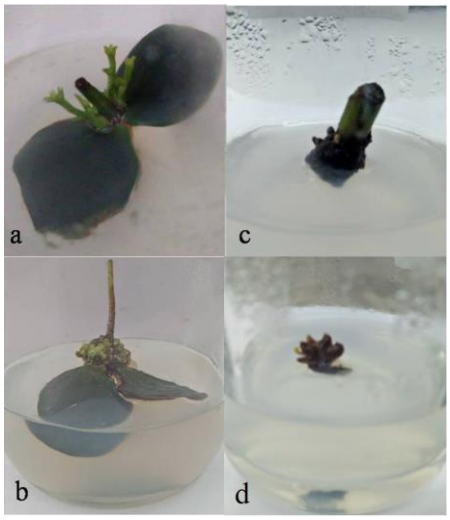

Figure 3. Effects of BAP to induce direct shoot regeneration from explants at four weeks; cotyledonary node was induced multiple shoots on MS medium supplemented with 2 mg/L (a) and callus formation on MS medium supplemented with 4 mg/L (b); turning brown of emerged shoots from nodal explants (c, d).

However, shoots emerged from shoot tips and nodal explants ceased growth, turned brown and finally died after four weeks (Fig. 3). It has been reported that the browning effect in nodal explants of *Bambusa balcooa* Roxb and *Psidium guajava* L. on the medium during *in vitro* culture [22], [23]. *In vitro* propagation of mature plants often results in browning of explants due to the accumulation of phenolic compounds. For example, explants from mature tissues of *Malosorbus florentina* displayed more browning and had higher content of phenolics than explants excised from juvenile tissues [24]. To prevent the browning effect, antioxidant and anti-browning additives including ascorbic acid, citric acid, activated charcoal and $AgNO_3$ are usually added to help controlling polyphenol secretion. According to achieve *in vitro* shoot multiplication of *Pterocarpus santalinus* from mature nodal, the culture medium must be supplemented with 250 mg/L L-ascorbic acid and 50 mg/L citric acid help to minimize medium browning and improve explant survival during shoot sprouting [25]. Furthermore, medium culture was further added with 1 g/L activated charcoal had effectively relieved

the browning problem of *Acacia auriculiformis* shoots [26].

IV. Conclusion

This study presents an effective protocol for surface sterilization of shoot tips and nodal explants of *P. sirindhorniae.* The cotyledonary nodes is a potential explant of choice for inducing multiple shoots and callus, while shoot tips and nodal explants should be avoided as they usually turned brown and finally died after a period of culture. These results can be used for developing a rapid and large-scale propagation for other *Phanera* species.

Acknowledgment

The authors would like to thank the Faculty of Applied Science and Engineering for providing the research facilities and we are grateful to Assistant Professor Supachai Vuttipongchaikij for language editing and proofreading. This work was supported by funds from Division of Research Administration, Khon Kaen University for research funding ID: 2561-KKU-NKC-01-004.

References

[1] K. Larsen and S. S. Larsen, "Bauhinia sirindhorniae sp. nov.(LeguminosaCaesalpinioideae) a remarkable new species from Thailand," *Nordic Journal of Botany*, vol. 17, no. 2, pp. 113-118, June 1997.

[2] C. M. Boo, N. P. Board, K. Omar-Hor, and C. L. Ou-Yang, *1001 Garden Plants in Singapore*, 3rd ed., Singapur: National Park Board, 2008.

[3] U. Quattrocchi, *CRC World Dictionary of Medicinal and Poisonous Plants: Common Names, Scientific Names, Eponyms, Synonyms, and Etymology*, CRC Press, 2016.

[4] B. Poonthananiwatkul, R. H. Lim, R. L. Howard, P. Pibanpaknitee, and E. M. Williamson, "Traditional medicine use by cancer patients in Thailand," *Journal of Ethnopharmacology*, vol. 168, pp. 100-107, Apr. 2015.

[5] S. Athikomkulchai, N. Sriubolmas, and N. Ruangrungsi, "Antibacterial activity of flavonoids from Bauhinia sirindhorniae," *Thai J Health Res*., vol. 19, no. 1, pp. 13-19, 2005.

[6] A. Akoumianaki-Ioannidou, M. Papafotiou, and I. Antoniou, "Studies on in vitro propagation of Bauhinia variegate," in *Proc. International Symposium on Woody Ornamentals of the Temperate Zone*, 2008, pp. 263-266.

[7] R. Naz, M. Anis, and I. Aref, "High frequency shoot regeneration through cotyledonary node explants of Bauhinia tomentosa L., a woody leguminous tree," *The Journal of Horticultural Science and Biotechnology*, vol. 86, pp. 37-42, Nov. 2015.

[8] I. Guti ́rrez, C. Nepomuceno, C. D. S. Ledo, and J. Santana, "Micropropagation and acclimatization of Bauhinia cheilantha (An important medicinal plant)," *African Journal of Biotechnology*, vol. 10, no. 8, pp. 1353-1358, Feb. 2011.

[9] T. Murashige and F. Skoog, "A revised medium for rapid growth and bio assays with tobacco tissue cultures," *Physiologia plantarum*, vol. 15, pp. 473-497, Jul. 1962.

[10] G. McDonnell and A. D. Russell, "Antiseptics and disinfectants: Activity, action, and resistance," *Clinical Microbiology Reviews*, vol. 12. pp. 147-149, 1999.

[11] S. S. Block, *Disinfection, Sterilization, and Preservation*, Lippincott Williams & Wilkins, 2001.

[12] N. Luddin and H. M. A. Ahmed, "The antibacterial activity of sodium hypochlorite and chlorhexidine against enterococcus faecalis: A review on agar diffusion and direct contact methods," *Journal of Conservative Dentistry*, vol. 16, pp. 9-16, Jan. 2013.

[13] Y. Homma and T. Asahira, "New means of Phalaenopsis propagation with internodal sections of flower stalk," *Journal of the Japanese Society for Horticultural Science*, vol. 54, pp. 379-387. 1985.

[14] O. Bello, O. Fajimi, E. Esan, and O. Obembe, "Callus and etiolation induction data from explants of Solanecio biafrae (Olive & Hierne) C. Jeffrey cultured in the dark," *Data in Brief*, vol. 20, pp. 113-117, 2018.

[15] N. Srikun, "In vitro propagation of the aromatic herb Strobilanthes tonkinensis Lindau," *Agriculture and Natural Resources*, vol. 51, pp. 15-19, 2017.

[16] T. Thonnalak, "Tissue culture of orchid tree (Bauhinia variegata L.)," *Fac Agric Techol*, pp. 12, Dec. 2012.

[17] K. Palme, *Signals and Signal Transduction Pathways in Plants*, Springer Science & Business Media, 2012.

[18] N. M. Moreno-Pachon, *et al.*, "Role of Tulipa gesneriana TEOSINTE BRANCHED1 (TgTB1) in the control of axillary bud outgrowth in bulbs," *Plant Reproduction*, vol. 31, pp. 145-157, 2018.

[19] A. H. F. Castro, H. D. S. Tavares, S. R. F. Pereira, P. A. Granjeiro, J. A. D. Silva, and A. S. Galdino, "Production and characterization of lectin from Bauhinia holophylla (Fabaceae: Cercideae) calli," *Plant Cell, Tissue and Organ Culture*, pp. 1-10, 2018.

[20] D. Barik, S. Naik, A. Mudgal, and P. Chand, "Rapid plant regeneration through in vitro axillary shoot proliferation of butterfly pea (Clitoria ternatea L.)—A twinning legume," *In Vitro Cellular & Developmental Biology-Plant*, vol. 43. pp. 144-148. 2007.

[21] P. Padmanabhan, M. R. Shukla, J. A. Sullivan, and P. K. Saxena, "Iron supplementation promotes in vitro shoot induction and multiplication of Baptisia australis," *Plant Cell, Tissue and Organ Culture*, vol. 129, pp. 145-152. 2017.

[22] K. D. Mudoi and M. Borthakur, "In vitro micropropagation of Bambusa balcooa Roxb. through nodal explants from field-grown culms and scope for upscaling," *Current Science*, pp. 962-966, 2009.

[23] I. Ahmad, M. J. Jaskani, M. Nafees, I. Ashraf, and R. Qureshi, "Control of media browning in micropropagation of Guava (Psidium Guajava L.)," *Pak J Bot.*, vol. 48, pp. 713-716, 2016

[24] A. N. Martini, M. Papafotiou, and S. N. Vemmos, "Season and explant origin affect phenolic content, browning of explants, and micropropagation of× Malosorbus florentina (Zucc.) Browicz," *HortScience*, vol. 48, pp. 102-107. 2013.

[25] E. Prakash, P. S. S. V. Khan, T. J. V. S. Rao, and E. S. Meru, "Micropropagation of red sanders (Pterocarpus santalinus L.) using mature nodal explants," *Journal of Forest Research*, vol. 11, pp. 329-335, 2006.

[26] R. Yadav, N. Yadav, and S. Kumar, "An improved micropropagation and assessment of genetic fidelity in multipurpose medicinal tree, Acacia auriculiformis," in *Proc. of the National Academy of Sciences, India Section B: Biological Sciences*, 2016, pp. 921-929.

Sukanya Sirimat was born on 10th November, 1979. She received her Master degree in Applied Statistics from Chiang Mai University, Thailand. Currently, she is working as lecturer in the Faculty of Applied Science and Engineering, Khon Kaen University, Nong Khai Campus. Her research interested is about application of Statistics. More information can be contact her at her official e-mail address: sukasi@kku.ac.th.

Arpakorn Sakulsathaporn, Ph.D. (Agricultural Biotechnology), is now working as an academic staff/lecturer, with Khon Kaen University, Nong Khai Campus, Thailand. Her research of interests includes plant tissue culture and molecular markers.