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Influence of Medium Component on *In vitro* Propagation of Thai's Endangered Orchid: *Bulbophyllum nipondhii* Seidenf.

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Abstract

In vitro propagation of a rare orchid, *Bulbophyllum nipondhii* was carried out. Five different media were tested to find the suitable medium for seed germination and seedling development. The tangible results were obtained on VW medium. To assess the effect of pollination types on seed germination and seedlings development, seeds derived from different self-, cross- and open-pollination were examined. Open-pollinated seeds produced the best germination and the highest seedling development, followed by cross- and self- pollination. VW medium was supplemented with 0, 25, 50 or 75 g/l potato extract (PE) and 0, 50, 100, 150 or 200 ml/l coconut water (CW) to identify their most suitable concentration. PE (75 g/l) with 100 ml/l CW was found to be best combination.

Introduction

An epiphytic orchid, *Bulbophyllum nipondhii* Seidenf., is classified in section *Chirropetalum* Lindl. The mature plant has small pseudobulbs (approximately 1 cm high of which each pseudobulb has one leaf, and inflorescences bearing 1 - 10 purple flowers. So for, this species has only been found in Thailand (Seidenfaden 1985, Pedersen 2005) and China (Ye and Li 2012). In Thailand, *B. nipondhii* is restricted for growing merely on hill evergreen forests in Phuluang Wildlife

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Sanctuary (PLWS), Loei Province. Nowadays, global warming seems to be the main influence on orchid survival, especially of the epiphytic orchid (Seaton et al. 2010). Many orchids are considered to be endangered species which may become extinct. Hence, appropriate conservation management is needed to recover their natural population levels (Cribb et al. 2003).

Many orchid species have been successfully propagated *in vitro* since Knudson (1922) showed that orchid seed can be germinated in artificial media. *In vitro* germination of orchid seed allows the production of a large number of seedlings. However, the seed germination rate of each orchid species is particularly depending on the composition of culture media (Arditti and Ernst 1993). The content of macro- and micronutrients in media is one of the important factors affecting the success of *in vitro* orchid propagation (Churchill et al. 1972). Additionally, the supplementation of culture media with undefined organic substances like potato extract (PE) and coconut water (CW) can increase the proliferation rate and growth of orchid seedling (Rahman et al. 2004, Kaur and Bhutani 2012, Chen et al. 2014). However, their effects are based on the concentrations of organic supplements, explants type, and orchid species (Thorpe et al. 2008, Molnár et al. 2011). In addition, the germination achievement of orchid seed often depends on pollination aspects. Seeds derived from self- and cross pollination particularly affect seed viability and enhance seed germination (Bellusci et al. 2009).

Although *Bulbophyllum* has a large number of species, only a few reports on *in vitro* culture have been published (Bhadra et al. 2004, Than et al. 2009). Furthermore, *in vitro* propagation of *B. nipondhii* has also never been published. Therefore, the objective of the present study was to establish an efficient micropropagation method of *B. nipondhii* for future re-introduction into their natural habitat.

Materials and Methods

Three progeny lines of *Bulbophyllum nipondhii* Seidenf. were produced through self-, cross-, and open-pollination at PLWS, Thailand. During the anthesis mature inflorescences were self-pollinated by using hand pollination and covered by fine nylon mesh. Meanwhile they also were emasculated and cross-pollinated from different plants. For the open-pollination, the progeny was allowed to randomly pollinate by natural pollinators. After 12 months of pollination, mature capsules were harvested and kept at 10°C before further use.

The capsules were washed in tap water, submerged in sterilized solution (5% v/v bleach solution and 1% v/v detergent) for 15 minutes and dipped in 70% (v/v) alcohol and finally flamed twice with a spirit burner in a laminar flow

cabinet. The seeds were aseptically taken out by scalpel and a pair of forceps and mixed together for use in the further experiments.

Two basal culture media, VW (Vacin and Went 1949) and MS were used to determine asymbiotic seed germination and seedling development on *in vitro*. The VW and half strength of VW media were modified by 50 g/l potato extract (PE) and 150 ml/l coconut water (CW). The pH was adjusted to 5.2. The PE was prepared from small pieces of peeled potatoes and then boiled in distilled water (200 ml) for 5 minutes. The supernatant was filtered through cheesecloth. The CW was filtered through cheesecloth before use. The pH of MS, ½MS and ¼MS were adjusted to 5.7. All media were poured in test-tubes, and autoclaved at 121°C, 1.05 kg/cm^2 pressures for 15 minutes. They were slanted at room temperature. Six developmental stages of asymbiotic germination of *B. nipondhii* seeds were recorded under a stereoscopic microscope following Yamasaki and Miyoshi (2006). Stages 1, 2, 3 and 4 were represented by no development of seed, by swollen embryo, embryo enlargement and testa rupture (germination stage), by protocorm formation with acute apex and rhizoid appearance, first leaf appearance, and by second leaf, root appearances, respectively (Fig. 1a, b). Data were analyzed using ANOVA, and means were compared by using DMRT.

To evaluate the most effective medium for promoting seed germination and seedling development, the cross-pollinated seeds were sown on five culture media with approximately 300 - 400 seeds per test tube and conducted with six replicates. These cultures were incubated at 25 ± 2°C, 40 µmol/m^2/s light intensity, and 12 hrs light photoperiod for 4 months. The seed germination and seedling development were recorded monthly after culturing. Data were analyzed as mentioned above.

For explant proliferation, the leaves and roots of plantlets with pseudobulbs 5 - 10 mm height, produced from cross pollination, were cut off. Only the pseudobulb was grown on VW (with 20 g/l sucrose, 7 g/l agar and 2 g/l activated charcoal) supplemented with the combination of PE and CW. Twelve treatment combinations of individual PE (25, 50 and 75 g/l) were combined with CW (50, 100, 150 and 200 ml/l), respectively and without PE and CW was used as a control. All treatments were incubated at 25 ± 2°C, 40 µmol m^2/s light intensity, and 12 hrs light photoperiod for 6 months. At the end of treatment, the seed germination and seedling development were recorded and analyzed.

Results and Discussion

Five modified media (VW, ½VW, MS, ½MS and ¼MS) were used to determine the most effective one for seed germination, and seedlings development of the cross-pollinated line of *Bulbophyllum nipondhii*. The results revealed that majority

seeds did not germinate (defined as the stage 1) in one month after culture. After two months most of the seeds (more than 80%) in all modified media germinated and developed, defined as the stages 2 and 3 (data not shown). Of five media, the number of seedlings developed (at stage 4) and cultured on VW (29%) and ½VW (24%) were higher than rest of the three MS media (range 1 - 2%) (data not shown).

At four months, the percentage of seed germination (from stages 2 - 5) cultured on the modified VW and MS basal media showed insignificant difference (Table 1). However, the percentage of seedling development (from stages 4 - 5) cultured on two VW basal media had higher than three MS basal media (Table 1). Of five media, the VW gave the highest seedling development at stage 5 (34%). Moreover, the results found that the ½VW, ¼MS, ½MS and MS showed (at stage 5) 24.0, 1.9, 1.8, and 0.9% of seedling development, respectively (Table 1). These results indicated that the VW medium was the most effective for seed germination and seedling development.

It had been reported that the different concentrations of nitrogen (N) and phosphorus (P) play an important role for growth and development of various orchid species (Dijk and Eck 1995). Our results showed that the VW, containing higher P level (3.77 mM) than other media (1.88, 1.26, 0.63, and 0.31 mM in ½VW, MS, ½MS, and ¼MS, respectively), was suitable for seedling development of *B. nipondhii*. This is in conformity with the previous reports that high P concentration in VW medium promoted seedling development in *Bletia purpurea* (Dutra et al. 2008). In contrast, this result indicated that high N concentration in MS decreased seedling development in *B. nipondhii* because the excess inorganic nitrogen in plant cells had been eliminated by nitrate reductase activity (Raghavan and Torrey 1964, van Waes and Debergh 1986).

To access the effect of pollination types on growth development of *Bulbophyllum nipondhii*, the seeds produced from self-, cross- and open-pollination were cultured on VW medium. After 4 months, results revealed that seed germination rate was up to 91% of seeds derived from open- and cross-pollination, but the seeds from self-pollination showed only 4.1% germination (Table 2). Moreover, the result showed that seeds derived from open-, cross-, and self-pollination had seedling development (in stage 5) approximately 44.0, 34.4 and 0.1%, respectively (Table 2).

These results suggested that the germination and development percentages of seeds of open- and cross-pollination had significantly higher than self-pollination. In agreement with previous studies, which reported that seed germination produced from cross-pollination was higher than self-pollination

Table 1. Effect of different strengths of MS and VW media of seedling development and seed germination of *Bulbophyllum nipondhii* after 4 months.

Medium	% seedling development						Germination[1]
	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	(%)
MS	6.2 ± 1.4 ab	7.9 ± 1.8 a	54.1 ± 10.4 a	26.0 ± 8.5 b	5.0 ± 1.1 c	0.9 ± 0.6 c	85.9 ± 1.6 ab
½ MS	5.2 ± 1.7 b	5.2 ± 1.6 ab	31.9 ± 4.1 b	32.8 ± 4.5 ab	23.0 ± 4.1 b	1.8 ± 1.0 c	89.6 ± 2.8 ab
¼ MS	5.2 ± 1.9 b	6.2 ± 2.1 ab	28.8 ± 5.0 b	41.0 ± 4.5 a	16.9 ± 3.9 b	1.9 ± 1.3 c	88.6 ± 3.5 ab
VW	5.6 ± 1.5 b	3.4 ± 0.8 b	6.1 ± 2.4 c	11.7 ± 3.1 c	38.8 ± 5.6 a	34.4 ± 4.6 a	91.1 ± 1.8 a
½ VW	9.3 ± 3.2 a	6.1 ± 2.9 ab	7.6 ± 2.1 c	16.8 ± 3.4 c	36.0 ± 6.8 a	24.1 ± 3.9 b	84.6 ± 5.9 b

Values are means ± Sd of six replicates (300 seeds per replicate). Different letters within the same column show highly significant differences analyzed by DMRT at $p \leq 0.01$. [1]Calculated from seedling development from stage 2 to 5.

Table 2. Effect of pollination type (POL) on percentage of seedling development and seed germination of *Bulbophyllum nipondhii* on VW medium after 4 months.

POL	% seedling development						Germination [1]
	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	(%)
Self	83.3 ± 2.6 a	12.7 ± 2.4 a	0.5 ± 0.6 b	1.7 ± 1.1 b	1.7 ± 0.7 b	0.1 ± 0.3 c	4.1 ± 2.1 b
Cross	5.1 ± 1.2 b	3.2 ± 0.7 b	5.8 ± 2.6 a	12.4 ± 3.0 a	40.1 ± 5.2 a	33.3 ± 4.2 b	91.7 ± 1.1 a
Open	3.5 ± 2.3 b	1.9 ± 0.6 b	8.1 ± 1.2 a	9.8 ± 2.7 a	32.7 ± 4.9 a	43.9 ± 4.8 a	94.5 ± 2.1 a

Values are means ± Sd of 5 replications (300 seeds per replication), different letters within the same column show highly significant differences analyzed by DMRT at $p \leq 0.01$. [1]Calculated from seedling developmental stage 2 to 5.

(Bellusci et al. 2009). Self-pollination may have caused inbreeding depression (Charlesworth and Charlesworth 1987, Irawati 2013), resulting in decrease rate of seed viability, seed germination and seed survival (Husband and Schemske 1995).

To examine the effect of organic supplement on proliferation of *B. nipondhii*, the pseudobulbs were cultured on PE (25, 50 and 75 g/l) and CW (50, 100, 150 and 200 ml/l) in VW media for 6 months. At the end of the experiment, the combination of 75 g/l potato extract (PE) and 100 ml/l CW gave the best result in *B. nipondhii* showing a high number of new pseudobulbs (3.5), leaves (4.4), and roots (8.1), including high length of leaves (19.2 cm), and roots (13.6 cm) (Table 3 and Fig. 1c,e). Since the fresh potato contains 0.7 - 1.5% of the amino acid compounds (Bartova and Barta 2009), and previous studies have reported the uses of PE for the enhancement of growth of *Vanda roxburgii* seedlings (Islam et al. 2011), and *Dendrobium officinale* (Chen et al. 2014). Moreover, previous reports stated that fresh CW comprised of rich sugars,

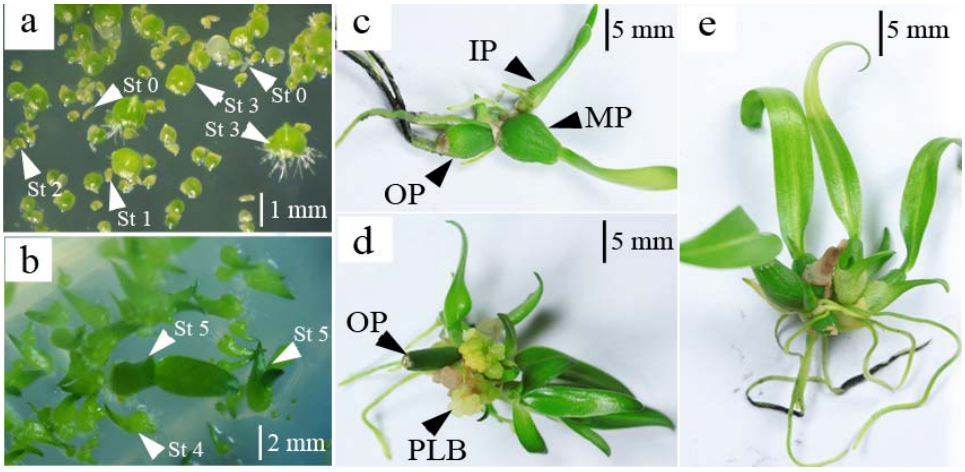

Fig. 1. Developmental stage of asymbiotic germination of *Bulbophyllum nipondhii* seed. a = Stage 0 (St 0), no development of seed; stage 1 (St 1), swollen embryo; stage 2 (St 2), embryo enlarge and testa rupture (germination stage); stage 3 (St 3), protocorm with acute apex and rhizoids. b = Stage 4 (St 4), emergent of first leaf; stage 5 (St 5), appearance of second leaf, root. c = New mature pseudobulb (MP) and new immature pseudobulb (IP) emergence from old pseudobulb (OP). d = Protocorm-like body (PLB) formation. e = Plantlet obtained from VW medium supplemented with 75 g/l PE and 100 ml/l CW.

vitamins, minerals, amino acids and phytohormones (Yong et al. 2009), and wildly used to enhance shoot multiplication of *Phalaenopsis violacea* (Gnasekaran et al. 2010) and *Cymbidium pendulum* (Kaur and Bhutani 2012). In the present study, the results also showed that the protocorm-like bodies were generated

Table 3. Effect of potato extract (PE) and coconut water (CW) on *in vitro* shoot regeneration of *Bulbophyllum nipondhii* after culturing for 6 months.

PE (g/l)	CW (ml/l)	Explant generates new pseudo-bulb (%)	No. of new pseudobulbs per explant	Explant showing PLBs (%)	No. of total leaves per explants	Mean of leaf length (mm)	The length of leaves (mm)	No. of root/ explants	Mean of root length (mm)	The length of roots (mm)
0	0	92.3	2.2 ± 1.2 ab[1]	0	3.9 ± 2.5 ab	6.3 ± 3.5 e	2 – 22	4.5 ± 2.6 b	7.4 ± 3.9 bcd	1 – 50
25	50	61.5	1.6 ± 1.7 ab	0	2.2 ± 2.5 b	7.9 ± 8.1 cde	2 – 29	2.9 ± 3.3 b	5.4 ± 6.5 cd	1 – 34
	100	92.3	3.4 ± 2.3 a	15.4	6.1 ± 4.9 a	9.6 ± 4.6 cde	2 – 25	5.2 ± 2.7 ab	8.4 ± 4.5 abcd	2 – 34
	150	76.9	1.4 ± 1.2 b	0	1.5 ± 1.2 b	10.4 ± 7.6 cde	5 – 23	3.2 ± 2.5 b	7.9 ± 6.3 abcd	1 – 31
	200	100.0	2.1 ± 1.4 ab	7.7	2.8 ± 2.2 b	10.2 ± 4.6 cde	2 – 21	4.6 ± 2.7 b	7.4 ± 2.5 bcd	2 – 20
50	50	92.3	1.7 ± 1.0 ab	7.7	2.2 ± 1.6 b	11.9 ± 5.2 abcde	3 – 23	3.8 ± 2.2 b	8.4 ± 3.6 abcd	2 – 30
	100	100.0	2.5 ± 1.3 ab	7.7	2.9 ± 2.1 b	18.7 ± 6.0 ab	3 – 32	5.2 ± 1.4 ab	12.9 ± 5.9 ab	2 – 43
	150	92.3	2.4 ± 2.1 ab	15.4	2.9 ± 2.7 b	14.6 ± 6.7 abcd	4 – 26	4.6 ± 2.7 b	6.9 ± 3.6 bcd	2 – 35
	200	76.9	2.0 ± 1.8 ab	7.7	2.2 ± 2.0 b	11.3 ± 8.2 bcde	4 – 29	4.7 ± 3.5 b	7.0 ± 5.5 bcd	2 – 32
75	50	84.6	1.7 ± 1.5 ab	7.7	2.2 ± 2.2 b	15.4 ± 9.0 abc	5 – 30	4.0 ± 3.0 b	9.6 ± 6.2 abcd	2 – 36
	100	100.0	3.5 ± 2.0 a	15.4	4.4 ± 3.6 ab	19.2 ± 8.2 a	3 – 39	8.1 ± 4.8 a	13.6 ± 5.9 a	1 – 44
	150	61.5	1.3 ± 2.2 b	0	1.7 ± 3.2 b	7.0 ± 6.4 de	2 – 21	1.9 ± 2.6 b	4.2 ± 5.1 d	1 – 23
	200	84.6	1.5 ± 1.0 ab	7.7	1.5 ± 1.0 b	14.3 ± 8.0 abcde	5 – 29	4.5 ± 2.6 b	11.0 ± 7.5 abc	2 – 50
Significant level [2]										
PE			NS		NS	**		NS	NS	
CW			***		**	**		***	***	
PE × CW			NS		NS	**		*	*	

[1]Values are means ± Sd of 13 replications. Different letters within the same column show highly significant differences analyzed by DMRT at $p \leq 0.01$.

[2]Significant levels as analyzed by two-way ANOVA. NS, *, ** and *** indicate non-significant or significant at level $p \leq 0.05$, 0.01 and 0.001, respectively.

approximately 15.4% at the cutting regions of the pseudobulb cultured in VM supplemented with the combinations of PE (25, 50, or 75 g/l) and CW (100, or 150 ml/l) (Table 3 and Fig. 1d).

The present study provided efficient methods to proliferate plantlets of Thai endangered orchid, *Bulbophyllum nipondhii*. Vacin and Went (VW) medium supplemented by PE (75 g/l) and CW (100 ml/l) combination was the most effective to proliferate plantlets from the orchid pseudobulbs.

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