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IN VITRO PROPAGATION OF INDIGENOUS PHU THO'S 5-PETALED WHITE ORCHID (*Dendrobium anosmum* Lindl.)

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Abstract

Indigenous Phu Tho's 5-petaled white orchid (Dendrobium anosmum Lindl.) was propagated by an in vitro method from keikis. Keikis were sterilized with HgCl2 0.1% for 12 minutes, the regeneration rate reached 24.44%. The percentage of budding from keiki parts reached 100% on ½ MS medium with 1.0 mg/L BAP. The best multi-shooting obtained on ½ MS medium with 1.5 mg/L BAP and 0.5 mg/L TDZ, shoot multiplication corresponded to 3.57 times. The ½ MS medium with 0.5 mg/L TDZ and 1.0 mg/L 2,4D was optimal for callus formation from stems (45.18%), shoot tips (85.93%) and leaves (17.04%). The ½ MS medium containing 1.0 mg/L BAP and 0.2 mg/L NAA induced the highest rate of rooting (95.56%). The best acclimatization substrate including 25% of coir, 75% of fir bark was found the most suitable medium for plantlets with a 77.04% survival rate, 4.24 cm in plantlet height, 6.3 number of leaves per plantlet among 8 weeks in the net house.

Keywords: *Coir, in vitro propagation, multi-shooting, orchid, Phu Tho's 5-petaled white orchid.*

1. Introduction

Orchids are flower species that are very diverse in size, shape, color, and fragrance. Orchid cultivation in Vietnam is currently bringing high economic value. Orchids have considerable ornamental, food, and medicinal values, so their demand in national and international markets is high [1]. The Orchidaceae is the largest plant family which comprises 30,000 - 35,000 species belonging to 850 genera [2]. In Vietnam, the botanists have counted more than 897 species of orchids belonging to 152 genera, of which the genus Dendrobium is a large genus with more than 100 species, distributed mainly in mountain areas from the North to the South and on some coastal islands [3]. Phu Tho's 5-petaled white orchid belongs to the genus Dendrobium anosmum. D. anosmum is an orchid species with a high economic value, wide distribution, and adapted to many different ecological regions. Especially in some ecological regions, they have changed structure that created diverse morphological characteristics in some parts such as stalks,

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leaves, and flowers. Therefore, they are called var orchids or mutated orchids. The mutated orchids created many types of flowers with very beautiful flower faces traded with high economic value such as Hong Yen Thuy orchid, Hien Oanh orchid in Hoa Binh, and Ha Tinh's 5-petaled white. Among them, Phu Tho's 5-petaled white orchid is an indigenous species that orchid players love it, and it has a high economic value. The plant has beautiful stems, leaves, white flowers. The flower shape is balanced with white top petal growing straight while the upper part of the top petal is slightly curved. The two horizontal petals are evenly arranged. The flower's lip is heart-shaped with snow velvets, two flower purple eyes, and a faint fragrance. Phu Tho's 5-petaled white orchid is often propagated through the axillary buds. The orchid players often cut stems on mother plants and place them directly on substrate or stimulate directly on the stem for shooting. At present, there has not been any in vitro propagation research for Phu Tho's 5-petaled white orchid. Therefore, we conducted an in vitro breeding study on Phu Tho's 5-petaled white orchid. The present study aimed to establish a complete micro-propagation protocol started with shoot induction from the keiki segment.

2. Methods

Plant materials: Keikis of Phu Tho's 5-petaled white orchid were used for the experiments in the biotechnological laboratory of the Institute of Applied Research and Development, Hung Vuong University (Vietnam).

Medium: ½ Murashige and Skoog (MS) medium supplemented with 20-30 g/L sucrose, 0.5 g/L activated charcoal, 100 ml/L coconut water, 6.5 g/L agar was tested with different concentrations of the growth regulators. The basal medium without plant growth regulators was used as a control. The pH of the media was adjusted to 5.8 after the addition of the growth regulators. Media were sterilized at 121°C for 20 min.

Culture conditions: The cultures were maintained at 25 ± 2°C in a photoperiod of 16 h light and 8 h darkness for the budding, shoot multiplication, rooting experiments, or completely dark for callus experiments. The growing conditions in the net house were maintained at 50% light, day, and night temperature from 25-35ºC, watering 2 times a day. The plantlets were planted in a plastic pot diameter of 7.5 cm and placed above the ground 0.8 m.

Experimental arrangement

Sterilization of plant materials: The Keikis placed under a running tap water for half an hour to remove dust and other foreign particles. After washing, they were thoroughly sterilized in 70% ethanol for 30 seconds, disinfected in 0.1% mercuric chloride (HgCl2) twice for 8, 10, 12, 14 minutes, and rinsed several times with sterilized distilled water. Keiki segments having axillary buds were given a final size by trimming up to 2-3 cm with subsequent inoculation in test tubes, containing basal medium supplemented with 1.0 mg/L 6-benzylaminopurine (BAP). Observations were taken after eight weeks for budding, infection, and death rate.

Shoot induction: In vitro shoots were cut off the top of the shoot and then it was placed on the surface of the basal medium with different concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L). Observations were taken after eight weeks for the shooting rate and characteristics of the shoot.

Shoot multiplication: The approximately 1 to 2 cm shoots were placed on basal

medium with combination of BAP (0.0, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 mg/L) and thidiazuron (TDZ) (0.5 mg/L.) After eight weeks, the shoot multiplication, the shoot height, and the number of leaves per shoot were recorded.

Callus induction via thin cell layer culture: The in vitro shoots were cut off into a thin cell layer approximately 5 mm. The stem, shoot tip, and leaf of the thin cell layer were placed on a basal medium with the combination of TDZ (0.5 mg/L) and dichlorophenoxyacetic acid (2,4D) (0.5, 1.0, 1.5, and 2.0 mg/L). After eight weeks, the callus formation rate was recorded.

The shoot formation from the callus: The yellowish and hard calli were placed on a basal medium with a combination of BAP (0.5 mg/L) and furfurylaminopurine (kinetin) (0.5, 1.0, 1.5, and 2.0 mg/L). After eight weeks, the germination percentage of callus, and the number shoot per callus were recorded.

In vitro rooting: Selected in vitro produced shoots were inoculated on the basal medium with a combination naphthalene acetic acid (NAA) (0.2 mg/L) and BAP (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L). After eight weeks, the number of rooting plantlets, the number of roots per plantlet, and root length were recorded.

Ex vitro acclimatization: Rooted shoots were removed from the rooting medium and washed thoroughly in distilled water to remove any adhering medium. The plantlets were then transferred to plastic pots containing a mixture of coir and small fir bark in rates 100:0, 75:25, 50:50, and 25:75 (v/v). The survival rate, number of leaves per plant, and plant height were recorded after eight weeks of growth.

Statistical analysis: Observed data were analyzed using Microsoft Excel and IRRISTART 5.0 software. Means and standard errors were applied to assess the experiment results using the ANOVA test at $p \leq 0.05$. Treatments were designed randomly with three replicates.

3. Results and discussion

Sterilization of plant materials

The results from Table 1 showed that four sterilization formulas exhibited a significant difference ($p \leq 0.05$). The highest percentage of budding explants (24.44%), the percentage of infection explants (57.04%), and the percentage of death explants (18.52) were observed when sterilization for 12 minutes. When increasing the sterilization time to 14 minutes, the percentage of budding explants, the percentage of death explants were decreased, and the percentage of infection explants were increased. The comparison of 8 and 10 min HgCl2 treatment showed that the percentage of budding explants decreased (8.89%, 13.33%), the percentage of death explants decreased (1.48%, 8.15%) and the percentage of infection explants increased (89.63%, 78,52%). The effect of sterilization times on the percentage of survival explants was also observed in various plants. The shoot tips of Bougainvillea spectabili treated with 0.05% HgCl2 for 3 minutes showed maximum survival (91.6%) while 0.05% and 0.25% HgCl2 for 5 and 3 minutes, respectively, gave (75%) survival [4]. The shoot tips of Psidium guajava L. were sterilized by HgCl2 at 0.05% for 5 minutes plus 70% ethanol which gave maximum survival percentage (67%) [5].

Table 1. Effects of various sterilization times on budding of Phu Tho's 5-petaled white orchid

Sterilization times (minute)	Budding explants rate (%)	Infection explants rate (%)	Death explants rate (%)
8	8.89a	89.63d	1.48a
10	13.33b	78.52c	8.15b
12	24.44d	57.04b	18.52c
14	19.26c	1.48a	79.26d

Different letters (a, b, c, d) indicate significant differences in the same column ($p \leq 0.05$).

In vitro shoot induction

The results from Table 2 showed that the effect of different concentrations of BAP varied significantly with shooting rate and characteristics of the shoot.

Table 2. Comparison of shoot induction of Phu Tho's 5-petaled white orchid at various BAP concentrations

BAP (mg/L)	Shooting rate (%)	Shoot characteristics
0.0	23.46^{a}	Small, light green
0.5	66.67^{b}	Normal, light green
1.0	100.0^{e}	Fat, dark green
1.5	98.52^{e}	Fat, dark green
2.0	78.52^{d}	Normal, dark green
2.5	73.33^{c}	Normal, dark green
3.0	63.70^{b}	Small, dark green

Different letters (a, b, c, d, e) indicate significant differences in the same column ($p \leq 0.05$).

The maximum percentage of shooting explants (98-100%) was observed in basal medium with 1.0-1.5 mg/L BAP. Shoots were big, strong, dark green (Fig. 1a). When low BAP concentration (0.5 mg/L), the percentage of shooting explants decreased (66.67%). When the increased concentration of BAP was adjusted to 2.0, 2.5 to 3.0 mg/L, the percentage of shooting explants were also decreased correspondingly to 78.52%, 73.33%, and 63.7%. The color of the shoots was also changed from dark green to light green. While the results obtained at control were opposite, the percentage of shooting explants was 23.46%, the shoots were small, light green. The result was in line with a study in D. nobile var. Emma White by Sana et al (2011) [6].

Shoot multiplication

The highest shoot multiplication (3.57) and the shoot height (1.1 cm) were observed in explants treated by the combination of 1.5 mg/L BAP and 0.5 mg/L TDZ (Table 3). Shoots were big, strong, dark green, good vitality (Fig.1b). The higher shoot multiplication (3.07), the shoot height (1.07 cm), and the number of leaves per shoot (3.02) were observed in explants treated by the combination of 2.0 mg/L BAP and 0.5 mg/L TDZ. When increased

or decreased concentration of BAP and kept the same concentration of TDZ, the shoot multiplication, the shoot height, and the number of leaves per shoot also substantially decreased. Many reports showed that the addition of BAP in medium cultures increased the shoot multiplication of D. moschatum Sw. [7], D. nobile orchids [6]. As well as the addition of TDZ in medium cultures increased the shoot multiplication of D. moschatum Sw. [7], D. moschatum [8]. However, there have not been yet published reports studying on the combination of BAP and TDZ in the propagation of orchids.

Table 3. Comparison of shooting in vitro of Phu Tho's 5-petaled white orchid at various concentrations of BAP with 0.5 mg/L TDZ

BAP (mg/L)	TDZ (mg/L)	Shoot multiplication	Shoot height (cm)	Number of leaves per shoot
0.0	0.5	1.55a	0.58a	1.64a
1.0	0.5	2.07c	0.71b	2.23b
1.5	0.5	3.57f	1.10e	3.24e
2.0	0.5	3.07e	1.07e	3.02d
2.5	0.5	2.39d	1.05e	2.38c
3.0	0.5	2.30d	0.89c	2.33bc
3.5	0.5	1.90b	0.95d	2.30bc

Different letters (a, b, c, d, e, f) indicate significant differences in the same column ($p \leq 0.05$).

Callus induction via thin cell layer culture and the shoot formation from the callus

The results from Table 4 showed that callus was formed via a thin cell layer of stem, shoot tip, and leaf within eight weeks. While there was no callus formation in the control. The highest percentage of callus formation from the stem, shoot tip, and leaf were observed in the medium containing 0.5 mg/L TDZ and 1.0 mg/L 2,4D respectively 45.18%, 85.93%, and 17.04%. In the medium, the remained concentration of TDZ (0.5 mg/L) and increased or decreased concentration of 2,4D, reduced the rate of callus samples in all three types of the stem, shoot tip, and leaf. The formed calli were yellowish or yellow and hard (Fig. 1c). The thin cell layer culture method was applied on a few different orchid species such as C. aloifolium [9], C. giganteum [10]. A combination TDZ and 2,4D was used to induce callus from the roots, stems, and leaves of C. ensifolium [11], Cymbidium Twilight Moon. [12].

Table 4. Effects of NAA and TDZ on callus formation from the stem, shoot tip, and leaf of Phu Tho's 5-petaled white orchid

TDZ (mg/L)	2,4D (mg/L)	Callus formation rate (%)			Callus color		
		Stem	Shoot tip	Leaf	Stem	Shoot tip	Leaf
0.0	0.0	0.00	0.00	0.00	-	-	-
0.5	0.5	29.63^{a}	57.04^{c}	8.89^{b}	Yellowish	Yellowish	Yellowish
0.5	1.0	45.18^{c}	85.93^{d}	17.04^{d}	Yellow	Yellow	Yellow
0.5	1.5	56.29^{d}	51.85^{b}	13.33^{c}	Yellow	Yellow	Yellow
0.5	2.0	31.85^{b}	48.15^{a}	6.67^{a}	Yellowish	Yellowish	Yellowish

Different letters (a, b, c, d) indicate significant differences in the same column ($p \leq 0.05$).

Calli were cultured on medium with BAP và Kinetin to stimulate germination (Fig. 1d). The results from Table 5 showed that the highest germination percentage of callus (71.85%) and the highest number shoot per callus (4.67) was observed in the medium containing 0.5 mg/L BAP and 1.5 mg/L Kinetin. When increased or decreased concentration of Kinetin and kept the same concentration of BAP (0.5 mg/L), the germination percentage of callus and the number of shoot per callus were decreased. In the control medium, the germination percentage of callus and number shoot per callus were the lowest with only 3.46% and 1.23, respectively. The combination of two growth regulators BAP and Kinetin were widely used in the in vitro culture of orchids, especially when used to induce seed germination, budding from protocorm-like bodies or callus. The combination of 2.0 mg/L BAP and 2.0 mg/L Kinetin gave the germination percentage of Vanda tessellata seeds (55.8%) [13]. The concentration of BAP 2.5 mg/L and Kinetin 1.5 mg/L gave the highest percentage of budding protocorm of Eulophia nuda orchid (69.9%), the number of shoots per sample (4.2), and the shoot length (5 cm) [14].

Table 5. Shoot induction from callus of Phu Tho's 5-petaled white orchid

BAP (mg/L)	Kinetin (mg/L)	Germination percentage of callus (%)	Number of shoots per callus
0.0	0.0	3.46^{a}	1.23^{a}
0.5	0.5	36.30^{b}	3.40^{b}
0.5	1.0	55.56^{d}	3.63^{d}
0.5	1.5	71.85^{e}	4.67^{e}
0.5	2.0	52.59^{c}	3.70^{c}

Different letters (a, b, c, d, e) indicate significant differences in the same column ($p \leq 0.05$).

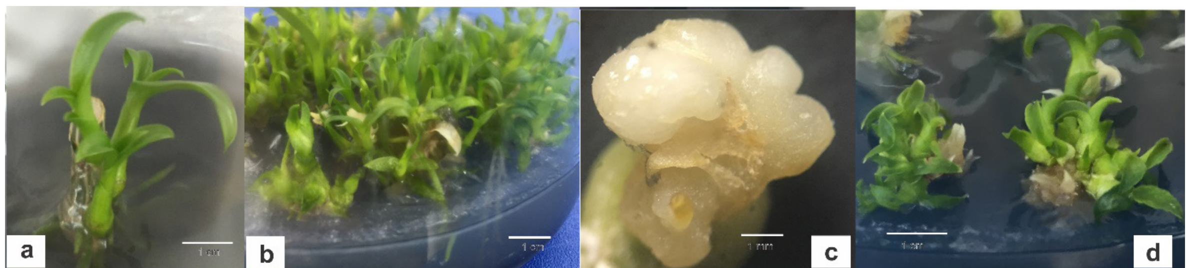

Figure 1. In vitro shooting of Phu Tho's 5-petaled white orchid

a) Shoot induction from shoot segments on 1/2MS with 1.0 mg/L BAP; b) Shoot multiplication from in vitro shoot 1/2MS medium with 1.5 mg/L BAP and 0.5 mg/L TDZ; c) Callus induction from shoot tip on 1/2MS medium with 0.5 mg/L TDZ and 1.0 mg/L 2.4D; d) Germination shoot from callus on 1/2MS medium with 0.5 mg/L BAP and 0,5 mg/L Kinetin.

In vitro rooting and Ex vitro acclimatization

Comparatively, the highest percentage of rooting plantlet (95.56%), the number (10.08) and length (2.81 cm) of roots was observed at 1.0 mg/L BAP and 0.2 mg/L NAA (Table 6), while the highest length (2.89 cm) of roots was found at 2.0 mg/L BAP and 0.2 mg/L NAA. When increased or decreased concentration of BAP and kept the same concentration of NAA (0.2 mg/L), the percentage of rooting plantlet in culture medium were decreased. In the culture

medium, the number of roots per plantlet and the root length did not change significantly about 8 to 10 roots and from 2 to 3 cm. The roots were big, strong, and opaque white (Fig. 2). Poor results were produced at control media, the percentage of rooting plantlet was 5.19%, the roots were short (0.58 cm) that did not require in acclimatization.

Rooted plantlets were transferred into the net house. Supporting media included coir and small fir bark. After eight weeks of acclimatization, the substrate medium showed the highest rate of survival (77.04%), the plant height (4.24), and the number leaf per plant (6.3) were observed in the substrate with 25% coir and 75% small fir bark (v/v) (Table 7). When increasing the coir rate and reducing the small fir bark rate, the efficiency of the acclimatization also decreased. The substrate with 50% coir and 50% small fir bark showed the rate of survival (68.89%), and 3,89 cm in plant height, and 6.10 in the number leaf per plant. For the substrates that used coir or small fir bark alone, the results of acclimatization were worse, specifically, the plants placed in the substrate with 100% coir, the rate of survival (51.11%), and 4.06 cm in plant height, and 5.92 in the number leaf per plant. The plants placed in the substrate with 100% small fir bark, the rate of survival (46.67%), and 4.03 cm in plant height, and 5.73 in the number leaf per plant. These differences were statistically different ($p \leq 0.05$).

Table 6. Effects of BAP and NAA on rooting of Phu Tho's 5-petaled white orchid

BAP (mg/L)	NAA (mg/L)	Percentage of rooting plantlet (%)	Number roots per plantlet	Length of root (cm)
0.0	0.0	5.19a	7.39a	0.58a
0.5	0.2	51.11c	9.04c	1.07b
1.0	0.2	95.56f	10.08e	2.81d
1.5	0.2	86.67e	8.97c	2.70c
2.0	0.2	79.25d	9.05c	2.89e
2.5	0.2	75.56d	8.08b	2.77d
3.0	0.2	45.19b	8.22b	288e

Different letters (a, b, c, d, e, f) indicate significant differences in the same column ($p \leq 0.05$).

Figure 2. Root induction from shoots cultured of Phu Tho's 5-petaled white orchid on 1/2MS media with 1.0 mg/L BAP and 0.2 mg/L NAA.

Table 7. Comparison of survival rate and growth of plantlets by the acclimatization medium used

Acclimatization medium	Survival rate (%)	Plant height (cm)	Number of leaves per plant
100% coir	51.11[b]	4.06[abc]	5.92[ab]
75% coir : 25% small fir bark	61.48[c]	4.20[bc]	6.16[bc]
50% coir : 50% small fir bark	68.89[d]	3.98[a]	6.10[bc]
25% coir : 75% small fir bark	77.04[e]	4.24[c]	6.30[c]
100% small fir bark	46.67[a]	4.03[ab]	5.73[a]

Different letters (a, b, c, d, e) indicate significant differences in the same column ($p \leq 0.05$).

4. Conclusions

The present study assessed for the first time on in vitro propagation of the indigenous Phu Tho's 5-petaled white orchid from Keikis. The study evaluated quite fully the stages of in vitro propagation, from the sterilization process of samples, to shoot multiplication, callus induction, callus germination, rooting plantlet, and acclimatization in the net house. The ½ MS medium with 1.0 mg/L BAP, and 1.5 mg/L BAP with 0.5 mg/L TDZ, and 0.5 mg/L TDZ with 1.0 mg/L 2,4D, and 1.0 mg/L BAP with 0.2 mg/L NAA were found to be optimal in vitro regulators for shoot induction, shoot multiplication, callus induction, callus germination, and rooting plantlet, respectively. The substrate with 25% coir and 75% small fir bark was also the most optimal to place plants in the net house.

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NHÂN GIỐNG IN VITRO LOÀI LAN BẢN ĐỊA 5 CÁNH TRẮNG PHÚ THỌ (*Dendrobium anosmum* Lindl.)

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Tóm tắt

Loài lan bản địa 5 cánh trắng Phú Thọ (*Dendrobium anosmum* Lindl.) đã được nhân giống in vitro từ các chồi non trên thân. Mẫu chồi non được khử trùng bằng HgCl2 0,1% trong 12 phút cho tỷ lệ mẫu tái sinh là 24,44%. Môi trường cơ bản bổ sung 1,0 mg/L BAP cảm ứng tạo chồi tốt nhất, tỷ lệ mẫu tạo chồi đạt 100%. Môi trường cơ bản bổ sung 1,5 mg/L BAP và 0,5 mg/L TDZ cho kết quả tạo đa chồi tốt nhất với hệ số nhân chồi đạt 3,57 lần. Môi trường cơ bản bổ sung 0,5 mg/L TDZ và 1,0 mg/L 2,4D cho kết quả tạo mô sẹo (callus) tốt nhất ở cả 3 mẫu thân, đỉnh chồi và lá, tỷ lệ tạo mô sẹo tương ứng đạt 45,18%; 85,93% và 17,04%. Môi trường cơ bản bổ sung 1,0 mg/L BAP và 0,2 mg/L NAA kích thích sự ra rễ tốt nhất, tỷ lệ cây ra rễ đạt 95,56%. Giá thể ươm cây gồm 25% mụn sơ dừa và 75% vỏ thông là thích hợp nhất, cho tỷ lệ cây sống là 77,04%, chiều cao cây trung bình 4,24 cm và số lá trung bình/cây 6,3 lá sau 8 tuần ra cây.

Từ khóa: *5 cánh trắng Phú Thọ, đa chồi, hoa phong lan, mụn sơ dừa, nhân giống in vitro.*