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In Vitro Propagation of a Vietnam Endemic Lady's Slipper Orchid (*Paphiopedilum vietnamense* O.Gruss & Perner)

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Abstract. *P. vietnamense* O. Gruss & Perner is one of the endemic lady's slipper orchids in Vietnam that has been known as an endangered species threatened with extinction due to over-collection. To protect *P. vietnamense* species, *in vitro* propagation method needs to be established for producing plantlets that can be introduced back in wild, as well as commercialized. In this study, we examined the effect of basal culture medium, plant growth regulators, and AC on the micropropagation ability of *P. vietnamense*, which were collected from wild population in Thai Nguyen province, Northeastern Vietnam. We determined that the 0.2% $HgCl_2$ can be used in within 5 to 10 min for sterilizing axillary buds of *P. vietnamense*, resulting in 65.55% of disinfected samples. Moreover, the 1/2 MS medium supplemented with 2.0 mg l^{-1} BA and 1.0 mg l^{-1} NAA, which gave about 2.85 shoots/sample, was the most suitable for shoot regeneration and multiplication. In addition, the result also indicated that 0.5 mg l^{-1} NAA and 1.0 g l^{-1} AC gave the best results (88.89%) for root induction and plantlets.

Abbreviations:

2,4-D	:	2,4-Dichlorophenoxyacetic acid
BA	:	N^6- Benzyladenine
NAA	:	Naphthaleneacetic acid
IAA	:	Indole-3-acetic acid
IBA	:	Indole-3-butyric acid
Kinetin	:	Kinetin 6-Furfurylaminopurine
MS	:	Murashige and Skoog
KC	:	Knudson C
RE	:	Robert Ernst
$HgCl_2$	:	Mercury(II) chloride

Introduction

Paphiopedilum orchids are widely known as a potted flower with magnificent beautiful and high-trade value. Although, the genus of *Paphiopedilum* is one of the biggest in the sub-family *Cypripedioideae*, comprised about 80-100 species, their distribution was very limited in Southeast Asia, southern China, India and mostly under the threat of extinction as a result of over-collection and loss of suitable habitats [1-2].

Paphiopedilum vietnamense O. Gruss and Perner (*P. vietnamense*) is one of the endemic orchids in Vietnam that was first found in Thai Nguyen province and described by Gruss and Perner [3], [4]. The population of *P. vietnamense* has been significantly reduced in recent decades due to over-collection for trade. The number of mature individuals is less than 50 and the population reduction is very high at over 95% [3-4], so The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has listed *P. vietnamense* orchid on Appendix I of the CITES [4].

Propagation of *P. vietnamense* for generating numerous plants that can be met the market demand is one of the best ways to protect this endemic orchid [2], [4-5]. However, in nature, *Paphiopedilum* seed germinates relatively slowly due to the absence of an endosperm [5-7]. In addition, the interval between seed germination and tiller production can take several years [6]. Because of these reasons, most of the *Paphiopedium* species are relatively rare or even extincted in wild population [1], [2], [4-5].

To overcome these limitations, some research groups have applied tissue culture technology in micropropagating *Paphiopedilum.* Of which, plantlets could be produced easier in short time by culturing on the artificial medium, including mature seed germination [1], [6-10], shoot induction from nodal segments, leaf explants [10-15]. Although, many studies have been reported for *in vitro* propagation of *Paphiopedilum* species as *P. wardii* [1], *P. ciliolare* [8], *P. insigne* [9], *P. hangianum* [10], *P. armeniacum* and *P. micranthum* [11], *P. delrosi* [14], *P. delenatii* [15], etc. However, *P. vietnamense* orchid has not been reported yet. In this study, we carried out a series of experiments as the effect of basal medium composition, plant growth regulators, activated charcoal to determine the suitable culture medium for *in vitro* propagation of *P. vietnamense.*

Materials and Methods

Plant materials

Paphiopedilum vietnamense O. Gruss & Perner plants were collected from wild populations in Thai Nguyen province (Northeastern Vietnam). The plants were transplanted into containers and grown under greenhouse conditions at Thai Nguyen University of Agriculture and Forestry in Thai Nguyen, Vietnam (Fig. 1a).

Culture medium and growth conditions

The basal culture medium used in this study is full-strength MS (Murashige and Skoog, 1962, [16]) micro- and macro-elements, 100 mg l^{-1} inositol, 120 ml l^{-1} coconut water (CW), sucrose 30 g l^{-1} and agar 5 g l^{-1}. The pH of the medium was adjusted to 5.8 with NaOH1N or HCl1N. Prior to use, the medium was autoclaved for 17 min at 121^{o}C. For each treatment in different media, thirty explants were tested in triplicate.

Explant preparation for experiments

Samples used are axillary buds detached from mother plants in the greenhouse. Firstly, samples were washed under tap water before sterilizing surface with 70% ethanol for 5 min and after that replacing by $HgCl_2$ 0.1% for 10 min. Subsequently, samples were washed thoroughly with autoclaved water for 3-4 times before placing on regeneration medium that prepared ahead.

Testing the effect of basal medium, BA and kinetin on shoot multiplication

In order to determine the effect of basal medium, BA and kinetin on shoot formation and multiplication of *P. vietnamense,* we tested basal medium of MS and RE (Robert Ernst, 1980) [6] which were supplemented with 100 mg l^{-1} inositol, 30 g l^{-1} sucrose, 120 ml l^{-1} CW. The best basal medium was then used for examination of BA, and kinetin. The efficiency of BA and kinetin was evaluated by shoot formation rate.

Testing the effect of NAA and activated charcoal on root formation

To determine the effect of NAA and activated charcoal (AC) on root formation, 3-4 cm shoots were transferred to rooting medium containing various concentrations, ranged from 0.5 to 2.0 mg l^{-1}. Number of shoots produced roots and morphology were recorded.

Statistical analysis

All of the experiments were carried out as a factorial experiment at 5 % probability level. Data obtained were statistically analyzed using IRRISTAT 5.0 software program (Duncan 1955) [17] for comparing among least significant difference (LSD).

Results

Effect of $HgCl_2$ on sterilization of *P. vietnamense* explants

To determine the most efficient procedure for initiation of micropropagation of *P. vietnamense* we used $HgCl_2$ as a surface sterilizing agent at various concentrations and duration of exposure. $HgCl_2$ was tested at a range from 0.1 to 0.2% for 5 to 15 min using axillary shoot as explants. The results showed that $HgCl_2$ has a positive impact on sterilization even at the low level of 0.1% $HgCl_2$. At this concentration, the survival rate was recorded from 16.56% to 41.11% when increasing duration exposure from 5 to 15 min, respectively. The percentage of disinfection explants was significantly increased from 52.22 to 58.88% when treated with 0.15% $HgCl_2$ for 5 to 10 min, respectively. However, when we increased the duration time exposure up to 15 min the result showing decrease the efficient sterilization (43.33%, Table 1). To test whether the rate of disinfection explant increase, we treated samples with a higher concentration of 0.2% $HgCl_2$, resulting in 65.55% disinfected explants after 5 min treatment and seem to be decreased after 10 and 15 min that showing 59.99% and 31.11%, respectively (Table 1, Fig. 1b). However, the survival rate and browning were significantly decreased when increasing duration treatment for all tested concentrations (Table 1). Overall, the results indicated that 0.2% $HgCl_2$ could be the best for sterilizing explants for *in vitro* propagation of *P. vietnamense.*

Table 1. Effect of $HgCl_2$ on sterilization of *P. vietnamense* explant after culturing for 4 weeks

Treatments	$HgCl_2$ concentration (%)	Time (min)	No. tested samples	Survival rate (%)	Dead rate (%)	Browning
T1	0.00	15	30	0.00	0.00	-
T2	0.10	5	30	16.56^{g}	0.00	-
T3		10	30	28.89^{f}	5.56^{g}	+
T4		15	30	41.11^{d}	20.00^{d}	+
T5	0.15	5	30	52.22^{c}	11.12^{f}	+
T6		10	30	58.88^{b}	16.68^{de}	+
T7		15	30	43.33^{d}	44.45^{b}	+
T8	0.20	5	30	65.55^{a}	17.90^{de}	+
T9		10	30	59.99^{b}	35.57^{c}	+
T10		15	30	31.11^{e}	68.89^{a}	+

Data are given as the mean of the frequency of survival and dead explants after 4 weeks of culture. Data expressed in different lower case letters are significantly different at $P< 0.05$ level by the LSD test (Duncan, 1955) [17]. (-) indicates not browning and (+) browning.

Basal culture medium on shoot formation

To determine the suitable culture medium for *in vitro* shoot regeneration of *P. vietnamense*, we tested explants on MS and RE medium with full- and half-strength of compositions. After 6 weeks

of culture, we calculated the frequency of new shoots regenerated on each media. The results showed that half-strength of composition medium of both MS and RE gave better shoot regeneration than full-strength composition. Of that, 1/2 MS and 1/2 RE gave the higher rate of shoot regeneration 66.67% and 62.22%, respectively (Table 2), whereas, MS and RE showed significantly lower frequency of shoot formation as 52.22% and 57.78 %, respectively. In addition, we observed that the growth pattern of shoots was difference from basal medium. While MS, RE and 1/2 RE showed slow growing pattern, 1/2 MS medium gave faster growing shoot with long leaf pattern (Table 2). These results suggested that 1/2 MS medium was suitable for *in vitro* shoot regeneration of *P. vietnamense*.

Table 2. Basal medium used for *in vitro* shoot regeneration of *P. vietnamense* after culturing for 6 weeks

Treatments	Medium	Tested samples	Shoot regeneration rate (%)	Growth pattern
T1	MS	30	52.22^{b}	Slow, short leaf
T2	½ MS	30	66.67^{a}	Fast, long leaf
T3	RE	30	57.78^{b}	Slow, short leaf
T4	½ RE	30	62.22^{ab}	Slow, short leaf

Data are given as the mean of shoot regeneration rate after 6 weeks of culture. Data expressed in different lower case letters are significantly different at $P< 0.05$ level by the LSD test (Duncan, 1955 [17]).

Effect of BA and NAA on shoot multiplication of *P. vietnamense*

In order to multiplication shoots of *P. vietnamense*, we transferred isolated shoots from regeneration medium to 1/2 MS medium that comprised various concentrations of BA and NAA. The result showed that the medium added BA alone produced high number of new shoots, range from 1.56 to 2.17 shoots per sample, whereas the 1/2 MS medium without BA showed 1.06 shoot per sample (Table 3). Of which, the highest rate of shoot formation was recorded at 2.0 mg l^{-1} BA (2.17 shoots/sample). In addition, in this treatment, we observed that shoots displayed healthily morphology with long leaf, whereas, the other treatments gave less number of shoots that showing shorter or browning pattern (data not shown). To increase the number of shoots, we have tested in a combination of 2.0 mg l^{-1} BA with a range from 0.5 to 1.0 mg l^{-1} NAA. The results showed that the number of shoots produced was from 2.06 to 2.83 shoots per sample that were no significant increase compared with using BA alone. However, the patterns of shoot showed better on medium consisted of both 2.0 mg l^{-1} BA and 1.0 mg l^{-1} NAA (Table 3, Fig. 1c).

Table 3. Effect of BA and NAA on *In Vitro* shoot multiplication of *P.vietnamense* after culturing for 5 weeks

Treatments	BA (mg l^{-1})	NAA (mg l^{-1})	No. tested samples	No. shoot formation	No. shoot/sample
T1	0	0	30	32	1.06^{c}
T2	1.0	0	30	47	1.56^{b}
T3	2.0	0	30	65	2.17^{a}
T4	3.0	0	30	57	1.90^{ab}
T5	5.0	0	30	39	1.30^{bc}
T6		0.5	30	62	2.06^{a}
T7	2.0	1.0	30	85	2.83^{a}
T8		1.5	30	74	2.46^{a}
T9		2.0	30	64	2.13^{a}

Data are given as the mean of shoot formation after 5 weeks of culture. Data expressed in different lower case letters are significantly different at $P< 0.05$ level by the LSD test (Duncan, 1955 [17]).

Effect of NAA and activated charcoal on root formation of *P. vietnamense*

Auxin has been known as a regulator for root induction, of which IBA and NAA have been widely applied. In this study, we examined the effect of NAA and activated charcoal (AC) on root induction of *P. vietnamense in vitro* at various concentrations as from 0.5 to 2.0 mg l^{-1}. After 6 weeks of culture, we observed that about 10.00% shoots could be produced roots in the medium without NAA, however, the rooting rate was significantly increased when applied NAA into medium, resulted in the range from 23.33 to 70.00 %. Of which, the highest rate (70%) was obtained at 0.5 mg l^{-1} NAA (Table 4). Interestingly, when we increased the concentrations of NAA to 1.0, 1.5 and 2.0 mg l^{-1}, the results showed that the root formation frequency of *P. vietnamense* was reduced as 60.00%, 43.33% and 23.33%, respectively. In addition, the induced roots displayed tiny morphology for all treatments (Table 4). To improve the root growth, we added AC into medium that having 0.5 mg l^{-1} NAA. The results indicated that AC significantly improved the root patterns and root induction rate, ranged from 74.44 to 88.89%. Of which, at low level of AC (0.5 g l^{-1}) gave 81.11% rooting shoots (Fig. 1d). The highest rate of root induction was obtained at treatment consisting of 0.5 mg l^{-1} and 1.0 g l^{-1} AC that showed 88.89% shoot produced roots (Table 4, Fig. 1e). Increasing AC concentration to 1.5 g l^{-1} AC gave 83.33% that was not significantly reduced root induction rate compared to the 1.0 g l^{-1} AC. Taken all indicated that medium with a combination of 0.5 mg l^{-1}NAA and 1.0 g l^{-1} AC was suitable for *in vitro* root induction of *P. vietnamense* (Table 4, Fig. 1d, e).

Table 4. Effect of NAA and activated charcoal on *in vitro* root induction of *P. vietnamense* after culturing for 6 weeks

Treatments	NAA (mg l^{-1})	AC (g l^{-1})	No. tested samples	No rooting samples	Rooting rate (%)	Root patterns
T1	0		30	3	10.00^{g}	Tiny
T2	0.5		30	21	70.00^{c}	Tiny
T3	1.0		30	18	60.00^{d}	Tiny
T4	1.5		30	13	43.33^{e}	Tiny
T5	2.0		30	7	23.33^{f}	Tiny
T6	0.5	0.5	30	23	81.11^{b}	Tiny
T7		1.0	30	27	88.89^{a}	Long hair
T8		1.5	30	24	83.33^{ab}	Long hair
T9		2.0	30	22	74.44^{c}	Tiny

Data are given as the mean of the rooting rate after 6 weeks of culture. Data expressed in different lower case letters are significantly different at $P< 0.05$ level by the LSD test (Duncan, 1955 [17]).

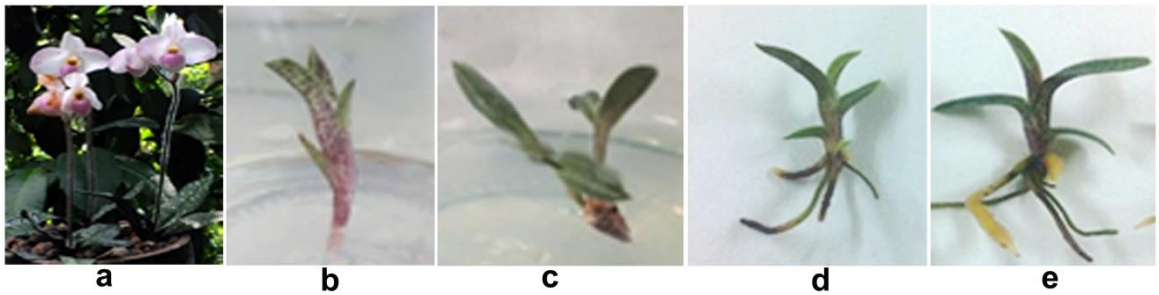

Figure 1. *In Vitro* propagation of *P. vietnamense* through axillary bud explants

a) *P. vietnamense* plants were collected in wild population in Thai Nguyen province and transplanted into containers and grown in the greenhouse; b) Sterilized buds were cultured on 1/2 MS for shoot induction; c) Shoot multiplication on 1/2 MS + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} NAA; d, e**)** Root induction on 1/2 MS + 0.5 mg l^{-1} NAA + 1.0 g l^{-1} AC.

Discussion

There are many reports of surface sterilization in plant tissue culture using $HgCl_2$ [18-20]**.** The effect of $HgCl_2$ on the efficiency of sterilization depends on plant and type of explants. For example, Firoz et al. (2016) compared the effect of various sterilization agents for *in vitro* seed germination and micropropagation of *Cucumis sativus* [20]. They concluded that $HgCl_2$ was the most effective on seed sterilization of cucumber. At low level of 0.1% $HgCl_2$ for 4 min treatment showed highest (84.44%) of survival cucumber seeds after 3 weeks of inoculation, whereas, sterilization with H_2O_2, NaOCl, and Bavistin did not give any satisfactory results [20]. Similarly, Sagaya and Divakar (2016) also indicated that 0.1% $HgCl_2$ was better than other agents for explant sterilization in *in vitro* propagation of *Dendrobium* orchid, however duration time treatment depended on types of explants as flower stalk (12 min), leaf segment (3 min). Interestingly, high concentration of $HgCl_2$ (20%) was required for sterilization of node and internode explants for 5 to 20 min [21].

In addition, to improve effectiveness in sterilization, ethanol that is a strong phytotoxic sterilizing agent is normally applied earlier to treatment with other compounds. In this study, before sterilizing with $HgCl_2$, we treated explants with 70% ethanol and afterward replacing by $HgCl_2$ solution with a various concentration of $HgCl_2$ (0.1, 0.15, 0.2) with different duration time treatment for each (5, 10, 15). The results showed that single treatment with 70% was not effective showing 100% of explants were contaminated (T1, Table 1). Previous reports indicated that ethanol is rapidly bactericidal rather than bacteriostatic against bacterial vegetative cells, fungicidal and virucidal but do not destroy spores [20], [22]. Although, $HgCl_2$ was a strong sterilizing agent, it also harmed with explants [20]. We recorded that the frequency of browning and death explants were increased when increasing concentration or duration exposure with $HgCl_2$ (Table 1). Only 65.55% survival explants were recorded, this might be due to bleaching action of two chloride atoms and also ions that combines strongly with proteins and causing death of organisms [18]. This phenomenon was also reported in previous studies [23], [24].

Furthermore, the effect of medium composition has been also tested on *Paphiopedilum* species by Zeng et al. (2016) [2]. Most studies concluded that the basal medium was affected not only on seed germination but also on seedling development. At high total mineral content in medium might cause the inhibition of seedling development in micropropagation of *Paphiopedilum* species. Most *Paphiopedilum* species prefer a low level of mineral composition of medium such that 1/2, 1/4, 1/5, 1/6 or 1/8 MS have been investigated to be more suitable [25-27]. However, the composition of basal medium for *in vitro* propagation of *Paphiopedilum* species was difference. For example, *P. wardii* showed higher seed germination rate on 1/2 MS than on MS or 1/4 MS medium [1], [7]. In contrast, *P. ciliolare* displayed highest seed germination on MS medium [8]. Long et al (2010) indicated that seed germination of *P. villosum* var. *densissimum* was higher on VW than on 1/4 MS, RE or KC media [28]. In this study, we also found that half strength of MS medium was suitable for *in vitro* propagation of *P. vietnamense*. Cytokinine and auxin combination has been widely applied in plant *in vitro* propagation, such as orchids. Martin et al. (2005) compared the effect of BAP and kinetin on shoot multiplication using *Dendrobium macrostachyum*, as a result 6.97 µM kinetin was the most efficient, of which, 50% explants showed 1.4 shoots per lower stalk node. While all tested BAP concentrations showed less than 1 shoot per explant after 60 days of culture. They also had tested the combination of BAP or kinetin with NAA but there were not significant differences for *D. macrostachyum* [29]. No influence of BAP or kinetin and NAA combination was also demonstrated in other *Dendrobium* species. In contrast, shoot induction of *Paphilopendilum* species was influenced by BAP rather than kinetin [28]. Interestingly, concentration of BAP used for shoot multiplication is different for species. For example, *P. villosum* var. *densissimum* was produced more shoots on medium having 3.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA [26]; *P. insigne* for the combinations of 0.2 mg l^{-1} BAP with 0.1 mg l^{-1} NAA [2]. *P. bellatulum*, the combination of 5.5 mg l^{-1} BAP with 0.5 mg l^{-1} NAA induced maximum shoot organogenesis [26].

Conclusions

P. vietnamense is one of endemic lady's slipper orchids in Vietnam that is distributed in narrow habitats and assumed that very fewer individuals in wild population as a result of over collection. In order to establish the procedure for propagation, we studied some factors that effected on the efficiency of *in vitro* propagation of *P. vietnamense*. The results indicated that axillary buds can be used as a good material for *in vitro* culture. The 0.2% $HgCl_2$ can be used as a suitable concentration for sterilizing *P. vietnamense* explants in within 5 to 10 min. Moreover, the 1/2 MS medium which comprised 100 mg l^{-1} inositol, 30 g l^{-1} sucrose, 120 ml l^{-1} CW, 2.0 mg l^{-1} BA and 1.0 mg l^{-1} NAA was the most suitable for shoot regeneration and multiplication. In addition, the results also indicated that 1/2 MS medium having 0.5 mg l^{-1} NAA and 1.0 g l^{-1} AC gave the best results for root induction and plantlets.

Conflict of Interest

The authors declare no conflicts of interest.

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