Tạp chí Khoa học Xã hội, Nhân văn và Giáo dục - ISSN: 1859 - 4603

UED JOURNAL OF SOCIAL SCIENCES, HUMANITIES & EDUCATION

IN VITRO PROPAGATION OF A RARE AND ENDANGERED ORCHID SPECIES *Hygrochilus parishii* Pfitz

Received:
05 – 09 – 2019
Accepted:
11 – 11 – 2019
http://jshe.ued.udn.vn/

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Abstract: In the study presented in this article, we found culture conditions for effective *in vitro* propagation of a rare and endangered orchid species *Hygrochilus parishii* Pfitz. Rapid multiplication of seed-derived protocorms was obtained on MS (Murashige và Skoog, 1962) medium containing 0.5 mg/L BA (6-benzylaminopurine) and 0.25 mg/L IBA (indole-3-butyric acid). The shoot generation and multiplication were optimum for protocorms cultured on the MS medium containing 1.5 mg/L BA, which generated 2.17 shoots/protocorm at the week eight. The shoot growth reached the highest value of 1.1 cm after 8 weeks cultivation on the medium containing 3 mg/L BA. The roots were induced in the presence of 1.5 mg/L NAA (1-naphthaleneacetic acid) which generated 2.6 roots/shoot and 2.3 cm root length at the week eight. The *in vitro* plantlets also acclimated well in greenhouse conditions.

Key words: *Hygrochilus parishii* Pfitz; protocorm; shoot generation; *in vitro* propagation; tissue culture.

1. Introduction

Vietnam is home to a large number of rare and endemic orchid species of Orchidaceae that is one of the largest and most diverse families of flowering plants [1, 2, 3]. However, some of these species are under threat of extinction due to extensive collection and habitat destruction by anthropogenic activities [4]. *Hygrochilus parishii* Pfitz, an epiphytic and monopodial orchid species, is naturally found in evergreen and semi-deciduous forests at elevations up to 2500 meters locating in North East India, Eastern Himalayas, Myanmar, Thailand, and Southern China [3, 5]. This species is also recognized at some forests that belong to Central and South Annamese, and South Indochinese floristic provinces including Vietnam [1, 3]. Due to its long flowering period and multicoloured and fragrant nature, *H. parishii* Pfitz has attracted the attention of floriculturists and orchid lovers [5]. Yet, the increasing market demand, together with the low multiplication rate of conventional propagation methods, has endangered the survival of this orchid. The International Union for Conservation of Nature (IUCN) has considered *H. parishii* Pfitz as an endangered species that needs to be protected [3]. Consequently, the plant cell tissue culture approach which produces a large numbers of plantlets within a short period has become an ideal solution for preserving *H. parishii* Pfitz from the extinction and commercialization purposes.

Shadang *et al*. (2007, 2017) reported effects of various culture media on *in vitro* plantlet regeneration from *H. parishii* Pfitz seeds, as well as on the protocorm and shoot formation from the cultured leaf and root explants; however, culture conditions that induce *in vitro* multiplication of this species remain to be investigated. Thus, in the study presented in this article, we investigated the effects of different culture conditions on the protocorm, shoot multiplication, and plantlet regeneration of *H. parishii* Pfitz.

2. Materials and Methods

2.1. Seed germination

Undihisced green capsules at age of 4-5 months, which were collected from *H. parishii* Pfitz grown in Kon Ka Kinh National Park (Pleiku, Vietnam), were

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surface sterilized by sequent washing steps of EtOH 70% (v/v) for 30 s, $HgCl_2$ 0.1% (v/v) for 5 min, and then NaOCl 5% (v/v) for 15 min [6]. The seeds were then distributed directly onto MS medium [7] and maintained for the seed's germination within 8 weeks [5].

2.2. Protocorm and shoot multiplication

Three-millimeter-diameter clusters of seed-derived protocorms were separated and cultured on the MS medium contained 0.5 mg/L BA (6-benzylaminopurine) combined with 0.1-0.5 mg/L IBA (indole-3-butyric acid) for the protocorm multiplication. The percentage of clusters induced new protocorms and their growth characteristics were observed in the fourth week after the onset of culture. Subsequently, the 3-mm-diameter clusters were cultured on the MS medium containing 0.5-2 mg/L KIN (kinetin) or 0.5-2 mg/L BA for the shoot regeneration and multiplication. The time of emerging shoots, numbers of shoots per a protocorm cluster, and shoot height were observed after 8 weeks culture.

2.3. Plantlet regeneration

The protocorm-derived shoots with 3 mm height were cultured on the MS medium containing 0.5 g/L active charcoal (AC) supplemented with 2-3.5 mg/L BA or 2.5 mg/L BA in combination with 0.5-2 mg/L NAA. The shoot growth was observed in the eighth week after the onset of treatment through number of leaves per shoot, shoot height, and shoot growth characteristics. Subsequently, the shoots growing up to the height of 1 cm were transferred to the MS medium containing 0.5 g/L AC in combination with 0.5-2 mg/L NAA for the rooting. The percentage of root-formed shoots, root length, and number of roots were observed after 8 weeks culture.

2.4. Growth conditions and greenhouse acclimation

All the culture media were added with 30 g/L sucrose and 8 g/L agarose as a carbon source and solidifying agent. Fifth milliliters of the media were delivered to a 250 mL vessels. pH of the media was adjusted to 5.8±1 prior to autoclaving at 121°C for 20 min. At least fifteen protocorm clusters or shoots were cultured on each the medium and three replicates were carried out. All the cultures were incubated at 25±1ºC under a 14/10 h (day/night) photoperiod and illumination of 2000 lux [5]. The obtained data were analyzed via an analysis of variance using Duncan's multiple range test. The data analysis was carried out using SAS version 9.3.

In vitro regenerated plantlets with one-cm-height shoots and 2-3 roots were removed from the vessels and transplanted to plastic trays with 5-cm-diameter holes containing sphagnum moss. The plantlets were acclimated under the condition of a greenhouse with average temperature of 28±5ºC and air humidity of 80-90%, at the Faculty of Biology and Environmental Science, University of Education and Science - The University of Danang. The plantlets were watered once per two days to maintain humidity of the sphagnum moss. The survival plantlet rate of one-hundred transplanted plants was measured at the second week after the transplantation.

3. Results and Discussion

3.1. Effects of BA and IBA combination on protocorm multiplication

A protocorm is an intermediate structure of orchid seeds and seedlings, which is an organized shape and developed from zygotic embryos [2]. A protocorm is able to produce new protocorms from itself once grown in a suitable culture medium and this ability may be promoted by plant growth regulators (PGRs) presenting in the medium. In this study, the addition of 0.5 mg/L BA and 0.1-0.5 mg/L IBA combination showed significantly positive effects on *H. parishii* Pfitz protocorm multiplication, compared to the MS medium without the PGRs (Table 1). Among them, the highest percentage of new protocorm-induced clusters of 100% was obtained via a combination of 0.5 mg/L BA and 0.25 mg/L IBA (Table 1). The protocorm clusters also showed green and vigorous growth with an approximate two-fold increase of biomass on the medium in the fourth week after the onset of culture (Figure 1a). Shadang *et al.* (2007) previously reported on the proliferation of *H. parishii* Pfitz protocorms cultured on the ½ strength MS medium containing 2 mg/L NAA [5]. Our results revealed that the combination of 0.5 mg/L BA and 0.25 mg/L IBA also enhanced the protocorm multiplication of this species. It is reported that the presence of BA and IBA in the culture medium induced

protocorm proliferation of numerous orchid species, such as *Dendrobium chrysotoxum* [8] and *Cephalanthera falcata* [9]. The multiplication of protocorms would increase *in vitro* propagation efficiency of the orchids.

Table 1. *Effects of BA and IBA combination on protocorm multiplication in the fourth week after the onset of culture*

BA (mg/L)	IBA (mg/L)	Percentage of protocorm-induced clusters (%)	Growth characteristics
0	0	0	Protocorms became brown and tended to die.
0.5	0.1	20	Protocorms became yellow and weak.
0.5	0.25	100	Protocorms having green and vigorous growth.
0.5	0.5	30	Protocorm became yellow and weak.

3.2. Effects of BA and KIN on shoot multiplication

BA and KIN are cytokinins that have been commonly used to induce shoot regeneration and promote growth of shoots from protocorms of different orchids [2, 10-12]. In this study, the presence of 0.5-2 mg/L BA in the MS medium also induced the regeneration of *H. parishii* Pfitz shoots. Meanwhile, the MS medium alone as well as the addition of KIN at 0.5-2 mg/L concentrations did not stimulate the shoot formation, and the cultured clusters tended to be gradually weak and died on these media after 8 weeks' culture (Table 2). The vigorous shoots were emerged on the medium containing 0.5-2 mg/L BA within 35-47 days after the onset of culture, and the shoot multiplication was best at 1.5 mg/L BA concentration in which 2.17 vigorous and green shoots per protocorm cluster were observed in the eighth week (Table 2 and Figure 1b), while one shoot was induced at lower or higher BA concentrations (Table 2). The regenerated shoots reached 0.3-0.74 cm in height. Shadang *et al.* (2007) also reported high rates up to 70% of shoot-induced protocorms of *H. parishii* Pfitz in the same culture medium, but the shoot multiplication rate was not determined [5]. Thus, the rates were revealed in our study. At the same BA concentration, the best shoot multiplication from protocorm-like bodies was also observed from *Dendrobium nobile* [13] and *Dendrobium primulinum* Lindl. [14]. In summary, these findings indicate that BA is a cytokinin suitable for the shoot regeneration and multiplication of these orchids, which would contribute to the enhancement of *in vitro* propagation efficiency.

Table 2. *Effects of BA and KIN on shoot multiplication 8 weeks after the onset of culture*

KIN (mg/L)	BA (mg/L)	Time of emerging shoots (d)	No. of shoots/protocorm cluster	Height (cm)	Shoot characteristic
0	0	ND	ND	ND	No shoots were observed.
0.5	0	ND	ND	ND	No shoots were observed.
1	0	ND	ND	ND	No shoots were observed.
1.5	0	ND	ND	ND	No shoots were observed.
2	0	ND	ND	ND	No shoots were observed.
0	0.5	35	1.00^{b}	0.52^{b}	Green and normal shoots.
0	1	36	1.00^{b}	0.40^{c}	Greenish and weak shoots.
0	1.5	21	2.17^{a}	0.74^{a}	Green and vigorous shoots.
0	2	47	1.00^{b}	0.30^{c}	Greenish and weak shoots.

**Different letters in a column indicate significant difference between mean values according to Duncan's multiple range test at $p \leq 0.05$.*

Table 3. *Effects of BA and NAA on shoot growth in the eighth week after the onset of culture*

BA (mg/L)	NAA (mg/L)	No. of leaves /shoots	Height (cm)	Shoot characteristics
0	0	4.80^{ab}	0.50^{c}	Light green and large leaves.
2	0	2.70^{d}	0.59^{c}	Light green and large leaves.
2.5	0	5.10^{a}	0.91^{ab}	Green and large leaves.
3	0	5.30^{a}	1.10^{a}	Green and large leaves.
3.5	0	3.90^{b}	0.68^{bc}	Greenish and large leaves.
2.5	0.5	4.90^{ab}	0.90^{ab}	Green and large leaves.
2.5	1	4.00^{b}	0.86^{b}	Green and large leaves.
2.5	1.5	4.00^{b}	0.69^{bc}	Green and large leaves.
2.5	2	3.40^{c}	0.62^{c}	Greenish and large leaves.

**Different letters in a column indicate significant difference between mean values according to Duncan's multiple range test at $p \leq 0.05$.*

3.3. Effects of BA and NAA on plantlet regeneration

Although the multiplication of *H. parishii* Pfitz shoots was obtained with the presence of 1.5 mg/L BA, the height of shoots obtained in the eighth week was not enough to generate *in vitro* whole plantlet for transplanting to greenhouse. Thus, the shoots with 0.5 cm in height were transferred to the MS medium containing various concentrations of BA and NAA to enhance the height. In addition, 0.5 g/L AC was also added to the medium to eliminate negative impacts caused by compounds that are separated from the shoots [5, 15, 16]. The results showed that the presence of 2-3.5 mg/L BA or 2.5 mg/L BA in combination with 0.5-2 mg/L NAA had positive effects on shoots growth. Compared to the MS medium, increasing the BA concentrations up to 2.5-3 mg/L or combination between 2.5 mg/L BA and 0.5-1.5 mg/L NAA significantly enhanced the growth of shoots, resulting in 0.69-1.1 cm in height and 4-5.3 green and vigorous leaves (Table 3). The highest growth of shoots was observed on the medium containing 3 mg/L BA, on which the shoots grew well with 1.1 cm in height and 5.3 vigorous leaves (Table 3, Figure 1c). In agreement with our results, Choopeng and Luksanasut showed the growth enhancement of *H. parishii* Pfitz shoots on the MS medium containing 2 mg/L BA and 0.5 mg/L NAA [10]. In addition, our study also showed that individual BA treatments at high concentrations also promote the growth of shoots.

The rooting is a necessary step to generate whole *in vitro* plantlets. In the present study, the one-cm-height shoots showed positive responses in the rooting when transferred to the medium containing 0.5 g/L AC and 0.5-2.5 mg/L NAA, but the levels were different depending on the NAA concentrations (Table 4). The rooting ability was gradually increased with increasing NAA concentrations from 0.5 to 2 mg/L, but it tended to be decreased at higher concentrations of 2 mg/L NAA (Table 4). The highest values of rooting were observed on the medium containing 2 mg/L NAA, which all the cultured shoots formed roots with 2.6 roots/shoot 8 weeks after the onset of culture. In addition, the shoots showed vigorous growth on this culture medium (Table 4, Figure 1d). In the previous study, Shadang *et al.* (2007) also revealed that the early rooting of *H. parishii* Pfitz seedlings maintained on the V&W medium having 15% coconut milk and 10% banana extract in place of sucrose [5]. In addition, the present study suggests that NAA promotes the rooting of cultured shoots of *H. parishii* Pfitz. The similar results were observed from other orchids, such as *Esmeralda clarkei* [17], *Anoectochilus setaceus* [18]. In addition, the regenerated plantlets of *H. parishii* Pfitz showed a good acclimation when being transferred to the condition of greenhouse. The survival rate of plantlets was above 83% after 2 weeks' transplantation (data not shown) and they maintained the growth under the culture condition (Fig 1).

Table 4. *Effects of NAA on rooting in the eighth week after the onset of culture*

NAA (mg/L)	Percentage of root-induced shoots (%)	No. of roots/ shoots	Length (cm)
0	50	1.20^{b}	0.50^{a}
0.5	84	0.70^{a}	0.55^{a}
1	92	1.00^{ab}	0.72^{a}
1.5	100	2.60^{d}	2.30^{c}
2	100	1.90^{c}	1.90^{b}
2.5	90	1.40^{b}	1.57^{b}

**Different letters in a column indicate significant difference between mean values according to Duncan's multiple range test at p≤ 0.05.*

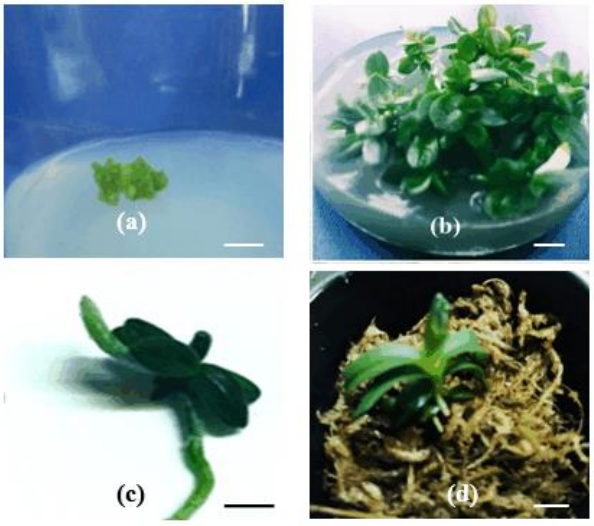

Figure 1. *Effects of culture conditions on in vitro propagation of H. parishii Pfitz.* Proliferated protocorms (a) and regenerated shoots (b) and roots (c) on the media in the eighth week after the onset of culture. Regenerated plantlets acclimated in greenhouse condition after 2 weeks transplantation. Bar is equal with 1 cm

4. Conclusion

The findings in this study revealed culture conditions which could be applied to the *in vitro* propagation and conservation of orchid species *H. parishii* Pfitz. The presence of BA and NAA in individuals or combinations at various concentrations promoted the protocorm and shoot multiplication which would enhance the efficiency of *in vitro* propagation of this orchid.

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NHÂN GIỐNG *IN VITRO* CÂY CẨM BÁO (*Hygrochilus parishii* Pfitz): MỘT LOÀI PHONG LAN QUÝ HIẾM

Tóm tắt: Trong nghiên cứu này, chúng tôi đã thiết lập các điều kiện nuôi cấy *in vitro* cho việc nhân giống hiệu quả cây lan Cẩm báo (*Hygrochilus parishii* Pfitz), một loài phong lan quý hiếm. Các protocorm hình thành từ hạt đã nhân nhanh tốt nhất trên môi trường MS (Murashige và Skoog, 1962) có bổ sung 0,5 mg/L BA (6-benzylaminopurine) và 0,25 mg/L IBA (indole-3-butyric acid). Các chồi đã được hình thành từ protocorm và nhân nhanh trên môi trường có bổ sung 1,5 mg/L BA, với hệ số nhân đạt 2,17 chồi/protocorm sau 8 tuần. Tiếp đó, sự tăng trưởng của chồi đạt được trên môi trường chứa 3,0 mg/L BA, với chiều cao 1,1 cm sau 8 tuần nuôi cấy. Toàn bộ các chồi đã tạo rễ với 2,6 rễ/ chồi sau 8 tuần trên môi trường có bổ sung 1,5 mg/L NAA (1-naphthaleneacetic acid). Cây con tái sinh cho thấy sự thích nghi tốt với điều kiện vườn ươm.

Từ khóa: lan Cẩm báo; protocorm; tái sinh chồi; nhân giống *in vitro*; nuôi cấy mô.