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Topical Review

SOME METHODS IN MICROPROPAGATION AND BREEDING OF *Paphiopedilum* spp.

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Abstract. *Paphiopedilum* orchids, the most popular and rare orchid genera, are used as potted flowers and cut flowers. In nature condition, the orchid population is threatened with extinction due to over-exploitation and altered habitat. Reduction in this orchids via large-scale micropropagation is a better option to reduce the wild harvest, meets the commercial needs as well as conserves this threatened orchid. This orchid can be propagated by germinating seeds under *in vitro* culture condition; however, they are difficult for micropropagation, especially regenerating plantlets from *in vitro* culture. This topical review aims to provide some techniques on *Paphiopedilum* spp. micropropagation as well as improve the culture process from *ex vitro*-derived explants of mature plants.

Keywords: *Paphiopedilum*, micropropagation, protocol.

Classification numbers: 1.3, 1.3.2.

1. GENERAL INTRODUCTION

Paphiopedilum spp. are often propagated via axillary shoots - derived mother plant; however, it is very inefficient and waste of time [1, 2]. In a natural condition, *Paphiopedilum* seeds were germinated very slowly cause of the lack of endosperm [3]. Knudson [4] first discovered the orchid germination of non-symbiotic seedlings, and germination of *in vitro* orchid seeds can be found in other studies [5, 6]. In *Paphiopedilum* spp., some seedlings germination processes have also been described [7 - 11]. However, the germination rate is low and affected by unknown factors [7, 12]. Success rate of *Paphiopedilum* micropropagation via *ex vitro*-derived explants is very low due to the rare sample source, re-infection of fungi and bacteria of *ex vitro*-derived cultures and poor growth of plantlets under *in vitro* condition [13, 14]. In addition, *Paphiopedilum* species and hybrids are still commercially viable and not asexual [1, 14, 15].

2. SOME TECHNIQUES IN MICROPROPAGATION AND BREEDING

2.1. Tissue culture

Paphiopedilum is an ornamental plant of high value and first micropropagation by Bubeck [16], while Morel [17] succeeded in cultured on stem apices of *Paphiopedilum*. Moreover, they were difficult to plantlet regeneration under *in vitro* conditions due to rare sample sources, difficulties due to fungal and bacterial re-infection of *ex vitro*-derived explants as well as poor growth of plantlets.

2.2. Explants

The morphogenetic response of *Paphiopedilum* depends on the explant as reviewed by some studies [18, 19]. Up to now, a few micropropagation studies from *ex vitro*-derived explants existed [13-15]. Flower stems (young and mature), tips of leaves and roots, stamens, ovaries and shoot tips of 3 *Paphiopedilum* species (*P. villosum*, *P. fairrieanum*, and *P. insigne*) were used as the materials for callus induction; shoot tips cultured on Heller medium adding 1.0 $mg.L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) with or without 0.5 $mg.L^{-1}$ N6-benzyladenine (BA) gave the highest callus induction rate [13]. Huang [14] indicated that a shoot tip meristems (2-3 mm) could be used as explants to effectively improve the success rate of disinfection, although the explants grew slowly, most of them were necrotic. Liao *et al*. [15] showed that transverse slices (flower buds) of *Paphiopedilum* hybrids (*P*. Deperle and *P*. Armeni White) could create secondary shoot induction and plantlet formation on modified (Murashige and Skoog (MS) [20]) medium containing 1.0 $mg.L^{-1}$ BA and 1.0 $mg.L^{-1}$ 2,4-D, or on modified MS medium supplemented with 10.0 $mg.L^{-1}$ BA and 5.0 $mg.L^{-1}$ α-naphthaleneacetic acid (NAA), respectively. This research found that flower buds (1.5 - 3.0 cm) from *Paphiopedilum* Deperle were able to produce shoots, but only sections of flower buds (> 2.5 cm) of *Paphiopedilum* Armeni White were regenerable [15].

Seeds, protocorm-like bodies (PLBs) and seedlings were used as initial materials for *Paphiopedilum* micropropagation studies, including that shoot apex [2, 21], internodes [1, 2, 22], PLBs [23, 24] or leaves [23, 25]. Huang [14] reported that secondary shoots and plantlets formation were obtained in cultured on shoot tips of *Paphiopedilum* hybrid (*P. philippinense* × *P.* Susan Booth) derived- *in vitro* seedlings. Chen *et al*. [8, 26] showed that secondary shoots and plantlets formation were induced by using internode and leaf explants of the hybrid PH59 and PH60 (*P. philippineese* hybrid). Lin *et al*. [23] reported callus induction and plantlet formation via leaves derived- *in vitro* seedlings of *P. callosum* 'Oakhi' × *P. lawrenceanum* 'Tradition'. The rate of shoot regeneration (75 %) of *P. delenatii* were induced by liquid culture medium of wounding nodal segments derived- *in vitro* seedlings [10, 22, 23].

Nhut *et al*. [10] reported that non-shoot formation in *P. delenatii* shoot non-wounded treatment in all types of media treatment. The green and vigorous shoots (2.3 shoots) were obtained when wounded seedlings were cultured on solid MS medium adding 0.25 $mg.L^{-1}$ thidiazuron (TDZ) combined with 0.5 $mg.L^{-1}$ NAA.

In *Paphiopedilum* micropropagation, the *Paphiopedilum* hybrids explants were used because of likely easier to micropropagation than those in native species [13 – 15, 26].

2.3. Basal media

The basal media used in the *Paphiopedilum* micropropagation including Heller [13], MS [10, 14, 22], modified MS [15, 25, 26] and ½ MS [1, 2, 23, 27], although no comparative experiment exists on the effect of media in the micropagation of *Paphiopedilum* spp.

2.4. Plant growth regulators

The *Paphiopedilum* spp. micropropagation via shoot regeneration and direct shoot-bud formation and the plantlet regeneration via PLB formation have been investigated as summarized in Table 1. The plant growth regulators (PGRs) used in *Paphiopedilum* micropropagation include auxin (2,4-D, IBA, and NAA), and cytokinin (BA, TDZ, Zeatin and Kinetin (KIN)). The types and concentrations of exogenous PGRs required for micropropagation of orchids are specific to a specific species [11].

Table 1. The PGRs were used in the micropropagation of *Paphiopedilum* spp.

PGRs	*Paphiopedilum* spp.	Basal medium	Explants	References
2,4-D	*P.* hybrids	Thomale	Stem apices	[17]
2,4-D, BA, NAA and pCPA	*P. villosum* (Lindl.) Stein, *P. fairrieanum* (Lindl.) Stein, *P. insigne* (Wall. ex Lindl.) Pfitzer	Heller	Stem apices	[13]
NAA, 2ip, BA	*P.* hybrids	MS	Shoot apices	[14]
2,4-D, TDZ, PBOA	*P. callosum* 'Oakhi' × *P. lawrenceanum* 'Tradition'	½ MS	Seed-derived Protocorms	[23]
TDZ, BA, NAA	*P.* hybrids	Modified MS	Seedlings *in vitro*	[21]
2,4-D, TDZ	*P. philippinense* hybrids (PH59, PH60)	Modified ½ MS	Nodal stem of seedlings *in vitro*	[26]
TDZ, BA, NAA	*P. delenatii* Guillaumin	½ MS, MS, Knudson C	Wounded seedlings	[10]
TDZ, BA, Zeatin	*P. delenatii* Guillaumin	Modified MS	Nodal stem of seedlings *in vitro*	[22]
2,4-D, TDZ, NAA	*P.* Alma Gavaert	½ MS	Seeds	[27]
2,4-D, Kn, TDZ, BA, NAA, IBA	*Paphiopedilum* spp.	¼ MS	Seed-derived protocorms	[11]
Kn, BA	*P. rothschildianum* (Rchb.f.) Stein	½ MS	Nodal stem of seedlings *in vitro*	[2]
2,4-D, BA	*P.* Deperle, *P.* Armeni White	Modified MS	Cross-sectioned flower buds	[15]

Nhut *et al*. [10] reported that the liquid or semi-solid media significantly affected the shoot regeneration in wounded *in vitro* seedlings. The wounded seedlings cultured on liquid medium could absorb medium and PGRs more easily than solid medium. In semi-solid media containing

0.5 mg.L^{-1} NAA, the number of newly shoots tended to decrease as the concentration of TDZ increased (0.25 - 2.5 mg.L^{-1}). In contrast, the number of shoots (1.2-3.0 shoots/explant) formed increased significantly in liquid medium with increased TDZ concentration. The results suggested that TDZ effected on explants depending significantly on the physical properties of the medium. On liquid or semi-solid media containing TDZ (0.5, 1.0, or 3.0 mg.L^{-1}), the effect of liquid medium on shoot regeneration was higher than semi-solid media. At 1.0 mg.L^{-1} TDZ, the number of shoots (5.2 shoots/explant) formed in liquid media was almost 5-fold greater than on semi-solid media (1.1 shoots/explant) and over 2-fold greater (2.2 shoots/explant) than those in others, suggested that auxin may play an inhibitory role in liquid media.

2.5. Callus or PLB induction and plant regeneration

Up to now, there have been some studies in *Paphiopedilum* callus induction, due to initial difficulties in self-induction, slowly growth rate as well as lowly regeneration rate, and browning of callus tissue [1, 11, 12, 23].

Lin *et al*. [23] reported that *Paphiopedilum* hybrid (*P. callosum* 'Oakhi' × *P. lawrenceanum* 'Tradition') callus induction from seed-derived protocorms cultured on ½ MS medium containing 1-10 mg.L^{-1} 2,4-D and 0.1-1 mg.L^{-1} TDZ in the darkness condition. On without PGRs in medium, sub-cultured callus showed poor growth and, eventually browning and necrotic. Similar results were observed in callus maintained on medium containing only TDZ or 2,4-D; the sub-cultured callus initially proliferated, increased in mass and then browned and necrotic. Callus cultured on media containing both 2,4-D and TDZ proliferated well and later regenerated on plantlet regeneration media. Medium supplemented with 5 mg.L^{-1} 2,4-D and 1 mg.L^{-1} TDZ was selected as the standard maintenance medium for callus proliferation. Callus could grow on this medium for 3-years without a loss of regeneration capacity; then, forming PLBs and eventually plantlets that could be transplanted to pots and grew well.

Hong *et al*. [27] indicated that seed derived- 5-month-old green capsules (*P.* Alma Gavaert) were calli induction on ½ MS medium supplemented with 5.0 mg.L^{-1} 2,4-D and 1.0 mg.L^{-1} TDZ in the darkness. Calli were further proliferated and maintained without any morphogenesis on the same medium with a 2-month sub-culture interval for more than 2 years. Sub-culture on ½ MS medium supplemented with 5.0 mg.L^{-1} NAA, PLBs/shoot buds (4.7 PLBs) formed from each explant after 120 days of culture.

Ng and Saleh [1] reported that nodal stem derived- *in vitro* seedlings of *P. rothschildianum* cultured on ½ MS supplemented with 0.86 mg.L^{-1} KIN and 2 g.L^{-1} peptone were highest callus induction than those others [1]. The callus induction could proliferation and PLBs formation from the surface of the proliferating callus (sub-cultured on the similar medium). The number of secondary PLBs (4.1 PLBs/explant) formed on ½ MS medium supplemented with 0.86 mg.L^{-1} KIN was highest as compared to others after 8 weeks of culture. Conversely, the addition of BA inhibited the secondary PLB induction. The secondary PLBs continued to proliferate further and formed new PLBs/secondary PLB (9.5 - 12.1 PLBs) after sub-culture on ½ MS medium supplemented with 60 g.L^{-1} banana homogenate (BH) and without PGRs.

2.6. Shoot multiplication

Shoot multiplication of *Paphiopedilum* spp. affected by species, explant type, PGRs, etc. is described in Table 2.

Table 2. Shoot multiplication of *Paphiopedilum* spp.

Species	Explants	Medium	PGRs	References
P. armeniacum, *P. insigne*, *P. villosum* var. *densissimum*, *P. bellatulum*	Seedlings	MS	BA, NAA	[11]
Paphiopedilum sp.	Axillary branches	MS	2iP, NAA, Adenine sulfate, CW	[14]
Paphiopedilum hybrids	Seedlings	MS	BA, NAA	[21]
P. delenatii	Nodal segments	Modified MS	TDZ	[22]
P. hangianum	Protocorm and seedling	½ MS	BA, NAA	[24]
P. philippinense hybrids	Stem nodal explants	Modified ½ MS	TDZ, 2,4-D	[26]
P. Alma Gavaert	Seedlings	MS	KIN	[27]
P. callosum, *P. gratrixianum*, ,*P. delenatii*	Shoot tip	Liquid SH	TDZ	[28]

2.7. Carbon source

Sucrose concentration (62 $g.L^{-1}$) was better for rooting of *Paphiopedilum* hybrid than those in other concentrations of sucrose and maltose [21]. No differences were observed between the two sugars regarding their effectiveness on shoot proliferation.

2.8. Organic amendments

The micropropagation of *Paphiopedilum* are also affected by adding natural complexes, including CW, BH, potato homogenate (PH), tomato homogenate (TH), tryptone and peptone, hydrolyzed casein (HC), and others [1, 21]. Huang *et al*. [21] reported that 15 % CW and 1.0 $g.L^{-1}$ HC were suitable to enhance both shoot regeneration and root formation. PH (10 $g.L^{-1}$) could promote the growth of adventitious buds, but had no effect on rooting. BH promoted the formation of adventitious buds, but inhibited rooting, especially at high concentrations (40 and 60 $mg.L^{-1}$); 20 $g.L^{-1}$ PH was suitable for the growth of adventitious buds.

Ng and Saleh [1] tested ½ MS medium adding BH, PH and TH (15 - 60 $g.L^{-1}$) or CW (5 – 20 %) on PLB formation. CW (20 %) was the most effective organic amendment to facilitate PLB formation of *P. rothschildianum* and subsequent differentiate into plantlets.

Ng *et al*. [2] reported that stem nodal and single shoot explants of *P. rothschildianum* cultured on ½ MS medium without PGRs and organic nitrogen additives could be successfully for shoot induction. The number of shoots were increased by supplementing the peptone and tryptone-peptone in culture medium. The highest number of shoots (2.9 shoots/explant) was obtained on stem nodal cultured on ½ MS medium supplemented with 1.0 $g.L^{-1}$ peptone after 16 weeks of culture; while the highest number of shoots (2.8 shoots) formed on single shoot cultured on ½ MS medium adding 2.0 $g.L^{-1}$ tryptone-peptone. In contrast, peptone could not effectively to shoot induction when single shoot explants were used, except at a low concentration (0.5 $g.L^{-1}$). The addition of higher peptone (1.0 and 2.0 $g.L^{-1}$) inhibited shoot multiplication.

2.9. Plant breeding

In order to commercialize this orchid, there have been a number of studies to propagate; however, the propagation efficiency of *Paphiopedilum* is relatively limited.

Furthermore, there has been little discussion about plant breeding in *Paphiopedilum.* The optimization of the *in vitro* conditions for the *P. delenatii* (Fig. 1) and *P. callosum* micropropagation as well as plantlets regeneration of these orchids by ionization radiation treatment in combination with micropropagation was reported [29]. Several studies investigating hybridization and self-fertilization in *Paphiopedilum* spp. were also carried out [23, 30 - 33].

Figure 1. Paphiopedilum delenatii – an endemic orchid of Viet Nam.

2.10. Internode tissue cultures

Paphiopedilum regeneration by internode segment is not easy. In a study, *P. callosum* young-plants (1.5 cm) were exposed to 8 darklight cycles (14-day dark combined with 1-day light) for elongated stem to increase the number of nodes due to obtain internode tissues [34]. In

this study, callogenesis, PLBs, rooting, and acclimatization were studied. The internode tissue culture presented in this research provides a new way for highly effective micropropagation of *Paphiopedilum* spp. using derived- *ex vitro* explants for conservation and horticultural.

2.11. Polyploid induction

Polyploid induction of *Paphiopedilum villosum* was investigated in the first time [35]. In this study, *P. villosum* shoots were used to material for inducing polyploid induction. The shoots were culture under darkness to form 3 stem-nodes which using nodal explants induced adventitious shoots. These shoots (1.5 cm) derived- 1st nodes culture on SH medium added 0.5 mg.L^{-1} TDZ were pretreated in colchicine solution (different concentrations and durations) and then transferred to rooting medium. Polyploid induction rate (19.88%) in 50 µM colchicine (6-day) treatment was obtained. Tetraploids rate (88.24%) and mixoploids were identified both by flow cytometric analysis (leaf) and chromosome counts (root tip). Shoot regeneration derived-stem nodes and colchicine treatment gave an effective method for the production of polyploid plantlets for further *P. villosum* breeding purposes.

3. CONCLUSION

The micropropagation techniques and breeding methods of *Paphiopedilum* spp. are new approaches contributing to the study of tissue culture techniques for efficient cloning of *Paphiopedilum* species. In this paper, some techniques (shoot tip removal, wounding manipulation, internode tissue cultures, polyploid induction, etc.) are exploited for effective *in vitro* culture of these crops with high multiplication rates. The innovation methods aimed to improve the callus induction, shoot regeneration, plant regeneration, set up protocol from explant sterilized surface, *in vitro* propagation and acclimatization at greenhouse of *Paphiopedilum* spp.

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