Multiplication of Paphiopedilums Using In Vitro Techniques

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ABSTRACT

Paphiopedilum orchids are among the world’s most popular orchid due to their impressively beautiful flowers. The genus derives its name from the Greek words paphos (Venus) and pedilon (sandal) in allusion to the lady’s slipper-like floral lip of the constituent species. In spite of their tremendous popularity in the floricultural trade, the paphs are often in short supply. The conventional propagation method through division of the axillary bud from the mother plant is very inefficient and time consuming because only one new growth can be obtained per year after a mature plant has flowered. Although propagation through in vitro asymbiotic seed germination had provided an alternative mode of propagating these orchids, seed setting and germination rate of many Paphiopedilum cultivars are extremely low and often affected by many unknown factors. This paper attempts to provide a brief review of the literature available on in vitro propagation systems for different species and hybrids of Paphiopedilum using seeds and other explants.

Keywords:
Slipper orchid, Micropropagation, Plantlet regeneration, 6-benzylaminopurine, 1-naphthaleneacetic acid, Kinetin

INTRODUCTION

The family Orchidaceae is a monocot family of herbaceous perennials that includes terrestrial, saprophytic, lithophytic and epiphytic species [1]. The orchids are primarily known for their intricately fabricated and stunningly beautiful flowers having unique shapes, curious ornamentation and prolonged shelf-life. They are highly prized in the floriculture industry as cut-flowers and pot-plants. Orchids inhabit fragile ecosystem and are extremely sensitive to their micro-environment; their reproductive success in nature largely depends on the availability of suitable pollinators and mycorrhizal association. In recent years, their wild populations have dwindled alarmingly due to ruthless collection by orchid enthusiasts and medicine traders, destruction of habitats by reclamation and shifting cultivation, and killing of their pollinators [2-3]. Consequently, many orchid species have already disappeared from their wild habitats and many more are on the verge of extinction. In fact, whole of family Orchidaceae is included in CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) Appendix II, where the international trade is strictly controlled and monitored. In India, the government has established Biosphere Reserves, National Parks, and Sanctuaries in the orchid rich regions of the country besides banning the export of orchids collected in wild. Incidentally, many orchids have been listed in Schedule VI of Wildlife Protection Act of India, which makes illicit collection of these orchids a cognizable offence [4]. However, the efforts are not as yet commensurate with the dimensions of the problem. The orchid piracy from the wild continues unabated and it is expected to continue as long as these plants enjoy an economic and commercial status [5].

Application of tissue culture techniques appears to be the best solution for mass propagation and conservation of this versatile group of plants. Although many propagation systems have been developed for the orchids, reliable protocols for many important species have remained elusive. The major obstacles in mass clonal propagation of orchids for conservation and commercial purposes are: 1) non-availability of efficient and reliable protocols for seed germination; 2) poor understanding of early seedling growth and development; 3) obligate mycorrhizal association for natural seed germination; 4) selection of most suitable explants for micropropagation, scaling up and automation of the techniques; 5) very slow and laborious vegetative propagation; 6) species specificity of culture medium; 7) limited germination success under controlled lab conditions of many rare and endangered orchid species, and 8) high mortality of in vitro seedlings during transplantation [6]. Therefore development of simple, reliable, economical, efficient and highly reproducible protocols is of utmost importance for the conservation and commercial cultivation of the orchids.

PAPHIOPEDILUM PFLITZ.

Paphiopedilum orchids are among the world’s most popular orchid due to their impressively beautiful flowers. This genus comprises 69 acaulous, terrestrial/saxatilic or sometimes epiphytic species and is distributed from Himalayas and South India through South-East Asia to Philippines, New Guinea, and Solomon Islands [1]. It derives its name from the Greek words paphos (Venus) and pedilon (sandal) in allusion to the lady’s slipper-like floral lip of the constituent species. Paphiopedilums (paphs) stand distinct in having persistent, waxy-leathery, and glossy-mottled green leaves; one- to two-flowered erect, hairy, and often purplish-brown scapes; and flowers with very distinctly patterned dorsal sepal, a vertical synsepal formed through fusion of two lateral sepals, and strikingly spotted or warted petals.

Paphs are amenable to frequent exchange of gene pools amongst themselves and related genera; hundred of artificial hybrids have been produced utilizing the showy character of the dorsal sepal in the wild species. In fact, the curious shape,
size, and colour of their long-lived flowers (4-6 wks) together with the ease with which they can be cultured have enabled paphs to compete successfully for admiration with cattleyas and cymbidiums in the cut-flower market.

In India, the genus is represented by 9 species [8] distributed primarily in the North-East Himalayan and adjacent (Khasia and Janitia) hills within an altitudinal range of 750-2000 m; *Paphiopedilum druryi* (Bedd.) Stein is, however, distributed in Travancore and Agasthamalai hills of South India. Most of the Indian species are getting rare in their natural habitats due to large-scale commercial exploitation and habitat destruction. In fact, species like *P. fairrieaum* (Lindl.) Stein, *P. hirsutissimum* (Lindl. ex Hook.) Stein, *P. insigne* (Wall. ex Lindl.) Stein, and *P. villosum* (Lindl.) Stein are endangered of survival and figure prominently in the Indian Red Data Book [9].

Paphs grow better on highly porous organic compost in well-drained pots. Their water requirements are very low during winter but they do not like to be dried out. The species with mottled leaves do well in warm and moist greenhouse, and the ones with glossy leaves generally prefer cooler conditions. These plants are usually propagated through division while repotting during early spring.

**PROPAGATION**

In spite of their tremendous popularity in the floricultural trade, the paphs are often in short supply. The conventional propagation method through division of the auxillary bud from the mother plant is very inefficient and time consuming because only one new growth can be obtained per year after a mature plant has flowered. Although propagation through in vitro asymbiotic seed germination had provided an alternative mode of propagating these orchids, seed setting and germination rate of many *Paphiopedilum* cultivars are extremely low and often affected by many unknown factors [10]. In what follows, is a brief review of literature available on in vitro propagation systems for different species and hybrids of *Paphiopedilum* using seeds and other explants.

**ASYMBIOTIC SEED GERMINATION**

Sharma [11] tested the germination potential of seeds of *Paphiopedilum spicerianum* and indicated the importance of developmental stage (age) of the capsules for harvesting responsive seeds besides an appropriate culture medium for their germination in vitro.

Hong et al. [12] germinated seeds from 5-month-old green capsules of a mauaddia type slipper orchid, *Paphiopedilum Alma Gavaeta* and induced totipotent callus from them on 1/2 strength Murashige and Skoog medium (MS) [13] supplemented with 22.60 microM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.54 microM 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (TDZ) in darkness. The callus was multiplied and maintained without any morphogenesis on the same medium for more than 2 years by subculturing at a 2-month interval. When transferred to 1/2 MS medium supplemented with 26.85 microM 1-naphthalene acetic acid (NAA), an average of 4.7 protocorm-like bodies (PLBs)/shoot buds formed from each explant after 120 days of culture. After another 72 and 240 days of culture on the same medium, 25 shoot buds and eventually 75 plantlets were obtained through shoot multiplication from the original culture. Kinetin at 4.65 microM was suitable for shoot multiplication and could induce an average of 3.0 shoots from a single young shoot after 60 days of culture. The regenerated plantlets grew normally when transplanted to containers with sphagnum moss in a shaded greenhouse.

Long et al. [14] carried out an extensive study on the effects of seed maturity, media type, carbon source, and organic nutrient additives on seed germination, protocorm development, and plant growth of *Paphiopedilum villosum var. densissimum*. Micropropagation frequency was enhanced through the use of 200-day-old seed, Knudson C medium (KC) [15], and the presence of both glucose and coconut milk in the medium. The effects of various plant growth regulators on the frequency of shoot organogenesis in four *Paphiopedilum* species were also investigated. Explants of *P. villosum var. densissimum* and *Paphiopedilum insigne* incubated in the presence of 5 mg/l 6-benzylaminopurine (BAP) with 0.5 mg/l NAA and 0.2 mg/l BAP with 0.1 mg/l NAA, respectively, showed a two-fold increase in the frequency of shoot organogenesis. In explants of *P. bellatulum* and *P. armeniacum*, the combination of 5.5 mg/l BAP with 0.5 mg/l NAA and 4 mg/l BAP with 0.1 mg/l NAA, respectively, resulted in the highest frequencies of shoot organogenesis.

Mao and Ranyaphi [16] reported in vitro seed germination of six species of *Paphiopedilum*, namely *P. fairrieaum, P. hirsutissimum, P. insigne, P. spicerianum, P. venustum* and *P. villosum*. Seeds from both immature (6 months old) and mature (10 months old) capsules were cultured on MS medium and Vacin and Went (VW) [17] medium and their various combinations with activated charcoal (1 g/l) and coconut milk (10%). The time taken for seed germination varied from 4-9 wks depending upon the species, and immature seeds germinated faster than the mature seeds. The seedlings when subcultured on medium supplemented with banana pulp (10%) grew rapidly and produced profuse rooting.

**CLONAL PROPAGATION**

Paphiopedilums have attained increasing demand in the flower industry but suffer from slow plant growth and difficulty of removing bacterial and fungal infections from explants originating from greenhouse plants had kept them in short supply. Huang [18] reported that most bacteria and fungi could be excluded by utilizing shoot tip explants that were considerably smaller than those usually employed for mericioning other orchids. However, the survival incidence of the smaller explants was low and the plant multiplication rate also remained slow. Huang et al. [19] modified the concentration of BAP and NAA in an attempt to simplify the above protocol so that shoot increase and rooting could be accomplished in a single step, thus shortening the time required for obtaining plants.

Lin et al. [20] induced totipotent calli from seed-derived protocorms of *Paphiopedilum hybrid* (*Paphiopedilum callosum ‘Oakhi’ x Paph. lawrenceae ‘Tradition’*) on a 1/2 strength MS medium supplemented with 1-10 mg/l 2,4-D and 0.1-1 mg/l TDZ. These calli grew well when subcultured on the same medium, but proliferated more on 1/2 MS medium
_plus 5 mg/l 2,4-D and 1 mg/l TDZ. Calli developed further and produced protocorm-like bodies, and eventually formed plantlets that could be transplanted to pots and grew well.

Chen et al. [21] reported multiple shoot formation and plant regeneration from stem nodal explants of *Paphiopedilum philippinense* hybrids (hybrid PH59 and PH60). The explants directly formed shoots when cultured on a modified half-strength MS basal medium supplemented with a combination of 2,4-D (4.52 and 45.25 microM) and TDZ (0.45 and 4.54 microM) for 6 months. On hormone-free basal medium, the percentages of explants with shoots were 33.3% and 0% and the shoot numbers per explant were 1 and 0 in hybrid PH59 and hybrid PH60, respectively. In hybrid PH59, 4.52 microM 2,4-D plus 0.45 microM TDZ induced a higher percentage of explants with shoots and shoot number per explant than did the hormone-free treatment. In hybrid PH60, although 4.52 microM 2,4-D and 0.45 microM TDZ promoted shoot formation, the highest shoot number was found with 4.52 microM 2,4-D alone. Plantlets, each having several roots, were obtained from regenerated shoots after transferring onto hormone-free basal medium for 3 months. The plantlets were potted in sphagnum moss and acclimatized well in a greenhouse.

Chen et al. [22] tested the regeneration potential of leaf explants of *Paphiopedilum philippinense* hybrids (hybrid PH59 and PH60). They observed the formation of adventitious shoots, without an intervening callus phase, from wound regions within 1 month, on modified MS medium (1/2-strength macron- and full-strength micro-elements) free of plant growth regulator in darkness. The combinations of 2,4-D (0, 4.52 and 45.25 microM) and TDZ (0, 0.45, 4.54 and 22.71 microM) were used to test their effects on direct shoot bud formation from two types of explants (1.5-cm long intact leaf explants and 0.5-cm long leaf segment explants). In hybrid PH59, 4.54 microM TDZ increased mean numbers of shoots per explant with leaf segment explants. In hybrid PH60, 4.52 microM 2,4-D plus 0.45 microM TDZ promoted direct shoot bud formation from leaf segment explants. In addition, three treatments (4.52 microM 2,4-D, 22.71 microM TDZ, 4.52 microM 2,4-D plus 4.54 microM TDZ) gave a higher response than control on mean numbers of shoots per explant with intact leaf explants. Healthy plantlets each with one to three roots were obtained from leaf-derived shoots after transfer onto a hormone-free medium for 22 months. These plantlets were acclimatized in a greenhouse and grew well with 100% survival rate.

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Ng and Saleh [23] reported in vitro propagation of *Paphiopedilum rothschildianum* through the formation of secondary protocorm-like bodies (PLBs) from the primary PLB that developed from stem-derived callus. The PLBs were cultured on half-strength MS medium supplemented with different concentrations (1.0, 2.0, 3.0, and 4.0 microM) of BAP and kinetin for the induction of secondary PLBs. The highest number of secondary PLBs formed was obtained on half-strength MS medium supplemented with 4.0 microM kinetin, with an average of 4.1 PLBs per explant after 8 weeks of culture. The secondary PLBs continued to proliferate further and formed 9.5–12.1 new PLBs per secondary PLB after being subcultured onto half-strength plant growth regulator-free MS medium supplemented with 60 g/L banana homogenate (BH). These tertiary PLBs were subcultured onto media containing different organic additives, such as BH, coconut water, potato homogenate, and tomato homogenate, for plantlet regeneration. Among the organic additives tested, the addition of 20% CW to half-strength MS medium resulted in the best average plantlet regeneration percentage from the PLBs, 67.9%, after 8 weeks of culture.

Liao et al. [24] used flower buds (FBs) for in vitro shoot induction and plant regeneration in two Paphiopedilum hybrids, a sequentially flowering *Paphiopedilum Deperle* and a single floral *Paphiopedilum Armeni White*. They used cross-sections of FBs as explants and observed that only the sections that contained the base tissue of FBs were able to produce shoots and plants. The size of the sections of FBs also affected the regeneration response. While sections ranging between 1.5 and 3.0 cm in *Paphiopedilum Deperle* were able to produce shoots, sections of FBs >2.5 cm only regenerated shoots in case of *Paphiopedilum Armeni White*. Microscopic examination of the explants revealed that the small bract at the FB base harbored a new miniature FB, which further harbored a primitive FB with dome-shaped meristem-like tissues that presumably led to the plant induction. The reiteration of this pattern resulted in a scorpioid cyme inflorescence architecture in the multifloral *Paphiopedilum* species, and its failure to
reiterate resulted in a single flower. The induction rates were 57–75%, and all plants survived in a greenhouse.

Borah et al. [25] reported in vitro propagation of *Paphiopedilum spicerianum* using leaf and shoot tip explants on MS medium with and without growth additives. The explants showed regeneration response via callusing, although development of the callus was extremely slow. The shoot tip explants gave better results as compared to the leaf explants. The initiation and development of callus was the maximum in basal medium supplemented with 0.20 mg/l TDZ.

**DISCUSSION**

Though *Paphiopedilum* orchids are propagated through the division of axillary buds from the mother plants, the process is time consuming, extremely unproductive and reliable for their conservation and commercialization. Although propagation through in vitro asymbiotic seed germination had provided an alternative mode of propagating these orchids, seed setting and germination rate of many *Paphiopedilum* cultivars are extremely low and often affected by many unknown factors [10]. Furthermore, given the fact that the seedlings are not able to retain the desired characters of the mother plant, it is almost impossible to obtain genetically similar plants through seed germination. This has always been the challenge faced by many commercial orchid growers in propagating these orchid genera since in vitro germinated seedlings are never uniform, and segregation of flower colors always occur in orchids [26].

Borah et al. [25] failed to achieve germination of seeds obtained from undehisced capsules in *P. spicerianum*. According to Arditti and Ernst [27], paphiopedilums have stringent nutritional requirements for their germination but studies on this aspect are however limited. The success of micropopagation of paphs via direct shoot regeneration largely depends on the optimization of culture medium [29]. MS and its modifications have been mainly used as the nutrient medium for in vitro propagation of paphiopedilums [12, 21, 22]. Shoot tip or nodal explants have been the popular explants for direct or indirect shoot-bud formation [21, 22, 29]. Addition of various growth hormones has generally improved the regeneration response and the number of regenerants, and TDZ has been particularly effective for the purpose [12, 25, 30].

The addition of organic additives to the culture medium to promote in vitro growth and proliferation of orchid is a common practice [10]. The presence of organic additives may contribute toward the development of a simple and economical plant culture media and at the same time minimize the used of exogenous PGRs, thereby possibly reducing the occurrence of undesired somaclonal variation [31]. Many orchid researchers favor the use of PGR-free media to obtain genetically stable PLBs [32, 33]. Although the effect of organic supplements is complex and their incorporation into the medium is not always effective, many organic forms of nitrogen and natural organic compounds are still being used in the micropropagation of orchids [27]. The variable effects of these organic supplements on different orchid species further emphasizes that the plant growth response depends largely on plant genotype and the formulation of the basal medium [31].

**CONCLUSION**

The genus *Paphiopedium* and its numerous hybrids have contributed significantly to the cut flower and pot plant trade. Like other orchids, they are also getting depleted in their natural habitats due to various anthropogenic pressures. Development of appropriate in vitro propagation protocols for these orchids is therefore highly desirable. Asymbiotic seed germination method is an effective tool for conservation of these orchids and their reintroduction into their natural habitats. In addition, variations in flowers produced from seed raised plants provide an opportunity to the grower to select the plants with most desirable flower character for commercial mass multiplication. The literature on in vitro clonal propagation of paphs using leaf/shoot explants is rather limited and restricted to a few species/hybrids. More studies are needed to identify the factors which could accelerate the multiplication of these beautiful and long-lasting orchids.

**REFERENCES**


