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STUDIES ON *IN VITRO* ASYMBIOTIC GERMINATION OF *PAPHIOPEDILUM* SPECIES OF NORTH EAST INDIA

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Abstract

Under the action plan of the department on micropropagation of rare, endangered and threatened plant species of North East India, studies on seed germination of *Paphiopedilum* species were taken up for large-scale rapid multiplication. The results on the *in vitro* seed germination studies of *P. fairrieanum, P. hirsutissimum, P. insigne, P. spicerianum, P. venustum and P. villosum* are presented in the paper.

Introduction

THE GENUS *Paphiopedilum* with *ca*. 70 species and numerous hybrids is well known in floricultural trade for its beautiful and long lasting flowers, having contributed significantly to cut flower and pot plant production. In Holland alone, the genus accounts for several millions dollar worth flower production. While the hybrids are more popular, the species command a high demand for their importance in developing new and novel hybrids besides reintroduction into the existing ones (hybrids) to regain vigor in the breeding lines. Consequently, they continue to be extensively collected from their natural habitats.

Pradhan (1979) reported nine species of *Paphiopedilum* from India. However, a detailed study of the subsequent literature reveals that the occurrence of 2 of these (*P. charlesworthii* and *P. wardii*) is, however, doubtful in the country (Joy, 1992; Kataki, 1984; Kumar *et al.*, 1995); these are Burmese in distribution (Cribb, 1998). Of the remaining seven species, *P. druryi* is confined to Agasthya Malai hills in Kerala, whereas *P. fairrieanum, P. hirsutissimum, P. insigne, P. specerianum, P. venustum* and *P. villosum* are NorthEast Indian in distribution; they grow along the base of Himalayan range and adjacent hills. Incidentally, all these species command a high price and demand in floriculture trade but their natural populations in the country have declined alarmingly in the recent years, due to the over collection and ecosystem disturbances resulting through various anthropogenic activities. They are listed as rare and endangered orchids in Red Data Book of Indian plants; some have even disappeared from their original habitats. It is therefore desirable to devise appropriate strategies for conservation of these species and their habitats in the region.

Although a variety of procedure and media are available for *in vitro* seed germination of different orchids (Arditti, 1982), there are only few reports on *in vitro* seed germination of Indian species of *Paphiopedilum* (Sharma and Chauhan, 1995). Botanical Survey of India, Eastern Regional Centre, Shillong has initiated an action plan for micropropagation of rare, endangered and threatened plants of NorthEast plant species for *ex situ* conservation using plant tissue culture techniques. This paper forms a part of studies done under the said plan and deals with *in vitro* seed germination of six species of *Paphiopedilum (P. fairrieanum, P. hirsutissimum, P. insigne, P. spicerianum, P. venustum* and *P. villosum)* found in NorthEast India, and may be useful to orchid growers and conservation biologists.

Materials and Methods

All the 6 *Paphiopedilum* species included under the scope of present studies were procured from natural conditions, grown in the National Orchidarium, Shillong and were hand pollinated. Both immature (6 months old) and the mature (10 months old) capsules were used for *in vitro* seed germination studies. Freshly harvested capsules were washed thoroughly in running tap water prior to surface sterilization. The sterilized capsules were split opened using a scalpel under aseptic conditions and the seeds were collected gently into a petridish. Two nutrient media namely Vacin and Went (VW; 1949) and Murashige and Skoog (MS; 1962) and their various combinations with activated charcoal (AC; 1gl^{-1}) and coconut milk (CM; 10%) were used as mixture. The seeds were spread thinly over the medium individually and using a spatula. The pH of the medium was adjusted to 5.8 before autoclaving. Aliquots (40 ml) of medium dispensed into 250 ml conical flasks and autoclaved at 121 °C and 1.1 Kg for 20 min along with all the equipments needed for the inoculation. The effect of AC (1gl^{-1}), coconut milk (10%) and banana pulp (BP; 10%), individually and in combination, in the

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medium, was also tested on seed germination. The effect of light and dark conditions on seed germination was also studied by keeping one set of replicates (5 Flasks) of each treatment in light and dark culture room condition. All the cultures maintained under warm white fluorescent lights at an irradiance of 160 mol m^{-2} s^{-2} with a 18 hr photoperiod and at 25 ± 2°C.

MS medium supplemented with 10% banana pulp (BP) was used as medium for subculturing of seedlings. The results of seed germination were scored by plus (+) signs based on the visual observations as the seeds are minute and it is difficult to count the number of the seeds sown per flask. For this reason, no percentage (%) was given in the results. The germinating seeds or protocorms like bodies (PLBs), when they developed 1-2 leaves, were subcultured on BP (10%) supplemented basal medium with or without AC (1 gl^{-1}). Subculturing was carried out after every three months for the rapid growth. Similarly, the growth of seedlings was also scored by (+) signs based on purely visual observation of growth and appearance. Sucrose (2%) was used as carbohydrates source and this media was gelled with 0.8% Difco-bacto agar powder.

Seedlings with fully developed leaves (4-6) obtained in about a year, were transferred from laboratory to community pots on mixture of soil: coarse sand: leaf mold (1:1:2) and kept under poly-house condition. The seedlings were washed in running tap water to remove the medium from the roots and then soaked in fungicide (0.1% Mancozeb WP, a contact fungicide) solution for 20 min and then planted into pots under poly-house conditions (80% RH; 25-30°C) for acclimatization. The plants were watered periodically to ensure the compost does not get dried up.

Results and Discussion

Presently, seeds in all the tested species germinated on both the MS and VW media. However, MS medium was used for further experimentation as it induced

Fig.1.a-c. *In vitro* culture of *Paphiopedilum* species: a, Seed germination; b, Seedlings ready for lab to land transfer; and c, Lab to land weaned plants in polyhouse.

better germination response than that of VW medium. In general, the basal medium supported a low frequency of seed germination. Additional use of CM in the medium improved germination frequency but the effect was less pronounced than that of AC enriched medium. Both the additives (AC and CM) acted synergistically in enhancing germination frequency. Basal medium supplemented with AC and CM was the best for seed germination followed by BM + AC and BM + BP. Seedlings produced profuse roots in the basal medium supplemented with 10% BP in all the species. Seed germination was obtained in all the six species, however, the species responded differently. In *P. fairrieanum, P. insigne, P. spicerianum, and P. villosum*, both immature and mature seeds germinated. On the other hand in *P. hirsutissimum* and *P. venustum*, the mature seeds failed to germinate even after a year; whereas the immature ones that were still white in color, germinated within 8-9 wks but the germinating entities failed to turn green and remained arrested at spherule stage. The germinated seeds remained white and perished despite subculturing on to fresh medium. It was also observed that the germination frequency varied with the batch of seeds. In *P. fairrieanum, P. insigne, P. spicerianum,* and *P. villosum,* the seeds began to swell within 3-4 wks after inoculation both in light and dark conditions and germinated within 4-6 wks after inoculation. Protocorms developed in the four species within 6-9 wks of seed germination (Fig.1a).

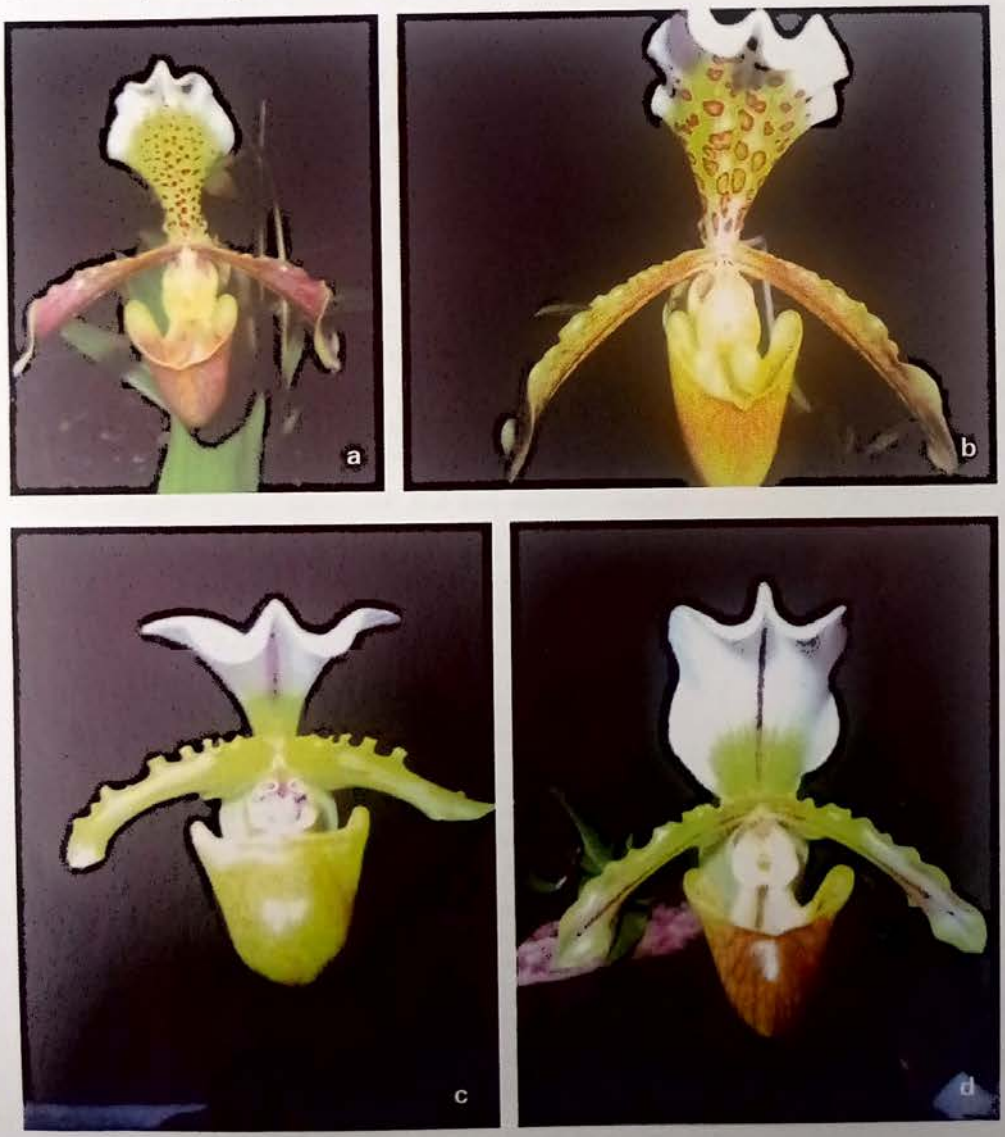

Fig.2.a-d. a-b: *Paphiopedilum insigne* showing *in vitro* raised plants variation in flower shapes: a, Flower with a normal shape and brownish-purple dots on dorsal sepals; b, Flower with much bigger size brownish-purple spots on dorsal sepal. c-d: *P. spicerianum*: c, Flower with a normal shape. d, Dorsal sepal much broader in shape and more or less like that of *P. insigne*.

The time taken for the seed germination in the six species varied from 4-9 wks depending on the species and the age of the seeds (Table 1). Some batches of the seeds induced very high seed germination while very low germination was recorded in others. This could be due to the quality of the seeds in different batches. It appears that immature seeds which were still white in colour *i.e.*, (6-7 months old capsule) germinated faster as reported in many other orchid species (Arditti *et al.*, 1982; Hossain *et al.*, 2009, 2010, 2012; Pathak and Vij, 2007; Pathak *et al.*, 2001, 2011; Piri *et al.*, 2013; Rao, 1977, Vij and Pathak, 1988, 2010). Of the species studied, the seeds of *P. fairrieanum, P. insigne, P. spicerianum,* and *P. villosum* germinated easily *in vitro*. Uses of activated charcoal and coconut milk in orchid culture and other have already been explained (Arditti *et al.*, 1982). On the effect of light and dark conditions on seed germination, it was found that there was no difference observed in all the species. The seedlings when subcultured into medium supplemented with 10% BP grew rapidly and also

Table. 1. Frequency and time taken for seed germination in different *Paphiopedilum* spp.

Species	Time taken for germination (wks)	Germination frequency
P. fairreanum	4-6	+ +
P. hirsutissimum	8-9	+ + +
P. insigne	4-6	+ + +
P. specerianum	4-6	+ + +
P. villosum	4-6	+ + +
P. venustum	8-9	+ + +

produced profuse rooting. From time to time depending on the need, the seedlings were thinned out by subculturing into fresh medium for rapid growth (Fig. 1b). Many seedlings produced side shoots (2-4 in numbers) and thus by regular subculture, it can be multiplied many folds. The seedlings normally took about 12-18 months for transplantation from lab to land (Fig.1b).

With the exception of *P. hirsutissimum* and *P. venustum*, over 1000 seedlings of the other four species were successfully transferred (Fig. 1c). The seedlings were potted in a mixture of leaf mould, coarse sand and loam soil (2:1:1) and cultivated in National Orchidarium, Shillong. The seedlings were hardy and no special treatment was needed for acclimatization; the survival rate was 90-95%. A few plants flowered after 3 years of cultivation and many more in the 4th year. It was observed that in *P. insigne* and *P. spicerianum*, there were a lot of variations in the flowers, some of which appeared to be interesting (Figs. 2a-d). Variations in the flowers produced from the seed raised plants are considered undesirable in commercial cut flower industry; however, it gives the growers the opportunity to select the plants with the most desirable flower character for commercial mass multiplication. The flower variation observed in the present study (bigger spots on dorsal sepal in *P. insigne* (Fig. 2b) and the larger dorsal sepal (Fig. 2d) in *P. spicerianum* are of desirable elite floral character, which are absent in normal plants. In addition, the variations observed are important for conservation of species as these maintained maximum genetic diversity.

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