J. Phytol. Res. 17(1) : 1-5, 2004

IN VITRO PROPAGATION OF A TERRESTRIAL ENDANGERED ORCHID *PAPHIOPEDILUM INSIGNE* (WALL. EX. LINDL) PFITZ.

MANASHI KALITA and C.M. SARMA
Department of Botany, Gauhati University, Guwahati- 781014, India.

Out of the four media (MS, B_5, VW, and KnC) tried , MS medium proved to be the best for seed germination and subsequent growth of the plantlets. Seed germination was highest (85%) with NAA, IBA (1.0 µg/ml each), Kn (0.1 µg/ml) and CH (100 µg/ml) . This medium was also best for development and proliferation of the plbs leading to plantlets formation. The regenerated plantlets were transferred to different potting media. Potting medium containing loamy soil, leaf mould, river sand and charcoal (1:1:1:1) exhibited best response for the highest rate of survival (90%) of plantlets and their growth.

Keywords : Casein Hydrolysate (CH); Indole-3-Butyric Acid (IBA); Kinetin (Kn); Naphthalene Acetic Acid (NAA); *Paphiopedilum insigne*; Plant growth regulators (PGRs); Protocormlike bodies (Plbs).

Introduction

Orchids are the largest groups of angiosperms comprising about 19500 species in about 750 genera world over[1]. This suggests that one in every 15 species of flowering plants is that of an orchid[2]. Orchids show different types of habits and habitats. North East India constitutes one of the important habitats of orchids. This region is alone represented by about 850 species in about 145 genera i.e about 73 per cent of the known orchid species of India[3]. The orchids have been occupying an important place in horticulture and flouriculture for their exquisite beauty, scent, medicinal values and long lasting quality as cut flowers. However due to destruction of natural habitats and over exploitation about 150 species are in the list of "Rare and Endangered" plants. This situation calls for micropropagation for rapid multiplication and conservation of orchids.

Paphiopedilum insigne is one of the best known endangered species of orchids. Its distribution is from high hills of North Eastern India (Khasi Hills) to the low lands of Phillipines. It is terrestrial with attractive yellow-greeen, brown spotted flowers. *Paphiopedilum* species are popularly known as "Ladies Slipper" orchid also.

Ever since the successful works of Knudson[4] *in vitro* propagation of orchids seed culture , has become a reality. Other media like Nt medium[5], Thomale medium[6] were also responded for asymbiotic seed germination in different species of *Paphiopedilum*. This paper reports the response of the seeds to Murashige and Skoog (MS), B_5, Knudson[4] (KnC), and Vacin and Went[7] (VW) media supplemented with different combinations of PGRs at various concentration and also the effect of potting media on the rate of survival and growth of the plantlets.

Materials and Method

Undehisced mature capsules of *P. insigne* were first washed with sterilized double distilled water containing a few drops 'Teepol". Finally the capsules were sterilized with 100% ethyl alcohol and again washed with DDH_2O. The pods were split open with a sterilized blade and the seeds were dispensed over the media under the laminar flow cabinet. MS, B_5, KnC, and VW media were tested for their effect on germination and organogenesis . On the basis of their performance on two media (MS and B_5) were further used for proliferation of plbs and development of shoots and roots. The media were supplemented with IBA, NAA, Kn and CH. Sucrose (3%) was added to the media and the pH was adjusted to 5.8 before autoclaving at 15 lb/inch2 pressure at 121° C for 20 minutes. The media were gelled with 0.8 per cent difcobactogen agar. The culture flasks were exposed to 16 hr light period / day at 2500 ± 3000 lux intensity after germination. The temperature of the culture room was maintained at 25 ± 1° C.

All the four media were supplemented with the following combination of concentrations of PGRs and other organic supplements:

(a) NAA and IBA (1.0 µg/ml each), Kn (0.1 µg/ml) and CH(100 µ//g/ml).
(b) NAA and Kn (0.1 µg/ml each) and CH (100 µg/ml).
(c) NAA(0.1 µg/ml), Kn(1.0 µg/ml).

Results and Discussion

In all the media tried, the combination (a) showed better response than the other two. The best results obtained on the four media with the same combinations are only presented (Table 1, Fig 1).

On the basis of early response, further subculture of the plbs with vegetative apices were done on both MS and B_5 media. The media were supplemented with various concentrations of IBA, NAA, Kn (0.1µg/ml each) and

Table 1. Germination of seeds, development of plbs and differentiation of *P. insigne* (Wall Ex.Lindl) Pfitz. on four media.

Media with NAA & IBA (1.0µg ml each) + Kn (0.1 µg/ml) +CH (100 µg/ml)	Observation			
	30 days (% germination)	60 days	90 days	Response
MS	85	Plbs became green, 0.2-0.3 mm in diameter	Plbs started forming plantlets, Shoot started emerging with minute leaves. Roots started emerging. Leaves not fully developed	++++
B_5	75	Plbs became green, 0.2-0.3 mm in diameter	Plbs started proliferating with emergence of minute shoot apices. Roots started emerging	+++
KnC	70	Plbs started greening and were 0.1 mm in diameter	Plbs green, swelled shoots with minute leaves developed	+++
VW	68	Plbs green 0.1 mm in diameter	Emergence of shoot apices with no roots	+++

Response : + Poor , ++ Satisfactory, +++ Highly Satisfactory, ++++ Excellent

Table 2. Effect of Plant Growth regulators and organic supplements on the growth of the seedling of *P. insigne* (Wall ex. Lindl.) Pfitz.

MS Medium supplemented with	Observation			
	30 days	60 days	90 days	Response
(i) IBA (1.0 µg/ml) NAA (1.0 µg/ml) Kn (1.0 µg/ml) CH (1.0 µg/ml)	Plantlets - 0.3-0.4 cm long, Leaf - 2nd leaf initiated, Roots - Emergence of single root, white	Plantlets - 0.4-1.5 cm long, Leaves - 3-4 in number, Roots - 2-3 in number became green, 0.4-0.5 cm long	Plantlets - 0.4-1.5 cm long Leaf - 3-4 in number Roots - 2-3 in number became green, 0.4-0.5 cm long	++
(ii) IBA (0.1 µg/ml) NAA (0.1 µg/ml) Kn (0.1 µg/ml) CH (100 µg/ml)	Plantlets - 0.1-0.3 cm long, Leaf - 2 in number, Roots - Single emergence of white root	Plantlets - 0.3-1.2 cm long, Leaves - 2-3 in number, light green Roots - 2-3 in number, green, 0.2-0.4 cm long	Plantlets - 1.5-3 cm long Leaves - 3-4 in number Roots - 3-4 in number, 0.4-0.5 cm long. Callus like structure formed	+++
(iii) IBA (1.0 µg/ml) NAA (1.0 µg/ml) Kn (5.0 µg/ml) CH (100 µg/ml)	Plantlets - 0.1-0.4 cm long, Leaf- 2 in number Roots - Just emerged	Plantlets - 0.4-0.5 cm long, Leaves - 2 in number ,Roots - Single white root	Plantlets - 0.4-1.5 cm long Leaves - 3 in number Roots - Not well developed	+
(iv) IBA (1.0 µg/ml) NAA (0.01 µg/ml) CH (1.0 µg/ml)	No proper developmen of plantlets leaves and roots	Plantlets - 0.1-0.2 cm long, Leaves - Single leaf initiated,Roots - not developed	Plantlets - 0.2-0.4 cm long Leaf - 2nd leaf initiated Roots - not developed	+

Response : + Poor , ++ Satisfactory, +++ Highly Satisfactory

Fig. 1-6 : *In vitro* propagation of *Paphiopedilum insigne* : 1. Plbs from seeds [NAA, IBA (1.0 µg/ml each), Kn (0.1 µg/ml) + CH (100 µg/ml)] 2. Plantlets on MS medium [IBA,NAA and Kn (0.1 µg/ml each)+CH (100 µg/ml)] 3. Plantlets on MS medium [IBA, NAA (1.0 µg/ml) , Kn (5.0 µg/ml) + CH (100 µg/ml)] 4. Plantlets on B_5 medium [NAA (0.01 µg/ml), BA (0.01 µg/ml) + CH (100 µg/ml) 5. Acclimatized plant in green house on potting medium [loamy soil + leaf mould + river sand + charcoal (1:1:1:1)] 6. Plantlets established in natural condition.

Table 3. Effect of Plant Growth Regulators and Organic Supplements on the growth of the seedling of *P. insigne* (Wall ex. Lindl.) Pfitz.

B_5 Medium supplemented with	Observation			
	30 days	60 days	90 days	Response
(i) IBA (1.0 µg/ml) NAA (1.0 µg/ml) Kn (0.5 µg/ml) CH (100 µg/ml)	Plantlets - 1 - 0.3 cm long, Leaf - 2-3 in number 0.2-0.4 cm long, Roots - Not well developed	Plantlets - 0.3-0.5 cm long, Leaves - 3 in number 0.4-0.6 cm long, Roots - 2 in number minute	Plantlets - 0.5-0.8 cm long Leaf - 3-4 in number Roots - 2-3 in number	++
(ii) IBA (0.1 µg/ml) NAA (0.1 µg/ml) Kn (0.1 µg/ml) CH (100 µg/ml)	Plantlets - 0.2-0.4 cm long, Leaf - 3 in numbei 0.3-0.5 cm long, Roots - No emergence of root	Plantlets - 0.4-0.8 cm long, Leaves - 3-4 in number 0.5-0.9 cm long, Roots - Single emergence of root	Plantlets - 0.1-0.3 cm long Leaves - 4-6 in number 0.7-1.4 cm long, Roots - 2 in number, light brown in colour	+++
(iii) IBA (1.0 µg/ml) NAA (1.0 µg/ml) Kn (5.0 µg/ml) CH (100 µg/ml)	No proper proliferation of plbs	Shoot started emerging with minute leaves with no development of roots	Plantlets - 0.1-0.3 cm long Leaves - 2 in number 0.2-0.6 cm long, Roots - Roots started emerging	+
(iv) NAA (0.01µg/ml) BA (0.01 µg/ml) CH (100 µg/ml)	Plbs started proliferating with minute emergence of shoot apices. No development of roots	Plantlets - 0.1-0.3 cm long, Leaves - 2 in number 0.2-0.4 cm long, Roots - 2 in number	Plantlets - 0.3-1.2 cm long Leaf - 2-4 in number 0.4-1.0 cm long Roots - 2-3 in number	++

Response : + Poor , ++ Satisfactory, +++ Highly Satisfactory

Table 4. Effect of potting media on Chlorophyll contents & growth and survivability of *Paphiopedilum insigne*

Potting media with composition	Chlorophyll contents (mg/gm) of leaves			Rate of survival (%)	Average plant (Average)	Leaf/Plant (Average)	Response
	Chl a	Chl b	Total Chl				
1. Loamy soil, leaf mould, river sand, charcoal dust (1:1:1:1)	0.446±0.002	0.606±0.001	1.053±0.002	90.1±0.006	6.2±0.041	7.1±0.038	++++
2. Leaf mould, vermiculite, perlite and dry *Sphagnum* (2:1:1:2)	0.326±0.001	0.495±0.001	0.821±0.001	79.8±0.072	5.07±0.068	6.07±0.062	+++
3. Loamy soil, river sand, tree fern pieces, charcoal dust (1:1:1:1)	0.326±0.001	0.495±0.001	0.821±0.001	60.07±0.020	4.5±0.047	5.1±0.044	+
4. Loamy soil and leaf mould (1:1)	0.345±0.001	0.544±0.001	0.888±0.001	70.07±0.018	4.7±0.046	6±0.041	++

Response : + Poor , ++ Satisfactory, +++ Highly Satisfactory, ++++ Excellent

CH (100µg/ml).

Highly satisfactory response was exhibited by MS and B_5 media supplemented with the above concentrations of PGRs. On MS medium after 30 days of subculture the plantlets attained a length of 0.1-0.3 cm with two leaves and emergence of one root . After 60 days the development of shoots and roots was conspicuous. After 90 days at the base of shoots callus developed with green colour. (Table 2, Fig. 2 & 3)

B_5 medium also with same concentration of PGRs and CH exhibited satisfactory response (Table 3, Fig. 4).

From the results (Table 2 and 3) it is clear that the combination of Kn, NAA, IBA at lower concentration was suitable for proliferation of plbs as well as for the growth of both root and shoot system. The interacting influence of kinetin and auxins (IBA, NAA) was significant in our investigations as has also been reported by some early workers[7, 8].

In the present experiment, CH promoted both seed germination as well as seedling growth. Similarly its addition in the medium has been emphasized for better seedling growth in vitro[9-11].

That light inhibits seed germination in many terrestrial orchid species like *Cypripedium, Paphiopedilum* has been reported by Voth[12] and Kano[13]. In this experiment also total absence of light (4 weeks) promoted rate of germination with the development of plbs. Here MS medium proved to be more effective for inducing early and better germination of seedlings than B_5 medium.

Axenic seedlings of *Paphiopedilum* derived from green pod culture were transplanted on different potting media . The results proved that the plantlets can be acclimatized with 90% servility. The growth of the plantlets were spectacular on the potting medium containing loamy soil, leaf mould, river sand , charcoal (1:1:1:1) in comparison to the other media (Table 4, Fig. 5 & 6).

The chlorophyll content of the leaves (Table 4) of the plants on this particular medium was higher than the plantlets on other potting media. More chlorophyll content reflected the healthy growth of the plants According to Sharma and Chauhan[14] for *P. spicerianum* the best compost comprised of leaf mould , perlite, vermiculite and dry *Sphagnum* in the ratio of 1:1:1:2 .

References

1. Hegde S N 1997, Orchid Wealth of India. *Proc. Ind. National Sci. Academy*. **B 63 No 3** 229-244
2. Chadha 1992, The Indian Orchid Scenario. *J. Orchid. Soc. India* **5(1,2)** 01-04.
3. Hegde SN 2001, *Orchids : Conservation, Culture, Farming and Trade*. (Ed.) S N Hegde, Himalayan Publishers, Delhi - Itanagar.
4. Knudson 1922, Non symbiotic germination of orchids. *Bot. Gaz.* **73** 1-25.
5. Tay L J, Takeno K and Hori Y 1988, Culture conditions suitable for *in vitro* seed germination and development of seedling in *Paphiopedilum. J. Jap. Soc. Hort. Sci.* **57(2)** 243-249.
6. Pierik R L M, Sprenkels P A, Harat B V D, Vonder Mays and Vander Q G 1988, Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz. *In vitro. Scient. Hort.* **34(1/2)** 139-153.
7. Vacin E F and Went F 1949, Some pH changes in nutrient solution. *Bot. Gaz.* **110** 605-13.
8. Viz S P, Sood A and Plaha K K 1981, *In vitro* : seed germination of some epiphytic orchid. *In : S.C. Science*, pp. 473-81. Kalyani Publisher, New Delhi.
9. Pierik RLM and Stegmans HHM 1972, The effect of benzylaminopurine on growth and development of Cattlaya seedling grown from unripe seeds. *Z. pftanzenphysiel* **68** 228-234.
10. Prasad R N and Mitra G C 1975, Nutrient requirements for germination of seeds and development of protocorms and seedling of *Cymbidium* in asceptic culture. *Ind. J. Exp. Bio.* **13** 123-126.
11. Rao A N 1977, Tissue culture in orchid industry. *In* : J Reinert and YPS Bajaj (eds.). *Applied and fundamental aspects of plat cell, tissue and organ culture.* Pp. 44-69. Springer Verlog, Berlin.
12. Vöth W 1976, Aussat and Kultar Von *Serpias parviflora* and *S. orientalis* pp 35. *In* : Proc. *8th world Orchids Conf. Frankfurt (1975).*
13. Kano 1965, Studies on the media for orchid seed germination. *Mem. Fac. Agr. Kagawa University* **20** 1-66.
14. Sharma J and Chauhan Y S 1995, Establishment of in vitro raised seedlings of *Dendrobium* and *Paphiopedilum. J. Orchid. Soc. India* **9(1-2)** 37-41.