

Original Research Article

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In vitro Mass Propagation of Endangered Terrestrial Orchid *Phaius tankervilleae* (L'Her.) Blume through Green Seed Pod Culture

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ABSTRACT

Phaius tankervilleae (L'Her.) Blume is one of the most attractive and horticulturally valuable ground orchids of the world. Due to illegal collection for trade and consumption and loss of habitat, the population of this species has decreased rapidly at an alarming rate in natural habitats. *In vitro* seed germination and seedling development of *Phaius tankervilleae* technique was successfully established for rapid multiplication using 0.8% (w/v) agar solidified MS medium supplemented with different concentrations and combinations of Kinetin (Kin) and NAA. MS medium supplemented with 1.0 mg L⁻¹ Kin + 1.0 mg L⁻¹ NAA was the most ideal condition for early seed germination (2.87 weeks), fastest protocorm formation (5.07 weeks), tallest shoots (6.85 cm) and maximum number of roots per plant (6.20). However, maximum number of shoots (52.27) per culture and maximum number of shoots with roots (50.27) were produced when the plantlets were transferred to MS basal medium supplemented with 1.5 mg L⁻¹ Kin + 1.0 mg L⁻¹ NAA. The well developed *in vitro* rooted plantlets were hardened successfully in the potting mixture containing only vermiculite (86.00 %) followed by vermiculite + vermicompost (71.00 %) at 1:1 ratio.

Keywords

Phaius tankervilleae,
in vitro, seed
germination,
Kinetin, NAA.

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Introduction

Phaius tankervilleae, commonly known as Nun's Orchid or Lady Tankerville's Swamp Orchid belongs to the sub-family Epidendroideae. It is a large terrestrial orchid with striking flowers that blooms from April to July. It is distributed from India, Sri Lanka and Southern China, through Thailand and Malaysia to Indonesia, Australia and the Pacific islands (Cheng *et al.*, 2012). *P. tankervilleae* has slender leaves, graceful greenery, and can be used as a potted plant, a garden plant, or as cut flowers or leaves (Mukherjee, 1979; Lee, 1989; Chang and Jian, 2010). Not only its ornamental values,

the leaves and flowers are also used to extract natural dyes (Mahanta and Tiwari, 2005). Paste prepared from the pseudobulb of this species is used to cure swellings of hands and legs (Buragohain *et al.*, 2015; De and Singh, 2016). Because of its high ornamental and medicinal properties, the plant is exploited illegally from the natural environment for trade and consumption. It has also been experiencing a steady decline due to destruction of natural forest areas. This orchid species has been categorized as endangered species, under the Environmental Protection and Biodiversity Conservation Act (Briggs

and Leigh, 1996) and has become one of the objects of concern to the conservationists due to high sensitivity to environmental changes (Rasmussen, 1995). Hence, it is essential to take necessary measures to conserve and propagate these endangered orchid species. For mass propagation of several commercially important orchids, tissue culture technique has been widely adopted (Malabadi *et al.*, 2005).

Orchid's seeds are very minute, numerous and non-endospermic. Germination rate is very slow (8-10 years) and need mycorrhizal association for germination in nature (Deb & Pongener, 2011). Tissue culture has become the standard method of propagation for the conservation of orchids. This problem can be overcome by tissue culture technique (Pradhan *et al.*, 2013). *In vitro* asymbiotic germination is possible only by tissue culture method because it substitutes the action of fungus with a nutrient medium (Pant *et al.*, 2011). Rapid *in vitro* germination of seeds and development take place in the controlled environment and requires no competition with fungi and bacteria. Asymbiotic seed germination of orchids is greatly influenced by several factors such as developmental stage of embryos, age of green pod and different nutrient media with adjuvant and plant growth regulators (Arditti, 1979).

With this view, the present study was carried out to establish an *in vitro* protocol for mass propagation and conservation of *Phaius tankervilleae* using axenic seed.

Materials and Methods

Plant materials

Seeds of *Phaius tankervilleae* (L' Her.) Blume were collected from Hans Florist, Kalimpong, West Bengal, India. Immature pods of *Phaius tankervilleae* were used as explants for the present study.

Sterilization

The seed pods were washed with running tap water along with 3-4 drops of Tween 20 for about half an hour. Then pods were then treated with Carbendazim 50 W.P. @ 1g L⁻¹ of water for half an hour followed by streptomycin sulphate (9%) + tetracycline hydrochloride (1%) [Plantomycin] @ 1g L⁻¹ for half an hour. The pods were then rinsed repeatedly using sterilized double distilled water in the laminar air flow chamber. The pods were again surface sterilized sequentially with 70% ethyl alcohol for 1 minute, 0.1% HgCl₂ (Mercuric Chloride) solution for 3 minutes and finally rinsed thoroughly 5-6 times with sterile distilled water. Sterilized capsules were cut horizontally by a sterile surgical blade. Around 200 mg seeds were inoculated per culture vessel.

Culture medium and incubation

In the present investigation, full strength of Murashige and Skoog (MS) medium supplemented with or without plant growth regulators viz. Kin (0.5 to 2.0 mg/l) either alone or in combination with NAA (1.0 mg L⁻¹) were used for *in vitro* seed germination and seedling development. Basal medium were fortified with 30 g L⁻¹ sucrose and with or without different plant growth regulators like kinetin and NAA. For gelling of the medium, agar (0.8% w/v) was used and was adjusted at pH 5.8 by using 0.1N NaOH or HCl. Agar was dissolved by boiling the mixture and about 100 ml of media was dispensed into each culture jar. The jars containing the media were autoclaved at 121°C for 20 min at 1.05 Kg cm⁻² pressure. All cultures were maintained at 25±2°C under 350-500 lux illumination for 16/8 hrs. (light/dark) photoperiod using white fluorescent tube lights (Philips, India).

Inoculation of seeds

The surface sterilized immature green pods were kept on the sterilized Petri dish containing sterilized blotting paper for absorbing the excess moisture from the pods. The pods were then cut longitudinally with the help of surface sterilized surgical blade and scooped out the immature seeds with the help of sterilized spatula. The seeds were then spread over the surface of the medium supplemented with or without, either in combination of kinetin and NAA or kinetin alone. Cultures were sub-cultured into fresh medium once in every eight weeks and were replicated thrice for each treatment. The initiation and rate of seed germination was recorded every week. To prevent microbial contamination, the entire experiment was performed under aseptic condition in the laminar air flow hood.

Data analysis

In the present experiment, ten culture jars were used in each treatment and each treatment were replicated thrice in case of seed germination, growth and development of the seedlings. For acclimatization of the seedlings, each treatment was replicated four times. The experiment was laid out using completely randomized block design and the percentile data of the experiments were assumed and subjected to square root transformations.

Results and Discussions

The seed germination rate of *P. tankervilleae* was found to be variable in hormone free MS medium and MS medium supplemented with various concentration of Kinetin and NAA (Table 1). Quickest germination of seeds was obtained on MS basal medium supplemented with 1.0 mg L^{-1} Kin + 1.0 mg L^{-1} NAA (2.87 weeks) which was also found to be the best

for fastest protocorm formation (5.07 weeks). The hormone free MS basal medium, though favored seed germination, took the longest day (16.47 weeks) for germination and protocorm formation (23.93 weeks) when compared to those basal medium supplemented with hormones.

The results obtained from the experiment shows that optimum amount of Kinetin (1.0 to 1.5 mg L^{-1}) when combined with 1 mg L^{-1} NAA was found to be more effectual for seed germination and subsequent development of this terrestrial orchid. MS medium supplemented with 2.0 mg L^{-1} Kin alone and combinations of Kinetin (0.5 - 1.5 mg L^{-1}) + NAA (1.0 mg L^{-1}) have the highest capability of forming protocorm-like bodies from the immature seeds as compared to the rest of the treatments (Table 1).

In the present study, MS medium alone and MS medium fortified with different concentrations and combinations of hormones were found to be efficient for immature seed germination upto the development of protocorms. This might be due to the chemical composition of MS medium which is highly enriched with macro and micro elements with different vitamins that favored seed development. MS basal medium fortified with different plant growth hormones improve the nutritional status of the basal medium and favored earlier seed germination and protocorm formation. The result is in conformity with the findings of Arditti *et al.*, (1981).

Protocorm undergoes further differentiation on MS medium alone and MS basal medium fortified with different concentrations and combinations of growth hormones. MS medium supplemented with 1.0 mg L^{-1} Kin + 1.0 mg L^{-1} NAA favored the development of complete seedlings directly from seeds after 20 weeks of culture.

Table.1 Effect of growth regulators in MS medium on seed germination and seedling development of *Phaius tankervilleae* (L'Her.) Blume

Treatment	Treatment combinations	Initiation of germination (weeks)	Development of protocorm (weeks)	Capability of immature seeds forming protocorm-like bodies	Number of shoots per bottle	Height of shoot (cm)	No. of shoots with roots	No. of roots per plant
M	MS + 0	16.47	23.93	+	-	-	-	-
MK ₁	MS + 0.5 mg/l Kin	8.73	11.07	++	35.33	3.03	26.07	3.07
MK ₂	MS + 1.0 mg/l Kin	7.00	9.40	++	38.13	4.99	29.20	4.47
MK ₃	MS + 1.5 mg/l Kin	6.40	8.73	++	32.47	4.75	22.73	4.40
MK ₄	MS + 2.0 mg/l Kin	4.73	7.20	+++	27.87	4.18	18.80	3.13
MK ₁ N ₁	MS + 0.5 mg/l Kin + 1.0 mg/l NAA	5.93	8.07	+++	43.13	6.00	36.80	5.13
MK ₁ N ₂	MS + 1.0 mg/l Kin + 1.0 mg/l NAA	2.87	5.07	+++	49.27	6.85	44.93	6.20
MK ₁ N ₃	MS + 1.5 mg/l Kin + 1.0 mg/l NAA	4.07	6.20	+++	52.27	5.67	50.27	5.67
MK ₁ N ₄	MS + 2.0 mg/l Kin + 1.0 mg/l NAA	5.33	7.47	++	46.87	3.75	41.67	5.20
SEm ±		0.33	0.38		1.28	0.16	1.25	0.23
CD at 5%		0.98	1.13		3.82	0.48	3.72	0.69

Table.2 Effect of different hardening media on percent survivability of seedlings of *Phaius tankervilleae* (L'Her.) Blume

Hardening media	Medium ratio	Survival (%)
Garden soil + sand + vermicompost	1:1:1	31.00 (33.75)
Garden soil + sand	1:1	53.00 (46.73)
Vermiculite	1	86.00 (68.18)
Vermiculite + vermicompost	1:1	71.00 (57.52)
Vermiculite + sand	1:1	59.50 (50.51)
SEm ±		1.85
CD at 5%		5.58



Fig.1. *Phaius tankervilleae* in full bloom



Fig.2. Seeds extraction from the green pod of *Phaius tankervilleae*



Fig.3. Hormone free MS media after 10 weeks of culture



Fig.4. Seed germination and PLBs formation after 7 weeks of culture in MS + 1.0 mg/l Kin + 1.0 mg/l NAA



Fig.5. Initiation of shoots after 12 weeks of culture



Fig.6. Shoot multiplication after 20 weeks of culture in MS + 1.5 mg/l Kin + 1.0 mg/l NAA



Fig.7. *In vitro* developed plantlets ready for acclimatization



Fig.8. Hardened plantlet on potting mixture

The same hormonal composition was found effective for production of tallest shoot height (6.85 cm) as well as maximum number of roots per plant (6.20). However, MS medium when supplemented with 1.5 mg L^{-1} Kin + 1.0 mg L^{-1} NAA produced maximum number of shoots per culture (52.27) and maximum number of shoots with roots (50.27) per culture.

Seeds could not undergo further differentiation in hormone free MS medium because terrestrial orchids require more regulated conditions of hormones for immature seeds to develop into seedlings (Pant *et al.*, 2011). The results from the above findings showed that hormone supplemented MS basal medium were found more effective for further growth and development of the

seedlings. The well rooted *in vitro* regenerated Phaius seedlings were later removed from the culture media, washed repeatedly under running tap water to remove the adhered traces of semisolid agar medium and then treated the seedlings with fungicide (Carbendazim 50 W.P.) @ 1g L⁻¹ for 30 minutes followed by bactericide (Plantomycin) @ 1g L⁻¹ for 30 minutes. Later, the seedlings were transferred in the plastic containers having different well sterilized growing medium consisting of garden sand, soil, vermiculite and vermicompost either alone or in combination at equal ratio. Among the different hardening media studied, vermiculite alone was found as suitable medium for hardening of Phaius seedlings in respect of survivability (86.00 %) (Table 2). The seedlings were kept inside the growth chamber maintaining a controlled temperature of about 25⁰C with a comparatively higher percentage of relative humidity (80%) and light density of 4000 lux.

In conclusion, the present investigation reports an efficient and easy protocol for *in vitro* seedling development of *Phaius tankervilleae* using immature seeds as explant. Optimal use of Kinetin and NAA (1.0-1.5 mg L⁻¹ + 1.0 mg L⁻¹) along with the MS basal medium was found to be the better hormonal combination in terms of seed germination, protocorm formation, growth and development of the seedlings. The well rooted *in vitro* plantlets can be successfully hardened *ex vitro* using well sterilized vermiculite alone. This protocol might be useful for mass propagation and *ex-situ* conservation of this rare and valuable orchid species.

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