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CONSERVATION OF IMPERIL ORCHIDS: *CLESIOCENTRON PALLENS* (CATHCART EX LINDL.) PEARCE & CRIBB AND *PHALEONOPSIS MANNII* RCHB.F. THROUGH *IN VITRO* SEED PROPAGATION

KUNTALA N. BARUA*[1], BONANYA BORA[2], SATYAM BORDOLOI[3] AND BEBIJA LOKTONGBAM[4]

Rain Forest Research Institute, Sotai, Jorhat 785 010, Assam, India,

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Abstract– *Cleisocentron pallens* and *Phalaenopsis mannii*, two spectacular orchids of North East India having highly medicinal and ornamental values with prominent existence in APENDIX-II of CITES. The present scenario of habitat destruction, unsustainable harvesting and global climate change is affecting the distribution of orchids, which leads to rapid depletion from nature. The study deals with mass multiplication of *C pallens* and *P mannii* through *in-vitro* seed propagation for future conservation strategies. In *C. pallens*, highest germination percentage (89.35%) of was observed in Mitra medium. The maximum number (11.2±1.75 cm) and length (4.75±0.58 cm) of shoot were observed in 1.5 mg l^{-1} BAP. Otherwise, medium containing 1.5 mgl^{-1} IBA showed highest number (7.3±1.33) with 4.67±1.71 cm length of root were recorded. MS media exhibited the best proliferation rates (88.6%) in *P. mannii,* although highest number (12.5 ±3.62) and (4.01±0.66) length of shoot found in Mitra medium supplemented with 1.5 mg/l BAP. Activated Charcoal powder plays an important role in root formation and phenolic compound exudation). Maximum (4.4±0.69) number with an average length (5.85±0.95) of root was observed in medium supplemented with 1.5mgl^{-1} IBA. The seedlings showed better survivality and growth increment in potting medium Brick chips: Charcoal: Sphagnum moss (1:1:2) for both the species in green house condition. Seed derived protocorm like bodies could be successfully applied for mass multiplication intended for *ex situ* conservation.

INTRODUCTION

North East India endowed with wide range of eco-climatic situation which is suitable to fulfill the specific micro- environment requirement for luxuriant growth of orchids. Presently many important orchid species are facing danger of extinction owing to destruction of habitats, over exploitation and various anthropogenic activities. Fruit setting of majority of the magnificent orchids are also very less. Nearly 151 genera with 876 species, about 73% of the total orchid species of India contributed from this region (Jain, 1985). Increasing vulnerability of these species it is a vital need to restore their habitat for conservation and has demanded technology for *ex situ* conservation. Mass multiplication through tissue culture is the most popular multiplication method of orchids and essential components of plant genetic resource management, conservation and restoration of rare and endangered plant species (Fay, 1992). *In vitro* seed propagation technique may be an efficient and alternative tools used for mass propagation.

Cleisocentron pallens (Cathcart ex Lindl.) Pearce & Cribb and *Phalaenopsis mannii* Rchb.f. are two spectacular orchid of North east region chosen for the study as of gradual depletion from their natural environment due to worthless collection, habitat destruction and mining activities. As monopodium orchids the multiplication rate of both the species are very low and fruit setting was also very insignificant. The species were recently observed in streamline of North Eastern Coalfields (NEC) covers forest areas viz. Tinkupani and Namphai Reserve

Forest hosted specially on *Lagerstroemia speciosa* and *Terminalia myriocarpa* trees. However, their distribution was inadequate. Hence, for successful multiplication, *in vitro* seed propagation technique was adopted for present investigation. Study was carried out to explore the effect of different *in vitro* propagation medium on germination, growth and development of plantlets, identified best potting medium for establishment of plantlets intended for mass multiplication in conservation point of view.

MATERIALS AND METHODS

Seed collection and surface sterilization

The fully mature capsules of *C. pallens* and *P. mannii* were collected from Tinkupani RF. and Namphai RF of Northern Eastern coal field areas under Digboi Forest division of Assam in the month of January and October 2019 respectively. The capsule were washed properly in tap water with 20% teepol solution for 15-20 min which was followed by surface sterilization for 7-8 min by treating in 0.4% $Hgcl_2$ solution with 1-2 drop of teepol solution as wetting agent. Subsequently the capsules were washed with sterile double distilled water for 4-5 times to completely remove $Hgcl_2$ from its surface and finally dripped in 70% ethanol for 15-20 min followed by flaming for 3-4 sec. The surface sterilized capsules were split open longitudinally by using sterile surgical blade to scoop out numerous minute exalbuminous seeds and spread out on the seed germination media. The whole procedure was performed in aseptic condition under laminar flow to prevent any kind of contaminations. In order to conserve the germplasm, the mother plant of both the species being maintained in Rain Forest Research orchidarium.

Seed culture, regeneration of multiple shoot and root

To study the seed germination and organogenesis rate of *C. Pallens* and *P. mannii* in different basal media, an initial trial was conducted by inoculating the mature seeds of both the orchids on gelled MS media (Murasgige and Skoog, 1962), 1/2 strength of MS media and Mitra media (Mitra *et al*., 1976). For *P. mannii*, a readymade commercial Phaleonopsis media (HIMEDIA) was used for comparison. The medium supplemented with various plant growth regulators like 6-Benzyl Amino Purine (BAP, 0.5-2 mgl^{-1}), Indole-3-butyric Acid (IBA, 0.5-2 mgl^{-1}), 1-Nathyl Acetic Acid (NAA) either in singly or in combination with different concentration to study the growth and development. The culture medium contains 0.8% of agar-agar and 0.2% of sucrose. In *C. pallens,* 0.2% of activated charcoal powder used as natural additives. The pH of the medium was adjusting to 5.8 with NAOH or HCL. All medium containing bottles were autoclaved at 121 ^{o}C for 15 min and finally allowed to gel in slanting position. The cultures after inoculation were maintained at 25 °C ± 2 °C with proper light illumination under 16/24 h photoperiod. Sub culturing was done at regular interval of one to eight week of culture and data were recorded. For induction of root, the well developed shoot of *C. pallens* was cultured on Mitra medium. Otherwise the shoots of *P. mannii* was cultured both Mitra and MS medium, supplemented with different concentration of rooting hormone like IBA and NAA (0.5-2 mg l^{-1}). The culture were maintained in laboratory condition and data recorded after 12 week.

Acclimatization and Hardening

Ex vitro hardening were done after the formation of complete rooted plants. The plantlets with well developed root and shoot were acclimatized in green house condition by gradually loosing the capes of the culture bottle for 7 days. Plantlets were thoroughly washed with double distilled water, treated with 0.1% (w/v) bavistin and rinse. Seedlings were planted in the potting mixture containing Brick chips, Charcoal, and sphagnum and covered with poly bag for at least 25 days to maintain low temperature and high humidity. The poly bags were removed gradually to reduce humidity. After 30 days plantlets were transferred to greenhouse condition for 4-6 weeks in three potting mixture viz. Brick chips: Charcoal: Coconut husk (1:1:2), Brick chips: Charcoal: Sphagnum Moss (1:1:2) and Brick chips: Charcoal: Leaf mould (1:1:2). Direct light can be progressively increased for photosynthesis of the seedlings. The survival percentage wear calculate after 90 days.

Data analysis

The experiments were conducted with 10 replicate per treatment and repeated twice. The results were calculated as mean ± standard deviation by application of Microsoft excels software.

RESULTS AND DISCUSSION

Cleisocentron is an incredible orchid genus of which

only six representatives have been documented so far, C. *pallens* is reported to be distributed in Eastern Himalayas (Sikkim, Bhutan, Assam) to Myanmer (Bruhl, 1926; Pridgeon *et al.*, 2014). The species was very sparse in tropical wet evergreen forest of upper Assam and presently reported Namphai RF. of Digboi Forest Division of Assam epiphytes on *Lagerstroemia speciosa,* and *Terminalia myriocarpa* trees. The orchid has sympodial branching system with fibrous sheath, divergent inflorescence, and flower white with pink dotted labellum with cylindrical spur, blooming during the month of June-July (Figure 1).

Phalaenopsis are monopodial epiphytes usually identified as *Moth Orchid* bear seventy species. Chen and Chang, 2006 stated that the genus *Phalaenopsis* was most familiar and profitable orchid in horticulture industry due to long lasting flower and their adaptability and the second most significant marketable orchid. Present study deals with *P. mannii,* one of the most admirable floricultural excellent species dispersed in eastern Nepal to South Yunnan at the elevation of 500 to 1500 meters (Sweet, Herman, 1980). Inflorescence is as long as the leaves, mandarin orange colour with brown stripes. The flowering and fruit setting period is limited to the month of April to January (Figure 1).

Successful germination of *C. pallens* was observed after 3 weeks of culture, evidence by the enlargement of the embryos and ultimately produces irregular shaped parenchymatous cell mass sphurels. The highest germination percentage (89.35%) of *C. pallens* was seen in Mitra medium and followed by MS medium (86.31%). Nongdam and Chongtham, (2011) also reported the maximum germination percentage in Mitra medium in case of *Cymbidium aloifolium*. The sphurels were transformed into round, oval, elongated, branched or spindle shaped protocorm like bodies (PLB) after five week of culture. On the basis of germination percentage, 7 week old PLB's were transferred to Mitra medium supplemented with different growth regulators like BAP (0.5, 1, 1.5, 2 mgl^{-1}), IBA (0.5 mgl^{-1}) at different concentration either in individual or in combination to observe the development of shoot. The positive response of BAP in plantlet regeneration from protocorm has been reported previously in *Coelogynae cristata* also (Naing *et al.,* 2011). In combination with different concentration of BAP and 0.5 mgl^{-1} IBA showed multiple shoot formation as well as root initiation. The highest number (11.2±1.75 cm) and length (4.75±0.58 cm) of shoot were recorded in 1.5 mg l^{-1} BAP and 0.5 mgl^{-1} IBA after 8 week of the culture (Table 2, Figure 2:3). The positive response of auxin and cytokines also reported in *Esmeralda clarkei* (Paudal and Pant, 2012) and in *vanda tessellata* (Rahman *et al.,* 2009).

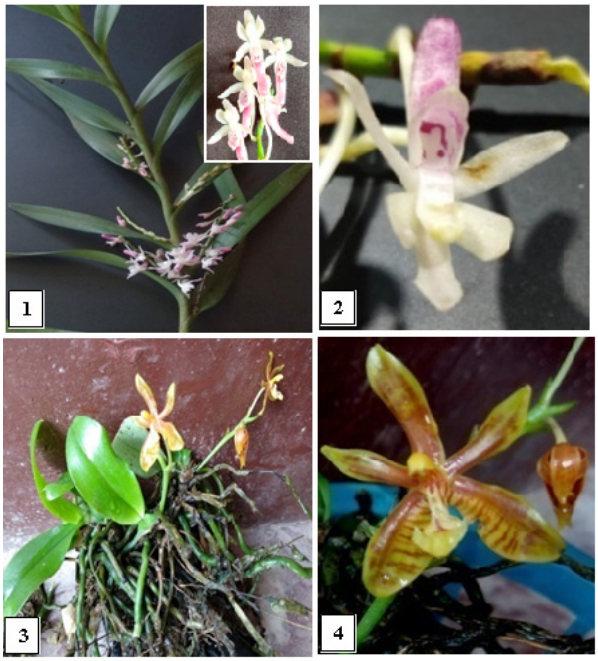

Fig. 1. 1 & 2-Flower of *Cleisocentron pallens* 3 & 4- Flower of *Phalaenopsis mannii*

Production of novel *Phalaenopsis* varieties the principle requirements were the composition of micro and macro elements in the culture medium (Tokuhara and Mii, 1993). Study revealed that, after 3 weeks of inoculation, seeds were started swelling becoming with yellowish green in colour indicating successful germination and spherule formation. 88.6% of seed germination was recorded in MS medium; otherwise 77.34% of seed germination was recorded in Mitra medium after 4 week and in commercial *phaleonopsis* medium 70.21% of germination was recorded (Table 1). The positive influence of basal MS medium on seed germination was also reported in *Esmeralda clarkei* (Paudal and Pant, 2012) and *Cymbidium mastersii* (Mohanty *et al.,* 2012). As germination percentage concern, the entire medium showed almost better response, however Mitra and commercial *phaleonopsis* medium take more time. In MS medium supplemented with 0.5 mgl^{-1} BAP was found most effective for transforming the spherules into fully developed protocorm like bodies (PLB's), observed after 5 week of culture. Otherwise, in Mitra medium required 6 week for PLB's formation. The difficulty comes

Table 1. Comparative effect of different culture medium on seed germination and Protocorm formation.

Name of species	Medium	Germination %	Average time taken (In weeks)	
			Spherule formation	Protocorm formation
C. pallens	MS	86.31%	4	6
	½ MS	70%	4	7
	Mitra	89.35%	3	5
	MS	88.6%	3	5
P. mannii	½ MS	75%	3	5
	Mitra	77.34%	4	6
	Commercial Phaleonopsis medium (HIMEDIA)	70.21%	4	7

across the culture of *Phalaenopsis* were the low PLB formation with long time requirement of multiplication from different explants previously reported by Thanka *et al*., 1975; Park *et al*, 2002; Tokuhara and Mii,2003; Chen and Chang, 2004, 2006; Harper, 2004; Khadazi, *et al*., 2020. Present

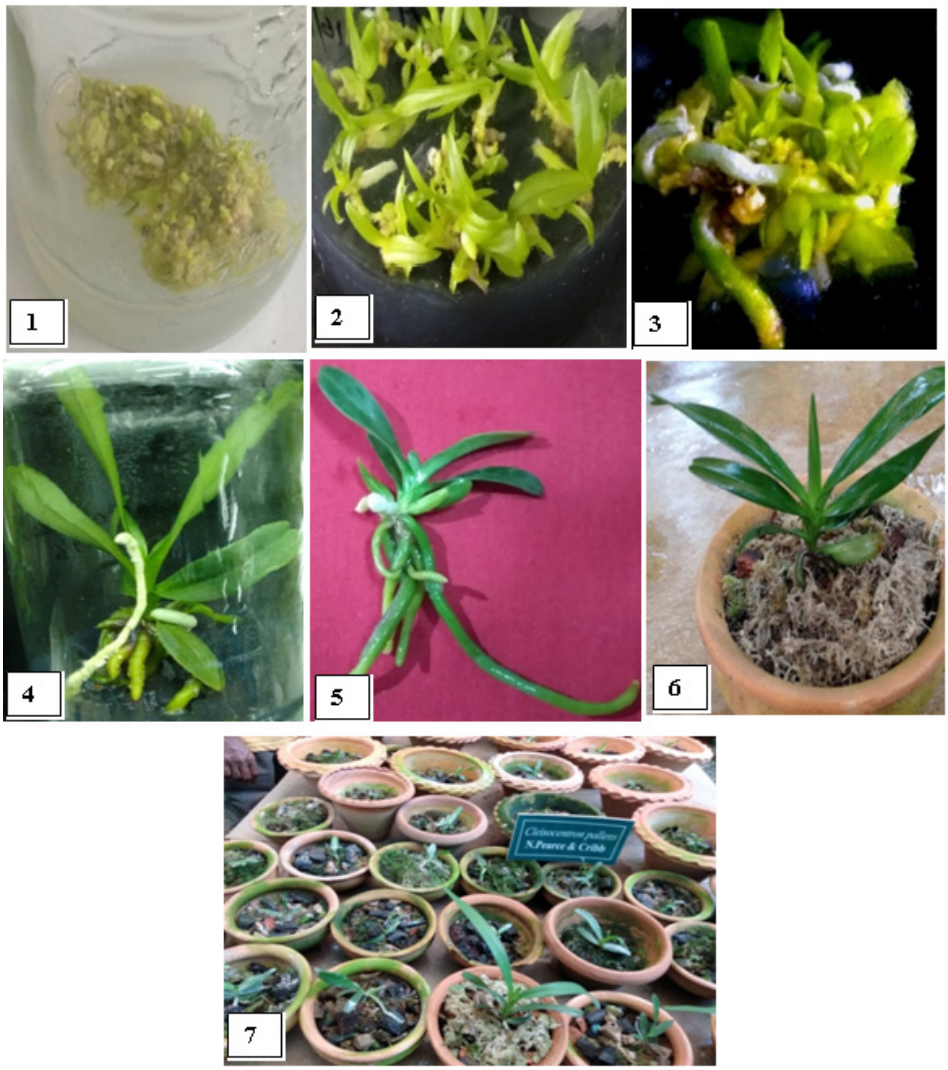

Fig. 2. In vitro seed propagation of *Cleisocentron pallens* 1- PLB formation 2 -shoot initiation 3- Multiple shoot & root development 4-Luxuriant growth of root 5- Plantlet ready to hardening 6- Best potting medium containing Brick chips: Charcoal: Sphagnum moss (1:1:2) after 30 days of transplantation 7-Mass multiplication

observation showed 70-88% of PLB formation in the entire basal medium.

The PLBs were transformed to small plantlets containing 3-4 leaves per shoot after sub culturing the medium with higher concentration of BAP (1, 1.5,2 mgL^{-1}). The maximum number (12.5 ± 3.62) and length (4.01±0.66 cm) of shoot were recorded in the medium containing 1.5 mgL^{-1} BAP (Table 2, Figure-3). Subramaniam *et al.,* (2009) discussed the effect of medium and different cytokinin concentration in propagation protocols of *Phalaenopsis violacea* through flower stalk cuttings. They also tested that MS media containing BAP in different concentration was better in inducing PLBs from leaf segment of *P. violacea*. Tokuhara and Mii (1993) study the Micropropagation of *Phalaenopsis* and *Doritaenopsis*

Table 2. Effect of different PGR's on shoot development after 12 week of culture

Name of species	IBA	BA	Mean no of shoot	Mean length of shoot	Mean no of root	Mean length of root cm
C. pallens	Mitra control	2.2 ± 0.82	1.65±0.70	3.1±0.99	2.1±0.58	
	0.5	-	4.3 ± 1.82	2.79±0.55	3.4±1.07	2.88±0.60
	-	0.5	3.5 ± 1.26	1.84±0.58	0	0
	-	1	5.5 ± 2.06	2.48±0.70	0	0
	-	1.5	10.5±1.77	3.78±0.46	0	0
	-	2	7.3 ± 1.49	4.15±0.58	0	0
	0.5	0.5	6.3 ± 1.49	2.53±0.66	0	0
	0.5	1	6.9 ± 1.66	3.61±0.33	3.81±0.46	1.82±0.77
	0.5	1.5	**11.2±1.75**	**4.75±0.58**	4.23±1.01	2.13±1.33
	0.5	2	7.3 ± 1.56	3.43±0.85	3.23±0.23	2.82±0.57
P. mannii	MS control	4.3 ± 0.82	3.18±0.16	3.1±0.99	2.1±0.58	
	0.5	-	3.8 ± 1.56	2.48±0.25	3.4±1.07	2.88±0.60
	-	0.5	3.5 ± 1.26	1.84±0.58	0	0
	-	1	4.5 ± 0.89	3.48±0.36	0	0
	-	1.5	**12.5 ±3.62**	**4.01±0.66**	0	0
	-	2	8.2 ± 1.03	3.03±0.73	0	0
	0.5	0.5	6.7 ± 1.41	2.53±0.66	0	0
	0.5	1	6.1 ± 1.1	3.61±0.33	2.3±0.46	1.82±0.77
	0.5	1.5	10.5±1.50	3.78±0.46	2.4±0.57	2.13±1.33
	0.5	2	7.4 ± 1.07	3.43±0.85	3.3±1.33	1.21±0.37

*Result based on average of 10 replicate per treatment denotes mean and ± standard deviation

Table 3. Effect of different plant growth regulator in multiple root development

Name of species	NAA	IBA	Mean No of roots per shoots	Mean Root length (cm)
C. pallens	0.5	-	3.1±0.82	1.76±0.81
	1.0	-	3.9±0.87	3.04±1.67
	1.5	-	6.3±0.82	4.17±1.54
	2.0	-	4.3±0.94	3.47±0.70
	-	0.5	3.4±0.84	1.9±0.75
	-	1.0	4.1±0.73	3.44±1.49
	-	1.5	**7.3±1.33**	**4.67±1.71**
	-	2.0	4.9±1.19	3.76±0.72
P. mannii	0.5	-	2.3±0.67	1.02±0.44
	1.0	-	2.9±0.73	1.62±0.24
	1.5	-	3.5±0.52	3.4±0.28
	2.0	-	3.9±0.99	4.9±0.85
	-	-	2.8±0.78	1.13±0.46
	-	0.5	3.2±0.73	1.75±0.19
	-	1.0	3.7±0.67	3.56±0.42
	-	1.5	**4.4±0.69**	**5.85±0.95**
		2.0	3.6±0.79	4.3±0.75

by culturing shoot tips of flower stalk buds. In After 12 week of culture, the maximum number (10.5±1.50) of multiple shoot per culture was found in the medium supplemented with 1.5 mgL^{-1} BAP and 0.5 and 1mgl^{-1} IBA (Figure 3: 4). The positive response on combine effect of auxin and cytokinin also reported in micro propagation of *Vanda testellata* (Rahman *et al.*, 2009); and in *Paphiopedilum* species (Long *et al.*, 2010).

For root initiation and development, the individual shoot (4-5 cm) were selected and transferred to Mitra medium for *C. pallens* and MS medium for *P. mannii* with addition of 0.2% activated charcoal powder. Both the medium were supplemented with different concentration of auxin as IBA (0.5,1, 1.5, 2 mgl^{-1}) and NAA(0.5,1, 1.5, 2 mgl^{-1}). After 12 week of culture, maximum number of root formation (7.3±1.33) with 4.67±1.71 cm length was observed in *C. pallens* (Table 3 Figure 2:4). In *P. mannii* the highest number of root (4.4±0.69) per shoot was recorded in medium supplemented with 1.5mgl^{-1} IBA with an average length of (5.85±0.95

Fig. 2. In vitro seed propagation of *Phalaenopsis mannii* 1 & 2- PLB formation in MS and Mitra medium 3 -shoot initiation 4- Multiple shoot & root development 5-Luxuriant growth of root 6- Plantlet ready to hardening 7- Best potting medium containing Brick chips: Charcoal: Sphagnum moss (1:1:2) after 30 days of transplantation 8-Mass multiplication

Table 4. Effect of potting media on growth and development of plantlets

Species name	Composition of potting media	Survival rate	Average leaf no	Average leaf length (cm)	Leaf width (cm)	*Response
C. pallens	Brick chips : Charcoal: Coconut husk (1:1:2)	80%	4.6±1.14	3.32±1.27	1.28±0.16	+++
	Brick chips : Charcoal: Sphagnum moss (1:1:2)	95%	5.8±0.8	3.35±1.07	1.35±0.42	++++
	Brick chips : Charcoal: Leaf mould (1:1:2)	70%	34.4±1.14	3.24±1.14	1.06±0.15	++
P. mannii	Brick chips : Charcoal: Coconut husk (1:1:2)	85%	5.2±1.48	3.02±0.63	1.45±0.20	+++
	Brick chips : Charcoal: Sphagnum moss (1:1:2)	90%	5.4±1.14	3.34±0.44	1.65±0.49	++++
	Brick chips : Charcoal: Leaf mould (1:1:2)	70%	3.8±0.83	2.86±0.40	1.25±0.25	++

*++ Moderate +++ Satisfactory ++++ Highly Satisfactory

cm) after 9 week of culture (Table 3, Figure-3: 5).

Activated charcoal plays an important role in root initiation and development. It helps to absorbing the toxic substance released in the medium due to germinating seed explants and also helps in absorbing the phenolic compounds, aeration and light absorption. Browning of the cultures and poor root formation was seen in this experiment before addition of charcoal powder. Poor regeneration in micropropagation of *phaleonopsis* due to phenolic compound exudation was reported by (Tanaka and Sakanishi, 1977). The positive response of activated charcoal powder in root development of *phaleonopsis* observed earlier by Hinnen *et al*., 1989; Eymar *et al*., 2000.

The plantlets having well developed root and shoot were transferred to green house condition with three potting medium at various concentration after the process of acclimatization. The seedlings of *C. pallens* showed excellent result with 95% of survival in potting medium containing Brick chips: Charcoal: Sphagnum moss (Table 4, Figure 2: 6&7) followed by the medium Brick chips: Charcoal: Coconut husk (1:1:2). The seedlings of *P. mannii* also exhibited better performance (90%) in the medium containing Brick chips: Charcoal: Sphagnum moss (Table 4, Figure 2: 7&8).

CONCLUSION

P. mannii is the most admired orchid and has immense ornamental value. As well as *C. pallens* is the most gorgeous rare wild individual. Seed derived propagation is very speedy and simple approach for mass multiplication. Study suggested that seed derived protocorm like bodies could be successfully applied for mass multiplication of both the orchids intended for *ex situ* conservation and reintroduction in their natural ecological niche. Even though, the species needed specific treatment and environmental condition in acclimatization.

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