

In vitro seed germination of *Cymbidium aloifolium* (L.) Sw., a potential medicinal Orchid from Eastern Ghats of Tamil Nadu, India

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Abstract *Cymbidium aloifolium* (L.) Sw. is an exquisite epiphytic orchid of the Kolli Hills (Eastern Ghats) of Tamil Nadu in Southern India. It is fast disappearing from its natural habitats due to deforestation and low germination rate in natural habitat. In the present study, an attempt was made to germinate the seeds from un-dehisced capsule of *Cymbidium aloifolium* (L.) Sw under *in vitro* condition. The seed germination and protocorm development were recorded in three different well known media namely Knudson C (KC), Half strength Murashige & Skoog (1/2 MS) and Vacin & Went (VW) media. The highest seed germination of 90% was observed KC basal media after 30th days whereas germination percentages were 40% and 30% on 1/2 MS and VW media respectively. The well-developed protocorm were transferred to KC media supplemented with 6-Benzyl Amino Purine (BAP) and Naphthalene acetic acid (NAA) where BAP (1.0 mg/l) and NAA (1.0 mg/l) together were found to be optimum for the highest shoot formation. About 90% of the shoots found to be well rooted after transfer to the KC medium differently supplemented with 1.5 mg/l Indole-3-acetic acid (IAA) and 1.0 mg/l Indole-3-butyric acid (IBA). Though rooting also took place in the two basic media but the duration was longer when compared with the hormone-supplemented media. The rooted plantlets were hardened and kept under greenhouse conditions which can be relocated in natural habitats.

Keywords Protocorm, Orchids, KC, 1/2 MS, VW media, NAA, BAP, IAA and IBA

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Introduction

Orchids are outstanding in many ways, like diverse shapes, colors and its medicinal values. They are marketed both as plants and as cut flowers and their production has been increased in recent years due to demand in the floriculture field (Tokuhara and Mii 1993, 2001; Chang and Chang 2000). *In vitro* culture is an important tool to save the species which faces various threats and extinction (Hey and Hey 1966). It can undermine the capacity of many developing countries to conserve resources and meet basic human needs. Increasing pressures due to population on land and other natural resources can aggravate the intensity of natural disasters such as flood and drought. Depletion of the Earth's biological diversity has much more profound consequences than some other environmentally alarming. Owing to its horticultural significance, it is collected indiscriminately from the nature and as a result it is prone to vulnerable. The present study was undertaken with a view to develop and establish an efficient protocol for mass propagation and consequently commercial exploitation and conservation of this important indigenous orchid.

Hard and intelligent decisions are needed to save the valuable taxa from the vastly changing landscape. As part of an *ex-situ* conservation strategy, artificial propagation including micro-propagation is successfully employed for recovering certain other species having specific problems in conventional horticulture (Fay, 1994). The advantage of plant tissue culture for the conservation of endemic and endangered species lies in the fact that it makes use of small units without sacrificing ex-plant takes pressure off the wild populations and makes available large number of plants for restoration as well as for horticultural applications. Symbiotic germination methods were only used for a short time before being replaced by non-symbiotic germination procedures (Zeng *et al.*, 2016). With non-symbiotic germination technology, thousands of seedlings could be raised to maturity from a single seed capsule. Besides the obvious potential in breeding, this technology is

also extremely important for vegetative propagation (Griesbach, 2002, Naing *et al.*, 2010). Propagation *in-vitro* has been successfully employed for the conservation of crop genetic resources, particularly with those crops which are vegetative propagated or possessing recalcitrant seeds, that cannot be stored under conventional seed bank conditions (Dodds, 1997, Villalolos *et al.*, 1991, Wilkins *et al.*, 1982, Paek *et al.*, 2011)) while tissue culture is essentially used as a crisis management tool for the multiplication of the existing genotypes.

Cymbidium aloifolium (L). Sw. is an exquisite and medicinally important epiphytic orchid of the Kolli Hills (Eastern ghats) of Tamil Nadu in Southern India. The leaf extract of the plant is also traditionally used to treat ear ache (Syndhya *et al.*, 2006). The leaf and root extracts are used to treat asthma and paralysis among the tribal peoples of Bangladesh (Mohammed *et al.*, 2010, Naing *et al.*, 2011). It is disappearing at a rapid rate from its natural habitats due to extensive collections by the orchid enthusiast. The plants produce very minute seeds and lacks endosperm. Wind acts as a vector for seed distribution and requires mycorrhizal association for their germination. Hence, a fast method of growing and conserving them in the green houses garners much attention.

In-vitro cultures of orchid seeds have shown that various species require different and often specific medium composition for optimum germination and growth. However, the sequential steps of development and histo-morphological changes from embryo to seedling have been traced only in a few species, including those of *Vanda* (Rao and Avadhani 1964), *Cattleya* (Shushan, 1959), *Cymbidium* (Vij, 1986) and *Geodorum* (Jon and Nirmalya, 2001).

Purpose of this article is to report our success in aseptic seed germination of *Cymbidium aloifolium* (L). Sw. and to trace the sequence of developmental changes that take place from the onset of seed germination and development of protocorm- like bodies (Plbs).

Materials and Methods

Preliminary assessment to select suitable medium for germination

The mature undehisced capsules of *Cymbidium aloifolium* (L). Sw. were collected from Kolli Hills (Eastern Ghats) of Tamil Nadu, India and stored at 4°C for 2 days. These capsules were utilized as seed resource for the further study. Initially it was washed with mild detergent and then soaked in 15% (v/v) NaOCl augmented with Tween-80 for 15 min.

Capsules were again washed with fungicide (Bavistin) for 10 min. and subsequently rinsed five times with sterile distilled water. The surface sterilized capsules were longitudinally dissected and around 100±10 seeds were transferred into a 200 ml sterile glass jar containing 50ml of 0.8% (w/v) agar-solidified KC, 1/2 MS and VW basal media. Effect of Coconut water (10% v/v) and Peptone (0.2% w/v) were studied in modified KC, 1/2 MS and VW media in which both were supplemented separately. Exact number of seeds was counted under a microscope after the successful transfer.

Plant cultures were maintained at 25°C±2°C for 12 h photoperiod provided by White fluorescent lights, M/s:Philips of 3000 lux intensity. Percentage of germination was calculated using the following formula:

$$\text{Percentage of Germination} = \frac{X - Y}{X} * 100\%$$

X = Number of seeds inoculated

Y = Number of seeds germinated

Assessment of suitable conditions for seedling and rooting formation

The KC was used for proliferation of the protocorm based on the obtained result (Table 1). In KC media 2% of sucrose served as carbon source and additionally two different plant growth regulators (PGRs) namely BAP and NAA were supplemented to each type of medium in different combinations as mentioned in the Table 2. The pH of the media was adjusted to 5.3, followed by an autoclave at 121°C for 15 min under 15 lbs pressure. These cultures were maintained under a 16 h-photoperiod at 25±2°C The experiment was performed in triplicate and average number of shoots per protocorm was determined after four weeks.

For the induction of root, the regenerated multiple shoots of *Cymbidium aloifolium* were excised and a single shoot was cultured on KC medium individually fortified with IAA and IBA (0.5 to 2.5 mg l⁻¹) as mentioned in Table 3. The observations were recorded with regular intervals of one week up to 16 weeks of culture and the obtained root number and their length was recorded.

Hardening of the seedlings

Well-developed rooted shoots were taken out from the culture vessels and washed under running tap water then the plantlets were washed with fungicide solution. Individual regenerates were placed onto net pots containing equal proportion of charcoal (0.5 ~ 1.0 cm each), brick-gravels and coconut husk as supporting materials. The plantlets were sprayed with

Table 1 Effect of different media on the germination and protocorm development in *Cymbidium aloifolium* (L)

Media employed for seed germination	% of germination	Time taken for initiation of germination (days)	Shoot growth (%)
1/2 MS – B	50	85	10
1/2 MS – CW	30	80	-
1/2 MS – P	20	90	-
KC – B	90	65	50
KC – CW	80	70	50
KC – P	70	75	40
VW – B	-	-	-
VW – CW	-	-	-
VW – P	-	-	-

B-Basal medium, CW-Coconut Water (10% v/v), P- Peptone (0.2%, w/v)

Table 2 Effect on BAP and NAA on the development of protocorm into microshoots and regeneration on KC medium

Hormone concentration (mg/l)		Mean number of shoots (M±SD)	Mean length (cm) of shoots (M±SD)	Mean number of roots (M±SD)
BAP	NAA			
0.5	-	1.3±1.34 lm	0.72±0.21 kl	0
1.0	-	2.0±1.34 i	0.54±0.37 mn	0
1.5	-	1.4±1.01 k	0.57±0.39 m	1.3±0.64 de
2.0	-	1.9±1.3 ij	0.80±0.30 h	1.4±0.48 cd
0.5	0.5	2.7±1.41 gh	0.73±0.34 ij	0
1.0	0.5	2.8±1.46 fg	1.32±0.14 de	0
1.5	0.5	4.8±1.46 b	1.46±0.28 c	1.6±0.19 c
2.0	0.5	3.2±2.00 ef	1.06±0.55 g	1.4±0.80 cd
0.5	1.0	4.5±1.24 bc	1.70±0.15 ab	2.5±1.02 b
1.0	1.0	6.1±1.13 a	1.79±0.19 a	3.1±1.64 a
1.5	1.0	3.8±0.97 d	1.21±0.31 f	1.6±0.66 c
2.0	1.0	3.4±1.42 de	1.40±0.26 cd	0

Table 3 Effect of IAA and IBA on root induction in KC medium after 25th day of inoculation

Medium+ Hormone (mg/l)	Concentration (mg/l)	Mean root number (M±SD)	Mean root (cm) length (M±SD)
IAA	0.5	1.7±0.90 cd	0.3 ±0.35 hi
	1.0	2.5±0.80 c	0.58±0.45 ef
	1.5	3.8±0.97 a	0.60±0.38 cd
	2.0	1.4±0.91 de	1.74±0.20 a
	2.5	1.2±0.60 ef	0.41±0.33 gh
	0.5	2.0±0.77 c	0.51±0.31 fg
IBA	1.0	3.8±0.97 a	1.3±0.13 b
	1.5	1.4±0.8 de	0.68±0.21 e
	2.0	0.6±0.66 g	0.26±0.16 jk
	2.5	0.3±0.64 gh	0.16±0.16 bc

balanced fertilizers (1 g/l 17N-17P-17k) twice weekly. The transplanted plants were finally kept in the shade house for further acclimatization.

Statistical analysis

Data were recorded on the basis of different parameters subjected to analysis of variance (ANOVA) and mean values

of treatments were compared by least significant difference (LSD).

Values are Mean ± SD (n = 10) of two independent experiments. Mean values followed by the same letter in a column are not significantly different as indicated by Duncan's multiple range test (P = 0.05). Values within a column having the same alphabet are not statistically significant and sharing at least one letter are no significantly



Fig. 1 Protocorm like bodies formation on KC- b medium



Fig. 3 Different stages of the protocorm like bodies (PLBs)



Fig. 2 Regeneration of plantlets and aerial root formation on 1/2 MS medium supplemented with 1.0 mg/l of BAP and 1.0 mg/l of NAA



Fig. 4 The seedlings before processed for hardening

different at $p < 0.05$ level according to Duncan's multiple range test.

Results and Discussion

The first sign of germination on the 30th day on KC basal medium was that the embryo turned into spherical form and was enclosed in dark yellow color. After a few days some adventive tissues appeared on their tips and the embryo ruptured at one of the poles and after a few days it showed 4-5fold increases in size with abundant chloroplasts and starch cells. The embryos by the 50th day exhibited a prominent zone of pro-meristematic cells and developed a pair of leaf primordia (Fig. 1).

The well-developed protocorm like bodies (PLBs) were inoculated with KC media augmented with BAP (1.0 mg/l) and NAA (1.0 mg/l) which yields High frequency of plant regeneration (Table 2). One of the primordial developed more rapidly than the other to produce an unequal pair of first embryogenic photosynthetic leaves. Simultaneously with the development of embryonic leaves at the proximal end of the embryo the marginal cells at the distal end of the embryo started giving rise to tubular and unicellular rhizoids.

The seeds are thin and transparent. The cells of seed coats



Fig. 5 Potted plant of *Cymbidium aloifolium* (L)

are varied in size and shape. Such variations that were observed in the present study are in agreement with the observations noted by the previous workers (Clifford & Smith 1969; Ekanthappa, 1981). It has been earlier established that the nature of seed coat is of great taxonomic value within the Orchidaceae (Stoutamire, 1963, Kalimuthu *et al.*, 2007)).

Thus the early concept that the orchid seeds are sterile and or at least incapable of germination is no more valid (Arditti, 1967). That the germination of seeds of epiphytic orchids posed no problem has been pointed out by various workers (Stoutamire, 1964a; Stoutamire 1964b; Warcap 1971; Mc Intyre *et al.*, 1972; Mc Intyre *et al.*, 1974). However, they have indicated that difficulty was encountered in the germination

of terrestrial orchid seeds in contrast to those of epiphytic taxa.

The well-developed shoots were removed from the culture tubes and transferred to the rooting media. About 90% of the shoots rooted well after transfer to the KC medium supplemented with 0.2 mg/l IBA.

In the present investigation the developed plantlets were transferred into KC medium supplemented with IAA and IBA separately which was more essential for regeneration of roots. The similar results were obtained in *Cymbidium kanran* (Shimasaki & Uemoto 1990) and *Dendrobium aqueum* (Philip et al., 2009). In *Vanda coerulea* the rooting efficiency varied with different concentrations of auxin such as IAA, IBA and NAA. The highest percentage of rooting was significant in 1/2 MS medium supplemented with IAA (1.5 mg/l) (Malabadi, 2004, Naing et al., 2010). In 1922, Lewis Knudson at Cornell University reported that the fungus was not required for germination if the seed was sown on agar containing appropriate salts and sugars. Hence Knudson C medium is still being used to germinate the seed of some species supplemented with the specific hormones.

Conclusion

The present investigation was mainly aimed at understanding the mode of seed germination and organogenesis of the developing seedling. Although different workers have suggested several nutrient media for orchids, three well-known media were tried in the present study to prove into the germination response of seeds selected species. While Knudson C medium promoted the germination of *Cymbidium aloifolium* better than other media like 1/2 MS and VC.

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