In Vitro Propagation Of The Endemic Orchid Ascocentrum Ampullaceum (Roxb.) Var. Auranticum Pradhan

Jina Heikrujam[1,2], Rajkumar Kishor[2] and Pranab Behari Mazumder[1]

[1]*Plant Biotechnology Laboratory, Department of Biotechnology, Assam University, Silchar, Assam, India*

[2] *Kwaklei and Khonggunmelei Orchids Pvt. Ltd., Sagolband Vijaygovind, Imphal-795001, Manipur,India.*

Abstract- *Ascocentrum ampullaceum var. auranticum is a popular albeit endangered orchid of Manipur, India, which would be desirable to propagate using regeneration methods. Of the three basal media tested for asymbiotic seed germination, half strength Murashige and Skoog medium was found to be the most effective. ½ MS medium supplemented with 0.5 mg/ L KIN induced the best seedling growth after 90 days of culture. Survival rate of the transplanted plantlets in the polyhouse condition grew best on brick chips:charcoal pieces (2:1) with 95% after 30 days of culture.*

Keywords- Ascocentrum ampullaceum var. auranticum; asymbiotic seed germination; Orchidaceae; protocorm.

Abbreviations- BAP: 6-Benzylaminopurine; KIN: Kinetin; MS: Murashige and Skoog medium; NAA: Naphthalene Acetic Acid; VW: Vacin and Went; INP: Ichihashi New Phalaenopsis ; PLB: Protocorm like bodies ; PM: Potting media; S.E.: Standard error; PGR: Plant growth regulator(s).

I. INTRODUCTION

The angiospermic family 'Orchidaceae' is reviewed as the most exceedingly developed group of flowering plant with near *ca.* 1000 genera and 25,000-35,000 species and this number is continually increasing with inclusion of more and more new ones every year which shows an incredible range of diversity in size, shape and color [1]. Orchidaceae is a cosmopolitan family and dispersed throughout the globe, except the hot desert and Antarctica.

Ascocentrum ampullaceaum (Roxb.) var. auranticum Pradhan [2] is one of the endemic orchid of Manipur with 6-10 cm high, simple and erect stem with fleshy cord like roots, 15 x 1.5 cm, dark green, invariably mottled with brown, lanceolate-oblong, apex unequally and acutely bifid or truncate and toothed in between leaves., sub-sessile, many flowered, 7-12 cm long, axillary, erect inflorescence, 1.8 cm across, deep-orange; *bracts* 2-3 mm long, greenish brown, ovate, acute; *pedicelled ovary* 0.9-1.0 cm long, orange; *sepals* elliptic-ovovate, obtuse or rounded 0.9 x 0.5 cm. *petals* similar to sepals but slightly longer, obtuse; *lip* 0.6-0.7 cm long, ligulate, orange, cylindric, obtuse; *spur* straight, longer than lip, slightly inflated towards the apex, 0.8 cm long; *column* 4-5 mm long, orange; *pollinia* globose, purplish flower and flowering time is March-May and a shelf life of approximately 25 days.

This orchid has rich floricultural traits and this being the reason why it have been collected from wild recklessly and therefore, becomes endangered. A conservation strategy has to be adopted for this beautiful orchid for its survival. So far, there has been no information regarding the development of *in vitro* propagation protocols of this endemic orchid.

II. MATERIALS AND METHODS

Plant materials of A. auranticum (Figure 1) were collected from their habitats. Morphological data were observed for identification of the genuine plant specimen. The flowers were allowed to self-pollinate. Matured seed pod 150 day-old containing seeds was collected from the plant. The sterilized capsules were dissected transversely with a sterile surgical blade. Immature seeds were scooped out of the capsules and small mass of the assembled seeds were innoculated on culture medium viz., ½ MS medium, VW modified medium, INP Medium to observe *in vitro* asymbiotic seed germination. The pH of the media was adjusted to 5.8, 5.2 and 5.6 respectively before autoclaving at 121 ° C for 20 min. the culture tubes were kept at 25±2ºC under light intensity of 3000 LUX (fluorescent tubes) for 16 hours photoperiod in a 24 hours cycle. Each treatment had 10 replicates and the experiment was repeated 3 times. The seeds were then counted using stereo zoom microscope. Germination percentage is calculated using the formula:

$$\text{Germination percentage} = \frac{\text{Number of enlarged seeds with developed chlorophyll}}{\text{Total number of seeds per microscopic field}} \times 100\ \%$$

½ MS medium was supplemented with varying concentrations of BAP (0.1, 0.5, 1.0, 2.0 and 4 mgL^{-1}), KIN (0.1, 0.5, 1.0, 2.0

and 4 mgL^{-1}) and NAA (0.1, 0.5, 1.0, 2.0 and 4.0 mgL^{-1} either individually or in combination to investigate PLB induction and seedling growth. Protocorm after 90 days of culture were taken for observing viz., PLB number, fresh weight and dry weight. PGR was investigated. Seedlings after 90 days of culture were taken for observing growth parameters viz., shoot height, leaf length, leaf number, root length and root number.

Well-rooted plantlets having an average of 5 leaves and 4 roots were washed thoroughly with water to remove the adhering agar medium and again treated with 5% (w/v) fungicide for 15 min for acclimatization. Transplantation of the plantlets was tried on three different potting media, viz., (i) brick chips:charcoal pieces (2:1) (PM1), (ii) brick chips:charcoal pieces (2:1) mulched with moss (Sphagnum sp.) (PM2) and (iii) brick chips only (PM3). The plantlets were introduced directly to the polyhouse condition with average temperature of 28ºC. The plantlets were watered on alternate days. Their survival percentage was calculated after 30 days of transfer.

Figure 1: Flowers of A. auranticum

III. RESULTS AND DISCUSSION

Asymbiotic seed germination is one of the methods of conservation and propagation of orchids [3]. The highest seed germination percentage was obtained in ½ MS medium followed by VW modified medium and no germination in INP medium (Table 1) (Figure 2). For the development of orchid seeds, a balanced supply of both organic and inorganic nutrients is needed [4]. PGRs i.e., cytokins are reported to play crucial role in orchid seed germination [5], however in our study, the seeds of A. auranticum germinated in medium devoid of PGRs. This could be due to the presence of sufficient endogenous growth regulators vital for the initial stages of germination [6]. In our present study, the germinating seeds took minimal time to reach globular (21 days), development of leaf primordial (49 days), first leaf and first root (97 days) on 1/2 MS medium. Similar to our study, Bebemcha et al., 2016 stated that seed germination of *Vanda stangeana* on 1/2 MS medium showed to be the most effective to reach globular (77 days), leaf primordia (113 days) and first leaf and root (171 days) [7].

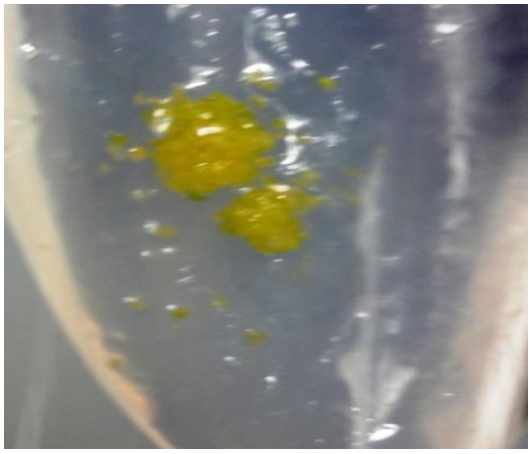

Figure 2: Seed germination on ½ MS medium

The highest PLB induction was obtained on ½ MS supplemented with 0.1 mg/L KIN (Table 2, Figure 3). This was correlated with the study of Devi et al., 2015 when *Taprobanea spathulata* was cultured in 1/2 MS medium enhanced with 0.1mg/L KIN was more effective for inducing optimal number of protocorms [8].

Figure 3: PLB induction in ½ MS supplemented with 0.1 mg/L KIN.

Development of protocorms into seedlings can further ascribed to the efficient assimilation and utilization of nitrogen in the form of ammonium nitrate present in the ½ MS medium. Optimal response of shoot height was observed in 1/2 MS medium supplemented with 0.5 mg/L KIN (Table 3, Figure 4) where average shoot height (3.2 cm), leaf number (9), leaf length (3 cm), root number (11) and root length (4 cm). Similar to our study, when Thokchom et al., 2017 innoculated *Phaius tankervilliae* in MS medium enhanced

with 0.5 mg/L KIN showed the average shoot height (3.03 cm) and number of root (3.07) [9] and also in the study of Devi et al., 2015 when *Taprobanea spathulata* was inoculated in 1/2 MS medium enhanced with 0.5 mg/L KIN showed average shoot length (0.66 cm), root number (1.5) and root length (0.41 cm) [8].

Figure 4: Seedling growth response in ½ MS medium supplemented with 0.5 mg/L KIN.

During the process of hardening, the transferred plants initially shed their leaves then produced new leaves. According to Preece and Sutter, in order to enable effective photosynthesis and growth plantlets must produce new leaves to adjust to new conditions [10]. Of the three different potting substrates used, survival percentage and the growth performance of the seedlings were found to be highest (95 %) in the community plastic bag having brick chips:charcoal pieces (2:1) as potting media (Table 4, Figure 5) after 30 days in poly- house condition. Similar to our study Kishor and Sharma, 2009 while transferring *Renanthera imschootiana X Vanda coerulea* plantlets to media containing brick chips:charcoal pieces (2:1) survival percentage was found to be 80% [11] and also Sunitibala and Kishor, 2009 while studying *D. transparens* when transferred to potting media brick:charcoal (2:1), the survival percentage was found to be greater than 90 % [12].

Figure 5: Transplanted seedlings in brick:charcoal (2:1) potting medium

Table 1: Comparative effects of ½ MS medium, VW modified medium and INP medium on asymbiotic seed germination of *Ascocentrum* auranticum from the day of culture to 200 days.

Media	Germination (%)	Response	Time taken for development (days)			
			Globular (mean ± S.E.)	Leaf primordial (mean ± S.E.)	First leaf (mean ± S.E.)	First root (mean ± S.E.)
½ MS	96	Yellowish green	21 ±0.77	49 ±1.54	64 ±1.80	97 ±3.61
VW	64.5	Yellowish green	35 ±0.51	89 ±0.51	125 ±1.54	162 ±0.77
INP	-	-	-	-	-	-

Table 2: Effect of PGRs viz., NAA, KIN and BAP on PLB induction of *Ascocentrum* auranticum.

NAA (mg/L)	KIN (mg/L)	BAP (mg/L)	No. of PLB (after 90 days) (mean ±S.E.)	Fresh weight of PLB (g) (mean ±S.E.)	Dry weight of PLB (g) (mean ±S.E.)
-	-	-	240 ±13.886	0.257 ±0.017	0.021 ±0.003
0.1	-	-	682 ±14.98	0.495 ±0.026	0.045 ±0.002
0.5	-	-	591 ±14.165	0.434 ±0.012	0.032 ±0.003
1.0	-	-	657 ±14.867	0.401 ±0.034	0.037 ±0.005
2.0	-	-	371 ±13.227	0.245 ±0.018	0.021 ±0.003
4.0	-	-	97 ±8.132	0.073 ±0.004	0.015 ± 0.002
-	0.1	-	**983** ±16.195	**1.065** ±0.073	**0.162** ±0.022
-	0.5	-	619 ±15.288	0.485 ±0.053	0.039 ±0.005
-	1.0	-	593 ±17.188	0.399 ±0.027	0.030 ±0.004
-	2.0	-	499 ±11.025	0.273 ±0.031	0.021 ±0.001
-	4.0	-	107 ±5.466	0.497 ±0.041	0.020 ±0.003
-	-	0.1	662 ±16.582	0.475 ±0.043	0.048 ±0.004
-	-	0.5	721 ±14.201	0.556 ±0.052	0.047 ±0.004
-	-	1.0	692 ±16.857	0.479 ±0.076	0.039 ±0.004
-	-	2.0	417 ±14.383	0.316 ±0.039	0.031 ±0.005
-	-	4.0	84 ±5.006	0.274 ±0.044	0.013 ±0.001

Table 3: PGRs viz., KIN, NAA and BAP on growth response of the seedlings of *Ascocentrum* auranticum on 1/2 MS medium.

KIN mg/L	NAA mg/L	BAP mg/L	Growth response of the seedlings (after 90 days)				
			Shoot height (cm)	Leaf		Root	
			(mean ±S.E.)	Number (mean ±S.E.)	Length (cm) (mean ±S.E.)	Number (mean ±S.E.)	Length (cm) (mean ±S.E.)
-	-	-	0.7 ±0.024	5 ±0.239	0.5 ±0.041	5 ±0.071	1.2 ±0.279
0.1	-	-	2.8 ±0.061	7 ±0.498	2.5 ±0.090	9 ±0.129	3.3 ±0.594
0.5	-	-	**3.2 ±0.058**	**9 ±0.596**	**3.0 ±0.096**	**11 ±0.207**	**4.0 ±0.610**
1.0	-	-	2.9 ±0.059	5 ±0.209	2.3 ±0.127	4 ±0.137	1.7 ±0.406
2.0	-	-	2.4 ±0.049	4 ±0.197	2.1 ±0.105	4 ±0.138	1.5 ±0.299
4.0	-	-	2.0 ±0.078	4 ±0.172	1.7 ± 0.110	3 ± 0.079	0.9 ±0.197

-	0.1	-	2.7 ±0.052	5 ±0.271	2.1 ±0.145	4 ±0.092	1.3 ±0.314
-	0.5	-	3.0 ±0.066	6 ±0.262	2.3 ±0.090	4 ±0.122	1.5 ±0.303
-	1.0	-	2.5 ±0.069	4 ±0.219	2.1 ±0.116	5 ±0.169	1.1 ±0.358
-	2.0	-	2.3 ±0.064	4 ±0.214	1.7 ±0.101	5 ±0.066	0.9 ±0.383
-	4.0	-	1.9 ±0.1	5 ±0.306	1.1 ±0.0447	4 ±0.086	0.7 ±0.295
-	-	0.1	2.6±0.058	6 ±0.368	2.3 ± 0.066	7 ±0.193	2.5 ±0.589
-	-	0.5	2.8±0.081	6 ±0.514	2.5 ±0.120	5 ±0.175	2.6 ±0.348
-	-	1.0	2.3 ±0.069	5 ±0.395	1.8 ±0.071	5 ±0.138	1.9 ±0.374
-	-	2.0	2.1 ±0.084	5 ±0.249	1.7 ±0.104	3 ±0.089	0.9 ±0.239
-	-	4.0	1.5 ±0.1	4 ±0.436	1.2 ±0.080	5 ±0.101	1.0 ±0.409
0.5	0.1	-	2.7 ±0.079	3 ±0.208	2.5 ±0.140	4 ±0.095	1.5 ±0.352
0.5	0.5	-	2.9 ±0.090	5 ±0.314	2.3 ±0.166	4 ±0.125	1.3 ±0.383
0.5	1.0	-	2.5 ±0.0942	3 ±0.179	1.9 ±0.132	3 ±0.082	1.1 ± 0.234
0.5	2.0	-	2.3 ±0.09	3 ±0.172	1.4 ±0.088	4 ±0.039	0.5 ±0.342
0.5	4.0	-	1.9 ±0.101	3 ±0.219	1.7 ±0.105	3 ±0.095	0.9 ±0.224
0.5		0.1	2.0 ±0.109	3 ±0.151	1.6 ±0.120	4 ±0.112	1.2 ±0.310
0.5		0.5	2.5 ±0.105	3 ±0.214	1.8 ±0.143	4 ±0.116	1.1 ±0.328
0.5		1.0	2.7 ±0.115	3 ±0.345	0.7 ±0.055	3 ±0.057	0.6 ±0.224
0.5		2.0	2.9 ±0.127	3 ±0.203	2.3 ±0.132	4 ±0.115	1.7 ±0.392
0.5		4.0	1.5 ±0.099	3 ±0.179	0.9 ±0.059	3 ±0.116	1.1 ±0.266
	0.5	0.1	1.7 ±0.132	3 ±0.253	0.6 ±0.050	4 ±0.100	0.8 ±0.383
	0.5	0.5	2.4 ±0.115	4 ±0.386	1.9 ±0.158	5 ±0.125	0.9 ±0.368
	0.5	1.0	2.1 ±0.101	3 ±0.244	1.6 ±0.131	3 ±0.133	1.3 ±0.271
	0.5	2.0	2.0 ±0.106	3 ±0.166	1.5 ±0.143	3 ±0.133	1.3 ±0.306
	0.5	4.0	1.5 ±0.115	3 ±0.185	1.1 ±0.129	4 ±0.102	0.8 ±0.392
0.5	0.5	0.5	3.0 ±0.145	5 ±0.374	2.5 ±0.138	8 ±0.181	2.7 ±0.491

Table 4: Effect of different potting media on growth response of seedlings of A. auranticum

Potting media	Survival (%)	Response of the seedlings			
		Leaf		Root	
		Number (mean±S.E.)	Length (cm) (mean±S.E.)	Number (mean±S.E.)	Length (cm) (mean±S.E.)
PM1	95	7.2±0.148	3.5±0.096	7±0.127	4.5±0.193
PM2	80	6.7±0.129	2.7±0.103	6.2±0.189	3.7±0.099
PM3	60	6±0.174	2.3±0.103	5.6±0.237	3.3±0.0814

IV. CONCLUSION

Since orchid seeds lacks endosperm, their germination in nature is very restricted. In the meantime, some of the orchid species have been obsolete and a large number of species have become limited and imperiled due to ruthless collections, destruction of habitats by restoration and deforestation, unauthorized trade have forced to decline in natural population of many orchid species.

This is the first report for *in vitro* propagation protocols of *Ascocentrum* auranticum. An attempt has been made to develop suitable protocols for mass multiplication. *In vitro* culture offers a sustainable and viable tool for rapid propagation. It can supply uniform and consistent plants materials in the market of floriculture. An efficient seed germination and acclimatization protocols that focus on proliferating orchid seedlings for reestablishing in their natural

habitats will help in the preservation of endangered or threatened orchid species has been developed.

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