International Journal of Botany Studies
ISSN: 2455-541X, Impact Factor: RJIF 5.12
www.botanyjournals.com
Volume 2; Issue 1; January 2017; Page No. 110-116

In vitro seed germination and rhizome based micropropagation of *Calanthe densiflora* Lindl: An indigenous terristerial orchid of Bangladesh

*** TK Bhowmik, MM Rahman**

Department of Botany, University of Chittagong, Chittagong, Bangladesh

Abstract
In vitro seed germination and rhizome based micropropagation protocol was established in *Calanthe densiflora* an indigenous terrestrial orchid of Bangladesh. The seeds were germinated on different media with three different sources of carbohydrates *viz.* sucrose, glucose and lactose. The germination rate was found to be better on PM medium. Among three carbon sources, the percentage of seed germination was higher in sucrose containing medium than glucose and lactose containing media. The germinated seedlings were cultured on solid & liquid medium. In liquid and solid culture the highest rate of elongation was achieved on MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP and 0.8% (w/v) agar solidified MS medium supplemented with 3% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP respectively. Elongation of shoot system was better in liquid media than 0.8% (w/v) agar solidified medium. It also proved that MS was better than PM for elongation of shoot system. Rhizome segments of aseptic seedlings were used for rapid micropropagation. Rhizome segments underwent direct organogenesis to produce multiple shoot buds and maximum average number of shoot buds (6.45 ± 0.41/segment) induced on MS medium supplemented with 3% (w/v) sucrose + 1.0 mg/l IAA + 2.0 mg/l BAP followed by 6.02 ± 0.35 shoot buds/segment on MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 2.0 mg/l BAP. Liquid media were more effective in SPSs induction than agar solidified medium. MS medium supplemented with 3% (w/v) sucrose and 0.5 mg/l IBA was best for induction of strong and stout root system. The rooted seedlings were finally transferred to outside natural environment by successive phases of acclimatization.

Keywords: *in vitro* germination, micropropagation, calanthe densiflora, PLBs, SPSs

1. Introduction
The orchidaceae is one of the highly specialized and largest families of flowering plants, comprising more than 17,000 species to which more and more new ones are being added every year [1]. In Bangladesh, it represents one of the largest group of flowering plants and 177 species with a variety under 70 genera [2] distributed throughout the country especially Chittagong, Chittagong Hill Tracts, Cox's Bazar, greater Sylhet, Gazipur and Sundarbans mangrove forest [3]. Loss of habitat, deforestation and destructive collection technique [4] and over exploitation of orchids with medicinal and ornamental values has depleted the orchid wealth of Bangladesh. Many orchids are now at the verge of extinction, so it is high time to conduct effective strategies to conserve this precious jewelry of nature. The presently investigated orchid species with spectacularly beautiful flowers belong to the genus *Calanthe*. The species of *Calanthe* are widely used as cut flowers and medicinal purposes.
Orchid seeds, unlike the seeds of other flowering plants are extremely small, dust like, produced in large numbers 1300-4,00,000 seeds per capsule [5] and with little or no stored food. Orchid seeds have unique physiology of germination. Under natural condition, fungal aid or mycorrhiza [6] usually species of *Rhizotectonia* is required. In nature, germination rate is very low (5%) and takes long time. Therefore highly exploited species immediately require *ex situ* conservation by tissue culture technique [7, 8]. During the last few years' tissue culture method have been extensively exploited for the large scale propagation of many orchid species. Use of exogenous plant growth regulators (PGRs) in artificial media at suitable concentrations and combinations stimulates zygotic embryo to initiate protocorms that develop into plantlets. However, very little work has been done for the germination, micropropagation and conservation of this species.

In the present study, *in vitro* seed germination, rhizome based micropropagation and seedling development method was investigated for the rapid propagation of *C. densiflora* Lindl.

2. Materials and Methods
Immature green capsules of *Calanthe densiflora* Lind. was collected from National Botanical Garden, Mirpur, Dhaka, Bangladesh which were used as plant materials for the present investigation.

2.1 Surface sterilization of plant materials
Immature green capsules of *C. densiflora* were used as explants for the present investigation. Young green capsules were first cleaned with detergent and rubbed with savlon soaked cotton and finally washed in running tap for 30 minutes till all the detergent was washed off clearly. After that, green pods were surface sterilized sequentially with 70% ethyl alcohol for 1 minute, 0.1% $HgCl_2$ solution for 10 minutes and finally rinsed thoroughly three times with sterile distilled water. Chemical surface sterilization process was carried out on laminar airflow cabinet.

2.2 Culture medium and incubation
In the present investigation, four basal media of KC, MS, MVW and PM with three types of carbohydrate source *viz.*

lactose (disaccharide), sucrose (disaccharide) and glucose (monosaccharide) were used for *in vitro* seed germination. Eighteen types of elongation media were prepared using MS and PM basal media (solid and liquid) with different concentrations and combinations of PGRs for enhancing elongation of seedlings. 0.8% (w/v) agar was also used in solid media but in liquid media no agar was added so that the medium remained liquid. Sixteen types of micro propagation media were prepared using MS basal media with different concentrations and combinations of PGRs for enhancing rhizome based micro propagation of seedlings. Ten types of half strength MS media and full strength auxin supplemented MS media were prepared for well-developed root system. Agar (0.8% w/v) was used as a gelling agent for all tested media. pH of the media was adjusted at 5.8 in case of MS and 5.4 in KC, MVW and PM by using 0.1N NaOH or HCl. Agar was dissolved by boiling the mixture and about 50 ml of media was dispensed into 100 ml each culture vessel and autoclaved at 121 ^{0}C for 20 minutes at 15 lb/cm^2 pressure. All cultures were maintained at 25±2 ^{0}C under 350-500 lux illumination for 14h photoperiod using white fluorescent tubes and 10h dark.

2.3 Seed culture

Surface sterilized immature green capsules were kept on sterilized petri dish containing sterilized filter paper for drying. They were cut longitudinally with the help of sharp sterilized surgical blade. The immature seeds were scooped out with the help of sterilized spatula and transferred to and spread over the surface of lactose, sucrose, glucose supplemented KC, MS, MVW and PM basal medium. Sub-culturing was carried out every six weeks into fresh medium and ten replicates were used for each treatment. The initiation and rate of seed germination was recorded regularly. The entire experiment was performed in aseptic condition under laminar air flow hood to prevent contamination.

2.4 Rhizome culture

In vitro grown seedlings of rhizome sections were used for micropropagation. MS media supplemented with various concentrations and combinations of PGRs were used for the purpose. Multiple shoot buds those produced from rhizome segments were subcultured in the same media for elongation and thereafter in rooting media for induction of well-developed root system.

2.5 Rooting

Half strength MS0 and nine different types of 0.8% (w/v) agar solidified MS medium supplemented with 3% (w/v) sucrose and three kinds of auxins *viz*. IAA, IBA, NAA were used for induction of strong and stout root system. The efficiency of the media in terms of enhancing the development of root system was assessed based on the increase in number and length of roots that developed within 30d of culture in rooting media.

2.6 Transplantation

In rooting medium the plantlets grew further and produced well developed root system. Those were taken out of the culture vessel and transferred to outside the culture room following successive phases of acclimatization. For the purpose, the mouth of the culture vessels was kept open for one day in the culture room and then kept outside of the culture room for 6h in the next day. Later on, those were kept outside of the culture room for 12h. Finally the seedlings were taken out of the culture vessels and rinsed with running tap water for removal of agar attached to the roots. Then the seedlings of *C. densiflora* were transferred to pots containing sandy loam soil. Transplanted seedlings were watered regularly for about 2-3 months where the seedlings established and grew well.

2.7 Conservation

In some cases, seedlings of *C. densiflora* produced shoot primodia like structures (SPSs) or protocorm like bodies (PLBs) at the base of the culture vessels. These SPSs or PLBs were taken as test materials for conservation. Seedlings were cultured in low nutrient supplemented or growth retardant containing media to retard their growth and by this way conserve them for long time.

3. Results and Discussion

The seeds of *Calanthe densiflora* were aseptically grown on 0.8% (w/v) agar solidified MS [9]; PM [10]; MVW [11] and 1.2% (w/v) agar solidified KC [12] media with three different sources of carbohydrates *viz.* sucrose, glucose and lactose. The results obtained are briefly summarized in Tables-1. The overall results revealed that PM was better than the other three media in respect of the percentage of germination and required time for germination of this orchid species (Fig.1a). Similar result was also noted by [13, 16] the references. The reference [17] reported that MS medium was found best for germination of *Aerides odorata orchid seeds.* Sugar is an important component of any kind of nutrient medium used in tissue culture studies. Generally sucrose is used in the medium but in some cases other carbohydrates such as lactose, glucose, maltose, fructose, dextrose, galactose, mannitol, cellulose, inulin, mannose have also been used [18, 19]. Carbon source has also great role for *in vitro* orchid seed germination. Among three carbon sources, the percentage of seed germination was higher in sucrose containing medium than in two glucose and lactose containing media.

In germination media, the germinated protocorms turned into mini seedlings and continued further growth. In order to induce rapid elongation and enhance growth, germinated seedlings were transferred to elongation media (Table-2) prepared with different combinations and concentrations of plant growth regulators (PGRs). Eighteen types of solid and liquid elongation media were prepared using MS and PM basal media with different concentrations and combinations of PGRs (BAP, Kn, NAA, IAA, IBA and Pic.) for enhancing elongation of seedlings. 0.8% (w/v) agar was also used in solid media but in liquid media no agar was added so that the medium remained liquid. The efficiency of a medium in terms of enhancing shoot elongation was determined based on the increase in length of shoot system within 30d of culture on elongation media. Different hormone combinations and culture condition were found to be better for elongation. The highest rate of elongation took place in liquid MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP (Fig.1b) followed by 0.8% (w/v) agar solidified MS medium supplemented with 3% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP (Fig.1c). Comparison of the results of liquid and solid media revealed that liquid culture was better than 0.8% (w/v) agar solidified

condition. The overall results indicate that MS based medium was better than PM based medium for enhancing elongation of shoot system of the seedlings. Similar findings have been recorded in many other studies on orchids [14, 15, 20, 23].

Source of explants, size of explants, media composition, pH and other environmental factors may play a significant role in mass scale clonal propagation of orchids. For rapid micropropagation, *in vitro* grown seedlings were used as source of rhizome explants [14, 21, 24, 27]. The rhizome explants were cultured on 0.8% (w/v) agar solidified MS media supplemented with various combinations and concentrations of PGRs and produced multiple shoot buds *via* direct organogenesis (Table-3). The efficiency of a medium was assessed on the basis of number of shoot buds produced from each explant. Direct organogenesis took place and that was dependent on combinations of the media. The rhizome segments of *C. densiflora* underwent direct organogenesis and maximum number of shoot buds (6.45 ± 0.41/ segment) were produced when cultured on 0.8% (w/v) agar solidified MS medium supplemented with 3% (w/v) sucrose + 1.0 mg/l IAA + 2.0 mg/l BAP (Fig.1d) followed by 6.02 ± 0.35 shoot buds/ segment on MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 2.0 mg/l BAP. Similar findings also noted by the reference [21] in *Geodorum densiflorum* and [24] in *Calopogon tuberosus*. In *Vanilla planifolia*, multiple shoot buds were produced from axillary bud explants on MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA [28].

Comparative study of the efficiency of the media in liquid and solid form was another aspect of the present investigation. PLBs (Protocorm Like Bodies) and SPSs (Shoot Primodia Like Structures) derived seedlings underwent elongation when grown individually on PGRs supplemented agar solidified and liquid elongation media. The elongation rate was different depending on PGR supplements liquid and solid media (Fig.1e) and liquid culture was best for elongation. Such better effect of liquid medium was also reported by the references [29, 30]. More surface exposure of cultured seedlings to liquid medium probably facilitated more uptakes of nutrients thereby contributing to better and prolific growth of seedlings.

In most of the cases higher concentration of cytokinin (BAP) and low concentration of auxins (IAA, NAA, Pic) was more effective for SPSs development at the base of the seedlings. On the whole, liquid media were more effective than agar solidified media. Most of the SPSs were greenish and a few were yellowish in color. MS based media was better than PM based media for induction of SPSs (Fig.1f). Similar finding was noted by the references [21, 30, 33].

The elongated Shoot buds and seed originated seedlings produced roots in elongation media but those were weak and few in number. Half strength MS0 and nine different types of PGR (IAA, IBA, NAA) supplemented MS media were used for induction of strong and stout root system (Table-4). The efficiency of the rooting media was evaluated based on the increase in length and number of roots developed per seedling within 30d of culture in rooting media. Induction of strong and stout root system of *C. densiflora* was best when cultured on MS medium supplemented with 3% (w/v) sucrose and 0.5 mg/l IBA (Fig.1g) followed by MS + 3% (w/v) sucrose and 1.0 mg/l IAA. The reference [34] shown that in *Acampe praemorsa*; [35] in *Cymbidium iridioides* observed that IBA was effective for rooting. But the reference [36] found that NAA was most appropriate in inducing roots in *Cymbidium*. The references [37, 38] also reported that IAA and auxin supplemented medium was more efficient for induction of strong and stout root system. It is noted that low concentration of auxin is more suitable than high concentration for induction of well developed root system.

Hardening of *in vitro* raised seedlings is an important aspect of plant tissue culture. Usually *in vitro* grown seed and shoot buds derived seedlings cannot adjust directly to outside natural environment. For that reason it is considered important to formulate efficient protocols for quick hardening technique. With this idea a number of mature *in vitro* developed seedlings were adjusted to outside natural environment through successive phases of acclimatization. It was possible to attain a considerable rate of survive of *in vitro* grown seedlings. The survival percentage of transplanted seedlings of *C. densiflora* was 70% (Fig.1h).

4. Conclusion

The existence of species medium specificity was therefore indicated in germination stage and PM was found superior then KC, MS & MVW media for promoting germination of orchid seeds. The effect of different carbohydrates namely, sucrose, glucose and lactose was also studied in terms of promoting seed germination and the overall results indicate that sucrose supplemented media was better for germination of orchid seeds. For comparing the efficiency in terms of enhancing seedling growth, the assessment was made to regard to shoot elongation dependent on the medium condition. It was found that a medium in liquid condition was better in terms of promoting shoot elongation than its solid counterpart. The plant growth regulator has an important role on the growth and differentiation of cultured tissues under *in vitro* condition and different kinds of PGRs of different nature are used in tissue culture media. The process of differentiation has been proved to be dependent of the ratio of auxins and cytokinins used in the culture media.The efficiency of a medium was assessed on the basis of number of shoot buds produced from each explant. The overall results indicated that increased in root length and number of roots is higher in shoot bud derived seedlings than that of seed originated seedlings. Furthermore, low concentration of auxin was more effective for enhancing rooting. Tissue originated plants has a great value in commercial firm and *ex situ* conservation. This clonal propagation technique created intense interest among the orchid growers and had a tremendous impact on the development of orchid industries.

Table 1: Results of *in vitro* germination of seeds *Calanthe densiflora*.

Medium	Carbohydrate	Number of culture vessels used	Number of culture vessels in which seeds germinated		Time (d) required for germination	Remarks
			No.	%		
KC	2% sucrose	10	05	50	45 - 50	Yellowish green PLBs
	2% glucose	10	04	40	48 - 50	Light green PLBs
	2% lactose	10	02	20	50 - 54	Light green PLBs
MS	3% sucrose	10	07	70	50 - 52	Brownish green PLBs
	3% glucose	10	05	50	48 - 52	Yellowish PLBs
	3% lactose	10	03	30	44 - 50	Greenish PLBs
PM	2% sucrose	10	09	90	42 - 45	Yellowish green PLBs
	2% glucose	10	02	20	48 - 52	Green PLBs
	2% lactose	10	04	40	50 - 55	Whitish green PLBs
MVW	2% sucrose	10	07	70	45 - 50	Green PLBs
	2% glucose	10	05	50	46 - 52	Whitish green PLBs
	2% lactose	10	03	30	48 -52	Light green PLBs

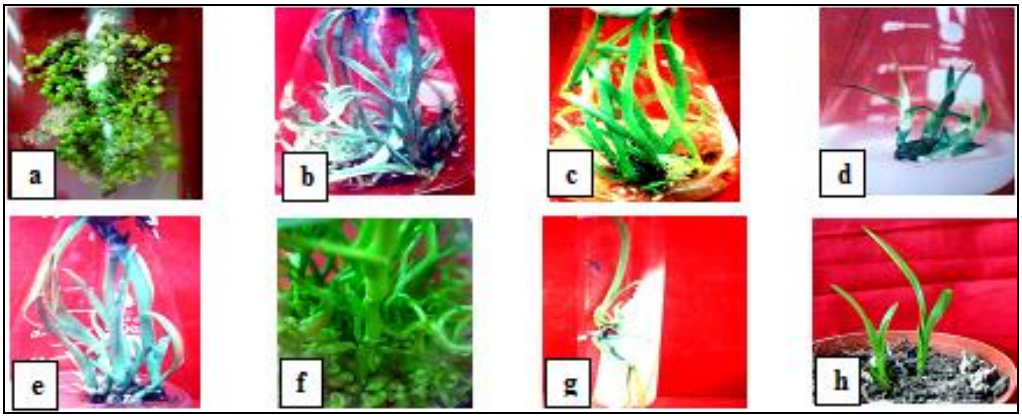

Fig 1: Different stages of *in vitro* seed germination and seedling development of *C. densiflora*: a. *In vitro* germination of *C. densiflora;* b. Elongation of germinated seedlings on liquid medium; c. Elongation of germinated seedlings on agar solidified medium; d. Development of multiple shoot buds on agar solidified medium; e. Elongation of multiple shoot buds derived seedlings on agar solidified medium; f. Development of SPS on liquid medium; g. Rooting of elongated seedling on agar solidified medium; h. Plantlets are growing in pot outside.

Table 2: Elongation of *in vitro* germinated seedlings of *C. densiflora* on 0.8% (w/v) agar solidified and liquid MS and PM media with different kinds of PGRs.

Culture medium with different combinations and concentrations of PGRs	Average initial length (cm) of seedlings after 60 days of culture on germination medium	Average length (cm) of seedlings after 30 days of culture on elongation medium	Increase in length** (cm) of seedling within 30 days of culture on elongation medium	Average initial length (cm) of seedlings after 60 days of culture on germination medium	Average length (cm) of seedlings after 30 days of culture on elongation medium.	Increase in length** (cm) of seedling within 30 days of culture on elongation medium
	Solid media			Liquid media		
MS+1.0 mg/l IAA+0.5 mg/l BAP	2.25	5.81	3.56	2.12	5.79	3.67
MS+0.5 mg/l IAA+1.0 mg/l BAP	2.24	5.67	3.43	2.15	5.60	3.45
MS+1.0 mg/l IAA+1.0 mg/l BAP	2.32	6.04	3.72	2.20	6.22	4.02
MS+1.0 mg/l NAA+0.5 mg/l BAP	2.30	5.93	3.63	2.25	6.03	3.78
MS+0.5 mg/l NAA+1.0 mg/l BAP	2.38	5.96	3.58	2.20	5.85	3.65
MS+1.0 mg/l NAA+1.0 mg/l BAP	2.20	6.05	3.85	2.17	6.04	3.87
MS+1.0 mg/l Pic+0.5 mg/l BAP	2.25	5.84	3.59	2.14	5.78	3.64
MS+ 0.5 mg/l Pic+1.0 mg/l BAP	2.32	5.84	3.52	2.23	5.79	3.56
MS+1.0 mg/l Pic+1.0 mg/l BAP	2.36	5.97	3.61	2.24	6.10	3.86

PM+1.0 mg/l IAA+0.5 mg/l BAP	2.25	5.79	3.54	2.15	5.87	3.72
PM+0.5 mg/l IAA+1.0 mg/l BAP	2.18	5.66	3.48	2.12	5.76	3.64
PM+1.0 mg/l IAA+1.0 mg/l BAP	2.35	5.93	3.58	2.14	5.92	3.78
PM+1.0 mg/l NAA+0.5 mg/l BAP	2.40	6.02	3.62	2.20	6.04	3.84
PM+0.5 mg/l NAA+1.0 mg/l BAP	2.22	5.78	3.56	2.22	6.03	3.81
PM+1.0 mg/l NAA+1.0 mg/l BAP	2.25	5.90	3.65	2.18	6.10	3.92
PM+1.0 mg/l Pic+0.5 mg/l BAP	2.30	5.75	3.45	2.25	6.08	3.83
PM+ 0.5 mg/l Pic+1.0 mg/l BAP	2.33	5.73	3.40	2.22	5.99	3.77
PM+1.0 mg/l Pic+1.0 mg/l BAP	2.24	5.77	3.53	2.25	6.16	3.91

*Based on observations from 50 seedlings taking five at random from each of ten culture vessels.

Table 3: Number of multiple shoot buds developed from rhizome segments on 0.8% (w/v) agar solidified PGRs supplemented MS media.

Combinations and concentrations of PGRs	% of induced multiple shoot buds per segment	Time (d) required for sprouting of multiple shoot buds	Number of multiple shoot buds produced per segment (Mean ± S.E.)
0.5 mg/l IAA + 1.0 mg/l BAP	55	35 - 38	5.44 ± 0.32
0.5 mg/l IAA + 1.0 mg/l Kn	45	35 - 40	4.11 ± 0.27
1.0 mg/l IAA + 2.0 mg/l BAP	65	28 - 32	6.45 ± 0.41
1.0 mg/l IAA + 2.0 mg/l Kn	50	35 - 38	4.75 ± 0.33
0.5 mg/l IBA + 1.0 mg/l BAP	35	35 - 40	3.26 ± 0.25
0.5 mg/l IBA + 1.0 mg/l Kn	35	34 - 38	3.29 ± 0.21
1.0 mg/l IBA + 2.0 mg/l BAP	45	35 - 38	4.05 ± 0.28
1.0 mg/l IBA + 2.0 mg/l Kn	40	35 - 40	3.89 ± 0.24
0.5 mg/l NAA + 1.0 mg/l BAP	55	30 - 35	5.42 ± 0.38
0.5 mg/l NAA + 1.0 mg/l Kn	50	32 - 35	4.68 ± 0.26
1.0 mg/l NAA + 2.0 mg/l BAP	60	30 - 34	6.02 ± 0.35
1.0 mg/l NAA + 2.0 mg/l Kn	50	30 - 36	4.78 ± 0.28
0.5 mg/l Pic + 1.0 mg/l BAP	40	34 - 38	3.77 ± 0.23
0.5 mg/l Pic + 1.0 mg/l Kn	45	32 - 36	4.16 ± 0.27
1.0 mg/l Pic + 2.0 mg/l BAP	50	30 - 35	4.71 ± 0.29
1.0 mg/l Pic + 2.0 mg/l Kn	55	30 - 32	5.53 ± 0.42

*Based on observations from 30 segments.

Table 4: Mean increased length (cm) and number of roots per seed originated and rhizome originated seedling of 30 days of culture on agar solidified ½ MS0 and Auxin supplemented MS rooting media.

Rooting medium			Average increased length and number of roots per seed derived seedling		Average increased length and number of roots per shoot bud	
			Mean length (cm) ± S.E.	Mean no. of roots/ seedling ± S.E.	Mean length (cm) ± S.E.	Mean no. of roots/ shoot bud ± S.E.
½ MS0			3.46 ± 0.19	2.13 ± 0.16	3.31 ± 0.18	2.07 ± 0.15
Auxin (mg/l)	IAA	0.5	3.41 ± 0.20	1.92 ± 0.13	3.02 ± 0.17	1.85 ± 0.13
		1.0	3.52 ± 0.19	2.28 ± 0.15	3.36 ± 0.21	2.24 ± 0.14
		1.5	3.13 ± 0.18	1.95 ± 0.12	2.82 ± 0.17	1.86 ± 0.11
	IBA	0.5	3.73 ± 0.23	2.47 ± 0.15	3.45 ± 0.23	2.37 ± 0.13
		1.0	3.39 ± 0.22	2.41 ± 0.14	3.27 ± 0.23	2.21 ± 0.12
		1.5	3.17 ± 0.22	2.36 ± 0.13	2.52 ± 0.18	1.98 ± 0.11
	NAA	0.5	2.41 ± 0.18	2.15 ± 0.12	2.32 ± 0.17	2.08 ± 0.13
		1.0	2.78 ± 0.19	2.25 ± 0.14	2.01 ± 0.15	2.22 ± 0.14
		1.5	2.05 ± 0.12	1.98 ± 0.13	1.93 ± 0.13	1.97 ± 0.14

*Based on observations from 50 seedlings/shoot buds taking five at random from each of ten culture vessels.

5. References

1. Chowdhery HJ. Orchid flora of Arunachal Pradesh. Bishen Singh, Mahaendra Pal Singh, Dehra Dun, India. 1998.
2. Huda MK. An updated enumeration of the family orchidaceae from Bangladesh. The Journal of the Orchid Society of India. 2007; 21(1-2):35-49.
3. Khanam M, Uddin MZ, Khan MS, Hasan MA. Our present knowledge on the terrestrial orchidaceous taxa from Bangladesh. Bangladesh Journal of Plant Taxonnomy. 2001; 8(2):35-49.
4. Bhatt A, Joshi SK, Gairola S. *Dactylorhiza hatagirea* (D. Don) Soo-A West Himalayan orchid in Peril. Current Science. 2005; 89(4):610-612.
5. Morel G. Tissue culture- a new means of clonal propagation of orchids. American Orchid Society Bulletin. 1964; 33:437-478.
6. Bernard N. La germination de orchidees. Compt. Rend. Academy Science, Paris. 1909; 137:483-485.
7. Chauhan S, Pathak P, Sharma S, Vij SP. *In vitro* asymbiotic seed germination of *Satyrium nepalense* D. Don. an endangered and medicinally important orchid. The Journal of the Orchid Society of India. 2010; 24(1-2):61-66.
8. Pathak P, Hossein P, Vij SP, Mahant KC, Chauhan S. *In vitro* propagation and mass scale multiplication of critically endangered epiphytic orchid, *Gastrochilus calceolaris* (Buch.-Ham ex.J. E. Sm.) D.Don. using immature seeds. Indian Journal of Experimental Biology. 2011; 49:711-716.
9. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum. 1962; 15:473-497.
10. Arditti J. Clonal propagation of orchids by means of tissue culture: A manual *In*: J. Arditti (ed.). Orchid Biology: Reviews and Perspectives, I. University Press, Ithaca, New York. 1977.
11. Vacin E, Went F. Some pH change in nutrient solution. Botanic Gardens, Conservation News. 1949; 110:605-613.
12. Knudson L. A new nutrient solution for germination of orchid seed. American Orchid Society Bulletin. 1946; 15:214-217.
13. Bhadra SK, Barua AK, Bhattacharjee DK, Hossain MM. *In vitro* germination and micropropagation of *Dendrobium aphyllum* G.E.C. Fischer. Bangladesh Journal of Genetics and Biotechnology. 2002; 3(1-2):47-50.
14. Bhadra SK, Bhowmik TK. Axenic germination of seeds and rhizome-based micropropagation of an orchid *Arundina graminifolia* (D. Don) Hochr. Bangladesh Journal of Botany. 2005; 34(2):59-64.
15. Bhadra SK, Bhowmik TK. *In vitro* seed germination of *Phaius tankervilleae* (Banks ex L'herit) Blume. The Chittagong University Journal of Science. 2005; 29(1):123-127.
16. Dasgupta P, Bhadra SK. *In vitro* production of seedlings in *Cymbidium aloifolium* L. SW. Plant Tissue Culture. 1998; 8(2):177-182.
17. Pant B, Gurrung R. *In vitro* seed germination and seedling development in *Aerides odorata* Lour. The Journal of the Orchid Society of India. 2005; 19(1-2):51-55.
18. Harvais G. Growth requirements and development of *Cypripedium reginae* in axenic culture. Canadian Journal of Botany. 1973; 51:327-332.
19. Tsutsui, Tomita. Suitability of several carbohydrates as the carbon sources for symbiotic seedling growth of two orchid species. Lindleyana. 1990; 5(2):134-139.
20. Barua AK, Bhadra SK. *In vitro* micropropagation of *Cymbidium aloifolium* L.SW. and *Spathoglottis plicata* Bl. Plant Tissue Culture. 1999; 9(2):133-140.
21. Bhadra SK, Hossain MM. *In vitro* germination and micropropagation of *Geodorum densiflorum* (Lam.) Scheltr., an endangered orchid species. Plant Tissue Culture. 2003; 13(2):165-171.
22. Clayton D, Cribb P. The Genus *Calanthe*. Natural History Publications (Borneo), Kota Kinabalu. 2013.
23. Geetha S, Shetty SA. *In vitro* propagation of *Vanilla planifolia*, a tropical orchid. Current Science. 2000; 79(6):886-889.
24. Kauth PJ, Vendrame WA, Kane ME. Micropropagation of *Calopogon tuberosus*, a native terrestrial orchid, by shoot production from corm explants. *In vitro* Cell Development Biology. 2006; 42:23-23.
25. Lee CL. Rapid propagation of *Nematanthus* hybrid *in vitro*. Acta. Horticulture. 1988; 226:635-638.
26. Sheelavantmath SS, Murthy HN, Pyati AN, Kumer HGA, Ravishankar BV. *In vitro* propagation of the endangered orchid, *Geodorum densiflorum* (Lam). Schltr. through rhizome section culture. Plant Cell, Tissue and Organ Culture. 2000; 60(2):151-154.
27. Takahashi CT. Establishment of axenic germination of seeds and micropropagation through rhizome tip culture in threatened *Pogonia japonica* Reichb. f. Conservation and propagation of the endangered wild orchids of the world. Proceedings of the International Orchid Wave in Shimanami. 1999, 22-28.
28. George PS, Ravishankar GA. *In vitro* multiplication of *Vanilla planifolia* using axillary bud explants. Plant Cell Reports. 1997; 16:490-494.
29. Hoque MI, Roy AR, Sarker RH, Haque MM. Micropropagation of *Cymbidium* bicolor through *in vitro* culture. Plant Tissue Culture. 1994; 4(1):45-51.
30. Watad AA, Gaba V, Alper Y, Levin R. Liquid culture as a route for high efficiency micropropagation. *In Vitro* Cell Development Biology. 1995; 31(3):30A Abstract.
31. Malabadi RB, Mulgund GS, Nataraja K. Efficient regeneration of *Vanda coerulea*, an endangered orchid using thidiazuron. Plant Cell, Tissue and Organ Culture. 2004; 76:289-293.
32. Martin KP. Cost effective *in vitro* conservation of the threatened *Ipsea Malabarica* Orchid using protocorm like bodies. Plant Cell Biotechnology and Molecular Biology. 2005; 6(1-2):57-60.
33. Sinha P, Roy SK. Regeneration of an indigenous orchid, *Vanda teres* (Roxb.) Lindl. through *in vitro* culture. Plant Tissue Culture. 2004; 14(1):55-62.
34. Nayak NRS, Pathak P, Rath SP. Direct shoot regeneration from foliar explants of an epiphytic orchids *Acampae praemorsa* (Roxb.) Blatter and Mccann. Plant Cell Report. 1997; 16:583-586.

35. Pant B, Swar S. Micropropagation of *Cymbidium iridioides* D. Don. The Journal of the Orchid Society of India. 2011; 25(1-2):9-12.
36. Banerjee B, Mandal AB. *In vitro* germination of immature *Cymbidium* seeds for rapid propagation of plantlets in lands. Cell and Chromosome Research. 1999; 21:1-5.
37. Barman D, Rajni, Das SP, Nagraju V, Upadhyaya RC. Growth regulators and regeneration of *Cymbidium* pseudobulbs. *In*: A study *in vitro* Proceedings Sixth National Seminar on Orchid Diversity in India, Science and Commerce and Orchid Show, Institute of Himalayan Bioresource Technology, Palampur, India 11-13 October. 2001; 63:78-78.
38. Gangaprasad AN, Decruse WS, Seeni S, Menon S. Micropropagation and restoration of the endangered Malabar daffodil orchid *Ipsea malabarica*. Lindleyana. 1999; 14(1):38-48.