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SEED GERMINATION, PROTOCORM MULTIPLICATION, AND SEEDLING DEVELOPMENT IN *DENDROBIUM FORMOSUM* ROXB. EX LINDL. OF BANGLADESH- A STUDY *IN VITRO*

Tapash Kumar Bhowmik and Md Mahbubur Rahman

Department of Botany, University of Chittagong, Chittagong- 4331, Bangladesh

Abstract

The germination potential of seeds procured from green capsules was evaluated on 0.8% (w/v) agar solidified half strength and, full strength basal nutrient media and PGRs (BAP and NAA; 0.5 mgl^{-1} each) supplemented full strength media [KC (modified Knudson C, 1946), MS (Murashige and Skoog, 1962), PM (Phytamax; Arditti, 1977), and VW (Vacin and Went, 1949)], in *Dendrobium formosum* Roxb. ex Lindl. Full strength PM medium supplemented with PGRs gave the cent per cent seed germination response whereas, minimum was observed on half strength basal KC (46.67%) medium. The minimum time required for initiation of germination (4.20±0.22 wks), development of protocorms (6.23±0.29 wks), differentiation of first leaf (9.33±0.31 wks) and root primordia (13.37±0.40 wks), and development of seedlings (18.20±0.36 wks) was recorded on full strength PGRs fortified PM medium. The highest rate of protocorm multiplication was observed on MS medium supplemented with 2,4-D (2.0 mgl^{-1}) and BAP (0.8 mgl^{-1}). Seedlings were transferred to a pot containing mixture of sterilized small bricks: activated charcoal and wooden coal: sawdust: coconut husk at the ratio of 1:1:1:1 and an average of 72.38% of seedlings survival rate was recorded.

Introduction

DENDROBIUM IS the largest genus of the Orchidaceae family containing more than 1,800 species that are found in diverse habitats throughout much of South, East and SouthEast Asia, including China, Japan, India, Philippines, Indonesia, Australia, New Guinea, Vietnam, and many of the islands of the Pacific. In Bangladesh, 27 species of *Dendrobium* are distributed throughout the country especially Chittagong, Chittagong Hill Tracts, Cox's Bazar, greater Sylhet, Gazipur, and Sundarbans forest (Huda, 2007). *Dendrobium* is the leading genus for phytochemicals content and 100 compounds including 32 alkaloids, 22 phenanthrenes, 15 bibenzyls, 7 sesquiterpenoids, 6 coumarins, and 4 fluorenones have been identified in 42 *Dendrobium* species (Hossain, 2015). *Dendrobium formosum* Roxb. ex Lindl. is an epiphytic pseudobulbous orchid found on trunks of large trees; it flowers in the month of May. The large attractive flowers of the species have horticultural importance. The biological status of this species is Vulnerable (VU) due to habitat destruction and mass collection by orchid hunters (Huda, 2008). In Bangladesh, the species was recorded from Sylhet, Chittagong, and Cox's Bazar district. The present study was therefore, designed to evaluate the *in vitro* asymbiotic germination potential of its seeds, growth and multiplication of protocorms, differentiation of first leaf and root primordia, and development of seedlings, with a view to developing mass propagation protocol for this species.

Material and Methods

Sterilization of Capsules

In the present study, seeds procured from green capsules (collected from Thanchi, Bandarban, Bangladesh) were used as explants. Collected capsules were first scrubbed with Teepol (0.01%) and washed thoroughly under the running tap water for 10-15 min and then washed with sterile distilled water, two times. Capsules were dipped in 70% ethyl alcohol for 30 sec and washed thrice with double distilled water. Then the capsules were treated with 0.1% (w/v) $HgCl_2$ for 10 min for surface sterilization and thereafter rinsed three times with double distilled water. The sterilized capsules were then split open longitudinally with a sterilized blade to scoop out the seeds, under aseptic condition, in a laminar airflow cabinet.

Culture Medium and Culture Conditions

The germination potential of seeds was assessed on 0.8% (w/v) agar (Fluka, USA) solidified half strength and full strength nutrient media and PGRs (BAP and NAA; 0.5 mgl^{-1} each) supplemented full strength media [KC (modified Knudson C, 1946), MS (Murashige and Skoog, 1962), PM (Phytamax; Arditti, 1977), and VW (Vacin and Went, 1949)]. Sucrose was used as a carbohydrates source; 3% (w/v) for MS and 2% (w/v) for KC, PM, and VW media. The pH was adjusted at 5.8 in MS and 5.4 in KC, PM, and VW media by using 0.1N NaOH or HCl. Agar was dissolved by boiling the

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mixture in the water bath and about 50 ml of medium was dispensed into 100 ml of each culture vessel and autoclaved (Hisense, South Korea) at 121°C for 30 min at 15 psi pressure. The cultures were maintained at 25±2°C temperature and exposed to 14 hrs illumination of 3500 lux intensity. The cultures were periodically observed (3-4 days intervals) and the responses were recorded on the basis of visual observations. Sub-culturing was carried after 6 wks into fresh medium.

Culture Multiplication

In vitro raised protocorms were cultured on 2,4-D individually and in combination with BAP or KN supplemented MS medium for increasing the weight and number of PLBs.

Transplantation

Healthy seedlings with 3-4 leaves and 2-3 roots were gradually hardened by successive phases of acclimatization. The hardened seedlings were washed thoroughly with sterile distilled water to remove agar and seedlings were transferred to a pot containing mixture of sterilized small bricks: activated charcoal and wooden coal: sawdust: coconut husk mixed at the ratio of 1:1:1:1.

Computation and Presentation of Data

The data on different parameters from different experiments were recorded after the required days of culture. The parameters were:

i) Per cent (%) of Culture Vessels Showing Seed Germination

The per cent of culture vessel showing seed germination in different media and conditions of four basal media was calculated using the following formula:

$$\% \text{ of culture vessel showing germination} = \frac{\text{Number of culture vessel showing germination}}{\text{Total number of culture vessel used}} \times 100$$

ii) Increased Weight of PLBs

The increased weight of PLBs after 30 and 60 days of inoculation was calculated using the following formula:

Increased weight of PLBs (30/60 days) = Weight of PLBs after inoculation (30/60 days) - Initial weight of PLBs

$$\text{Increased weight of PLBs (g/vessel)} = \frac{\text{Total increased weight of PLBs (30/60 days)}}{\text{Number of cultured vessels}}$$

iii) Increased Number of PLBs (number/vessel)

The increased number of PLBs after 30 and 60 days of inoculation were calculated using the following formula:

Increased number of PLBs (30/60 days) = Number of PLBs after inoculation (30/60 days) - Initial number of PLBs

$$\text{Increased number of PLBs (number/vessel)} = \frac{\text{Total increased number of PLBs (30/60 days)}}{\text{Number of cultured vessels}}$$

iv) Per cent of Seedlings Survived

The percent of seedlings that survived was calculated using the following formula:

$$\% \text{ of seedlings survived} = \frac{\text{Number of seedling survived}}{\text{Total number of transplanted seedlings}} \times 100$$

Statistical Analysis

The experiments were conducted thrice, designed by Completely Randomized Design (CRD), using the different number of replicates per treatment and data was presented as means ± standard error (mean±SE) (Tables 1-2). Standard deviation (SD) was also calculated with Microsoft Excel 2013 software. The data was subjected to analysis of variance (ANOVA) and the significant differences were determined by employing Duncan's Multiple Range Test (Gomez and Gomez, 1984) at a 5% level of significance ($P<0.05$). The data analysis was performed using the IBM SPSS (Statistical Product and Service Solutions) Statistics software program.

Results and Discussion

The nutritional necessities of diverse orchid species depend upon their innate hereditary makeup; some have very rigorous requirements whereas others can grow on a broad range of nutritional systems (Arditti, 1967). The nutrient necessities often vary considerably, throughout the different developmental stages in the similar taxon (Bhattacharjee and Hossain, 2015; Bhatti *et al.*, 2017; Borah *et al.*, 2015; Kaur *et al.*, 2017). After Knudson's discovery (1922), the technique of asymbiotic seed germination has been positively tested in many orchid species (Anuprabha and Pathak, 2012; Bhowmik and Rahman, 2020; Chen *et al.*, 2015; Decruse and Gangaprasad, 2018; Gurudeva, 2019; Franceschi *et al.*, 2019; Kumari and Pathak, 2021; Lalduhsanga *et al.*, 2021; Mohanty and Salam, 2017; Parmar and Pant, 2016; Piri *et al.*, 2013; Rao and Barman, 2014; Sunita *et al.,* 2021; Thakur and Pathak, 2020, 2021; Vasundhra *et al.,* 2021).

During the present investigation, the seeds of an epiphytic orchid *Dendrobium formosum* were aseptically grown on 0.8% (w/v) agar solidified half and full strength

Table 1. Effect of different strengths of KC, MS, PM, and VW media with or without PGRs on *in vitro* seed germination and seedling development in *Dendrobium formosum.*

Nutrient medium	Strength of medium	Time taken in wks					% of culture vessel showed seed germination	Remarks
		Initiation of germination	Development of protocorms	Differentiation of 1st leaf primordium	Differentiation of 1st root primordium	Development of seedings		
KC	Half without PGRs	11.43± 0.37^{i}	13.40±0.38^{f}	18.37±0.37^{f}	23.47±0.37^{e}	28.47±0.37^{e}	46.67	+
	Full without PGRs	8.43±0.37^{g}	11.10±0.36^{de}	15.47±0.37^{e}	19.43±0.37^{d}	24.30±0.36^{d}	53.34	++
	Full with PGRs	6.40±0.36^{e}	9.20±0.32^{c}	13.47±0.35^{d}	17.47±0.42^{c}	22.30±0.33^{c}	66.67	++
MS	Half without PGRs	7.37±0.35^{f}	10.23±0.33^{d}	15.20±0.36^{e}	19.33±0.37^{d}	24.13±0.35^{d}	53.34	++
	Full without PGRs	5.33±0.38^{cd}	8.53±0.31^{c}	12.43±0.37^{c}	17.30±0.44^{c}	22.27±0.32^{c}	80.00	+++
	Full with PGRs	4.30±0.24^{ab}	6.17±0.29^{a}	10.17±0.35^{ab}	14.27±0.33^{a}	19.37±0.36^{b}	93.34	+++
PM	Half without PGRs	6.27±0.34^{de}	9.13±0.32^{c}	14.17±0.31^{d}	19.23±0.40^{d}	23.93±0.34^{d}	66.67	++
	Full without PGRs	5.23±0.30^{bc}	7.33±0.36^{b}	11.10±0.39^{b}	16.23±0.35^{b}	21.63±0.35^{c}	80.00	+++
	Full with PGRs	4.20±0.22^{a}	6.23±0.29^{a}	9.33±0.31^{a}	13.37±0.40^{a}	18.20±0.36^{a}	100.00	+++
VW	Half without PGRs	10.47±0.32^{h}	13.17±0.36^{f}	18.50±0.39^{f}	23.37±0.36^{e}	29.17±0.35^{e}	53.34	++
	Full without PGRs	9.17±0.35^{g}	11.47±0.37^{e}	15.37±0.40^{e}	20.10±0.35^{d}	24.23±0.39^{d}	66.67	++
	Full with PGRs	6.33±0.39^{e}	8.33±0.33^{c}	12.30±0.34^{c}	16.17±0.34^{b}	21.57±0.42^{c}	73.34	++

PGRs (BAP + NAA; 0.5 mgl^{-1} each); +, Minimum germination (0% ≤ + ≤ 49%); ++, Medium germination (50% ≤ ++ ≤ 74%); +++, Maximum germination (75% ≤ +++ ≤ 100%). Values represent mean ± SE of each experiment consist of 15 replicates. Mean values followed by different superscript letters within a column are significantly different at P<0.05 according to DMRT.

basal nutrient media (KC, MS, PM, and VW) and full strength media supplemented with PGRs (BAP and NAA; 0.5 mgl^{-1} each) for *in vitro* germination, growth and development of protocorms, and development of seedlings (Table 1; Figs. 1-6). The seeds showed the first signs of germination *i.e.* swelling of embryos in all the tested combinations. Amongst the all tested media, full strength PM medium with PGRs gave cent per cent germination response (100.00%; Fig. 1) followed by PGRs supplemented MS (93.34%), VW (73.34%), and

Table 2. Effect of 2,4-D used individually or in combination with BAP/or KN on agar solidified MS medium on PLBs multiplication in *Dendrobium formosum*.

PGRs concentration (mgl^{-1})			Increased weight of PLBs (g/vessel)		Increased number of PLBs (Number/vessel)		Quantity of PLBs	Colour and texture of induced PLBs
2,4-D	BAP	KN	30 DAI	60 DAI	30 DAI	60 DAI		
0.5	-	-	0.49±0.03^{abc}	0.94±0.04^{b}	21.0±0.71^{b}	47.2±0.73^{c}	Few	GYC
1.0	-	-	0.53±0.04^{abcd}	1.04±0.05^{bcde}	24.2±0.86^{cde}	59.0±0.89^{f}	Moderate	GF
1.5	-	-	0.57±0.04^{bcde}	1.22±0.04^{fgh}	28.4±0.51^{gh}	70.2±0.86^{j}	Many	GYC
2.0	-	-	0.63±0.03^{de}	1.51±0.04^{kl}	34.0±0.71^{k}	87.0±0.71^{o}	Many	YGC
2.5	-	-	0.60±0.04^{cde}	1.39±0.04^{ijk}	31.4±0.93^{ij}	79.2±0.86^{m}	Many	WGC
0.5	0.2	-	0.54±0.03^{bcde}	1.08±0.05^{cde}	25.2±0.86^{def}	61.8±0.58^{g}	Moderate	YGF
1.0	0.4	-	0.58±0.04^{bcde}	1.28±0.04^{ghi}	29.8±0.66^{hi}	73.0±0.71^{k}	Many	WGC
1.5	0.6	-	0.61±0.04^{cde}	1.45±0.05^{ijk}	32.6±0.51^{jk}	83.2±0.66^{n}	Many	YGC
2.0	0.8	-	0.67±0.04^{e}	1.62±0.04^{l}	37.2±0.58^{l}	94.4±0.93^{p}	Many	GC
2.5	1.0	-	0.55±0.04^{bcde}	1.12±0.04^{def}	26.4±0.93^{efg}	64.0±0.71^{h}	Moderate	GYF
0.5	-	0.2	0.47±0.02^{ab}	0.91±0.05^{ab}	20.8±0.86^{b}	43.6±0.93^{b}	Few	GYC
1.0	-	0.4	0.52±0.04^{abcd}	1.01±0.04^{bcd}	23.6±0.75^{cd}	55.2±0.86^{e}	Moderate	YGF
1.5	-	0.6	0.56±0.04^{bcde}	1.17±0.05^{efg}	27.0±0.71^{fg}	66.4±0.75^{i}	Moderate	GYC
2.0	-	0.8	0.59±0.04^{bcde}	1.33±0.05^{hij}	30.8±0.58^{ij}	76.2±0.86^{l}	Many	WGC
2.5	-	1.0	0.51±0.04^{abcd}	0.97±0.04^{bc}	22.4±0.93^{bc}	51.0±0.71^{d}	Moderate	YGC
MS (Control)			0.41±0.04^{a}	0.79±0.04^{a}	16.6±0.75^{a}	35.4±0.51^{a}	Few	WGF

DAI, Days After Inoculation; Few (0 ≤ Few ≤ 49 number); Moderate (50 ≤ Moderate ≤ 69 number); Many (70 ≤ Many ≤ Above 70 number); GYC, Greenish Yellow Compact; GYF, Greenish Yellow Friable; YGC, Yellowish Green Compact; YGF, Yellowish Green Friable; WGC, Whitish Green Compact; WGF, Whitish Green Friable; GC, Greenish Compact; GF, Greenish Friable. Values represent mean±SE of each experiment consist of five replicates. Mean values followed by different superscript letters within a column are significantly different at P<0.05 according to DMRT.

KC (66.67%) media, respectively. The minimum percentage of seed germination was observed on half strength basal KC medium (46.67%; Fig. 2). The frequency and onset of germination and related morphogenic changes leading to seedling development differently affected by the chemical stimulus *in vitro.* During germination, the embryos swelled and emerged out of the seed coats through lateral slits as spherules. The germinating entities turned green and developed into protocorms. Chlorophyll production was invariably a pre-protocorm phenomenon. According to Stoutamire (1974) and Pathak *et al.* (2001), pre-protocorm development of chlorophyll is almost universal in epiphytic orchids. The minimum time required for initiation of germination was recorded on full strength PGRs fortified in descending order were PM (4.20±0.22 wks), MS (4.30±0.24 wks), VW (6.33±0.39 wks), and KC (6.40±0.36 wks) media. Half strength KC medium needed the maximum time (11.43±0.37 wks) for seed germination. Full strength PGRs supplemented PM medium proved beneficial for growth and development of protocorms (6.23±0.29 wks; Fig. 3), and early differentiation of first leaf primordia (9.33±0.31 wks; Fig. 4), first root primordia (13.37±0.40 wks), and seedling development (18.20±0.36 wks; Fig. 6), followed by full strength PGRs supplemented MS medium (Fig. 5). Different media with or without PGRs showed significant differences ($P<0.05$) in the time taken for differentiation of first root primordia and development of seedlings. Again, the initiation of seed germination and differentiation in PGRs supplemented different media also showed significant variation ($P<0.05$).

PM medium enriched with vitamins and organic additives proved more effective for inducing early and better germination of orchid seeds (Bhattacharjee and Islam, 2014; Bhowmik and Rahman, 2017; Hossain and Dey, 2013). Peptone in the PM medium strongly accelerated during the early stages of seed germination (Anuprabha and Pathak, 2019). A perusal of literature revealed that peptone favoured germination, protocorm multiplication, differentiation, and seedling growth in *Cymbidium aloifolium* (Hossain *et al.*, 2009); *C. macrorhizon* (Vij and Pathak, 1988); *Gastrochilus calceolaris* (Pathak *et al.*, 2011); and *Phalaenopsis* hybrid (Shekarriz *et al.*, 2014). In PM medium, peptone also favoured the highest germination frequency in *Cymbidium giganteum* (Hossain *et al.*, 2010); *Dendrobium aphyllum* (Hossain *et al.*, 2012); and *Paphiopedilum liemianum* (Utami *et al.*, 2015) promoted the growth and advanced development of protocorms in *Epidendrum ibaguense* (Hossain *et al.*, 2008). Presently, the additional presence of PGRs (BAP, NAA; 0.5 mgl^{-1} each) in all the nutrient media proved beneficial for enhancing the frequency of seed germination and early seedling development. Similar observations were made earlier by a few authors (Bhadra and Bhowmik, 2015; Pant and Swar, 2011; Pradhan *et al.*, 2013).

For culture multiplication, *in vitro* raised *D. formosum* protocorms were presently cultured on MS medium supplemented with 2,4-D alone (0.5-2.5 mgl^{-1}) or in combination with BAP/or KN (0.2-1.0 mgl^{-1} each) with a view to increasing the weight and number of PLBs (Table 2; Figs. 7-8). The highest rate of PLBs

Figs. 1-8. Seed germination and seedling development in *Dendrobium formosum*: 1, Protocorms (Full strength PM medium); 2, Seed callusing (half strength KC); 3-4, Protocorm multiplication and shoot development (PM medium); 5-6, Seedlings (MS; PM+$BAP_{0.5}$+ $NAA_{0.5}$); 7, Profuse multiplication of protocorms (MS+2,4-$D_{2.0}$+$BAP_{0.8}$); 8, Multiplication of PLBs *via* callusing (MS+2,4-$D_{2.0}$).

multiplication (0.67±0.04 g, 37.2±0.58 number after 30 days; 1.62±0.04 g, 94.4±0.93 number after 60 days) was observed on MS medium supplemented with 2.0 mgl^{-1} 2,4-D and 0.8 mgl^{-1} BAP (Table 2; Fig. 7) followed by MS + 2.0 mgl^{-1} 2,4-D (0.63±0.03 g, 34.0±0.71 number after 30 days; 1.51±0.04 g, 87.0±0.71 number after 60 days; Fig. 8); the PLBs were compact and green in colour. The weight and number of PLBs showed significant variations ($P<0.05$) in MS medium with and without PGRs. PLBs weight in general, increased significantly ($P<0.05$) at 60 DAI (days after inoculation). PLBs number at 60 DAI showed significant variation ($P<0.05$) amongst all treatments while individual treatment of 2,4-D at 30 DAI showed significantly difference ($P<0.05$). These observations may be due to the fact that 2,4-D strongly stimulates cell proliferation but inhibits organogenesis and maintains the growth of PLBs as also indicated earlier by Ariati (2012). The benign role of 2,4-D in inducing high percentage of PLBs formation was also obtained earlier in pencil orchid (*Papilionanthe hookeriana*) by Romeida and Ganefianti (2016). Presently, medium supplemented with 2,4-D and cytokinins (BAP or KN) in general, proved better for PLBs multiplication. Amongst the two cytokinins used, BAP proved more efficient than KN in promoting number and weight of PLBs. Lee and Lee (2003) also observed that PLBs proliferation in *Cypripedium formosum* was better on MS medium supplemented with 2,4-D and BAP.

Healthy seedlings/plantlets of *D. formosum* were hardened by successive phases; *t*hese were subsequently washed thoroughly with sterile distilled water so as to remove agar and transferred to pots containing a mixture of sterilized small bricks: activated charcoal and wooden coal: sawdust: coconut husk at the ratio of 1:1:1:1, in a humidity chamber. The appearance of new roots and leaves was taken as a sign for successful acclimatization. Plantlets were frequently irrigated and sprayed with nutrient solutions and these showed 72.38% survival rate.

Conclusion

On the basis of the present study, PM medium is recommended for effective *in vitro* seed germination, growth and development of protocorms, differentiation thereof, and seedling development in *D. formosum* followed by MS, VW, and KC media. Moreover, PGRs fortified basal media responded effectively from germination to seedlings development. To increase the number and weight of PLBs, the combined effect of 2,4-D and BAP in MS medium was the best for PLBs multiplication.

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References

Anuprabha and Promila Pathak. 2012. Green pod culture in *Dendrobium chrysanthum* Lindl.: A study *in vitro. J. Orchid Soc. India,* **26**(1-2): 105-09.

Anuprabha and Promila Pathak. 2019. *In vitro* asymbiotic seed germination and seedling development in *Coelogyne fimbriata* Lindl. *J. Orchid Soc. India,* **33**: 83-89.

Arditti, J. 1967. Factors affecting the germination of orchid seeds. *Bot. Review,* **33**(1): 1-97.

Arditti, J. 1977. Clonal propagation of orchids by means of tissue culture: A manual. *In*: *Orchid Biology: Reviews and Perspectives* I (ed. J. Arditti) pp. 114-1255. University Press, Ithaca, New York, U.S.A.

Ariati, S. 2012. Induksi Tanaman kakao pada medium MS dengan penambahan 2,4-D. *J. Natural Sci.*, **1**(1): 74-84.

Bhadra, S. K. and T. K. Bhowmik. 2015. *In vitro* seed germination and micropropagation of *Dendrobium lindleyi* Steud. an indigenous orchid of Bangladesh. *J. Asiatic Soc. Bangladesh (Science)*, **41**(2): 163-71.

Bhattacharjee, D. K. and M. M. Hossain. 2015. Effect of plant growth regulators and explants on propagation of a monopodial and sympodial orchid: A study *in vitro. J. Orchid Soc. India,* **29**: 91-102.

Bhattacharjee, B. and S. M. S. Islam. 2014. Development of an efficient protocol for *in vitro* germination and enhancing protocorm like body development in three indigenous orchid species in Bangladesh. *Asia Pac. J. Mol. Biol. Biotechnol.*, **22**(3): 209-18.

Bhatti, S. K., J. Verma, Jaspreet K. Sembi, and Promila Pathak. 2017. Symbiotic seed germination of *Aerides multiflora* Roxb.- A study *in vitro. J. Orchid Soc. India,* **31**: 85-91.

Bhowmik, T. K. and M. M. Rahman. 2017. *In vitro* seed germination and flowering of *Dendrobium palpebrae* Lindl. orchid. *J. Global Biosci.,* **6**(1): 4748-57.

Bhowmik, T. K. and M. M. Rahman. 2020. *In vitro* seed germination and micropropagation of *Dendrobium chrysotoxum* Lindl. (Golden Bow): A highly fragrant orchid species of Bangladesh. *J. Orchid Soc. India,* **34**: 69-77.

Borah, N. J., S. Chakraborty, R. Choudhary, and B. K. Dutta. 2015. *In vitro* propagation of *Paphiopedilum spicerianum* (Reichb. f.) Pfitz.- A rare and endangered orchid species from North East India. *J. Orchid Soc. India,* **29**: 85-90.

Chen, Y., U. M. Goodale, X. L. Fan, and J. Y. Gao. 2015. Asymbiotic seed germination and *in vitro* seedling development of

Paphiopedilum spicerianum: An orchid with an extremely small population in China. *Glob. Ecol. Conserv.,* **3**: 367-78.

Decruse, S. W. and A. Gangaprasad. 2018. Restoration of *Smithsonia maculata* (Dalz.) Saldanha, an endemic and vulnerable orchid of Western Ghats through *in vitro* propagation. *J. Orchid Soc. India,* **32**: 25-32.

Franceschi, C. R. B., E. C. Smidt, L. Vieira, and L. L. F. Ribas. 2019. Storage and *in vitro* germination of orchids (Orchidaceae) seeds from Atlantic Forest, Brazil. *An. Acad. Bras. Ciênc.,* **91**(3): 227-33.

Gomez, K. A. and A. A. Gomez. 1984. *Statistical Procedures for Agricultural Research*. John Willey and Sons, New York, U.S.A.

Gurudeva, M. R. 2019. *In vitro* seed germination and developmental morphology of seedlings in *Dendrobium ovatum* (L.) Kraenzl. *J. Orchid Soc. India*, **33**: 31-41.

Hossain, M. M. 2015. *Orchid: Biology and Biotechnology.* Protiva Prokash, Century Arched, Dhaka, Bangladesh.

Hossain, M. M. and R. Dey. 2013. Multiple regeneration pathways in *Spathoglottis plicata* Blume - A study *in vitro*. *South Afr. J. Bot.,* **85**: 56-62.

Hossain, M. M., M. Sharma, and Promila Pathak. 2008. *In vitro* mass propagation of an economically important orchid, *Cymbidium aloifolium* (L.) Sw. *J. Orchid Soc. India,* **22**: 91-95.

Hossain, M. M., M. Sharma, and Promila Pathak. 2009. Cost effective protocol for *in vitro* mass propagation of *Cymbidium aloifolium* (L.) Sw. - A medicinally important orchid. *Eng. Life. Sci.*, **9**: 444-53.

Hossain, M. M., M. Sharma, and Promila Pathak. 2012. *In vitro* propagation of *Dendrobium aphyllum* (Orchidaceae) seed germination to flowering. *J. Plant Biochem. Biotechnol.*, **22**: 157-67.

Hossain, M. M., M. Sharma, and J. A. Teixeira da Silva. 2010. Seed germination and tissue culture of *Cymbidium giganteum* Wall. ex Lindl. *Sci. Hortic.*, **123**: 479-87.

Huda, M. K. 2007. An updated enumeration of the family Orchidaceae from Bangladesh. *J. Orchid Soc. India,* **21**(1-2): 35-49.

Huda, M. K. 2008. Orchidaceae. *In*: *Encyclopedia of Flora and Fauna of Bangladesh* Vol. 12. *Angiosperms: Monocotyledons* (eds. Z. U. Ahmed, M. A. Hassan, Z. N. T. Begum, M. Khondker, S. M. H. Kabir, M. Ahmad, A. T. A. Ahmed, A. K. A. Rahman, and E. U. Haque) pp. 1-149. Asiatic Society of Bangladesh, Dhaka, Bangladesh.

Kaur, S., Promila Pathak, Ankush Prakash, Anamika, and Aakanksha Sharma. 2017. *Ex situ* conservation of floriculturally and medicinally important endangered orchid, *Coelogyne cristata* Lindl. *J. Orchid Soc. India,* **31**: 15-22.

Knudson, L. 1922. Non symbiotic germination of orchid seeds. *Bot. Gaz.,* **73**: 1-15.

Knudson, L. 1946. For orchid seedlings in culture. *Am. Orchid Soc. Bull.,* **15**: 214-17.

Kumari, Anamika and Promila Pathak. 2021. *De novo* plantlet regeneration from leaf explants of *Rhynchostylis retusa* (L.) Blume: A study *in vitro*. *J. Orchid Soc. India*, **35**: 47-53.

Lalduhsanga, R. Jayanthi, B. N. Sathyanarayana, K. S. Nirmala, and Vena S. Anil. 2021. A comparative study of different nutrient media for *in vitro* asymbiotic seed germination of two threatened wild orchids. *J. Orchid Soc. India*, **35**: 109-13.

Lee, Y. L. and N. Lee. 2003. Plant regeneration from protocorm derived callus of *Cypripedium formosum*. *In vitro Cell. Dev. Biol. Plant*, **39**(5): 475-79.

Mohanty, C. R. and P. Salam. 2017. *In vitro* seed culture studies in *Dendrobium* orchid cv. Banyat Pink. *J. Orchid Soc. India,* **31**: 93-96.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.,* **15**: 473-97.

Pant, B. and S. Swar. 2011. Micropropagation of *Cymbidium iridioides* D. Don. *J. Orchid Soc. India,* **25**(1-2): 9-12.

Parmar, G. and B. Pant. 2016. *In vitro* seed germination and seedling development of the orchid *Coelogyne stricta* (D. Don) Schltr. *Afr. J. Biotechnol.,* **15**(5): 105-09.

Pathak, Promila, K. C. Mahant, and Ashish Gupta. 2001. *In vitro* propagation as an aid to conservation and commercialization of Indian orchids: Seed culture. *In: Orchids: Science and Commerce* (eds. P. Pathak, R. N. Sehgal, N. Shekhar, M. Sharma, and A. Sood) pp. 319-62. Bishen Singh Mahendra pal Singh, Dehradun, India.

Pathak, Promila, H. Piri, S. P. Vij, K. C. Mahant, and S. Chauhan. 2011. *In vitro* propagation and mass scale multiplication of a critically endangered epiphytic orchid, *Gastrochilus calceolaris* (Buch.-ham ex J. E. Sm.) D. Don. using immature seeds. *Indian J. Exp. Biol.,* **49**: 711-16.

Piri, H., Promila Pathak, and R. K. Bhanwra. 2013. Asymbiotic germination of immature embryos of a medicinally important epiphytic orchid *Acampe papillosa* (Lindl.) Lindl. *Afr. J. Biotechnol.,* **12**(2): 162-67.

Pradhan, S., T. Regmi, G. Parmar, and B. Pant. 2013. Effect of different media on *in vitro* seed germination and seedling development of *Cymbidium aloifolium* (L.) Sw. *Nepal J. Sci. Technol.,* **14**(1): 51-56.

Rao, S. and B. Barman. 2014. *In vitro* micropropagation of *Dendrobium chrysanthum* Wall. ex Lindl. - A threatened orchid. *Sch. Acad. J. Biosci.,* **2**(1): 39-42.

Romeida, A. and D. W. Ganefianti. 2016. Embryogenic callus induction of pencil orchid (*Papilionanthe hookeriana* Rchb.f.) through *in vitro* culture. *Int. J. Adv. Sci. Eng. Info. Technol.*, **6**(2): 196-200.

Shekarriz, P., M. Kafi, S. D. D. Deilamy, and M. Mirmasowni. 2014. Coconut water and peptone improve seed germination and protocorm like body formation of hybrid *Phalaenopsis*. *Agric. Sci. Dev.,* **3**(10): 317-22.

Stoutamire, W. P. 1974. Terrestrial orchid seedlings. *In: The Orchids- Scientific Studies* (ed. C. L. Withner) pp. 101-28. Wiley-Interscience, New York, U.S.A.

Sunita, Promila Pathak, and K. C. Mahant. 2021. Green pod culture of an endangered and medicinally important orchid, *Vanda*

cristata Wall. ex Lindl. from Himachal Pradesh. *J. Orchid Soc. India,* **35**: 25-33.

Thakur, Babita and Promila Pathak. 2020. *In vitro* propagation of *Herminium lanceum* (Thunb. ex Sw.) Vuijk (Orchidaceae), through asymbiotic seed germination: A therapeutically important and endangered orchid from NorthWestern Himalayas. *J. Orchid Soc. India,* **34**: 61-67.

Thakur, Babita and Promila Pathak. 2021. Application of organic additives for the enhancement of seed germination and seedling development in an endangered and medicinal orchid, *Rhynchostylis retusa* (L.) Blume through asymbiotic culture. *J. Orchid Soc. India*, **35**: 99-107.

Utami, E. S. W., H. Purnobasuki, T. Soedarti, and S. Hariyanto. 2015. Asymbiotic seed germination and *in vitro* seedling development of *Paphiopedilum liemianum* Fowlie, an endangered terrestrial orchid in Northern Sumatra, Indonesia. *J. Plant Sci.,* **10**(1): 25-34.

Vacin, E. and F. Went. 1949. Some pH change in nutrient solution. *Bot. Gard. Cons. News*., **110**: 605-13.

Vasundhra, Promila Pathak, and Anuprabha. 2021. *In vitro* asymbiotic seed germination and regeneration competence of leaf explants in *Satyrium nepalense* D.Don, A medicinally important, and endangered terrestrial orchid of Kasauli Hills, Himachal Pradesh (NorthWestern Himalayas). *J. Orchid Soc. India,* **35**: 73-82.

Vij, S. P. and Promila Pathak. 1988. Asymbiotic germination of the saprophytic orchid, *Cymbidium macrorhizon:* A study *in vitro. J. Orchid Soc. India,* **2**(1-2): 25-32.