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In vitro mass scale propagation and seedlings development of medicinal orchid *Dendrobium transparens* Wall ex Lindl

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Abstract
In the present investigation, *in vitro* seed germination and seedlings development of *Dendrobium transparens* Wall ex Lindl. Was assessed on agar solidified KC; MS; PM and MVW media with three different sources of carbohydrates *viz.* sucrose, glucose and lactose. PM medium proved to be best for early germination and PLBs formation followed by MS medium. Sucrose supplemented media was found to be best whereas lactose containing medium gave at least performance. Germinated PLBs were cultured on agar solidified or liquid MS and PM media supplemented with different concentrations and combinations of auxins, cytokinins. Highest shoot growth was found on liquid PM medium supplemented with 1.0 mg/l IAA and 1.0 mg/l BAP (3.58 ± 0.12 cm) followed by agar solidified PM + 1.0 mg/l NAA + 1.0 mg/l BAP (3.35 ± 0.13 cm). PM was found better than MS medium and liquid culture was superior than agar solidified condition for elongation of *in vitro* grown germinated seedlings. BAP, IAA was better in elongation media than Kn, NAA, Picloram. Agar solidified full strength MS with 1.0 mg/l IAA produced highest increased length and number of roots (3.27 ± 0.20 cm/shoot bud and 1.74 ± 0.13 no/shoot bud) followed by half strength hormone free MS medium (2.18 ± 0.15 cm/shoot bud and 1.56 ± 0.12 no/shoot bud). The well developed seedlings were acclimatized subsequently transferred to pots in green house and watered regularly.

Keywords: *Dendrobium transparens*, *in vitro* germination, medicinal orchid, PGRs, PLBs

Introduction
Orchids are exhibiting a wide range of diversity in form, size, colour and texture of flowers beyond the imagination of human mind [1]. The orchid species belonging to the family Orchidaceae are represented mostly in the tropical parts of the world by 880 genera and about 26567 species [2] and a few in the arctic regions. In Bangladesh, the family is rich with 187 species [3]. In Bangladesh; Chittagong, Chittagong Hill Tracts, Cox's Bazar, greater Sylhet, Gazipur and Sundarbans mangrove forest are rich in orchid flora [4]. Loss of habitat, deforestation, destructive collection technique and over exploitation of orchids with medicinal and ornamental values has depleted the orchid wealth of Bangladesh [3]. Many orchids are now at the verge of extinction, so it is high time to conduct effective strategies to conserve in nature.

Medicinally and horticulturaly important orchid *Dendrobium transparens* Wall ex Lindl., found in Bangladesh, Bhutan, India, Myanmar, Nepal and Vietnam in dense wet forests at elevations of 500 to 2100 meters [5]. Critically endangered orchid *D. transparens* flowered in May [6] and grow as vines producing aerial shoots along their stems. Paste is used to treat fractured and dislocated bones [7].

Orchid seeds, unlike the seeds of other flowering plants are extremely small, dust like, produced in large numbers (1300-4,00,000/capsule) and with little or no stored food [8]. Orchid seeds have unique physiology of germination. Under natural condition, fungal aid mycorrhiza usually species of *Rhizotectonia* is required. Germination rate in nature is very poor (5%) and takes long time [8]. Therefore highly exploited species immediately require *ex situ conservation* by tissue culture method. During the last few years tissue culture technique have been extensively exploited for the large scale propagation of many orchid species. However, very little work has been done for the propagation and conservation of this species.

In the present study *in vitro* seed germination and seedlings development method was investigated for the rapid propagation of medicinally and horticulturaly important orchid *Dendrobium transparens*.

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Materials and Methods

Studies on the seed germination of indigenous orchid species, *Dendrobium transparens* were carried out in the Plant Tissue Culture and Biotechnology Laboratory, Department of Botany, University of Chittagong. The materials used for the present investigation were the immature green capsules of *D. transparens* which were collected from Bandarban, Bangladesh.

The dust in the pods collected from the naturally grown orchid was removed. The young green capsules was washed with tap water containing few drops of teepol solution for few minutes and washed under running tap water for five minutes. The capsules were surface sterilized by immersing it in the solution of 0.1% $HgCl_2$ for 10 minutes followed by 70% ethanol for 30 second and finally rinsing three times with sterile distilled water.

Four basal media namely, KC [9]; MS [10]; PM [11] and MVW [12] with three different sources of carbohydrates *viz.* sucrose, glucose and lactose were used for germination. The pH of all media was adjusted to 5.8 using 0.1N NaOH or HCl before mixing agar. 0.8% (w/v) agar was added as a gelling agent and dissolved by boiling. 100 ml of the media were dispensed into 250 ml culture bottles and autoclaved at 121^0C for 20 minutes at 15 lbs pressure. The experiment was conducted under aseptic condition and the cultures, incubated at 25 ± 2 ^{o}C were subjected to 14 hr photoperiod at 4000-5000 lux intensity and 60% humidity level were maintained regularly [13]. For subsequent development of the seedlings, they were subcultured on respective media at different intervals. Hormones *viz.* BAP, Kn, Pic, NAA, IAA and IBA were freshly prepared.

For the inoculation of seeds, immature green capsule was put on sterile petridish containing sterile filter paper and cut longitudinally using a sharp sterile blade under laminar air flow cabinet. The very tiny seeds were scooped out with the help of sterile forceps and spread over the surface of the germination media. Eighteen types of full strength MS and PM based solid & liquid elongation media were prepared using with different concentrations and combinations of PGRs. 0.8% (w/v) agar was also used in solid media but in liquid media no agar was added. For *in vitro* rooting of *D. transparens*, half strength MS0 with 1.5% (w/v) sucrose and nine different types of 0.8% (w/v) agar solidified MS medium supplemented with 3% (w/v) sucrose with three auxins *viz.* IAA, IBA and NAA were used for induction of strong and stout root system. The well developed seedlings were taken out of the culture vessels and successfully transferred to outside the culture room following successive phases of acclimatization. Transplanted seedlings were watered regularly for about 2-3 months where the seedlings established and grew well in the Orchidarium.

Results and Discussions

Table-1 represents the response of *Dendrobium transparens* seed cultured on KC, MS, PM and MVW media supplemented with diverse carbohydrate source *viz.* glucose, lactose and sucrose. Remarkable differences showed on the cultured media in terms of frequency of germination and quality of the protocorms. Lactose supplemented all of the basal media, the frequency of seeds germination was very poor and protocorms were chlorotic which failed to continue their growth after germination. But in the PM medium, the germinated protocorms continued normal growth and produced healthy seedlings after subsequent subcultures. Agar solidified PM medium with 2% (w/v) sucrose was proved to be best for germination (Fig.1a) followed by MS medium with 3% (w/v) sucrose (Fig.1b). Similar result was also found in *Cymbidium aloifolium* [14]; *Dendrobium aphyllum* [15]; *Arundina graminifolia* [16]; *Calanthe densiflora* [17] orchid species. PM media is enriched with vitamins and organic additives. Addition of vitamins and additives into the medium was reported to be enhanced for seed germination and seedling growth of many orchids. Peptone in media enhances the germination rate and also favours the healthy protocorm development. MS medium was found best for germination in *Aerides odorata* [18]; *Vanda tessellata* [19]. Sugar is an important component of any kind of nutrient medium used in tissue culture studies. Carbon source has also great role for *in vitro* orchid seed germination. Sugar is an important and effective component than glucose and lactose as a source of carbon used in tissue culture media [20-21]. Our results indicate that selection of medium is an important aspect of success in asymbiotic germination of this orchid species.

Table 1: *In vitro* germination of seeds of *Dendrobium transparens* Wall ex Lindl.

Nutrient medium	Carbohydrate source with concentration (w/v)	Number of culture vessels used	Number of culture vessels in which seeds germinated		Time (d) required for germination	Remarks
			No.	%		
KC	2% glucose	10	03	30	40 - 42	Green PLBs
	2% lactose	10	02	20	42 - 45	Whitish PLBs
	2% sucrose	10	06	60	33 - 35	Yellowish green PLBs
MS	3% glucose	10	04	40	32 - 35	Green PLBs
	3% lactose	10	02	20	38 - 40	Whitish PLBs
	3% sucrose	10	07	70	32 - 36	Light greenish PLBs
PM	2% glucose	10	05	50	36 - 40	Green PLBs

	2% lactose	10	02	20	38 - 40	Whitish PLBs
	2% sucrose	10	09	90	32 - 35	Greenish yellow PLBs
MVW	2% glucose	10	04	40	38 - 42	Greenish PLBs
	2% lactose	10	02	20	40 - 42	Whitish PLBS
	2% sucrose	10	08	80	33 - 36	Yellowish green PLBs

The *in vitro* developed protocorms on the basal media produced mini seedlings on subsequent subculture in the same media and their growth rate was slow. But when the protocorms were grown on eighteen different kinds of solid & liquid MS and PM media with various combinations, concentrations of PGRs (BAP, IAA, NAA, and Picloram) gave differential responses. In liquid media no agar was added. Different hormone combinations and culture condition were found to be better for elongation of seed originated tiny plantlets (Table-2). The rate of elongation of seedlings after 30d of culture was dependent on the PGRs combinations, concentrations and culture conditions. Maximum elongation took place on liquid PM medium fortified with 2% (w/v) sucrose + 1.0 mg/l IAA and 1.0 mg/l BAP (3.58 ± 0.12 cm; Fig. 1c) followed by agar solidified PM medium with 2% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP (3.35 ± 0.13 cm; Fig. 1d). It is reputable that elongation of seed originated seedlings was better in liquid media than agar solidified condition. Further PM was superior to MS for elongation of shoot bud. BAP, IAA was better in elongation media than Kn, NAA, Picloram [16-17, 22-25].

The elongated seedlings at a height of 3-5 cm length were individually grown on 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-3). MS medium fortified with 3% (w/v) sucrose + 1.0 mg/l IAA produced highest increased length and number of roots (3.27 ± 0.20 cm/shoot bud; 1.74 ± 0.13 no/shoot bud and Fig. 1e) followed by half strength hormone free MS medium + 1.5% (w/v) sucrose (2.18 ± 0.15 cm/shoot bud and 1.56 ± 0.12 no/shoot bud) was proved best for induction of strong and stout root system within 30d of culture. Similar result was found in *Dendrobium* hybrid, *Dendrobium thyrsiflorum* respectively [26-27]. IBA was effective for rooting in *Ilex khasiana* and *Cymbidium finlaysonianum* respectively [28-29]. The opposite result was also noted that NAA was most appropriate in inducing roots in *Esmeralda clarkei* and *Vanda tessellata* [30-31]. Combine effect of IAA, IBA or NAA induced excellent rooting response in *Rhyncostylis retusa* and *Aerides ringens* [32-33] orchid species.

The well developed rooted plantlets were transferred from culture room to the green house during successive phase of adaptation (Fig. 1f). For this purpose, the culture vessels were kept open for one day in the culture room and then kept outside of the culture room for 6h in the next day. On the third day 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.

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

Culture medium with different combinations and concentrations of PGRs	**Average initial length (cm) of *in vitro* germinated seedlings**	**Average length (cm) of germinated seedlings after 30d of culture on elongation medium**	**Increase in length (cm) of germinated seedlings within 30d of culture on elongation medium**	**Average initial length (cm) of *in vitro* germinated seedlings**	**Average length (cm) of germinated seedlings after 30d of culture on elongation medium**	**Increase in length (cm) of germinated seedlings within 30d of culture on elongation medium**
	Solid media			**Liquid media**		
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.55±0.11	4.49±0.10	2.94±0.13	1.40±0.10	4.85±0.12	3.45±0.11
MS + 3% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	1.58±0.10	4.70±0.15	3.12±0.12	1.35±0.18	4.89±0.14	3.54±0.13
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	1.52±0.13	4.74±0.17	3.22±0.15	1.45±0.12	4.97±0.17	3.52±0.14
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.60±0.15	4.48±0.14	2.88±0.17	1.48±0.15	4.90±0.13	3.42±0.16
MS+3% (w/v) sucrose + 0.5 mg/l NAA+1.0 mg/l BAP	1.65±0.08	4.58±0.12	2.93±0.11	1.62±0.13	5.10±0.11	3.48±0.15
MS + 3%(w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	1.46±0.12	4.75±0.14	3.29±0.13	1.55±0.14	5.07±0.12	3.52±0.11
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.50±0.13	4.55±0.09	3.05±0.10	1.60±0.10	4.97±0.15	3.37±0.13
MS+3% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.60±0.16	4.61±0.12	3.01±0.15	1.55±0.14	4.99±0.10	3.44±0.12
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.55±0.14	4.81±0.13	3.26±0.18	1.45±0.11	4.94±0.13	3.49±0.10
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.45±0.17	4.68±0.12	3.23±0.15	1.40±0.09	4.78±0.12	3.38±0.13
PM + 2% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	1.60±0.12	4.81±0.13	3.21±0.14	1.42±0.18	4.85±0.14	3.43±0.12
PM + 2% (w/v) sucrose +1.0 mg/l IAA + 1.0 mg/l BAP	1.62±0.13	4.90±0.15	3.28±0.09	1.55±0.13	5.13±0.10	3.58±0.12
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.56±0.14	4.58±0.11	3.02±0.10	1.42±0.09	4.83±0.13	3.41±0.15
PM + 2% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	1.48±0.15	4.62±0.09	3.14±0.12	1.45±0.12	4.91±0.11	3.46±0.09
PM + 2% (w/v) sucrose +1.0 mg/l NAA + 1.0 mg/l BAP	1.62±0.14	4.97±0.10	3.35±0.13	1.40±0.11	4.93±0.14	3.53±0.12
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.52±0.17	4.64±0.14	3.12±0.11	1.38±0.16	4.89±0.11	3.51±0.13
PM + 2% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.56±0.13	4.72±0.17	3.16±0.16	1.35±0.13	4.83±0.17	3.48±0.14
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.45±0.11	4.68±0.13	3.23±0.14	1.40±0.14	4.94±0.12	3.54±0.17

All the values are mean ± SE, shoot length of each treatment contains 10 replicates.

Germination of seeds on agar solidified PM medium

Germination of seeds on agar solidified MS medium

Elongated seedlings on liquid PM + 1.0 mg/l IAA. + 1.0 mg/l BAP

Elongated seedlings on agar solidified PM + 1.0 mg/l NAA + 1.0 mg/l BAP

Strong and stout root system on agar solidified MS + 1.0 mg/l IAA

In vitro developed plantlets growing in pot outside of the culture room

Fig (1a-1f): *In vitro* germination, elongation, rooting and hardening of *Dendrobium transparens* Wall ex Lindl.

Table 3: Increased length (Mean ± SE) and number of roots in seed derived seedlings of *D. transparens* in half strength MS0 and auxin supplemented MS rooting media.

Culture medium			Average increased length and number of roots per seedling	
			Mean length (cm) ± SE	Mean no. of roots/ shoot bud ± SE
½ MS0			2.18 ± 0.15	1.56 ± 0.12
Auxin (mg/l)	IAA	0.5	2.11 ± 0.14	1.43 ± 0.10
		1.0	3.27 ± 0.20	1.74 ± 0.13
		1.5	2.16 ± 0.13	1.45 ± 0.10
	IBA	0.5	1.97 ± 0.14	1.23 ± 0.08
		1.0	2.06 ± 0.15	1.31 ± 0.09
		1.5	2.14 ± 0.13	1.44 ± 0.11
	NAA	0.5	2.13 ± 0.12	1.53 ± 0.09
		1.0	2.07 ± 0.11	1.39 ± 0.08
		1.5	2.02 ± 0.12	1.27 ± 0.07

Root length and number of roots of each treatment contains 10 replicates

of agar attached to the roots. Then the seedlings were transferred to plastic pots containing a potting mixture of sterilized small brick, coal pieces, saw dust and peat moss at a ratio of 1 : 1 : 1 : 0.5 and kept in the green house (at 25-30 ^{0}C and RH 60-70%). Transplanted seedlings were watered regularly for about 2-3 months where the seedlings established and grew well.

Conclusions

PM was found better than KC, MS & MVW media for promoting germination of orchid seeds. Sucrose supplemented media was found to be best whereas lactose containing medium gave at least performance. For comparing the effectiveness in terms of enhancing seedling, liquid culture was better than agar solidified condition. Increased in root length and number of roots is higher in IAA supplemented full strength MS media. However, the *ex situ* conservation of this species is highly recommended not only for its conservation but also to best utilized its commercial demand.

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