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Multiple Shoot buds formation through *in vitro* pseudobulb culture of medicinal orchid *Dendrobium transparens* Wall ex Lindl

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Abstract
An efficient protocol for micropropagation of *Dendrobium transparens* Wall ex Lindl. using the axenic pseudobulb segments, derived from *in vitro* germinated seedlings, was developed. For multiple shoot induction, the axenic pseudobulb segments excised from 120d old seedlings were cultured on MS medium supplemented with auxins (IAA, IBA, NAA, Picloram) and cytokinins (BAP, Kinetin). *In vitro* grown pseudobulb segments both upper and lower part directly produced multiple shoot buds *via* organogenesis. Average highest number of MSBs (7.42 ± 0.36/segment in lower part; 5.56 ± 0.32/segment in upper part) produced on MS medium with 1.0 mg/l NAA + 2.0 mg/l BAP followed by (7.24 ± 0.41/segment in lower part; 5.96 ± 0.37/segment in upper part) MS medium supplemented with 1.0 mg/l NAA + 2.0 mg/l BAP. Highest individual shoot bud increased length was recorded on 1.0 mg/l IAA + 1.0 mg/l BAP supplemented in both liquid PM (3.27±0.14 cm) and followed by agar solidified PM (3.15±0.10 cm) medium. Elongation of shoot bud was better in liquid media than agar solidified condition and PM was found better than MS. Shoot bud derived seedlings were best responses as increase in length as well as the number of roots developed on agar solidified full strength MS medium with 1.0 mg/l IAA (4.06 ± 0.22 cm/shoot bud and 1.87 ± 0.14 no/shoot bud) followed by half strength hormone free MS medium with 1.5% (w/v) sucrose (3.75 ± 0.23 cm/shoot bud and 1.64 ± 0.10 no/shoot bud). The plantlets were hardened off and were released to the shade house.

Keywords: *Dendrobium transparens*; micropropagation; MSBs; pseudobulb segment; SPSs

1. Introduction
Orchids are not only important in terms of economical aspects but also attractive in terms of ecological issues, like the diversity of species. Flowering plants, orchids are commercially grown worldwide as cut flower and potted plants in floriculture trade. Orchidaceae is the largest, including most multifariousness family of flowering plants, consisting of more than 35,000 species belonging to 700-800 genera [1]. It included both terrestrial and epiphytic orchids. Due to their ornamental and medicinal importance they demand a very high price in the international market. Due to their ornamental and medicinal importance they demand a very high price in the international market. They are well known for their strange shaped, longevity and beautiful looking flowers. Many indigenous valued orchids are found in Bangladesh; mainly in Chittagong Hill Tracts, Chittagong, Cox's Bazar, greater Sylhet, Gazipur and Sundarbans mangrove forest [2].

The genus *Dendrobium* under Orchidaceae family exhibits a vast diversity in vegetative and floral characteristics and is of considerable interest due to its broad geographic distribution and high value of hybrids as a floricultural commodity [3]. Dendrobium genus has in excess of 1100 species of epiphytic orchids with a wide distribution from Central Asia throughout Australasia [4-5]. This genus is one of the largest among the Orchidaceae, the largest family of angiosperms [6-7]. In Bangladesh, about 27 species of *Dendrobium* are distributed throughout the country. *D. transparens* is a mildly fragrant, sympodial epiphytic orchid, having a geographical distribution ranging from North-east India, Nepal and Myanmar to a few countries of South-east Asia [8]. *D. transparens* is important ornamental orchid having pendulous racemes of medium-sized flowers, usually of pristine white, except that of a purple blotch at the tip of sepals, petals and the throat of the labellum. *D. transparens* flowered in May [8] and grow as vines producing aerial shoots along their stems. The plant is generally propagated through the division of pseudobulb but the rate of multiplication is extremely slow, normally giving rise to 2-4 plants per year. Paste is used to treat fractured and dislocated bones [9]. Bangladesh are facing the grim possibility of extinction of *D. transparens* for intense biotic pressures like jhum cultivation, forest fires, indiscriminate wild collection and illegal trade by the local people. Hence, conservation and sustainable utilization assume greater importance to save the dwindling orchids [10]. Satisfying the interest of the hobbyist and demand of the traders through large-scale micropropagation is one of the preferable options to prevent illegal collection from wild.

A perusal of available literature reveals that micropropagation has been achieved using immature or mature embryos, protocorms, shoot tip explants and from axenic nodal segments in *D. candidum* [11-13]. *In vitro* seeds germination [14] and clonal propagation by pseudobulb explant [15] of *D. transparens* has been reported. In this study, authors report the development of an efficient and reproducible protocol for multiple shoot induction form axenic of *D. transparens* seedlings, elongation, rooting and SPSs development of the micro shoots; and successful transplantation.

2. Materials and Methods

The experiment was performed with pseudobulb segments of upper and lower part excised from the one-year old *in vitro* raised seedlings of *D. transparens*. Any leaves or roots, if present, were removed from the segments prior to inoculation. *In vitro* grown pseudobulbs were cut 0.5 to 1.0 cm size using sterilized surgical blade and forceps in the laminar air flow cabinet. Then the cuttings of upper and lower part were put into the culture vessel containing 0.8% (w/v) agar solidified MS based micropropagation media supplemented with sixteen different concentrations and combinations of PGRs. Morphogenetic response of the treatments was evaluated in terms of frequency of shoot bud from the pseudobulb segments. Media was heated till the agar is dissolved. Hormones *viz.* 6-benzyl Amino Purine (BAP), Kinetin (Kn), Picloram (Pic), Napthelene Acetic Acid (NAA), Indole Acetic Acid (IAA) and Indole Butaric Acid (IBA) were freshly prepared. 100 ml of the media were dispensed into 250 ml culture bottles and autoclaved at 121^0C for 20 minutes at 15 lbs pressure. p^H of the medium was set at 5.8 using 0.1N NaOH or HCl prior to gelling with agar. The culture vessels were capped with two layered aluminium foil before autoclaving and sealed. The growth room was maintained at temperature 25±2 ^{0}C; 50-60% humidity and 4000-5000 lux light illumination from cool white fluorescent to set 14/10 h (Light/Dark) photoperiod [16]. Cultures were sub cultured regularly and observed twice in a week. Eighteen types of elongation media were prepared using full strength [17-18] based solid & liquid media supplemented with different concentrations and combinations of PGRS.

Full strength MS and based solid & liquid eighteen types of elongation media were prepared using with different concentrations and combinations of PGRs. In solid media 0.8% (w/v) agar was used 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.

For hardening, 90 days old plantlets with good rooted 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%).

3. Results and Discussions

Pseudobulbs of *Dendrobium transparens* were collected from *in vitro* derived seedlings developed in the Laboratory of Plant Tissue Culture and Biotechnology, Department of Botany, University of Chittagong, Bangladesh for rapid micropropagation [19-20]. Two types of pseudobulb segments; the upper part and the lower part were cultured on 0.8% (w/v) agar solidified MS media supplemented with various combinations and concentrations of PGRs and produced multiple shoot buds (MBSs) *via* direct organogenesis (Table-1). The efficiency of a medium was assessed on the basis of number of shoot buds produced from each explant. Average highest number of MSBs (7.42 ± 0.36/segment in lower part; 5.56 ± 0.32/segment in upper part) produced on MS medium with 1.0 mg/l NAA + 2.0 mg/l BAP (Fig.1a) followed by (7.02 ± 0.31/segment in lower part; 5.11 ± 0.27/segment in upper part) MS medium supplemented with 1.0 mg/l NAA + 2.0 mg/l Kn [16, 20-21]. It's also noted that lower part of pseudobulb segments showed better response than upper part. The ratio of auxins and cytokinins for shoot bud formation varies from species to species. Exogenous application of cytokinins and auxins has been known to be important for shoot induction and elongation of many plant species *in vitro* [22]; while BAP and NAA, respectively, are the two most commonly used cytokinin and auxin for multiple shoot induction [23]. In the present study, authors obtained a relatively higher number of regenerated micro shoot and similar result was noted by Sunitibala and Kishor in *D. transparens* [15]; but differ to the report of Vij *et. al.* [24] who obtained only a single shoot per pseudobulb segment of *D. chrysanthum* cultured on MS medium. The synergistic effect of BAP and NAA towards multiple shoot formation from axillary buds without intermediate callusing is evident from the present study and it is in conformity with the findings of Herrera *et. al.* [25] who reported that exogenous auxin could be effectively employed along with cytokinin for better response of multiple shoot development of *Digitalis thapsi*. BAP was best for shoot bud formation in *Vanda spathulata* and *Dendrobium bensoniae* respectively [26-27].

Then the multiple shoot buds developed from pseudobulb segment culture were put on eighteen different kinds of solid & liquid media with various combinations and concentrations of PGRs for better elongation. Nine were prepared using MS basal medium and the rest of others were prepared using PM basal medium for enhancing elongation of MSBs. Agar was not added in liquid media. 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.

Table 1: Development of multiple shoot buds from pseudobulb explant of *Dendrobium transparens* when grown on 0.8% (w/v) agar solidified MS media supplemented with different PGRs.

Combinations and concentrations of PGRs	Explants	% of induced multiple shoot buds per segment	Time (d) required for sprouting of multiple shoot buds	Number of multiple shoot buds/ PLBs produced per segment (Mean ± S.E.)
0.5 mg/l IAA + 1.0 mg/l BAP	PSU	40	35 - 40	4.22 ± 0.32
	PSL	45	35 - 38	3.64 ± 0.26
0.5 mg/l IAA + 1.0 mg/l Kn	PSU	35	35 - 40	3.45 ± 0.23
	PSL	45	33 - 36	3.73 ± 0.23
1.0 mg/l IAA + 2.0 mg/l BAP	PSU	45	32 - 35	4.79 ± 0.28
	PSL	50	30 - 35	4.67 ± 0.25
1.0 mg/l IAA + 2.0 mg/l Kn	PSU	35	35 - 40	3.39 ± 0.19
	PSL	50	32 - 36	4.55 ± 0.32
0.5 mg/l IBA + 1.0 mg/l BAP	PSU	35	35 - 40	3.41 ± 0.24
	PSL	45	30 - 35	3.68 ± 0.26

0.5 mg/l IBA + 1.0 mg/l Kn	PSU	35	35 - 40	3.36 ± 0.22
	PSL	40	33 - 36	3.12 ± 0.25
1.0 mg/l IBA + 2.0 mg/l BAP	PSU	45	32 - 35	4.78 ±0.29
	PSL	50	30 - 35	4.63 ± 0.27
1.0 mg/l IBA + 2.0 mg/l Kn	PSU	40	32 - 36	3.96 ± 0.28
	PSL	45	33 - 36	3.71 ± 0.28
0.5 mg/l NAA + 1.0 mg/l BAP	PSU	40	32 - 35	3.84 ± 0.29
	PSL	55	28 - 32	5.23 ± 0.37
0.5 mg/l NAA + 1.0 mg/l Kn	PSU	35	35 - 40	3.46 ± 0.19
	PSL	50	30 - 35	4.62 ± 0.26
1.0 mg/l NAA + 2.0 mg/l BAP	PSU	55	28 - 32	5.56 ± 0.32
	PSL	70	25 - 30	7.42 ± 0.36
1.0 mg/l NAA + 2.0 mg/l Kn	PSU	50	28 - 32	5.11 ± 0.27
	PSL	60	30 - 32	7.02 ± 0.31
0.5 mg/l Pic + 1.0 mg/l BAP	PSU	40	33 - 36	3.87 ± 0.29
	PSL	45	30 - 35	3.83 ± 0.27
0.5 mg/l Pic + 1.0 mg/l Kn	PSU	35	35 - 40	3.43 ± 0.22
	PSL	45	30 - 35	3.78 ± 0.23
1.0 mg/l Pic + 2.0 mg/l BAP	PSU	45	32 - 36	3.31 ± 0.25
	PSL	50	28 - 32	4.56 ± 0.32
1.0 mg/l Pic + 2.0 mg/l Kn	PSU	35	35 - 40	3.02 ± 0.23
	PSL	50	28 - 32	4.35 ± 0.31

PSU = Pseudobulb Segment Upper part; PSL = Pseudobulb Segment Lower part; Based on observations recorded from 10 cultured segments in each medium.

Different hormone combinations and culture condition were found to be better for elongation of multiple shoot buds originated tiny plantlets (Table-2). Highest individual shoot bud increased length was recorded 2% (w/v) sucrose, 1.0 mg/l IAA and 1.0 mg/l BAP supplemented on both liquid PM (3.27 ± 0.14 cm; Fig. 1b) followed by agar solidified PM (3.15 ± 0.10 cm; Fig. 1c) medium. It is proved that elongation of shoot bud was better in liquid media than agar solidified condition. Further PM was found better than MS for elongation of shoot bud [28, 30]. The elongation rate was different depending on PGR supplements liquid and solid media and solid culture was best for elongation [31, 32].

The elongation media are not excellent for rooting; so the seedlings were put on rooting media in order to create strong and stout root system. Half strength MS0 and nine different types of PGRs (IAA, IBA, NAA) supplemented MS media were used for induction of strong and stout root system (Table-3). 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. Increased in length as well as the number of roots developed shoot bud derived seedlings were more on full strength MS medium supplemented with 3% (w/v) sucrose + 1.0 mg/l IAA (4.06 ± 0.22 cm/shoot bud and 1.87 ± 0.14 no/shoot bud and Fig.1d) followed by half strength hormone free MS medium (3.75 ± 0.23 cm/shoot bud and 1.64 ± 0.10 no/shoot bud). Similar result was found in *Dendrobium* hybrid and *Dendrobium thyrsiflorum* respectively [33, 34]. IBA was effective for rooting in *Ilex khasiana* and *Cymbidium finlaysonianum* respectively [35, 36]. The opposite result was also noted that NAA was most appropriate in inducing roots in *Esmeralda clarkei* and *Vanda tessellata* [37, 38]. Combine effect of IAA, IBA or NAA induced excellent rooting response in *Rhyncostylis retusa* and *Aerides ringens* [39, 40] orchid species.

Mass scale propagation was done with the use of shoot primodia like structures (SPSs) of *D. transparens* when the tiny seedlings of this species were subcultured in both agar solidified and liquid MS & PM media supplemented with different kinds of PGRs those produced SPSs at the base. These SPSs were used as mass scale production of seedlings. Liquid media were more effective in SPSs development (Fig. 1e). It was revealed that PM medium was more effective than MS medium for SPSs induction [31, 41].

Table 2: Elongation of multiple shoot buds developed from pseudobulb explant 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 individual shoot bud	Average length (cm) of individual shoot bud after 30d of culture on elongation medium	Increase in length (cm) of shoot bud within 30d of culture on elongation medium	Average initial length (cm) of individual shoot bud	Average length (cm) of individual shoot bud after 30d of culture on elongation medium	Increase in length (cm) of shoot bud 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	1.50±0.12	3.64±0.09	2.14±0.14	1.60±0.14	3.88±0.09	2.28±0.12
MS + 3% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	1.52±0.14	4.27±0.12	2.75±0.17	1.48±0.11	4.35±0.16	2.87±0.13
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	1.45±0.10	4.54±0.14	3.09±0.09	1.40±0.15	4.56±0.11	3.16±0.17
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.50±0.15	3.78±0.19	2.28±0.16	1.55±0.17	3.90±0.14	2.35±0.11
MS+3% (w/v) sucrose + 0.5 mg/l NAA+1.0 mg/l BAP	1.70±0.16	4.41±0.13	2.71±0.11	1.65±0.09	4.59±0.12	2.94±0.10
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	1.42±0.11	4.54±0.18	3.12±0.15	1.45±0.19	4.69±0.14	3.24±0.16
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.55±0.13	4.02±0.10	2.47±0.14	1.50±0.10	3.98±0.16	2.48±0.13
MS+3% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.40±0.08	4.23±0.11	2.83±0.09	1.38±0.18	4.31±0.15	2.93±0.17
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.52±0.07	4.58±0.11	3.06±0.14	1.40±0.13	4.62±0.19	3.22±0.15
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.38±0.18	3.87±0.17	2.49±0.13	1.45±0.09	3.91±0.10	2.46±0.12

PM + 2% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	1.70±0.19	4.61±0.15	2.91±0.17	1.35±0.08	4.27±0.12	2.92±0.11
PM + 2% (w/v) sucrose +1.0 mg/l IAA + 1.0 mg/l BAP	1.65±0.14	4.80±0.12	3.15±0.10	1.45±0.12	4.72±0.09	3.27±0.14
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.55±0.08	3.91±0.13	2.36±0.11	1.40±0.16	3.81±0.13	2.41±0.14
PM + 2% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	1.50±0.12	4.27±0.16	2.77±0.15	1.42±0.13	4.29±0.11	2.87±0.09
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	1.52±0.11	4.50±0.08	2.98±0.12	1.55±0.15	4.71±0.17	3.16±0.13
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.60±0.13	3.86±0.17	2.26±0.16	1.42±0.08	3.76±0.12	2.34±0.10
PM + 2% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.65±0.16	4.51±0.19	2.86±0.18	1.45±0.11	4.36±0.09	2.91±0.12
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.40±0.17	4.44±0.18	3.04±0.14	1.48±0.14	4.70±0.16	3.22±0.11

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

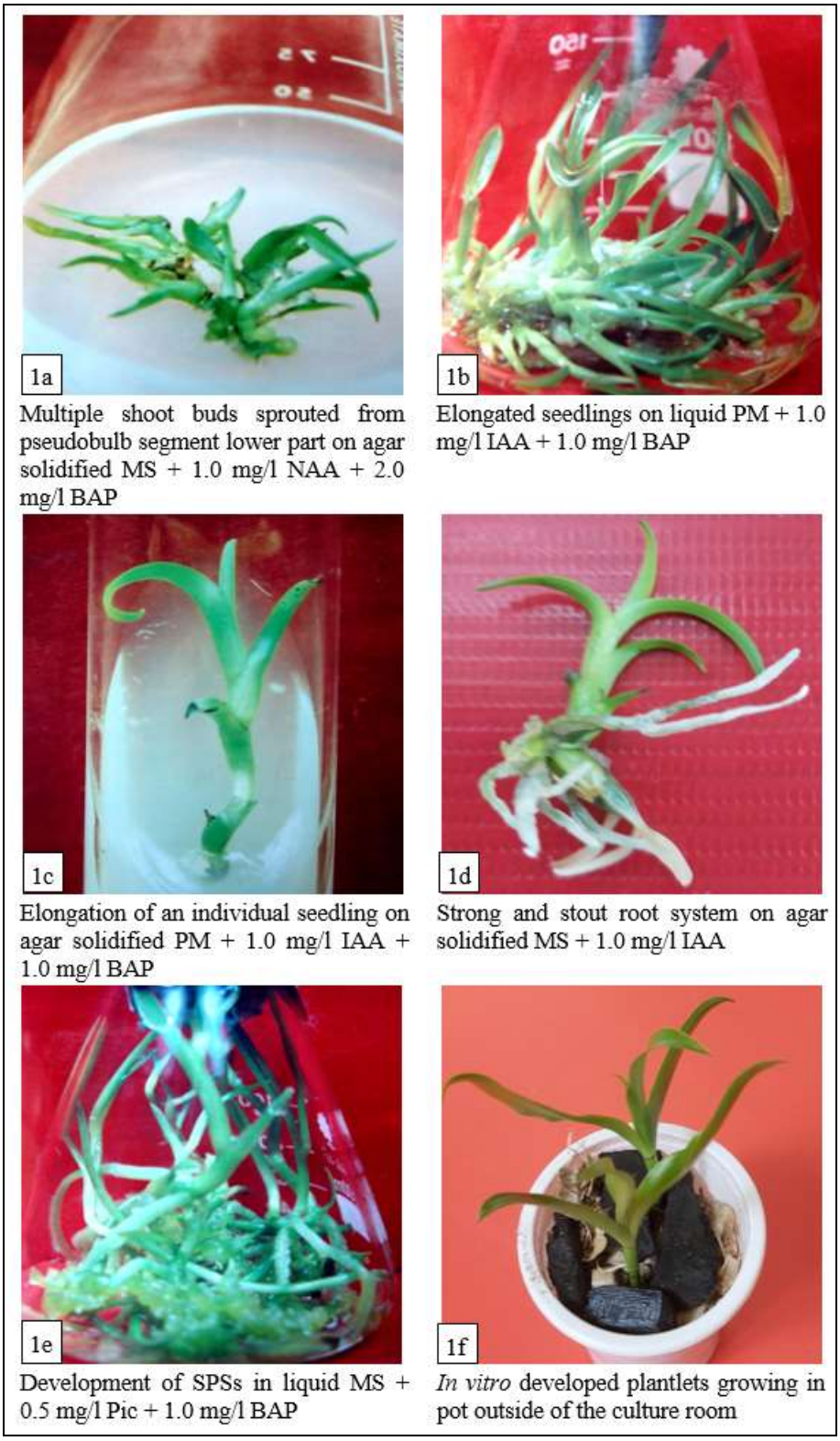

Fig 1: (1a-1f): *In vitro* micropropagation, Shoot bud elongation, SPSs development, rooting and hardening of *Dendrobium transparens* Wall ex Lindl.

The well-developed plantlets were transferred from culture room to the outside environment through successive phase of acclimatization (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 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 and established in the green house.

Table 3: Mean increase in length (cm) and number of roots of *D. transparens* shoot bud originated seedlings in half strength MS0 and auxin supplemented MS rooting media.

Culture medium			Average increased length and number of roots per shoot bud	
			Mean length (cm) ± S.E.	Mean no. of roots/ shoot bud ± S.E.
½ MS0			3.75 ± 0.23	1.64 ± 0.10
Auxin (mg/l)	IAA	0.5	3.64 ± 0.20	1.33 ± 0.09
		1.0	4.06 ± 0.22	1.87 ± 0.14
		1.5	3.71 ± 0.24	1.41 ± 0.08
	IBA	0.5	3.02 ± 0.21	1.25 ± 0.07
		1.0	3.26 ± 0.25	1.37± 0.07
		1.5	3.52 ± 0.25	1.53 ± 0.08
	NAA	0.5	3.67 ± 0.26	1.56 ± 0.09
		1.0	3.55 ± 0.22	1.44 ± 0.09
		1.5	3.34 ± 0.19	1.21 ± 0.07

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

4. Conclusions

Lower part of pseudobulb segments showed better response than the upper part. MS media supplemented with PGRs like NAA, BAP and Kn were very effective for MSBs and SPSs development. In elongation media, liquid media were better than agar solidified condition and PGRs supplemented PM media were more effective than MS media. IAA fortified MS rooting media were more effective than half strength MS0 and NAA, IBA supplemented full strength MS media. This micropropagation technique can be used by commercial firm for mass scale seedlings production to fulfill demand in international floriculture market and pharmaceutical industries. Furthermore, the protocol may facilitate conservation of this potential orchid from extinction in the natural population.

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