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Micropropagation of commercially important orchid *Dendrobium palpebrae* Lindl. through *in vitro* developed pseudobulb culture

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ABSTRACT: *Dendrobium palpebrae* Lindl. an epiphytic fragrant indigenous orchid species of Bangladesh and distributed in Bangladesh, eastern Himalayas, Myanmar, Thailand, Laos and Vietnam at altitudes of 800 to 2500 meters. From *in vitro* growing plantlets, upper and lower part of pseudobulb segments were aseptically 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 (8.21 ± 0.44/segment in lower part; 6.43 ± 0.40/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 Picloram + 2.0 mg/l BAP. Highest individual shoot bud increased length was recorded on agar solidified MS medium with 1.0 mg/l Picloram + 1.0 mg/l BAP (3.76 ± 0.14 cm) followed by liquid MS + 0.5 mg/l NAA + 1.0 mg/l BAP (3.11 ± 0.12 cm) medium. Elongation of shoot bud was better in agar solidified media than liquid condition and MS was found better than PM. Shoot bud derived seedlings were best responses as increase in length as well as the number of roots developed on agar solidified MS with 0.5 mg/l NAA (4.82 ± 0.22 cm/shoot bud and 2.75 ± 0.17 no/shoot bud) followed by MS with 0.5 mg/l IBA supplemented medium (4.49 ± 0.12 cm/shoot bud and 2.57 ± 0.08 no/shoot bud). The plantlets were successfully transferred to outside environment by successive phases of acclimatization.

KEYWORDS: *Dendrobium palpebrae*; micropropagation; MSBs; PGRs; SPSs.

INTRODUCTION

Orchids are flowering plants, commercially grown worldwide as cut flower and potted plants in floriculture trade. Orchidaceae is considered as the most advanced and largest among monocotyledons, including most multifariousness family of flowering plants, consisting of more than 35,000 species belonging to 700-800 genera [1]. Due to their ornamental and medicinal importance they demand a very high price in the international market [1]. They are well known for their strange shaped, longevity and beautiful looking flowers. They are cosmopolitan but primarily distributed in tropical areas. They are rare in arctic regions [1]. 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].

Dendrobium is one of the largest genera of orchidaceae and contains about 1184 species worldwide [3] and has high horticultural value. They are widely distributed and cultivated in the world ranging from southern Asia to New Guinea and Australia [4]. In Bangladesh, about 27 species of *Dendrobium* are distributed throughout the country. *Dendrobium palpebrae* Lindl. distributed in Bangladesh, eastern Himalayas, Myanmar, Thailand, Laos and Vietnam at altitudes of 800 to 2500 meters. This fragrant indigenous epiphytic species blooms from spring to late summer and for commercially important as cut flower [2].

Orchids can be easily propagated through *in vitro* propagation or tissue culture technique by using different plant parts as explants such as seeds, shoot tip, flower bud segment, lateral bud, young inflorescence, inflorescence, node, root, leaf and pseudobulb [5]. Through micropropagation technique we can produce large number of disease-free plantlets at a very low cost. Micropropagation technique is appropriate for multiplication rather than *in vivo* [6]. There is a great scope for large scale production of *Dendrobium* orchid in Bangladesh to meet the demand of international market and to earn foreign currency through export [7]. The current study was intending to develop a reliable, reproducible and efficient protocol for mass multiplication of commercially high demanding orchid *D. palpebrae*.

MATERIALS AND METHODS

Explant and culture conditions

In vitro developed seedlings of *Dendrobium palpebrae* Lindl. were used for micropropagation. For agar solidified media, agar (Himedia, India) was heated till is dissolved and finally mixing with stock solution gently. Plant Growth Regulators (Merck, Germany) *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 (Duran, Germany) and autoclaved (HYSC, Korea) at 121^0C for 20 minutes at 15 lbs pressure. p^H (Fisons, UK) of the medium was set at 5.8 using 0.1N NaOH or HCl prior to gelling with agar and the culture temperature was maintained at 25±2 ^{0}C. Humidity level was between 50-60% and light was maintained between 4000-5000 lux illumination from cool white fluorescent to set 14/10 h photoperiod [8]. Cultures were sub-cultured regularly and observed once in a week.

Micropropagation of pseudobulb segments

In vitro grown pseudobulbs were cut 0.5 to 1.0 cm size using sterilized surgical blade and forceps under the laminar air flow cabinet (HYSC, Korea). 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.

Elongation of multiple shoot buds (MSBs)

Eighteen types of elongation media were prepared using full strength [9-10] 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. 0.8% (w/v) agar was also used in solid media but in liquid media no agar was added.

Rooting of multiple shoot buds derived seedlings

For *in vitro* rooting of *D.* palpebrae, 0.8% (w/v) agar solidified half strength MS0 (Hormone free Murashige and Skoog medium) with 1.5% (w/v) sucrose and nine different types of MS medium supplemented with 3% (w/v) sucrose with three different concentrations of auxins *viz.* IAA, IBA and NAA were used for induction of strong and stout root system.

Hardening and transplantation

For hardening 90 days old plantlets with good rooting and 3-4 leaf conditions were selected. A gradual system of hardening was taken place in order to grow healthy plantlets. In this process, cultured vessels were kept open in the culture room for several hours, and then it was exposed to natural light for a day. Further, plantlets were washed by double distilled water to remove the adhering agar. Plants were treated with auxins to induce *ex vitro* rooting and roots were treated with fungicide. Then the seedlings of *D. palpebrae* 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 25-30^0C and RH 60-70%). Transplanted seedlings were watered regularly for about 2-3 months where the seedlings established and grew well.

Computation and presentation of data

In vitro culture experiments were carried out systematically with the use of sufficient no of explant.

Different growth parameters were considered to record data on morphogenic responses of explant under different conditions. The data on different parameters from different experiments were recorded after required days of culture.

Statistical analysis

All experiments were conducted in triplicate and data were presented as means ± standard error (mean ± SE). Standard deviations (SD) was calculated with Microsoft Excel 2013. In the table the mean ($\bar{x}$) data of different replication of each treatment are accompanied by standard error of mean (SE) which was calculated as follows:

$$\text{Standard error (SE)} = \frac{SD}{\sqrt{N}}$$

Where, SD = Standard deviation, N =Number of observations.

RESULTS

In vitro micropropagation of pseudobulb segments

Pseudobulbs were collected from *in vitro* derived seedlings as a source of explant of *Dendrobium palpebrae* for rapid micropropagation [11-13] and result is shown on Table-1. 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. The efficiency of a medium was assessed on the basis of number of shoot buds produced from each explant. Average highest number of MSBs (8.21 ± 0.44/segment in lower part; 6.43 ± 0.40/segment in upper part) produced on MS medium with 1.0 mg/l NAA + 2.0 mg/l BAP (Fig.1a) 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 Picloram + 2.0 mg/l BAP. It's also noted that lower part of pseudobulb segments showed better response than upper part.

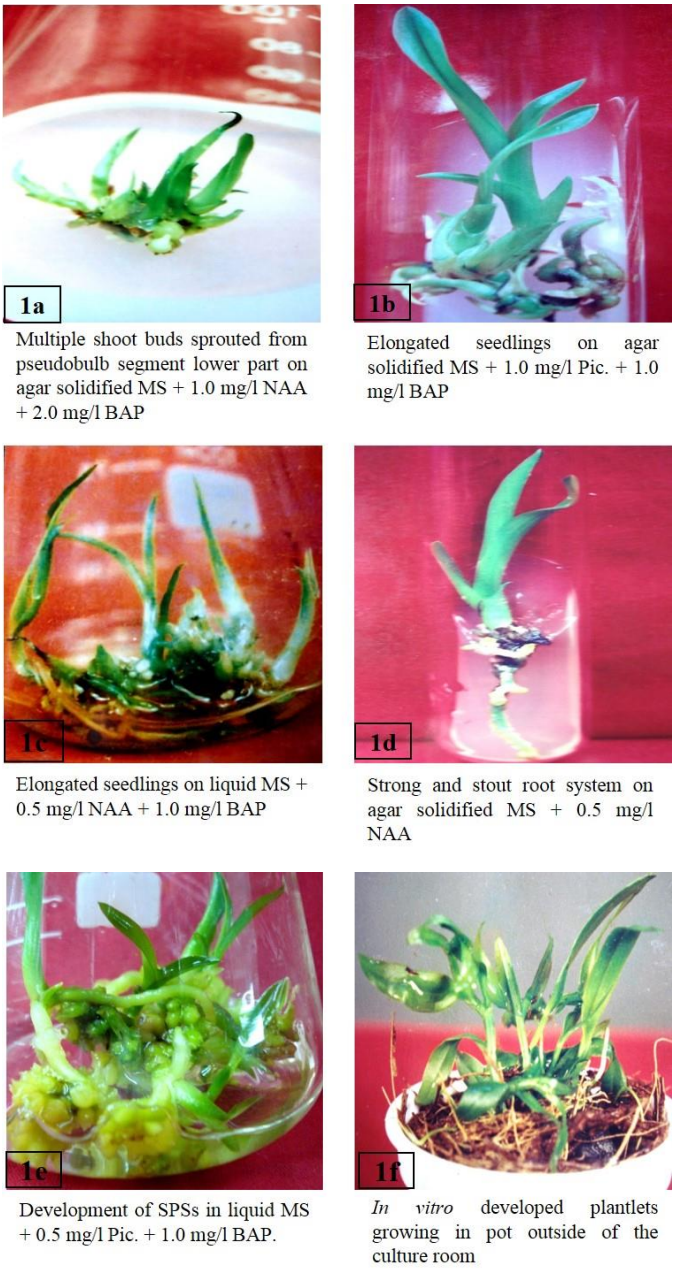

Figure 1. (1a-1f): *In vitro* micropropagation, shoot bud elongation, rooting, SPSs development and hardening of *Dendrobium palpebrae* Lindl.

Elongation of multiple shoot buds (MSBs)

Then the multiple shoot buds developed from pseudobulb segment culture were put on elongation media. Eighteen different kinds of solid & liquid media with various combinations and concentrations of PGRs were used for the purpose. Of these, nine were prepared using MS basal medium and the rest nine were prepared using PM basal medium for enhancing elongation of MSBs. In liquid media no agar was added. 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. 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 on agar solidified MS medium with 1.0 mg/l Picloram + 1.0 mg/l BAP (3.76 ± 0.14 cm; Fig. 1b) followed by liquid MS + 0.5 mg/l NAA + 1.0 mg/l BAP (3.11 ± 0.12 cm; Fig. 1c) medium.

Table 1. Development of multiple shoot buds from pseudobulb explant of *D. palpebrae* 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 - 38	3.81 ± 0.29
	PSL	55	30 - 35	6.72 ± 0.48
0.5 mg/l IAA + 1.0 mg/l Kn	PSU	40	35 - 40	3.73 ± 0.24
	PSL	45	35 - 38	5.36 ± 0.33
1.0 mg/l IAA + 2.0 mg/l BAP	PSU	45	35 - 40	5.07 ± 0.31
	PSL	55	25 - 30	6.62 ± 0.44
1.0 mg/l IAA + 2.0 mg/l Kn	PSU	45	32 - 36	5.11 ± 0.30
	PSL	50	30 - 35	6.17 ± 0.34
0.5 mg/l IBA + 1.0 mg/l BAP	PSU	35	35 - 40	3.68 ± 0.28
	PSL	30	35 - 40	3.03 ± 0.21
0.5 mg/l IBA + 1.0 mg/l Kn	PSU	35	36 - 40	3.75 ± 0.25
	PSL	40	32 - 36	4.11 ± 0.25
1.0 mg/l IBA + 2.0 mg/l BAP	PSU	45	32 - 35	5.03 ± 0.31
	PSL	55	28 - 32	6.78 ± 0.39
1.0 mg/l IBA + 2.0 mg/l Kn	PSU	45	33 - 36	5.12 ± 0.28
	PSL	50	32 - 35	6.08 ± 0.35
0.5 mg/l NAA + 1.0 mg/l BAP	PSU	40	30 - 35	3.69 ± 0.23
	PSL	50	30 - 35	6.21 ± 0.41
0.5 mg/l NAA + 1.0 mg/l Kn	PSU	35	35 - 40	3.86 ± 0.25
	PSL	40	35 - 38	4.23 ± 0.30
1.0 mg/l NAA + 2.0 mg/l BAP	PSU	55	25 - 30	6.43 ± 0.40
	PSL	70	25 - 28	8.21 ± 0.44
1.0 mg/l NAA + 2.0 mg/l Kn	PSU	40	35 - 40	4.23 ± 0.32
	PSL	50	32 - 35	5.97 ± 0.42
0.5 mg/l Pic + 1.0 mg/l BAP	PSU	35	35 - 40	3.78 ± 0.25
	PSL	45	32 - 35	4.66 ± 0.29
0.5 mg/l Pic + 1.0 mg/l Kn	PSU	30	35 - 40	3.22 ± 0.19
	PSL	45	33 - 36	4.56 ± 0.26
1.0 mg/l Pic + 2.0 mg/l BAP	PSU	50	25 - 30	5.96 ± 0.37
	PSL	60	25 - 28	7.24 ± 0.41
1.0 mg/l Pic + 2.0 mg/l Kn	PSU	40	30 - 35	4.37 ± 0.25
	PSL	50	28 - 33	6.02 ± 0.40

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

Table 2. Elongation of multiple shoot buds developed from pseudobulb explant of *D. papebrae* on 0.8% (w/v) agar solidified and liquid media with different kinds of PGRs.

Culture medium with different combinations and concentrations of PGRs	Solid media			Liquid media		
	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
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.65±0.15	3.78±0.09	2.13±0.12	1.40±0.11	3.64±0.08	2.24±0.14
MS + 3% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	1.58±0.09	4.49±0.12	2.91±0.16	1.35±0.13	4.36±0.16	3.01±0.11
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	1.40±0.11	3.86±0.14	2.46±0.10	1.52±0.12	4.25±0.11	2.73±0.14
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.45±0.19	3.50±0.19	2.05±0.13	1.45±0.18	4.07±0.14	2.62±0.16
MS+3% (w/v) sucrose + 0.5 mg/l NAA+1.0 mg/l BAP	1.70±0.11	4.68±0.10	2.98±0.17	1.52±0.08	4.63±0.17	3.11±0.12
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	1.55±0.14	4.17±0.18	2.62±0.08	1.70±0.14	4.75±0.19	3.05±0.17
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.65±0.17	4.02±0.09	2.37±0.11	1.65±0.12	4.27±0.16	2.62±0.19
MS+3% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.52±0.09	4.54±0.15	3.02±0.19	1.52±0.17	4.56±0.10	3.04±0.16
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.48±0.09	5.24±0.11	3.76±0.14	1.45±0.15	4.36±0.09	2.91±0.10
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.45±0.15	3.47±0.07	2.02±0.12	1.50±0.10	3.95±0.06	2.45±0.07
PM + 2% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	1.40±0.10	4.25±0.19	2.85±0.16	1.65±0.07	4.61±0.12	2.96±0.08
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	1.55±0.17	4.04±0.14	2.49±0.11	1.70±0.09	4.27±0.15	2.57±0.11
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.60±0.18	3.76±0.15	2.16±0.13	1.45±0.17	3.73±0.14	2.28±0.13
PM + 2% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	1.65±0.13	4.56±0.16	2.91±0.18	1.70±0.10	4.71±0.17	3.01±0.8
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	1.70±0.08	4.35±0.07	2.65±0.17	1.75±0.18	4.49±0.11	2.74±0.9
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.52±0.19	3.67±0.17	2.15±0.13	1.80±0.12	4.21±0.09	2.41±0.7
PM + 2% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.50±0.16	4.26±0.13	2.76±0.17	1.52±0.13	4.53±0.07	3.01±0.13
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.65±0.12	4.07±0.12	2.42±0.14	1.55±0.08	4.30±0.16	2.75±0.10

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

Rooting of individuals shoot bud

The elongation media are not good enough for root development of young seedlings. So, the developed 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 MS medium supplemented with 3% (w/v) sucrose + 0.5 mg/l NAA (4.82 ± 0.22 cm/shoot bud and 2.75 ± 0.17 no/shoot bud and Fig.1d) followed by MS with 0.5 mg/l IBA supplemented medium (4.49 ± 0.12 cm/shoot bud and 2.57 ± 0.08 no/shoot bud).

Development of shoot primordia like structures (SPSs)

Mass scale propagation was also done with the use of shoot primodia like structures (SPSs) of D. palpebrae. When the masses of the in vitro grown tiny seedlings of this species were subcultured in MS & PM media both solidified and liquid condition supplemented with different kinds of PGRs those produced SPSs at the base. These SPSs were used for mass scale production of seedlings (Fig. 1e).

Hardening and transplantation

The well-developed plantlets were transferred from culture room to the outside environment through successive phase of acclimatization. Sixty-four per cent of the in vitro grown seedlings survived and continued to grow in pots in the green house. Then, they were finally established in Orchidarium of the Botanical Garden of Chittagong University (Fig. 1f).

Table 3. Mean increase in length (cm) and number of roots of *D. palpebrae* 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			4.22 ± 0.20	2.43 ± 0.14
Auxin (mg/l)	IAA	0.5	3.73 ± 0.21	1.98 ± 0.16
		1.0	4.26 ± 0.27	2.17 ± 0.15
		1.5	4.11 ± 0.22	2.45 ± 0.12
	IBA	0.5	4.49 ± 0.12	2.57 ± 0.08
		1.0	3.61 ± 0.20	1.83 ± 0.12
		1.5	4.12 ± 0.22	2.18 ± 0.10
	NAA	0.5	4.82 ± 0.22	2.75 ± 0.17
		1.0	3.46 ± 0.20	2.21 ± 0.15
		1.5	3.14 ± 0.16	1.76 ± 0.12

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

DISCUSSION

Because of long lasting property of *Dendrobium palpebrae* flowers are commonly used as cut flowers and for that quality these orchids are now used commercially. Some countries such as Thailand, Singapore and Malaysia took the advantage of mass production of commercially important orchids by tissue culture and earn a good amount of foreign currency by exporting it to other countries [12].

Moreover, 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 [8]. For micropropagation, noted that lower part of pseudobulb segments showed better response than upper part [13-14]. The cytokines, like BAP and Kn; auxins like IAA, IBA, NAA and Picloram are involved in the process [12]. The requisite of auxins and cytokinins for regeneration of multiple shoot buds and seedlings development has been find out of many orchid species [15-17]. On the other hand, the combinations, concentrations and media type are usually critically important [18]. The ratio of auxin and cytokinins for shoot bud formation varies from species to species. BAP was best for shoot bud formation in *Cymbidium gigantean*, *Vanda spathulata* and *Dendrobium bensoniae* respectively [17-19].

In elongation of multiple shoot buds (MSBs) was better in agar solidified media than liquid condition. Further MS was found better than PM for elongation of shoot bud [11, 20-25]. The elongation rate was different depending on PGR supplements liquid and solid media and solid culture was best for elongation [26-27].

For induction of strong and stout rooting, auxin supplemented medium was more efficient [28-29]. In some cases, IBA was effective for rooting [30]. Combined effect of BAP (0.5 mg/l) and IBA (1.0 mg/l) was more effective of induced rooting in *Dendrobium bensoniae* [19]. It is noted that low concentration of auxin is more suitable than high concentration for induction of well-developed root system.

Liquid media were more effective in SPSs induction. Comparison of the efficiency of media combination in terms of SPSs induction revealed that liquid media were more effective than agar solidified media. It was further revealed that MS medium was more effective than PM medium for SPSs induction [25-26, 31-32].

CONCLUSIONS

Lower part of pseudobulb segments showed better response than the upper part. MS media supplemented with PGRs like NAA, BAP and Picloram were very effective for MSBs and SPSs development. In elongation media, agar solidified media were better than liquid condition and PGRs supplemented MS media were more effective than PM media. NAA containing MS rooting media were more effective than half strength MS0 and IAA, 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.

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AUTHOR CONTRIBUTIONS

TKB and MMR were involved in conception and design of the experiments. TKB contributed to perform the experiments. TKB analyzed data. TKB contributed to drafting the article. MMR contributed to revising it critically for important intellectual content. TKB and MMR made the final approval of the version to be published.

CONFLICTS OF INTEREST

Authors declared that they have no conflict of interest.

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