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Multiple regeneration pathways in *Spathoglottis plicata* Blume — A study *in vitro*

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ABSTRACT

Asymbiotic germination of immature seeds (embryos), and mature seeds and micropropagation of *Spathoglottis plicata* were described. Effects of three nutrition media namely, Murashige & Skoog (MS); Phytamax (PM); and Phyto-Technology orchid seed sowing medium (P_{723}), two carbon sources such as glucose and sucrose at 2–3% (w/v), two plant growth regulators such as 6-benzylaminopurine (BAP; 0.5–3.0 mg l^{-1}) and α-naphthalene acetic acid (NAA; 0.5–2.0 mg l^{-1}) and peptone (2.0 g l^{-1}) were examined on seed germination, early protocorm development and micropropagation. The maximum germination of mature seeds (95%) was recorded in PM medium supplemented with 2% (w/v) sucrose + 2.0 g l^{-1} peptone. For germination of embryos P_{723} medium supplemented with 1.0 mg l^{-1} BAP proved best. Multiple shoot buds or protocorm-like bodies (PLBs) were produced from stem segments of *in vitro* raised seedlings. Both direct organogenesis and embryogenesis were observed and the morphogenetic response was initiated by different concentrations and combinations of PGRs. The optimum PGR combination for maximal PLB regeneration was 1.0 mg l^{-1} NAA + 2.5 mg l^{-1} BAP, while 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BAP for shoot bud development. Strong and stout root system was induced in half strength PM medium supplemented with 0.5 mg l^{-1} IAA. The well-rooted plantlets were transferred to pots containing a potting mixture composed of saw dust, coconut coir, humus, and coal pieces at 1:1:1:2 (w/w) with 80% survival in outside environment and flowered after two years of transfer.

1. Introduction

Spathoglottis plicata Blume known as 'Garden orchid' is a terrestrial species very popular for its gorgeous, perpetual, magenta purple flowers on 70–90 cm long erect sprays with a large head of flowers and lengthy blooming period. The species originated from tropical Asia and distributed throughout Southeast Asia, China, Malay Archipelago, New Guinea, Thailand, Borneo, the Philippines, Australia and the islands of the Southwest Pacific Ocean (Teng et al., 1997; Beltrame, 2006; Thakur and Dongarwar, 2012). The plants grow at low to moderate altitudes in grassland and open forests in moist areas. In Bangladesh this plant is generally found in the home garden and as potted plant at the lobby and rarely found in nature. The plant produces flowers all the year round and the flower stalks carry 10–50 flowers which bloom continually for several months and the flower, remains open for several weeks. *Spathoglottis* has a great interest because of its high value as ornamental commodity. It is conventionally propagated through separation of pseudobulbs; however, the proliferation rate is very low necessitating rapid and reliable protocol for mass propagation. To propagate threatened and rare orchids many authors have suggested *in vitro* seed germination as a suitable propagation method (Arditti, 1967; Arditti et al., 1981, 1982a, 1982b, 1982c; Ballard, 1987; Zettler and McInnis, 1992, 1993; Thompson et al., 2006; Hossain et al., 2009, 2010). However, most terrestrial orchid seeds are difficult to germinate *in vitro* and *ex vitro* due to specific nutrient and environmental requirements (Van Waes and Debergh, 1986; Thompson et al., 2006). The research on the *in vitro* culture of *S. plicata* is very limited. Beechey (1970) reported regenerating plantlets from root culture. Bapat and Narayanaswamy (1977) reported callus and adventitious root induction from leaves and secondary roots of seedlings. A general method of hand pollination and *in vitro* seed culture of *Spathoglottis* have been described by Kauth et al. (2008) and Thakur and Dongarwar (2012). Shoot bud induction from nodal explants was also reported (Barua and Bhadra, 1999; Sinha et al., 2009). Interestingly, the available information failed to provide a comprehensive protocol for micropropagation of *S. plicata*. In the present study, we report a complete, easy, fast, efficient and reliable *in vitro* regeneration system for mass propagation of *S. plicata* through culture of embryos (immature seeds), mature seeds and nodal segments of *in vitro* raised seedlings. The protocol established in this species will facilitate *in vitro* propagation for commercial purpose and conservation of economically important orchids.

2. Materials and methods

2.1. Explants, nutrient media and culture conditions

Four–five week old immature pods of *S. plicata* Blume collected from the hand pollinated plants maintained in the Orchid House of the Botanical Garden of the Chittagong University of Bangladesh

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were used for embryo culture, and 8–9 week old undehisced green pods were used as a source of mature seed culture. Three different nutrient media namely, MS (Murashige and Skoog, 1962), PM (Phytamax®, Sigma Chemical Co. USA), and P_{723} (PhytoTechnology Orchid Seed Sowing Medium) supplemented with 2–3% (w/v) sucrose/glucose and with or without peptone (2.0 g l^{-1}) and PGRs (BAP, NAA) were used for embryos or seed culture. Nodal segments (0.5–1.0 cm in size having 1–2 buds) of *in vitro* raised seedlings were used for micropropagation. PM medium fortified with BAP (0.5–2.0 mg l^{-1}) and NAA (0.5–2.0 mg l^{-1}) at different concentrations and combinations were used for micropropagation. The pH of the medium was adjusted at 5.8 before autoclaving at 121 °C at 117 kPa for 20 min. Different types of glass vessels including test tubes (1.5×15 cm), culture bottles, and conical flasks (100–150 cm^3) were used. Culture vessels with inoculated explants were maintained in a culture room where a cycle of 14/10 h light–dark at 60 mmol m^{-2} s^{-1} was provided by cool white fluorescent lamps (Philips Truelight 36w/86 65001 K B7, Philips, India), and 60% RH at 25±2 °C. Regular subculturing was done at 20–25 days interval.

2.2. Establishment of axenic culture

The pods were washed with a hair brush under running tap water to remove dust particle and then surface sterilized by 0.1% (w/v) $HgCl_2$ solution for 10 min with occasional agitation and washed thoroughly with sterile distilled water. Finally the capsules were dipped in 70% ethanol for 1 min followed by flaming for 1–2 s. The surface sterilized pods were placed on a sterile filter paper and sliced with a sterile surgical blade at 1 mm thickness and cultured on the surface of the agar-gelled medium. For seed culture the sterilized pods were dissected longitudinally and the seeds were scooped with the help of forceps and inoculated on the surface of sterile medium. All the operations were performed in a laminar air-flow cabinet. When seeds germinated and protocorms came out, these were taken out aseptically from the culture vessels and the masses of protocorms were sub-cultured to fresh culture media for further growth and the nodal segments from these seedlings were used for micropropagation. The protocom-like bodies (PLBs) or shoot buds developed from the nodal explants were subcultured regularly to fresh nutrient media at one month interval.

2.3. Percent germination and seedling development

After two weeks of inoculation, some of the seeds were taken out and dispersed in one drop of water on a glass slide and observed under light microscope. Percent germination was calculated employing following formula:

$$\frac{\text{No. of seeds showing swelling of the embryo} \times 100}{\text{Total no.of seeds}}$$

Once the spherules were formed, observations were recorded at an interval of one week to trace different stages of protocorm development. These were observed using stereozoom microscope.

2.4. Rooting and transplantation of seedlings

Seedlings grown in *in vitro* culture conditions exhibited fewer roots, which may not support successful acclimatization once it is transferred to *ex vitro* conditions. On the other hand, shoot buds that produced from nodal explants did not produce any roots. Thus for induction of stout root system these were grown on different rooting media made up of half strength PM medium supplemented with 0.5–1.5 mg l^{-1} IAA. The well-rooted plants were taken out from the cultural vessels and washed thoroughly under running tap water for removal of agar medium attached to the root surface and transferred to pots containing a potting mixture of saw dust, coconut coir, humus and coal pieces, at 1:1:1:2 (w/w).

2.5. Data collection and statistical analysis

The experiments were designed following Complete Randomize Block Design (CRD). Five replicates were taken per treatment for seed and embryo culture whereas for micropropagation and rooting 10 replicates were used. For studying multiple shoot buds or PLBs production 20 shoot segments were used while for rooting, 10 shoots were used for each treatment. The effects of different media on germination of seeds, induction of shoot buds, PLBs and roots in the *in vitro* experiments were tested applying Duncan's multiple range test ($P>0.5$) in one way ANOVA. The statistical analyses were performed using the Statistica ver. 7 (Statsoft, Tulsa, USA). The experiments were repeated thrice.

3. Results and discussion

This is the first complete protocol that has ever been developed for mass propagation of *S. plicata*. Different routes for plantlets production were developed including *in vitro* germination of immature seeds (embryos), mature seeds (Fig. 1); and induction of multiple shoot buds and PLBs from nodal segments of *in vitro*-raised seedlings (Fig. 2). The different morphogenetic pathways of regeneration are described below.

3.1. Immature seed (embryo) culture

Within two weeks of culture the embryos started swelling and subsequently produced green protocorms on cut surface of the immature pods. The frequency of protocorm production varied depending on media combinations and orientation of explants on the medium. The pod discs placed horizontally on the surface of the medium gave superior response than vertically oriented ones. The highest number of protocorms per pod disc (70±2.3) were recorded on P_{723} medium supplemented with 1.0 mg l^{-1} BAP and the quantity of protocorms rapidly increased in subsequent subculture to same fresh medium (Fig. 1A) and subsequently developed into seedlings (Fig. 1C,D). BAP in the medium enhances germination of embryos in terms of germination frequency and production of healthy protocorms. The embryos in general, germinate readily and much better than the mature seeds as they possess distended testa cells which readily absorb nutrients from the culture medium, and metabolically awakened embryos free from any dormancy and/or inhibitory factors (Yam and Weatherhead, 1988; Pathak et al., 2001; Sharma et al., 2005). Moreover, the nutritional requirements of embryos during the initial stages of development are quite simple facilitating their germination (Stoutamire, 1974). The tendency of BAP impairing the germination response and stimulating protocorm proliferation has been reported in a large number of orchids including *Cattleya aurantiaca* (Pierik and Steegmans, 1972), *Cyprepedium* spp. (Depauw et al., 1995), *Aerides multiflorum*, *Dendrobium chrysotoxum*, *Eulophia dabia*, *Pachystoma senile* and *Cymbidium pendulum* (Pathak et al., 2001), *Epidendrum ibaguense* (Hossain, 2008), *Cymbidium aloifolium*, *C. giganteum* and *Dendrobium aphyllum* (Hossain et al., 2009, 2010, 2012), *etc*.

3.2. Mature seed culture

The seeds were germinated in all the three media (MS, PM and P_{723}) but germination percentages varied in different media (Table 1; Fig. 1B). The seeds began swelling within three weeks of inoculation, and germination commenced within five weeks after inoculation. The highest germination of seeds with numerous hairy structure in protocorms was observed on PM (95.00±1.10%) followed by P_{723} (90.00±1.80%) medium supplemented with 2% (w/v) sucrose and 2.0 g l^{-1} peptone. In the presence of 2% (w/v) glucose in the same basal medium it showed poor germination (60%) and the protocorms developed in this medium were

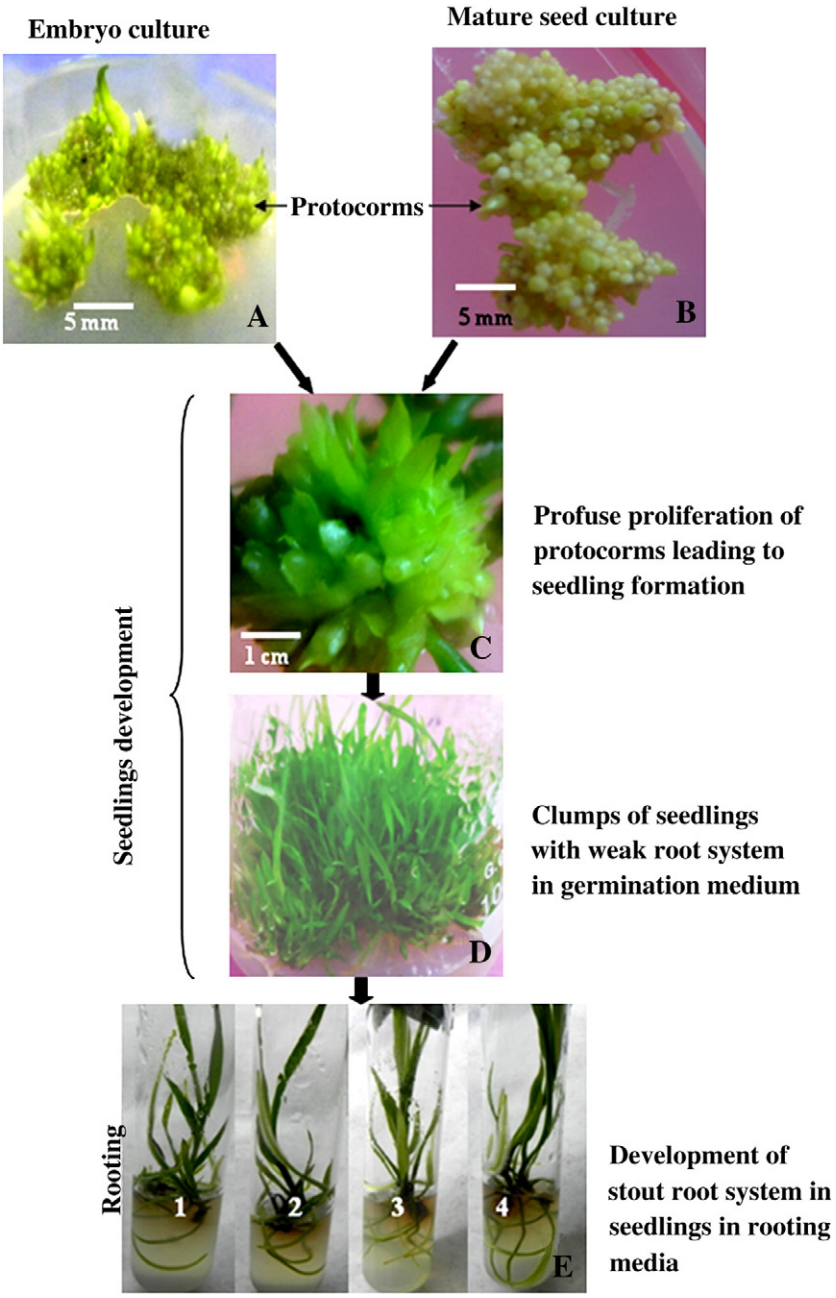

Fig. 1. *In vitro* germination of embryos and mature seeds of *Spathoglottis plicata*: A, protocorms derived from immature embryo; B, protocorms derived from mature seeds; C, protocorms underwent profuse proliferation through induction of secondary protocorms; D, seedlings developed from embryos/seeds; and G, rooting in seedlings [1 = PM, 2 = PM + 0.5 mg l^{-1} IAA, 3 = 1/2PM + 5.0 mg l^{-1} IAA, 4 = 1/2PM + 1.0 mg l^{-1} IAA].

light green with no hairy structures. Furthermore, on MS medium with glucose, germination was observed only after 9–10 weeks of inoculation. These findings revealed that sucrose was more efficient than glucose and PM medium supplemented with 2% (w/v) sucrose was more efficient for germination of seeds in this orchid.

Species-specific media for seed germination have been reported in orchids (Arditti and Ernst, 1984; Hossain et al., 2009, 2010; Paul et al., 2012). The specificity was reported even within species of same genus (Devi et al., 1990; Vij and Pathak, 1988; Jamir et al., 2002; Sharma and Tandon, 1990) and germination of seeds was greatly influenced by the quality and quantity of the nutrients of the culture medium (Pathak et al., 2001). All the presently employed media differ from one another in their chemical compositions (Table 2). MS is highly enriched with macro and micro elements and lacked peptone, PM contained approximately half of MS, while P_{723} contained comparatively low amount of both macro and micro nutrients than MS or PM. But both PM and P_{723} were enriched with 2.0 g l^{-1} peptone. The maximum per cent germination of seeds in PM or P_{723} medium could be attributed to the fact that this medium is enriched with peptone. The beneficial effect of peptone on seed germination and growth of protocorms is attributed to its amino acids, amides, minor elements and vitamin constituents (Oliva and Arditti, 1984). Peptone favoured seed germination, protocorm formation, protocorm multiplication, and differentiation into seedlings in many orchids including *Paphiopedilum* and *Vanda* species (Curtis, 1947), *Acampe praemorsa* (Krishna-Mohan and Jorapur, 1986), *Aerides multiflora*, *Rhynchostylis retusa*, *Saccolabium calceolare* and *Vanda testacea* (Vij et al., 1981), *Calopogon tuberosus* (Kauth et al., 2006), *C. aloifolium* (Hossain et al., 2009) and *C. giganteum*

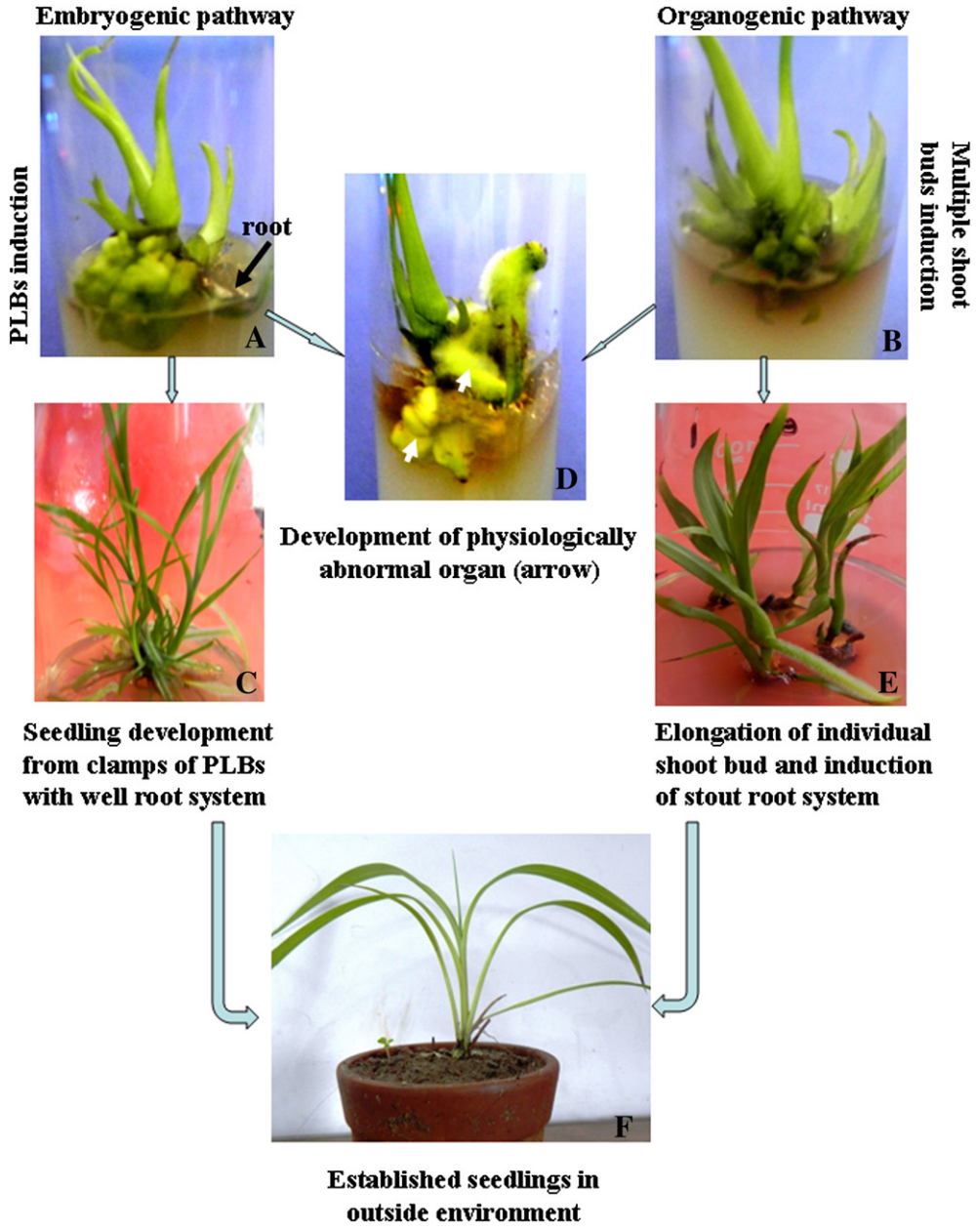

Fig. 2. Micropropagation of *Spathoglottis plicata* through nodal segment culture.

(Hossain et al., 2010). In case of *Kingidium taenialis* seed germination, peptone was found to be obligatory (Pathak et al., 2001). It is responsible for more synchronous protocorm and seedling development (Kauth et al., 2006).

From the beginning of orchid tissue culture sugar has been used as a source of carbon and its utility has been stressed by many workers in germination of orchid seeds and seedling development. Literature studies on different orchids suggested that sugar was obligatory for orchid seed germination (de Melo Ferreira et al., 2011). The orchid seeds lack an appropriate machinery (glyoxysomes) to utilize lipidaceous food reserves (Harrison, 1977) and they depend upon mycorrhizal endophytes for their requirements for sugars and other nutrients in nature (Poole and Sheehan, 1982). The mycorrhizal fungi break down the complex carbohydrates into simpler acceptable form for germinating seeds. According to Smith (1973), *Bletilla striata* seeds do not germinate well on sucrose and it may be advisable to use glucose or trehalose. Similar results obtained by Ichihashi and Yamashita (1977) and Yam and Weatherhead (1988) and the latter authors suggested that the specific preference for glucose/trehalose may have been a reflection of the geographical origin of the seeds. But most of the earlier reports favour sucrose rather than other carbohydrates (Arditti et al., 1982c; Mitra, 1987; Han and Stephens, 1992; Sharma, 1996). The concentrations of sucrose were also emphasized by many workers and reported that 2–3% (w/v) is effective concentration for germination of seeds and healthy growth of germinating entities (Arditti et al., 1982c; Sharma, 1996; de Melo Ferreira et al., 2011) whereas at higher levels (4–5%) are detrimental for germination and growth behaviour and lower levels are inhibitory (Pathak et al., 2001). In the present investigation 2% sucrose was optimum for better germination of seeds and growth of germinating protocorms of *S. plicata*.

3.3. Nodal segment culture

Both shoot buds and PLBs were induced in nodal segments. The morphogenetic response was reliant to the concentrations and combinations of BAP and NAA. The requirement of exogenous NAA and/or BAP for induction of shoot buds or PLBs has been reported in many

Table 1
Comparative effect of culture media on asymbiotic germination of seeds of *Spathoglottis plicata* Bl.

Basal medium	Carbohydrate % (w/v)		Peptone (g l^{-1})	Time (weeks) for germination of seeds		% seed germination (Mean ± SE)*
	Sucrose	Glucose		Spherule formation	Protocorm formation	
PM	2.0	–	–	4–5	5–6	75.50 ± 1.30^{abc}
	–	2.0	–	4–5	7–8	52.00 ± 1.30^{ef}
	2.0	**–**	**2.0**	**4–5**	**5–6**	$\mathbf{95.00 \pm 1.10^{a}}$
	–	2.0	2.0	4–5	6–7	60.00 ± 1.69^{cd}
MS	3.0	–	–	5–6	6–7	70.00 ± 1.26^{bc}
	–	3.0	–	6–7	8–9	65.00 ± 1.35^{cd}
	3.0	–	2.0	4–5	7–8	85.00 ± 1.09^{abc}
	–	3.0	2.0	5–6	9–10	55.00 ± 1.25^{def}
P_{723}	2.0	–	–	4–5	6–7	70.00 ± 1.40^{bc}
	–	2.0	–	6–7	8–9	50.00 ± 1.20^{f}
	2.0	–	2.0	4–5	6–7	90.00 ± 1.80^{ab}
	–	2.0	2.0	5–6	7–8	60.00 ± 1.50^{cd}

Bold data indicated for the best media compositions.
* Mean values within the column followed by the same letters are not significantly different at P = 0.05 according to Duncan's multiple range test; n = 5 per treatment, conducted in triplicate.

orchid species (Arditti and Ernst, 1993; Tokuhara and Mii, 1993; Melissa et al., 1994; Le et al., 1999; Decruse et al., 2003; Hossain et al., 2010, 2012). However, the combinations, concentrations, and the ratio between them are usually critically important (Hossain et al., 2010). In *S. plicata*, the optimum PGR combination for maximal shoot bud regeneration (8.30 ± 0.36 shoot buds per explants) was 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} BAP, while for induction of PLBs the best combination of PGRs was 1.0 mg l^{-1} NAA + 2.5 mg l^{-1} BAP (10.80 ± 0.44 PLBs per explants) (Table 3; Fig. 2A, B). The ratio of auxin to cytokinin for shoot buds or PLB formation varies from species to species (Teng et al., 1997; Hossain et al., 2012). A striking synergic effect of BAP and NAA in inducing shoots has been reported for many orchids including *Dendrobium fimbriatum* (Roy and Banerjee, 2003), *Rhynchostylis gigantea* (Le et al., 1999), *Vanda spathulata* (Decruse et al., 2003), *Cattleya* (Melissa et al., 1994), *Phalaenopsis* and *Doritaenopsis* (Tokuhara and Mii, 1993), *Renanthera imschootiana* and *Vanda coerulea* (Seeni and Latha, 2000), *Vanilla planifolia* (Tan et al., 2011), *etc.* Janarthanam and Seshadri (2008) reported that only shoot buds were formed in *V. planifolia* when the medium was supplemented with BAP in addition to NAA, and no shoots were induced when BAP or NAA were applied alone. Both shoot buds and PLB formation from same explant were also reported (Bhadra and Hossain, 2004). The PLB regeneration was greatly influenced by the specific BAP and NAA combination. The combination of BAP with NAA switched the morphogenetic pathway, *i.e.* PLBs were produced instead of shoot buds and a higher concentration of BAP along with lower concentration of NAA stimulated PLB induction (Table 3). The PLBs profusely proliferated on subsequent subculture forming a shoot apex which eventually developed into a complete plantlet (Fig. 2C). Similar results were also observed in *Micropera pallida* (Bhadra and Hossain, 2004), *Cymbidium giganteum* (Hossain et al., 2010), *Lycaste aromatica* (Martín et al., 2010), *Paphiopedilum rothschildianum* (Ng and Saleh, 2011) and in other orchids. Malformation or hyperhydricity (development of physiologically abnormal organ or proliferating bodies) was also observed in 1.5 mg l^{-1} NAA + 3.0 mg l^{-1} BAP which was enriched with high concentration of BAP (Fig. 2D). Similar results were observed by Vasudevan and Van Staden (2011) during *in vitro* regeneration of *Ansellia africana* through culture of protocorm sections. Hyperhydricity is a common disadvantage of *in vitro* propagation (Debergh et al., 1992; Kevers et al., 2004; Coste et al., 2011; Appleton et al., 2012). Accumulation of high level of CO_2 released by growing cultures stimulates ethylene synthesis, which in turn affects transpiration and photosynthesis negatively that may be one of the causes for hyperhydricity (Grodzinski et al., 1981; Lai et al., 2005). Generally hyperhydric tissues/organs are unable to regenerate true plantlets.

Table 2
Composition of different basal media.

Constituents	MS	PM	P_{723}
Macronutrients (mg l^{-1})			
KNO_3	1900	950	475.00
NH_4NO_3	1650	825	412.50
KH_2PO_4	170	85	–
K_2PO_4 (Monobasic)	–	–	42.50
$MgSO_4 \cdot 7H_2O$	370	90.35	75.18
$CaCl_2 \cdot 2H_2O$	440	166	–
$CaCl_2 \cdot$ Anhydrous			83.00
Iron source			
Na2-EDTA	37.3	37.3	18.65
$FeSO_4 \cdot 7H_2O$	27.8	27.8	13.93
Micronutrients (mg l^{-1})			
KI	0.83	0.415	0.2075
$MnSO_4 \cdot 4H_2O$	22.3	8.45	4.23
$ZnSO_4 \cdot 7H_2O$	8.6	5.30	2.65
H_3BO_3	6.2	3.1	1.65
$CuSO_4 \cdot 5H_2O$	0.025	0.0125	0.0063
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.125	0.0625
$CoCl_2 \cdot 6H_2O$	0.025	0.0125	0.0063
Vitamins and organics (mg l^{-1})			
Glycine	2	–	–
Thiamine-HCl	0.50	1.00	10
Pyridoxine-HCl	0.50	0.50	1.0
Nicotinic acid/niacin	0.50	0.50	1.0
myo-inositol	100	–	100
Peptone		2000	2000
Carbon source			
Sucrose[a]	30,000	20,000	20,000

[a] During experiments sucrose was replaced with glucose and used as modified medium.

3.4. Rooting and acclimatization of plants

The *in vitro* germinated seedlings produced weak root system on germination medium which was not sufficient for establishment in outside environment. On the other hand shoot buds developed from nodal explants did not produce any roots. Thus for induction of well developed root system these were grown on half strength PM media supplemented with 0.5–1.5 mg l^{-1} IAA. Medium supplemented with 0.5 mg l^{-1} IAA proved effective for induction of strong and stout root system (>6/seedling) (Table 4, Figs. 1G, 2E). In the presence of 1.0–1.5 mg l^{-1} IAA the seedlings produced roots but the roots were very thin and long. This finding indicated that enhancement of rooting in 1/2 PM medium was due to low nutrition ions concentration and striking effects of IAA. Similar response was also reported by Hossain et al. (2009, 2010, 2012) in *C. aloifolium*, *C. giganteum* and *D. aphyllum*. Development of roots is

Table 3
Development of multiple shoot buds (MSBs) or protocorm-like bodies (PLBs) in nodal segments of *Spathoglottis plicata* on agar solidified PM medium supplemented with different concentrations and combinations of BAP and NAA.

PGR combination (mg l^{-1})		No. of MSBs/nodal segment (Mean ± SE)*	No. of PLBs/nodal segment (Mean ± SE)*	Length of shoots after 30 days (cm) (Mean ± SE)*
BAP	NAA			
Control		1.00 ± 0.00^{g}	–	0.88 ± 0.33^{f}
0.5		3.25 ± 0.40^{ef}	–	1.60 ± 0.55^{e}
1.0		5.00 ± 0.22^{bcde}	–	2.29 ± 1.01^{cd}
1.5		5.50 ± 0.20^{bcd}	–	2.82 ± 1.42^{bcd}
2.0		5.40 ± 0.00^{bcd}	–	2.44 ± 1.02^{cd}
2.5		6.20 ± 0.25^{bc}	–	2.67 ± 1.12^{bcd}
3.0		5.22 ± 0.20^{bcd}	–	2.11 ± 0.90^{de}
	0.5	3.50 ± 0.55^{def}	–	2.48 ± 0.67^{bcd}
	1.0	3.70 ± 0.17^{def}	–	2.25 ± 0.98^{cde}
	1.5	3.30 ± 0.32^{def}	–	2.76 ± 1.23^{bcd}
	2.0	2.50 ± 0.40^{fg}	–	2.22 ± 0.97^{cde}
	2.5	1.70 ± 0.06^{fg}	–	2.28 ± 0.44^{bcde}
	3.0	1.50 ± 0.15^{fg}	–	2.56 ± 0.52^{bcd}
0.5	0.5	7.50 ± 0.40^{ab}	–	2.27 ± 0.99^{bcde}
1.0	1.0	**8.30 ± 0.36^{a}**	–	2.92 ± 1.22^{ab}
1.5	1.5	–	4.00 ± 0.25^{cd}	2.95 ± 1.29^{ab}
0.5	2.0	–	9.20 ± 0.27^{ab}	2.22 ± 0.95^{cd}
1.0	2.5	–	**10.80 ± 0.44^{a}**	3.04 ± 0.93^{a}
1.5	3.0	–	3.70 ± 0.35^{d}**	2.66 ± 0.75^{bcd}

Bold data indicated for the best media compositions.
* Mean values within a column followed by the same letters are not significantly different at $P = 0.05$ according to Duncan's multiple range test; $n = 20$ per treatment.
** Malformation of PLBs was observed.

an innate nature of plants which is controlled by endogenous level of hormones (Jarvis, 1986). Exogenous supply of hormones in combination with endogenous ones can effectively enhance rooting. The present study suggested that combined effects of nutritional stress with IAA enhanced the development of strong and stout root system in *S. plicata*. The well-rooted plants were then transferred to greenhouse where 80% seedlings survived (Fig. 2F) and some of the plants flowered after two years of transfer to pots.

4. Conclusions

Although extensive work has been initiated on micropropagation of many orchids from mature seeds, root, leaf, nodal and protocorm section culture and vast number of literatures have been accumulated in this area, culture of immature seeds for micropropagation of orchids is limited which has several advantages. Firstly, the germinating embryos show induction and profuse proliferation of protocorms. Secondly, embryos in general germinate readily and much better than the mature seeds and during seed development only 1/2 to 2/3 of the developing embryos get opportunity to maturity. Moreover, embryo culture reduces the time period for plant production remarkably as they are harvested at early stage of seed development. Thirdly, it can assist in obtaining seedlings from wide crosses where embryos often abort before reaching maturity. Therefore, this protocol can be extended to other economically valuable and rare and endangered orchids for mass propagation and conservation.

Table 4
Rooting response in seedlings or shoot buds of *Spathoglottis plicata*.

Culture medium	No. of roots/seedling or MSB (Mean ± SE)*	Length of roots (cm) after 30 d of culture (Mean ± SE)*
PM	2.22 ± 0.21^{e}	2.31 ± 0.15^{e}
PM + 0.5 mg l^{-1} IAA	3.50 ± 0.33^{cd}	3.45 ± 0.18^{b}
PM + 1.0 mg l^{-1} IAA	4.10 ± 0.25^{bd}	3.25 ± 0.17^{bc}
1/2 PM	2.95 ± 0.18^{ce}	2.73 ± 0.13^{de}
1/2 PM + 0.5 mg l^{-1} IAA	6.10 ± 0.28^{a}	4.44 ± 0.15^{a}
1/2 PM + 1.0 mg l^{-1} IAA	5.30 ± 0.26^{ab}	4.60 ± 0.33^{e}

* Mean values within a column followed by the same letters are not significantly different at $P = 0.05$ according to Duncan's multiple range test. $n = 10$ per treatment.

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