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Effect of different basal media and PGRs on asymbiotic seed germination of *Spathoglottis plicata* Blume: A highly ornamental and medicinal orchid

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Abstract
Spathoglottis plicata Blume is an ornamentally and medicinally significant terrestrial orchid of Bangladesh. This orchid seeds can be asymbiotically germinated by *in vitro* process for fast propagation. Varied response was found in terms of seed germination, protocorm like bodies formation and seedling development was observed on four different basal media. Medium supplemented with PGRs favored optimum condition for the germination of seeds followed by full strength and half strength on KC, MS, PM and VW media. Based upon the results, it was found that MS medium was more effective than KC, PM and VW media for germination, PLBs and plantlet formation. PM medium with PGRs combination gave the maximum percentage response (93.34%) followed by PGRs supplemented MS (80.00%), VW (73.34%) and KC (73.34%) media. Minimum percentage of seed germination was observed on half strength PGRs free VW (26.67%) medium. Minimum time needed for initiation of germination in full strength PGRs supplemented PM (8.67 ± 0.36^{a} weeks) medium, whereas, maximum time required (20.17 ± 0.38^{h} weeks) in PGRs free half strength KC medium.

Keywords: Medicinal orchid, PGRs, PLBs, *Spathoglottis plicata*

1. Introduction
The orchids, flowers of exquisite beauty belong to one of the largest families of angiosperms, the Orchidaceae, which represents the most highly evolved family amongst monocotyledons with 28,237 species [1]. In a more recent report, however, the Royal Botanic Garden of Kew lists 880 genera and nearly 22,000accepted species, but the exact number is unknown because of taxonomic disputes [2]. Orchids are undoubtedly the ornamental elite because of their perplexingly complex flowers of exquisite beauty. Reason being, orchids nowadays became an object of multibillion dollar business [3]. Apar from their ornamental value, many orchids also have apparent medicinal importance [4, 6].
In Bangladesh, the family is rich with 187 species [7]. In Bangladesh; Chittagong, Chittagong Hill Tracts, Cox's Bazar, greater Sylhet, Gazipur and Sundarbans mangrove forest are rich in orchid flora [8]. Loss of habitat, deforestation, destructive collection technique and over exploitation of orchids with medicinal and ornamental values has depleted the orchid wealth of Bangladesh [7]. Many orchids are now at the verge of extinction, so it is high time to conduct effective strategies to conserve in nature.
Spathoglottis plicata Blume, is a terrestrial orchid, naturally distributed in Bangladesh, Malaysia, the Philippines and New Guinea. The species is Vulnerable (VU) in the wild, but common in cultivation and flowered in February-June [9]. It is floricultural important species bearing long lasting, fragrant and deep violet coloured flowers. The leaves are used as a packing material and wrap parcels. Leaves also are used as a filling material in Taiwan [9]. The species is vulnerable in nature due to habitat destruction and heavy collection pressures. Hence, present study was planned to assess the asymbiotic germination potential of seeds *in vitro* and subsequent protocorms development, differentiation and seedlings development with a view to increasing mass propagation protocol for the species.

2. Materials and Methods
2.1 Source and Collection of capsules
The materials used for the present investigation were the mature green capsules of *S. plicata* which were collected from the forest area of Manikchari, Khagrachari, Bangladesh during the months of August and September.

2.2 Sterilization of Capsules
The dust in the capsules collected from the naturally grown orchid was removed. The mature green capsules was washed with tap water containing few drops of teepol solution for few minutes and washed under running tap water for ten 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.

2.3 Culture medium and incubation
In the present study, half strength, full strength and plant growth regulators (PGRs) *viz.* BAP (0.5 mg/l) and NAA (0.5 mg/l) fortified media of KC [10], MS [11], PM [12] and VW [13] were used for *in vitro* seeds germination, protocorms development, differentiation and seedlings development. Basal medium were fortified with 30g/l sucrose for MS (Murashige and Skoog) and 20g/l sucrose for KC (Knudson c), PM (Phytamax), VW (Vacin and Went) and with or without different plant growth regulators (PGRs) like BAP & NAA (Table 1). 0.8% agar (w/v) was used as a gelling agent for all of the tested media. The pH of the media was adjusted at 5.8 in case of MS and 5.4 in KC, PM and VW by using 0.1N NaOH or HCl before mixing agar. Agar was dissolved by boiling with distilled water. 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 [14]. For subsequent development of the seedlings, they were subcultured on respective media at different intervals. PGRs *viz.* BAP and NAA were freshly prepared.

2.4 Seed culture and Sub-culturing
For the inoculation of seeds, surface sterilized mature 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. Twelve types of full or half strength with or without PGRs fortified KC, MS, PM and VW media were used for that experiment. Sub-culturing was carried out every 4-6 weeks into fresh medium till the protocorms grew and formed complete seedlings.

2.5 Hardening and Transplantation
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.

2.6 Computation and presentation of Data
The experiments were conducted thrice using 15 replicates per treatment. Different strength of basal media and PGRs combinations were considered to record data on morphogenic responses of explant under different conditions. The data on different parameters were recorded after required days of culture.

2.7 Statistical analysis
Experiment was set up as a randomized complete design and all graphs were prepared with using Microsoft Excel 2013. The data were statistically analyzed, using SPSS software package. ANOVA and mean comparison were carried out by DMRT at 5% level of significance (P=0.05).

3. Results and Discussion
Table-1 represent the response the germination pattern of *Spathoglottis plicata* orchid seeds cultured on half or full strength 0.8% (w/v) agar solidified KC, MS, PM and MVW media supplemented with or without PGRs (0.5 mg/l BAP and 0.5 mg/l NAA). Remarkable differences showed on the cultured media in terms of frequency of germination, quality of the protocorms formation, leaf and root differentiation and seedlings development. PGRs free half strength all of the basal media responded slowly in the frequency of seeds germination as well as protocorms formation. Whereas, PGRs fortified full strength all basal media gave maximum responses in the medium, the germinated protocorms continued normal growth and produced healthy seedlings after subsequent subcultures.

Amongst four basal media used, full strength PM medium with PGRs combination gave the maximum percentage response (93.34%, Fig. 1a) followed by PGRs supplemented MS (80.00%, Fig. 1b), VW (73.34%) and KC (73.34%) media. Minimum percentage of seed germination was observed on half strength PGRs free VW (26.67%) medium. Minimum time required for initiation of germination in full strength PGRs fortified PM (8.67 ± 0.36^a weeks) medium, followed by (10.47 ± 0.34^b weeks) MS, (12.47 ± 0.31^{cd} weeks) VW and (13.27 ± 0.28^{de} weeks) KC media. Similar result was also found in *Cymbidium cyperifolium* [15]; *Dendrobium aphyllum* [16]; *Arundina graminifolia* [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.

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 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. PGRs free half strength VW medium needed maximum time (19.3 ± 0.40^h weeks) for seeds germination.

Table 1: Effect of different strength of KC, MS, PM and VW media with plant growth regulators on *in vitro* seed germination, differentiation and seedling development of *Spathoglottis plicata* Blume

Medium	Culture condition	Time taken in weeks					% of culture vessel germinated	Remarks
		Initiation of germination (Mean ± SE)	Development of protocorm (Mean ± SE)	Differentiation of 1st leaf primodia (Mean ± SE)	Differentiation of 1st root primodia (Mean ± SE)	Development of seedling (Mean ± SE)		
KC	*	20.17 ± 0.38^h	24.53 ± 0.35^j	29.37 ± 0.41^i	35.10 ± 0.28^i	40.27 ± 0.29^h	33.34	+
	**	16.30 ± 0.35^g	20.50 ± 0.41^h	25.43 ± 0.30^g	31.57 ± 0.37^g	37.40 ± 0.31^g	66.67	++
	***	13.27 ± 0.28^{de}	16.17 ± 0.38^e	21.13 ± 0.31^d	27.40 ± 0.32^e	34.47 ± 0.34^e	73.34	++
MS	*	16.70 ± 0.37^g	20.57 ± 0.32^h	25.33 ± 0.29^g	30.20 ± 0.34^f	36.13 ± 0.32^f	46.67	+
	**	12.20 ± 0.32^c	15.33 ± 0.30^{de}	21.13 ± 0.31^d	26.33 ± 0.30^d	33.33 ± 0.39^d	73.34	++

	***	10.47 ± 0.34[b]	13.53 ± 0.24[c]	18.23 ± 0.33[c]	24.07 ± 0.31[c]	30.13 ± 0.37[b]	80.00	+++
PM	*	14.17 ± 0.31[ef]	17.17 ± 0.34[f]	22.20 ± 0.39[e]	27.60 ± 0.36[e]	33.17 ± 0.35[d]	53.34	++
	**	10.30 ± 0.27[b]	12.27 ± 0.33[b]	17.13 ± 0.33[b]	23.07 ± 0.34[b]	30.20 ± 0.33[b]	86.77	+++
	***	8.67 ± 0.36[a]	10.33 ± 0.35[a]	14.30 ± 0.29[a]	20.20 ± 0.29[a]	26.07 ± 0.32[a]	93.34	+++
VW	*	19.3 ± 0.40[h]	23.40 ± 0.41[i]	28.13 ± 0.32[h]	33.67 ± 0.30[h]	40.27 ± 0.31[h]	26.27	+
	**	14.73 ± 0.35[f]	18.37 ± 0.36[g]	24.17 ± 0.34[f]	31.33 ± 0.31[g]	37.10 ± 0.30[g]	46.67	+
	***	12.47 ± 0.31[cd]	15.13 ± 0.31[d]	20.17 ± 0.32[d]	26.17 ± 0.31[d]	32.13 ± 0.36[c]	73.34	++

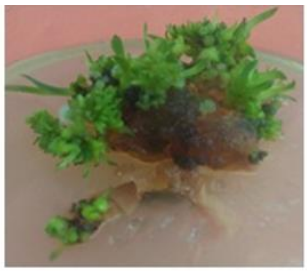
*Half strength without PGRs, **Full strength without PGRs, *** Full strength with PGRs (0.5mg/l BAP + 0.5mg/l NAA); + = Minimum germination (0% ≤ + ≤ 49%), ++ = Medium germination (50% ≤ ++ ≤ 74%), +++ = Maximum germination (75% ≤ +++ ≤ 100%). Values represent mean ± SE of each experiment consist of 15 replicates. Mean values followed by different superscript letters within a column are significantly different at p = 0.05 according to DMRT.

First visible greenish yellow spherule like protocorms were develop after seed inoculation in PGRs supplemented full strength PM (12.27 ± 0.33[b] weeks) medium followed by MS (13.53 ± 0.24[c] weeks), VW (15.13 ± 0.31[d] weeks) and KC (16.17 ± 0.38[e] weeks) media respectively. Differentiation of first leaf primodia was shown on after 14 weeks in PGRs fortified PM (14.30 ± 0.29[a] weeks) subsequently MS (18.23 ± 0.33[c] weeks), VW (20.17 ± 0.32[d] weeks, Fig. 1c) and KC (21.13 ± 0.31[d] weeks, Fig. 1d) media. Whereas, first root primodia was exposed on PGRs supplemented PM (20.20 ± 0.29[a] weeks) medium followed by MS (24.07 ± 0.31[c] weeks, Fig. 1e), VW (26.17 ± 0.31[d] weeks) and KC (27.40 ± 0.32[e] weeks) media. Within 26 weeks of culture, complete seedlings were first showing on PGRs fortified PM (26.07 ± 0.32[a] weeks, Fig. 1f) medium followed by MS (30.13 ± 0.37[b] weeks), VW (32.13 ± 0.36[c] weeks) and KC (34.47 ± 0.34[e] weeks) media.

With or without PGRs supplemented full strength KC, MS, PM and VW media was found to be the most suitable culture condition for mature seed germination of *S. plicata* up to seedling development. Here also distinguished those PGRs supplemented media took lesser time for germination (Fig. 2), protocorm formation (Fig. 3), first leaf and root primodia differentiation and seedlings development (Fig. 4) than full or half strength of PGRs free medium. The number of protocorms was few on Knudson medium and highest in PM medium but size of protocorms was superior on Knudson medium. Low amount of macro and micro elements of Knudson medium could have been effective for enlargement of protocorms due to nutritional stress but not for increasing their number. Production of larger size of protocorms indicates that culture seeds require sufficient amount of nutrients. Thus, the nutrient system for orchid culture is specific species to species and no particular culture medium is generally relevant for all the orchid species. From above result, it was consummate that PM medium enriched with Peptone, which was suitable for earlier germination, large number of protocorm formation and seedling development rather than MS, VW and KC media. Similar findings were reported in *Dendrobium transparens* [18]; *Micropera obtusa* [14]; *Cymbidium aloifolium* [19]; *Calanthe densiflora* [20] where PM medium was shown to be the most suitable medium over other nutrient media. In the present research, a successful attempt was made to assess the *in vitro* seed germination and their subsequent differentiation of *S. plicata* on four media *viz.* KC, MS, PM and VW. This protocol might be helpful for option of best state for mass propagation and *ex situ* conservation of this valuable orchid species.

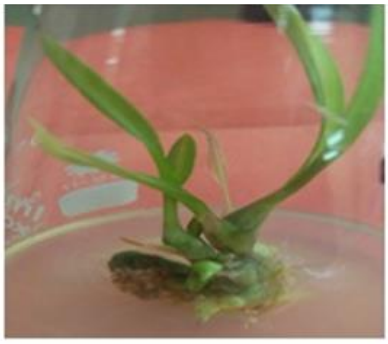
a. Germination of *S. plicata* seeds on PGRS fortified full strength agar solidified PM medium.

b. Germination of *S. plicata* seeds on PGRS supplemented full strength agar solidified MS medium.

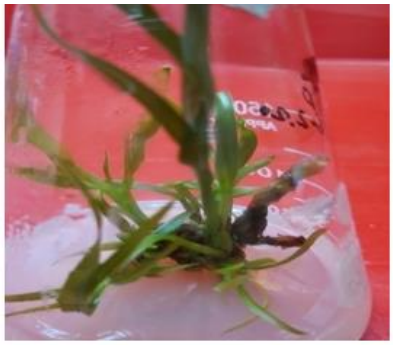


c. Germinated PLB's *S. plicata* turned into small shoots on PGRs supplemented full strength agar solidified VW medium.

d. Germinated PLB's *S. plicata* turned into small shoots on PGRS fortified full strength KC medium.



e. Development of seedlings and small root of *S. plicata* on PGRS fortified full strength MS medium.



f. Plantlets of *S. plicata* developed on full strength PM + 0.5 mg/l BAP + 0.5 mg/l NAA.

Fig 1.

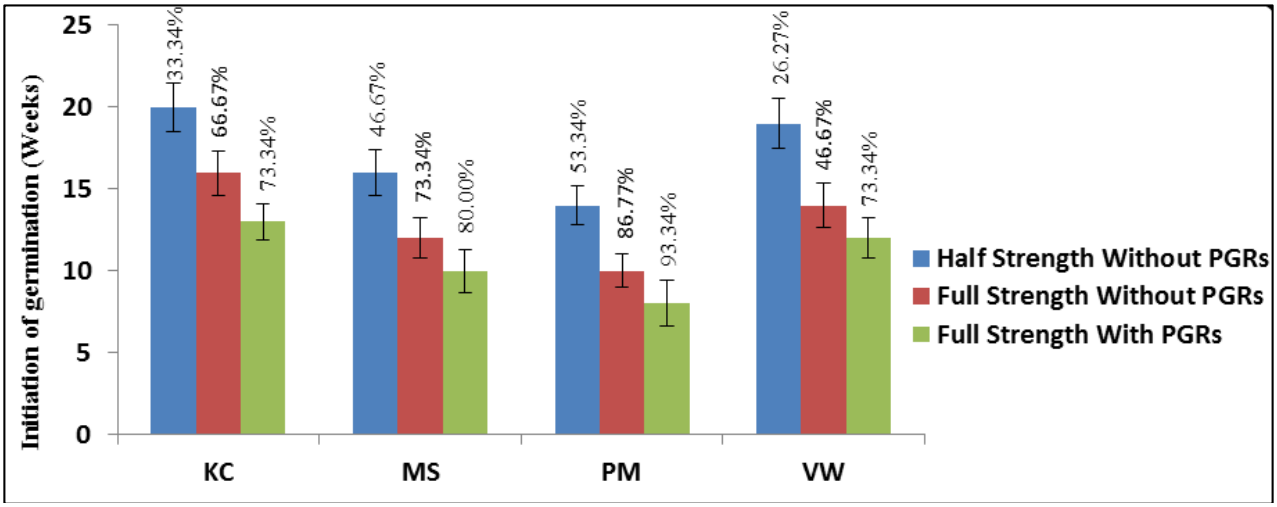


Fig 2: Initiation of germination pattern of *Spathoglottis plicata* Blume

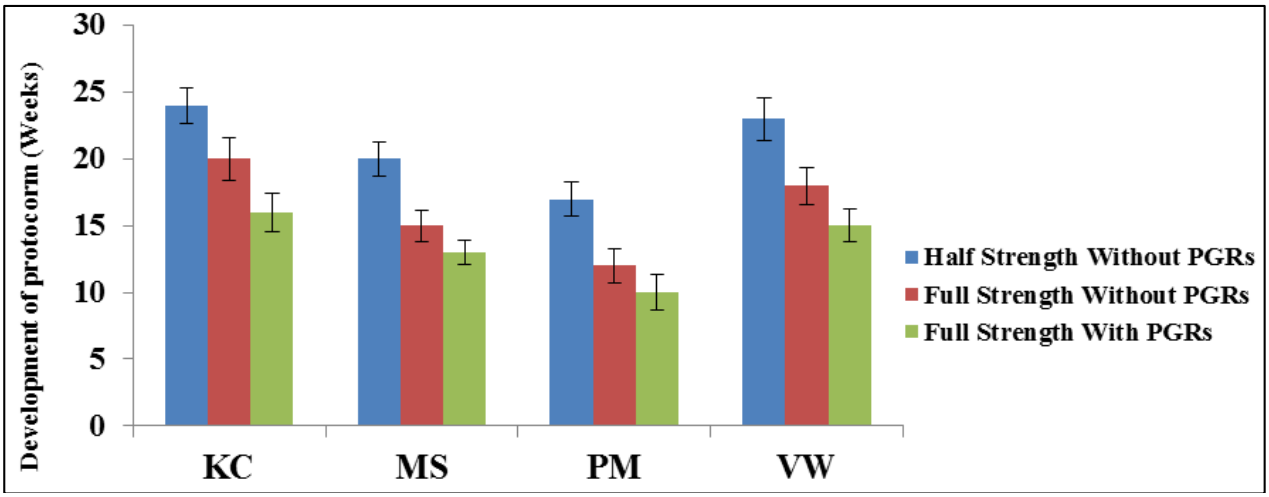
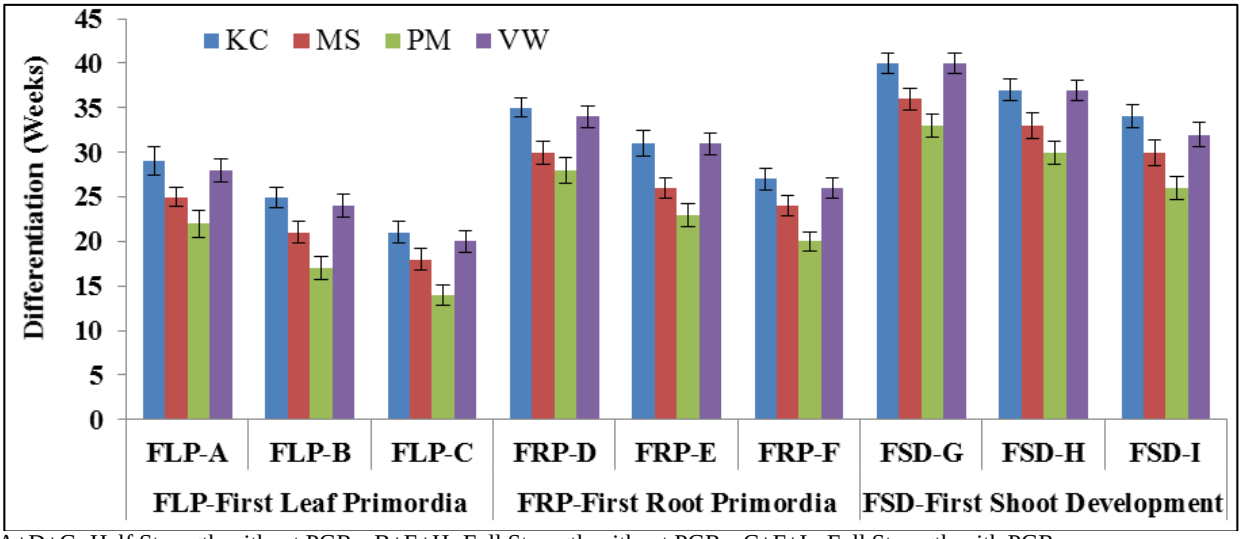


Fig 3: Development of protocorm pattern of *Spathoglottis plicata* Blume



A+D+G=Half Strength without PGRs; B+E+H=Full Strength without PGRs; C+F+I= Full Strength with PGRs

Fig 4: Differentiation pattern of *Spathoglottis plicata* Blume

Four different basal media using in present study, which were different from each other in their chemical composition. There are numerous reports explaining perfection of germination and seedling growth and improvement by vitamins on different orchids. Addition of various vitamins into the medium was reported to be promoting for seeds germination and seedlings development of *Cymbidium elegans* and *Coelogyne punctulata* [21]. Mariat [22] reported that vitamin B enhanced for germination and differentiation in *Cattleya* seedlings. He showed that thiamine, nicotinic acid and biotin were most efficient in *Cattleya* hybrids production. In other study reported that, Pyridoxine was shown to be vital for chlorophyll synthesis and combination of nicotinic acid and biotin favored for better germination of *Orchis laxiflora* seeds [23]. All of the basal media of full strength gave acceptable result but the time taken for germination and

seedling development was quite longer than PGRs supplemented medium. PGR like BAP was known to increase the germination frequency of *Habenaria macroceratitis*; *Cypripedium candidum*; *Erythrodes humilis* [24, 26]; protocorm multiplication and shoot formation in *Erythrodes humilis* and *Cymbidium pendulum* [26, 27]. In present investigation, protocorms were globular and chlorophyllous in all testing conditions of PM and MS medium; whereas, in KC and VW medium, they were light yellowish in color.

The well developed rooted plantlets were transferred from culture room to the green house during successive phase of adjustment. 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 Soil, Sand, Activated Charcoal and Pit Moss, Vermicompost at a ratio of 1: 1: 1: 1 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 standard and grew well.

4. Conclusion

Full strength PGRs fortified PM medium was found better followed by MS, VW and KC media for promoting germination of *S. plicata* orchid seeds. PGRs free half strength VW media was found to be at least performance. For comparing the effectiveness; in terms of enhancing seeds germination, protocorm development, differentiation and seedlings development full strength PGRs supplemented media was gave superior responses than other conditions. However, the *ex situ* conservation of this species is highly suggested not only for its conservation but also to best utilized its commercial demand.

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