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Research Article

Asymbiotic seed germination and Micropropagation of *Spathoglottis plicata* Blume.

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Abstract

The effect of different nutrient solution, organic supplement and plant growth regulators on *in vitro* seed germination and plantlet development of *Spathoglottis plicata* were studied. Seed germination was higher in Knudson (1946) medium (KnC) (90%) and lower in Vacin and went medium (VW) (1949). The seedling was grown well in the Knudson medium. The plantlets were allowed to grow on that medium until the plantlets get matured in that medium. For micro propagation the pseudobulbs were used from in *vitro* developed matured plants. The Half strength MS medium (1962) (HMS) was good for Protocorm like bodies (PLBs) development from the pseudobulb explant. The HMS supplemented with BAP (6-Benzylamino purine) and KIN (6-furfuryl amino purine) alone and combined with NAA (α- naphthalene acetic acid) hormone. The BAP + NAA (100+50µl/l) was good for maximum (12.8 shoots) multiplication. The matured rooted plantlets were acclimatized by using paper cup containing Garden soil+ Coconut fiber + Sand (1:1:1 ratio) for hardening. The soil was wetted with distilled water periodically for plantlets development.

Key words: Spathoglottis plicatta, Asymbiotic seed germination, Protocorm like bodies, Acclimatization.

INTRODUCTION

Spathoglottis plicatta Blume. is a handsome terrestrial orchid species, with long grassy leaves and about 7.5 cm wide flowers on erect spikes, Orchids are the most exquisite and wondrous plants of nature. It is the third largest family of flowering plants in India having 1141 species in 168 genera, of these 657 species in 86 genera is epiphytes and 484 species in 82 genera are terrestrial [1]. Orchid seeds are extremely small, usually undifferentiated and are produced in large numbers around 1,300 – 4,000,000 seeds per capsule. Each seed contains an undifferentiated embryo composed of 80 – 100 cells enclosed within a more or less transparent seed coat. A few fine strands of testa attach the embryo. The testa cells vary in size and assume a netted appearance due to their variously thickened longitudinal and transverse walls. Endosperm development is suppressed and the embryo remains arrested at the 'globular' or 'pre-heart' shaped stage. In spite of this poor organization they can germinate in nature in association with a suitable fungus (mycorrhiza). Most of the mycorrhizal fungi of the orchids were identified as *Rhizoctonia*, the major species being *Rhizoctonia languinosa*, *Rhizoctonia mucoroides* and *Rhizoctonia repens* [2]. The pioneering work of Bernard [3] is important in the development of *in vitro* culture technique of orchids since he successfully isolated the root infecting fungi helpful in orchid seed germination.

The first *in vitro* studies on the development of orchidaceae seedlings were conducted in asymbiotic conditions[3]. The asymbiotic culture of the seeds of the orchidaceae has provided physiological morphological and histological data on the development of the protocorm of various species [4, 5, 6,7].

Spathoglottis plicata is conventionally propagated through separation of pseudobulbs but the proliferation rate is very low[8]. A more efficient approach for its regeneration is *in vitro* seed culture [9,10] .For mass clonal propagation, regeneration from nodal segments and axillary bud explants is superior to seed culture due to availability of plant materials round the year, an exponential propagation rate and sustaining the parental characteristics in regenerated plants. Reports on the *in vitro* culture of *Spathoglottis plicata* are limited [11, 12, 13]. The available information fails to provide a comprehensive protocol and understanding of micropropagation of *Spathoglottis plicata.* The present investigation was, therefore, undertaken to develop a suitable protocol for *in vitro* seed

germination and rapid propagation of plantlets from the *in vitro* Pseudobulbs expalnts and successful hardening system of this indigenous terrestrial orchid.

MATERIALS AND METHODS

Mature undehised capsule of *Spathoglottis plicata* were collected from Narikkuni village, Calicut District in Kerala and used as a source material. Then the plant materials were surface sterilized with running tap water for 10 minutes followed by rinse the capsule using 5-6 drops of Teepol solution for 5 minutes. Then the capsule was taken into the laminar air flow chamber followed by rinse the capsule using 0.1% Mercuric chloride solution for 5 minutes. The capsule was rinse with sterilized distilled water for 3-5 times. After sterilization the treated capsule was cut longitudinally, then using the sterile spatula scooped out the seeds and spreading over the surface of the culture medium. The medium was Knudson C medium[14], Half Strength MS medium [15], and Vacin and Went medium [16]. The pH of the medium was adjusted to 5.2 -5.3 and the cultures were maintained in 16 hrs. photoperiod from cool dry light florescent tubes giving a total intensity (2000 lux) and proper temperature (25 ± 2ºC) should be provided. Till they get their proper growth they should be in aseptic condition. The chemicals used were of analytical grade (Himedia Laboratories, Mumbai, India).

For micropropagation the pseudobulbs were cut aseptically from the *in vitro* raised plantlets and transferred into the HMS supplemented with BAP and KIN alone and combined with NAA hormone. The cultures were examined the emergence of protocorm like bodies (PLBs) from the base of the pseudobulbs. After 20 days shootlets were emerged out from the PLBs.

***In vitro* acclimatization:**

The rooted plantlets were removed from the culture tubes and were washed thoroughly with running tap water to remove any traces of the medium. Then they were treated with 0.1% (w/v) Bavistin (fungicide) and again washed with sterilized distilled water. The rooted plantlets were planted in paper cubs filled with Garden soil + Coconut fiber+ Sand (1:1:1) ratio. The plants were covered with plastic bags for 30 days maintained under humidity (67%). Plants became acclimatized to a reduced relative humidity by gradually opening the plastic cover and after 30 days plantlets were completely uncovered and hardened to greenhouse condition.

Statistical analysis:

The protocorm multiplication from the pseudobulb explant achieved using HMS medium supplemented with cytokinin (BAP and KIN) and combined with auxin NAA. The multiplication of the protocorm was assessed using the standard deviation (SD) methods.

RESULTS AND DISCUSSION

***In vitro* seed germination:**

The onset of germination and the percentage of seed germination on three basal media were recorded periodically at every 10-day interval after the day of initial inoculation. In this experiment, seeds were cultured using 16/8 light/dark photoperiod. Seed germination was first observed after 20 days, 30 days, and 40 days of inoculation on KnC, VW and HMS media respectively. The highest seed germination of 90% was observed in KnC basal medium. The germination percentage was 50% in HMS and 0% in VW respectively. In KnC basal medium complete seed germination was achieved by the 25th day after inoculation (Table-1). Among the basal media tested, KnC was the most effective. All the media contain mineral salts that vary not only in their concentration but also in their availability of nutrients [17]. Nitrogen sources vary in all tested media. While KnC contain only inorganic forms of nitrogen (ammonia and nitrate) MS medium contain a mixture of both organic and inorganic sources. Inorganic forms of nitrogen may inhibit germination due to low nitrate reductase activity during germination and protocorm development [18, 19]. According to previous reports, organic nitrogen sources (i.e., amino acids) as opposed to inorganic forms may have a positive effect on seed germination in orchid [20, 21].

In this experiment, seeds were cultured using 16/8 light/dark photoperiod. The negative effects of light application on seed germination have been reported by several researchers Van Waes and Debergh [22]; Yamazaki and Kazumitsu [23]; however responses may vary [24].In another study, seed germination in terrestrial orchid *Bletia purpurea* was tested under different photoperiod conditions, and 16/8 light/dark was found to be suitable for germination and seedling development [25]. According to Valletta[26], the seed germination was not observed under continuous darkness, and the best photoperiod regime for germination was 16/8 light/dark, which is similar to our findings.

PLB's multiplication from the Pseudobulb explants:

About 5 mm long *in vitro* pseudobulb explants were cultured on a HMS basal medium supplemented with BAP and Kin. The morphogenetic response to various concentrations of plant growth regulators showed variation in initiating the meristematic activity of the explants. The explant showed swelling initially at cut ends after 15 days of culture and subsequently along the entire surface. The swelling eventually converted too many shoot buds. It was observed that the whole pseudobulb yielded better result over the segments. The HMS media with 3% sucrose was

found most suitable for pseudobulb culture. Treatment with BAP 2.0 mg/l alone respond well. Then combined treatment with BAP with NAA (2.0 + 0.6 mg/l) respectively induced a high rate of shoot development (Table-2). *in vitro* sourced pseudobulb segments showed better response on HMS medium supplemented with BAP + NAA (2.0 + 0.6 mg/l) induced maximum number of shoots.

Teng [11,12] was obtained PLBs from nodal and leaf explants of eight-month-old seedlings cultured on MS supplemented with 5.37 µM NAA and 0.44 µM BA and 2 g/l charcoal and the best combination of Plant growth regulators for plantlet development was 2.69 µM NAA + 8.88 µM BA. Earlier researchers used modified MS medium for induction of PLBs from the explants [11,12, 13]. In the present experiments, HMS medium was found to be suitable in relation to number of micro-shoots formed per culture. Half strength MS was also found suitable for *in vitro* regeneration of other orchids, like *Epidendrum radicans* [27]. *Paphiopedilum* [28]and *Phalaenopsis* [29,30,31,32].

Rooting:

To obtain rooted plantlets the shoots were taken out from the culture vessels and cultured on half strength of MS supplemented with IBA (Indole-3-butyric acid) and IAA (0.50 to 4.00 mg/l). The HMS medium with IAA 2.0 mg/l with 2% sucrose (w/v) was good for root induction and this combination induced an average number of 6 roots in 90% cultures within in 10 days (Table 3). For rooting, Barua and Bhadra (1999) cultured regenerated shoots of *Spathoglottis plicata* on MS medium with 0.5 mg/l IAA, on which 3.2 roots were induced from each shoot.

Hardening:

After rooted the plantlets were planted in decomposed paper cups containing garden soil, coconut fiber, sand (1:1:1 ratio), was used for hardening the plantlets. From these soil mixtures the soil was wetted with distilled water. The plants were watered periodically and they were covered with polythene bags for maintaining humidity. Sinha[8] was successfully hardened the plantlets using plastic basket containing coconut husk (10 mm^2). After 30 days the plantlets were implanted in clay pots containing alluvial soil and compost (3: 1) and kept under shade net. The plants were watered every seven days and they were fertilized with a mixture of urea, triple super phosphate (TSP) and muriate of potash (MOP) (2: 1: 1) at 25 g/plant at three months intervals.

In three months the *Spathoglottis plicata* increased in height, and rooted well from the soil mixture. As in the present study plants were directly regenerated through micro-shoot formation from the *in vitro* explants without the intervention of callus. The growth regulators BAP + NAA (2.0 + 0.6 mg/l) were used in small doses for protocorm inducing. From the protocorm plantlets were regenerated, there was no risk of genetic variation in the cultured plants. This protocol will help to conserve the wild variety without any variations. In *in vitro* culture, the excessive use of growth regulators and especially during the intermediary callus phase is undesirable as those are thought to be the main causes of variation in plants cultured *in vitro*, it was avoided during this work[33].

CONCLUSION

Asymbiotic seed germination was higher in Knudson medium (90%) and lower in Vacin and Went medium. The *in vitro* pseudobulb explants BAP+NAA (2.0+0.6mg/l) was good for maximum (12.8 shoots) number of shoots. The rooted plants were transferred to decomposed paper cups containing garden soil, coconut fiber, sand (1:1:1 ratio) was good for hardening the plants. The protocol as developed in the present study was through adjusting and determining the optimum concentrations and combinations of plant growth regulators, organic supplements and other medium adjuncts. The protocol is reproducible and would be utilized in high frequency regeneration of *Spathoglottis plicata* for commercial as well as conservation aspects.

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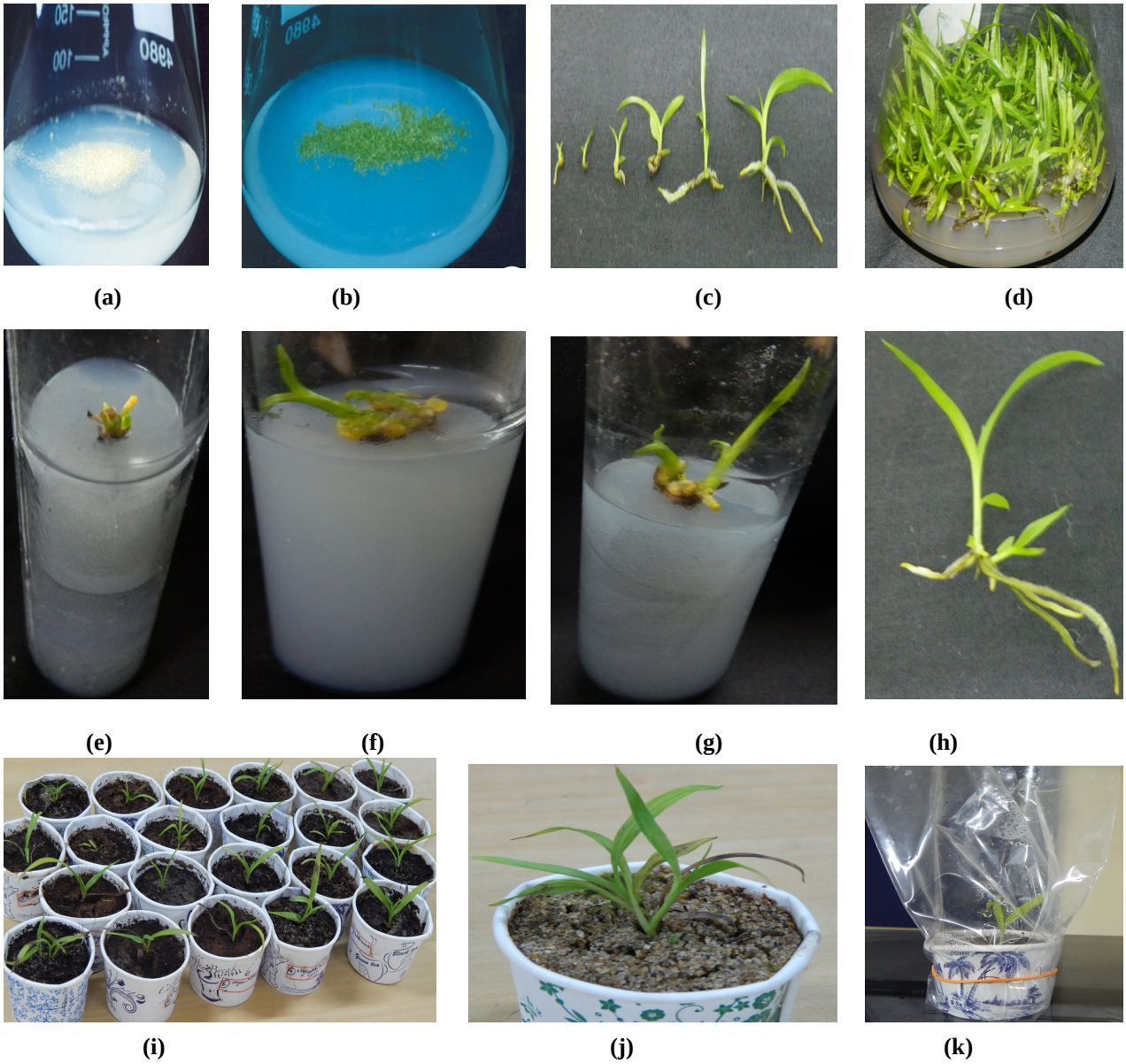
Table – 1
Media employed for seed germination of *Spathoglottis plicata*

Media employed for seed germination	% of germination	Time taken for initiation of germination (days)	Shoot growth (%)
MS	20%	45 days	30%
HMS	50%	40 days	50%
KnC	90%	25 days	90%
VW	-	-	-



a. *Spathoglottis plicata* seeds in KnC medium.
b. Germination of seeds in Knudson C medium after 25 days.
c. Different stages of plantlets development from the PLBs in knudson C medium.
d. Matured plantlets in knudson C medium.
e. PLBs developed from HMS medium with BAP+NAA (2.0+0.6mg/l) Pseudobulb explant.
f-g. Plantlets developed from HMS medium with BAP+NAA (2.0+0.6mg/l) Pseudobulb explant.
h. Rooted plants from HMS medium with IAA (2.0 mg/l).
i-k. Hardening and acclimatization of *in vitro* developed plantlets.

Fig 1
Asymbiotic seed germination and micropropagation of *Spathoglottis plicata*

Table – 2

Effects of HMS medium supplemented with plant growth regulators for micropropagation and shoot multiplication from the psuedobulb explant of *Spathoglottis plicata*

Plant growth regulators (mg/l)	% of response	No of Plantlets multiplication from Pseudobulb explant (mean ± SD)	Regeneration pathway
BAP			
0.50	60%	4.2±1.2	PLB-SP
1.00	70%	6.8±1.2	PLB-SP
2.00	90%	9.7±1.2	PLB-SP
3.00	70%	7.6±1.0	PLB-SP
4.00	60%	5.2±1.2	PLB-SP
BAP+NAA			
2.00+0.2	60%	6.2±1.9	PLB-PL
2.00+0.4	70%	7.4±1.9	PLB- PL
2.00+0.6	90%	12.8±1.2	PLB- PL
2.00+0.8	70%	7.8±1.2	PLB- PL
2.00+1.0	60%	6.4±1.9	PLB- PL
KIN			
0.50	20%	1.4±1.9	PLB
1.00	30%	2.5±1.7	PLB
2.00	60%	4.0±1.4	PLB
3.00	30%	2.5±1.7	PLB
4.00	20%	2.0±1.4	PLB
KIN+NAA			
2.00+0.2	30%	4.0±1.2	PLB-CA
2.00+0.4	30%	4.2±1.4	PLB-CA
2.00+0.6	50%	4.8±1.7	PLB-CA
2.00+0.8	40%	4.2±1.4	PLB-CA
2.00+1.0	20%	3.0±1.9	PLB-CA

***** PLB-Protocorm-like bodies development; SP-Shoot primordium development;
PL-Plantlet development; CA-Callus development;

Table 3

Effect of different concentration of IBA and IAA for root induction of shoots derived from Pseudobulb explants of *Spathoglottis plicata.*

Hormone	Concentration	% of response	No.of roots developed from shootlets (mean ± SD)
IBA	0.50	30	1.4±1.8
	1.00	50	3.2±1.0
	2.00	**70**	4.4±1.6
	3.00	50	3.8±1.4
	4.00	30	2.4±1.2
IAA	0.50	50	4.4±1.2
	1.00	70	5.8±1.6
	2.00	**90**	**6.3±1.6**
	3.00	80	5.3±1.4
	4.00	30	3.5± 0.8

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