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RESEARCH ARTICLE

IN VITRO PROPAGATION OF SPATHOGLOTTIS PLICATA BLUME VIA ASYMBIOTIC SEED GERMINATION.

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Abstract

The loss of plant genetic resources has necessitated the development of many *ex situ* conservation techniques. The application of plant tissue culture techniques in orchid conservation and propagation requires an efficient *in vitro* regeneration protocol. This study reports the development of such highly efficient protocols for the *in vitro* asymbiotic seed germination of *Spathoglottis plicata*. The seeds were successfully germinated asymbiotically on Gamborg B5 (1968). Various growth regulators such as 2,4-Dichlorophenoxyacetic acid (2,4-D), Indole acetic acid (IAA) Benzyladenine (BA) and Kinetin individually were used for callus induction and multiple shoot initiation from the protocorms. B5 medium supplemented with 2,4-D (9.03 μ M) was suitable for callus induction. Calli developed a route of production of protocorm-like bodies and eventually develop into plantlets on transfer to growth regulator free half strength basal medium. The well rooted plants were hardened successfully in the potting mixture containing coconut husk, sand, charcoal, and brick pieces in the ratio 1:1:1:1.

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Introduction:-

Orchidaceae is a highly successful family, with representatives capable of occupying almost every ecological situation, apart from marine environments and habitats characterized by extreme cold throughout the year. Orchids exhibit an incredible range of diversity in size, shape and colour of their flowers and as a result, these plants have great ornamental value (Kasulo *et al.*, 2009). In India, orchids form 9% of the flora. It is estimated that about 1300 species belonging to 140 genera are present in the Himalayas with others scattered in Eastern and Western Ghats (Jain, 1980). Around 10% (3000) of the world's total orchid species are believed to be endangered in their native habitats. Orchids are subject to high levels of threat, through both natural and anthropogenic causes. The greatest threat to orchid diversity is habitat loss. Clearance of natural vegetation for ranching, monocrop agriculture, mining, logging, burning and urban development, has decimated many orchid species. Habitat destruction triggers the loss of pollinators, other plants and fungi on which the orchids mostly depend for their survival (Hagsater and Dumont, 1996).

Micropropagation has major advantages over conventional methods of plant propagation. It is an invaluable aid in the multiplication of elite clones of recalcitrant species, and is important in terms of multiplying plants throughout the year, with control over most facets of production. It is possible to generate pathogen free plants, even from explants of infected mother plants, plant materials such as male sterile, fertility maintainer and restorer lines can be cloned; and it enables the production of large number of plants in a short time from a selected number of genotypes,

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where the traditional methods of multiplication are either not available or are ineffective in large scale multiplication systems (Debergh and Zimmerman, 1991).

The genus *Spathoglottis* is a member of the subfamily Epidendroideae. All members of this genus are terrestrial and most grow at low moderate altitudes, although a few taxa occur at high altitudes in grasslands and open forests in moist areas (Dressler, 1993; Beltrame, 2006). Whole plants as well as their different parts, viz., roots, rhizomes, pseudobulbs, stems and leaves are used as medicinal products. These are used for treatment of different diseases such as general debility, stomachache, bone fractures, colds, and wound healing, general weakness and to cure various other diseases (Subedi et al., 2013; Pant and Raskoti, 2013). *S. plicata* is a handsome terrestrial orchid species with nodes borne thickened bulb like stem (pseudobulb). The ground orchid *S. plicata* is one of the most commonly cultivated orchids in the Southeast Asia and could be found in gardens throughout the region. It is suitable as a potted orchid because it is very attractive, fast growing, easy to grow and with rapid fruit maturation. At maturity, plants flower almost throughout the year (Kheawwongjun and Thammasiri, 2008).

Materials and Methods:-

Explants source:-

Asymbiotic seed germination:-

The immature capsules of *S. plicata* (naturally pollinated) collected from Kakkavayal, Wayanad, Kerala were used for asymbiotic seed germination. The plant was authenticated and identified by Dr. K. Kishore Kumar, Head, Department of Botany, Farook College, Calicut, Kerala

Callus induction:-

40 days old protocorms (before the emergence of first leaves) of *S. plicata* were used as explants for callus.

Sterilization:-

The freshly collected capsules were surface sterilized in sodium hypochlorite solution (NaClO, 0.6 % w/v) for 2 minutes, rinsed thrice with sterile distilled water, dipped in 70 % (v/v) ethanol for 60 seconds and flamed. Seeds from the surface sterilized capsules were extracted by splitting the capsule longitudinally with a sharp sterilized surgical blade. The seeds were then spread as thin film in the test tube containing 10 ml of solid culture medium.

Culture media and initiation of culture:-

Five different basal media such as Murashige and Skoog (1962), Linsmaier and Skoog (1965), Gamborg B5 (1968), Schenk and Hildebrandt (1972) and Knudson C (1946) were tested to select a suitable medium for asymbiotic seed germination of *S. plicata*. Gamborg B5 with growth regulators such as 2,4-D (2.26, 4.52 and 9.03 μM), IAA (2.9, 5.7 and 11.4 μM) BA (2.22, 4.44 and 8.88 μM) and Kinetin (2.32, 4.64 and 9.29 μM) individually were used to assess the effect on callus induction.

All the media were supplemented with 3 % (w/v) sucrose and solidified with 0.8 % (w/v) agar (Hi media-India). The pH of the media was adjusted to 5.6–5.8 with 1 N NaOH or HCl. Around 10–15 ml of the medium was dispensed into 250 mm \times 150 mm culture tubes (Borosil). The mouth of the tubes was covered with aluminium foil and was autoclaved at 1.06 kg pressure for about 20 minutes at 121°C. The autoclaved medium in the culture tubes was cooled and allowed to solidify as slants. The inoculations were done after four days to ensure that the media were free from contamination. Five replicates were used for each treatment and maintained at $25 \pm 2^\circ\text{C}$ in culture room under a 12 hrs photoperiod of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance provided by white fluorescent tubes and with relative humidity of 70 %. All the experiments were repeated three times with 5 replicates per treatment.

Rooting:-

For root development, individual shoots were inoculated on half strength liquid basal medium supplemented with auxins such as IAA (2.9, 5.7, and 11.4 μM), IBA (2.46, 4.92, and 9.84 μM) and NAA (2.69, 5.38 and 10.76 μM) individually. The microshoots were supported on filter paper bridges (Whatman's No. 1).

Hardening of plantlets and transferring to community potting mix:-

About 2–4 cm long rooted plantlets (with 2–3 roots) were placed in perforated plastic cups containing charcoal pieces (\approx 2–4 cm), brick pieces (\approx 2–4 cm), coconut husks, and sand in the ratio of 1:1:1:1 and covered with holed transparent poly bag. The plantlets were maintained for 3–4 weeks in normal laboratory condition and irrigated at regular intervals. The potted plants were exposed to normal day light for about 1 hr in a day for the first week and

subsequently the exposure period was increased by 2 hrs and finally after 1 month the plantlets were placed in full day light condition.

Data Analysis:-

Asymbiotic seed germination:-

Bursting of the seed coat and emergence of the enlarged embryo i.e. the protocorm was considered as germination. The germination of seeds was recorded and percentage of seed germination was calculated. The seeds were scooped out and scrutinized randomly and observed under the microscope. The seeds were classified as germinated/non-germinated and the germination frequency was calculated by counting the total number of seeds germinated with that of total number of seeds observed.

$$\% \text{ of germination} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds observed}} \times 100$$

Callus induction:-

The induction period, frequency of callus induction survival rate and conversion frequencies on various hormonal treatments were tabulated.

Results:-

Asymbiotic seed germination:-

The immature pods of *S. plicata* showed differential response (Table 1) when cultured on different basal media. Greening and swelling of seeds was found to be the first significant change after 8 weeks of culture.

Among the five different basal media used, B5 medium was found to be most effective in inducing a germination frequency of 95 % in *S. plicata* (Fig. 1) where as KC and MS induced 90 % and 25 % of seed germination.

Callus induction:-

80 days old protocorms were inoculated on the B5 basal medium supplemented with 2,4-D and IAA alone. Callus was initiated from the protocorms became visible within 30 days. Two morphologically distinct types of callus are observed. The first type was pale yellow in colour and compact in texture. The second type of callus was appeared slightly later and developed more rapidly than the first with translucent in colour and friable.

Callus induction was observed directly from seed derived protocorms of *S. plicata*. B5 medium supplemented with various concentrations of 2,4-D (2.26, 4.52 and 9.03 μM) were found to be efficient in inducing callus from protocorms of *S. plicata* (Table 2). 2,4-D (9.03 μM) supplemented B5 basal medium promoted 70 % callus induction within 45 days (Fig. 2 A&B). The well developed calli were transferred to hormone free basal medium for further differentiation. After 50 days with an intermediate subculture, the calli transformed into PLBs. These PLBs eventually develop into plantlets. The protocorm derived calli induced in the lower concentration of 2,4-D (2.26) shows 60 % frequency of plantlet conversion with the maximum of 4.6 ± 0.50 plantlets.

Rooting and Hardening:-

IAA significantly promoted rooting in *S. plicata* within 15 days. Number of days required for initiation was less, frequency of root induction was maximum, roots were longer and number of roots was more when compared to other hormonal treatments (Table 3; Fig 3 A&B). Higher root numbers were recorded in 5.7 μM of IAA (4.4 ± 0.92) with the maximum root length of 1.49 ± 0.34 . The well rooted shoots were washed thoroughly under sterile distilled water and transplanted into pots with a potting mixture of charcoal pieces, brick pieces and chopped mosses (at 1:1:1 ratio). The cultures were maintained for 3–4 weeks in normal laboratory condition before transferring to perforated plastic pots with potting mixture containing charcoal, brick pieces, coconut husks, and sand (at 1:1:1:1 ratio) for hardening (Fig 3 C,D&E).

Table 1:- Effect of various basal media on asymbiotic seed germination of *S. plicata*.

S.no	Basal Medium	No. of days taken for seed germination	Frequency of germination [%]
1	MS	30	25
2	LS	-	-
3	B5	30	95
4	SH	-	-
5	KC	30	90

MS - Murashige and Skoog (1962) medium, LS - Linsmaier and Skoog (1965), B5 - Gamborg B5 medium (1968), SH - Schenk and Hildebrandt medium (1972) and KC - Knudson C medium (1946).

Table 2: Effect of plant growth regulators on callus induction from 40 days old protocorms of *S. plicata*

Hormone (μM)		Number of days taken for callus induction ^a	Frequency of callus induction (%) ^a	Frequency of plantlet conversion (%) ^a	Average number of plantlets ($\pm\text{SE}$) ^{ab}
2,4-D	IAA				
2.26	-	30	64	60	4.6 \pm 0.50
4.52		30	44	45	3.4 \pm 0.38
9.03		30	70	30	3.6 \pm 0.38
	2.9	30	-	-	7.0 \pm 1.2
	5.7	30	-	-	7.2 \pm 1.2
	11.4	30	-	-	7.2 \pm 1.1

^a Data represents the mean of five replicate

^b \pm SE: Standard error

Table 3: Effect of auxins on rooting of *S. plicata* in B5 liquid medium

Growth regulators (μM)			Time taken for root initiation (days)	Frequency of root formation (%)	Average number of roots \pm SE ^a	Average root length (cm) \pm SE ^a
IAA	IBA	NAA				
2.9			15	50	3.2 \pm 0.73	0.82 \pm 0.22
5.7			15	55	4.4 \pm 0.92	1.49 \pm 0.34
11.4			15	40	3.0 \pm 0.31	1.06 \pm 0.10
	2.46		15	30	2.6 \pm 0.50	1.14 \pm 0.08
	4.92		15	27	2.0 \pm 0.31	0.69 \pm 0.12
	9.84		15	29	2.5 \pm 0.41	0.96 \pm 0.19
		2.69	15	25	2.0 \pm 0.44	0.68 \pm 0.14
		5.38	15	32	3.0 \pm 0.54	1.09 \pm 0.29
		10.76	15	40	3.4 \pm 0.4	1.14 \pm 0.17

Only the significant treatments are computed here

Data represents the mean of five replicates

^a \pm SE: Standard error

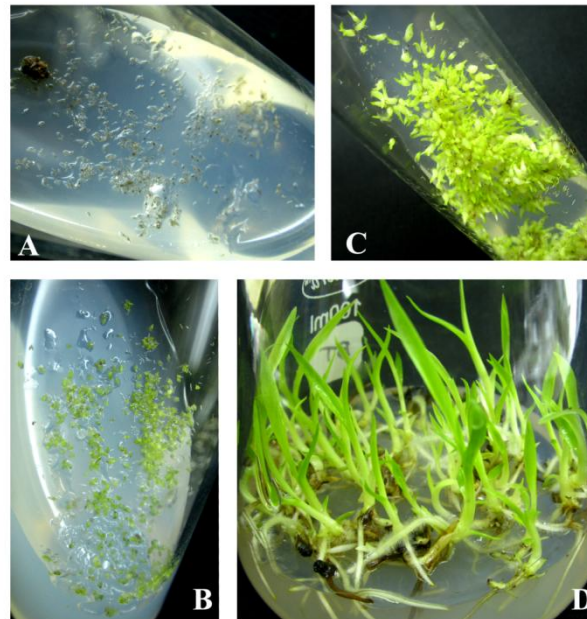
Figure 1. Asymbiotic seed germination of *S. plicata*

Figure 1:- Asymbiotic seed germination of *S. plicata*, A-Seeds of *S. plicata* in B5 medium, B- Protocorm formation, C- First leaf emergence from protocorms, D- Plantlet development.

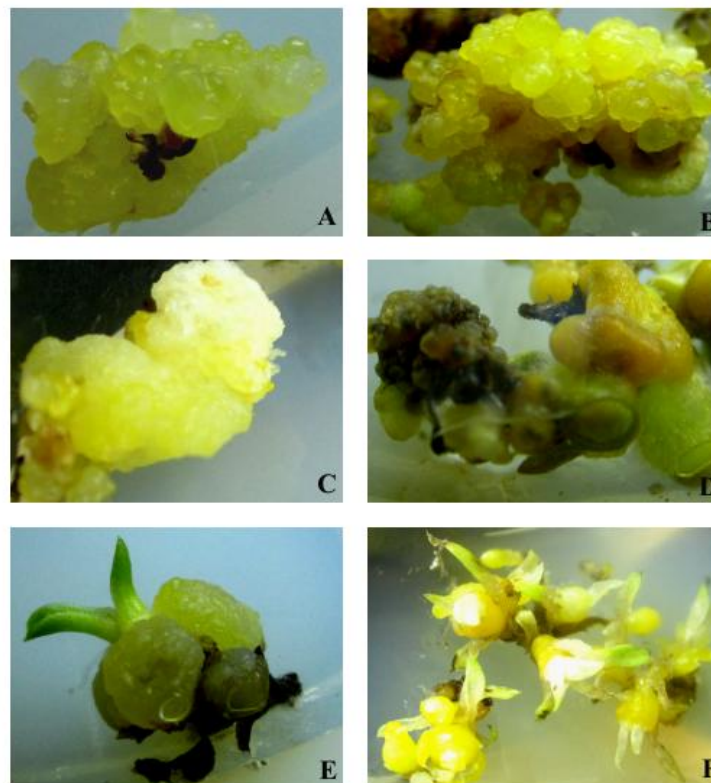
Figure 2. Callus induction of *S. plicata*

Figure 2: Callus induction of *S. plicata*, A&B - B5 medium+ 2,4-D (9.03 µM), C&D- B5 medium + 2,4-D (2.26 µM), E&F- B5 medium+ 2,4-D (2.26 µM)

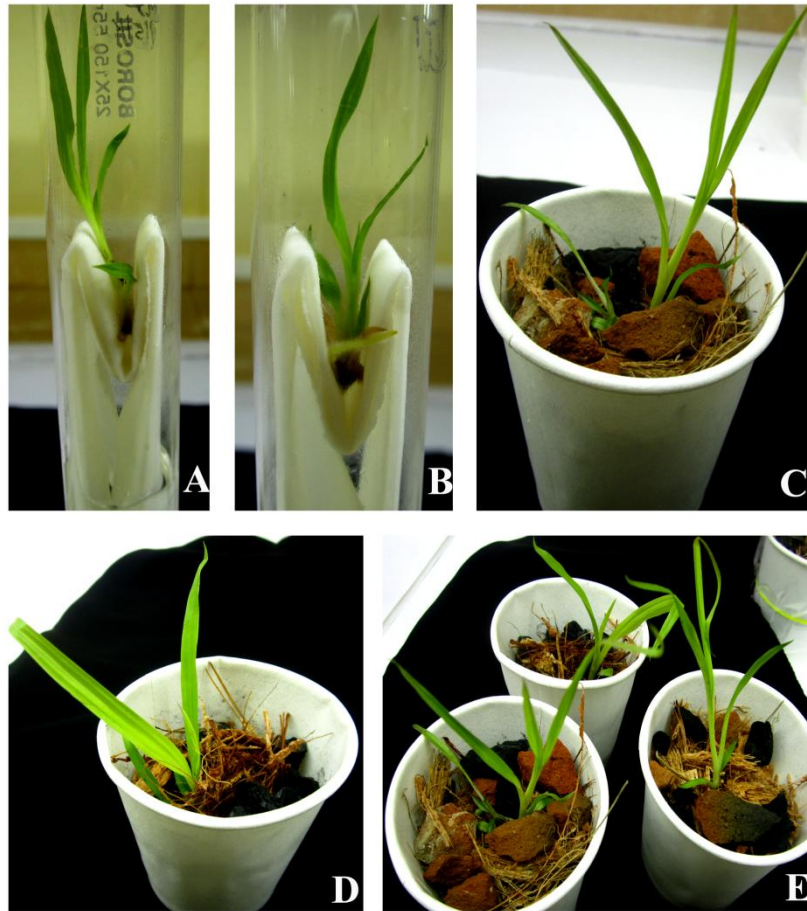
Figure 3. Rooting and Hardening of *S. plicata*

Figure 3:- Rooting and Hardening of *S. plicata*, A&B - Rooting [B5 liquid medium + IAA (5.7 µM)], C, D&E – Hardening.

Discussion:-

Many terrestrial and epiphytic orchids have been successfully propagated using *in vitro* asymbiotic seed germination techniques (Malmgren, 1992; Arditti and Ernst, 1990; Chou and Chang, 2004). Species specific media for germination of seeds have been reported in many orchids (Arditti and Ernst, 1984; Johnson *et al.*, 2007). This study reports the standardization of suitable seed germination medium for *S. plicata*. In the present study, B5 medium was found to be the best suitable medium for the germination of *S. plicata*. Similar results were observed with the early findings in *Calopogon tuberosus* (Kauth *et al.*, 2006) and *Habenaria macroceratitis* (Stewart and Kane, 2006). The nutrient requirement for orchid seed germination differs from species to species as well as there is no universal medium for all the orchid species (Stewart and Kane, 2006). The germinated seeds continued to develop into complete plants on the same respective seed germination medium (Fig. 1 C and 1 D).

Malmgren (1992) found that the asymbiotic seed germination of terrestrial orchids were higher on media containing amino acids because this form of nitrogen can more readily be assimilated by the germinating seeds than inorganic nitrogen due to its simplified form. However, glycine, an amino acid added to MS medium was reported to inhibit germination compared to ammonium nitrate (Spoerl and Curtis, 1948). Hence, in the present study, it is inferred that the low germination frequencies observed for *S. plicata* (25 %) on MS medium may be due to the inhibitory effects of glycine (2 mg/L) present in the medium. Effects of various amino acids on germination frequencies differ among orchid species (Kauth *et al.*, 2006). The nutritional requirement for seed germination and seedling development varies with genus, species and locality. A number of standard media such as KC, Vacin and Went, Raghavan and

Torrey, MS, Hyponix medium etc have been formulated for orchid culture. Several species of orchid show specific requirement.

Generally callus induction in orchids is rather difficult due to its slow growth and necrotic tendency (Naing *et al.*, 2011A). Recently, combinations of 2,4-D and TDZ have been reported for the callus induction of ornamental plants including some orchid genera, *Cypripedium formosanum* (Lee and Lee, 2003), *Vanda coerulea* (Lang and Hang, 2006). However, BA alone or in combination with 2,4-D totally inhibited callus induction in *Paphiopedilum* hybrid (Lin *et al.*, 2000). Similarly Ishii *et al.* (1998) mentioned that the combination of 2,4-D and BA could not effectively induce callus from leaf segment in *Phalaenopsis*. This shows that 2,4-D at certain concentration induced the callus formation in orchids. Similarly the ratios between concentrations of 2,4-D, BA and Kinetin were significantly associated with the percentage survival of protocorm and callus formation. Of the combinations tested, the lower concentration of 2,4-D (2.26 μ M) was found as optimal concentrations for the best callus induction in both the orchids. Finally it revealed that addition of exogenous hormones to the medium is quite important for callus induction.

In the present study higher concentration of 2,4-D (9.03 μ M) successfully induced callus from the protocorms within a short period of time. When PLB were transferred into different kinds of basal media, formation of shoots appeared on all media but conversion frequencies of PLBs to shoot and average number of shoots were ultimately low (Naing *et al.*, 2011B). In this study, transfer of callus obtained from lower concentration of 2,4-D (2.26) to hormone-free medium stimulated more PLB development and eventually allowed to produce plantlets.

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