

## Inbreeding and *in vitro* seed germination in *Spathoglottis albida* Kraenzl.

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**Abstract:** Development of inbreds, for selecting as parents for hybridisation, *in vitro* seed germination and its establishment in *Spathoglottis albida* Kraenzl. was achieved. The pods obtained through selfing were harvested after 30 days and cultured in different media supplemented with individual and combinations of 6-benzylamino purine (BAP) and 1-naphthalene acetic acid (NAA) Seed germination was higher in MS medium (93%) and lower in Mitra medium (87%). The seedlings were grown well in the MS medium supplemented with 1.5 ppm BAP and 0.5 ppm NAA. The plantlets obtained in this medium were healthy and vigorous with 16.8 cm height at the time of deflasking for hardening. The matured rooted plantlets were acclimatized by using potting mixture at 1:1:1 proportion for hardening. The survived plantlets were transferred to field establishment and its growth was successful.

**Keywords:** Orchid, Inbreeding, *Spathoglottis albida*, Seed culture, Pollen, Protocorm

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### I. Introduction

*Spathoglottis albida* Kraenzl. is one of the important wild terrestrial species belonging to the family Orchidaceae and it is widely distributed in Northern India, Southern Japan and China [1]. The plant is a native of Thailand. In Kerala it is gaining popularity as an ornamental plant because of its large attractive white beautiful flowers, nature of continuous flowering throughout the year and also due to the long duration of inflorescence with large number of flowers which are opening continuously from base to top. Moreover, it is a good perennial herb for landscaping also. *Spathoglottis* species can be differentiated by their morphological characteristics based on their flower colour and shape of the lip. It produces flowers with varying colours ranging from white to mauve and in shades ranging from yellow to golden yellow. Based on the mode of pollination, orchids are grouped under highly cross pollinated plant species. So naturally this will lead to considerable genetic variability in the seedling progenies. Hence, it is very difficult to get true to type plants through natural seed propagation. Moreover, it is very difficult to get plants through seed germination in the natural condition.

*Spathoglottis* species are reported to be freely inter-fertile. These are cross pollinated orchids but to induce new variation through cross breeding is difficult [2]. Most hybrids, either it is self or cross pollinated with parent or other species, are nearly sterile [3, 4]. Selfing is done to confirm whether the desired traits are present in double dose or not. So the population may have desirable and undesirable types. If they are well separated geographically the two populations may respectively be homozygous but if there has been interbreeding between populations they may be heterozygous. The only way to tell whether an individual plant will breed true for desirable type is to self the plant and if the progeny are all similar the plant is homozygous or uniform. Selfing is a way of proving that the plant is a suitable parent for whatever desirable trait the hybridizer is pursuing.

It is widely cultivated and have been successfully used for hybridization [5, 6,7] and therefore preservation of its germplasm is worthy for breeding. An attempt was made to utilize it as one of the parents in a distant hybridization programme. In this context, it is essential to develop inbred line to avoid segregation due to cross pollination. *S. albida* is conventionally propagated through separation of pseudobulbs but the proliferation rate is very low [8]. *In vitro* seed culture technique is an efficient approach for its regeneration [9,10]. Seed culture is not preferred particularly in cross pollinated species because true to type plants cannot be obtained. *In vitro* seed germination is the best option for the rapid development of inbreds. But reports on the *in vitro* culture of *S. albida* are limited [11,12, 13]. Most of the orchids flowered regularly but fail to set pods due to the lack of particular pollinating insects. Hence hand pollination technique should be employed for fruit setting. With this objective, the present investigation was carried out at JNTBGRI, Puthenthope, Thiruvananthapuram during the period 2015-2016.

## II. Materials And Methods

*Spathoglottis albida* Kraenzl, white flowered wild type orchid species (Fig. 1a) available at JNTBGRI, Puthenthope, Thiruvananthapuram was selected for the development of inbred lines. The healthy, vigorous, pest and disease free plants were selected as parent plants for inbred development. Plants with inflorescence axis containing unopened flower buds were selected. Mature flower buds which would be opened on the next day were selected in the evening and labelled. On the next day carefully anthers (pollinia) were removed from the opened and labelled flower with needle without rupturing anther wall. Pollinia taken were placed over the stigmatic surface at the same time. After pollination it was labelled. Mature and undehisced green capsules or pods were harvested after 30 days and used for *in vitro* seed culture.

Harvested mature and green capsules were subjected to surface sterilization using running tap water for one minute followed by rinsing it in detergent solution (Savlon antiseptic liquid, Johnson & Johnson, India) for ten minutes. Again the capsules were washed with running tap water for one minute. Capsules were brought to the laminar air flow chamber and immersed the capsule in 70% ethyl alcohol and flamed (5-10 seconds) without damaging the capsule coat. Then the pods were cut longitudinally with sterilized knife and split opened with sterilized forceps. Seeds were scooped out and put it into the culture media. Different types of basal media were prepared for germination such as Murashige and Skoog (MS) medium [14], Mithra medium [15] and half strength of MS medium [14]. The pH of the medium was adjusted to 5.6 to 5.8. Inoculated explants were kept in the culture room for germination which is maintained at  $25 \pm 2$  °C and under 12 hrs. photoperiod ( $50-60 \text{ mol m}^{-2} \text{ s}^{-1}$ ). Protocorm developed were subcultured for regeneration of shoots and roots into different media (Table 1). Observations were recorded frequently.

*In vitro* grown seedlings were deflasked and washed well in tap water to remove media adhered to the seedlings and planted out in pots filled with potting mixture and kept it in the growth chamber for hardening for one month. Then seedlings were transferred into large pots filled with potting mixture for the field establishment. All experiments were done following CRD and significance determined following ANOVA [16].

## III. Results

Pollen viability and pollen grain germination evaluation were carried out in order to ascertain capsule development (Fig. 1b). At shedding, the pollen grains are two-celled and the tetrad of pollen grains was enclosed in thick walls. The mature pollen grains are irregular or polygonal in shape and appear in tightly packed tetrads. The pollen viability and pollen germination test showed 100% viability and germination (Fig. 1 c, d, e).

Ovary started swelling four days after pollination and it was the indication of capsule (pod) setting and development. Capsules attained maturity one month after pollination. The microscopic view of seed morphology is depicted in (Fig. 1f). Orchid seeds were very small in size and have no endosperm [17]. The micromorphology of the seeds revealed that they were minute, dust-like and fusiform in shape. They were brown and have an aperture in the posterior. The testa cells were longitudinally oriented, tetragonal or polygonal in outline with no intercellular spaces between them. Inner to seed coat there was a golden brown embryo occupied at the central area, conforming to the widest zone of the seed forming an ovoid shape.

Seed germination initiated after 18 to 21 days inoculation and started to produce green coloured protocorm within one month (Fig. 2a, b). Three types of basal media were used for seed germination. Of the different media tried for germination, both MS and Mitra medium were showed better response (Fig. 3). The highest seed germination percentage was noticed in MS medium and lowest germination was observed in MS (half) media. Protocorms were transferred for shoot and root regeneration in different media under *in vitro* condition. Highest percentage of shoot and root regeneration were observed in the MS media supplemented with BAP and NAA. The results are depicted in table 1 and figure 2. Analysis of variance (Table 2) revealed statistically significant differences for the different traits of the seedlings produced through *in vitro* culture.

Of the different media tried for protocorm development and shoot as well as root regeneration under *in vitro* condition, MS medium supplemented with BAP and NAA exhibited statistically significant and superior influence for the traits such as seedling height (Fig. 4), number of roots, length of root, length of leaf and width of leaf. But number of seedlings was maximum in MS media supplemented with 2.5 ppm BAP whereas for number of leaves it was maximum in the Mitra media supplemented with 1.5 ppm BAP and 0.5 ppm NAA.

After 3 months, rooted seedlings were taken out from culture bottles and planted in community pots containing potting mixture soil, sand and cow dung in 1:1:1 ratio and kept in polyhouse chamber for hardening. Later after four weeks, seedlings survived (80%) were planted out into large pots containing potting mixture for further establishment and development of inbreds or true to type plants (Fig 2e) as parents for multiplication and hybridization.

The plants were irrigated daily once in a day. In three months the plantlets were well established in the field. In the present study plants were directly regenerated through micro-shoot formation from the *in vitro* seedlings without the intervention of callus. The growth regulators BAP and NAA (1.5 ppm + 0.5 ppm) were used in small doses for protocorm induction. 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 variation.

#### IV. Discussion

Selfing was done to determine species status. If the offspring produced is relatively uniform or not depending on the degree of variation exhibited, a hybridizer can conclude that the plant is not a pure species but rather a natural hybrid or a species where introgression has occurred if the progeny of a selfing shows a high degree of variation. Pollen viability has been investigated in terms of its contribution to incompatibility and fertility studies or crop improvement and breeding projects [18]. Additionally, it has been documented that internal, morphological and environmental factors all play a role in determining the duration of pollen viability [19]. Pollen grains are surrounded by pollen kit or elastoviscin [20,21] and commonly packaged into dispersal units [22] and hence viability will be more. Present findings revealed 100 % of pollen viability as well as pollen germination in *Spathoglottis albida* Kraenzl.

All the media contain mineral salts that vary not only in their concentration but also in their availability of nutrients [23]. Nitrogen sources vary in all tested media. MS medium contains 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 [24,25]. 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 [26,27]. Hence, the results obtained in the present study are in confirmation with the report of [26] and [27].

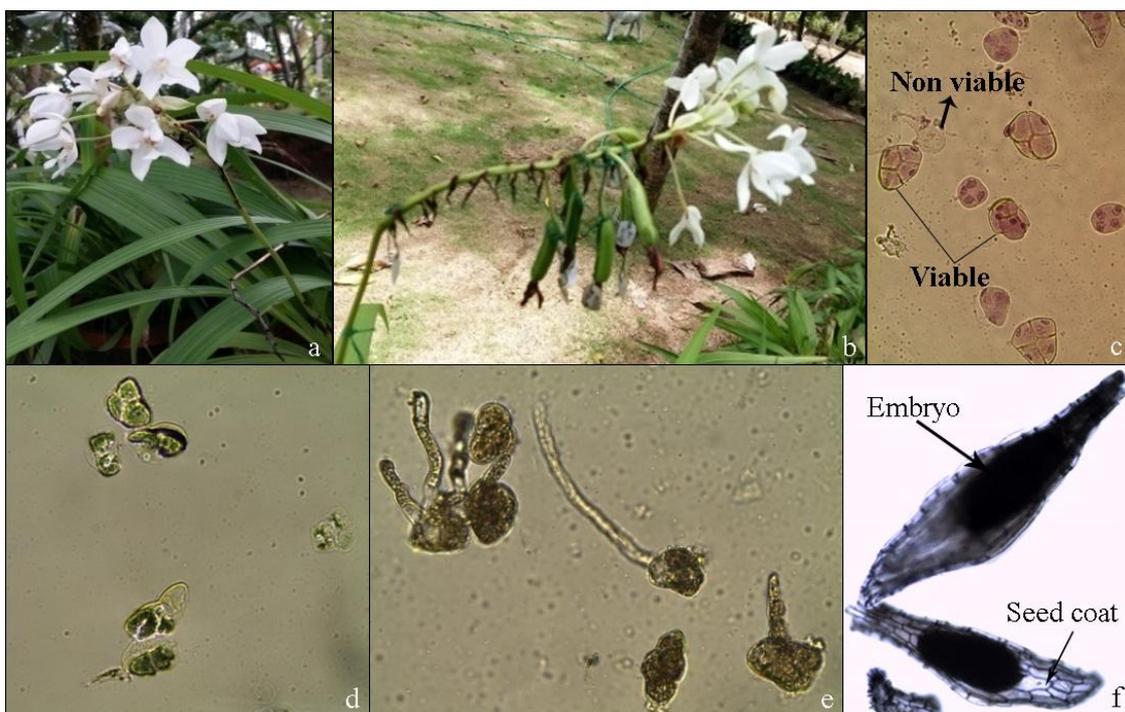
Since the seeds of orchid are without endosperm it needs specific nutritional and environmental conditions [28]. Present study revealed that both auxin and cytokinin together enhanced the proliferation of shoots as well as roots from protocorm in *Spathoglottis albida*. The type and concentration of plant growth regulators play an important role during micropropagation of many orchids [29]. The growth regulators BAP and NAA (1.5 ppm + 0.5 ppm) were used in small doses for protocorm induction in MS medium. Similar results were reported earlier by the researchers in different orchids. The positive effect of BAP for maximum protocorm multiplication and BAP acts more efficiently when used in combination with NAA. This finding is in agreement with some other findings obtained in micropropagation of orchids [30, 31].

Regenerated shoots of *Spathoglottis albida* on MS medium with 0.5 mg/l IAA, on which 3.2 roots were induced from each shoot [13]. According to the present study on an average maximum of 7.5 roots were induced per seedlings on MS media supplemented with BAP and NAA. But without the application of NAA resulted numerous small seedlings with reduced root growth at the same time profuse callus growth. As the concentration of cytokinin increased, shoot growth and number of seedlings increased proportionately and addition of both cytokinin and NAA promoted shoot growth and root growth in a balanced proportion.

Protocorms are being applied by many researchers as explants for micropropagation of many rare and endangered orchid species [32, 34, 35, 36, 37, 33]. Orchids need auxins and cytokinins for plantlets development [33]. BAP and NAA are most applicable plant growth regulators for micropropagation of most orchids [38]. BAP in combination with NAA had been suggested by some studies to obtain the maximum number of protocorm like bodies [39]. Present finding is corroborated with the earlier reports. NAA was found more effective for root induction.

In *in vitro* culture, the excessive use of growth regulators and especially during the intermediary callus phase is undesirable as those induce variation [40].

V. Figures and Tables



**Figure 1.** a. *Spathoglottis albida* Kraenzl. - flowering plant, b. Pod development after selfing, c. Pollen viability, d. Pollen germination showing tetrad e. Pollen tube elongation, f. Seeds



**Figure 2.** a. *Spathoglottis albida* Kraenzl. – seed cultured in liquid medium, b. Protocorms, c. Shoot initiation, d. Multiple shoots with roots e. *In vitro* seed germinated plants established in pots

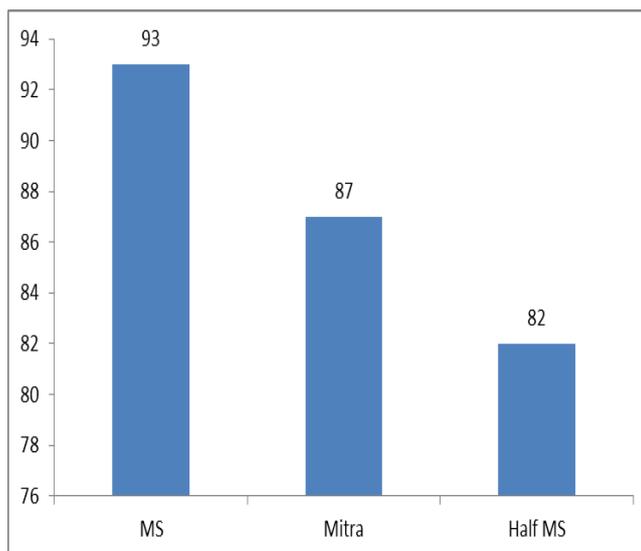


Figure 3. *Spathoglottis albida* percentage seed germination in different media

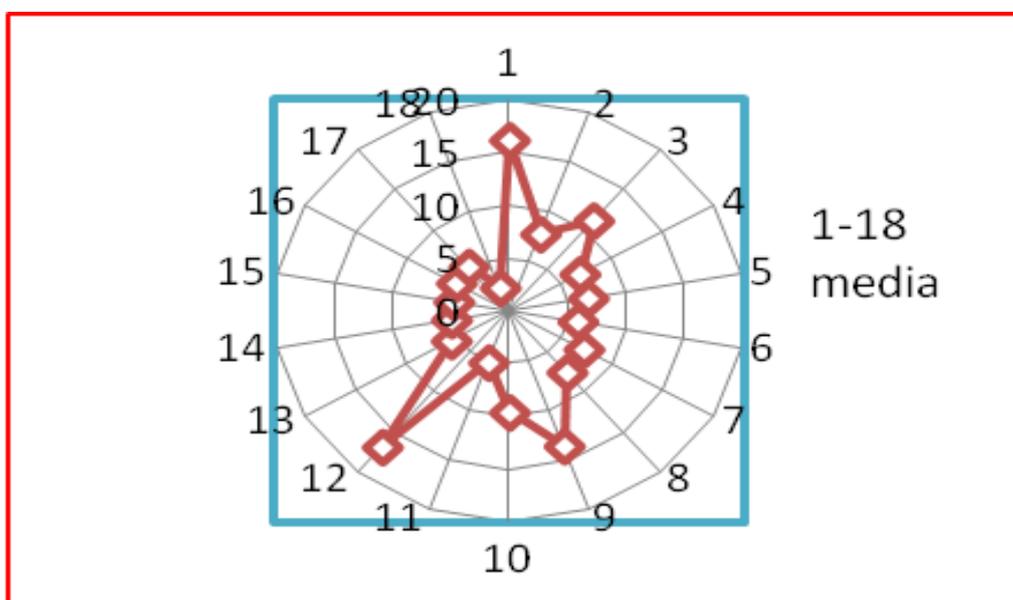


Figure 4. Seedling height in 1-18 media composition (see table 1)

Table 1. Observations of seedlings during deflasking for hardening

Media	BAP (ppm)	NAA (ppm)	No. of Seedlings	Seedling height (cm)	No.of roots	Length of root	No.of leaves	Leaf length (cm)	Leaf width (cm)
MS	-	-	45	16.10	4.50	7.9	7.5	15.72	0.24
MS	2.5	-	250**	7.70	2.00	3.2	4.0	7.12	0.23
MS	5.0	-	175	11.10	5.50	5.1	4.5	9.90	0.58
MS	7.5	-	44.5	6.90	3.50	3.9	3.5	6.47	0.27
MS	10.0	-	100	6.80	3.00	3.1	4.0	6.45	0.24
MS	12.5	-	100	5.90	3.00	3.1	4.0	6.40	0.23
MS	7.5	2.5	62.5	7.30	2.50	3.6	3.5	6.12	0.24
MS	0.5	-	66	7.60	1.00	2.0	7.0	7.23	0.23
MS	1.0	-	30	13.60	1.50	3.1	10.5	11.84	0.64
MS	1.5	-	90	9.60	7.00	3.3	6.5	9.05	0.25
MS	2.0	-	135	5.20	2.50	2.9	5.5	5.00	0.24
MS	1.5	0.5	75	16.80**	7.50**	8.1**	7.5	15.8**	0.67**
Mitra	-	-	60	5.70	3.00	2.2	4.5	5.10	0.23
Mitra	0.5	-	50	5.00	3.00	2.6	4.5	4.83	0.23
Mitra	1.0	-	211	4.80	2.50	2.6	3.5	4.30	0.20
Mitra	1.5	-	70	5.20	2.50	2.6	3.5	4.60	0.20
Mitra	2.0	-	6	5.40	2.50	2.5	5.0	5.20	0.23

Mitra	1.5	0.5	29	2.30	3.00	3.2	15.0**	5.70	0.24
CD			94.92	4.49	1.917	0.835	4.8	3.787	0.1757
**			Significant at 1% level						

**Table 2. ANOVA for seedling traits in *in vitro* method**

Source of Variation	Degrees of freedom	No. of seedlings	Seedling height (cm)	No of roots	Length of root (cm)	No of leaves	Leaf length (cm)	Leaf width (cm)
MSE	18	2041.167	4.57	0.83	0.15	5.22	3.25	0.0073
CD (0.01)		94.92	4.49	1.917	0.835	4.8	3.787	0.1757

## VI. Conclusion

Selfing is essential for the development of inbreds for hybrid development. Since normal seed germination delays in orchids *in vitro* seed culture is an easiest method for seedling development. The present study revealed that the capsule developed within one month after pollination in *Spathoglottis albida* Kraenzl. and the seeds were germinated at 100% viability in MS hormone free medium. Subsequently, protocorm development and shoot and root regeneration were maximum in MS media supplemented with BAP and NAA (1.5 ppm +0.5 ppm). The rooted plants were successfully acclimatised and transferred to field. The protocol developed in the present study is reproducible and would be utilized in high frequency regeneration of selfed progenies for commercial as well as breeding aspects in *S. albida*.

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