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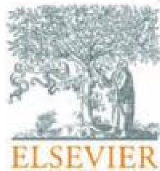

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In vitro asymbiotic seed germination, mycorrhization and seedling development of *Acampae praemorsa* (Roxb.) Blatt. & Mc Cann, a common south Indian orchid

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ABSTRACT

Objective: To develope a conservation strategies for orchid species. **Methods:** Seeds of *Acampae praemorsa* (Roxb.) Blatt. Mc Cann (*A. praemorsa*) were obtained from mature pods collected from Velliangiri hills and germinated on Murashige and Skoog (MS) medium supplemented with various concentration of Benzylaminopurine (BAP). **Results:** Maximum seed germination (85%) was observed on MS media supplemented with 2 mg/LBAP. Seed germination percentage increased with increasing concentrations of BAP (0.5 mg/L to 2 mg/L), but 3 mg/L of BAP inhibited seed germination. Variations observed were significantly (P<0.01) different for concentrations of growth regulators, days and their interactions. The embryos in the seeds swelled prior to germination. Fifty days after inoculation, well developed greenish porotocorm like bodies (PLBs) were formed and by 80th day, 85% (maximum) of the PLBs were observed in MS supplemented with 2 mg/L BAP. Eighty days after seed sowing, the germination rate decreased and the PLBs were transformed into first and second leaf stages in media supplemented with Naphthaleneacetic acid (NAA). Maximum seedling numbers (84%) were observed in MS medium supplemented with 1 mg/L NAA at 110th day. After 110 days, the seedlings were transferred to 0.6 g/L MS medium containing activated charcoal and similar concentration of growth regulators for root induction. After rooting, the seedlings were transferred to *ex vitro* conditions. **Conclusions:** In present study all the mycorrhizal seedlings survived, because orchid mycorrhizal fungi enhance growth of orchid plantlets and present study gives an effective protocol for seed germination and plantlet regeneration from immature seeds which can be used for establishing *A. praemorsa* populations in Velliangiri Hills and elsewhere.

1. Introduction

Orchid seeds are minute with limited food reserves and are produced in large numbers [1]. In nature, their germination depends upon association with suitable mycorrhizal fungi, which provides a stimulus for initiating seed germination [2]. Non-symbiotic seed germination of orchids are greatly influenced by several factors like seed age, different nutrient media with adjuvant and plant growth regulators (PGRs) [3-6]. *In vitro* seed germination is one of the most efficient methods for orchid propagation for conservation purpose [7-9]. It enables large number of seedlings to be produced within a short span of time. For commercial requirement and the conservation of orchid species, it is desirable to establish an *in vitro* protocol for rapid and large scale propagation [10]. Plant development in orchids has three

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successive stages, seed germination, formation of protocorm-like bodies (PLB's) and seedling development [11]. Swelling of the embryo is the first visible sign of seed germination which later turns green to form protocorms [11]. After successive cell divisions various structures like rhizoids, shoot primordia and leaves are formed.

The Velliangiri hills, popularly known as "Thenkailaya Malai" , are the holy hills of Southern India, and form a major range in Western Ghats and has rich biodiversity. The cave "Panchalingas" and Velliangiri Andavar temple are popular pilgrims within the Velliangiri hills (11°10′ N & 76°10 E′), a Nilgiri Biosphere Reserve of south Western Ghats. The altitudes ranging from 520 to 1 800 m. a. s. l leads to a wide variability in rainfall (mean annual rainfall 500 to 7 000 mm) and temperature (0 ℃ in winter to 41 ℃ in summer). The wide variability in climatic conditions has resulted in different vegetation types such as grassland, sholas and tropical rainforests. This mountain range serves as a habitat for wide variety of flora and fauna [12]. The 48 sq. km mountain range consists of around 1 715 species of angiosperms including 439 endemics [13, 14]. Of these, more than 61 plant species from these hill ranges including many endemics, rare and threatened orchids are commercially exploited in an unsustainable manner for their therapeutic value [15]. In addition, human interference during festival seasons is a major threat to flora and fauna of Velliangiri hills. Therefore, to conserve these endemic, endangered, rare and threatened plant species, *ex situ* or *in situ* conservation strategies needs to be developed urgently [12].

Acampae praemorsa (Roxb.) Blatt. & Mc Cann (*A. praemorsa*) is a common epiphytic orchid (sometimes exists as lithophytes) distributed in plains of southern districts of Tamil Nadu and Kerala. The flowers are long lasting because of its waxy texture. Population of *A. praemorsa* distributed in Velliangiri hills faces threat due to overexploitation of the forest biodiversity resources and anthropogenic activities as mentioned earlier. So the objective of the study was to standardize an effective protocol for *in vitro* seed germination and seedling development of *A. praemorsa*.

2. Materials and methods

2.1. Seed source and capsule preparation

Naturally pollinated, immature pods of *A. praemorsa* were collected from Velliangiri hills, Coimbatore. The pods were washed in rinsing tap water and surface sterilized using 1% sodium hypochloride solution for one minute, followed by rinsing in sterile distilled water. Final disinfection was done by submerging the capsules in 70% ethanol for one minute, and washed thoroughly in sterile distilled water for five times. The sterilized capsules were then flamed, split longitudinally with a sterile scalpel and the seeds were used for inoculation.

2.2. Seed germination medium and inoculation

Murashige and Skoog [16] (MS) media supplemented with Benzylaminopurine (BAP), Naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) individually in various concentrations were used for assessing seed germination. The pH of the medium was adjusted to 5.8 prior to solidification with 0.8% agar. Ten milliliters of the media were poured in 50 mL test tubes, plugged with cotton and autoclaved at 120 ℃ for 20 minutes at 15 psi. Approximately 100 seeds were inoculated onto the MS medium prepared with various concentrations of growth regulators. The culture tubes were maintained in 18 h light, 6 h dark, at 18–20 ℃ and light intensity of 1 200 lux.

2.3. Percent germination and seedling development

Thirty days after inoculation, some of the seeds were scoped out, dispersed in a drop of water on a glass slide and observed under light microscope. Germination percentage was calculated employing the following formula.

After the initiation of germination, observations were recorded at 50th, 80th and 110th day to trace the germination, protocorms and seedlings in first and second leaf stages. These were observed using a stereo zoom microscope (Carl Zewiss, West Germany).

2.4. Rooting, mycorrhizal inoculation and transfer to ex vitro establishment

Full strength MS medium with similar concentrations of growth regulators, plus 0.6% activated charcoal (AC) were used for rooting. After rooting, the tubes were inoculated with 1 cm^3 block of fungal mycelium (*Epulorhiza* sp. isolated from *Eulophia epidendreae*; Accession No. JF499918). Thirty days after fungal inoculation, the roots were sectioned with a razor blade, stained with trypan blue (0.05% trypan blue in lactic acid: glycerol; 1:1 ratio) and examined for the presence of pelotons. The colonized plantlets were transferred to suitable medium for *ex vitro* establishment.

2.5. Ex vitro–establishment of seedlings

All the mycorrhizal seedlings were transferred to plastic pots containing coconut pith, charcoal, sand and tile bits (3:1:1:1 w:w:w:w) for hardening and after hardening all the plantlets were transferred to *ex vitro* conditions.

2.6. Statistical analysis

Analysis of Variance (ANOVA) was performed on all data

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to compare the concentration effect of growth regulators and influence. Means were separated using Duncan's Multiple Range Test (DMRT).

3. Results

Seeds germinated on all the concentrations of growth regulators (BAP, NAA and 2,4-D) tested and the germination was maximum (85%) in MS medium supplemented with BAP. Seed germination percentage increased with increasing concentrations of BAP (0.5 mg/L to 2 mg/L), but at 3 mg/L there was a decline in the percentage of seeds germinated. The percentage of seed germination significantly varied with the concentration of growth regulators used, days taken for seed germination and their interaction (P<0.01) (Table 1). Initially, the embryos in the seeds swelled in response to hydration prior to germination. At the initial stage of germination, white colored PLBs were formed. After 50 days, the well developed PLBs turned green in MS medium supplemented with BAP, and at 80th day, maximum number of the PLBs (85%) were observed in MS medium supplemented with 2 mg/L BAP. Some of the PLBs enlarged and formed rhizoids in their basal portions.

After 80 days, the PLBs with rhizoids were transferred onto fresh media containing the same concentrations of growth regulators and the MS medium supplemented with NAA produced first leaf primordia. Eighty days after seed sowing the germination rate decreased and PLBs in 0.5 and 1mgL^{-1} NAA developed their first and second leaves. Maximum seedling numbers (84%) were observed in MS medium supplemented with 1 mg/L NAA at 110th day. After 110th day, the seedlings transferred to MS medium containing 0.6 g/L activated charcoal produced roots.

Seedlings inoculated with mycorrhizal fungi had their roots colonized and all the mycorrhizal seedlings survived in pot and produced new leaves. The seedlings in pots were transferred to *ex vitro* conditions.

4. Discussion

Conservation and propagation of orchids via in vitro germination and plant development is a more appropriate method [17]. Arditii [18] and Rasmussen [19] suggested that

Table 1

Seed germination and the various developmental stages of *A. praemorsa* in different hormonal concentrations.

Days	Hormone concentration (mg/L)	Germination (%)	Protocorm (%)	Two leaf stage (%)
50th day	MS+0.5BAP	24.60 ± 1.08$^{d\Delta}$	4.40 ± 0.51^{b}	0.00 ± 0.00^{a}
	MS+1BAP	44.80 ± 2.69^{b}	6.00 ± 0.71^{a}	0.00 ± 0.00^{a}
	MS+2BAP	85.00 ± 0.71^{a}	6.20 ± 0.66^{a}	0.00 ± 0.00^{a}
	MS+3BAP	42.00 ± 1.52^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}
	MS+0.5NAA	0.00 ± 0.00^{g}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}
	MS+1NAA	0.00 ± 0.00^{g}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}
	MS+0.52,4-D	2.40 ± 0.51^{f}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}
	MS+1 2,4-D	6.00 ± 1.58^{e}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}
80th day	MS+0.5BAP	0.00 ± 0.00^{c}	26.60 ± 1.03^{d}	0.00 ± 0.00^{a}
	MS+1BAP	0.00 ± 0.00^{c}	51.00 ± 1.05^{b}	0.00 ± 0.00^{a}
	MS+2BAP	0.00 ± 0.00^{c}	84.60 ± 0.93^{a}	0.00 ± 0.00^{a}
	MS+3BAP	0.00 ± 0.00^{c}	37.40 ± 0.51^{c}	0.00 ± 0.00^{a}
	MS+0.5NAA	0.00 ± 0.00^{c}	2.40 ± 0.51^{e}	0.00 ± 0.00^{a}
	MS+1NAA	17.00 ± 0.71^{a}	0.00 ± 0.00^{f}	0.00 ± 0.00^{a}
	MS+0.5 2,4-D	3.00 ± 0.71^{b}	0.00 ± 0.00^{f}	0.00 ± 0.00^{a}
	MS+1 2,4-D	3.40 ± 0.51^{b}	0.00 ± 0.00^{f}	0.00 ± 0.00a
110th day	MS+0.5BAP	0.00 ± 0.00^{a}	4.40 ± 0.51^{a}	0.00 ± 0.00^{c}
	MS+1BAP	0.00 ± 0.00^{a}	6.00 ± 0.71^{a}	0.00 ± 0.00^{c}
	MS+2BAP	0.00 ± 0.00^{a}	6.20 ± 0.66^{a}	0.00 ± 0.00^{c}
	MS+3BAP	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}
	MS+0.5NAA	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}	47.40 ± 0.81^{b}
	MS+1NAA	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}	83.60 ± 1.72^{a}
	MS+0.5 2,4-D	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}
	MS+1 2,4-D	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}
Concentration (C)		412.88**	1 900.50**	2 237.54**
Days (D)		2 475.68**	7 956.13**	4 378.09**
C × D		541.43**	1 518.96**	2 237.82**

MS, Murashige and Skoog medium; BAP, Benzylaminopurine; NAA, Naphthaleneacetic acid; 2,4-D, 2.4-dichlorophenoxyacetic acid.

$^{\Delta}$Mean± S.E, ** Significant at P<0.01 level. Means in a column followed by a same letter(s) for a days are not significantly (P<0.05) different according to Duncan's Multiple Range Test.

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asymbiotic seed germination is the most reliable method for commercial production of orchids. In the present study, MS media promoted seed germination. Nitrogen is essential for plant growth and nitrogen source has been shown to have an effect on the germination of different orchid species [7]. Murashige and Skoog medium contains nitrogen both in ammonium and nitrate forms that are known to stimulate seed germination [20]. The efficiency of plant growth regulators tested varied with type and their concentrations used. In the present study, seed germination and PLB formation response was higher in MS supplemented with BAP. Hossain [21] reported that 75% of the *Eulophia ibaguense* seeds germinated on MS medium supplemented with 1 mgL-1 BAP, but with increasing concentrations of BAP, the seed germination percentage tended to decline. In the terrestrial orchid, *Cypripedium* sp. BAP enhanced the seed germination [22]. Enhanced seed germination, and protocorm multiplication in *Eulophia dabia* and *Pachystoma senile* as well as shoot formation in *Cymbidium pendulum* [6] and *Cattleya aurantice* [23] with increased concentrations of BAP have been reported. Luo et al. [24] also showed that the presence of BAP was essential for enhancing the percentage of explants producing PLBs, as well as the number of PLBs per stem segment in *Dendrobium densiflorum*. Both high and low concentrations (0.5 and 1 mg/L) of 2, 4-D failed to stimulate seed germination and protocorm development in the present study. This is in line with the observations of Fornnesbech [25], Kusumoto [26] and Sharma and Tandoon [27] where 2,4-D inhibited seed germination and protocorm development in species of *Cymbidium* and *Coelogyne*. However, Huan et al [28] reported that a low concentration (0.001 mg/L) of 2, 4-D with or without 0.001 mg/L TDZ and 0.25 mg/L 2, 4-D resulted in the formation of PLBs from Cymbidium explants. Pedroza-Mantrique and Mican-Gutierrez [10] observed that higher concentrations of NAA (5.37 μm) failed to stimulate seed germination in *Odontoglossum gloriosum*. Similar results were also observed in the present study where very low seed germination was observed in media containing NAA.

In the present study almost all PLBs developed into individual plantlets on MS basal medium containing various concentrations of NAA. At 1 mg/L of NAA, 84% of the seedlings entered the two leaf stage. According to Zhao et al [29] lower concentrations of NAA stimulates shoot development. Luo *et al* [24] in his study on *D. densiflorum* recorded conversion rate of 35% of PLBs to shoots at 1 mg/L NAA. Earlier studies have shown that NAA supplementation successfully initiated the shoot bud formation and development in *Oncidium varicosum* [30], *Cleisostoma fordii*, *Pholidota chinensis*, *Bletilla stricta* [31] and *Cymbidium* [32]. Sharma *et al* [33] reported that 0.1 mg/L NAA combined with 15% coconut milk and 20 g/L sucrose enhanced the protocorm and seedling development. In the present study shoot formation failed in BAP supplemented medium which is supported by the observations of Lugo *et al* [24] where higher concentrations of BAP were effective in protocorm formation, but significantly inhibited the conversion of PLBs to shoots in *D. densiflorum*.

In general orchid propagated through asymbiotic seed germination have very low survival rate and re-establishment in natural habitats upon transferring to *ex vitro* conditions. In present study all the mycorrhizal seedlings survived, because orchid mycorrhizal fungi enhance growth of orchid plantlets, through supplying N and stimulate root growth [34]. Six month of orchid cultivation with mycorrhizal endophytes mimics the effects of two year cultivation under absence of fungal endophytes [35].

This study gives an effective protocol for seed germination and plantlet regeneration from immature seeds which can be used for establishing *A. praemorsa* populations in Velliangiri Hills and elsewhere.

Conflict of interest statement

We declare that we have no conflict of interest.

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