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Direct somatic embryogenesis of *Malaxis densiflora* (A. Rich.) Kuntze

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Abstract A protocol for induction of direct somatic embryogenesis and subsequent plant regeneration for the medicinally important and endangered plant of *Malaxis densiflora* has been developed for the first time. In the present study, *in vitro* seed derived protocorm explants were cultured on half strength Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), Picloram and Dicamba individually and in combination with cytokinins BAP, TDZ and Kn for its effectiveness to induce the differentiation of somatic embryos. The best response was observed in protocorms cultured half strength MS medium supplemented with 2,4-D at 3.39 μM and TDZ at 6.80 μM. Both epidermal and sub epidermal cells were involved in the formation of embryos. The proembryos developed into globular stage and subsequently developed into protocoms. Complete plantlets were formed after 60 days of culture. The plantlets were acclimatized in plastic pots containing sterilized vermiculite. The survival rate was 76%.

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1. Introduction

The Orchidaceae is the largest, most highly evolved and most diverse family of flowering plants, and is comprised of 30,000–35,000 species belonging to 850 genera, accounting for almost 30% of monocotyledons or 10% of flowering plants [1]. About 70% of orchids are epiphytic which comprise approximately two thirds of the world's epiphytic flora [2]. On the other hand, 25% orchids are terrestrial and the remaining 5% can be found on various supports [3]. While the majority of temperate orchids are terrestrial, tropical orchids are epiphytic or lithophytic [4]. These ornamental plants are widely distributed, cultivated for their beautiful flowers and are of economic importance. In addition to their ornamental value, orchids are also well known for their medicinal usage especially in the traditional folk medicine [5].

The orchid genus *Malaxis* comprising about 300 species has distribution throughout the tropical to temperate climate regions of the 19 species of the genus represented in India. In the Ayurvedic branch of traditional medicine, a group of eight drugs, known as ''Astavarga'', provide important ingredients for different types of tonics. Dried pseudo-bulbs of

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Malaxis species serve as important sources of Astavarga utilized in the preparation of the Ayurvedic tonic 'Chyavanprash'. The latter is one of the most widely used Ayurvedic preparations for promoting human health and preventing disease [5,6].

Malaxis densiflora (A. Rich.) Kuntze is an erect herb. Its leaves are long, five to seven nerved at the base, and acute or acuminate. Its flowers are purple and fragrant. *M. densiflora* is extensively used for curing various ailments, including wound healing, tuberculosis, cough and hepatic disorders [7,8]. Orchids are among the most vulnerable plant families with almost all orchid species forming a strong association with mycorrhizal fungi for development [9]. Due to the economic importance of pseudobulbs of orchids, plants have been harvested excessively and beyond sustainable levels.

Tissue culture provides an alternate method for large-scale propagation of threatened and endangered plants, including orchid micropropagation using various explants. Somatic embryogenesis is one of the most promising approaches for plant propagation due to the production of large numbers of plantlets [10], the possibility of producing synthetic seeds [11,12], the ability to store and rapidly mobilize germplasm for cryopreservation [13], the opportunity for genetic manipulation [14] and production of bioactive compounds within a short period of time [15,16]. It is necessary to develop a method for mass clonal propagation and conservation to satisfy the pharmaceutical demand of this high value medicinal plant. The present investigation was undertaken with the objective of developing an efficient *in vitro* somatic embryogenesis protocol for *M. densiflora*.

2. Experimental

2.1. Plant material, explant preparation and surface sterilization

Green capsules of *M. densiflora* (A. Rich.) Kuntze were collected from Vellingiri Hills (longitude 60–40′ and 70–10′E and latitude 10°-55 and 11°-10′N 1200) at an altitude of 1650–1750 m a.s.l. Tamil Nadu, India. Freshly collected green pods were washed thoroughly under running tap water. The capsules were immersed in 3–5% (v/v) Teepol for 2–5 min under continuous shaking and then rinsed three times with double distilled water; they were then pretreated with 0.1% (w/v) Bavistin, a fungicide, for 5 min and then rinsed in double distilled water. Then the capsules were surface sterilized in 0.01% mercuric chloride solution for 5 min and rinsed thoroughly with sterile distilled water (5–7 times). The capsules were dipped in 70% ethanol for 30 s and flamed. The surface sterilized pods were cut opened with sterile blade and seeds were extracted using sterile forceps and spread as thin film in test tubes containing 20 ml of culture media.

2.2. Optimization of culture medium for asymbiotic seed culture and culture condition

Immature seeds of *M. densiflora* were inoculated on Knudson C modified Morel (KCM) [17], Lindemann orchid medium [18], Mitra medium (M) [19], Knudson C medium (KC) [20], Murashige & Skoog medium (MS) [21] and BM-1-Terrestrial orchid medium [22] (Procured from Hi-Media Laboratories Mumbai, India) initially to find out the suitable medium for maximum seed germination. The best medium for seed germination was selected for further studies. All media contained 2% sucrose and were solidified with 0.8% agar (Hi Media Laboratories, India). The pH of the media was adjusted to 5.6–5.8 with 1 N NaOH or HCl before autoclaving at 121 °C, 105 kPa for 20 min. All the cultures were maintained at 25 ± 1 °C with photoperiod of 16-h using a photosynthetic photon flux density (PPFD) of 50 $\mu mol^{-2}\,s^{-1}$ provided by cool white fluorescent lamps (Philips, India) for 60 days.

2.3. Induction of embryogenesis from seed derived protocorms

Protocorms, developed on MS medium sowed as explants (Fig. 1(A)). Murashige and Skoog [21] medium containing half-strength macro, micro-elements and vitamins (Thiamine HCl (0.625 mg/L), Pyridoxine HCl (0.15 mg/L) and Nicotinic acid (0.15 mg/L)) supplemented with peptone (1.0 g/L) and NaH_2PO_4 (170 mg/L) was used as the basal medium. Basal medium was supplemented with 2,4-D (1.13, 2.26, 3.39, 4.52, 5.56 and 6.78 μM), Picloram (1.20, 2.41, 3.62, 4.82, 6.03 and 7.24 μM), Dicamba (1.10, 2.21, 3.31, 4.42, 5.52 and 6.63 μM), BAP (1.10, 2.20, 3.30, 4.40, 6.60 or 8.80 μM), TIBA (2.49, 4.98, 7.47, 9.96, 12.45 and 14.94 μM), (TDZ (1.1, 2.2, 3.3, 4.5, 6.8 and 9.0 μM) and Kn (1.15, 2.32, 3.45, 4.64, 6.90 or 9.20) individually or in combination for the induction of direct somatic embryogenesis.

2.4. Experimental design and data analysis

Number of embryos were recorded after 12 weeks of culture. Each treatment was repeated twice and each treatment consisted of 5 replicate culture tubes, each containing three protocorms. Data were subjected to analysis of variance (ANOVA) and comparisons between the mean values of treatments were made by the Duncan multiple range test calculated at the confidence level of $P < 0.05$. The statistical package SPSS (Version-17) was used for the analyses (see Tables 1 and 2).

2.5. Hardening

Well-developed plantlets were rinsed thoroughly with tap water to remove residual nutrients and agar from the plant body and transplanted to plastic pot containing vermiculite. The paper pots were covered by polyethylene bag and maintained two months inside the culture room for acclimatization under cool white tubular fluorescent lights (40 W, 220 V, Philips Electronics India Ltd.) at 50 $\mu\,mol^{-1}\,m^{-2}\,s^{-1}$ with a 16 h photoperiod at 25 ± 2 °C.

3. Results

In the present study, an efficient and highly reproducible system for *M. densiflora* somatic embryogenesis was developed (Fig. 1 (A)–(K). Somatic embryogenesis was achieved from seed derived protocorm explants on half strength MS medium, 2% (w/v) sucrose and PGRs: 2,4-D (1.13–6.78 μM), Picloram (1.20–7.24 μM), Dicamba (1.10–6.63 μM), TIBA (2.49–14.94 μM), BAP (1.10–8.80 μM), TDZ (1.0–9.0 μM) and Kn (1.15–9.20 μM). Embryos formed on protocorm explants after 2 week in culture and later globular embryoids developed directly from protocorm explants in all treatments except the

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