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Journal of Genetic Engineering and Biotechnology (2016) **14**, 77–81

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Journal of Genetic Engineering and Biotechnology

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

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Received 14 August 2015; revised 20 October 2015; accepted 7 November 2015
Available online 31 December 2015

KEYWORDS

Malaxis densiflora;
Somatic embryogenesis;
Histological analysis;
Plant growth regulators;
Plantlet regeneration;
Scanning electron microscopy

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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Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2015.11.003
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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

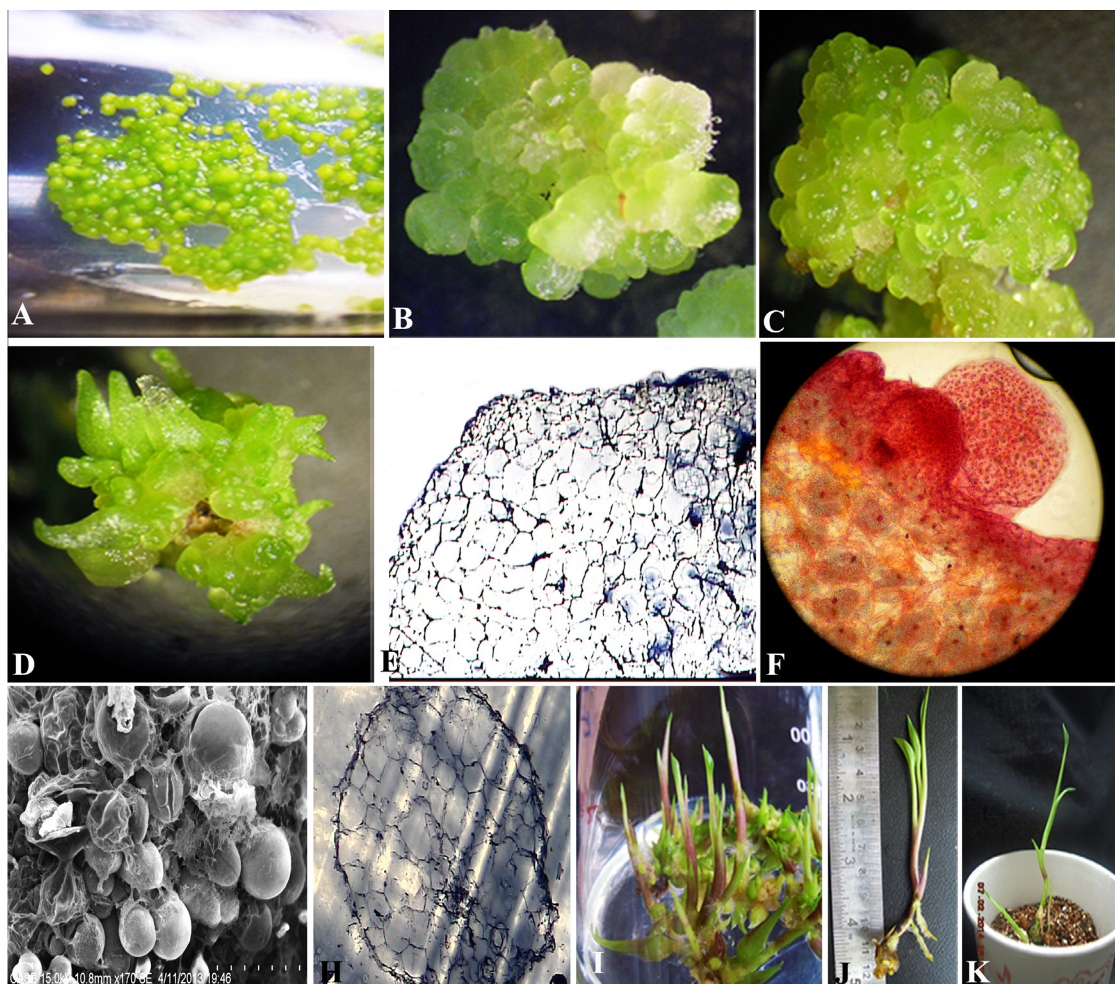

Figure 1 Plant regeneration and histological origin of direct somatic embryogenesis from seed derived protocorm of *Malaxis densiflora*. (A) Zygotic protocorms developed on MS medium. (B) Stereo microscopic view of somatic embryo formation from seed derived protocorm. (C) Development of globular stage embryos on half strength MS supplemented with (3.39 μM) 2,4-D and (6.80 μM) TDZ. (D) Formation of protocorms with first leaf. (E) Embryogenic cell originating from epidermal cell of seed derived protocorms. (F) Globular embryos with densely stained embryogenic cells. (G) SEM view of globular embryos. (H) Microtome section of globular embryo. (I) Regenerated plantlet derived from somatic embryos from half strength MS medium supplemented with (3.39 μM) 2,4-D and (6.80 μM) TDZ. (J) Plantlets with pseudo-bulb. (K) Hardening.

control after 5 weeks (Fig. 1(B)). Auxins stimulated swelling explants within one week of culture and yellowish to light green embryos started to differentiate from surfaces of seed derived protocorms. The inductions of globular embryoids were observed after 3 weeks of culture and later shoot and root apical meristems were also observed. Among various concentrations of different auxins tested, the lower concentrations of 2,4-D were not much effective and at 1.13 μM (2,4-D) exhibited the induction of 23.10 ± 3.10 embryos with the differentiation of plantlets after 10 weeks. The most effective was (3.39 μM) 2,4-D, inducing 60.12 ± 1.91 embryos/explants (Fig. 1(C)). Picloram (4.82 μM), Dicamba (6.63 μM) and TIBA (9.96 μM) were found to be the least effective and exhibited only 23.26 ± 3.61, 33.60 ± 1.87 and 22.15 ± 1.49 respectively.

The optimal concentration 3.39 μM of 2,4-D was also tested with three different cytokinins at various concentrations to produce embryos. The addition of cytokinins along with 2,4-D improved the rate of embryogenesis and also facilitated the germination of embryoids on the same medium. The best embryo induction was obtained on an MS medium amended with (3.39 μM) 2,4-D and (6.80 μM) TDZ producing a maximum of 65.15 ± 0.34 embryoids per explants. A further increase in the concentration of TDZ resulted in reduction in the rate of embryogenesis to 40.10 ± 1.02 on MS + (3.39 μM) 2,4-D + (9.00 μM) TDZ.

In *M. densiflora*, clusters of nodular masses protruded from the surfaces of seed derived protocorms after 10 days of culture on medium containing 2,4-D. No such nodular masses formed on the explants grown on media devoid of growth regulators. The development of these nodular masses was followed by somatic embryo production which became visible within the next 15–20 days. Initially, the embryos appeared as light green small globular masses (Fig. 1(B) and (C)) which passed through successive developmental stages, ultimately giving rise to protocorms with sheath leaves and absorbing hairs (Fig. 1 (D)). Histological examination (Fig. 1(F)) showed that the cell

Table 1 Effect of auxins on direct somatic embryos induction from seed derived protocorms of *Malaxis densiflora*.

2,4D (μM/l)	Picloram (μM/l)	Dicamba (μM/l)	TIBA (μM/l)	Number of somatic embryos/explants
1.13	–	–	–	23.10 ± 3.10[f]
2.26	–	–	–	41.17 ± 4.12[c]
3.39	–	–	–	60.12 ± 1.91[a]
4.52	–	–	–	53.00 ± 1.23[b]
5.56	–	–	–	20.55 ± 4.17[g]
6.78	–	–	–	10.01 ± 2.11[k]
–	1.20	–	–	09.22 ± 1.21[k]
–	2.41	–	–	13.32 ± 1.98[j]
–	3.62	–	–	16.57 ± 0.92[i]
–	4.82	–	–	23.26 ± 3.61[g]
–	6.03	–	–	17.18 ± 2.12[h]
–	7.24	–	–	11.15 ± 1.14[k]
–	–	1.10	–	08.23 ± 1.13[l]
–	–	2.21	–	13.21 ± 1.19[ij]
–	–	3.31	–	21.16 ± 1.00[g]
–	–	4.42	–	28.22 ± 1.43[e]
–	–	5.52	–	30.18 ± 1.20[d]
–	–	6.63	–	33.60 ± 1.87[d]
–	–	–	2.49	03.87 ± 0.11[m]
–	–	–	4.98	10.13 ± 0.29[k]
–	–	–	7.47	16.04 ± 1.00[i]
–	–	–	9.96	22.15 ± 1.49[g]
–	–	–	12.45	19.83 ± 1.23[h]
–	–	–	14.94	16.21 ± 1.29[i]

Values are mean of five replicate determinations ($n = 5$) ± standard error. Mean values followed by different superscripts in a column are significantly different according to DMRT ($P < 0.05$).

Table 2 Effect of cytokinin and auxin on direct somatic embryos induction from seed derived protocorms of *Malaxis densiflora*.

2,4D (μM/l)	BAP (μM/l)	Kn (μM/l)	TDZ (μM/l)	Number of somatic embryos/explants
3.39	1.10	–	–	50.10 ± 1.80[b]
3.39	2.20	–	–	33.17 ± 3.10[d]
3.39	3.30	–	–	26.80 ± 1.61[e]
3.39	4.40	–	–	13.43 ± 5.27[g]
3.39	6.60	–	–	07.78 ± 3.27[h]
3.39	8.80	–	–	03.15 ± 1.14[i]
3.39	–	1.15	–	18.10 ± 1.23[f]
3.39	–	2.32	–	23.31 ± 2.99[e]
3.39	–	3.45	–	28.56 ± 1.21[e]
3.39	–	4.64	–	33.39 ± 4.21[d]
3.39	–	6.90	–	20.00 ± 1.15[f]
3.39	–	9.20	–	10.60 ± 1.87[g]
3.39	–	–	1.10	20.15 ± 1.91[f]
3.39	–	–	2.20	24.35 ± 1.28[e]
3.39	–	–	3.30	36.12 ± 1.20[d]
3.39	–	–	4.50	45.33 ± 1.17[c]
3.39	–	–	6.80	65.15 ± 0.34[a]
3.39	–	–	9.00	40.10 ± 1.02[c]

Values are mean of five replicate determinations ($n = 5$) ± standard error. Mean values followed by different superscripts in a column are significantly different according to DMRT ($P < 0.05$).

division originated from the epidermal cells of the seed derived protocorm explants. These embryogenic cells formed directly from the explants cells, without an intervening callus phase. The embryogenic cells were clearly distinguishable from the surrounding cells by the thickness of the cell wall dense cytoplasm and conspicuous nucleus. These isolated zones displayed attributes of pre-embryo structures (Fig. 1(F)). The pro-embryos developed in globular embryos (Figs. 1(F)–(H)) and ultimately developed into protocorm and seedlings (Fig. 1(I)).

The plantlets (Fig. 1(J)) were transferred to the potting medium containing vermiculite (Fig. 1(K)). After 2 months, the cover was gradually loosened, thus dropping the humidity (65–70%). This procedure subsequently resulted in *in vitro* hardening of the plants. The survival rate was 76% when maintained in culture room condition (25 ± 2 °C).

4. Discussion

Somatic embryogenesis is a process where a bipolar structure resembling a zygotic embryo develops from a non-zygotic cell without vascular connection with the original tissue [23]. In the present study, embryos were induced from seed derived protocorm of *M. densiflora*. Somatic embryos production followed by somatic development has been reported in several orchid plants using different explants, such as protocorms in *Cymbidium* [24], *Phalaenopsis amabilis* var. *formosa* [25], *Rhynchostylis gigantea* [26], *Cymbidium bicolor* [27], *Phalaenopsis* Richard Shaffer Santa Cruz [28], *Cattleya maxima* [29] and *Phalaenopsis aphrodite* [30].

In this work, all tested concentrations of 2,4-D, picloram, dicamba and TIBA were able to induce somatic embryogenesis from seed derived protocorm explants in *M. densiflora*. However, the number of somatic embryo was significantly higher using 2,4-D when compared with medium containing other auxins. Generally, auxin like 2,4-D is considered essential for the induction and maintenance of embryogenic cultures [31], however, a combination of auxin and cytokinin can be the best to induce embryos in orchids [32,33]. In the present investigation, various auxins were tried and among them, 2,4-D proved to be the best. Similarly in *Vanda coerulea*, *Paphiopedilum* Alma Gavaert and *Coelogyne cristata*, the role of 2,4-D in the production of embryo has been emphasized [33–35] and the same was corroborated by our study. The capability of 2,4-D in activating the embryogenic pathway may be related to its capacity to induce stress genes which have been shown to contribute to the cellular reprogramming of the somatic cells toward embryogenesis [36]. All other auxins tested proved to be less effective than 2,4-D, whereas Picloram was efficient for the germination of embryoids on the same medium, although it produced a lesser number of embryoids.

The presence of cytokinin in the induction medium proved to be crucial for a high frequency of somatic embryos. Accordingly, the augmentation of different cytokinins with optimal concentration of 2,4-D (3.39 μM) enhanced the rate of embryogenesis and facilitated the germination of embryoids. The maximum number of embryos was obtained on a medium containing (3.39 μM) 2,4-D and (6.80 μM) TDZ. However, in an earlier report of somatic embryogenesis in *C. cristata* and *C. maxima* [29,35] a lesser number of embryoids were produced compared to our study. Thus, our protocol proved to be more effective for efficient embryogenesis in *M. densiflora*. The combined favorable influence of auxin and cytokinins

observed in the present study is in accordance with reports on *Oncidium* [36], *Phalaenopsis* [37], *Dendrobium* [38], *Rhynchostylis gigantea* [26], *C. cristata* [35], *C. bicolor* [27]. Conversely, the addition of 2,4-D alone or with TDZ was not favorable for somatic embryo formation in *Oncidium* [39,40].

The globular embryo like structures of PLBs were induced via embryogenesis as suggested by Begum et al. [25,41], Huan et al. [42], Su et al. [43] and Mahendran and Narmatha Bai [27]. It was also suggested that the process of somatic embryogenesis was involved in PLBs formation [44] which is clearly evident in the present study.

5. Conclusion

In conclusion, the present study reported direct somatic embryogenesis results in *M. densiflora* for the first time. Individual auxins and cytokinins represented effective for direct somatic embryos induction factors. Healthy plants developed through somatic embryogenesis survived well when transplanted in the greenhouse. This protocol is simple, easy to carry out and can provide a large number of embryos and plants for mass propagation in a short period of time. We expect that this ability will also open up the prospect of using biotechnological approaches for *M. densiflora* improvement.

Acknowledgment

This work was financially supported by University Grants Commission, New Delhi [F. No. 37-97/2009(SR)]. The author (G. Mahendran) would like to thank UGC for providing Dr. D.S. Kothari Postdoctoral Research Fellowship (BSR/BL/14-15/0100).

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