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BRIEF COMMUNICATION

In vitro propagation of *Ophiorrhiza prostrata* through somatic embryogenesis

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Abstract

In vitro propagation of an anticancerous drug synthesizing plant, *Ophiorrhiza prostrata* D. Don, was established through indirect somatic embryogenesis. Friable embryogenic calluses were initiated from *O. prostrata* leaf and internode explants on Murashige and Skoog (MS) media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) either alone or in combination with N^6-benzyladenine (BA) or kinetin (KIN). Somatic embryos were developed after subculture of the friable calluses onto half strength MS media containing 0.45 or 2.26 μM 2,4-D alone or in combination with BA or KIN. Medium supplemented with 2.26 μM 2,4-D and 2.22 μM BA was optimal, supporting the production of a mean of 5.8 globular embryos. Subculture of globular embryo-bearing calluses on half strength MS medium without growth regulators produced the highest embryo frequency, and the majority of them developing to early torpedo stage. Somatic embryos underwent maturation and converted to plantlets at high frequency (90 %) on half strength MS medium supplemented with 0.44 μM BA. Somatic embryo-derived plantlets with well-developed roots were established in field conditions with a 90 % survival rate.

Additional key words: auxins, camptothecin-synthesizing plant, cytokinins, embryogenic callus, plantlets.

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Ophiorrhiza prostrata D. Don (family *Rubiaceae*) is a tropical herbaceous medicinal plant which accumulates camptothecin (CPT), an anticancer drug mainly in its roots. CPTs are modified monoterpene indole alkaloids, originally isolated from *Camptotheca acuminata* (*Nyssaceae*). CPTs are also reported from members of *Icacinaceae*, *Olacaceae*, *Rubiaceae*, and *Apocynaceae*. The demand for CPTs makes the propagation of *O. prostrata* essential in order to meet the requirement of CPTs. Low seed viability and germination rate in *O. prostrata* curtail the propagation of this important plant from seed. In addition, the seed progenies are heterozygous and do not breed true-to-type with respect to these specific metabolites. The difficulty in obtaining sufficient numbers of stem cuttings restricts vegetative propagation approaches. Due to the presence of the anticancer drugs in the root, bulk harvesting of plants for roots without simultaneous re-cultivation could endanger the long-term survival of this plant.

Somatic embryogenesis represents the most efficient *in vitro* plant propagation system. Procedures for induction of somatic embryogenesis have been developed for most of the commercially important plants (Martin 2004, Chithra *et al.* 2005, Wang *et al.* 2006, Chen and Chang 2006). No plant regeneration through somatic embryogenesis has been reported to date for this important medicinal plant. The present study describes an efficient plant regeneration protocol for *O. prostrata* through somatic embryogenesis which will help to keep the pressure off the wild population, and enable the continuous production of CPTs from this plant.

Young leaves and internode segments detached from shoots of *Ophiorrhiza prostrata* D. Don from potted plants were used as the source of explants. They were washed separately under running tap water and then submerged in 5 % solution of *Extran* (a neutral liquid detergent, *Merck*, Mumbai, India) for 5 min. The tissues were then surface sterilised using 0.1 % mercuric chloride. Leaf explants were sterilized for 7 - 9 min while internode explants were treated for 10 - 12 min. After

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Abbreviations: BA - N^6-benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; KIN - kinetin; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid; PGRs - plant growth regulators.
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repeated washing in sterile water the tissues were cut into appropriate sizes (10 mm^2 for leaf explants and 7 - 15 mm length for internode). The explants were then cultured on MS (Murashige and Skoog 1962) media supplemented with different levels of plant growth regulators (PGRs; Table 1). Subcultures were carried out on media containing half or full strength MS medium without or with various concentrations of PGRs as mentioned in the text. The media were adjusted to pH 5.8, solidified with 0.8 % (g dm^{-3}) agar, and autoclaved at a pressure of 1.06 kg cm^{-2} at 121 °C for 20 min. All cultures were incubated at 25 ± 2 °C with 16-h photoperiod (at an irradiance of 25 μmol m^{-2} s^{-1}).

Callus growth was evaluated based on the fresh mass after 40 d of culture. The histology of somatic embryogenesis was studied by taking random sections at different developmental stages. Different embryonal stages fixed in formalin (10 cm^3) : acetic acid (5 cm^3) : alcohol (50 cm^3 of 95 % ethyl alcohol) were dehydrated in a graded series of tertiary butyl alcohol (TBA), followed by infiltration in paraffin. Serial sections of 10 μm, taken in a rotary microtome (*Leitz*, Wetzlar, Germany) were deparaffined with xylene and dehydrated in ethanol series. The sections were stained with haematoxylin and mounted in synthetic resin DPX. Photographs were accomplished using a *Leitz Dialux 20* microscope.

Plantlets were transferred to small pots containing sand and soil (1:1) and subsequently to field conditions.

The experimental design was completely randomized. Twenty replicates were used for each treatment, and all the best treatments were repeated twice. The mean values of different treatments were compared using Duncan's multiple range test.

Leaf and internode explants cultured on PGR-free MS medium did not show callus formation. MS media supplemented with different concentrations of α-naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with N^6-benzyladenine (BA) or kinetin (KIN) facilitated callus formation from both leaf and internode explants (Table 1). Depending on the PGRs and their concentrations, the explants developed calli, roots, and shoots. The amount, texture, and colour of the produced calluses varied with the type, concentration, and combination of PGRs.

Besides callus formation, the leaf and internode explants cultured on MS media supplemented with different concentrations of NAA (2.69 - 8.06 μM) alone or in combination with BA or KIN developed shoots and roots. Of the different concentrations of NAA, 5.37 μM induced the highest amount of callus (Table 1). The calluses initiated within 12-d culture from both internode and leaf explants were yellowish and semi-hard. The callus later developed some roots also. On leaf explants, the calluses were mainly induced from the cut ends and also on the mid-rib region. Low concentrations of NAA showed initiation of 2 - 5 shoots as the culture period went on. MS medium with 5.37 μM NAA and 2.22 μM BA was most beneficial for callus induction. The concentrations of BA or KIN determined the number of shoots. In this medium, as the culture period progresses, the leaf and internode explants developed 2 - 5 and 4 - 7 small shoots, respectively. The ratios of NAA and BA determined the texture and colour of the calli. At high concentrations of NAA, the calluses were semi-hard and pale green, while with the increase of BA concentration the calluses become green and hard.

Table 1. Callus induction from leaf and internode explants of *O. prostrata* on MS media supplemented with different growth regulators. Data represents the mean of 20 replicates. Mean values followed by the same letters within columns are not significantly different at 5 % level. Growth period was 40 d. Initial mass of the leaf and internode explants was 60 ± 15 mg and 95 ± 10 mg, respectively.

PGRs [μM]				Callus fresh mass [mg $explant^{-1}$]	
2,4-D	NAA	BA	KIN	internode	leaf
2.26				156^{f}	120^{f}
4.52				245^{cd}	229^{c}
6.78				223^{d}	199^{d}
9.05				194^{e}	158^{e}
4.52		2.22		293^{a}	248^{b}
4.52		4.44		277^{b}	215^{c}
4.52			2.32	257^{c}	209^{cd}
4.52			4.65	239^{cd}	199^{d}
	2.69			144^{f}	109^{f}
	5.37			219^{d}	188^{d}
	8.06			161^{f}	140^{e}
	5.37	2.22		310^{a}	295^{a}
	5.37	4.44		297^{a}	259^{b}
	5.37		2.32	278^{b}	249^{b}
	5.37		4.65	268^{bc}	226^{c}

Leaf and internode explants cultured on MS media containing various concentrations of 2,4-D (2.26 - 9.05 μM) alone or in combination with BA or KIN favoured induction of callus and root. Calluses were initiated within 15-d culture. In the case of leaf explants, the callus mainly confined to the adaxial surface. MS medium supplemented with 4.52 μM 2,4-D was superior for the induction and proliferation of calluses (Table 1). Calluses developed on 2,4-D supplemented media were friable and golden yellow, and later became dark red (Fig. 1*A*). Some calluses developed small root like structures. Combinations of 4.52 μM 2,4-D and BA (2.22 μM) or KIN (2.32 μM) were the best for callus induction and proliferation. The 2,4-D, BA or KIN ratios determined the texture of calluses. High concentrations of 2,4-D along with BA or KIN changed friable and pale yellow calluses to dark yellow. The roots induced on this PGR combination changed the colour from yellowish to reddish.

The colour of the calluses in almost all cases turned to golden yellow and finally scarlet red. This reflects the accumulation of secondary metabolites, probably camptothecin in particular.

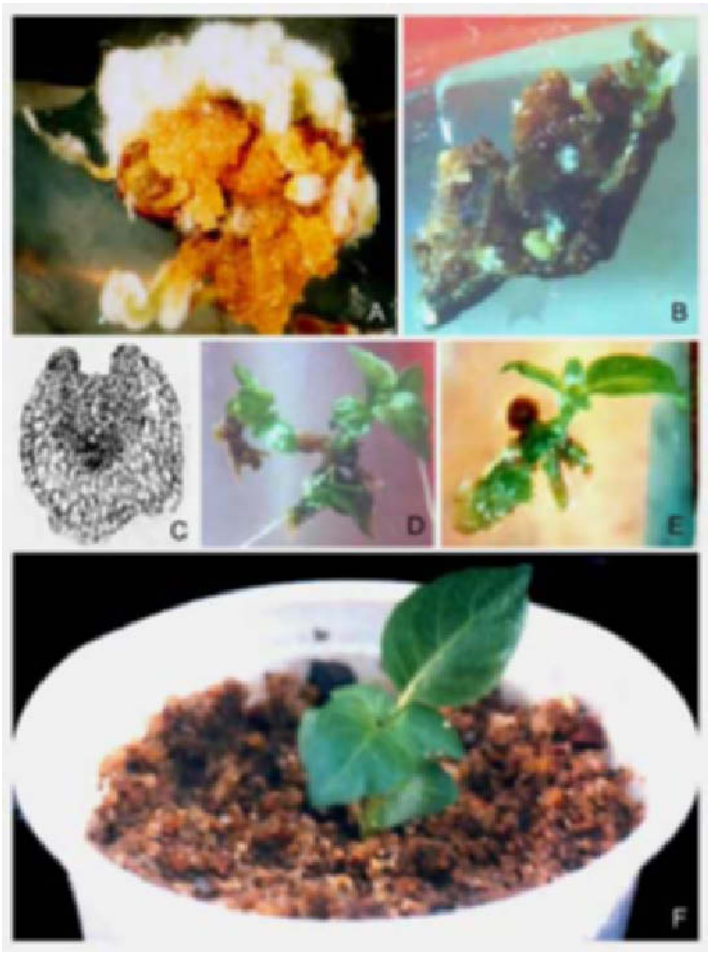

Fig. 1. Somatic embryogenesis and plant regeneration in *O. prostrata. A* - Friable embryogenic callus grown on MS medium supplemented with 4.52 μM 2,4-D. *B* - Somatic embryos developed on medium containing 2.26 μM 2,4-D and 2.22 μM BA. *C* - Section of the late globular stage embryo. *D* - Converted somatic embryos. *E* - Converted somatic embryo with rootlets on taproot. *F* - Somatic embryo-derived plantlet (35 d).

The PGRs used for inducing callus influenced somatic embryogenesis from those calli. Calluses developed on 5.37 μM NAA either alone or in combination with low concentrations of KIN or BA during subculture favoured no embryogenesis. 2,4-D used for callus induction was mandatory for somatic embryogenesis. Calluses produced on MS media supplemented with 2,4-D either alone or in combination with BA or KIN were efficient for the induction of somatic embryos. Transfer of friable callus from MS media with 4.52 μM 2,4-D alone or in combination with BA or KIN onto half strength MS media with reduced concentrations of 2,4-D (0.45 - 2.26 μM) alone or with BA or KIN facilitated the development of somatic embryos. Half strength MS medium with 2.26 μM 2,4-D and 2.22 μM BA was superior for the induction of somatic embryos at a mean value of 5.8 embryos (Fig. 1*B*). It was noticed that some embryos showed a tendency to develop roots with a dormant shoot apex. Subculture of the calluses bearing globular embryos onto half strength MS media with or without PGRs developed embryos at different frequencies (Table 2). Half strength PGR-free MS medium favoured the development of the highest number of somatic embryos (a mean value of 28.3 per 250 mg calli), on which embryos developed up to the early torpedo stage (and rarely to the early cotyledonary stage). On medium with 0.23 μM 2,4-D and 2.22 μM BA less than 10 % of the embryos were converted to plantlets. However, on

Table 2. Effects of 2,4-D and BA on induction of somatic embryos (number of embryos per 250 mg callus) on half strength MS medium. Data represents the mean of 20 replicates. Mean values followed by the same letters are not significantly different at 5 % level. Growth period was 40 d from the transfer of callus on induction medium.

2,4-D	BA	Somatic embryos
0.0	0.0	28.3[a]
0.23	0.0	11.7[c]
0.45	0.0	7.4[d]
0.23	0.22	19.4[b]
0.23	0.44	12.2[b]
0.23	2.22	6.7[d]

half strength MS medium, a few of the embryos were transformed to roots.

Histological studies confirmed the embryonic stages of somatic embryos (Fig 1*C*). The somatic embryos were initiated from the periphery of the callus and exhibited progressive development from globular to cotyledonary stage.

Somatic embryos underwent maturation and converted to plantlets at a high frequency (90 %) on medium supplemented with 0.44 μM BA (Fig 1*D*). At 0.22 μM and 0.66 μM BA conversion rates were 75 and 60 %, respectively. Increasing the concentration of BA further reduced the conversion rates of embryos to plantlets. Half strength PGR-free MS medium exhibited low frequency of embryo maturation and conversion. The embryos transferred onto PGR-free MS medium exhibited a greater tendency for root development. The embryo-derived plantlets grew well and developed numerous roots (Fig 1*E*).

In the present study, 2,4-D was necessary for the induction of somatic embryos in *O. prostrata*. The efficacy of 2,4-D as the most reliable plant growth regulator for the induction of somatic embryogenesis has been well documented in several plants *viz*. *Euphorbia nivulia* (Sunandakumari *et al.* 2005), *Rotula aquatica* (Chithra *et al.* 2005), and *Areca catechu* (Wang *et al.* 2006*)*. In the view of Zimmerman (1993), medium supplemented with an auxin generally stimulates the expression of all of the genes necessary to complete the globular stage of embryogenesis. Contrary to this view, several species such as peanut and sugar beet only require cytokinin, *e.g.*, BA or thidiazuron for somatic embryogenesis (Zhang *et al.* 2001, Martin 2004, Gairi and Rashid 2005, Chen and Chang 2006).

Somatic embryos underwent maturation and converted to plantlets at high frequencies on medium containing low concentrations of BA. In the present study, the embryos induced by 2,4-D underwent low frequency of maturation and conversion on medium without PGRs. According to Stuart *et al.* (1985), somatic embryos under the influence of an auxin failed to accumulate storage proteins which are a prerequisite for the progression to other developmental stages. The failure to accumulate storage products due to the persistence of auxin may be the reason that the low frequencies of maturation even on medium without growth regulators were obtained. High frequencies of embryo maturation and conversion to plantlets may be the result of negation of the auxin effect caused by the added BA.

Somatic embryo-derived plantlets transferred *ex vitro* showed 88 % (44 out of 50) survival. The plantlets revived growth 9 to 16 d after transfer and grew well (Fig 1*F*). Morphological characteristics of the plantlets established in field conditions were identical to that of mother plants.

The protocol described in this study facilitates development of 90 plantlets per g callus of *O. prostrata via* somatic embryogenesis through the subculture of 2,4-D induced callus onto half-strength MS medium with 2.26 μM 2,4-D and 2.22 μM BA and subsequently to half strength PGRs-free MS medium and medium with 0.44 μM BA.. It takes about 5 months from explant inoculation to plantlets established in soil. The protocol will be very useful for elite cultivar propagation, conservation, and performance improvement through modern genetic transformation approaches.

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