In Vitro Cell. Dev. Biol.—Plant 39:510–513, September–October 2003
DOI: 10.1079/IVP2003468
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1054-5476/03 $18.00+0.00

IN VITRO PROPAGATION OF THE RARE MEDICINAL PLANT *CEROPEGIA CANDELABRUM* L. THROUGH SOMATIC EMBRYOGENESIS

M. R. BEENA[1†] AND K. P. MARTIN[2*]

[1]*Department of Botany, University of Calicut, Kerala-673 635, India*
[2]*Department of Biotechnology, University of Calicut, Kerala-673 635, India*

(Received 16 July 2002; accepted 23 May 2003; editor T. A. Thorpe)

Summary

Efficient *in vitro* propagation of *Ceropegia candelabrum* L. (Asclepidaceae) through somatic embryogenesis was established. Somatic embryogenesis depended on the type of plant growth regulators in the callus-inducing medium. Friable callus, developed from leaf and internode explants grown on Murashige and Skoog (MS) medium supplemented with 4.52 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D), underwent somatic embryogenesis. Compared to solid media, suspension culture was superior and gave rise to a higher number of somatic embryos. Transfer of the friable callus developed on MS medium containing 4.52 μ*M* 2,4-D to suspension cultures of half- or quarter-strength MS medium with lower levels of 2,4-D (0.23 or 0.45 μ*M*) induced the highest number of somatic embryos, which developed up to the torpedo stage. Somatic embryogenesis was asynchronous with the dominance of globular embryos. About 100 mg of callus induced more than 500 embryos. Upon transfer to quarter-strength MS agar medium without growth regulators, 50% of the somatic embryos underwent maturation and developed into plantlets. Plantlets acclimatized under field conditions with 90% survival.

Key words: Ceropegia candelabrum; endangered; somatic embryos; suspension culture.

Introduction

Ceropegia candelabrum L., the 'glabrous goglet flower' is an endangered, twiny, perennial medicinal herb found at the edges of moist deciduous forests. The officinal part of the plant, the root tuber, is rich in the alkaloid ceropegine (Nadkarni, 1976). The tubers are valuable constituents in many traditional Indian ayurvedic drug preparations against many diseases, such as diarrhea and dysentery (Kirtikar and Basu, 1935). The tubers that contain starch, sugar, gum, albuminoids, fats, and crude fiber are also useful as a nutritive tonic (Kirtikar and Basu, 1935).

Propagation of *C. candelabrum* through seed is held back by a low span of viability and a low germination rate of seeds, and scanty and delayed rooting of seedlings. Seed-derived progenies are not true-to-type due to cross-pollination. Vegetative propagation by root tubers is onerous, and is too low to meet the commercial needs. The development of an efficient method for rapid clonal propagation is important to meet the pharmaceutical needs and for conservation of this valuable rare medicinal plant. Micropropagation via somatic embryogenesis, as reported in many medicinal plants, is a proxy method to obtain clonal plants. Somatic embryogenesis is reported as non-chimeric and upholds clonal fidelity. The true-to-type nature of somatic embryo-derived plantlets has been well established in many plants (Jayanthi and Mandal, 2001; Tokuhara and Mii, 2001). Plant regeneration through indirect somatic embryogenesis has been reported in Asclepiadacean plants such as *Tylophora indica* (Rao and Narayanaswamy, 1972; Manjula et al., 2000; Jayanthi and Mandal, 2001), *Hemidesmus indicus* (Sarasan et al., 1994), *Ceropegia* spp. (Patil, 1998), and *Holostemma ada-kodien* (Martin, 2003). In *C. candelabrum*, micropropagation through axillary bud proliferation has been reported in our laboratory (Beena et al., 2003). However, there is no report on plant regeneration through somatic embryogenesis for *C. candelabrum*. In this study, we describe the *in vitro* propagation of *C. candelabrum* through somatic embryogenesis.

Materials and Methods

Leaf and internode segments of *C. candelabrum* were collected from the young shoot tips of mature plants grown in Calicut University Botanical Garden. These segments were washed under running tap water, followed by a detergent, extran (5%, v/v) for 5 min. After repeated washing in double-distilled water, surface sterilization was done with mercuric chloride (0.5%, w/v) solution for 12–14 min, and washed thoroughly (three times, 5 min each) with sterile double-distilled water. The segments, cut into appropriate sizes (leaf 1 cm^2; internode 1 cm), were cultured on MS (Murashige and Skoog, 1962) medium without or with N^6-benzyladenine (BA), kinetin (Kn), α-naphthaleneacetic acid (NAA), or 2,4-dichlorophenoxyacetic acid (2,4-D), each at different concentrations (Table 1). The effect of combinations of 2,4-D and BA or Kn was also studied. The medium, pH 5.8, was solidified with 0.8% (w/v) agar, except for liquid medium, and autoclaved at a pressure of 1.06 kg cm^{-2} for 20 min at 121°C.

For suspension culture, 100 mg of friable callus were transferred to 100 ml conical flasks each containing 25 ml of liquid medium. The suspension cultures were incubated in the dark on a rotary shaker at 120 rpm. Solid medium cultures were kept in a 16 h light/8 h dark cycle at 25 ± 2°C. Cultures were transferred to fresh medium at 40 d interval.

*Author to whom correspondence should be addressed: Email marttin@sify.com

†Present address: Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Andhra Pradesh-500 046, India.

TABLE 1

CALLUS INDUCTION FROM LEAF AND INTERNODE EXPLANTS GROWN ON MS MEDIUM WITH OR WITHOUT GROWTH REGULATORS AFTER 40 d OF CULTURE

Growth regulators (μ*M*)				Callus fresh weight (mg)[z]	
2,4-D	NAA	BA	Kn	Leaf	Internode
Basal medium				610 k	730 k
2.26	0	0	0	1630 d	1695 d
4.52	0	0	0	1675 bc	1770 c
6.78	0	0	0	1695 bc	1810 c
9.05	0	0	0	1665 cd	1785 c
0	2.69	0	0	960 j	1155 h
0	5.37	0	0	1320 g	1445 f
0	8.06	0	0	1670 bc	1790 c
0	10.74	0	0	1485 f	1575 e
0	0	2.22	0	1050 i	1120 h
0	0	4.44	0	1655 cd	1530 e
0	0	6.66	0	1785 a	1730 cd
0	0	8.87	0	1710 b	1870 b
0	0	0	2.32	920 j	970 j
0	0	0	4.65	1245 h	1360 g
0	0	0	6.97	1550 e	1675 d
0	0	0	9.29	1250 h	1475 f
4.52	0	0.44	0	1710 b	1890 b
4.52	0	2.22	0	1820 a	1960 a
4.52	0	0	0.46	1575 e	1720 cd
4.52	0	0	2.32	1650 cd	1790 c

Mean of 20 replicates. Means with the same *letters* are not significantly different at the 5% level.

[z] The weight includes that of explants, whose initial weight per explant was 150 ± 25 mg for leaf and 100 ± 15 mg for internode.

Callus growth was evaluated in terms of fresh weight after 40 d of culture. The experiments were conducted in a completely randomized design. Twenty replicates were used for each treatment, and all the best treatments were repeated twice. Means were compared using Duncan's multiple range test (Duncan, 1955). Plantlets were transferred to small cups containing sand and soil (1:1) and subsequently to field conditions.

Results and Discussion

Internode and leaf explants of *C. candelabrum* grown on hormone-free MS medium formed callus. Callus formation on hormone-free MS medium has also been reported in *Erysimum scoparium* (Pérez-Francés et al., 1995) and *Melia azedarach* (Handro and Floh, 2001). According to Kahl (1983), callus formation in the absence of exogenous plant growth regulators was due to the wound reaction, where the cells at the cut ends underwent mitosis, resulting in callus formation.

The use of various concentrations of NAA, 2,4-D, BA, and Kn alone, and combinations of 2,4-D and BA or Kn was efficient in inducing callus from both internode and leaf explants (Table 1), but the texture, amount, and color varied depending on the type and concentration of the growth regulators. Callus developed on MS medium containing 2.26–9.05 μ*M* 2,4-D alone or in combination with 2.22 μ*M* BA or 2.32 μ*M* Kn was friable and creamy colored, while the callus grown on other media was hard or semihard and pale green to dark green. Development of callus on medium containing BA alone has also been reported on *Tylophora indica* (Manjula et al., 2000), another member of the Asclepiadaceae.

Callus grown on MS medium containing growth regulators other than 2,4-D alone did not form somatic embryos. Friable callus developed on MS medium supplemented with 4.52 μ*M* 2,4-D turned embryogenic upon subculture onto the solid half-strength MS medium with 2.26 μ*M* 2,4-D (Fig. 1*A*). The embryogenic callus (80 d from establishment) transferred to solid or liquid medium developed somatic embryos at different frequencies (Table 2). Of all the treatments, half- or quarter-strength MS medium containing 0.23 or 0.45 μ*M* 2,4-D induced somatic embryo formation at the highest frequency. The concentrations of 2,4-D higher than 0.45 μ*M* resulted in callus proliferation (Table 2). It was found that the presence of low concentrations of 2,4-D (0.23 or 0.45 μ*M*) was mandatory for embryo formation, whereas elimination of 2,4-D was not beneficial for the induction of embryos. Suspension and solid cultures displayed significant difference in the induction of embryos (Table 2). Suspension cultures induced the highest number of somatic embryos. Similar results have been reported in black pepper (Joseph et al., 1996) and *Holostemma ada-kodien* (Martin, 2003), where high-frequency embryo formation occurred in suspension cultures compared to a lower frequency in static cultures. In the present study a 100 mg callus induced more than 500 somatic embryos (5000 embryos per g callus) (Table 2). The cultures showed globular- to early cotyledonary-stage embryos (Fig. 1*B*), but the globular-stage embryos dominated in culture. Late globular embryos showed a small suspensor-like structure (Fig. 1*C*), which persisted up to an early heart stage. Upon transfer to quarter-strength MS agar medium without growth regulators, 50% of somatic embryos underwent maturation and developed into plantlets. It was observed that a few embryos underwent dedifferentiation. The embryo-derived callus later developed somatic embryos (secondary embryogenesis).

The promoting effect of 2,4-D on somatic embryo induction and effect of reduced salt levels in the consecutive development and conversion of somatic embryos has been reported in several members of Asclepiadaceae such as *Tylophora indica* (Rao and Narayanaswamy, 1972), *Ceropegia* spp. (Patil, 1998), and *Holostemma ada-kodien* (Martin, 2003). Nevertheless, elimination of 2,4-D was indispensable for the maturation and conversion of plantlets. In view of Zimmerman (1993), removal of auxin from the

TABLE 2

RESPONSE OF CALLUS (INITIATED ON MS MEDIUM WITH 4.52 μ*M* 2,4-D) GROWN IN SUSPENSION AND STATIC MS MEDIUM WITH OR WITHOUT 2,4-D AFTER 40 d

MS medium	2,4-D (μ*M*)	Number of embryos per 100 mg callus	
		Suspension	Static
Half-strength	4.52	Callus proliferation	Callus proliferation
Half-strength	2.26	Callus proliferation	Callus proliferation
Half-strength	0.45	498.8 a	15.7 b
Half-strength	0.23	507.5 a	21.9 a
Half-strength	0	6.7 b	4.4 c
Quarter-strength	4.52	Callus proliferation	Callus proliferation
Quarter-strength	2.26	Callus proliferation	Callus proliferation
Quarter-strength	0.45	501.2 a	16.2 b
Quarter-strength	0.23	510.8 a	23.6 a
Quarter-strength	0	7.7 b	3.4 c

Data are means of 20 replicates.

Means with the same *letters* are not significantly different at the 5% level.

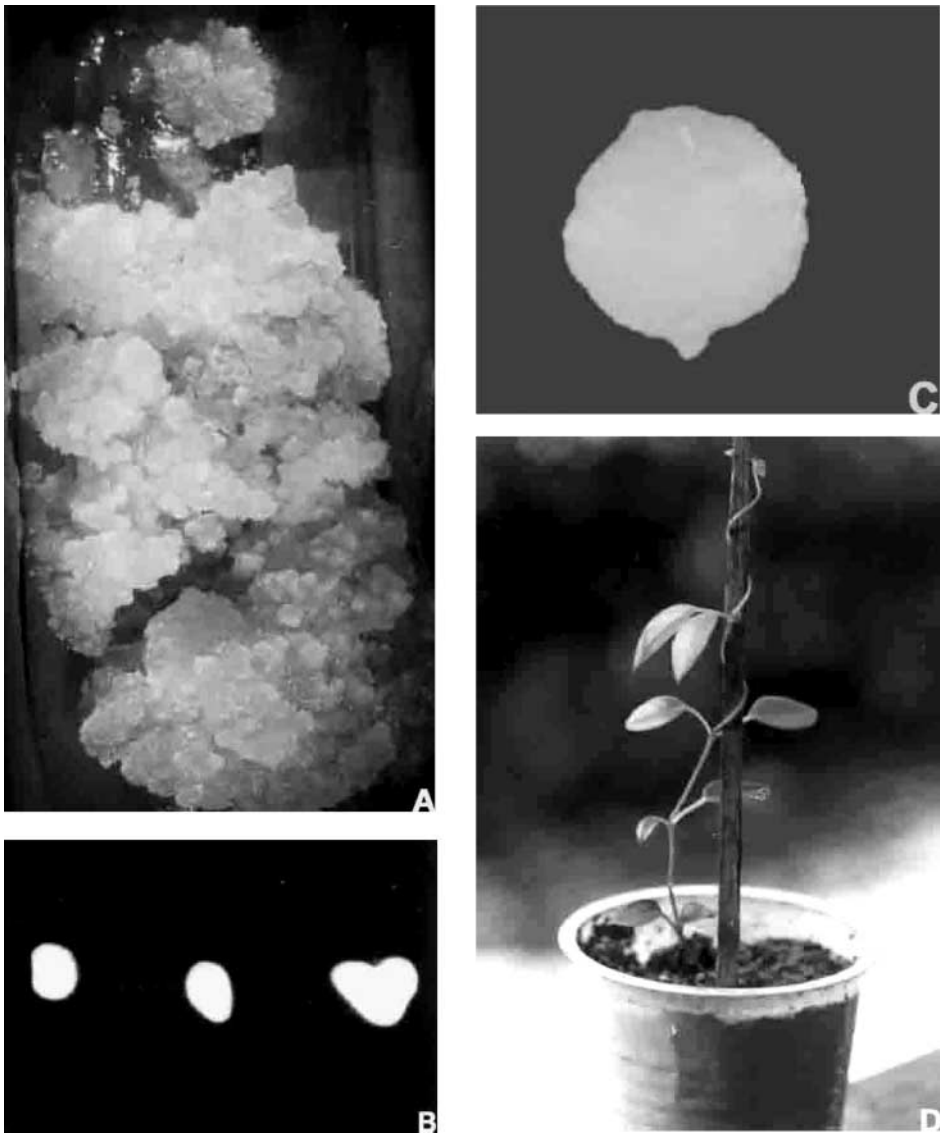

FIG. 1. Somatic embryogenesis and plant regeneration of *Ceropegia candelabrum*. *A*, Friable embryogenic callus grown on half-strength MS medium with 2.26 μ*M* 2,4-D. *B*, Globular to heart-shaped embryos. *C*, Globular embryo with small suspensor-like structure. *D*, Somatic embryo-derived plantlet (45 d).

culture medium is vital to inactivate several genes or to synthesize new gene products essential for the successful completion of embryo development. According to Stuart et al. (1985), failure in the accumulation of storage proteins necessary for the sequential development of embryos due to the exposure to auxin during development results in low frequency germination. Low frequency of embryo germination in the present study may be due to the persistence of auxin, which may also be the cause of dedifferentiation of embryos.

Somatic embryo-derived plantlets were transferred to small cups containing soil and sand (1:1) and acclimatized within 10 d (Fig. 1*D*). Plantlets were transferred to the field and 90% (54 out of 60) survived. Established plantlets were morphologically similar to the source plant.

The protocol described in this study facilitates development of 2500 *C. candelabrum* plantlets per g callus via somatic embryogenesis within 6 mo. This efficient tissue culture method will be useful to conservation, and to the improvement of *C. candelabrum* using gene transfer technologies.

ACKNOWLEDGMENTS

We gratefully acknowledge the Department of Botany, University of Calicut for the facilities provided, and the Art and Photography unit, especially Mr. K. Narendranath, for photographic assistance.

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