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Efficient clonal propagation method for *Decalepis hamiltonii,* an endangered shrub, under the influence of phloroglucinol

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A highly efficient two stage protocol was developed for induction of multiple shoots from single node *in vitro* shoot tip explants of *Decalepis hamiltonii*. It was found that phloroglucinol (PG) had synergistic effect on shoot multiplication when added with N^6-benzyladenine and gibberellic acid. This protocol uses PG for both multiple shoot induction from nodal explants, elongation of primary shoots and initiation of adventitious shoot formation from primary shoots , which was more in presence of triacontanol (TRIA). Maximum number of shoots per culture was observed on the medium containing N^6-benzyladenine (1.1 μM; BA), GA_3 (5.8 μM) and PG (800 μM). Sub-culturing of the shoots onto MS medium containing optimum concentration of BA (5.6 μM), PG (200 μM) and TRIA (0.011 μM) produced elongated shoots along with secondary shoot formation. The long shoots were rooted on α -naphthalene acetic acid (5.38 μM; NAA) and PG (400 μM) containing medium. The rooted plantlets were hardened and their field survival rate was 80 -90%.

Keywords: Axillary bud, Clonal propagation, *Decalepis hamiltonii,* Multiple shoots, Phloroglucinol
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The swallow root *Decalepis hamiltonii* Wight & Arn. is a monogeneric climbing shrub endemic to Deccan peninsula of India, which is used as a culinary spice due to its highly priced aromatic root[1]. It is useful as an appetizer, blood purifier, preservative[2] and as a source of bioinsecticide for stored food grains[3]. Over-exploitation of these plants by injudicious harvesting for the aromatic roots almost endangered this plant in its natural habitat. Measures to develop micropropagation protocols for this endangered shrub with high field survival are essential. Reports about the regeneration of plantlets from leaf callus[4] *of Decalepis hamiltonii* along with protocols for *in vitro* rooting [5] are available. Although, cultural requirements for Decalepis nodal explants are not very strict, they grow very slowly in cultures. In addition, the *in vitro* shoot tips do not readily root in the proliferation or differentiation medium. Growth regulating properties of phenolic compounds and their glycosides are well documented in plant tissue cultures[6]. Phloroglucinol which is 1,3,5 trihydroxybenzene, one of the degradation products of phloridzin, is known for its growth regulating property[7,8]. It even acts as an auxin synergist during the auxin-sensitive phase of root initiation[9]. However, the most interesting aspect of this phenolic compound is its reported ability to enhance survival of, and shoot formation from small tissue explants like meristems, and or shoot tips *in vitro*[10]. There are no reports on the effect of phloroglucinol on *Decalepis hamiltonii* nodal explants culture vis-a-vis micropropagation. The present report describes a highly efficient and reproducible method for clonal propagation of plants from axillary bud explants of *Decalepis hamiltonii* using BA and PG.

Materials and Methods

Preparation of explants—Healthy plants of *Decalepis hamiltonii* Wight & Arn., were collected from Gumballi forest range located between 11° to 13° N and 77° to 78° S in B. R. Hills, Mysore, India. Axillary buds of *D. hamiltonii* were washed under running tap water to remove soil and other superficial contamination. Single bud explants (1 cm each) with upper portion were washed with Tween 20 (5% v/v) for 5 min followed by thorough washing under running tap water for 15 min. The explants were surface sterilized with 0.15% (w/v) mercuric chloride for 3 to 5 min and later rinsed 4 or 5 times with sterile distilled water. Earlier, *in vitro* shoots of *D. hamiltonii* have been regenerated from leaf-derived callus and are maintained in the laboratory[4]. Some of these shoots were maintained on Murashige and Skoog (MS basal medium)[12] for one month. Nodal explants containing

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axillary bud (1 cm each) were cut from the shoots and used for the experiment.

Media preparation and culture conditions—For this experiment in stage I (shoot induction stage), MS basal medium with 3% of sucrose (w/v) was used. The *p*H was adjusted to 5.8 ± 0.2 before gelling with 0.8% (w/v) of agar (Hi Media, India). Explants were cultured in conical flasks (150 ml) covered with non-absorbent cotton plugs and the medium was subsequently autoclaved under 1.06 kg/cm^{-2} at a temperature of 121°C for 15 min. The growth regulators, N^6-benzyladenine (BA; 1.1 μ*M*), gibberellic acid (GA_3; 5.8 μ*M*) were added to MS basal medium and then phloroglucinol (PG; 80-1600 μ*M*) tried individually and in combination to obtain the most suitable level for proliferation of shoots in established explants. The cultures were kept at an incubation temperature of 25° ± 2°C and light (45 μmol m^{-2} s^{-1}) for 16 hr/day using fluorescent lights (Philips India Ltd) for 30 days. PG or BA alone did not induce shoot multiplication or growth. The shoots obtained from nodal explants in stage I were sub-cultured after 30 days onto MS basal containing BA (5.6 μ*M*), PG (200 μ*M*) and triacontanol (TRIA; 0.002-0.114 μ*M*) in stage II. Single shoot explant (2 cm) with a node or nodal explant was kept in each flask and 20 replicates were maintained. The experiment was repeated twice and the observations were made for 45 days for shoot elongation and adventitious shoot formation. To obtain *in vitro* rooting, shoots longer than 4 cm were transferred to the rooting medium (RM) containing MS salts and vitamins along with NAA (2.69-10.76 μ*M*) and PG (200-400 μ*M*) supplemented with sucrose (3%) and agar (0.8% w/v). The experiment was repeated twice with 5 replicates each. Rooted plantlets after 45days growth were removed from the medium, freed of agar by washing in running tap water and planted in sand:compost mixture (1:2) for hardening for 30 days at about 80% RH under polyethylene hoods in the greenhouse and then transplanted in the field. Data were analyzed ursing a one-way analysis (ANOVA) and comparison between the mean values of treatment was made by the least significant difference (LSD) test.

Results and Discussion

It was found that MS medium containing BA and GA_3 was less effective for shoot multiplication (Table 1) as compared to BA (1.1 μ*M*), GA_3 (5.8 μ*M*) and PG (800 μ*M*) that gave rise to a maximum number of shoots per culture (4.0 ± 0.85) with the longest shoot length (5-8 cm), maximum number of nodes (3.0 ± 0.60) and maximum number of leaves (6.0 ± 0.40) after 30 days of culture (Fig. 1a). The proliferation of shoots on the medium with BA + GA_3+ PG started after 6-8 days of culture on this medium. The nodal explants produced multiple shoots from the nodal portion. Only a single shoot per explant was obtained on BA (1.1 μ*M*) and GA_3 (5.8 μ*M*) containing medium. The combination of BA + GA_3+ PG was found to be good, which initiated a maximum of 4-5 shoots which varies with concentration of PG used (Table 1, Fig. 1a). Even the combination of GA_3 and PG, and also PG alone was not at all effective for shoot production from nodal explants. When shoot tip explants without node were inoculated on shoot induction medium the linear growth of shoots was only noticed (data not shown). Similarly, when shoot tip explants with single node inoculated only 1-2 shoots per explant were produced (data not shown). In general, the presence of shoot tip hinders the proliferation of axillary bud for shoot formation. But this study indicates that, in presence of PG the apical dominance

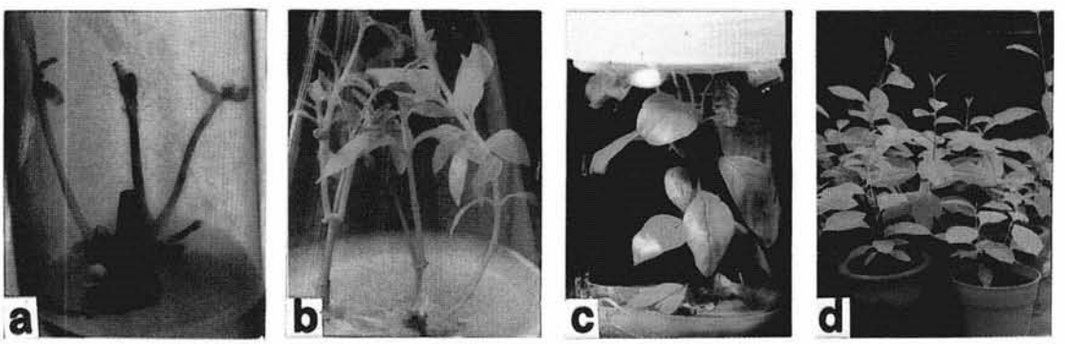

Fig.1— (a) Multiple shoot formation (4-5) from nodal explant on medium containing BA (1.1 μ*M*)+ GA_3 (5.6 μ*M*) + PG (400 μ*M*); (b) Adventitious shoot formation from primary shoot in stage II on medium containing BA (5.6 μ*M*)+ PG (200 μ*M*) + TRIA (0.011 μ*M*); (c) Rooting of *in vitro* shoots on MS medium containing NAA (5.6 μ*M*) + PG (400 μ*M*); and (d) Potted plant ready for field transfer.

of shoot tip explants having single node was overcome, and were able to produce 1-2 shoots from node.

The shoots formed in the primary induction medium with BA (1.1 μ*M*) + GA_3 (5.8 μ*M*) and PG (400-800 μ*M*) were sub-cultured onto MS medium supplemented with BA (5.6 μ*M*) in combination with PG or TRIA alone or in combination (Table 2). Considerable elongation of shoots was achieved on medium with PG (200 μ*M*) in combination with BA and also in presence of TRIA, though lower (100 μ*M*) and higher (300 μ*M*) concentration of PG were not effective (data not shown). On the medium with BA (5.6 μ*M*) in combination with PG (200 μ*M*) and triacontanol (0.011 μ*M*) the formation of more adventitious shoots along with shoot elongation was achieved (Fig. 1b). But only 3-4 shoots were produced on medium containing BA+GA_3+PG. Further, sub-culturing of these shoots onto the shoot elongation medium containing BA (1.1 μ*M*), TRIA (0.011 μ*M*) and PG (200 μ*M*) led to differentiation of additional shoots. Subculturing of both shoot tips having single node and nodal segments containing axillary buds responded well on this medium by producing multiple shoots. Callus formation from base of the explants was mini-

Table 1—Influence of phloroglucinol on shoot formation from nodal explants of *D. hamiltonii in vitro*

MS medium + Hormones (μ*M*)			% response	No. of shoots per explant	% of shoots with shoot length in the range			No. of nodes per explant	No. of leaves per explant
BA	GA_3	PG			A	B	C		
-	-	-	-	-	-	-	-	-	-
1.1	5.8	-	75	1.0 ± 0.0	100.0	-	-	0.5 ± 0.04	1.0 ± 0.04
1.1	5.8	80	90	1.3 ± 0.47[a]	100.0			1.67 ± 0.45[a]	3.33 ± 0.97[c]
1.1	5.8	400	100	1.65 ± 0.58[b]	50.0	50.0	-	2.05 ± 0.39[a]	3.85 ± 0.93[c]
1.1	5.8	800	90	4.0 ± 0.85[b]	16.6	44.4	38.9	2.0 ± 0.60[b]	4.0 ± 0.28[b]
1.1	5.8	1200	30	1.16 ± 0.40[a]	100.0	-	-	1.16 ± 0.40[b]	2.3± 0.40[a]
1.1	5.8	1600	-	-	-	-	-	-	-
-	5.8	80	50	1.0 ± 0.2[a]	100.0	-	-	1.2 ± 0.42[a]	2.4 ± 0.84[b]
-	5.8	400	60	1.1 ± 0.05[a]	100.0	-	-	0.75 ± 0.45[b]	1.5 ± 0.90[c]
-	5.8	800	-	-	-	-	-	-	-
-	5.8	1200	-	-	-	-	-	-	-
-	5.8	1600	-	-	-	-	-	-	-
1.1	-	80	45	1.0 ± 0.2[a]	100.0	-	-	-	-
1.1	-	400	15	1.0 ± 0.2[a]	100.0	-	-	-	-
1.1	-	800	-	-	-	-	-	-	-
1.1	-	1200	-	-	-	-	-	-	-
1.1	-	1600	-	-	-	-	-	-	-
-	-	80	-	-	-	-	-	-	-
-	-	400	-	-	-	-	-	-	-

Shoot length range A= 0.5-3.0 cm; B= 3-5 cm; and C=5-8 cm. *P* values[a] < 0.01,[b] < 0.05,[c] < 0.001 compared to explants cultured on medium with BA (1.1 μ*M*) + GA_3 (5.8 μ*M*), data recorded after 45 days

Table 2—Secondary shoot formation from primary shoots of *Decalepis hamiltonii in vitro*

MS medium + Hormones (μ*M*)			% response	No. of shoots per explant	Shoot length (cm)	No. of nodes per explant
BA	PG	TA				
0	0	0	-	-	-	-
5.6	0	0	60.0	1.0 ±0.2	1.8 ±0.32	1.2 ±0.28
5.6	200	0	60.0	1.0 ±0.05[a]	1.6 ±0.54[a]	1.0 ±0.12[a]
5.6	0	0.002	70.0	1.0 ±0.2[a]	2.0 ± 0.45[b]	1.0 ±0.40[b]
5.6	0	0.004	70.0	2.2 ±0.5[b]	2.2 ±0.75[b]	1.0 ±0.05[a]
5.6	0	0.011	70.0	1.5 ±0.42[a]	1.8 ±0.24[a]	1.0 ±0.20[b]
5.6	0	0.114	40.0	1.0 ±0.64[b]	2.1 ±0.32[a]	1.0 ±0.20[a]
5.6	200	0.002	80.0	3.5 ±0.85[c]	4.2 ± 0.54[c]	3.0 ±0.34[a]
5.6	200	0.004	80.0	4.8 ±0.35[c]	4.9 ±0.62[a]	4.0 ±0.72[c]
5.6	200	0.011	100.0	6.0 ±0.28[a]	8.5 ±0.44[c]	4.8 ±0.45[c]
5.6	200	0.114	60.0	2.5 ±0.12[a]	4.2 ±0.62[c]	3.2 ±0.52[b]

P values:[a] < 0.01,[b] < 0.05,[c] < 0.001 compared to explants cultured on medium with BA (5.5 μ*M*), data recorded after 45 days

Table 3—*In vitro* rooting of regenerated shoots of *D. hamiltonii*

MS medium + Hormones (μ*M*)		% response	No. of roots per explant	Root length (cm)	% field survival
NAA	PG				
0	0	-	-	-	-
2.69	0	40	2.0 ± 0.20	0.8 ±0.05	40-50
5.38	0	60	2.4 ±0.28[a]	1.2 ±0.09[a]	50-60
10.76	0	70	2.2 ±0.16[a]	1.6±0.12[b]	50-60
5.38	200	100	4.0 ±0.12[b]	2.0 ±0.22[b]	80-90
5.38	400	100	3.5 ±0.24[b]	3.2 ±0.28[a]	70-80
5.38	800	60	2.0 ±0.09[a]	1.5 ±0.11[a]	50-60

P values:[a]< 0.01,[b]< 0.05,[c]< 0.001 compared to shoots cultured on medium with NAA (2.69 μ*M*). Data was recorded after 45 days

mal in case of a medium containing optimum concentration of PG (200 μ*M*) + BA (5.6 μ*M*) against higher concentration of PG (800 μ*M*).

Elongated shoots (6-8 cm) were transferred to MS medium supplemented with NAA (2.69-10.76 μ*M*) alone or in combination with PG (20-400 μ*M*) for rooting. It was observed that the shoots were rooted after 25-30 days with good root proliferation (Table 3). NAA at 5.38 μ*M* concentration in combination with PG (400 μ*M*) promoted thick roots (Fig. 1c), with better rooting response and complete plantlets developed in 5-6 weeks in 100% of the shoots. The rooted plants were transplanted *ex vitro* and raised in pots under green house conditions for one month, followed by their field transfer (Fig. 1d). Approximately 80-90% of the plantlets survived. The present study showed that PG had a beneficial effect on *Decalepis hamiltonii* nodal explants. It induced multiple shoot formation with enhanced axillary shoot proliferation from nodal explants in stage I and shoot elongation in stage II of growth. The effectiveness of certain phenolic substances in enhancing shoot formation is well established in plant tissue cultures[6,11,13]. In all these experiments, the results were based on testing of only single concentration (0.5-1.0 m*M*) of PG. In comparison, a wide range of PG was tested in the present experiment. For the first time the present study clearly demonstrated that PG significantly interacted with BA and GA_3 for axillary shoot proliferation during Decalepis nodal explant cultures *in vitro*. In general, auxins such as NAA and IAA in combination with cytokinin (BA) are required to achieve shoot initiation and shoot elongation as described in various plant systems[14,15]. In the present study, influence of PG and BA+ GA_3 combination was found to be superior for shoot multiplication and shoot growth, with least callusing from base of the explant within 4-6 weeks. According to earlier report[4], a maximum of 3-4 shoots have been obtained from nodal explants by using both cytokinin and auxins. Apart from this basal callusing from explants hindered further growth of shoots. In the present report, the optimum concentration of PG in combination with BA+TRIA employed, curtailed to a greater extent callus formation from the base of the explants, moreover explants produced more number of multiple shoots. So the results of the present study indicated that PG at lower concentration appeared to induce shoot bud growth and in combination with BA+GA_3 yielded the best results, shoot initiation and their subsequent elongation upon subculturing in *D. hamiltonii*. Another breakthrough in this study was elongation of shoot along with adventitious shoot formation from primary shoots in presence of low concentration of BA+PG during shoot elongation stage that was further increased in presence of low concentration of TRIA (stage II). The promotive effect of PG on rooting has been identified in several plant species[7,8,16,17]. In all these reports, PG favoured rooting only in the presence of an auxin, which was similar in case of *Decalepis hamiltonii* too. The beneficial effect of PG, a tri-hydroxy phenol, on rooting of *Decalepis* shoots, as presented in the study may be due to its ability to depress the peroxidase activity within the cultured shoot tips, thereby protecting the auxin from peroxidase-catalyzed oxidation[18]. Similarly, the growth promoting nature of TRIA in this study is further supported by earlier reports on other plant systems[19,20]. From the present experiment, it was clear that PG could favourably be used to promote axillary shoot proliferation of nodal explants in culture and also *in vitro* rooting. The obvious effect of PG in producing multiple shoots, subsequent elongation of the shoots along with adventitious shoots formation in presence of TRIA in D. hamiltonii has been reported here for the first time and this clonal propagation method is highly useful for providing large number of plantlets.

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