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Micropropagation prospective of cotyledonary explants of *Decalepis hamiltonii* Wight & Arn.—An endangered edible species

P Samydurai[1]*, M Saradha[2], R Ramakrishnan[1], S Santhosh Kumar[1] and V Thangapandian[1]

[1]PG and Research Department of Botany, Kongunadu Arts and Science College (Autonomous), Coimbatore 641 029, India
[2]Department of Botany, Nirmala College for Women (Autonomous), Coimbatore 641 018, India

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The study was undertaken to standardize the development of callus, shoot and root regeneration from cotyledonary explant of *Decalepis hamiltonii* Wight & Arn. through the tissue culture techniques. The MS medium supplemented with 6-benzyl amino purine (BA), 2,4-dichlorophenoxy acetic acid (2,4-D), kinetin (Kn), gibberelic acid (GA_3), indole acetic acid (IAA), indole butyric acid (IBA) and 1-naphthalene acetic acid (NAA) was used for callus, shoot and root regeneration. The maximum percentage (82.0%) of callus formation was achieved on 0.5 mg/L BA in combination with 0.05 mg/L Kn, followed by 78.5% of callus formation on 0.5 mg/L 2,4-D fortified with 0.05 mg/L Kn. The highest shoot proliferation (4.6 shoots/callus) and shoot length (6.9 cm) was achieved on 1.0 mg/L BA combined with 0.1 mg/L GA_3, followed by 3.8 shoots per callus and 5.8 cm shoot length on 1.0 mg/L IAA combined with 0.1 mg/L GA_3. The highest root formation (38.2 roots/shoot) and root length (11.8cm) was achieved on ½ strength MS medium fortified with 0.4 mg/L IBA, followed by 36.5 roots per shoot and root length of 10.7 cm on 0.4 mg/L NAA. The well-developed rooted plantlets were hardened in the mixtures of forest soil, soil and vermiculite (1:1:1) and 97.5% plantlets survived after hardening.

Keywords: Benzylamino purine, callus induction, dormancy, germination, stratification

Introduction

Global concern about loss of valuable genetic resources prompted international action. Thus programmes for conservation of plant genetic resources for food and agriculture had been initiated and gene banks were established in many countries[1]. The main objective was to maintain genetic diversity in order to ensure its continuous availability to meet the needs of different users. The concept of germplasm conservation demands that the collection methods initially capture maximum variation and subsequently conservation and regeneration techniques minimize losses of wild resources[2]. Advancement in plant biotechnology provides new options for collection, multiplication and short to long-term conservation of plant biodiversity by using *in vitro* culture techniques. Significant progress has been made for conserving endangered and rare ornamental crops, medicinal and forest species, especially for non-viable seeds, and vegetative propagation of plants of temperate and tropical origin[3]. Cell and tissue culture techniques ensure rapid multiplication and production of plant material under aseptic conditions. It ensures safe and cost-effective long-term conservation of a wide range of plant species. These techniques were established and validated on large scale by genetically diverse accessions, which is increasing steadily[4].

Decalepis hamiltonii Wight & Arn. (Family: Asclepiadaceae) is an endemic and endangered traditional food resource plant in Deccan peninsular of Central Tamil Nadu, Andra Pradesh and Karnataka states of India. The plant is distributed at an elevation of 720-1200 m and growing in the crevices of rocks. The monoliform tuberous roots of the plant are highly aromatic and the native tribes use it as an effective appetizer, blood purifier, rejuvenating tonic, and in peptic ulcer and cancer-like affliction[5]. Recent pharmacological investigations on the root extract revealed its antioxidant, anti-inflammatory and anticancer properties[6,7]. The phytochemical constituents of the tuberous roots are closely similar to that of *Hemidesmus indicus* an allied species of *D. hamiltonii*. Both are widely used in the traditional system of medicine as a blood purifier and as a flavoring agent for the preparation of soft drinks and bakery products[8]. They are not only used as food but also consumed to maintain health status and are being staple foods of inhabitant tribes of the Kolli hills[9]. It is interesting to note that a recent investigation has revealed the potent insecticidal activity of tuberous roots of *D. hamiltonii*[10].

*Author for correspondence:
samydurai.bio@gmail.com

The destructive harvesting of the tuberous roots of *D. hamiltonii* for vegetative propagation seems not be a feasible strategy because there is always a possibility of losing the mother plant during tribal collection. Further, seeds show less viability and dormancy, and poor seed germination because of a fungal infection[11]. Considering the urgent need for *ex situ* conservation, an *in vitro* propagation system was recently achieved through shoot formation from field-derived nodal explants of *D. hamiltonii*[12]. The present investigation was carried out to develop a standard procedure for *in vitro* production of callus, shoot and root regeneration from cotyledonary explants of *D. hamiltonii*.

Materials and Methods

Plant Material

The matured fruits of *D. hamiltonii* plants were collected from the different localities of Eastern Ghats of Tamil Nadu, India. After drying, the fruits were broke-open, seeds were collected in glass bottles and stored at 23°C.

Surface Sterilization of Seeds

The matured seeds were thoroughly washed under running tap water for 20 min, followed by 1 mL/L Tween 20 for 15 min, 3-5 rinses with sterile double distilled water for every 3 min, finally using 0.1% (w/v) mercuric chloride for 5 min. After sterilization, the seeds were cut longitudinally. The excised cotyledons were used as explants.

Culture Medium and Conditions

The MS medium[13] was prepared from stock solutions of macro, micro, iron, vitamin and plant growth regulators. Further, it was supplemented with 30 g/L sucrose. The medium was adjusted to pH 5.7 before adding agar 7.0 g/L or phytagel 2.5 g/L. The medium was then autoclaved at 121°C and 1.5 kg cm^2 for 25 min. The cultures were kept at 24±2°C under a 12 h photoperiod at a light intensity of 37.037 µmol m^{-2} s^{-1} from cool florescent light tubes (Model LIFEMAX-A 73, Philips India Ltd., India).

Callus Induction

Cotyledon explants were inoculated on MS medium containing different concentrations (0.5-4.0 mg/L) of growth regulators, 6-benzylaminopurine (BA), 2,4-dichlorophenoxy acetic acid (2,4-D) and thidiazuron (TDZ) in combination with (0.05-0.4 mg/L) of kinetin (Kn) for callus induction. After 4 wk of inoculation, the culture response was recorded.

Shoot Regeneration

The callus was subcultured on MS medium supplemented with different concentrations (0.5-4.0 mg/L) of BAP and indole acetic acid (IAA) in combinations with (0.05-0.4 mg/L) GA_3 for shoot regeneration. After 8 wk of inoculation, the number and length (cm) of shoots were recorded.

Root Induction and Acclimatization

Actively growing shoots were inoculated at various concentrations (0.2-1.0 mg/L) of indolebutyric acid (IBA) and naphthalene acetic acid (NAA) for root induction. After 8 wk of incubation, the number and length (cm) of roots were recorded. The rooted plantlets were removed from the cultures and washed several times to remove the adhering medium. Then plantlets were individually potted in the hardening media as given in the Table 4 and placed in growth room with about 80-90% relative humidity for 2 wk. The plantlets were kept under greenhouse for 2 wk for acclimatization and transferred to field.

Statistical Analysis

All the concentrations were replicated in 20 culture bottles and experiments were repeated at least three times. Analysis of variance (ANOVA) of different parameters was performed and mean and SD were compared by the Duncan's Multiple Range Test (DMRT).

Results and Discussion

Indirect Organogenesis of Callus

The lower concentrations of auxins and cytokinins could induce a number of calli from the cotyledonary explant of *D. hamiltonii* (Figs 1A & B). The maximum percentage (82.0%) of callus induction was obtained at 0.5 mg/L BA in combination with 0.05 mg/L Kn (Table 1). It was followed by 78.5% of callus formation at 0.5 mg/L 2,4-D with 0.05 mg/L Kn. The moderate percentage (68.1%) of callus formation was observed on MS medium fortified with 0.5 mg/L TDZ in combination with 0.05 mg/L Kn (Table 1). It has been widely reported that organogenesis from the cotyledons was better in a wide variety of plant species compared to any other older parts of the plant, as they have young and actively dividing cells in their cotyledons[14]. In the present study also, maximum percentage of callus and multiple shoots proliferation was achieved through cotyledon-derived callus of *D. hamiltonii*. Similarly, cotyledonary explants of chilli produced highest percentage of callus (96.28%) on MS medium supplemented with 1.0 mg/L Kn and 3.0 mg/L 2, 4-D within short period of 12.66 d[15].

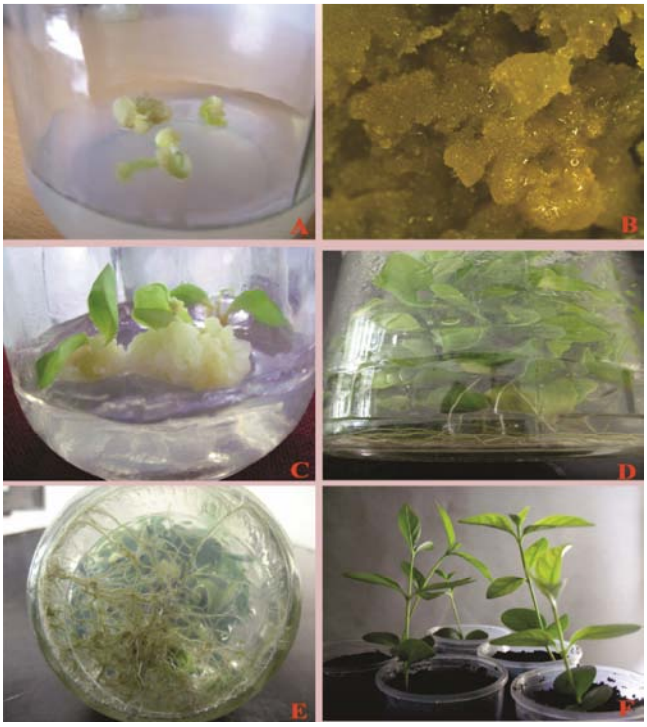

Fig. 1 (A-F)—*In vitro* plant regeneration of *D. hamiltonii* from the cotyledonary explants: A. Initial stage of cotyledonary explants; B. Microscopic view of cotyledonary callus; C. Shoot regeneration from cotyledonary calli; D. Multiple shoot formation; E. Root formation on shoots; & F. Plantlets transferred to pots under controlled condition.

Table 1—Effect of different concentrations and combinations of auxins and cytokinins on callus induction from the cotyledonary explants of *D. hamiltonii*

MS medium plus plant growth regulators (mg/L)				% callus formation
BA	2,4-D	TDZ	Kn	
0.5	0.0	0.0	0.05	82.0 ±0.49^{a}
1.0	0.0	0.0	0.1	72.8±0.83^{cd}
2.0	0.0	0.0	0.2	73.7±0.95^{bc}
3.0	0.0	0.0	0.3	74.2±0.39^{bc}
4.0	0.0	0.0	0.4	73.8±1.46^{bc}
0.0	0.5	0.0	0.05	78.5±0.92^{ab}
0.0	1.0	0.0	0.1	75.3±0.84^{b}
0.0	2.0	0.0	0.2	73.2±0.75^{bc}
0.0	3.0	0.0	0.3	71.9±0.97^{cd}
0.0	4.0	0.0	0.4	69.1±1.05^{e}
0.0	0.0	0.5	0.05	68.1±0.21^{ef}
0.0	0.0	1.0	0.1	65.6±0.40^{g}
0.0	0.0	2.0	0.2	63.2±1.59^{h}
0.0	0.0	3.0	0.3	51.8±0.65^{i}
0.0	0.0	4.0	0.4	49.5±0.91^{j}

Values are mean±SD (*n=20*)
Mean values followed by different superscripts in a column are significantly different (*P<0.05*) according to Duncan's multiple range tests (DMRT)

Shoot Organogenesis

Influence of different concentrations and combinations of BA, IAA and GA_3 were studied on shoot proliferation from the cotyledon-derived callus of *D. hamiltonii* (Figs 1C & D). The maximum number of shoot proliferation (4.6 shoots/callus) and shoot length (6.9 cm) was observed from the callus cultured on MS medium fortified with 1.0 mg/L BA in combination of 0.1 mg/L GA_3, followed by 3.8 shoots per callus and 5.8 cm shoot length on 1.0 mg/L IAA combined with 0.1 mg/L GA_3 (Table 2). Rajanna *et al*[16] achieved maximum number of multiple shoot regeneration from cotyledonary nodal segments of *Bauhinia racemosa* using lower concentrations of cytokinins, 1.5 mg/L BA with 1.0 mg/L Kn. Singh[17] reported that the combination of two synthetic cytokinins, BA and Kn, promoted cell division and performed other growth regulatory functions in the cultures. Improvement in shoot regeneration with cytokinin and auxin combination was proved in several Asclepiadaceae species, such as, *D. hamiltonii*[18], *Pergularia pallida*[19], *Gymnema elegans*[20] and *Holostemma ada-kodien*[21]. The best efficiency of BAP for maximum shoot induction was also observed in *H. indicus*[22].

Root Organogenesis

The regenerated shoots were transferred onto ½ strength MS medium supplemented with different concentrations of IBA and NAA for root formation

Table 2—Shoot regeneration and proliferation ability of cotyledonary callus of *D. hamiltonii* subcultured on different concentrations of BA, IAA in combination with GA_3.

MS medium plus plant growth regulators (mg/L)			% culture response	No. of shoots/callus	Av. shoot length (cm)
BA	IAA	GA_3			
0.5	0.0	0.05	67.8±0.69^{g}	3.1±0.68^{de}	5.7±0.093^{d}
1.0	0.0	0.1	94.1±0.34^{b}	4.6±0.35^{a}	6.9±0.58^{a}
2.0	0.0	0.2	96.8±1.90^{a}	4.3±0.53^{ab}	6.7±0.63^{ab}
3.0	0.0	0.3	78.6±0.53^{d}	3.5±0.90^{c}	5.8±0.51^{cd}
4.0	0.0	0.4	78.1±0.76^{d}	3.0±0.48^{de}	4.1±0.96^{g}
0.0	0.5	0.05	82.9±0.92^{cd}	3.5±0.67^{cd}	5.7±0.59^{d}
0.0	1.0	0.1	83.1±1.70^{c}	3.8±0.72^{bc}	5.8±1.49^{cd}
0.0	2.0	0.2	73.9±0.09^{e}	3.4±0.058^{cd}	5.3±0.38^{e}
0.0	3.0	0.3	72.1±0.38^{ef}	3.5±0.85^{cd}	5.3±1.72^{ef}
0.0	4.0	0.4	67.2±0.65^{gh}	2.8±0.82^{f}	3.8±0.49^{h}

Values are mean±SD (*n=20*)
Mean values followed by different superscripts in a column are significantly different (*P<0.05*) according to Duncan's multiple range tests (DMRT)

Table 3—Regeneration of roots from the cotyledon derived callus shoots of *D. hamiltonii* on different concentrations of auxins

½ MS medium plus plant growth regulators (mg/L)		% rooting response	No. of roots/shoot	Av. root length (cm)
IBA	NAA			
0.2	0.0	89.7±0.94^{c}	33.3±0.06^{bc}	11.2±0.72^{ab}
0.4	0.0	93.3±1.95^{a}	38.2±0.49^{a}	11.8±0.62^{a}
0.6	0.0	75.6±1.50^{f}	30.1±1.94^{cd}	7.3±0.078^{d}
0.8	0.0	63.1±0.32^{h}	28.0±0.43^{ef}	7.1±0.49^{ef}
1.0	0.0	55.7±0.84^{i}	23.2±1.84^{h}	6.8±0.72^{g}
0.0	0.2	84.1±0.49^{d}	31.3±1.80^{cd}	9.7±1.80^{cd}
0.0	0.4	91.1±0.084^{ab}	36.5±0.43^{ab}	10.7±1.40^{b}
0.0	0.6	90.2±0.39^{bc}	34.3±1.72^{b}	10.1±0.09^{bc}
0.0	0.8	78.9±0.71^{ef}	30.3±0.59^{c}	9.9±0.39^{c}
0.0	1.0	71.3±0.49^{g}	28.3±0.93^{g}	7.2±0.75^{e}

Values are mean±SD (*n=20*)
Mean values followed by different superscripts in a column are significantly different (*P<0.05*) according to Duncan's multiple range tests (DMRT)

(Fig. 1E). The maximum number of roots/shoot (38.2) and length of roots (11.8 cm) were recorded on 0.4 mg/L IBA, followed by 36.5 roots/shoot with 10.7 cm root length on 0.6 mg/L NAA (Table 3). It is clear from present results that lower concentrations of IBA and NAA induced the maximum number of roots and root length of *D. hamiltonii*. Another Asclepiadaceae member, *H. annulare* was cultured on ½ strength MS medium supplemented with 1.48 µM IBA for the best root proliferation[23]. Root sprouting quality of IBA was also proved in *Ceropegia bulbosa*[24], *C. bulbosa* var. *lushi*[24], *C. jainii*[24], *H. indicus*[22] and *Hoya* w*ightii* f. sp. *palniensis*[25]. Similarly Krishnakumar *et al*[26] cultured *in vitro* regenerated shoots of *Calamus travancoricus* on the ½ strength MS medium supplemented with 1.5 mg/L IBA for subsequent root sprouting.

Acclimatization

Hardening is a vital process for transplantation of plant from laboratory to land. The well-developed regenerated plantlets of *D. hamiltonii* were acclimatized (Fig. 1F). The highest survivability rate (97.5%) was observed in cotyledonary callus derived plantlets on the hardening medium composed of forest soil, soil and vermiculite in the ratio of 1:1:1 (Table 4).

Conclusion

The present study highlights the need to investigate the effective and efficient micropropagation protocols for commercially and medicinally important, especially for endangered, edible ethnomedicinal plant species of *D. hamiltonii*. Optimizing environmental conditions could increase growth rate, reduce labour costs and thereby reduces the production costs. It is expected that the increase in multiplication rate within a short period and easy acclimatization process would make this protocol as a highly advantageous one.

Table 4—The survivability of the plantlets derived from the cotyledon callus of *D. hamiltonii* on different hardening media

No.	Hardening medium	Composition (v/v/v) or (v/v)	Total no. of plantlets potted	No. of plantlets survived	% survivability
1	Sand:Soil: ermiculite	1:1:1	40	37	92.5±1.34^{b}
2	Forest soil:Soil: Vermiculite	1:1:1	40	39	97.5±1.21^{a}
3	Garden soil:Soil: Vermiculite	1:1:1	40	36	90.0±0.94^{bc}
4	Sand:Soil	1:1	40	33	82.5±1.29^{c}
5	Forest soil:Sand	1:1	40	37	92.5±0.87^{b}

Values are mean±SD (*n=40*)
Mean values followed by different superscripts in a column are significantly different (*P<0.05*) according to Duncan's multiple range tests (DMRT)

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