ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 13, Issue 4, 2020

Online - 2455-3891
Print - 0974-2441

Research Article

IN VITRO SHOOT REGENERATION OF SWALLOW ROOT (*DECALEPIS HAMILTONII*) – A STENO-ENDEMIC RED LISTED MEDICINAL PLANT

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Received: 27 December 2019, Revised and Accepted: 14 February 2020

ABSTRACT

Objective: *In vitro* shoot regeneration of *Decalepis hamiltonii* Wight & Arn. is an endangered endemic medicinal plant using biotechnological interventions and to conserve this threatened species.

Methods: In the present study, various explants such as shoot tip, leaf, and nodal segments were inoculated on Murashige and Skoog media augmented with different hormonal regimes of auxin and cytokinin combinations, namely, naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), benzyl adenine (BAP), 6-(γ,γ-Dimethylallylamino)purine (2iP), and triacontanol (TRIA).

Results: Direct regeneration of shoots obtained in 3.0 mg/l 2iP alone and in combination with 0.1 mg/l IAA and/or 1.0 mg/l BAP exhibited the best response with average shootlet length being 6.5±0.17–8.0±0.92 cm, respectively, and percentage response was between 68% and 75%. The callus induced regeneration was obtained from both nodal and leaf explants with maximum response (85%) observed in combination of (2.0 mg/l) 2iP, (1.0 mg/l) IAA and (2.0 mg/l) kinetin with multiple shoots showing mean shoot number of 1.83 and average shootlet length of 6.3±0.19 cm.

Conclusions: The current research provides a competent *in vitro* propagation method for *Decalepis* which could be commercialized for developing identical plants with good mass multiplication rate and for better conservation of the germplasm.

Keywords: Regeneration, *Decalepis*, 2-Hydroxy-4-methoxybenzaldehyde, Apocynaceae, 6-(γ,γ-Dimethylallylamino)purine.

 DOI: http://dx.doi.org/10.22159/ajpcr.2020.v13i4.36714

INTRODUCTION

In recent years, *in vitro* plant regeneration or biotechnological interventions are exceptionally benevolent in conserving endangered, endemic medicinal plants with high prudent significance. *Decalepis hamiltonii* Wight & Arn. (Apocynaceae) is a monotypic species of the genus *Decalepis*, steno-endemic to Western Ghats of India and the Deccan Peninsula. The wild destructive harvesting of medicinally significant, tuberous fleshy roots of *D. hamiltonii* has threatened its extinction and has been included recently in IUCN Red List [1,2].

D. hamiltonii is a conglomeration of biomolecules with antioxidant activity and a well-known source for edible plant nutraceuticals. *Decalepis* is well recognized for its potential application as antidiabetic, hepatoprotective, antiatherosclerotic, antimicrobial, and in the treatment of other ailments [3,4]. The bioactive compounds present in *D. hamiltonii* have not been explored [5]. The dry roots of *Decalepis* sp. is envisaged for the production of various bioactive compounds; key component being a flavoring compound, 2-Hydroxy-4-methoxybenzaldehyde (2-HMB) (96.29%) with other essential oils [6] and adjuvants in traces [4]. In recent years, many efforts have been documented on micropropagation and regeneration of this important genus, but the recalcitrant nature has led to meager reproducibility. *In vitro* regeneration [7-9] in *D. hamiltonii* is meager because of its genotype specificity. Tissue culture plants of *D. hamiltonii* have been reported to enhanced levels of 2-HMB in the roots [9]. Hence, the current research was undertaken to demonstrate the *in vitro* regeneration capability of *D. hamiltonii* through direct and indirect regeneration methods.

METHODS

Culture conditions for callus induction, direct and indirect regeneration

D. hamiltonii is an endangered plant, endemic to Western Ghats of India. *Decalepis* mother plant was obtained from the Foundation for Revitalization of Local Health Traditions, Bengaluru. For *in vitro* regeneration healthy nodal segments, shoot tip and young tender leaves were used as explants. The explants were initially washed under running tap water for 30 min with few drops of Tween 20 and were treated with 1% Bavistin (carbendazim 50% WP) for 30 min. After thorough washing in distilled water to remove fungicide, explants were surface sterilized aseptically for a minute with mercuric chloride (0.1%) followed by several washes with autoclaved distilled water. The explants were finally sterilized with 70% alcohol (v/v) for 30 s along with thorough wash in sterile distilled water. The explants were further cultured on Murashige and Skoog (MS) medium supplemented with various hormonal regimes [10].

Direct regeneration

For direct regeneration, nodal and shoot tip explants were selected and cultured initially on basal MS medium supplemented with varied concentrations of benzyl adenine (BAP) 0–5 mg/l, 0.1 mg/l naphthalene acetic acid (NAA), and 5.0 µg/l triacontanol (TRIA) (Table 1). All the cultures were maintained at 26±2°C under white fluorescent light with 16/8 h photoperiod and subcultured regularly at 3 weeks interval.

Indirect regeneration

For indirect regeneration, young tender leaf and nodal explants were used as the explants and were cultured on MS basal medium augmented with different concentration of auxins, 1 mg/l indole-3- acetic acid (IAA), 0.1 mg/l NAA, and cytokinins 2–4 mg/l 6-(γ, γ-Dimethylallylamino) purine (2iP), 2 mg/l kinetin, 1–7 mg/l BAP, and 6 mg/l zeatin. The callus obtained from leaf and nodal explants after inoculation for a period of 20–25 days was further subcultured on shoot regeneration medium with MS medium supplemented with various concentrations of plant growth regulators (Table 2). Initially, cultures were maintained in dark for a week and later shifted to white fluorescent light with 16/8 h photoperiod at 26±2°C with regular subculturing at every 3 weeks.

Table 1: Effect of plant growth regulators on *in vitro* direct regeneration of *Decalepis hamiltonii* from nodal explants

Plant growth regulators				% response	Mean length of shoot (cm)
NAA (mg/l)	BAP (mg/l)	2iP (mg/l)	Zeatin (µg/l)		
0.1	-	-	-	48	4.0±0.42
0.1	0.1	-	-	65	4.8±0.52
0.1	0.5	-	-	70	6.7±0.45
0.1	1.0	-	-	68	4.8±1.13
0.1	2.0	-	-	67	3.2±0.45
0.1	3.0	-	-	55	1.8±0.24
0.1	4.0	-	-	40	1.6±0.38
0.1	5.0	-	-	57	5.0±0.29
0.1	1.0	3.0	-	75	8.0±0.92
-	7.0	-	-	35	2.1±0.02
0.1	-	3.0	-	68	6.5±0.17
-	-	2.0	-	70	6.8±0.34
0.1	-	-	6.0	42	8.3±0.88

Mean length of shoot (cm): Mean±standard error after 8 weeks of inoculation. NAA: Naphthalene acetic acid, BAP: Benzyl adenine, 2iP: 6-(γ,γ-Dimethylallylamino)purine

Table 2: Effect of plant growth modulators on *in vitro* indirect regeneration of *Decalepis hamiltonii* from leaf and nodal explants

Plant growth regulators							% response	Mean number of shoot	Mean length of shoot (cm)
NAA (mg/l)	IAA (mg/l)	BAP (mg/l)	TRIA (µg/l)	2iP (mg/l)	Zeatin (mg/l)	Kinetin (mg/l)			
0.1	-	1.0	-	-	-	-	75	1	6.0±0.92
0.1	-	2.0	-	-	-	-	63	1	4.3±0.21
0.1	-	3.0	-	-	-	-	45	1	2.8±0.13
0.1	-	0.2	5.0	-	-	-	79	1	5.0±0.25
0.1	-	0.3	5.0	-	-	-	82	1	5.5±0.41
0.1	00	7.0	-	-	-	-	35	1	2.1±0.02
0.1	-	-	-	-	6.0	-	45	1	6.0±0.88
-	-	-	-	2.0	-	-	84	1	6.5±0.05
-	-	-	-	3.0	-	-	68	1	6.3±0.87
-	-	-	-	4.0	-	-	72	1	5.5±0.28
-	1.0	-	-	2.0	-	2.0	85	1.8	6.2±0.19
-	1.0	2.0	-	-	-	2.0	66	1	4.2±0.21

Mean length of shoot (cm): (Mean±standard error) after 8 weeks inoculation to shooting medium. NAA: Naphthalene acetic acid, IAA: Indole-3-acetic acid, BAP: Benzyl adenine, TRIA: Triacontanol, 2iP: 6-(γ,γ-Dimethylallylamino)purine

Statistical analysis

The experiments were repeated thrice with 10 explants for each combination. Analysis of variance and mean separations were carried out using Tukey's test at 0.05% level of significance.

RESULTS AND DISCUSSION

In recent years, *D. hamiltonii* has been red listed as endangered and endemic plant. The presence of economically important bioactive compounds in *Decalepis* has been the reason for its continuous and extensive exploitation. Hence, the need of an hour is to conserve this important medicinal plant from being extinct, so *in vitro* culture technique offers an alternative strategy to conserve this endangered endemic plant [11]. The development of efficient *in vitro* micropropagation method emphasizes a prime role in crop improvement. In the current manuscript, a reproductive, commercially viable plant regeneration method was optimized for *D. hamiltonii* using cytokinins and auxins based plant growth regulators.

The direct plant regeneration was observed from both nodal explants and shoot tip explants. Shoot induction was recorded after 8 weeks of culturing. All the hormonal compositions studied, responded to shoot regeneration on MS medium along with supplements. The different concentrations of 0.1–7 mg/l BAP were used in combination with 0.1 mg/l NAA (Fig. 1). The BAP concentrations of 0.1 and 0.5 mg/l along with NAA 0.1 mg/l resulted in 65% and 70% regeneration response with mean shoot length of 6.7±0.45 cm and 4.8±1.13 cm, respectively (Table 1), and BAP is the most frequently used synthetic cytokinins supplement in plant regeneration protocols. The combinations of cytokinins are reported to be best in many studies for the induction of axillary shoots [11]. Veerabathini *et al*. [12] observed maximum friable callus development in *Catharanthus roseus* on the MS medium supplemented with 1.0 mg/L BAP+1.0 mg/L NAA. The combination of BAP with indolebutyric acid is proved best in direct regeneration response in micropropagation of many plants like *Aloe vera* [13]. In line with our findings, Saritha and Naidu [14] also obtained adventitious shoots directly from leaf explants of *Spilanthes acmella*, on the MS media incorporated with 1.0 mg/l BAP and 0.1 mg/l NAA combination and similarly in *Ceropegia bulbosa* Roxb. (Asclepiadaceae) [15]. Correspondingly, multiple shoot regenerations were observed in 3.0 mg/l BAP and 0.5 mg/l NAA from leaf and nodal explants in *Centella asiatica* L. [16]. With the increased concentration of 7 mg/l BAP, the percentage of shoot response was decreased (42%) (Table 1). The shoot tip culture when inoculated on MS medium with 6 mg/l zeatin (Fig. 1e), the length of the shoot reported was 8.3±0.88 cm, but the percentage of response was low (42%). Ravanfar *et al*. [17] obtained a direct *in vitro* shooting in cabbage with 2 mg/l zeatin and in sweet potato cv. Brondal using 0.2 g/l zeatin [18]. Augmentation of 2.0 mg/l 2iP alone (Fig. 1a) and in combination with 1.0 mg/l BAP and/or 0.1 mg/l NAA (Fig. 1c and 1f) in the regeneration media showed best results in terms of average shoot length being 6.5±0.17–8.0±0.92 cm and also percentage response 68–75% both single and multiple shoots from a single node (Table 1). Shinde *et al*. [19] observed that MS media incorporated with 2.5 µM BAP and 7.5 µM 2iP produced highest frequency 83.3% of regenerated adventitious shoots from callus cultures of *Artemisia nilagirica* var. *nilagirica* (Indian wormwood), and in *Pterocarpus santalinus*, 2.5 µM BAP and 2 µM 2iP gave the same trend for regeneration [20].

Callus-based indirect regeneration was obtained from nodal and leaf explants of *D. hamiltonii* (Table 2). The maximum response toward

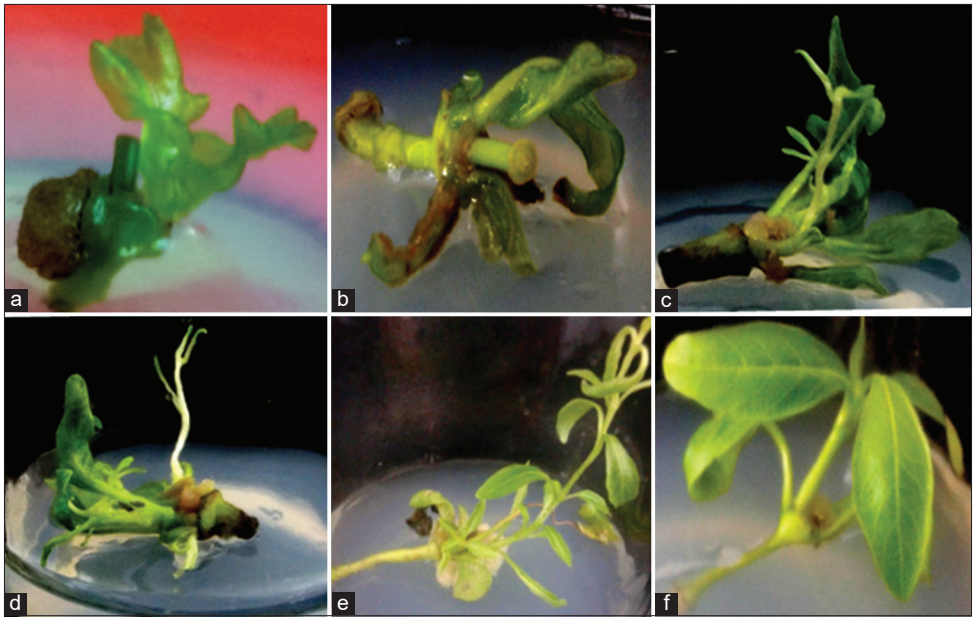

Fig. 1: *In vitro* direct regeneration of *Decalepis hamiltonii* from shoot tip and nodal culture. (a) Shoot regeneration from nodal culture on 2 mg/l 6-(γ,γ-Dimethylallylamino)purine (2iP) supplemented Murashige and Skoog (MS) medium, (b) adventitious shoot induced from nodal explant grown on MS medium supplemented with 7 mg/l benzyl adenine (BAP), (c) multiple shoot induction from shoot tip explant on MS medium with 1 mg/l BAP+3 mg/l 2iP, (d) direct regeneration from shoot tip explant grown on MS medium 3 mg/l 2iP+0.1 mg/l NAA, (e) 6 mg/l zeatin+0.1 mg/l NAA, (f) 3 mg/l 2iP+0.1 mg/l NAA+1 mg/l BAP

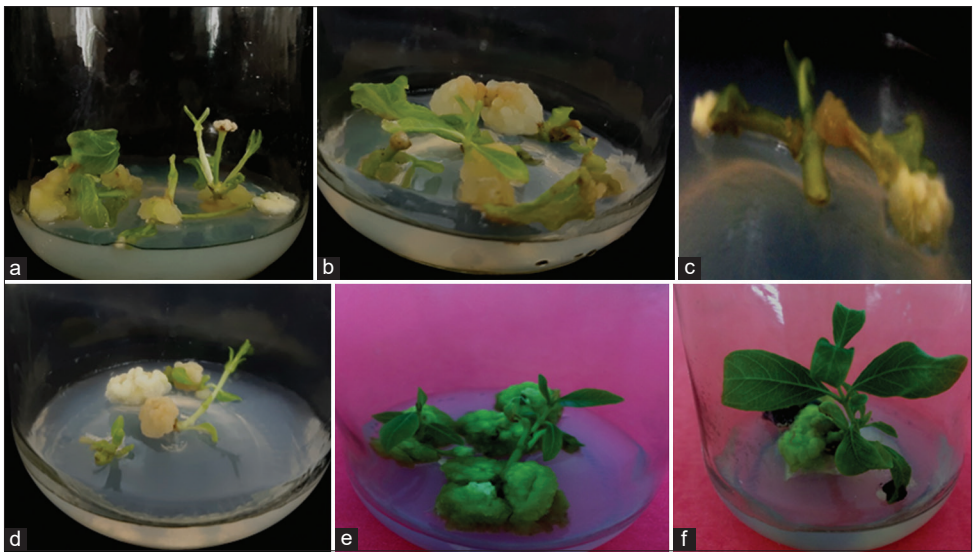

Fig. 2: In direct regeneration in *Decalepis hamiltonii* from leaf and nodal explants from MS basal media augmented with various hormonal regime, (a) multiple shoots from 2 mg/l 6-(γ,γ-Dimethylallylamino)purine (2iP)+2 mg/l Kin+1 mg/l indole-3-acetic acid (IAA), (b) shoot induction from nodal explant on 2 mg/l 2iP, (c) nodal explant grown on Murashige and Skoog medium with 5 µg/l triacontanol and 0.3 mg/l benzyl adenine (BAP) shows shoot initiation and callus induction, (d) shoot initiation from leaf callus on 2 mg/l BAP+2 mg/l Kin+1 mg/l IAA, (e) and (f) shoot regeneration from nodal tissue callus on 3 mg/l 2iP and 4 mg/l 2iP, respectively

regeneration (82%) was observed in combination of 0.1 mg/l NAA, 0.3 mg/l BAP, and 5 µg/l TRIA with little growth (Fig. 2 c and d). Verma *et al*. [21] have evaluated good impact of 2 mg/l TRIA in combination with 3 mg/l BAP on *in vitro* shoot multiplication potential of *Arachis hypogaea* L. Many studies have reported TRIA-mediated complete crop improvement in terms of growth, enhanced enzyme activities, photosynthesis, yield, and nitrogen fixation [22]. In the present study, MS media with 0.1 mg/l NAA and 1.0 mg/l BAP gave a single shoot per explant with 75% of response with mean length of 6.0±0.92 cm. Among various concentrations of 2iP, namely, 2, 3, and 4.0 mg/l for shoot bud regeneration, shooting response exhibited was 8484%(Fig. 2b), 68% (Fig. 2e) and 72% (Fig. 2f), respectively. The combination of 2.0 mg/l kinetin and 1.0 mg/l IAA with 2.0 mg/l 2iP (Fig. 2a) produced multiple shoots with an average shoot of 1.83 per explants and response of 85% showing mean length of 6.3±0.19 cm on par with the other combinations studied (Table 2). In line with our results, shoot regeneration of wheat (*Triticum aestivum* L.) was maximum in MS media containing hormonal combination of 0.5 mg/l 2iP, 0.4 mg/l kinetin, and (0.1 mg/l) IAA [23]. Kinetin has proved to increase the shoot number alone or in combination with BAP or IAA in an important nootropic plant Bacopa monnieri (L) Pennell [24]. The fortification of 2.0 mg/l kinetin and 1 mg/l BAP in MS medium produced maximum shoots in *Gymnema sylvestre* Br. [25]. However, similar composition with 2 mg/l BAP had regeneration response of 66% proving that the

addition of two cytokinins aids in indirect regeneration (Fig. 2d). *In vitro* organogenesis depends on the application of exogenous plant growth regulators. The cytokinin BAP individually 1.0 mg/l also proved 100% efficient in producing maximum shoot proliferation response in *Thevetia neriifolia* [26]. The efficiency of BAP in inducing adventitious shoots over kinetin was reported in *Hemidesmus indicus* [27] and *C. bulbosa* [28]; auxins and cytokinins are able to bring shoot or root formation from callus, but the effective concentrations of these growth regulators may vary. The combination of auxin and cytokinin was found to be effective for shoot bud formation from callus in *Tylophora indica* [29] and *C. bulbosa* [28].

CONCLUSIONS

This investigation provides a competent protocol for genetically reliable shoot induction by supplementation of cytokinins, auxin, and other adjuvants along with callus initiation and proliferation of *D. hamiltonii* a recalcitrant and steno-endemic plant. The current research bestows a proficient *in vitro* propagation method which could be commercialized for developing identical plants with good mass multiplication rate and to conserve germplasm. Further, this study can be extended to overexpress the biosynthetic gene in callus or suspension culture to enhance pharmaceutically and nutraceutically valuable specific secondary metabolites.

ACKNOWLEDGMENT

The authors would like to thank the Department of Biotechnology (DBT), Government of India, for financial support and extend concede to RSST and Rashtreeya Vidyalaya College of Engineering, Bengaluru (affiliated to Visvesvaraya Technological University, Belagavi), for providing R&D support and encouragement.

AUTHORS' CONTRIBUTIONS

Nagashree N Rao and Ashwani Sharma designed research; Manjula Ranganatha and Annapurna AS performed research; Nagashree N Rao, Ashwani Sharma, and Manjula R analyzed data; Nagashree N Rao, Manjula R, and Ashwani Sharma wrote the paper.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SOURCE OF FUNDING

DBT – Government of India.

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