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Evaluation of Genetic Fidelity of in-*Vitro* propagated *Decalepis hamiltonii* Wight & Arn. using DNA Based Marker

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Abstract: *In-vitro culture stress might result in breakdown of control mechanisms causing instability of the genome in tissue cultured plantlets and hence these plantlets have to be subjected to assessment of genetic fidelity. We have used a DNA based molecular marker to assess the genetic stability of in - vitro regenerated Decalepis hamiltonii. The nodal explant responded satisfactory in terms of growth related traits when inoculated in the MS medium supplemented with BA(0.886mg/l)+2ip(0.24mg/l). When screened with 12 Random Amplified Polymorphic DNA (RAPD) primers, it produced clear reproducible and scorable bands. All banding profiles from micropropagated plants were monomorphic and similar to that of the mother plant. This study is of high significance as these could be commercially utilized for large scale production of true-to-type plantlets in D. hamiltonii.*

Key words: *Decalepis hamiltonii, in-vitro propagation, genetic fidelity, RAPD marker.*

I. Introduction

Decalepis hamiltonii Wight & Arn also known as swallow root, is a woody climber belonging to the family Asclepiadaceae. It is found in moist as well as dry deciduous forests of peninsular India (4). It is seen growing along rocky slopes and rock crevices in wild (14). In local languages, this plant is known as Maredu Kommulu, Nannari Kommulu, Madina Kommulu, Barre Sugandhi and Maredu Gaddalu. *D. hemiltonii* roots are known to contain abundant quantity of 2-hydroxy-4-methoxy benzaldehyde (HMB) which has been widely used in many of the medicinal applications (9). *D. hamiltonii* is commonly used in blood purification, wound healing, bronchial asthma, fever, intrinsic haemorrhage, kushtha, erysipelas, poisoning, paediatric rejuvinative and as a general vitaliser in Ayurveda, Siddha and Folk systems of medicines (12). Chewing the roots and drinking Nannari -herbal drink prepared from roots of *D. hemiltonii* is considered as a good digestive aid and appetizer (15). Due to the health promoting properties of its roots, pickles and juices are prepared out of its roots and consumed (6). In various food and pharmaceutical applications, it is used as a preservative due to the bacteriostatic properties of its volatile principle compound HMB (10). Due to the similar aromatic properties, its roots are used as a substitute for *Hemidesmus indicus* in ayurvedic preparation of ancient Indian medicine. It is also used to cure skin diseases, nutritious disorders (2), epilepsy and central nervous system disorders (8). The roots are used as a flavouring principle (16), preservative (11), and is demulcent, diaphoretic and diuretic. It is also used to treat diarrhoea and used as bioinsecticide for stored food grains (5).

With such a wide range of uses, *D. hemiltoni* is being commercially exploited; the plants are being uprooted for their aromatic roots. Hence, the population of this plant has decreased exponentially in the last few years in their natural habitat. Due to several medicinal uses of *D. hemiltonii*, the conservation of this plant is very essential. Further cultivation of these plants is of urgent need to ensure their availability to the industry as well as to people associated with traditional system of medicine. *In- vitro* conservation of these plants is a safe method to protect the species from risk of natural disasters as well as increase their population (1). If steps are not taken for their, mass propagation, cultivation and conservation, they may be lost from the natural habitat forever. Genetic fidelity is one of the most important pre-requisites in the micropropagated plant species. The occurrence of genetic instability arising due to somaclonal variation in the regenerates can seriously limit the utility of the micropropagation system (13). Hence, it is important to establish genetic homogeneity of micropropagated plants to confirm the quality of the plantlets for its commercial utility. In order to help us with such an assessment, in recent years molecular markers such as RAPD are used to analyze any somoclonal variations in the *in- vitro* propagated plants. RAPD markers are universal, ten base pair length primers, are cost effective and easy to use.

II. Materials and Methods

Plant material and cultural conditions

The nodal explants collected from disease-free plants of *D. hamiltonii* raised in glasshouse were thoroughly washed in running tap water with a neutral liquid detergent for 3 to 5 minutes. These explants were

cut into convenient sizes after removal of the leaf sheaths. The cut pieces were surface sterilized with 0.1% mercuric chloride for 4 to 6 minutes in a laminar flow cabinet and rinsed 3 to 4 times in sterile distilled water to remove the traces of sterilants prior to inoculation. Surface disinfected explants were inoculated on basal Murashige and Skoog (MS) medium supplemented with combination of BA, 2ip and NAA. The pH of the medium was adjusted to 5.7 before adding agar and was autoclaved at 121^0 C and 105 kg/cm^2 of pressure for 20 minutes. All the cultures were incubated at 25 ± 10^0 C under white fluorescent light with 50µ mole m^{-2} s^{-2} light intensity during a photoperiod of 16:8 h light and dark cycles.

DNA extraction and PCR amplification conditions

The genomic DNA of *D. hamiltonii* was isolated by following the protocol of Doyle and Doyle (1990) from both *in- vitro* grown plants and *ex- vitro* grown mother plants. Genetic fidelity of *in -vitro* raised plantlets was tested using RAPD marker. For this purpose, 15 *in-vitro* raised and hardened plants were chosen randomly from the population and compared with the mother plant. 12 RAPD primers were used for screening. PCR amplification were carried out in a total volume of 25µl containing 25ng of genomic DNA as template, 2.5ml of 10x assay buffer (100 mM Tris Hcl, pH 8.3, 500mM KCl and 0.1% gelatin), 1.5 mM $MgCl_2$, 200µM dNTPs, 0.5 unit (U) of Taq polymerase and 15ng of primer. The primers showing polymorphic bands were used to analyse the genetic fidelity of *in-vitro* raised plants. PCR amplification was performed in a DNA thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, CA, USA), which was programmed for initial DNA denaturation at 94^0C for 5 min, followed by 30 cycles of 45 seconds denaturation at 94^0C, 45 seconds annealing (temperature specific to the primer) at 37^0C and 45 seconds extension at 72^0C, with a final extension at 72^0C for 8 min. Amplified products were resolved by electrophoresis on 1.8% agarose gel in TAE buffer (40mM Tris base, 20mM, sodium acetate, glacial acetic acid to pH 7.2) stained with ethidium bromide (0.5µg µl-1) for 3h at 60 volts and photographs were taken by using the Gel Documenting system (Bio-Rad, USA).

III. Results and Discussion

***In -vitro* shoot multiplication**

Nodal segments from glasshouse plants of *D. hamiltonii* were used as explant and inoculated to MS media containing varying combinations and concentrations of BA, 2ip and NAA. Effect of different plant growth regulators on growth related traits like shoot length, number of shoots and number of leaves were observed and recorded. Estimation of growth parameters was done after 8 weeks of inoculation. Data were statistically analysed by analysis of variance (ANOVA) and significance was calculated. MS medium supplemented with BA (0.886 mg/l)+2ip(0.24 mg/l) showed significantly high shoot length (2.9 ± 0.18). The same hormonal combination showed significantly high number of shoots (2.7 ± 0.94) as well as high number of leaves per explant (5.8 ± 1.61) (Table. 1 and Fig. 1).

Table. 1: In vitro shoot multiplication in *D. hamiltonii* using different plant growth regulators

Serial No.	MS Media + Growth regulators (mg/l)	Shoot length (Mean ± SD)	No. of shoots/explant (Mean ± SD)	No. of leaves/explant (Mean ± SD)
1	BA(0.443)	2.2 ± 0.14	1.3 ± 0.48	2.7 ± 0.67
2	BA(0.443)+2ip(0.24)	2.5 ± 0.15	1.4 ± 0.69	3.2 ± 0.78
3	BA(0.443)+NAA(0.27)	2.6 ± 0.18	1.4 ± 0.51	3.6 ± 1.26
4	BA(0.886)	2.5 ± 0.20	1.3 ± 0.67	3.8 ± 0.78
5	BA(0.886)+2ip(0.24)	2.9 ± 0.18	2.7 ± 0.94	5.8 ± 1.61
6	BA(0.886)+NAA(0.27)	2.8 ± 0.07	1.5 ± 0.52	4.4 ± 1.07
7	BA(2.22)	2.5 ± 0.17	1.3 ± 0.48	4.2 ± 1.13
8	BA(2.22)+2ip(0.24)	2.8 ± 0.13	2 ± 0.94	5.4 ± 1.83
9	BA(2.22)+NAA(0.27)	2.5 ± 0.19	1.9 ± 0.87	4 ± 1.15
10	BA(5.37)	2.4 ± 0.17	1.7 ± 1.06	4.2 ± 1.03
11	BA(5.37)+2ip(0.24)	2.4 ± 0.16	1.7 ± 1.06	4.2 ± 1.22
12	BA(5.37)+NAA(0.27)	2.4 ± 0.19	1.6 ± 0.96	3.7 ± 0.94
13	BA(10.74)	2.3 ± 0.12	1.8 ± 1.03	4.7 ± 1.33
14	BA(10.74)+2ip(0.24)	2.5 ± 0.11	1.8 ± 0.91	4 ± 1.05
15	BA(10.74)+NAA(0.27)	2.5 ± 0.20	1.7 ± 0.67	4.8 ± 1.03

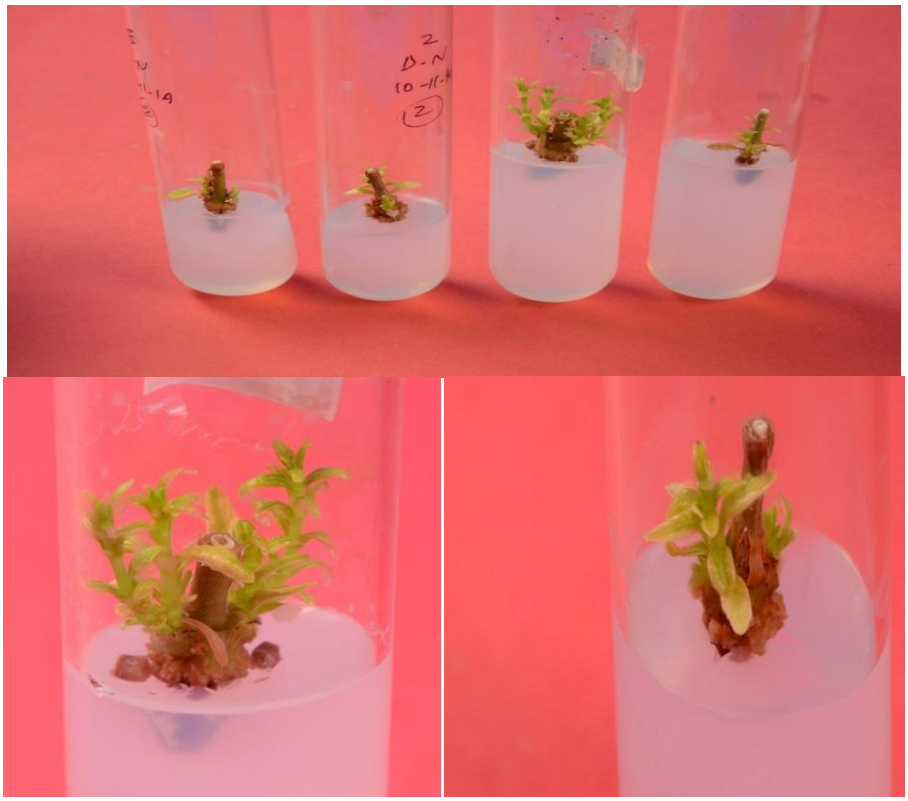

Fig. 1: *In-vitro* raised plantlets of *D. hamiltonii* using nodal explants.

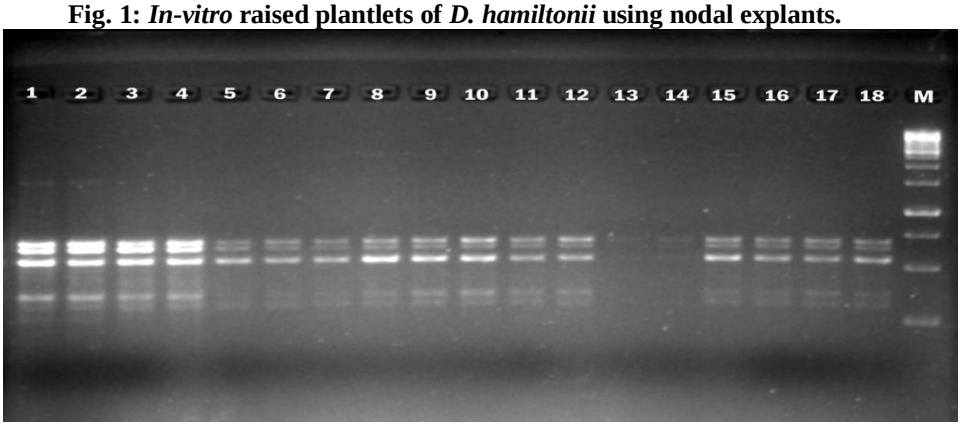

Fig. 2: RAPD banding pattern in both micropropagated and glasshouse grown mother plants of *D. hamiltonii* (Lane 1-3: Mother plant, Lane 4-18: Micropropagated plants and M: Marker)

Table. 2: List of primers, total number of bands and size of amplified fragments generated by RAPD primers in both micropropagated and glasshouse grown mother plants of *D. hamiltonii.*

Sl. No.	Primers	No. of bands produced	Range of amplicons [bp]
1.	OPB-08	5	900-10000
2.	OPC-06	4	450- 3000
3.	OPC-11	6	400- 1500
4.	OPC-16	3	300-1000
5.	OPD-08	8	300-10000
6.	OPD-20	12	250-2300
7.	OPE-01	6	300-10000
8.	OPF-10	4	650-2300
9.	OPG-02	6	650-10000
10.	OPG-06	5	300-650
11.	OPG-14	7	300-10000
12.	OPG-18	4	550-1450
Mean		**5.83**	
Total		**70**	

Assessment of genetic stability

RAPD analysis was done to ascertain the genetic stability of *in-vitro* raised plants of *D. hamiltonii*. RAPD gel profile amplified by the primers OPB-08, OPC-06, OPC-11, OPC-16, OPD-08, OPD-20, OPE-01, OPF-10, OPG-02, OPG-06, OPG-14 and OPG-18 and their size is given in Table.2. All the tried 12 primers gave amplification and total of 70 RAPD bands were generated, which were monomorphic indicating that micropropagated plants were similar to mother plant (Fig. 2). The number of bands resolved per amplification were primer dependent and varied from 3 (OPC-16) to 12 (OPD-20) with an average of 5.83 bands per primer. The size range of amplification products also varied with selected primers as well as the plant from which DNA was isolated and ranged from 250bp to 10000bp (Table 2). Number of monomorphic bands was highest (12) in case of primer OPD-20 (ranging from 250-2300bp in size) and lowest (3) in case of primer OPC-16 (ranging from 300-1000bp in size). The micropropagated plants of *D. hamiltonii* showed a similar profile to that of its mother plant (Fig 2).

Randomly Amplified Polymorphic DNA (RAPD) markers are used to estimate the genetic makeup of micropropagated plants because of its simplicity and cost effectiveness. The present study provides the first report on the genetic fidelity of micropropagated *D. hamiltonii* obtained from nodal explants using RAPD marker. A total of 12 RAPD markers were employed to assess the genetic stability. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant indicating no variation among the *in-vitro* raised plants. The result obtained in our experiment suggest that *in-vitro* shoot multiplication using nodal segment as explant may be used for rapid clonal propagation and conservation with a low risk of somaclonal variation.

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