RESEARCH ARTICLE

GENETIC FIDELITY STUDIES OF *HOLOSTEMMA ADA-KODIEN* SCHULT.– A VULNERABLE MEDICINAL PLANT

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Abstract: *Holostemma ada-kodien* a species indigenous to India and popularly known as Jivanti, is a twiny, laticiferous perennial medicinal shrub belongs to the family Asclepiadaceae. The occurrence of *in-vitro* culture stress might results instability of the genome in tissue cultured plantlets and hence these plantlets have to be subjected to assessment of genetic fidelity using DNA based molecular marker in *in- vitro* regenerated *H. ada-kodien* plantlets. The nodal explants responded satisfactory in terms of growth related traits when inoculated in the MS medium supplemented with KIN (1.50 mg/l) + NAA (0.50 mg/l). When screened with 12 Random Amplified Polymorphic DNA (RAPD) primers, it produced clear reproducible and scorable bands. All banding profiles from *in-vitro* raised 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 *H. ada-kodien*.

Keywords: *Holostemma ada-kodien*, *In-vitro* propagation, Genetic fidelity, RAPD marker

INTRODUCTION

Holostemma ada-kodien a twiny, laticiferous perennial medicinal shrub belonging to the family Asclepiadaceae (Martin, 2002) and It has several vernacular names in different languages, in Sanskrit, it is known as Jivanti; Arane beeru, Jeeva haale, Maruligana kasa in Kannada; Holostemma in English; Chirvel, Kanju in Hindi and Adapathian, Atapatiyan in Malayalam (Joy *et al*., 1998). The species is widely distributed in the tropical rain forests of the world including India, West peninsula, Srilanka and China (Sivarajan and Balachandran, 1994). In India, maximum distribution is seen in the forests of Andhra Pradesh, Tamil Nadu and Western Ghats of Karnataka and Kerala. Though distributed widely throughout Southern India, the population in wild is gradually reducing due to the destructive and ruthless collection of root tubers for ayurvedic drug preparations and fruit set is a major problem in multiplying the species in wild, which has led to the species being listed as vulnerable medicinal plant in FRLHT red list (Ravikumar and Ved, 2000).

Traditionally the plant is used as an alternative, astringent to the bowels; cures ulcers, diseases of the blood, itching, leucoderma, gonorrhoea and it has ability to maintaining vigour, strength and vitality (Gamble, 1967; Irimpan, 2011). The root and leaves are used in the form of powder and juice to treat spider-poisoning. The roots rubbed into a mash are used in cold milk as a curare to diabetes (Kirtikar and Basu, 1975). The tuberous roots are useful in, intestinal disorders galactogogue, orchitis, pain, stomach ache, in ophthalmic disorders and acts as a stimulant, rejuvenative, aphrodisiac and expectorant (Singh *et al*., 2012; Warrier, *et al*, 1995; Chopra, *et al*, 1956). Apart from all these traditional uses, it has some proven medicinal activity *viz.*, anti-diabetic (Janapati *et al*., 2009) antipyretic, anthelmintic (Sadasivam *et al*., 2014), antioxidant (Mallikarjuna *et al*., 2011), hepatoprotective (Junapudi *et al*., 2015) activity.

The tuberous roots are uprooted and used in several Ayurvedic drug preparations and fruit set is a major problem in multiplying the species in wild. Hence, the population of this plant has decreased simultaneously in the last few years in their natural habitat, that's the reason conservation of this species is of almost importance. *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 (Borthakur *et al*., 1999). If steps are not taken for their, mass propagation, cultivation and conservation, they may be lost from the natural habitat forever. However genetic fidelity is one of the most important pre-requisites in the micro propagated plant species. The occurrence of genetic instability arising may be due to somaclonal variation in the regenerates, which can seriously limit the utility of the micro propagation system (Salvi *et al*., 2001). Therefore it is very important to establish genetic homogeneity of *in-vitro* plants to confirm the quality of the plantlets for its commercial utility. The RAPD molecular markers are used to analyze any somoclonal variations in the *in- vitro* propagated plants.

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MATERIAL AND METHOD

Plant material and cultural conditions

Nodal bud explants collected from *H. ada-kodien* raised in ICAR-Indian Institute of Horticulture Research, Field Gene Bank were thoroughly washed in running tap water along with neutral liquid detergent for 4 to 5 minutes. These explants were cut into convenient sizes after removal of the leaves along with petiole. The nodal explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 4 to 5 minutes and rinsed 3 to 5 times in sterile distilled water to remove sterilants before the inoculation. The whole procedure is performed in the laminar hood, in sterile conditions. After that surface disinfected, explants were inoculated on basal MS medium containing different combinations of growth regulators. All the cultures were incubated at 25 ± 100 ºC under white fluorescent light with 50µ mole m^{-2} s^{-2} light intensity during a photoperiod of 16 hour light and 8 hour dark.

DNA extraction and PCR amplification conditions:

Genomic DNA of *H. ada-kodien* was isolated by using the protocol of Doyle and Doyle (1990) from both *in- vitro* grown plants and field grown mother plants. Genetic fidelity of *in -vitro* raised plantlets were tested using RAPD markers. For this study, seven *in-vitro* raised and hardened plants were chosen and compared with the mother plant. 12 Random Amplified Polymeric DNA (RAPD) primers were used for screening and assessment. PCR amplification were carried out in a 25µl of PCR reaction mixture containing 2.5 µl of 10mM concentration dNTPs, 3µl of complete buffer, 1 µl of taq polymerase, 13.5 µl of nucleus free water, 2.5 µl of DNA sample and 2.5 µl of primer. PCR amplification was performed in a thermal cycler, which was programmed for initial denaturation at 94°C for 5 min, followed by 40 cycles of 45 seconds denaturation at 94°C, 45 seconds annealing at 37°C and 45 seconds extension at 72°C, with a final extension at 72°C for 8 min. Amplified products were resolved by electrophoresis on 1.5% agarose gel in TAE buffer stained with ethidium bromide for 3h at 60 volts and photographs were taken by using the Gel Documenting system.

RESULT AND DISCUSSION

In -vitro shoot multiplication

Nodal bud segments from IIHR- Field Gene Bank plants of *H. ada-kodien* were used as explant and inoculated to MS media containing different combinations and concentrations of growth regulators *viz.,* KIN, NAA, BA, IBA and IAA. Effect of varying plant growth regulators on growth related characters like shoot length, number of shoots and number of leaves was observed and recorded. Estimation of growth parameters was done after 8 weeks of inoculation. Data were statistically analysed by analysis of variance and significance was calculated. MS medium supplemented with KIN (1.5 mg/l) + NAA (0.50 mg/l) shown significantly high shoot length (3.20 ± 0.20), number of shoots (2.80 ± 0.45) as well as high number of leaves per explant (4.00 ± 0.71) followed by BA (2.0 mg/l) +IBA (0.50 mg/l) which shoot length (2.90 ± 0.10), number of shoots (2.40±0.55) and number of leaves per explant (3.60±0.55) (Table. 1, Plate. 1) whereas in a study reported by Pushparajan and Surendran (2014) who conducted similar experiments observed that shoot tips inoculated in MS medium supplemented with NAA (0.5mg L-1) and KIN (1.5mg L-1) showed highest percentage of regeneration.

Assessment of genetic stability

The RAPD analysis was done to ascertain the genetic stability of *in-vitro* raised plants of *H. ada-kodien*. RAPD gel profile amplified by the primers OPC-2, OPC-05, OPD-10, OPD-12, OPE-14, OPE-17, OPF-11, OPF-13, OPG-04, OPG-06, OPG-08 and OPG-09 and their size is given in Table 3. All the tried twelve primers gave amplification and a total of 83 RAPD bands were generated, which were monomorphic indicating that micro propagated plants were similar to mother plant (Plate 2). The number of bands resolved per amplification were primer dependent and varied from 5 (OPD-12 and OPG-8) to 10 (OPF-11) with an average of 6.92 bands per primer. The range of amplification products varied with selected primers and from 125bp to 950bp (Table 2).

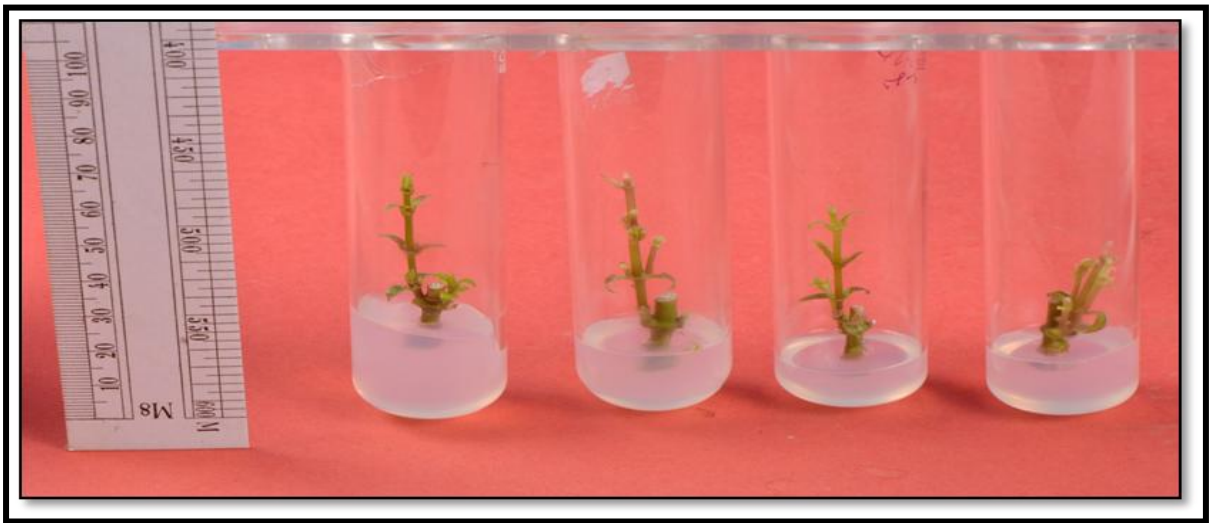
Number of monomorphic bands was highest (10) in case of primer OPF-11 ranging from 125-900bp and lowest (5) in case of primer OPD-12 and OPG-8, ranging from 150-900bp and 375-900bp in size respectively). The micropropagated plants of *H. ada-kodien* showed a similar profile to that of its mother plant (Plate. 2). 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. Kumar *et al.* (2015) came out with similar results after the assessment of the genetic fidelity of *in-vitro* plants *of Decalepis hamiltonii*, an endemic threatened medicinal plant, and found that amplified products were monomorphic and *in-vitro* raised plants were similar to the mother plant after RAPD analysis. Similar results were obtained after evaluating the genetic fidelity of *in-vitro* raised plant of greater galangal (*Alpinia galanga* L.), and found after RAPD and ISSR analysis that all the micropropagated plants were monomorphic and similar to mother plant (Parida *et al.*, 2011). Tyagi *et al*. (2010) also found no variation among the micropropagated plants *Capparis deciduas*, after the assessing the clonal fidelity by using RAPD. Similar results after assessing the genetic fidelity of micropropagated plants of *Arnebia hispidissima*, which belongs to the family Boraginaceae and is an

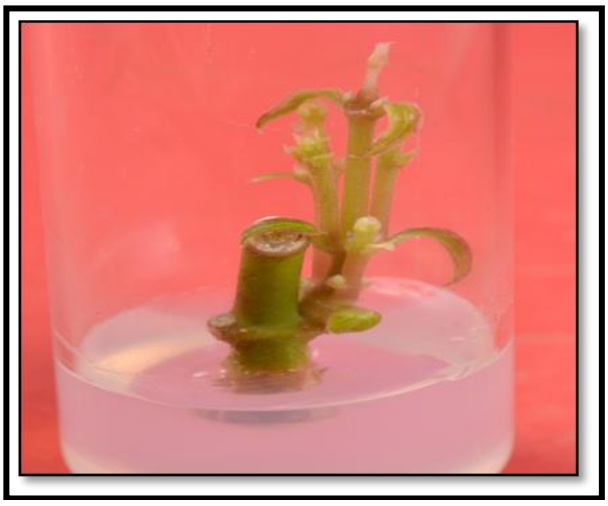
important medicinal plant and found that amplified product were monomorphic and *in-vitro* plants were similar to mother plant after RAPD analysis (Phulwaria *et al*. 2013).

Table 1. *In-vitro* shoot multiplication in *Holostemma ada–kodien* using different plant growth regulators after 8 weeks of inoculation

Sl. No.	MS Media + Growth regulators (mg/l)	Shoot length (cm) (Mean ± SD)*	Number of shoots per explant (Mean ± SD)*	Number of leaves per explant (Mean ± SD)*
1	BA (0.443)	2.17 ± 0.15	1.20 ± 0.45	2.00 ± 0.71
2	BA (0.443)+KIN(0.24)	2.30 ± 0.10	1.40 ± 0.55	2.20 ± 0.45
3	BA (0.443)+IAA(0.27)	2.48 ± 0.13	1.80 ± 0.84	2.60 ± 0.55
4	BA (0.886)	2.22 ± 0.08	1.20 ± 0.45	2.20 ± 0.45
5	BA (0.886)+KIN(0.24)	2.67 ± 0.12	1.80 ± 0.45	2.80 ± 0.84
6	BA (2.0)+IBA(0.50)	2.90 ± 0.10	2.40 ± 0.55	3.60 ± 0.55
7	KIN(1.50)+NAA(0.50)	3.20 ± 0.20	2.80 ± 0.45	4.00 ± 0.71

* Mean value of growth parameters of 10 plantlets per treatment.





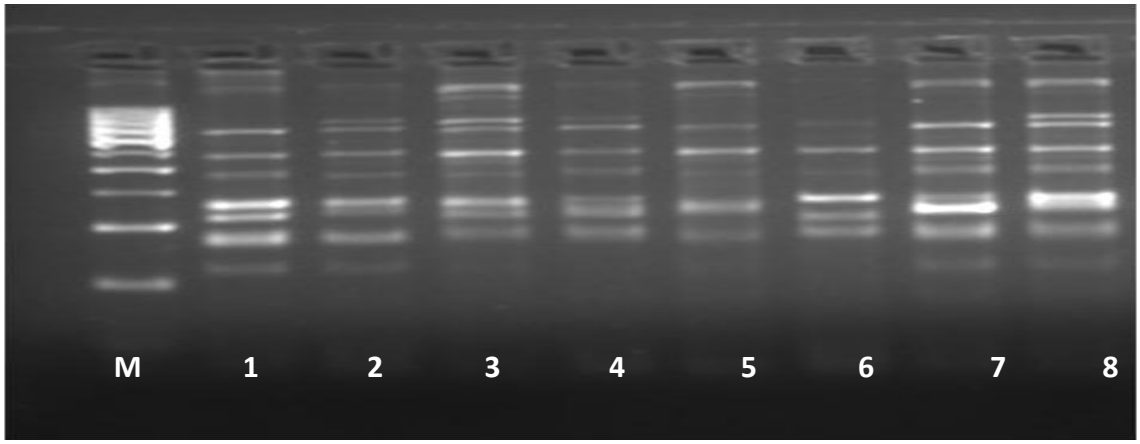


Plate 1. *In-vitro* raised plantlets of *H. ada-kodien* using nodal explants.
Plate 2. RAPD banding pattern in both micropropagated and field grown mother plants of *H. ada-kodien* (Lane 1: Mother plant, Lane 2-8: Micropropagated plants and M: Marker)

Table 2. List of primers, total number of bands and size of amplified fragments generated by RAPD primers in both micropropagated and Field grown mother plants of *H. ada-kodien* accessions

Sl. No.	Primers	No. of bands produced	Range of amplicons [bp]
1.	OPC-02	06	175-950
2.	OPC-05	06	175-950
3.	OPD-10	06	150-900
4.	OPD-12	05	150-900
5.	OPE-14	08	125-600
6.	OPE-17	09	125-600
7.	OPF-11	10	125-900
8.	OPF-13	08	125-900
9.	OPG-04	06	275-600
10.	OPG-06	06	350-800
11.	OPG-08	05	375-900
12.	OPG-09	08	230-700
Mean		6.92	
Total		83	

CONCLUSION

The present study provides the first report on the genetic fidelity of micropropagated *H. ada-kodien* obtained from nodal explants using RAPD marker. Our experiment proves that *in-vitro* shoot multiplication using nodal segment as explant can be used for rapid clonal propagation and conservation work with very low risk of somaclonal variation

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