



## Original article

Auxin-cytokinin synergism *in vitro* for producing genetically stable plants of *Ruta graveolens* using shoot tip meristems

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## ABSTRACT

An efficient micropropagation protocol was developed for *Ruta graveolens* Linn. using shoot tip meristems derived from a 4-month-old field grown plant. *In vitro* shoot regeneration and proliferation was accomplished on Murashige and Skoogs (MS) semi-solid medium in addition to different doses of cytokinins viz. 6-benzyl adenine (BA), Kinetin (Kn) or 2-isopentenyl adenine (2iP), singly or in combination with auxins viz. indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or  $\alpha$ -naphthalene acetic acid (NAA). Highest regeneration frequency (27.6%) was obtained on (MS) medium composed of BA (10  $\mu$ M) with maximum number (9.4) of shoots and 4.3 cm shoot length after 4 weeks of incubation. Among various combinations tried best regeneration frequency (71%) of multiple shoot formation with highest number (12.6) of shoots per shoot tip explants were achieved in MS medium augmented with a combination BA (10.0  $\mu$ M) and NAA (2.5  $\mu$ M) after 4 weeks of incubation. The optimum frequency (97%) of rhizogenesis was achieved on half-strength MS medium having 0.5  $\mu$ M IBA after 4 weeks of incubation. Tissue culture raised plantlets with 5–7 fully opened leaves with healthy root system were successfully acclimatized off in Soilrite™ with 80% survival rate followed by transportation to normal soil under natural light. Genetic stability among *in vitro* raised progeny was evaluated by ISSR and RAPD markers. The entire banding pattern revealed from *in vitro* regenerated plants was monomorphic to the donor. The present protocol provides an alternative option for commercial propagation and fruitful setting up of genetically uniform progeny for sustainable utilization and germplasm preservation.

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## 1. Introduction

*Ruta graveolens* Linn (Rutaceae) is a multipurpose perennial medicinal herb, commonly known as 'rue' or 'herb of grace', native to Mediterranean region. It is also grown worldwide as an ornamental plant, due to its bluish green foliage with yellow flowers and for its tolerance in dry soil and hot climatic condition (Bohidor et al., 2008). The herb has resolving, diuretic, antispasmodic, emmenagogue, stimulant, antispasmodic properties and useful in hysteria, amenorrhoea, earache and toothache. The rue plant has drawn special attention due to an essential oil called as

'rue oil' extracted by steam distillation of fresh materials of this plant. The rue oil contains several biologically active constituents like bergaptene, butanone, nonanone, nonyl acetate, psoralen, undecanone, and xanthotoxin (Anonymous, 2003).

Conventional cultivation via seeds or shoot cuttings is not adequate to accommodate the growing need of this species. The pharmaceutical companies are excavating the herb from the wild posing a threat to its existence and disturbing the ecological balance. Also, the variability in the constituents and chances of weed contamination reduce the profitability of the companies. Therefore, it is essential to develop an effective and reproducible method for clonal propagation for *Ruta graveolens*, which can provide contamination free supply of the herb that could suffice the demand.

Nowadays, tissue culture technique is proving to be very useful tool for mass propagation and conservation of various important medicinal species and the modern approaches of this technique emerge as an important production link between multiplication, conservation and sustainable utilization. Plant tissue culture technique is a key technology for production of large quantities of planting material of selected genotypes and chemotypes (Anis

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et al., 2008). Shoot tip culture technique is a very interesting tissue culture approach, as it eliminates the probability of endogenous contamination and provides a high frequency shoot regeneration system.

Clonal nature of regenerants is the most essential requirement in micropropagation of selected medicinal plant. Genetic variations in terms of somaclonal variation is a hindrance in attempts to maintain the advantages of elite germplasm. Consequently, it is crucial to ascertain genetic stability of the *in vitro* regenerated plantlets in order to substantiate the superiority of the regenerants.

Recently, PCR (Polymerase chain reaction) based molecular markers viz. RAPD (Random amplified polymorphic DNA) and ISSR (inter-simple sequence repeat) are extremely functional in establishing genetic constancy among micropropagated plantlets in many plant species (Ahmad and Anis, 2011; Faisal et al., 2012). RAPD and ISSR techniques are very simple, rapid, consistent, extremely discriminative and commercial. These necessitate only a very minute quantity of DNA sample and also, they do not need any prior sequence information to design the primer. Since, it is immensely essential to maintain genetic uniformity of *in vitro* raised progenies, therefore, we have adopted DNA based RAPD/ISSR molecular techniques for the appraisal of inherited stability among *in vitro* regenerated progeny.

Clonal propagation through shoot apices is advantageous over other explants as it eliminates any chance of contamination. Owing to the importance of the species *in vitro* regeneration has been attempted using nodal segments (Faisal et al., 2005) and leaf explants (Ahmad et al., 2010). However, to our knowledge there is no data published on direct regeneration from shoot tip meristems of this plant.

Hence, considering an enormous potential exhibited by the relevance of *in vitro* propagation procedures and existing situation of *R. graveolens*, the current research work was attempted to examine the effects of different concentration of cytokinins and auxin: cytokinin interactions on shoot regeneration using shoot tip meristems and determination of genetic fidelity of the regenerant progenies using molecular markers.

## 2. Materials and methods

### 2.1. Plant material establishment of *in vitro* cultures

Juvenile and fresh shoots of *R. graveolens* procured from a healthy plant were rinsed properly with normal laboratory tap water for 20 min, followed by 10 min treatment with a liquid detergent 5% (v/v) Labolene™ (a liquid detergent; Qualigens Fine Chemicals, Mumbai, India and cleansing thrice in tap water. After proper washing the explants were sterilized by 0.1% (w/v) mercuric chloride (Central Drug House, New Delhi, India) under Laminar Air Flow (Macro Scientific Works, Pvt. Ltd. New Delhi, India) for 5 min. After washing (5 times) with distilled water (sterile), the shoots were trimmed-off and shoot tip meristems (0.3–0.6 cm) were exterminate aseptically and cultured on pre-sterile culture medium (20.0 ml) in culture tubes (125 mm × 25 mm; Borosil, Agra, India). The culture medium contained MS nutrients (Murashige and Skoog, 1962) with 3% sucrose (a carbon source) and 1.0% (w/v) agar-agar (a gelling agent; Thermo Fisher Scientific India Pvt. Ltd, Mumbai, India) supplemented with 6-benzyladenine (BA), 6-furfurylaminopurine (Kn), 2 isopentenyl adenine (2iP) either singly or in different amalgamation at various doses of auxins viz. indole-3-acetic acid (IAA), indole-3-butyrac acid (IBA) or  $\alpha$ -naphthalene acetic acid (NAA). The pH of the medium was fixed to 5.8 with 1N NaOH before sterilization (autoclaving) at 121 °C and 15 psi for 15 min. The cultures were incubated in a cul-

ture room at 25 ± 2 °C with 16/8 h light/dark cycle. The light intensity of 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  provided by 40 W fluorescent Lamps (Philips Electronics India Ltd. Kolkata, India) and the relative humidity was about 50–60%.

### 2.2. Sub-culturing

4 weeks after incubation, responded shoot tip meristems were sub-cultured to the culture media composed of same concentrations and combinations of plant growth regulators (PGRs) as was used in the respective inoculation media, after collecting the initiated shoots from cultures. The frequency of responded explants, the shoot number with length were documented after 8 weeks of culture.

### 2.3. *In vitro* rooting and hardening

*In vitro* propagated microshoots (approx. 4–5 cm) were removed from shoot cultures and submitted to full and half strength MS basal medium augmented with 0.5  $\mu\text{M}$ . After proper root formation, plantlets were washed cautiously with normal water, transferred to pots composed of sterile potting mixture, Soilrite™ (Keltech Energies Ltd., Bangalore, India), moisturized with 0.5x MS, lacking organic supplements, and covered with clear polyethylene covers to ensure a high humidity. The covers were evacuated gradually after 2 weeks, in order to hardened the plantlets. The acclimatized plants were then shifted to a green-house, net house and finally to the field condition.

### 2.4. Genetic stability analysis

The genetic constancy of the *in vitro* raised plants was examined by RAPD and ISSR techniques. Randomly selected 10 *in vitro* raised plants alongwith mother plant was investigated for genetic integrity. Genomic DNA from young leaves of selected plants was isolated following the cetyltrimethylammonium bromide (CTAB method) defined by Doyle and Doyle (1987). The isolated genomic DNA was certified for purity (A260/280 ratio) on a Nanodrop Spectrophotometer (UV-1700 Implen, Germany).

A set of 10 RAPD primers (Kit C, Operon Technologies, California, USA; Table 1) and 10 ISSR primers (UBC, Vancouver, BC, Canada; Table 2) were used for initial screening. PCR reactions for ISSR/RAPD marker based amplification were executed on a PCR Machine (Biometra, T Gradient Thermoblock, Germany). The mixture (20  $\mu\text{l}$ ) for PCR contains 10X buffer (2  $\mu\text{l}$ ), 25 mM  $\text{MgCl}_2$  (1.2  $\mu\text{l}$ ), 10 mM dNTPs (0.4  $\mu\text{l}$ ), 2  $\mu\text{M}$  primers, 3 Unit Taq polymerase (0.2  $\mu\text{l}$ ) and 25 ng Template DNA. The PCR amplification schedule contains 45 cycles inclusive of 94 °C denaturation segments of 5 min, an annealing (35 °C) for 1 min and a 72 °C extension of 1 min. A final extension was followed at 72 °C for 10 min. The amplification results of DNA were separated by electrophoresis on agarose gels (0.8%) with 4  $\mu\text{l}$  ethidium bromide in TAE buffer

**Table 1**  
RAPD primers used for the assessment of genetic stability of *R. graveolens* plantlets.

S. no.	Name of the primer	Sequence
1.	OPC-1	TTCGAGCCAG
2.	OPC-2	GTGAGGCGTC
3.	OPC-3	GGGGGTCTTT
4.	OPC-4	CCGCATCTAC
5.	OPC-5	GATGACCGCC
6.	OPC-6	GAACGGACTC
7.	OPC-7	GTCCCGACGA
8.	OPC-8	TGGACCGGTG
9.	OPC-9	CTCACCGTCC
10	OPC-10	TGTTCTGGTG

**Table 2**  
ISSR primers used for the assessment of genetic stability of *R. graveolens* plantlets.

S. no.	Name of the primer	Sequence
1.	UBC-801	ATA TAT ATA TAT ATA TT
2.	UBC-811	GAG AGA GAG AGA GAG AC
3.	UBC-825	ACA CAC ACA CAC ACA CT
4.	UBC-827	ACA CAC ACA CAC ACA CG
5.	UBC-834	AGA GAG AGA GAG AGA GYT
6.	UBC-841	GAG AGA GAG AGA GAG AYC
7.	UBC-855	ACA CAC ACA CAC ACA CYT
8.	UBC-866	CTC CTC CTC CTC CTC CTC
9.	UBC-868	GAA GAA GAA GAA GAA GAA
10	UBC-881	GGG TGG GGT GGG GTG

(pH 8.0) run at 50 V for 2 h and pictured on a UV transilluminator (Bio Rad, Hercules, CA, USA). To confirm the consistency in banding pattern, the DNA isolated and PCR reaction experiment was performed thrice. Only well characterized and reproducible bands were properly scored. Bands with the identical movement were considered to be autologous segments, regardless the magnitude.

### 2.5. Statistical analysis

All experimentation was repeated thrice, with 20 explants per treatment. Collected data were analyzed by SPSS version 11 (SPSS Inc. Chicago, USA) statistical software and the means values compared using Duncan's multiple range test (DMRT) at  $P \geq 0.05\%$  level of significance. All data were represented as means  $\pm$  standard error.

## 3. Results and discussion

### 3.1. Effect of cytokinin

Cytokinins are N<sup>6</sup> substituted adenine derived molecules, have the capability to enhance cell division in tobacco tissue culture and have been playing an important role in the developmental process including shoot and root development (Moubayidin et al., 2009). Therefore, in present investigation the morphogenetic response of shoot tip meristems to various cytokinins (BA, Kn or 2iP) was evaluated. Shoot tips failed to show any morphogenic response on control MS medium devoid of any growth regulator. But multiple shoot buds were induced in all the tried concentrations in which MS medium was augmented with PGRs. However, the response of the explant varied with the concentration and type

of the cytokinins (Table 3) used. BA supplemented medium exhibited the prime response of shoot induction, and could be regarded as a useful cytokinin for direct shoot growth from shoot tips. Multiple shoot buds were observed after 2–3 weeks of incubation and the maximum regeneration frequency ( $27.6 \pm 1.45\%$ ) was documented on MS medium enriched with  $10 \mu\text{M}$  BA, which also recorded the greatest number ( $9.4 \pm 0.43$ ) of shoots with maximum shoot length ( $4.3 \pm 0.29$  cm). The relative effectiveness of different cytokinins in inducing multiple shoots revealed the order as  $\text{BA} > 2\text{iP} > \text{Kn}$ . Corresponding conclusion were also obtained in *Artimesia vulgaris* (Sujatha and Ranjitha Kumari, 2007), *Psoralea corylifolia* (Baskaran and Jayabalan, 2008), *Vitex trifolia* (Ahmad et al., 2013)

### 3.2. Effect of auxins and cytokinin

Combination of auxins and cytokinins has been recognized long back as potential signaling molecules controlling growth and development. High levels of cytokinins along with low levels of auxins synergistically affect the cell division and *in vitro* plant regeneration (Fatima et al., 2011). In the current experimentation, the efficiency of shoot initiation from shoot tip meristems on MS medium supplemented with cytokinin and auxins (IAA, IBA or NAA) was also evaluated (Table 4). A synergetic influence of BA and NAA was, however, noticeable when amalgamation of most favorable concentration of BA ( $10 \mu\text{M}$ ) with variant concentration of NAA (0.5, 2.5 or  $5.0 \mu\text{M}$ ) were tested. Supplementation of NAA amplified the shoot proliferation rate and the best regeneration frequency ( $71.0 \pm 2.08\%$ ) of shoot formation with mean number ( $12.6 \pm 0.87$ ) of shootlets per explant was achieved in a combination  $10.0 \mu\text{M}$  BA with  $2.5 \mu\text{M}$  NAA (Fig. 1A). Thus, it was considered as the optimal treatment for maximum shoot induction among all the treated explants. However, presence of IAA or IBA ( $0.5$ – $5 \mu\text{M}$ ) with BA ( $10 \mu\text{M}$ ) supplemented medium; was less effective than NAA for shoot multiplication. A maximum number of  $10.6 \pm 0.87$  and  $9.0 \pm 0.63$  shoots per explant were observed at  $2.5 \mu\text{M}$  IAA and IBA respectively (Table 4). The obtained results are in consonance with the earlier reports on *Ruta graveolens* (Faisal et al., 2005) *Vitex trifolia* (Ahmad et al., 2013).

### 3.3. In vitro rooting and acclimatization

The achievement of *in vitro* regeneration relies on the percent rooting of rejuvenated shootlets and their continuity in the normal environmental conditions. Rooting in isolated shootlets did not

**Table 3**  
Influence of different cytokinins on shoot regeneration from shoot apex of *Ruta graveolens* on MS medium, after 8 weeks of culture.

Cytokinins ( $\mu\text{M}$ )			% Response	Mean no. of shoots	Mean shoot length (cm)
BA	Kn	2iP			
0.0	0.0	0.0	0.0 <sup>i</sup>	0.0 <sup>h</sup>	0.0 <sup>h</sup>
0.5	–	–	11.3 $\pm$ 1.76 <sup>fg</sup> <sup>h</sup>	1.8 $\pm$ 0.37 <sup>g</sup>	2.2 $\pm$ 0.33 <sup>efg</sup>
2.5	–	–	15.0 $\pm$ 2.51 <sup>defg</sup>	3.4 $\pm$ 0.67 <sup>fg</sup>	2.5 $\pm$ 0.31 <sup>defg</sup>
5.0	–	–	17.3 $\pm$ 1.76 <sup>cdef</sup>	6.0 $\pm$ 0.54 <sup>bcd</sup>	3.1 $\pm$ 0.22 <sup>bcde</sup>
10.0	–	–	27.6 $\pm$ 1.45 <sup>a</sup>	9.4 $\pm$ 0.74 <sup>a</sup>	4.3 $\pm$ 0.29 <sup>a</sup>
20.0	–	–	22.6 $\pm$ 2.67 <sup>abc</sup>	7.4 $\pm$ 0.50 <sup>b</sup>	4.0 $\pm$ 0.31 <sup>ab</sup>
–	0.5	–	8.0 $\pm$ 1.15 <sup>h</sup>	2.0 $\pm$ 0.31 <sup>g</sup>	1.8 $\pm$ 0.33 <sup>g</sup>
–	2.5	–	10.6 $\pm$ 1.33 <sup>gh</sup>	3.6 $\pm$ 0.60 <sup>efg</sup>	2.0 $\pm$ 0.35 <sup>fg</sup>
–	5.0	–	15.3 $\pm$ 2.67 <sup>defg</sup>	5.4 $\pm$ 0.74 <sup>cde</sup>	2.8 $\pm$ 0.37 <sup>defg</sup>
–	10.0	–	22.6 $\pm$ 1.76 <sup>abc</sup>	7.6 $\pm$ 0.60 <sup>b</sup>	3.5 $\pm$ 0.28 <sup>abcd</sup>
–	20.0	–	18.0 $\pm$ 2.30 <sup>cde</sup>	7.0 $\pm$ 0.54 <sup>bc</sup>	3.8 $\pm$ 0.20 <sup>abc</sup>
–	–	0.5	7.6 $\pm$ 1.20 <sup>h</sup>	2.4 $\pm$ 0.87 <sup>g</sup>	2.0 $\pm$ 0.33 <sup>fg</sup>
–	–	2.5	10.3 $\pm$ 1.67 <sup>gh</sup>	3.0 $\pm$ 0.89 <sup>g</sup>	2.1 $\pm$ 0.29 <sup>fg</sup>
–	–	5.0	12.0 $\pm$ 1.15 <sup>efgh</sup>	4.6 $\pm$ 0.67 <sup>def</sup>	2.5 $\pm$ 0.27 <sup>efg</sup>
–	–	10.0	25.0 $\pm$ 2.88 <sup>ab</sup>	7.4 $\pm$ 0.50 <sup>b</sup>	2.9 $\pm$ 0.38 <sup>cdef</sup>
–	–	20.0	21.0 $\pm$ 2.08 <sup>bcd</sup>	6.4 $\pm$ 0.50 <sup>bcd</sup>	2.7 $\pm$ 0.34 <sup>defg</sup>

Data shows mean  $\pm$  standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same alphabets are not significantly different ( $P = 0.05$ ) using Duncan's multiple range test.

**Table 4**  
Influence of different auxins with optimal concentration of BA on direct shoot regeneration from shoot apex of *Ruta graveolens* on MS medium, after 8 weeks of culture.

Auxins ( $\mu\text{M}$ )				% Response	Mean No. of shoots	Mean shoot length (cm)
BA	IAA	IBA	NAA			
10	0.5	–	–	38.0 $\pm$ 1.15 <sup>c</sup>	9.0 $\pm$ 0.54 <sup>b</sup>	3.4 $\pm$ 0.33 <sup>de</sup>
10	2.5	–	–	62.0 $\pm$ 2.30 <sup>bc</sup>	10.6 $\pm$ 0.87 <sup>b</sup>	4.4 $\pm$ 0.25 <sup>abcd</sup>
10	5.0	–	–	39.3 $\pm$ 1.76 <sup>c</sup>	9.4 $\pm$ 0.67 <sup>b</sup>	4.0 $\pm$ 0.31 <sup>bcde</sup>
10	–	0.5	–	31.0 $\pm$ 2.08 <sup>d</sup>	8.4 $\pm$ 0.50 <sup>b</sup>	3.0 $\pm$ 0.50 <sup>e</sup>
10	–	2.5	–	57.0 $\pm$ 1.52 <sup>c</sup>	9.0 $\pm$ 0.63 <sup>b</sup>	3.8 $\pm$ 0.20 <sup>cde</sup>
10	–	5.0	–	36.3 $\pm$ 1.85 <sup>cd</sup>	8.8 $\pm$ 0.48 <sup>b</sup>	3.3 $\pm$ 0.33 <sup>e</sup>
10	–	–	0.5	42.0 $\pm$ 2.30 <sup>bc</sup>	9.2 $\pm$ 0.86 <sup>b</sup>	5.0 $\pm$ 0.44 <sup>ab</sup>
10	–	–	2.5	71.0 $\pm$ 2.08 <sup>a</sup>	12.6 $\pm$ 0.87 <sup>a</sup>	5.2 $\pm$ 0.25 <sup>a</sup>
10	–	–	5.0	46.0 $\pm$ 1.85 <sup>ab</sup>	10.0 $\pm$ 0.70 <sup>ab</sup>	4.8 $\pm$ 0.46 <sup>abc</sup>

Data shows mean  $\pm$  standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same alphabets are not significantly different ( $P = 0.05$ ) using Duncan's multiple range test.



**Fig. 1.** A. Multiple shoot regeneration from shoot tip meristem of *R. graveolens* on MS medium containing 10.0  $\mu\text{M}$  BA and 2.5  $\mu\text{M}$  NAA; B. Regenerated shoot rooted on  $\frac{1}{2}$ MS medium containing 0.5  $\mu\text{M}$  IBA.

achieved on the shoot regeneration medium even after a long incubation gap of culture >8 weeks. Therefore, the regenerated shoots were excised from shoot clusters and shifted to MS medium augmented with 0.5  $\mu\text{M}$  IBA and well developed roots were achieved after 4 weeks. Similar observations were also stated in *Psoralea corylifolia* (Baskaran and Jayabalan, 2008) and *Dracaena sanderiana* (Aslam et al., 2013).

A discrete study was also taken out to standardize the application of IBA in half and full and full strength MS culture medium. Individual microshoot of appropriate size (4–5 cm) were excised from shoot clusters and shifted to  $\frac{1}{2}$ MS and MS media enriched with 0.5  $\mu\text{M}$  IBA. Rhizogenesis was recorded in about 97% in produced microshoots on half concentrated culture medium containing 0.5  $\mu\text{M}$  IBA (Fig. 1B). However reduced root induction response (89%) was obtained at the equivalent dose of IBA with full strength MS medium.

Tissue culture raised plantlets with 5–7 fully expanded leaves and well grown roots were isolated from the culture vials, washed carefully with sterile water, transplanted to various planting substrates and hardened off, adopting the procedure given in material and methods. Of various type of potting mixture tested, % age survival of *in vitro* grown plants was about 70% in vermicompost, however more than 80% plantlets survived following transfer from Soilrite<sup>®</sup> to garden soil after 2 weeks. The plantlets thus obtained were symmetrical and uniform to the donor mother plant in relation to development, vegetative growth, flower shape and size, fruiting twigs and seeds.

#### 3.4. Genetic stability

Potential to regenerate elite plants makes micropropagation an essential part of plant biotechnology. However, commercialization any micro-propagation system increases the risk of somaclonal variations amongst the sub-clones of the selected parental line (Larkin and Scowcroft, 1981). Hence, it becomes crucial to frequently screen for genetic variations among *in vitro* raised plantlets in order to generate look alike descendants. The plants obtained from organized meristems antagonistic susceptible to genetic modifications since the organized meristems are resistant to genetic changes (Shenoy and Vasil, 1992). In the present study, ISSR and RAPD profiles of ten randomly selected *in vitro* regenerated *R. graveolens* plants and the mother plant was carried out. All the primers produced well-resolved, clear and distinct banding patterns, which were corresponding to the donor plant, validating the genetic fidelity of the micropropagated plants. ISSR primers were found to be more suitable to determine the genetic stability on the basis of generation of bands. PCR (Polymerase chain reaction) based technique such as random amplified polymorphic DNA (RAPD) and ISSR are enormously useful in constituting the genetic fidelity of *in vitro* raised plantlets by many authors (Joshi and Dhawan, 2007; Ahmad and Anis, 2011). ISSR and RAPD markers are technically fast, very simple, cost-effective, reliable, highly discriminative, requires a small and relatively crude amount of DNA and do not require any prior information about the genome (Waugh and Powell, 1992).

#### 4. Conclusion

The presently developed protocol followed and the techniques implemented (sub-culturing of shoots) would further support persistent inexhaustible *in vitro* preservation of shoot tip meristems produced clones devoid of any modification or variation of DNA. The established *in vitro* system could reveal the significance of shoot tip meristems in maintaining their regeneration frequency with morphogenetic competence. The developed protocol will

provide an alternative approach for large-scale multiplication and successful establishment of genetically stable plants before it is released for commercial exploitation.

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