Indian Journal of Science and Technology Vol. 4 No. 10 (Oct 2011) ISSN: 0974- 6846

Identification of DNA elements involved in somaclonal variants of *Rauvolfia serpentina* (L.) arising from indirect organogenesis as evaluated by ISSR analysis

S. Saravanan[1], R. Sarvesan[2] and M.S. Vinod[3]

[1]*Post-graduate & Research Dept. of Plant biology and Biotechnology, Pachaiyappa's College, Chennai-600030, India*
[2]*Post-graduate & Research Dept. of Plant biology and Biotechnology, Presidency College, Chennai-600005, India*
[3]*M.S.Swaminathan Research Foundation, Chennai-600 113, India*
rajasarvesan@gmail.com

Abstract

Rauvolfia serpentina an endangered medicinal plant was chosen for *in vitro* propagation using modified MS medium. Genetic fidelity study of the regenerated plants were analysed with 18 ISSR markers. A total of 159 monomorphic bands were obtained; one ISSR marker HB-12 showed a polymorphic band among the callus regenerants. The specific polymorphic fragment was then gel eluted, cloned and sequenced.

Keywords: *Rauvolfia serpentina*, ISSR, Somaclonal variation**,** Medicinal plant, India

Introduction

Rauvolfia serpentine (L.) Benth. ex Kurz (syn *Ophioxylon serpentinum* L.) (Family: Apocynaceae) commonly known as Sarpagandha in India is a perennial under-shrub. This hermaphroditic plant is indigenous to India, Andaman Islands, Bangladesh and South-East Asia. It is an important medicinal plant normally found in forests but also cultivated for its roots which are used for the extraction of therapeutically active alkaloids like Reserpine, Ajmaline, Serpentine, Ajmalicine etc. Moreover it is extensively used in traditional medicine for the treatment of insomnia, hypochondriasis, irritable condition of the central nervous system and high blood pressure. *Rauvolfia* is threatened with extinction in India due to indiscriminate collection, over exploitation and limited cultivation for commercial purposes to meet the requirements of pharmaceutical industry (Richa Bhatt *et al*., 2008; Sihag & Nidhi Wadhwa, 2011). It has been included in the Appendix II of The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and in the negative list of exports-plants by the Government of India (Notification no.24(RE-98)/1977-2002).

Poor seed germination (due to stony endocarp) coupled with absence of embryos possibly due to parthenocarpy and with the viability of the seeds decreasing drastically with increase in the time interval between collection and sowing, its propagation using the root cuttings for tissue culture is an attractive option (Goel *et al*., 2009). *In vitro* micropropagation has a number of advantages over the sexual reproduction in a large-scale propagation program as it offers the possibility of speeding up the breeding of elite, pathogen-free cultivars. By means of micropropagation, superior gene combinations can be preserved practically unaltered as a consequence of direct organogenesis.

On the contrary tissue culture also induces variations in regenerated plants, which are commonly referred to as somaclonal variation (Larkin & Scowcroft, 1981). This can result in an assortment of genetically stable variations, useful in crop improvement (Skirvin *et al*., 1993; Jain *et al*., 1998), similar to that induced with chemical and physical mutagens. On the other hand it can also lead to chromosomal rearrangements detrimental to the plant in question. Somaclonal variation is unpredictable in nature, and can be both heritable and non-heritable (epigenetic). The occurrence of somaclonal variation is associated with point mutations, chromosomal rearrangements and recombination, DNA methylation, altered sequence copy number, transposable elements and is reportedly influenced by the genotype, explant type, culture medium and age of the donor plants (Jain, 1997; Jain *et al*., 1998; Veilleux & Johnson, 1998). Depending on the plant type, the number of sub cultures is another important aspect that can lead to further variations (Larkin & Scowcroft, 1981).

Many strategies are available for detecting genetic variation, including phenotypic identification and DNA analysis techniques. Phenotypic identification based on description of the morphological and physiological traits can be used, although this method requires an extensive observation of the plants until maturity. Furthermore, some changes induced by *in vitro* culture cannot be observed because the rearrangement of the gene or its product may not always alter its expression to such a degree that it can be visualized phenotypically. When this occurs, somaclonal variability can be evaluated by DNA analysis techniques. With the availability of different DNA based molecular markers, somaclonal variation can be accurately assessed using a combination of two or more types/class of markers (Rani *et al*., 1995; Schneider *et al*., 1996; Hashmi *et al*., 1997; Goto *et al*., 1998; Vendrame *et al*., 1999; Tiwari & Jatav, 2008). Random amplification of polymorphic DNA (RAPD) markers are the most commonly employed markers used to detect variations (Nitish Kumar *et al*., 2010). RAPD markers are extensively used to assess genetic variations generated by *in vitro* techniques (Hashmi *et al*., 1997; Goto *et al*.,

Indian Journal of Science and Technology Vol. 4 No. 10 (Oct 2011) ISSN: 0974- 6846

1998) to measure genetic similarity or dissimilarity. These classes of markers offer the advantage of being simpler to use, less expensive and less time consuming than restriction fragment length polymorphism (RFLP) or amplified fragment length polymorphism (AFLP).

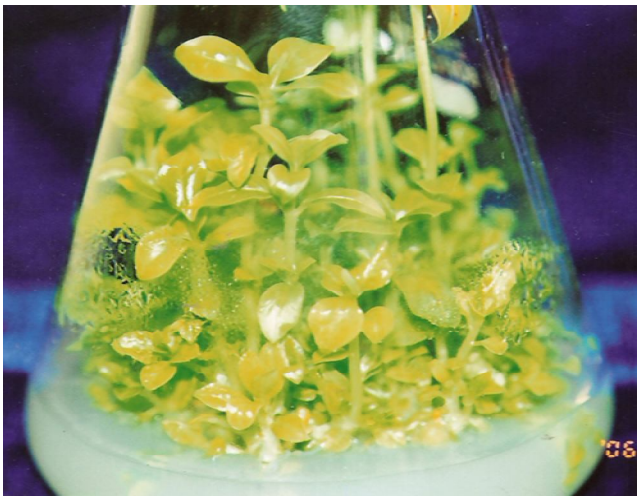
With the aim of studying the genetic fidelity of *Rauvolfia*, leaves, nodal segments and shoot tip of the plant were *in vitro*-regenerated primarily on MS medium with certain variations. To screen for potential somaclonal variants arising as a consequence of tissue culturing and to identify the specific DNA segments involved, inter-simple sequence repeat (ISSR) markers were used in the present study. One ISSR primer HB-12 consistently amplified a polymorphic banding profile in the in *vitro* regenerated plants. The specific polymorphic fragment was then gel eluted, cloned and sequenced.

Materials and methods

Plant material

Rauvolfia serpentina was collected from the foothills of Western Ghats in India and maintained in a green house. For surface sterilization the shoots were defoliated and nodal segments with 2-3 nodes were selected. They were then cleaned thoroughly under a continuous stream of running tap water for 60 mins and washed with Tween 20 for 15 mins. The leaves, shoot apices and nodal segments were then washed repeatedly with distilled water and finally treated with mercury chloride (0.1%) for 10 mins in a laminar flow cabinet and washed 3 times with autoclaved doubled distilled water to remove any trace of mercury chloride. After surface sterilization, shoot apices and the nodal cuttings were trimmed as explants of size 25-30mm with atleast one node in each explant. MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of BAP + NAA, BAP + ADS and ½ MS BAP + ADS were used for *in vitro* propagation. For shoot induction from leaf induced calli, the highest shoot induction (Fig.1) was observed on MS medium supplemented with BAP (22.19µM) and ADS (86864µM). The highest shoot induction from direct regeneration was on MS medium fortified with BAP (17.74µM) and ADS (32.57µM). The cultures were maintained at 25^{0}C under a 16 h photoperiod and light intensity of 40 µmol m^{-2} s^1 provided by cool white fluorescent tubes. Six direct regenerated plantlets from the explants and four indirect regenerated plants from the calli with the mother explant were chosen for the genetic fidelity studies.

Fig.1. Multiple shoot induction on MS medium fortified with BAP(22.19µM) +ADS(86864 µM)



Genomic DNA extraction and PCR amplification profile

Genomic DNA was isolated from the leaves of individual plants by using the standard CTAB method (Rogers & Bendich, 1988) followed by an RNAse treatment. Quantification of DNA was performed both spectrophotometrically and electrophoretically using λ DNA as a standard for the latter.

PCR for ISSR was performed in a total volume of 25µl containing 10X assay buffer (1X contains 10mM Tris Cl, pH 8.8 at 25^{0}C, 50mM KCl, 1.5mM $MgCl_2$), 25pmol of primer (Sigma Genosys), 40ng of template DNA, 80µm of each of the four dNTPs and one unit of *Taq polymerase* (Bangalore Genei). Thermal conditions such as initial denaturation at 94^{0}C for 5 mins, followed by 35 cycles at 94^{0}C for 1 min, 72^{0}C for 1.30 mins and a final extension of 72^{0}C for 10 mins were common to all the employed markers. For the ISSR markers, depending on the nature of the individual primers, variations were observed for the annealing temperature that ranged from 37ºC to 53ºC.The reactions were carried out in an ABI 9700 GeneAmp® PCR system. Amplified products were resolved on 1.2% agarose gels stained with ethidium bromide.

Cloning and sequencing the ISSR marker fragments

A total of 18 ISSR markers were used to generate 154 fragments between the 11 samples (Table1). ISSR marker (HB-12) consistently polymorphic in the samples was identified and the specific fragment gel eluted using Perfectprep® Gel cleanup kit (Eppendorf). The isolated fragment was cloned into a T vector (pTZ57R/T; MBI Fermentas) and transformed into competent *E. coli* XL-1 blue cells. The plasmids were then sequenced using an ABI 3130 DNA sequencer using the BigDye Terminator Cycle Sequencing Kit v3.1 (ABI). All the steps were performed according to the respective manufacturer's protocol.

Results and discussion

A total of 18 ISSR primers were employed in the present study all the primers gave reproducible results. The primer sequence, the number of amplification products and the number of polymorphic fragments are given in the Table 1. The amplification profile of each marker was visualized on a 1% agarose gel and documented by photographing the gels using a Pharmacia Biotech documentation system. The number and size of bands were scored from the photographs. The result was scored by comparing the bands obtained

Indian Journal of Science and Technology Vol. 4 No. 10 (Oct 2011) ISSN: 0974- 6846

Table 1. The primer sequence of the 18 ISSR primers used for analyzing the genetic fidelity of the micropropagated plants

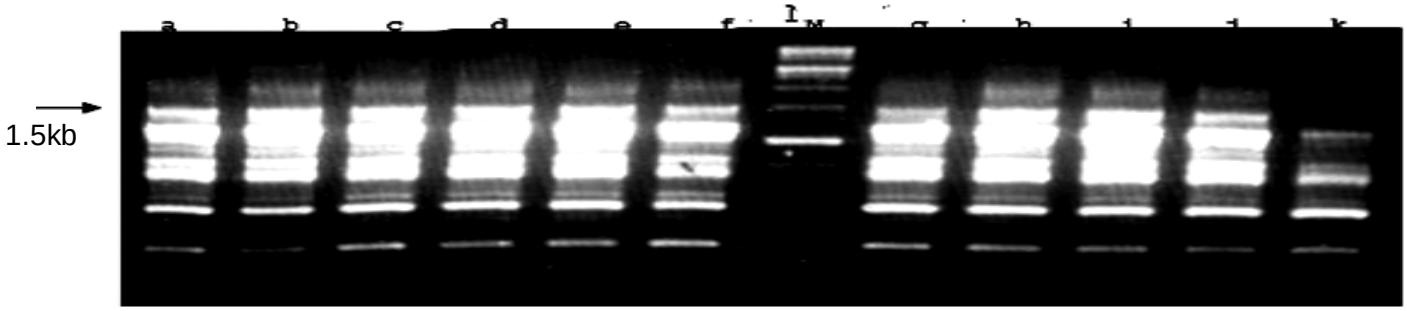
Primer	Sequence (5' - 3')	Total amplified fragments	Polymorphic bands	Size in bp
814	CTCTCTCTCTCTCTCTTG	9	0	
844A	CTCTCTCTCTCTCTCTAC	10	0	
844B	CTCTCTCTCTCTCTCTGC	10	0	
17898A	CACACACACACAAC	4	0	
17899A	CACACACACACAAG	14	0	
17899B	CACACACACACAGG	11	0	
(GATA)4	GATAGATAGATAGATA	11	0	
(CAGG)5	CAGGCAGGCAGGCAGGCAGG	5	0	
HB-12	CACCACCACGC	11	1	1400
HB-13	GAGGAGGAGGC	7	0	
HB-14	CTCCTCCTCGC	12	0	
HB-15	GTGGTGGTGGC	12	0	
P-01	GAGCAACAACAACAACAA	15	0	
P-03	AGAGAGAGAGAGAGAGTG	9	0	
P-04	ATGATGATGATGATG	4	0	
P-06	CCACCACCACCACCA	7	0	
P-08	CACCACCACCACCAC	5	0	
P-09	GCCGCCGCCGCCGCC	4	0	
	Total	160	1	

in the micropropagated plants to the pattern of bands obtained from the source plant, and recording them as present or absent. The bands that appeared very weak were not counted. The experiment was performed thrice for all the primers. All the 18 ISSR primers gave amplification products ranging from 4 bands (17898A, P-04, P-09) to 15 bands (P-01) with an average of 8.9 bands per primer. The size of bands ranged from150bp to 4.3kb. The ISSR finger prints of 10 micropropagated plants randomly collected from the different stages of micropropagation (b, c, d, e, h and j - direct regenerants; i, f, g, and k - callus regenerants) and the mother plant (a) are shown in the (Fig.2).

Among 18 ISSR primers tested 17 primers produced 160 bands that were monomorphic across all the micropropagated plants. One ISSR marker HB-12 produced a polymorphic band in the sample f (callus regenerant) (Fig.2). The size of the polymorphic band was 1400bp.

The sample (callus regenerants) containing polymorphic fragments amplified with HB-12 ISSR was cloned in T -vector and transformed in competent *E.coli* DH5α cells. The presence of insert was confirmed by performing colony PCR with M13forward and reverse primers. The colony PCR results showed that the polymorphic fragments amplified with ISSR primer HB-12 was successfully transformed into the cells. Once it was established that the specific polymorphic fragments were cloned, the *E.coli* cells were grown overnight at 37ºc in LB broth containing ampicilin as antibiotic and the following day the respective plasmids were isolated using a plasmid isolation kit as mentioned earlier.

Fig.2.Molecular profile of the polymorphic ISSR (HB12) marker for the regenerated Plantlets of Rauvolfia serpentina



Lane M:1Kb ladder: a: Mother plant; b,c,d,e,h,j: Direct regenerants; i,f,g,k: Callus regenerants

Indian Journal of Science and Technology Vol. 4 No. 10 (Oct 2011) ISSN: 0974- 6846

Fig. 3. Sequence information of the polymorphic fragment present only in the regenerated sample (F) of Rauvolfia serpentina amplified by ISSR (HB-12)

HB12-F (393bp)

5'-CTCGCGATGCATCTAGATTCACCACCACGCACTCCC CACTTTTTTCATCC

TCCAAATCCTCGTGGAAATCGG ATT GAAAGCTAAAACGGGAGAATTCAA

GGGTGATTAGTCCATTCAGCTTCCTTCAAATAAAsTTTCCCAGCAAGTTAAT

TCTTTTTAAACTGAAAGACACAGAAAATCTTTTCCTCTTTACAAGAAATCTTTT

TCAGCTGTTCTCTTGAACACAGAAATTTTTTTTTCCTCTTTACAGTAAATCTTT

TTCAGCTCTTCTTTATAAAGAAGATATTCGTGTTTCAATAAGATATCAGCAAAT

TTGGAGGGACGTTTGGTTTATGGTTTTGGCATTAAATCTTGAAAAAAGATTGG

AATTCCCCTGTTCATTCCGAAAAGTAA-3'

HB12- R (721bp)

5'-TCACCACCACGCACTCCCCACTTTTTTCATCCTCCAA ATCCTCGTGG

AAATCGGATTGAAAGCTAAAACGGGAGAATTCA AGGGTGATTAGTCCATT

CAGCTC CCTTCAAA TAAATTTCCCAGCAAGTTAATTCTTTTTAAACTGAAA

GACACAGAAAATCTTGTTCCTCTTTACAAGAAATCTTTTTCAGCTGTTCTCTTGA

ACACAGAAATTTTTTTTTCCTCTTTACAAGTAAATCTTTTTCAGCTCTTCTTTATAA

AGAAGATATTCGTGTTTCAATAAGATATCAGCAAATTTGGAGGGACGTTTGTTTA

TGGTTTTGGCATTAAATCTTGAAGAAAGATTGGAATTTCTCTGTTCAATTCTGAG

AGTTAATGGGCGGGATTGCTCTAGTATTGGATCTATTGAAGAAAAATCCAAGCT

TGATAAGGCCGTCTCTGCATTCATATGGACTCTTCTCAGCTAAAGCCGCTGCTT

CCACAGCTGCCGCTTCTGCTGCTGTGGCTGCCCCTTTTGCCACTTGGGCTTTT

CTTGGGTAAGCTATTGGCTTTTTTGGTACTCTTATCATTGTTTTTGTGTTATGAA

AACTATTGCACGGTAGATTTATTTATGCCAGTGAGCTAGTTTTCGTTGATTTTGA

GTTGAACCCAATACTTATTGGAATCAGTGAAAGCTGAAGTTTTTAAAAGTCCGT

GCCTTTTTCATTTCTTTTTTTTGGA-3'

The following polymorphic band was chosen for automated sequencing; A 1.5kb fragment amplified by ISSR primer HB-12 (Sample f, Fig.2). Sequence information for the above fragments was obtained (Fig.3) The sequences were then subjected to BLAST analysis for homology with known DNA sequences using the BLASTn option of the NCBI database, after eliminating any vector backbone contamination using the NCBI Vecscreen tool. The sequences were tested for homology against all mapped DNA sequences of plants listed in the database. A BLASTn search was done where nucleotides are compared with nucleotides directly. The expected value was set to E=1 in all the searches. The primer ISSR HB-12 containing polymorphic band of size 1.4kb produced 393bp from the 5'prime end and 721bp from the 3' prime end. Clone f (ISSR HB -12) containing 1114 sequenced nucleotides, showed homology to exportin 6 with a E value of 0.84. Exportin belong to a group of proteins that are primarily involved in nuclear export of proteins in plants. The sequence information of the polymorphic fragments generated by the primer HB-12 revealed that these genes are involved in protein transport.

ISSRs have been used to analyze the genetic fidelity of micropropagated plants in many species. ISSR markers reportedly reveal substantially higher levels of polymorphism than RFLP markers in maize (Kantety *et al*., 1995). ISSR fingerprinting has been previously found to be useful for detecting somaclonal variation among microprogagated plants of coffee (Rani *et al*., 2000). In the present study, the amplified products obtained through ISSR analysis exhibited monomorphism among all *in vitro* raised direct regenerated plants. These results indicate that the direct regenerated micropropagated plants of *Rauvolfia serpentina* are genetically stable and that the micropropagation protocol is working successfully. Whereas callus derived regenerants were found to show polymorphism. The occurrence of genetic changes among callus culture regenerants is a well established phenomenon (Skirvin & Janick 1976; Larkin & Scowcroft 1981). Among 18 ISSR markers tested 17 markers produced 159 bands that were monomorphic across all the micropropagated plants. One ISSR marker namely HB-12 produced a polymorphic band among callus regenerants subcultured on MS media supplemented with (BAP44.38 µM +NAA21.48 µ*M).*

References

1. Ahuja MR (1987) *In vitro* propagation of poplar and aspen. In: Cell and tissue culture in forestry. Bonga JM & Durzan DJ (eds) Vol 3. Martinus Nijhof, Dordrecht, pp: 207- 223.
2. Damasco OP, Graham GC, Henry RJ, Adkins SW and Godwin ID (1996) Random amplified polymorphic DNA (RAPD) detection of dwarf off-types in micropropagated Cavendish (*Musa* spp.AAA) bananas. *Plant Cell Rep.* 16,118-1123.
3. Goel MK, Mehrotra S, Kukreja AK, Shanker K and Khanuja SPS (2009) *In vitro* propagation of *Rauwolfia*

Indian Journal of Science and Technology Vol. 4 No. 10 (Oct 2011) ISSN: 0974- 6846

serpentina using liquid medium, assessment of genetic fidelity of micropropagated plants, and simultaneous quantitation of reserpine, ajmaline, and ajmalicine. *Methods in Mol. Biol. Clifton Nj*. 547, 17-33.

4. Goto S, Thakur RC and Ishii K (1998) Determination of genetic stability in long- term micro propagated shoots of *Pinus thunbergii* parl. using RAPD markers. *Plant Cell Rep*.18, 193- 197.
5. Hashmi G, Huettel R, Meyer R, Krusberg L and Hammerschlag F (1997) RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. *Plant Cell Rep.* 16, 624-627.
6. Iriondo JM and Perez C (1996) Somaclonal variation in Lavatera. In: Somaclonal variation in crop improvement. Bajaj YPS (Ed.), Vol II, Springer-Verlag, Berlin. pp:280-295.
7. Jain SM (1997) Somaclonal variation and mutagenesis for crop improvement. *Maat- alouden tutkimuskuksen*, *Sirkka Immonen* (Ed.). 18, 122-132.
8. Jain SM, Brar DS and Ahloowalia BS (Eds.) (1998) Somaclonal variation and induced mutation in crop improvement. Kluwer Academic Publ., UK.
9. Kaushal Bindiya and Kamlesh Kanwar (2003) RAPD markers for genetic analysis in micropropagated plants of *Robinia pseudoacacia* L. *Euphytica* 132, 41-47.
10. Larkin PJ and Scowcroft SC (1981) Somaclonal variation- a novel source of variability from cell culture for plant improvement. *Theor. Appl. Genet.* 60,197-214.
11. Maria Antonietta Palombi, Beatrice Lombardo and Emilia Caboni (2006) *In vitro* regeneration of wild pear (*Pyrus pyraster* Burgsd) clones tolerant to Fe-chlorosis and somaclonal variation analysis by RAPD markers. *Plant cell reports, genetics and genomics*. online date: Wednesday, 15th Nov.
12. Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*. 15, 473-497.
13. Nitish Kumar, Modi AR, Singh AS, Gajera BB, Patel AR and Patel MP (2010) Assessment of genetic fidelity of micropropagated date palm (*Phoenix dactylifera* L.) plants by RAPD and ISSR markers assay. *Physiol. Mol. Biol. Plants.*16 (2), 207-213.
14. Rani V, Parida A and Raina SN (1995) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. *Plant Cell Rep.* 14, 459-462.
15. Richa Bhatt, Mohd Arif, Gaur AK and Rao PB (2008) *Rauwolfia serpentina*: Protocol optimization for *in vitro* propagation**.** *Afr. J. Biotechnol*. 7 (23), 4265-4268.
16. Rival A, Betrand L, Beale T, Combes MC, Trouslout P and Leshermes P (1998) Suitability of RAPD analysis for detection of somaclonal variation in oil palm (*Elaeis guineenis* Jacq.) *Plant Breed*. 117, 73-76.
17. Rogers SO and Bendich AJ (1988) Extraction of DNA from plant tissues. In: Plant molecular biology. Manual A6. Kluwer Academic publishers, Dordrecht. pp:1-10.
18. Rout GR, Das P, Goel S and Raina SN (1998) Determination of genetic stability of micropropagated plants of ginger using Random Amplified Polymorphic DNA (RAPD) markers. *Botanical Bull. Acad. Sinica.* 39, 23-27.
19. Schneider S, Reustle G and Zyprian E (1996) Detection of somaclonal variation in grapevine regenerants from protoplast by RAPD- PCR. *Vitis.* 35, 99-100.
20. Shu QY, Liu GS, Qi DM, Chu CC, Liu J and Li HJ (2003) An effective method for axillary bud culture and RAPD analysis of cloned plants in tetraploid black locust. *Plant Cell Rep.* 22, 175-180.
21. Sihag RC and Nidhi Wadhwa (2011) Floral and reproductive biology of Sarpagandha *Rauvolfia serpentina* (Gentianales: Apocynaceae) in semi-arid environment of India*. J. Threatened Taxa* (JoTT) Short Commun. 3(1), 1432-1436.
22. Skirvin RM and Janick J (1976) Tissue culture - induced variation in scented *Pleargonium* spp. *J. Am. Soc. Hort. Sci.* 101, 281-290.
23. Skirvin RM, Nortonand M and McPheeters KD (1993) Somaclonal variation: has it proved useful for plant improvement. *Acta Hort.* 336, 333-340.
24. Tiwari S and Jatav DS (2008) Detection of somoclonal variations aamong micropropagated population of some important medicinal plants. *Afr. J. Traditional, Complementary & Alternative Med. (AJTCAM),* Abstracts of tongress on Medicinal and aromatic Plants, Cape Town, Nov.
25. Varshney A, Lakshmikumaran M, Srivastava PS and Dhawan V (2001) Establishment of genetic fidelity of *in vitro* raised *Lilium bulblets* through RAPD markers. *Special Issue of In Vitro Cellu. Dev. Biol-Plant.* 37(2), 227-231.
26. Veilleux RE and Johnson AAT (1998) Somaclonal variation: Molecular analysis, transformation, interaction, and utilization. *Plant Breed Rev.* 16, 229-268.
27. Vendrame WA, Kochert G and Wetzstein HY (1999) AFLP analysis of variation in pecan somatic embryos. *Plant Cell Rep.* 18, 853-857.
28. Wang P-J and Charles A (1991) Micropropagation through meristem culture. In: High tech and micropropagation. Biotechnology in agriculture and forestry. Bajaj YPS (ed), Vol.17, Springer, BerlinHeidelberg, NY. pp: 32-52.