Regular

International Journal of Integrative Biology
A journal for biology beyond borders

IJIB
ISSN 0973-8363

Assessment of genetic stability of micropropagated plants of *Curcuma longa L.* by cytophotometry and RAPD analyses

Manoj Kumar Panda, Sujata Mohanty, Enketeswar Subudhi, Laxmikanta Acharya, Sanghamitra Nayak [*]

[1] *Centre of Biotechnology, Siksha 'O' Anusandhan University, Orissa, India*

Submitted: 12 Nov. 2007; Accepted: 15 Dec. 2007

Abstract

A protocol was developed for *in vitro* propagation of an elite genotype of *Curcuma longa* (cv Roma) using axillary buds from unsprouted rhizomes. The explant produced multiple shoots when cultured on MS basal medium supplemented with varying combinations and concentrations of Benzyladenine, Kinetin,α Napthalene, Acetic acid, Indole-3- acetic acid and Adenine sulphate. Medium containing BA (3mg/l) was found to be optimum for micropropagation and conservation of *C. longa* plantlets. The genetic stability of the micropropagated clones was evaluated over 26 months in culture using cytophotometry and Random Amplified Polymorphic DNA (RAPD) analysis. Cytophotometry analysis revealed unimodal DNA distribution with 4C peak and RAPD analysis revealed monomorphic bands in all *in vitro* grown plants of *C. longa*, thus confirming genetic uniformity among somaclones of *C. longa*. This study is of high significance as these cultures are currently being used as source of disease free planting material for commercial utilization.

Keywords: Curcuma longa, Micropropagation, Cytophotometry, RAPD, genetic stability.

INTRODUCTION

Curcuma longa (turmeric) of family Zingiberaceae is an important plant valued all over the world as a spice and also for its medicinal properties (Roses, 1999). Turmeric powder significantly increases the mucus content in gastric juices, no wonder that Indian cuisine lays emphasis on turmeric's therapeutic effect against gastric disorders. Curcuma oil, curcumin and its alkali salts prevent histamine induced gastric ulceration. In traditional systems of medicine, turmeric is known as a stomachic and blood purifier. It is useful for common cold, leprosy, intermittent fevers, infections of the liver, dropsy, purulent ophthalmia, otorrhea, indolent ulcer, pyogenic affections, wound healing and inflammation. The rhizome of turmeric is highly aromatic and antiseptic. Its paste is used in cleansing and disinfecting the skin and skin ulcer without drying out its natural oil. Curcumin and other curcuminoids found in turmeric inhibit growth of various bacteria (Tiwari and Agrawal, 2003).

Slow multiplication rate, limited availability of high yielding genotypes, expensive field maintenance of planting material and high susceptibility of turmeric to rhizome rot diseases necessitate application of tissue culture techniques as a solution to these problems (Khader *et al.*, 1994, Nayak and Naik, 2006). Tissue culture has long been recognized as an efficient means for rapid clonal multiplication and conservation of important plant species. However, for use of tissue culture as continuous source of disease free planting material for commercial utilization, periodic monitoring of the degree of genetic stability among *in vitro* grown plantlets is of utmost importance. The chances of inclusion of variable genotypes that might arise due to occurrence of somaclonal variation at early or late phase of culture, can be

[*]***Corresponding author:***
Sanghamitra Nayak, Ph.D.
Centre of Biotechnology,
Siksha 'O' Anusandhan University,
Bhubaneswar, 751030, Orissa, India.
Email: sanghamitran@yahoo.com

Figure 1: (a) Axillary bud explant of rhizome sprouting *in vitro* (b) Multiple shoot and root formation in *Curcuma longa* in medium containing BA (3mg/l); (c) Micro-propagated plantlets of *Curcuma longa*.

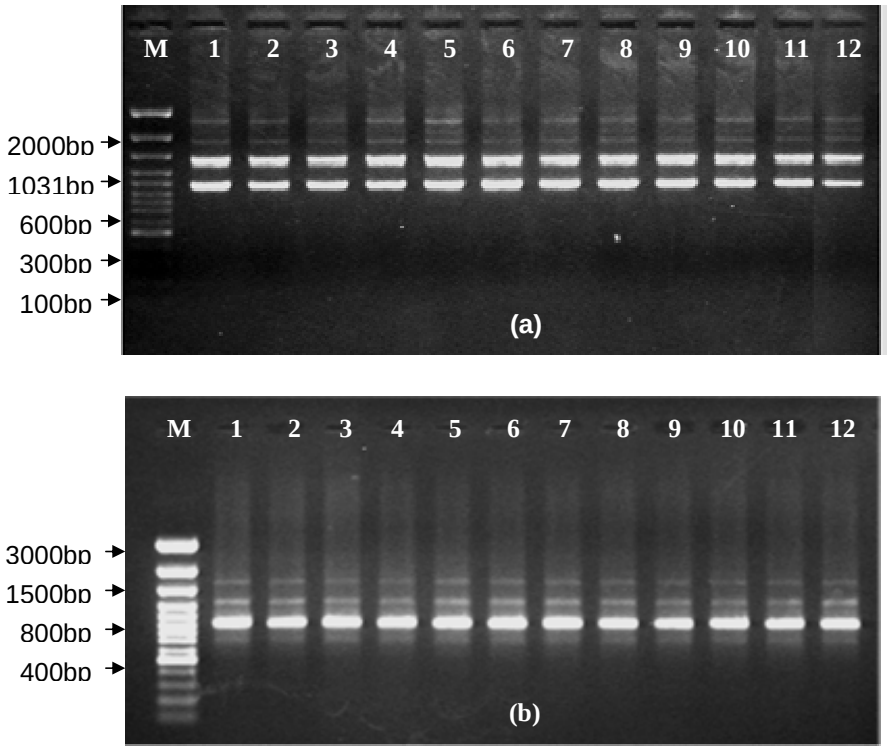

Figure 2: RAPD banding pattern with primers (a) OPC12 and (b) OPN15 in both micropropagated and field grown mother plant of C. longa (lane 1 mother plant and lane 2-12 micropropagated plants).

minimized by assessment of genetic stability at regular intervals.

Cytological and cytophotometric analysis for *in vitro* assessment of clonal fidelity has been reported in several species (Nayak and Sen, 1995, 1997; Al-Zahim *et al.*, 1999). Of several molecular markers used for such assessment, Random Amplified Polymorphic DNA (RAPD) is the simplest, cheapest and appears to be a useful tool for the analysis of genetic fidelity of *in vitro* propagated plants (Williams *et al*., 1990; Rout and Das, 2002; Martins *et al*., 2004). In the present paper, we report rapid micropropagation of an elite cultivar (cv Roma) of *C. longa* using dormant axillary buds of rhizomes and periodic evaluation of genetic homogeneity of *in vitro* conserved plantlets through cytophotometry and RAPD analysis, not included in earlier studies on micropropagation of this species. Cultivar 'Roma' is a high curcumin yielding genotype, released from High Altitude Research Station (HARS), Pattangi, Orissa (India) in 1988 and is currently under cultivation in limited places in Orissa.

Table 1; Effect of various growth regulators on *in vitro* shoot multiplication of *C. longa* (cv Roma)

Sl. No.	MS Media + Growth regulators (mg/l)	% of explants forming shoot buds (Mean ± SE)*	No. of shoots per explant (Mean ± SE)*
1	BA (1.0)	22.6 ± 0.9	1.6 ± 0.3
2	BA (3.0)	85.8 ± 0.7	7.6 ± 0.3
3	BA (5.0)	15.8 ± 0.8	2.0 ± 0.5
4	Kin (2.0)	66.0 ± 0.7	4.3 ± 0.8
5	BA (3.0) + IAA (2.0)	25.0 ± 1.3	1.3 ± 0.3
6	Kin (2.0) + NAA (2.0)	58.6 ± 0.6	4.0 ± 0.5
7	BA (3.0) + IAA (1.0) + Ads (100)	43.0 ± 1.1	4.0 ± 0.5
8	BA (5.0) + IAA (1.5) + Ads (100)	62.8 ± 0.6	3.6 ± 0.8
F value		754.5	11.41
P value			< 0.001

The differences in mean value among the treatment are statistically significant ($p < 0.001$)
*data represents the mean of 15 replicates for each treatment

Table 2: Nuclear DNA content of in vitro grown plantlets of C. longa at different culture periods.

Period of culture			
Control (from field)	10	Exclusively Diploid	8.55-8.63
2 months	30	Exclusively Diploid	8.56-8.61
8 months	33	Exclusively Diploid	8.55-8.60
14 months	27	Exclusively Diploid	8.53-8.59
20 months	25	Exclusively Diploid	8.56-8.62
26 months	31	Exclusively Diploid	8.54-8.61

MATERIALS AND METHODS

Plant material

Rhizome of *Curcuma longa* (cv Roma) was collected from the High Altitude Research Station (HARS), Pattangi of Orissa and was grown in medicinal plant garden of Centre of Biotechnology, Bhubaneswar, Orissa (India). Rhizome segments were used as explants and were thoroughly washed with water and the scale leaves were carefully cleaned so that impression of embedded buds could be marked without being damaged. Those explants were kept under running water for 10-15 minutes and then dipped in liquid detergent (Extran, Merck, Germany) for 3-5 minutes. They were thoroughly washed with distilled water to remove the last drop of detergent. Surface sterilization was done by 0.1% mercuric chloride solution for 8-10 minutes. After sterilization the explants containing buds were washed several times with sterile distilled water under aseptic condition prior to inoculation.

Plant multiplication and Culture conditions

Explants were inoculated to the basal medium of Murashige and Skoog (MS) with varying combination of 1–5 mg/l of 6-benzylaminopurine (BA), 1–2 mg/l of indole-3-acetic acid (IAA), 1-2mg/l of 1-napthaleneacetic acid (NAA), 1-2mg/l of kinetin (Kin) and 50-100 mg/l of Adenine sulphate (Ads). 15 replicas were used for each treatment for recording data on percent of explants forming shoots, number of shoots per explants and formation of roots. Prior to inoculation, MS media containing hormones were autoclaved at 121° C and 1.05 kg/cm^2 of pressure for 20 minutes. pH of the media was adjusted to 5.7. Culture tubes containing the

Table 3: RAPD banding pattern of micropropagated and field grown mother plants of C. longa.

Primers	Sequence	Range of amplicons (in bp)	Total bands
OPA02	5′TGCCGAGCTG3′	300-850	4
OPA03	5′AGTCAGCCAC3′	375-2000	9
OPA04	5′AATCGGGCTG3′	375-1800	6
OPA06	5′GGTCCCTGAC3′	475-1100	2
OPA10	5′GTGATCGCAG3′	600	1
OPC02	5′GTGAGGCGTC3′	650-1400	2
OPCO5	5′GATGACCGCC3′	950-2400	5
OPCO6	5′GAACGGACTC3′	550	1
OPC12	5′TGTCATCCCC3′	1000-2700	4
OPD03	5′GTCGCCGTCA3′	600-1250	4
OPD07	5′TTGGCACGGG3′	400-2500	5
OPD08	5′GTGTGCCCCA3′	375-1400	4
OPD18	5′GAGAGCCAAC3′	400-2000	6
OPD20	5′ACCCGGTCAC3′	450-2000	7
OPN05	5′ACTGAACGCC3′	800-1750	3
OPN06	5′GAGACGCACA3′	300-1700	7
OPN07	5′CAGCCCAGAG3′	550-2600	4
OPN08	5′ACCTCAGCTC3′	450-2200	4
OPN15	5′CAGCGACTGT3′	750-1750	4
OPN16	5′AAGCGACCTG3′	425-1050	4
		Total	**86**

inoculated explants were kept under white fluorescent light with 55 μ mole m^{-2} s^{-2} light intensity.

Field transfer of plantlets

In vitro grown plantlets with well developed roots and shoots were transferred to pots containing soil, cow dung and sand mixture in 1:1:1 ratio after ninety days of culture. These were kept in green house for acclimatization. After thirty days, the pots were transferred to the normal field condition and grown to maturity.

Cytophotometric analysis

For analysis of 4C nuclear DNA content through cytophotometry, root tips of *in vitro* grown plantlets were collected aseptically from culture tubes and were fixed over night in 1:3 propionic acid: ethanol. This treatment was followed by hydrolysis in 1N HCl of 60°C for 12 min, washed in distilled water and stained in Schiff's reagent for 2hr at 14°C and squashed with 45% acetic acid. The DNA content of nuclei was measured with Leitz Wetzler micro-spectrophotometer using the single wavelength (550 nm) method (Sharma and Sharma, 1980). *In situ* DNA values were obtained on the basis of optical densities which were converted to picograms (pg) by using Van't Hof's (1965) 4C nuclear DNA value of *Allium cepa* (67.1pg) as standard.

RAPD analysis

The protocol of Doyle and Doyle (1987) was followed with some modifications for isolation and purification of genomic DNA from both micropropagated and field grown mother plants. The crude DNA was purified by giving RNaseA treatment (@ 60μg RNaseA for 1ml of crude DNA solution) followed by three washes with Phenol: Chloroform: Iso-Amyl-Alcohol (25:24:1) and subsequently three washes with Chloroform: Iso-Amyl-Alcohol (24:1). The upper aqueous phase was separated after centrifugation and mixed with $1/10^{th}$ volume of 3M-sodium acetate. DNA was precipitated by adding 2.5 volume of chilled absolute ethanol, pelleted, dried in vacuum and dissolved in $T_{10}E_1$ buffer. Quantification of DNA was accomplished by analyzing the purified DNA on 0.8% agarose gel alongside uncut lambda DNA as standard. DNA was diluted in $T_{10}E_1$ to 25ng/μl for PCR analysis.

A total of 30 random primers were utilized for RAPD analysis, out of which 20 random decamer primers (Operon Tech., USA) from A,

C, D and N series (OPA02, 03, 04, 06, 10; OPC02, 05, 06, 12; OPD03, 07, 08, 18, 20 and OPN05, 06, 07, 08, 15, 16) were selected on the basis of the clarity of banding patterns. The protocol for RAPD analysis was adapted from that of Williams *et al.* (1990). PCR was performed in a volume of 25 µl containing 25ng of template DNA, 2.5 ml of 10X assay buffer (100mM Tris HCl, pH 8.3, 500 mM KCl and 0.1% gelatin), 1.5 mM $MgCl_2$, 200µM each of dNTPs, 15ng of primer (Genei, Bangalore, India), 0.5 U Taq DNA Polymerase (Bangalore Genei Pvt. Ltd, India). DNA amplification was performed in thermal cycler (Applied Biosystems, Model Gene amplification PCR System 9700, USA). The first step consists of holding the samples at 94ºC for 5 minutes for complete denaturation of the template DNA. The second step consists of 42 cycles having three ranges of temperature i.e. at 92ºC for 1 minute for denaturation of template DNA, at 37ºC for 1 minute for primer annealing and at 72ºC for 2 minutes for primer extention followed by running the samples at 72ºC for 7 minutes for complete polymerization. The PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide @ 0.5µg/ml of gel solution in TAE buffer (40 mM Tris base, 20 mM sodium acetate, 20 mM EDTA, glacial acetic acid to pH 7.2) for 3 h at 60 volts. The size of the amplicons was determined using size standards Generuller 100bp DNA Ladder Plus (MBI Fermentas, Lithuania). DNA fingerprints were visualized under UV light and photographed using Gel Documenting System. RAPD analysis using each primer was repeated at least twice to establish reproducibility of banding pattern of different DNA samples of turmeric.

RESULTS AND DISCUSSION

Rapid plant multiplication

The dormant axillary buds from rhizome fragments of *in vivo* grown plants were used as explant and inoculated to MS media containing varying combinations of BA (1-5mg/l), Kin (1-2mg/l), NAA (1-2mg/l), IAA (1-2mg/l) and Ads (50-100mg/l). Percentage of response of the explants to selected combinations was shown in Table 1. Medium containing BA (3mg/l) was optimum for shoot multiplication of *C. longa*. Dormant axillary bud of rhizome became active and sprouted on this media (Fig. 1a) with subsequent production of multiple shoots. Root and shoot development occurs in same media (Fig. 1b). Shoot multiplication was maximum in media containing BA 3mg/l producing 6-8 shoots per culture within 4 weeks (Table 1). Approximately 400 million plantlets could be produced from a single explant in a year where as multiplication rate through traditional method is maximum 8 plants per annum. Multiplication rate remained unchanged even after 2 years of culture. The cultures were maintained in the same media with regular sub-culturing at 3months interval (Fig. 1c).

During planting season, some plantlets grown *in vitro* were transferred to the field out of which about ninety percent of plants survived successfully with normal growth after transfer to field condition (Fig. 1d).

The axillary buds of rhizome used as explants were available throughout the year for explantation for initiating tissue culture. In all the earlier reports on micropropagation of *C. longa* direct shoot multiplication and plant regeneration were achieved using active sprouted shoot buds of rhizome, which were available during planting season. (Nadgauda *et al*. 1978; Balachandran *et al.* 1990; Salvi *et al.* 2002; Nayak and Naik, 2006).

Cytophotometric analysis

The ploidy status of *in vitro* grown regenerants of *C. longa* was determined by *in situ* estimation of nuclear DNA content at 6 months interval. 146 plants analyzed over a period of 26 months in culture were exclusively diploid revealing unimodal distribution of a peak in each case corresponding approximately to 4C value (8.58pg) as in control. The range of mean DNA content in root tip of *in vitro* conserved plantlets of different ages was almost similar to the range obtained in root tip of 10 control plants from field (Table 2). Stable nature of regenerates of *C. longa* was thus ascertained by cytophotometric estimation of 4C nuclear DNA content. Determination of stability of tissue cultured plants through cytophotometry has earlier been reported in species of *Ornithogalum thyrsoides* (Nayak and Sen, 1991) and *Ornithogalum umbellatum* (Nayak and Sen, 1995). Diploidy was confirmed with unimodal distribution with a peak at 4C DNA value in all regenerates in *O. thyrsoides* after 3 years and in *O.* umbellatum after 5 years of culture *in vitro.*

RAPD analysis

In order to confirm the genetic integrity of micropropagated turmeric maintained in culture over a period of more than 2 years, RAPD analysis was carried out at 6 months interval starting from 2 months old plantlets. A total of 65 plantlets over a period of 26 months were analyzed taking a minimum of 10 plants randomly at each culture period.

Twenty selected RAPD primers utilized in this study gave rise to a total of 86 scorable bands, ranging from 300 bp to 2700 bp in size (Table 3). The number of bands for each primer varied from 1 to 9, with an average of 3.95 bands per RAPD primer. A total of 5590 bands (number of plantlets analyzed X number of bands with all primers) were generated by the RAPD technique, giving rise to monomorphic patterns across all 65 plantlets analyzed. Number of monomorphic bands was highest i.e. 9 in case of primer OPA03 (ranging from 375- 2000bp in size) and was lowest i.e. 1 in case of both the primers OPA10 (600 bp) & OPC06 (550bp). The RAPD banding pattern showing monomorphic bands obtained among 11 regenerates from 26 months old culture is shown in Fig. 2 for RAPD markers. No RAPD polymorphism was observed in the micropropagated plants (Fig. 2).

RAPD analysis of micropropagated plants of turmeric showed a profile similar to that of the control indicating that no genetic variation had occurred *in vitro*. RAPD based assessment of genetic stability of *in vitro* grown micropropagated plants has been reported in many other plant species (Rout *et al.*, 1998; Rout and Das, 2002, Martins *et al*. 2004, Venkatachalam *et. al*., 2007). We have used axillary buds as explants for micropropagation of *Curcuma longa* because it lowers the risk of genetic instability. The result concur with those of Shenoy and Vasil (1992) who earlier reported that the micropropagation through explants containing organized meristem is generally associated with low risk of genetic instability because they are generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* condition.

In *C. longa* the length of the culture period (for more than 2 years) with regular sub culturing did not seem to affect their genetic integrity. Similar results were also reported by Martins *et al.* (2004) in almonds plantlets and Angel *et al.* (1996) in cassava plantlets. Some authors, however, have reported that the time in *in vitro* culture could promote somaclonal variation (Orton 1985; Hartmann *et al.* 1989; Nayak and Sen 1991). Venddrame *et al*. (1999) concluded that, genetic variation in a culture line could be affected more by the genotype than by the period in culture. Hammerschlag *et al*., (1987) suggested that the genotype and the nature of the explants could influence the phenotypic stability of the plants obtained, in studies performed with micropropagated peach plantlets. Culture time does not seem to be the only parameter affecting genetic stability (Gould, 1986).In the study reported here, RAPD analysis was used to evaluate the genetic homogeneity of turmeric plantlets after 2 years of *in vitro* culture.

The results obtained in our experiment suggest that *in vitro* shoot multiplication using axillary buds of the rhizomes may be used for rapid clonal propagation and conservation with a low risk of generating somaclonal variations, particularly for elite clone of turmeric (cv Roma). These cultures are now being used as a source of disease free planting material having several commercial implications.

Acknowledgement

The authors are grateful to Dr. S. C. Si, Dean, School of Pharmaceutical Sciences, and Dr. M. R. Nayak, Chancellor, Siksha 'O' Anusandhan University, Bhubaneswar, for providing facilities and continuous encouragement.

References

Al-Zahim MA *et al.*, (1999) Detection of somaclonal variation in garlic (*Allium cepa*) using RAPD and cytological analysis. *Plant Cell Reports*. **18**: 473-477.

Angel F *et al.*, (1996) Stability of cassava plants at the DNA level after retrival from 10 Years of *in vitro* storage. *Euphytica*.**90**:307-313.

Balachandran SM *et al.*, (1990) *In vitro* clonal multiplication of turmeric (curcuma spp.) and ginger (*Zingiber officinale* Rosc.) *Plant cell Reports.* **8**:521-524.

Gould AR (1986) Factors controlling generation of variability *in vitro*. In:Vasil IK (ed) Cell culture and somatic cell genetics in plants. 3. Plant regeneration and genetic variability. Academic, Orlando. pp 549-567.

Doyle JJ and Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*. **19**: 11-15.

Hammerschlag FA *et al.*, (1987) Factors influencing in vitro multiplication and rooting of peach cultivars. *Plant Cell Tissue Organ Culture*. **8**: 235-242.

Hartmann C *et al.*, (1989) identification of new mitochondrial genome organizations in wheat plants regenerated from somatic tissue cultures. *Theortical and Applied Genetics*. **77**:169-175.

Khader *et al.* (1994) Improvement of Turmeric, Advances in Horticulture Vol 9 – Plantation and Spice Crops Part 1. Eds: K.L. Chadha and P. Rethinam, Malhotra Publishing House, New Delhi-110064, India.

Martins M *et al.*, (2004) Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers, *Plant Cell Reports.* **23**: 492-496.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures, *Physiologia Plantarum*. **15**: 473-97.

Nadgauda RS *et al.*, (1978) Rapid clonal multiplication of turmeric (*Curcuma longa*), plants by tissue culture. *Indian Journal of Experimental Biology*. **16**:120-122.

Nayak S and Naik PK (2006) Factors affecting *in vitro* microrhizome formation and growth in *Curcuma longa* L. and improved field performance of micropropagated plants. *Science Asia*. **32**:31-37.

Nayak S & Sen S (1991) Cytological and cytophotometric analysis of direct explant and callus derived plants of *Ornithogalum thyrsoides* Jacq. *Cytologia*. **56**: 297-302.

Nayak S & Sen S (1995) Rapid and stable propagation of *Ornithogalum umbellatum* in long term culture. *Plant Cell Report*. **15**: 150-153.

Nayak S and Sen S (1997) Cytological and cytophotometric analysis of callus and regenerated plants of *Ornithogallum virens*. *Cytobios*. **91**: 135-142.

Orton TJ (1985) Genetic instability during embryogenic cloning of celery. *Plant cell Tissue Organ Culture*. **4**:159-169.

Roses IA (1999) *Curcuma longa* in Medicinal Plants of the World, Chemical Constituents, Traditional and Modern Medicinal Uses. Human Press, Totowa, New Jersy, pp 139-153.

Rout GR and Das G (2002) An assessment of genetic integrity of micropropagated plants of *Plumbago zeylanica* by RAPD markers, *Biologia Plantarum*. **45** (1):27-32.

Rout GR *et al.*, (1998) Determination of genetic stability of micropropagated plants of ginger using Rapid Amplified Polymorphic DNA (RAPD) markers. *Botanical Bulletin of Academia Sinica*. **39**:23-27.

Salvi ND *et al.*, (2002) Micropropagation and field evaluation of micro-propagated plants of turmeric, *Plant Cell Tissue and Organ Culture*. **68**: 143-151.

Sharma AK and Sharma A (1980) Chromosome Techniques: Theory & Practice. Third Edition, Butterworths, London.

Shenoy VB and Vasil IK (1992) Biochemical and molecular analysis of plants derived from embryogenic tissue cultures of napiergrass (Penisetum purpureum K. Schum.). *Theoritical and Applied Genetics*. **83**:947-955.

Tiwari L and Agrawal DP (2003) All Purpose Turmeric: Uses, Chemistry, IPR. Essay in 'History of Indian Science and Technology'.

Van't Hof J (1965) Relationships between mitotic cycle duration, S period duration and the average rate of DNA synthesis in the root meristem cells of several plants. *Experimental Cell Research*. **39**: 48-58.

Venddrame WA *et al.*, (1999) AFLP analysis of variation in pecan somatic embryos. *Plant Cell Report*. **18**:853-857.

Venkatachalam L *et al.*, (2007) Genetic analysis of micropropagated and regenerated plantlets of banana as assessed by RAPD and ISSR markers. *In vitro Cellular Developmental Biology*. **43(3)**:267-274.

Williams JGK *et al.*, (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*. **18**: 65.