

Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers

Venkatachalam Lakshmanan

Plant Cell Biotechnology Department
Central Food Technological Research Institute
Mysore - 570 020, India
Tel: 91 821 2516502
Fax: 91 821 2517233
E-mail: genevenki@yahoo.com

Sreedhar Reddampalli Venkataramareddy

Plant Cell Biotechnology Department
Central Food Technological Research Institute
Mysore - 570 020, India
Tel: 91 821 2516502
Fax: 91 821 2517233
E-mail: rvsree@rediffmail.com

Bhagyalakshmi Neelwarne*

Plant Cell Biotechnology Department
Central Food Technological Research Institute
Mysore - 570 020, India
Tel: 91 821 2516502
Fax: 91 821 2517233
E-mail: blakshmi_1999@yahoo.com

Financial support: Junior and Senior Research Fellowship from the Council of Scientific and Industrial Research, India.

Keywords: Growth regulators, *Musa*, protocol, shoot cultures, somaclonal variation.

Abbreviations: AS: Adenine Sulphate
BAP: 6-Benzylaminopurine
ISSR: Inter Simple Sequence Repeats
Kn: Kinetin
MS: Murashige & Skoog
NR: Nanjanagudu Rasabale
RAPD: Random Amplified Polymorphic DNA

A large number of micropropagated plantlets of banana, *Musa acuminata* var. Nanjanagudu Rasabale (NR), that were developed from axillary shoot bud explants over 10 years ago were screened for genetic variation, if any, using RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeats) markers. Of the 4000 *in vitro* plantlets, 11 were used for screening that involved shoot cultures with distinct variation in morphological characteristics (morphotypes). Similarly, the mother maintained in the field was also subjected for genetic analysis. Out of the 50 RAPD and 25 ISSR primers screened, 30 RAPD and 5 ISSR primers produced totally 424 clear, distinct and reproducible band classes resulting in a total of 5088 bands where the banding patterns for each primer was highly uniform and comparable to the field-grown

mother clone from which the cultures had been established. These results indicate that the micropropagation protocol developed by us for rapid *in vitro* multiplication is appropriate and applicable for clonal propagation of banana var. NR over a long period. This is the first report on the use of genetic markers to establish genetic fidelity of long-term micropropagated banana using RAPD and ISSR.

Banana belongs to the genus *Musa* and is the most important of the tropical fruits accounting for the world production of 40 million tonnes (FAO, 2004). Most of the edible cultivars of banana are sterile triploids or tetraploids, propagated mainly by vegetative means. Tissue culture propagation of banana has gained attention due to its potential to provide genetically uniform, pest- and disease-

*Corresponding author

free planting materials. Among the delicious dessert bananas, *Musa acuminata*, var. Nanjanagudu Rasabale (NR) (group “Silk” having genotype AAB) which is grown in Mysore district of Karnataka, India, has very high commercial demand due to its inviting aroma and white fluffy sweet pulp. This variety is highly susceptible to bacterial and viral diseases reducing the area of cultivation to only 5 hectares and hence recently considered as an endangered one.

Propagation of banana through *in vitro* techniques has been reported by several researchers using different explant sources as well as regeneration pathways (Novak et al. 1989; Bhagyalakshmi and Singh, 1995; Venkatachalam et al. 2006). A very few exogenous hormones and growth regulators have been reported useful for the micropropagation of banana (Novak et al. 1989; Bhagyalakshmi and Singh, 1995) where the sub- and supra-optimal levels of plant growth substances, especially the synthetic ones, have been associated with somaclonal variation (Martin et al. 2006). Even at optimal levels, long-term multiplication often may lead to somaclonal or epigenetic variations in the micropropagated plants questioning the very fidelity of their clonal nature. Therefore, it is necessary to establish genetic uniformity of micropropagated shoots by recent molecular techniques.

Analyses of isozymes patterns of specific enzymes provide a convenient method for detection of genetic changes but they are subjected to ontogenic variations. Such methods have limited applications due to their limited numbers and only those DNA regions coding for soluble proteins can be sampled. Tissue and environment independence in expression of DNA based markers have made them more reliable over morphological and isozyme markers. Among various DNA-based markers, though Restriction Fragment Length Polymorphism (RFLP) can be used for screening

genetic stability of tissue cultured plants, the method involves use of expensive enzymes, radioactive labeling and extensive care, therefore, appears unsuitable. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers, on the other hand, require only small amount of DNA sample without involving radioactivity tests and are simpler as well as faster. RAPD has proven to be quite efficient in detecting genetic variations (Williams et al. 1990), even in closely related organisms such as two near isogenic lines (NIL). ISSR technique is also very simple, fast, cost-effective, highly discriminative and reliable (Pradeep et al. 2002). At present, RAPD and ISSR markers have been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in various plants (Carvalho et al. 2004; Martins et al. 2004; Ramage et al. 2004; Modgil et al. 2005). The study presented here was undertaken to monitor the genetic stability of long term micropropagated shoots of a banana clone using the RAPD and ISSR technique. To our knowledge, this is the first assessment of DNA sequence variation in the long term micropropagules of any *Musa* species. An *in vitro* clonal propagation protocol has been developed for efficient multiplication of banana in our lab (Bhagyalakshmi and Singh, 1995) and has been used continuously as an initial step for the production and maintenance of shoot cultures - the latter are often used for other biotechnological studies. The aim of the present study was to assess the impact of the protocol and long term *in vitro* effects on the induction of somaclonal variation in banana var. NR.

MATERIALS AND METHODS

Plant material

A healthy and high yielding banana clone of var. Nanjanagudu Rasabale was collected and maintained as

Table 1. Composition of media used at various stages of micropropagation of NR banana.

Medium	Composition with growth regulators in mg l ⁻¹	Stages
M1	MS* + BAP (0.2) + Sucrose (3%) + Gelrite® (0.2%)	Meristem culture
M2	MS* + BAP (2) + Kn (1) + AS (80) + Sucrose (2.5%) + Gelrite® (0.25%)	Shoot multiplication
M3-A	MS* + NAA (0.5) + Sucrose (2.5%) (Liquid medium)	Elongation and rooting
M3-B	MS* + NAA (0.5) + Sucrose (2.5%) + Agar (0.8%) (Solid medium)	Semi-hardening and further elongation and rooting
M4	½ MS + Agar (1.6%)	Hardening

* Modified MS where the concentration of ammonium nitrate was reduced to 75% of original MS medium (Murashige and Skoog, 1962).



Figure 1. *In vitro* multiplied plants under hardening. The plantlets were obtained after repeatedly sub-culturing in M2 medium for 150 times, each after 4 weeks period. Further elongation and rooting were done as mentioned in Table 1 and in section Material and Methods.

mother plant at the back yard of authors' laboratory. The rhizomes of sword suckers were used to excise the meristem and establish shoot cultures as explained earlier (Bhagyalakshmi and Singh, 1995).

Culture medium and incubation conditions

The aseptic shoot cultures used in the present study were established as reported in an earlier study (Bhagyalakshmi and Singh, 1995) and were maintained on M1 medium containing $\frac{3}{4}$ strength Murashige and Skoog (Murashige and Skoog, 1962) basal salts (MS) with additional 1 g l^{-1} of potassium nitrate and 1 ml l^{-1} of vitamin mixture (Novak et al. 1989) supplemented with ascorbic acid (100 mg l^{-1}), BAP (1 mg l^{-1}), indole butyric acid (0.2 mg l^{-1}) and gelrite® (Sigma, USA) (2.5 g l^{-1}). The pH was adjusted to 5.8 before autoclaving at 121°C and 15 lbs for 20 min. Cultures were maintained at $25 \pm 1^\circ\text{C}$ under a 16 hrs light ($320\ \mu\text{mol.m}^{-2}\ \text{S}^{-1}$) / 8 hrs dark photoperiod. The *in vitro* multiple shoot cultures were established and maintained on M2 medium (Table 1).

Elongation and rooting

Multiple shoot/buds proliferated from each segment were separated and sub cultured twice with a 4 week interval in liquid medium containing MS basal nutrient with 0.5 mg l^{-1} NAA for further elongation (M3-A in Table 1). After 8 weeks, individual shoots with 4-5 cm in length were separated and transferred to solid MS basal medium with 0.5 mg l^{-1} NAA (M3-B) for 4 weeks to induce further shoot elongation and root formation. Further hardening before transferring to soil was done by briefly culturing the plantlets in M4 medium (Table 1).

Greenhouse transfer and morphological analysis

The rooted shoots were washed in running tap water and were planted in polythene bags containing mixture of sand, vermicompost and garden soil in equal proportions. The plants were incubated in a mist chamber maintained at 70-80% RH for 3-4 weeks and subsequently maintained in

greenhouse for one month (Figure 1) before planting in the field.

Plant material for DNA extraction

The mother NR plant collected from Nanjanagudu, Karnataka, India was maintained in our department garden and the fresh leaves from this were used as a source of DNA. For testing the long-term effects, the plantlets obtained after repeatedly sub-culturing in M2 medium for 150 times (about 10 years), each after 4 weeks period, were used. Eleven plantlets, including certain morphological variants were chosen from over 4000 micropropagated plantlets (Figure 1) for studying DNA fingerprints.

Preparation of template DNA

The genomic DNA from the cultures was extracted at the end of last subculture. The DNA was extracted by using the GenElute™ Plant Genomic DNA Mini prep kit supplied by Sigma (USA). The RNA contamination in all the samples was removed by digesting the extract with 100 RNase-A ($100\ \mu\text{g ml}^{-1}$; Bangalore Genei, India) for 30 min at 37°C . Quality and quantity of DNA preparations were checked by standard spectrophotometry and the samples were diluted to a concentration of $25\text{ ng }\mu\text{l}^{-1}$ before use.

DNA amplification

Initially, optimum PCR conditions for both RAPD and ISSR were standardized with various quantities of template DNA (12.5, 25 and 50 ng), dNTPs (100, 200 and 300 μM) and MgCl_2 (0, 1, 2 and 3 mM). Later, RAPD amplifications were performed routinely using PCR mixture (25 μl) which contained 25 ng of genomic DNA as template, 1X PCR buffer (Fermentas GMBH, Germany), 200 μM dNTPs (Fermentas GMBH, Germany), 1 unit (U) of *Taq* DNA polymerase (Bangalore Genei, India), 1 μM of each primer with various concentrations of MgCl_2 (Fermentas GMBH, Germany) (Table 2) depending on the primer (Sigma-Aldrich, India). PCR was performed at initial denaturation at 93°C for 4 min followed by 40 cycles of 1 min denaturation at 94°C , 1 min annealing at 36°C and 2 min extension at 72°C with a final extension of 72°C for 10 min using a thermal cycler (Eppendorf Thermal cycler).

In case of ISSR primers, optimal annealing temperature was found to vary according to the base composition of the primers. PCR mixture (25 μl) which contained 25 ng of genomic DNA as template, 1 x PCR buffer (Fermentas GMBH, Germany), 200 μM dNTPs (Fermentas GMBH, Germany), 1 unit (U) of *Taq* DNA polymerase (Bangalore Genei, India), 1 μM of each primer (Sigma Aldrich, India) with various concentrations of MgCl_2 (Table 2) depending on the primer. PCR was performed at initial denaturation of 94°C for 4 min followed by 40 cycles of 1 min denaturation at 94°C , 1 min annealing at 2°C lower than melting point for each primer and 2 min extension at 72°C with a final extension of 72°C for 10 min using a thermal cycler.

Table 2. List of primers, their sequences and size of the amplified fragments generated by 30 RAPD (SI. No. 1-30) and 5 ISSR (SI. No. 31-36) primers.

Sl. No.	Primer	Primer sequence (5' – 3')	G + C Content (%)	Mg Cl ₂ Concentration (mM)	Number of scorable bands	Size range (bp)
1	OPA-04	AATCGGGCTG	60	2	12	300-3000
2	OPA-09	GGGTAACGCC	70	0	8	400-1800
3	OPA-11	CAATCGCCGT	60	2	18	300-3100
4	OPA-14	CTCGTGCTGG	70	0	13	200-3000
5	OPA-20	GTTGCGATCC	60	1	14	200-2600
6	OPC-01	TTCGAGCCAG	60	1	12	280-1800
7	OPC-02	GTGAGGCGTC	70	2	12	300-2800
8	OPC-04	CCGCATCTAC	60	0	15	220-2000
9	OPC-05	GATGACCGCC	70	0	14	320-1900
10	OPC-07	GTCCCGACGA	70	0	16	300-2600
11	OPC-08	TGGACCGGTG	70	1	14	290-3100
12	OPC-09	CTCACCGTCC	70	1	10	480-1800
13	OPC-13	AAGCCTCGTC	60	0	8	480-1800
14	OPD-07	TTGGCACGGG	70	1	13	380-2500
15	OPD-08	GTGTGCCCCA	70	0	7	290-2400
16	OPD-16	AGGGCGTAAG	60	1	14	400-2000
17	OPJ-07	CCTCTCGACA	60	1	11	300-2000
18	OPJ-08	CATACCGTGG	60	0	6	400-3000
19	OPJ-09	TGAGCCTCAC	60	2	8	400-2000
20	OPL-14	TCGTGCGGGT	70	1	8	1031-2400
21	OPM-12	CACAGACACC	60	0	12	300-2600
22	OPM-16	GTAACCAGCC	60	2	18	400-2000
23	OPM-20	AGGTCTTGGG	60	1	22	320-2000
24	OPM-18	CACCATCCGT	60	0	7	400-2000
25	OPN-03	GGTACTCCCC	70	0	18	300-2600
26	OPN-04	GACCGACCCA	70	0	12	400-2800
27	OPN-06	GAGACGCACA	60	2	11	340-2000
28	OPN-09	TGCCGGCTTG	70	1	18	240-3000

29	OPN-10	ACAACCTGGGG	60	1	19	240-3000
30	OPN-12	CACAGACACC	60	0	7	240-2400
Total					377	
31	UBC-811	(GA) ₈ C	-	0	7	250-2500
32	UBC-817	(CA) ₈ A	-	1	8	400-2800
33	UBC-820	(GT) ₈ T	-	0	11	200 - 2000
34	UBC-826	(AC) ₈ C	-	0	8	400- 2000
35	UBC-834	(AG) ₈ YT	-	1	13	1031 - 3000
Total					47	

The PCR products obtained were separated on 2% agarose gel (ICN, USA), stained with ethidium bromide (0.001%) and documented in a gel documentation system (Hero-Lab GMBH, Germany). The size of the amplification products was estimated from 100 bp DNA ladder (Fermentas GMBH, Germany).

Data analysis

All treatments tested for the shoot cultures had at least five replicates and the data presented as an average of mean of replicates of two separate experiments with standard deviation. For both RAPD and ISSR profiles, the well-resolved and consistently reproducible fragments ranging from 200 bp to 3.0 kb were scored as present or absent. For detecting any genetic change, all the RAPD and ISSR results were compared with each other for all the DNA samples.

RESULTS AND DISCUSSION

True-to-type clonal fidelity is one of the most important prerequisites in the micropropagation of any crop species. A major problem encountered with the *in vitro* culture is the presence of somaclonal variation amongst sub-clones of one parental line, arising as a direct consequence of *in vitro* culture of plant cells, tissues or organs. A better analysis of genetic stability of plantlets can be made by using a combination of two types of markers which amplify different regions of the genome (Martins et al. 2004). Palombi and Damiano (2002) suggested the use of more than one DNA amplification technique as advantageous in evaluating somaclonal variation while working on micropropagated plants of kiwi fruit. Hence, in the present study, two PCR based techniques, RAPD and ISSR were adopted for evaluation of clonal fidelity of banana plantlets. PCR - based techniques such as RAPD, ISSR, and AFLP have been found immensely useful in establishing the genetic stability of cultivated as well as *in vitro* regenerated

plants such as *Prunus dulcis* (Martins et al. 2004), apple (Modgil et al. 2005), *Pinus thunbergii* (Goto et al. 1998), cauliflower (Leroy et al. 2001) and *Digitalis obscura* (Gavida et al. 1996). Cultures of *Anigozanthos viridis* (Turner et al. 2001) and *Foeniculum vulgare* (Bennici et al. 2004) that were kept under cryo-preservation, where the latter is known to cause high variation and instability due to long-term exposure to extreme conditions, the PCR-based techniques unequivocally proved to be useful for establishing the genetic stability.

In the present study, DNA extraction by GenElute™ Plant Genomic DNA Mini prep kit from Sigma (USA) resulted in the preparation of DNA samples of good quality and quantity in comparison to CTAB method that was tested primarily. Also the conditions of PCR amplification such as magnesium chloride, template DNA, *Taq*-polymerase concentration and annealing temperature were different and most crucial for each primer. The method used for standardizing the annealing temperature by calculating the

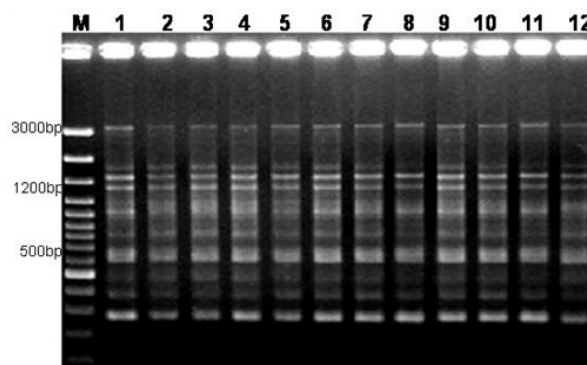


Figure 2. Randomly Amplified Polymorphic DNA (RAPD) amplification pattern obtained for mother plant (lane 1) and long-term micropropagated shoot cultures (lanes 2-12) generated by primer OPJ 09. M: GeneRuler™ 100 bp DNA Ladder Plus.

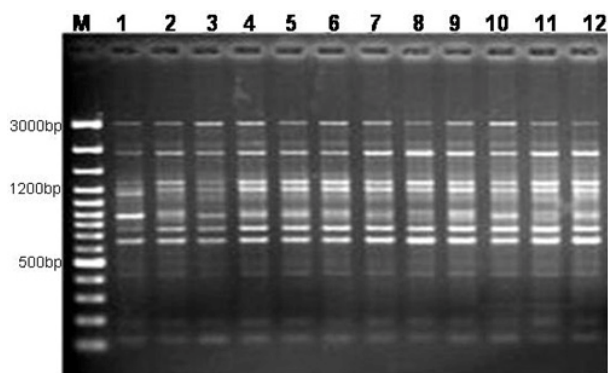


Figure 3. Randomly Amplified Polymorphic DNA (RAPD) amplification pattern obtained for mother plant (lane 1) and long-term micropropagated shoot cultures (lanes 2-12) generated by primer OPN 10. M: GeneRuler™ 100 bp DNA Ladder Plus.

melting point ($T_M = 4(G+C) + 2(A+T)^{\circ}C$) of primers and the selection of $2^{\circ}C$ lesser than the T_M appeared suitable.

In order to confirm the genetic fidelity a comparison of RAPD and ISSR patterns of 11 plants chosen from 4000-plus plantlets (Figure 1) and a control plant (mother plant) was carried out. Of the 50 arbitrary RAPD primers initially screened, 30 produced clear and scorable amplification products. The 30 RAPD primers resulted in 377 scorable band classes, ranging from 200 bp to 3100 bp in size. The number of bands for each primer varied from 6 (OPJ 08) to 22 (OPM 20) with an average of 12.6 bands per RAPD primer (Table 2). The screening with 5 ISSR primers generated 47 scorable band classes, ranging in size from 200 bp to 2200 bp. The number of bands for each primer varied from 7 (UBC 811) to 13 (UBC 834) with an average of 9.4 bands per ISSR primer. A total of 5088 bands (numbers of plantlets analysed x number of band classes with all the ISSR and RAPD primers) were generated, giving rise to monomorphic patterns across all the plantlets analysed. Samples of the monomorphic band classes obtained for RAPD (Figure 2 and Figure 3) and ISSR (Figure 4) markers are shown. No polymorphic bands were observed.

Reliable monitoring of variability in DNA sequences of plants has been achieved using PCR based molecular markers like RAPD, SSR, ISSR and AFLP. Absence of genetic variation using RAPD has been reported in several cases such as micropropagated shoots of *Pinus thunbergii* (Goto et al. 1998), axillary bud proliferation of chestnut root stock hybrids (Carvalho et al. 2004) and almond plantlets (Martins, 2004). In contrast, somaclonal variations were reported in micropropagated plants of *Pinus tremuloides* (Rahman and Rajora, 2001) and *Actinidia deliciosa* (Palombi and Damiano, 2002) using PCR based RAPD and ISSR markers.

Dwarf somaclonal variants in *in vitro* cultures of banana var. Williams (Cavendish banana), have been reported

(Israeli et al. 1996) and such dwarf somaclones were relatively stable and did not generally revert to a normal phenotype (Ramage et al. 2004). It has been reported in *Musa* that the extent of instability caused by *in vitro* culture was related to cultivar rather than the culture condition (Ray et al. 2006). Whereas, in plantain, Krikorian et al. (1993) reported that frequency of variants in a certain cultivar was confined to individual primary explant rather than clones, where same chimeric heterogeneity of the primary explant was retained (Reuveni and Israeli, 1990). Another common undesirable somaclonal variant is the mosaic type heterogeneity and such an incident of variation was reported in Cavendish banana (Reuveni and Israeli, 1990). Thus the observations made in various studies indicate that variability can occur within the clone, different explants within a plant, inter-clonal and inter-variatal.

In the present study, we adopted the use of two PCR-based techniques, RAPD and ISSR, for the identification of somaclonal variation of in NR banana plantlets because of their simplicity and cost-effectiveness. The use of two types of markers, which amplify different regions of the genome, allows better chances for identification of genetic variation in the plantlets. Although this study has not detected any genetic change, it is possible that some changes might have occurred that go undetected as there is a possibility of point mutations occurring outside of the priming sites.

The exact cause of somaclonal variation in *in vitro* cultures are still unknown, although it is believed that alterations in auxin-cytokinin concentrations and their ratio, duration of *in vitro* culture, *in vitro* stress due to unnatural conditions, altered diurnal rhythm and nutritional conditions (Modgil et al. 2005) together or independently are responsible. Cultured plant tissues are also known to undergo high levels of oxidative stress due to reactive oxygen species formed within the cells and the latter is known to cause DNA damage, including that of microsatellite instability (Jackson et al. 1998). Probably due to some of such

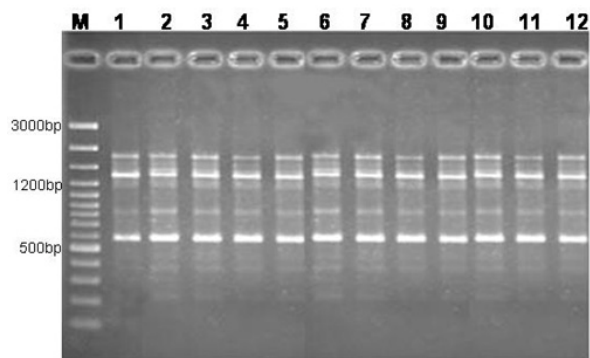


Figure 4. Inter Small Sequence Repeats (ISSR) amplification pattern obtained for DNA of mother plant (lane 1) and long-term micropropagated shoot cultures (lanes 2-12) generated by primer UBC 811. M: GeneRuler™ 100 bp DNA Ladder Plus.

reasons, morphological variations have been a common feature as long as the shoot/plantlet cultures are under *in vitro* conditions, with reversal to normal state when such plantlets are transferred to soil. Development of different morphotypes during prolonged *in vitro* culturing was also observed by Goto et al. 1998 and Ishii et al. 1987 while working on micropropagated morphotypes of *Pinus thunbergii* and *Pinus radiata* respectively. In these studies, as well as in several other studies, the use of cytokinins, especially the exposure to BAP coupled with altered diurnal rhythm and continuous availability of high levels of nutrients have been noted to induce hyper-hydricity and alterations in morphology. Hyper-hydricity, otherwise known as vitrification phenomenon, has also been prevalent in xerophytic plants. Such morphological changes, however, were found un-associated with the genetic change (Goto et al. 1998), as also observed in NR banana of the present study. Even at post-transcriptional level, the absence of any change in isozyme patterns in such morphological off-type plants (Ishii et al. 1987) indicates that there could be temporary alterations in the physiological states of the shoots. Therefore, even in the case of NR banana, though certain morphotypes were observed during routine multiplication, the tissue culture protocol developed by us resulted in clonal plantlets with no detectable genetic change.

In conclusion, this study has screened a large number of random primers that are common for higher plants, and some of them are prevalent in monocots. Since there were no changes in the banding pattern observed in tissue culture plants as compared with that of mother plant, we conclude that that our micropropagation protocol for banana var. Nanjanagudu Rasabale can be carried out for a considerable length of time without much risk of genetic instability.

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