

Original Research Article

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Analysis of the Genetic Stability of Banana Tissue Culture Propagated Plantlets cv. Ney Poovan (AB) Using Morphological and Molecular Markers

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ABSTRACT

The present study was carried out to monitor somaclonal variations of banana cv. Ney Poovan (AB) during micropropagation using random amplified polymorphic DNA (RAPD). Sword suckers were used as explants and regenerated on MS media supplemented with BAP alone, BAP + Kinetin and TDZ at varying levels of concentrations. The regenerated shoots were again subcultured on MS medium fortified with plant growth regulators at different concentrations. Rooting was obtained on ½ MS medium supplemented with 2.00 mg/l IBA and 2 g/l of activated charcoal. Rooted plantlets were observed visually for phenotypic appearances and then transferred in greenhouse for hardening. After one month the hardened plants of banana were screened for genetic stability using RAPD primers. For molecular analysis 6 operon primers were used and all of them showed amplification. 04 primers (OPC 04, OPC 20, OPK 14, OPM 12) amplified unambiguous, readable and showed monomorphic bands, whereas, 02 primers (OPB 04 and OPD 07) gave polymorphic banding patterns. A total of 296 scorable band classes were produced by 6 RAPD primers tested in banana cv., Ney Poovan. The number of bands for each primer varied from 28 to 80 with an average of 49.33 bands per primer. The results obtained using RAPD showed recordable monomorphic and polymorphic band patterns. RAPD had shown 94.10 % monomorphism and 5.90 % polymorphism. In the present investigation, the percentage of polymorphism was 5.90 % which is under permissible limit. In banana, up to 10% variation is permitted (as practiced by commercial micropropagation outfits) owing to the flexible genetic make-up of the crop.

Keywords

Somaclonal variations, BAP, Kinetin, TDZ, Phenotypic appearances, Genetic stability

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Introduction

Bananas (*Musa* spp.) represent one of the most important crops in the world as well as India which is known as fruit of tropics (Purseglove,

1988). Banana and plantains together rank as the fourth most important food commodity in the world only after rice, wheat, milk or milk products (Amar, 2000). *In vitro* micropropagation has several advantages which

include high rates of multiplication, the small space required to multiply large numbers of plants in a short duration, production of diseases free planting material and the homogeneity of their growth.

True to type clonal fidelity is one of the most important prerequisites in the micropropagation of any crop species. Appearance of off-types during the *in vitro* multiplication process is an important drawback for mass propagation of bananas.

The types of cytokinins, concentrations, their combinations and type of banana cultivars significantly influences regeneration of morphological abnormal plants (Arinaitwe *et al.*, 2000; Gubbuk and Pekmezci, 2004). Whereas, some researchers (Vuylsteke *et al.*, 1991; Martin *et al.*, 2006) reported that the extent of instability in *Musa* was found to depend on the interactions between the genotype and the tissue culture conditions.

The *in vitro* multiplication of plants involves the application of plant growth regulators and changes to these phytohormone habituations are known to be associated with genetic instability in plants. Somaclonal variation in tissue culture is a common phenomenon, which makes it mandatory to check the genetic stability of *in vitro* raised plants. The genetic integrity of the micropropagated plants may lead to changes either in the phenotype or the genotype that can be determined with by different techniques. These variations are often undesirable, but still heritable (Brieman *et al.*, 1987).

Presently, there are various methods available which can be used to detect the tissue culture derived somaclonal variants. The most reliable methods are the molecular marker techniques that identify the variance depending on the plant proteins, which are expressed from defined regions of DNA, or DNA

polymorphisms. RAPD (random amplified polymorphic DNA's) is a powerful technique for identification of genetic variation (Welsh *et al.*, 1990). It has the distinct advantage of being technically simple and quick to perform, requiring only small amounts of DNA. Hence in the present study, RAPD were used to analyze the genetic stability of plantlets that were produced through tissue culture and compared with the genetic constitution of the mother plant.

Materials and Methods

The banana plants used in the present study was *Musa paradisiaca* cv. Ney Poovan (AB). The experiments were conducted at the Centre for Horticulture Biotechnology, Directorate of Research, University of Horticultural Sciences, Bagalkot, during 2016-17. Sword suckers were used as explants and regenerated on MS media supplemented with BAP alone (at 5,6,7,8 mg/l), BAP + Kinetin (at 5+1, 6+1, 7+1, 8+1 mg/l) and TDZ (at 0.20, 0.40, 0.60, 0.80, 1.00 mg/l). Two subculturing was carried out every after 30 days of inoculation.

Separated elongated shoots were inoculated on ½ strength of MS medium supplemented with 2.0 mg/l IBA and 2 gm/l activated charcoal for rooting. The rooted plantlets were observed visually for phenotypic appearances and then transferred in greenhouse for hardening. The hardened plantlets were again observed visually for phenotypic appearances viz., normal, subnormal and abnormal plantlets. They are identified on the basis of the following criteria:

Normal plantlets

These were sturdy, green, taller than subnormal and abnormal plantlets, in good health, having alternate leaf arrangement with better leaf area and having well developed roots.

Subnormal plants

These plantlets were shorter than the normal plantlets but taller than abnormal plantlets, having thinner shoots, poorer leaf area with poorer root development.

Abnormal plantlets

These plantlets were having more number of adventitious buds during multiplication, stunted growth, very thin shoot, very poor leaf and root development during multiplication as well as hardening stages.

Molecular analysis of plantlets

The hardened plants were then screened genotypically and compared with the mother plant for genetic stability using 10 RAPD markers.

Preparation of template DNA

DNA sample was extracted from the leaf of mother plant as well as tissue cultured multiplied plantlets. Two hundred mg of young leaves were collected. The leaves were first washed with distilled water and these leaf samples were used immediately for extraction or stored at -20°C for short term, for future use. These fresh leaves were ground to fine power with liquid nitrogen in a mortar and pestle.

The powder was transferred to a 50 ml sterile polypropylene tube containing 5ml of extraction buffer (200 mM NaCl, 25 mM EDTA, 200mM Tris-HCl, pH 8.0, 0.5% SDS, 0.2 % (v/v) β mercaptoethanol (BME), 0.2 % (v/v) Bovine serum albumin and 1% polyvinyl pyrrolidone (PVP). Banana being high polyphenol containing plant, additional measure was adopted in the extraction buffer with addition of BME and PVP. The homogenate was centrifuged at 10000 g for 10

minutes. To the supernatant, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 10000 g for 10 minutes. The DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol and kept at 20°C overnight followed by washing with 70% ethanol. The DNA pellet was then air dried and resuspended in sterile distilled water. The RNase treatment at a concentration of 1 mg/ml was given by incubating the solution at 37°C for 20 minutes. The quality and quantity of purified total DNA was estimated by taking absorbance at 260 and 280 nm using Nanodrop Spectrophotometer (NanoDrop 1000 spectrometer, Thermo Scientific Corp., USA). The DNA was then separated on 0.8% agarose gel using ethidium bromide and photographed in a Gel Documentation System.

RAPD analysis

A total of 10 RAPD markers were used to assess the genetic fidelity of plantlets with the mother plant.

Results and Discussion

Phenotypic appearance of plantlets at the end of rooting stage, primary and secondary hardenings

At the end of rooting stage, the plantlets exhibited different kinds of responses viz., normal (+), subnormal (++) and abnormal (+++) (Table 1 and Plate 1). All the plantlets derived from the MS Basal media without hormone, BAP (T₂ to T₅) alone at different concentrations and BAP with kinetin (T₆ to T₉) at varying levels showed normal growth. Whereas, those plantlets derived from TDZ supplemented media exhibited both subnormal (T₁₀, T₁₁ and T₁₂) as well as abnormal (T₁₃ and T₁₄) phenotypic appearances with stunted growth, thinner shoots and abnormal adventitious buds.

All the plantlets which appeared to be subnormal and abnormal during multiplication as well as after rooting stages could not revert back to normal plantlets upon primary and secondary hardening (Table 1 and Plates 2, 3).

Assessment of genetic stability of micropropagated plants using Randomly Amplified Polymorphic DNA (RAPD) marker

It was difficult to distinguish the somaclonal variants from the normal plants using traditional morphological markers. Random amplified polymorphic DNA markers (RAPD) were, therefore, used in the present study to determine the genetic variation among the *in vitro* regenerated plantlets. For molecular analysis 10 operon primers were used and 6 of them showed amplification. 04 primers (OPC 04, OPC 20, OPK 14, OPM 12) amplified unambiguous, readable and showed monomorphic bands, whereas, 02 primers (OPB 04 and OPD 07) gave polymorphic banding patterns. A total of 296 scorable band classes were produced by 6 RAPD primers tested in banana cv., Ney Poovan. The number of bands for each primer varied from 28 to 80 with an average of 49.33 bands per primer. The results obtained using RAPD showed recordable monomorphic and polymorphic band patterns. RAPD had shown 94.10 % monomorphism and 5.90 % polymorphism. Samples with monomorphic and polymorphic band classes obtained from cultivar 'Ney Poovan' is shown in Table 2 and Plates 4, 5, 6, 7.

Phenotypic appearance of plantlets at the end of rooting stage, primary and secondary hardenings

In the present investigation, all the plantlets derived from MS B without hormone (Control), BAP treated media as well as a combination of BAP and Kn appeared to be

phenotypically normal. The present study was in agreement with the finding of Ray *et al.*, (2006). They reported that there was no morphological variation among the regenerated clones during *in vitro* micropropagation with BAP, acclimatization and transfer to the field conditions.

The plantlets derived from full strength MS Basal media supplemented with TDZ 0.02, 0.04 and 0.06 mg/l appeared to be subnormal. Whereas, the plantlets derived full strength MS Basal media containing TDZ 0.08 and 0.10 mg/l were abnormal in appearance. The phenotypic appearance of these off-types include stunted growth, thinner shoots, production of more number of abnormal adventitious buds. It is evident that the appearance of phenotypic variant plants is attributed to the inhibitory effects of TDZ at higher concentrations. Huetteman and Preece (1993) stated that thidiazuron (TDZ) may inhibit shoot elongation. Lee (2005) reported that at higher concentration of TDZ (9.1 μM), elongation of shoots was inhibited and clumps of small globular buds appeared at the base of shoots. Farahani *et al.*, (2008) observed that with high concentrations of TDZ the number of normal shoots were reduced and abnormal shoots were observed. Strosse *et al.*, (2008) stated that in contrast to BAP being the most effective at high concentrations, TDZ should be applied at lower concentrations. Also, Shirani *et al.*, (2009) reported that cultivars having 'B' as one of its genomic constitutions are more sensitive to higher concentrations of TDZ as compared to BAP. Arinaitwe *et al.*, (2000) reported that at 16.8 μM equimolar concentrations shoot proliferation on TDZ supplemented media was manifested by the appearance of numerous fleshy bulbous structures each of them producing several stunted tiny adventitious buds on their surfaces. This high cytokinin activity of TDZ has been reported by several workers (Thomas and Katterman, 1986; Fellman *et al.*, 1987;

Mok *et al.*, 1987). This behavior is attributed to TDZs ability to accumulate endogenous cytokinins in cultured tissues (Huetteman and Preece, 1993) (Fig. 1–3).

List of primers for screening

Sl. No.	Primers	Nucleotide sequence 5'-3'
1	OPB 04	GGACTGGAGT
2	OPC 04	CCGCATCTAC
3	OPC 20	ACTTCGCCAC
4	OPD 07	TTGGCACGGG
5	OPK 14	CCCGCTACAC
6	OPM 12	CACAGACACC
7	OPA 20	GTTGCGATCC
8	OPC 02	GTGAGGCGTC
9	OPC 08	TGGACCGGTG
10	OPN 10	ACAACTGGGG

Table.1 Effect of Cytokinins on phenotypic appearance of *in vitro* regenerated plantlets of banana cv. Ney Poovan (AB)

Treatment	<i>In vitro</i> rooted plantlets	Primary hardened plantlets	Secondary Hardened plantlets
T ₁ - MS B without hormone (Control)	+	+	+
T ₂ - MS B + BAP 5.00 mg/l	+	+	+
T ₃ - MS B + BAP 6.00 mg/l	+	+	+
T ₄ - MS B + BAP 7.00 mg/l	+	+	+
T ₅ - MS B + BAP 8.00 mg/l	+	+	+
T ₆ - MS B + BAP 5.00 mg/l + Kn 1mg/l	+	+	+
T ₇ - MS B + BAP 6.00 mg/l + Kn 1mg/l	+	+	+
T ₈ - MS B + BAP 7.00 mg/l + Kn 1mg/l	+	+	+
T ₉ - MS B + BAP 8.00 mg/l + Kn 1mg/l	+	+	+
T ₁₀ - MS B + TDZ 0.02 mg/l	++	++	++
T ₁₁ - MS B + TDZ 0.04 mg/l	++	++	++
T ₁₂ - MS B + TDZ 0.06 mg/l	++	++	++
T ₁₃ - MS B + TDZ 0.08 mg/l	+++	+++	+++
T ₁₄ - MS B + TDZ 0.10 mg/l	+++	+++	+++

+ = Normal plantlets; ++ = Subnormal plantlets; +++ = Abnormal plantlets

Table.2 Comparative RAPD analysis on *in vitro* derived plantlets regenerated by different concentrations of Cytokinins and mother plants of banana cv. 'Ney Poovan'

Primers	Nucleotide sequence 5'-3'	Number of monomorphic bands	Number of polymorphic bands	Total number of bands	Per cent Monomorphism	Per cent Polymorphism
OPB 04	GGACTGGAGT	65	15	80	81.25	18.75
OPC 04	CCGCATCTAC	42	0	42	100	0
OPC 20	ACTTCGCCAC	56	0	56	100	0
OPD 07	TTGGCACGGG	40	8	48	83.33	16.67
OPK 14	CCCGCTACAC	42	0	42	100	0
OPM 12	CACAGACACC	28	0	28	100	0
Total		273	23	296	564.58	35.42
Average		45.50	3.83	49.33	94.10	5.90

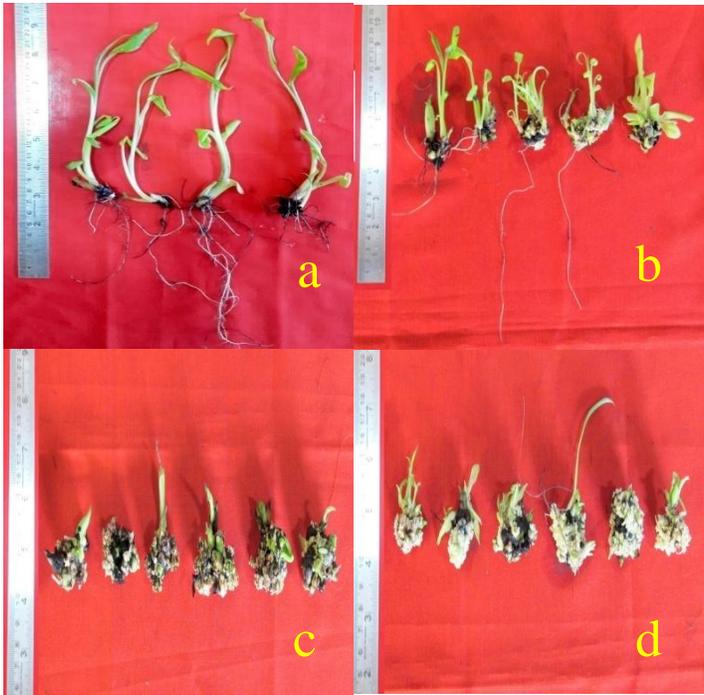


Fig.1 (a-d) Phenotypic appearance of *in vitro* rooted plantlets of banana cv. Ney Poovan (AB)

- a) Normal plantlets obtained from MS B without hormone (Control)
- b) Subnormal plantlets obtained from MS B + TDZ 0.06 mg/l
- c) & d) Abnormal plantlets obtained from MS B + TDZ 0.08 and TDZ 0.10 mg/l



Fig.2 (a-d) Phenotypic appearance of plantlets of banana cv. Ney Poovan (AB) after primary hardening

- a) Normal plantlets obtained from MS B without hormone (Control)
- b) Subnormal plantlets obtained from MS B + TDZ 0.06 mg/l
- c) & d) Abnormal plantlets obtained from MS B + TDZ 0.08 and TDZ 0.10 mg/l



Fig.3 (a-d) Phenotypic appearance of plantlets of banana cv. Ney Poovan (AB) after secondary hardening

- d) Normal plantlets obtained from MS B without hormone (Control)
- e) Subnormal plantlets obtained from MS B + TDZ 0.06 mg/l
- f) & d) Abnormal plantlets obtained from MS B + TDZ 0.08 and TDZ 0.10 mg/l

Plate.4 RAPD profile of mother plant and plantlets obtained with OPC 04

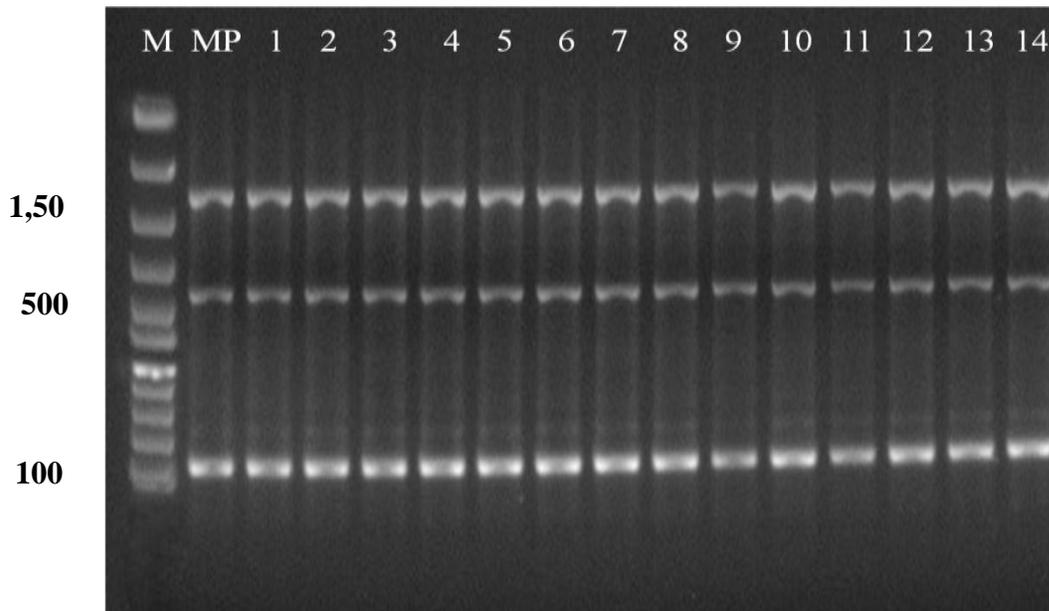


Plate.5 RAPD profile of mother plant and plantlets obtained with OPC 20

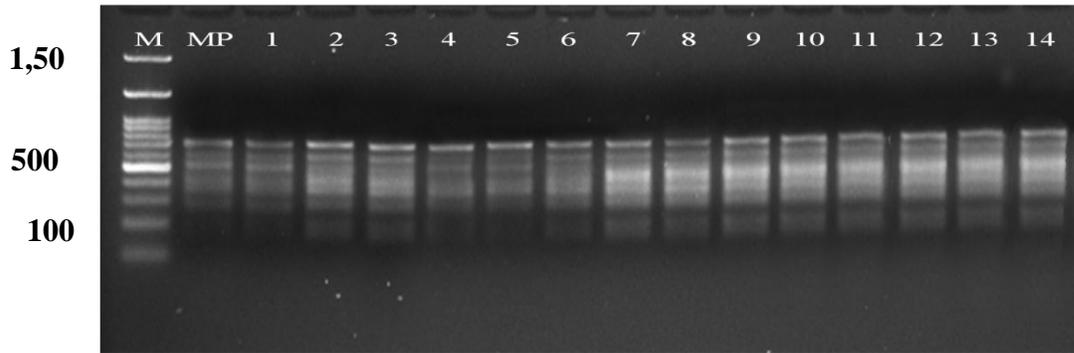


Plate.6 RAPD profile of mother plant and plantlets obtained with OPK 14

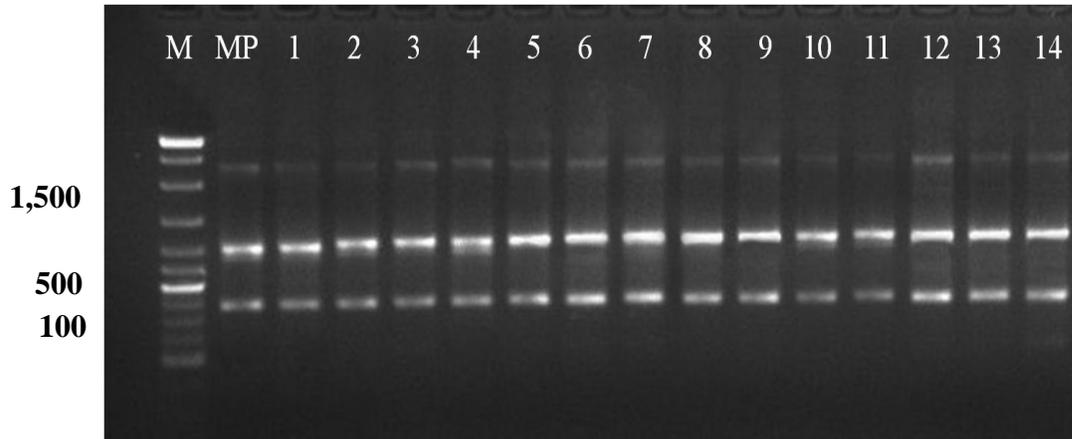
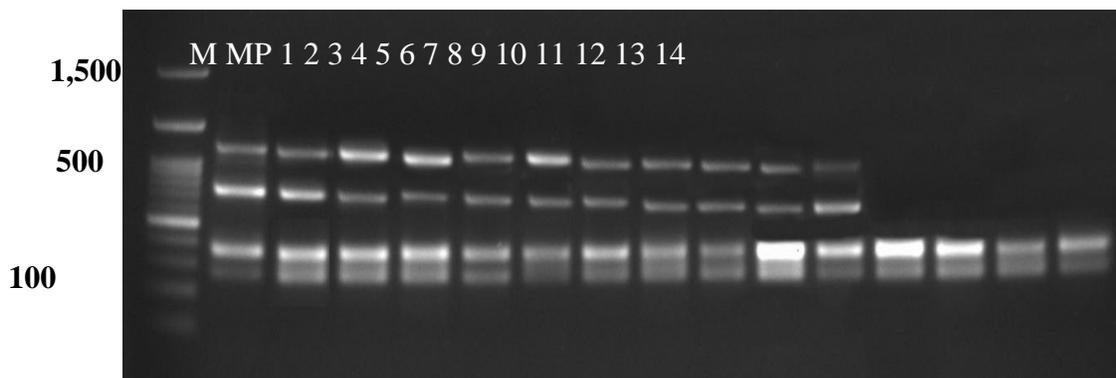


Plate.7 RAPD profile of mother plant and plantlets obtained with OPD 07



Lane M: 100 bp DNA ladder

MP: Mother Plant

1: MS B without hormone (Control)

2: MS B+ BAP 5.00 mg/l

3: MS B + BAP 6.00 mg/l

4: MS B + BAP 7.00 mg/l

5: MS B + BAP 8.00 mg/l

6: MS B + BAP 5.00 mg/l + Kn 1mg/l

7: MS B + BAP 6.00 mg/l + Kn 1mg/l

8: MS B + BAP 7.00 mg/l + Kn 1mg/l

9: MS B + BAP 8.00 mg/l + Kn 1mg/l

10: MS B + TDZ 0.02 mg/l

11: MS B+ TDZ 0.04 mg/l

12: MS B + TDZ 0.06 mg/l

13: MS B + TDZ 0.08 mg/l

14: MS B + TDZ 0.10 mg/l

Smith and Smith (1989) opined that identification of the variants based on the morphological appearance of the plantlets does not reflect the genetic relationship. The plantlets appeared to be subnormal and abnormal during multiplication stage could not revert back to normal upon primary and secondary hardening (Table 1 and plate 2 and 3). This might be due to the reason that the present variation obtained during multiplication and rooting stages might be due to genetic variations which are caused by changes in DNA nucleotide sequence. These findings are in agreement with the reports of Ramage *et al.*, (2004) who reported that some dwarf somaclonal variants in *in vitro* cultures of banana var. Williams (Cavendish banana) were relatively stable and did not generally revert to a normal phenotype with time. Vuylsteke (2001) noted that variations found in inflorescence were stable over bananas tubers and suggested that this variation is genetic in nature.

Assessment of genetic stability of micropropagated plants using Randomly Amplified Polymorphic DNA (RAPD) marker

An important problem associated with plant propagation through tissue culture is the genetic stability among sub-clones derived from the original parents. The genetic integrity of the micropropagated plants may lead to changes either in the phenotype or the genotype that can be determined with by different techniques. These variations are often undesirable, but still heritable.

The results obtained using RAPD showed recordable monomorphic and polymorphic band patterns. 04 primers (OPC 04, OPC 20, OPK 14, OPM 12) showed monomorphic bands, whereas, 02 primers (OPB 04 and OPD 07) gave polymorphic banding patterns. RAPD had shown 94.10 % monomorphism

and 5.90 % polymorphism. Presence of negligible variation in the banding pattern of banana cv. Ney Poovan derived plants clearly indicated that the Cytokinins with high suppression of shoot growth and exudation of phenolics do not have much effect on the genetic level. In the present investigation, the percentage of polymorphism was 5.90 % which is under permissible limit. In banana, up to 10% variation is permitted (as practiced by commercial micropropagation outfits) owing to the flexible genetic make-up of the crop (Smith, 1988; Cote *et al.*, 1993).

In present study, it was observed that shoot tip derived plants were genetically identical to the sucker derived plantlets. Shoot tips, maintain strict genotypic stability under tissue culture conditions (Bennici *et al.*, 2004), which appears applicable also to present results because little variations were observed for the primers tested which were under permissible limit. The polymorphism assessed through RAPD markers in cv. Ney Poovan clones might be due to the use of higher concentrations of TDZ. Variations observed in plantlets derived from tissue culture of cv. Ney Poovan was negligible compared to the results obtained by Grapin (1995) who reported 16-22% somaclonal variants among plants regenerated from a 'French Sombre' suspension, whereas all plants obtained via *in vitro* clonal propagation were normal.

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, some researchers (Vuylsteke *et al.*, 1991; Martin *et al.*, 2006) reported that the extent of instability in *Musa* was found to depend on the interactions between the genotype and the tissue culture conditions. *Musa* cultivars exhibit differences in genetic stability, with some cultivars being more stable than others (Martinez *et al.*, 1998). Variability in the

presence of the A genome is high while in the presence of the B genome it is lower (Pancholi *et al.*, 1996). The cultivar used in the present investigation belongs to AB genomic group with 'B' as one of its genomic constitutions revealed 5.90 % polymorphism. Ventura *et al.*, (1988) reported that micropropagation in the genome ABB resulted in 10.4% somaclonal variation exhibited in bunch characters (hands per bunch and atrophied fruits).

Venkatachalam *et al.*, (2007) established that tissue culture conditions do not induce somaclonal variation in banana cv. Nanjanagudu Rasabale (AAB) as screened by RAPD markers despite exposure to high levels of cytokinins during micropropagation. Choudhary *et al.*, (2015) reported that no polymorphism was detected during RAPD analysis of *in vitro* raised clones of banana cv. Robusta (AAA) indicating the genetic stability. Ray *et al.*, (2006) highlighted the genetic stability of the micropropagated plants of three banana cv. Robusta (AAA), Giant Governor (AAA) and Martaman (AAB) by using 21 RAPD and 12 ISSR markers. Izquierdo *et al.*, (2014) obtained 27 bands with RAPD marker in which all are monomorphic during the genetic stability analysis of the *in vitro* regenerated banana cv. FHIA-18 (AAAB). Similar results were also observed by Kajla *et al.*, (2014) in banana cv. Grand Naine using RAPD and ISSR markers.

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