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Rapid Plant Regeneration And Molecular Assessment Of Genetic Stability Using ISSR And RAPD Markers In Commercial Banana Cv. Grand Naine (G-9)

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ABSTRACT

The investigation was carried out to assess the genetic stability in tissue culture raised plants of banana cv. G-9 using random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers.

Aims: Molecular assessment of genetic stability of tissue culture raised plants of banana cv. G-9 using molecular markers.

Material and Results:

Apical shoots were established on medium EM_4 (MS + BAP 4.0 mg L^{-1}) with maximum of 3.8 buds/explants in 2.6 days. The maximum bud multiplication with 16.5±0.06 shoots was observed on medium Ma_3 (MS medium+ 5.0 mg L^{-1} BAP + 0.25 mg L^{-1} NAA of + 30 mg L^{-1} $AdSO_4$). The maximum rooting response (100%) was observed on 1/2 MS medium supplemented with 2.0 mg L^{-1} NAA in 12.2 days. After acclimatization the hardened plants were examined for genetic stability using RAPD and ISSR primers. Total forty six (twenty six RAPD and twenty ISSR) markers were used. RAPD primers produced 87 distinct and scorable bands, with an average of 3.34 bands per primer and the amplification products range was from 100-1200 bps. The number of scorable bands for RAPD primer varied from 2 to 5 with an average of 3.34 bands per primer. ISSR primers produced 71 distinct and scorable bands in the range of 100-1000 bps and the number of scorable bands for each primer varied from 2 to 6 with an average of 3.55 bands per primer.

Conclusion:

Similar profile with monomorphic bands was observed for all the tissue culture raised plants when compared to mother plant in both types of markers used. These results corroborate the fact that plant tissue culture technology has immense importance for production of true to type of planting material.

Indexing terms/Keywords

Banana cv Grand naine (G-9); genetic stability; RAPD and ISSR Markers

Academic Discipline and Sub-Disciplines

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Subject Classification

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Type (Method/Approach)

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INTRODUCTION

Banana is a basic food source for millions of people in developing countries in the tropics and subtropics [1]. The plant belongs to family Musaceae and is one of the oldest fruits of the world [2] .The crop ranks fourth in terms of production in the world and is second most significant fruit crop in India next to mango [3]. Fruits of the plant are good source of proteins, carbohydrates, vitamins, minerals such as potassium, sodium, calcium and magnesium along with trace amounts of iron, zinc and carotenoids [4].

Conventional propagation generally leads to production of 4-5 suckers from single plant but through *in vitro* propagation, a large amount of true to type and disease free planting material can be obtained [5, 6, 7, 8, 9, 10]. The tissue culture technique was successfully used for *in vitro* multiplication of a particular genotype [11, 12, 13, 14, 15]. The application of plant growth regulators during *in vitro* multiplication of plants may lead to genetic instability in plants. Hence it is mandatory to check for genetic stability of *in vitro* raised plants. The precise monitoring of quality control during *in vitro* multiplication and analysis of tissue culture raised plants by using simple and routine method is prerequisite step in micropropagation [16].

During large scale *in vitro* production, the plants may lead to genetic changes and the product may not be identical to the mother plant [17, 18]. The failure to assess the morphological polymorphism in plants does not negate the possibility of genetic variations and it requires critical analysis. In order to assess genetic variability, reliable methods of identification are also required for the establishment of plant variety rights [19]. Unambiguous identification is especially important in a clonally-propagated crop such as banana [20]. Commercial cultivators need to be sure that they are investing their time and money in propagating the specific cultivar which are true to type in nature. Various methods can be used to detect and monitor *in vitro* raised plants. The most reliable method is the use of molecular marker techniques [18]. The molecular marker technologies have become a powerful tool in crop improvement through their use in germplasm characterization and fingerprinting, genetic analysis, linkage mapping, and molecular breeding. Identification of possible somaclonal variants at an early stage of development is considered to be very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants [21]. Genetic stability of micropropagated plants and morphologically similar genotypes using molecular markers have been reported in many plants like *Chlorophytum borivilianum* [22] *Curcuma* sp. [23], *Zingiber rubens* [24] , strawberry [25], wheat [26], rice [27], eastern cottonwood [28] and date palm [29, 30] including banana [31]. RAPD and ISSR markers being simple and less laborious can be used for genetic stability testing of micropropagated plants. Sheidai *et al.* [32] used RAPD markers to detect the somaclonal variations in the tissue culture raised plants of banana cultivar Cavendish Dwarf. In the present study, the genetic stability and uniformity of the important banana cultivar Grand naine (G-9) produced through tissue culture technique was examined by using RAPD and ISSR markers.

MATERIALS AND METHODS

1- Microporpagation

In the present study, MS medium comprising of 30 g L^{-1} sucrose was used for *in vitro* establishment and multiplication and 50 g L^{-1} for rooting. The pH of the medium was adjusted to 5.8 prior to autoclaving, using 0.1 N HCl or 0.1 N NaOH. Agar-agar (0.8%) was added as a gelling agent. Medium was sterilized at 121°C and15 psi pressure in an autoclave for 15 minutes. Sterilized apical shoots were cultured on MS medium [33] supplemented with different concentrations of BAP and kinetin for initiation of buds under aseptic conditions. MS medium fortified with 5.0 mg L^{-1} BAP + 0.25 mg L^{-1} NAA and different concentrations of adenine sulphate ($AdSO_4$) was used for *in vitro* multiplication. Elongated shoots were cultured on 1/2 MS medium supplemented with different concentrations of NAA for rooting. The culture vessels were kept in growth chambers at 25±2 ^oC and light intensity of 100 μEM^{-2} sec^{-1} (1000 lux) for 16/8 (light/dark) hours. The plantlets were transferred in green house for hardening. The hardened plants were screened for genetic stability using twenty six RAPD (Table 4) and twenty ISSR (Table 5) primers (IDT make).

2- Genomic DNA isolation

Genetic stability of tissue culture raised plants was tested using RAPD and ISSR markers. For this study, nine plants were selected randomly from the tissue cultured raised population of thousand hardened plants and mother plant. Young leaf tissue was used for extraction of total genomic DNA using the modified cetyl trimethyl ammonium bromide (CTAB) method as described by Murray and Thompson [34]. DNA amplification was carried out after standardizing the required amplification conditions viz. template DNA concentrations, primers, $MgCl_2$, Taq DNA polymerase and annealing temperature. RAPD-PCR reactions were carried out in 20µl of reaction mix containing 1 X PCR buffer, 250 µM dNTPs mix, 0.6 µM primers, 1.5 mM $MgC1_2$, 0.5U Taq DNA polymerase and 50 ng of template DNA. ISSR-PCR reaction was conducted in 10µl of reaction mix containing l X PCR buffer, 500 µM dNTPs mix, 0.5 µM primers, 1.5 mM $MgCl_2$, 1U Taq DNA polymerase and 25 ng of template DNA.

3- PCR reaction

3.1- RAPD-PCR reaction

For RAPD primers, PCR was initiated by a denaturation step at 94°C for 3 min followed by the reaction comprising of 40 cycles at 94°C for 1 min, 36°C for 1 min, 72°C for 1 min, with a final extension step of 5 min at 72°C.

3.2- ISSR-PCR reaction

For ISSR primers, PCR was initiated for denaturation at 94°C for 4 min followed by the reaction comprising of 40 cycles at 94°C for 1 min, 50-65°C for 1 min, 72°C for 2 min, with a final elongation step of 8 min at 72°C. The amplified products were stored at -20˚C till further use.

4- Visualization and products analysis

The amplified products were resolved by electrophoretically using 2% agarose gel with ethidium bromide (5µg mL^{-1}) stain. Products were viewed using Alpha Digi Doc Pro™ documentation system under UV light. Unambiguous and clear amplification profiles of RAPD and ISSR markers were scored based on presence and absence of bands. Size of amplicons was recorded based on their migration relative to molecular size marker (l00 bps ladder).

5- Data analysis for genetic fidelity

During data analysis each band was treated as a marker and well-resolved each fragment in the size range of 100 bps was manually scored. The scoring of RAPD and ISSR amplicons was done on the basis of their presence ('1') or absence ('0') in the gel. Further the profiles were compared with each other for all the DNA samples to detect any genetic variation.

RESULTS

The experiments were conducted to study the *in vitro* multiplication and to access the genetic stability of micropropagated plants of banana cv. G-9.

1- Micropropagation of cultivar G-9

Effect of plant hormones viz BAP and kinetin was observed on *in vitro* establishment of banana cultivars G-9 and data were recorded for buds break and time required for bud initiation (Table 1, Fig. 1A). The cultivar varied in bud proliferation response to different hormones used and showed best response on EM_4 (MS + BAP 4.0 mg L^{-1}) medium with maximum of 3.8 buds/explants in 2.6 days. The sprouted buds were further cultured on MS medium supplemented with 5.0 mg L^{-1} BAP + 0.25 mg L^{-1} NAA and varied concentrations of adenine sulphate ($AdSO_4$) for *in vitro* multiplication (Table 2). The maximum bud multiplication was reported on medium Ma_3 comprising MS medium+ 5.0 mg L^{-1} BAP + 0.25 mg L^{-1} NAA of + 30 mg L^{-1} $AdSO_4$ (Fig. 1B). The elongated shoots were transferred on MS medium supplemented with various concentrations of auxins (NAA, IBA) and maximum rooting (100%) was reported on RM_4medium (MS basal+ 2.0 mg/l NAA) in 12.2 days (Table 3, Fig. 1C). The rooted plants were then transplanted in green house for hardening and genetic fidelity testing (Fig. 1D).

Table 1: Effect of growth hormones on i*n vitro* establishment of banana cultivar G-9 (average bud break & average days required for bud break).

Media code	Average no. of buds /explants	Average days required for bud break
EM_0 (control)	1.0±0.00	10.6±0.24
EM_1 (BAP 1.0 mg L^{-1})	1.6±0.08	7.5±0.49
EM_2 (BAP 2.0 mg L^{-1})	1.9±0.10	6.6±0.63
EM_3 (BAP 3.0 mg L^{-1})	2.4±0.21	5.8±0.40
EM_4 (BAP 4.0 mg L^{-1})	3.3±0.17	2.6±0.37
EM_5 (BAP 5.0 mg L^{-1})	1.4±0.15	4.7±0.17
EM_6 (KIN 1.0 mg L^{-1})	1.4±0.15	6.6±0.63
EM_7 (KIN 2.0 mg L^{-1})	1.6±0.06	5.8±0.40
EM_8 (KIN 3.0 mg L^{-1})	1.8±0.08	6.9±0.36
EM_9 (KIN 4.0 mg L^{-1})	1.2±0.13	5.8±0.40
EM_{10} (KIN 5.0 mg L^{-1})	1.2±0.13	7.3±0.36

*Mean of three replicates, ± = Standard error (mean)

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Table 2: Effect of growth hormones on i*n vitro* multiplication of banana cultivar i.e. G-9 (average no. of shoots)

Media code	Media combinations	$AdSO_4$ (mg L^{-1})	Average no. of shoots on	
			16th day	24th day
Ma_1	BAP 5.0+NAA 0.25 mg L^{-1}	10.0	5.4±0.06	12.5±0.36
Ma_2	BAP 5.0+NAA 0.25 mg L^{-1}	20.0	6.6±0.63	15.1±0.16
Ma_3	BAP 5.0+NAA 0.25 mg L^{-1}	30.0	7.3±0.36	16.5±0.06
Ma_4	BAP 5.0+NAA 0.25 mg L^{-1}	40.0	6.9±0.36	13.3±0.63

*Mean of three replicates, ± = Standard error (mean)

Table 3: Effect of growth regulators on i*n vitro* rooting of banana cultivar G-9 (number of days taken for visible root formation from regenerated shoots)

Media Code ½ MS + 50g L^{-1} Sucrose	Percentage rooting response	No. of days taken for visible root formation* G-9
RM_0 (control)	20	17.0± 0.31
RM_1 (NAA0.5)	40	13.7± 0.47
RM_2 (NAA1.0)	50	13.7± 0.49
RM_3 (NAA1.5)	70	12.6 ±0.32
RM_4 (NAA2.0)	100	12.2 ±0.30
RM_5 (IBA0.5)	30	15.1 ±0.56
RM_6 (IBA1.0)	60	14.3 ±0.44
RM_7 (IBA1.5)	60	12.7 ±0.35
RM_8 (IBA2.0)	70	12.6 ±0.37

*Mean of three replicates, ± = Standard error (mean)

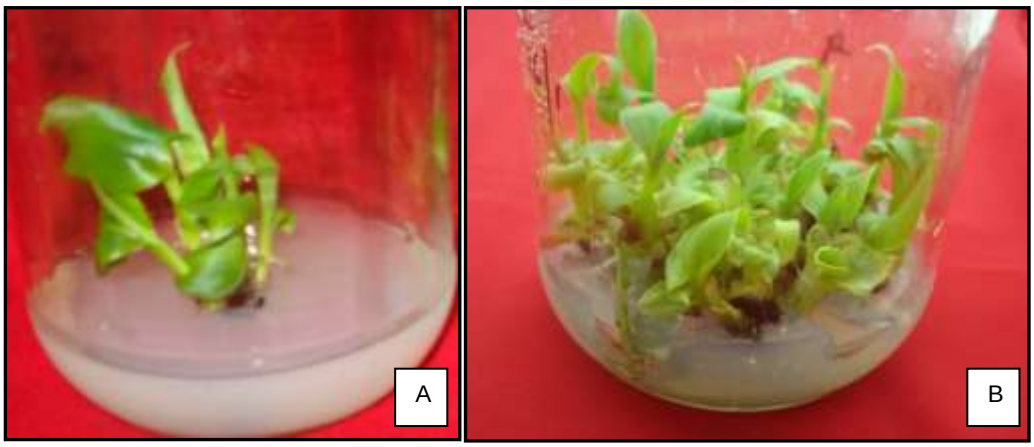

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Fig 1: Micropropagation of banana cultivar G-9. A- Establishment. B- Multiplication. C- Rooting. D- Hardened plants

2- Genetic stability testing of *in vitro* raised plants of banana cv. G-9 using RAPD & ISSR primers

For this study, mother plant and nine *in vitro* raised plants were selected randomly from the population of thousand of plants and screened for genetic stability using twenty six RAPD (Table 4) primers which produced 87 distinct and scorable bands, with an average of 3.34 bands per primer. The number of amplified bands for RAPD primers was varied between 2 (MA1, MA3, MA10, MA13, MA14, MA18, MA20) to 5 (MA2, MA4, MA8, MA9, MA21, MA25) (Table 4). The size of amplicons varied from 100 bps (MA12) to 1200 bps (MA4). No polymorphism was detected during the RAPD analysis of *in vitro* raised plants (Fig. 2). Likewise twenty ISSR primers were used to detect variation in *in vitro* raised plants which produced 71 distinct and scorable bands in the size range of 100 bps (MM2, MM14) to 1000 bps (MM17). The number of amplified bands for each primer varied from 2 (MM12, MM13, MM16, MM19, MM20) to 6 (MM2) with an average of 3.55 bands per primer (Table 5). Banding pattern of all tissue culture raised plants was similar to mother plant and was monomorphic (Fig. 3).

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Table 4: DNA amplification profile generated for clonal fidelity testing of *in vitro* propagated banana cultivar G-9 using RAPD primers.

Sr. no.	Primer	Sequence (5'-3')	Molecular weight in bps (range)	No. of scorable bands per primer
1	MA1	TGCCGAGCTG	600-700	2
2	MA2	AATCGGGCTG	150-350	5
3	MA3	AGGGGTCTTG	400-700	2
4	MA4	GGTCCCTGAC	350-1200	5
5	MA5	GAAACGGGTG	120-450	4
6	MA6	GTGACGTAGG	300-700	3
7	MA7	GGGTAACGCC	500-1000	3
8	MA8	GTGATCGCAG	650-1100	5
9	MA9	AGCCAGCGAA	640-850	5
10	MA10	GACCGCTTGT	600-700	2
11	MA11	AGGTGACCGT	250-350	4
12	MA13	CCGAACACGG	100-450	2
13	MA14	TCGTTCCGCA	800-900	2
14	MA15	CCTCTCGACA	600-820	3
15	MA16	TGAGCCTCAC	650-900	3
16	MA17	CCCAAGGTCC	140-400	4
17	MA18	GGTGCGGGAA	400-500	2
18	MA19	CCAGATGCAC	500-800	3
19	MA20	GTGACATGCC	600-700	2
20	MA21	TCAGGGAGGT	300-1000	5
21	MA22	AAGACCCCTC	400-1000	3
22	MA23	AGATGCAGCC	400-1000	3
23	MA25	GAGTCTCAGG	200-500	4
24	MA26	GGTGACTGTG	150-600	5
25	MA27	GGCACGTAAG	550-800	3
26	MA28	AAGTCCGCTC	300-500	3
Total				87
Mean				3.34

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Table 5: DNA amplification profile generated for clonal fidelity testing of *in vitro* propagated banana cultivar G-9 using ISSR primers.

Sr. no.	Primer	Sequence (5'-3')	Molecular weight in bps (range)	No. of scorable bands per primer
1	MM1	AGAGAGAGAGAGAGAGYT	200-450	3
2	MM2	GAGAGAGAGAGAGAGAA	100-600	6
3	MM3	GAGAGAGAGAGAGAGAC	600-850	4
4	MM4	GAGAGAGAGAGAGAGAT	450-700	3
5	MM5	CTCTCTCTCTCTCTCTCTA	250-350	4
6	MM6	CTCTCTCTCTCTCTCTRC	200-400	5
7	MM7	ATGATGATGATGATGATG	700-900	4
8	MM8	GACAGACAGACAGACA	400-600	2
9	MM9	TCTCTCTCTCTCTCTCG	300-800	4
10	MM10	GAGAGAGAGAGAGAGAYT	400-800	4
11	MM11	GAGAGAGAGAGAGAGAYG	500-900	4
12	MM12	CACACACACACACACARG	400-600	2
13	MM13	ACACACACACACACACYT	400-500	2
14	MM14	CTCCTCCTCCTCCTCCTC	100-800	5
15	MM15	VDVCTCTCTCTCTCTCT	200-900	5
16	MM16	DBDACACACACACACAC	400-500	2
17	MM17	VHVGTGTGTGTGTGTGT	200-1000	3
18	MM18	HVHTGTGTGTGTGTGTG	200-800	5
19	MM19	TGGATGGATGGATGGATGGA	400-600	2
20	MM20	AGGGAGAGGAGGAGGAGG	400-500	2
Total			71	
Mean			3.55	

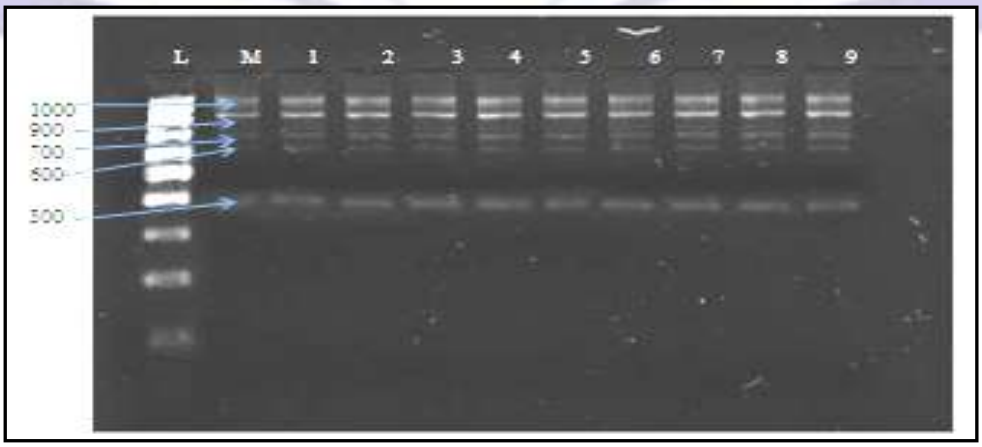

Fig 2: RAPD profiles of mother plant (M) and tissue culture raised (1-9 lanes) plants using primer MA-21.

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Fig 3: ISSR profiles of mother plant (M) and nine tissue culture raised (1-9 lanes) plants using the primer MM-2.

DISCUSSION

Venkatachalam [35] reported the similar results on MS medium supplemented with 5.0 mg L^{-1} BAP. Effect of different combination of growth hormones on *in vitro* propagation of banana genotypes was also observed by many workers [36, 37, 38, 39, 40, 14, 15].The elongated shoots were cultured on 1/2 MS liquid media fortified with 50.0 g L^{-1} sucrose and different concentrations of NAA (Table 3). The maximum rooting response (100%) was reported on 1/2 MS liquid media supplemented with 2.0 mg L^{-1} NAA (Fig 1*C*) in 12.2 days. Al-Amin *et al*. [41] and Ganapathi *et al.* [42] also reported that root numbers varied with different concentrations of IBA, IAA and NAA and results obtained were in similar pattern. *In vitro* rooted shoots were successfully transferred in green house without any mortality (Fig. 1D).

Molecular markers are considered to be reliable in monitoring genetic variability in plants [43].RAPD technique was used by several authors to investigate the genetic variability and found it to be very efficient and reliable[26, 22, 23, 24].Results obtained using RAPD were similar with those obtained with restriction fragment length polymorphism (RFLP) and isozymes [44]. Banding patterns obtained using RAPD technique can be used to develop genotype-specific cultivar identification. DNA profiling and reproducibility of RAPD technique is influenced by any variation in the methods used for isolation of genomic DNA [45], template DNA, primer, Taq-DNA polymerase concentration, annealing temperature, number of thermal cycles and $MgCl_2$ concentration[46, 47]. Many reports are available which suggest that the majority of RAPD bands are reproducible if standard protocol is strictly followed in each reaction [48, 49].

Genetic fidelity assessment of banana cv. G-9 using RAPD and ISSR primers reveals that all the micropropagated plants were true to type. The uses of two types of markers, which amplify different regions of the genome, allow better chances for identification of genetic variations in the plantlets. In this study RAPD's and ISSR markers have been used for analysis of variants as they offer several advantages over other conventional methods. These molecular markers are technically simple, inexpensive, require very little plant material, quickly to perform and yields true genetic markers study [50]. Shenoy and Vasil [51] reported that micropropagation through meristem culture are generally less subjected to genetic changes that might occur during cell differentiation under *in vitro* conditions. Ray *et al*. [52] also observed the genetic stability of the tissue culture raised plants of three banana cultivars i.e. G-9 (AAA), Giant Govenor (AAA) and Martaman (AAB) by using 21 RAPD and 12 ISSR primers. And found three somaclonal variants from 'G-9' and three from 'Giant Govenor". Harirah and Khalid [53] used eighteen arbitrary decamer primers to study the genetic fidelity of banana cv. Berangan and found that all the regenerated plants were monomorphic in nature. Similarly, Choudhary *et al.* [54] also reported genetic stability of banana cultivar-Robusta using RAPD and ISSR markers. In some cases, regeneration process is prone to somatic variation resulting in off-types as in case of *Populus termuloides* [55] and tea clones [56]. Variations are induced by different genetic and epigenetic mechanisms that are likely to be reflected in the banding pattern developed by employing different marker system. However, the reliability and efficiency of molecular markers in detecting large scale genome arrangements have been frequently questioned. Since simple sequence repeat based primer target the fast evolving and hyper variable DNA sequence. ISSR markers are considered suitable to detect variation among micropropagated plants [55, 52, 57]. RAPD and ISSR markers have also been successfully used to determine genetic diversity and relationships amongst *Musa* germplasm [58, 59, 60, 61].The use of ISSR & RAPD to discriminate between somatic mutants and the clone from which the mutants originated have also been studied in banana [35].

CONCLUSIONS

In the present study, no polymorphism was detected during the RAPD & ISSR analysis of i*n vitro* raised clones. A homogenous amplification profile was observed for all the micropropagated plants when compared to mother plant in both types of markers used. The results corroborate the fact that *in vitro* multiplication is the safest mode for production of true

to type of plants. So this methodology not only provide the homogenous planting material but also useful in conservation of elite traits of specific planting material. It can be used to provide disease free and quality planting material (banana plants) to the farmers which will help in the upliftment of economic status of the farmers as well as the country.

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Competing of interest

The author(s) declare that they have no competing interests.

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