Deciphering the Genetic Identity and Fidelity of Banana Genotype Musa Acuminata through Molecular Fingerprinting

ABSTRACT

Micropropagation for extensive production of elite planting material ~~clone~~s is an effective and superior alternative to suckers and conventional means of propagation of Musa ~~Spp~~spp. Th~~e present investigation was consummate to appraise~~s study assessed the genetic identity and fidelity of different micropropagated banana genotypes Grand Naine, Shrimantee and Basrai. Further micropropagated banana genotypes Grand Naine comparing with mother plant during different stages of subculture using ~~two molecular markers SSR (~~Simple Sequence Repeat (SSR) and ~~ISSR (~~Inter Simple Sequence Repeat (ISSR). Moreover, for micropropagation explants (suckers) were inoculated on initiation medium containing MS (Murashige and Skoog) fortified with 5 mg/l BAP (Benzyl amino purine) and 39 mg/l $AdSO_4$ (Adenine sulphate). The second, third and fourth subcultures were carried out to separate all multiple shoots. Furthermore, for rooting MS media fortified with growth hormones viz., 2 mg/l BAP, 1mg/l IBA (Indole-3-butyric Acid) and 200mg/l activated charcoal were used under present investigation. Findings revealed that ~~The~~ 90~~-~~% and 75~~-~~% plantlets were successfully established during primary hardening in controlled ~~as well as in~~and natural field condition~~s~~. Further, 26 primers were screened from that 20 primers (ISSR and SSR) showed 146 clear, reproducible and scorable bands ranging from 100-2000 bp (Base Pair) produced. The banding pattern of these randomly selected plants was monomorphic with mother plant of all selected genotype that confirmed the clonal uniformity.

Keywords: Musa spp., Micropropagation, Plant harmones, Genetic fidelity, Molecular markers

1. INTRODUCTION

Banana is a monocotyledonous, perennial stooling herb that contains a wide range of triploid (2n=3x=33) cultivars ~~that evol~~develop~~v~~ed from interspecific and intraspecific ~~hybrids~~ hybridization of the two wild diploid species *M. acuminate* Colla and *M. Balbisisana* Colla [1]. Edible bananas (*Musa acuminata*) are the major staple fruit and vegetable crops for urban consumers in the tropical and subtropical countries. It is an important source of rural income [2]. World banana production is around 103,000 thousand tons annually [3]. India ~~gain top slot by~~ranks first producing ~~the manifold of bananas with~~ 27,575 thousand ton~~ne~~s of bananas, followed by China with 12,075 thousand ton~~ne~~s. Tamil Nadu is the top banana producing state of India producing 8016.35 thousand ton~~ne~~s, followed by Maharashtra 4,100 thousand ton~~ne~~s [4]. Banana has multiple uses so popularly known as Kalpataru [2][5]. Grand Naine an introduction from France is the most wide spreading commercial banana cultivar of India. It grows to 5-7 feet and solid green in colour. ~~It~~ The crop is a tall mutant derived from Dwarf Cavendish and is grown on huge plantations in Central America, resistant to blossom end rot that can occur on Dwarf Cavendish. It is one of the most

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important commercial cultivar worldwide due to its resistance to wind throw and production of large bunches and fingers despite its relatively small stature compared to other cultivars of banana. ~~Plant~~ Phenotypic characters of Grand Naine resembles ~~with~~ those of Dwarf Cavendish, except for its robustness, finger of bigger size and heavy bunches. It bears bunches ~~of weight~~ weighing 25-30 kg with uniform long fingers. It is very attractive for its landscaping potential and good wind resistance [2].

The transportation of bulky suckers for commercial planting is not economical, and offers introduction of deadly diseases of banana. Nevertheless, in the banana production system, the non-availability of diseases-free, true to type planting material, low fertility due to triploidy, slow propagation, long time span from one generation to the next and the soil borne diseases transmitted through suckers and bunchy top diseases are the major constraints [Ref???]. Tissue culture offers methods for faster multiplication of superior clones and production of diseases free propagules through meristem shoot tip culture. In vitro propagation has many advantages such as higher rates of multiplying clean (pest and disease free) planting materials and the small amount of space required to multiply large number of plants [5]. Micropropagation [6][7][8][9][10] has major supporting role in biotechnology for rapid multiplication of rare, exotic, endangered species and genetically engineered plants, large production of superior propagules and conservation of economically important ornamental, horticultural, plantation crops and medicinal plants. Genetic fidelity is the maintenance of the genetic constitution of a particular clone through its life span. Clonal uniformity confirming genetic fidelity in all micropropagated plants is an important part of quality assurance for commercial production [Ref??]. Undesirable and non-uniformity (somaclonal variants) in plants can result from stressful condition including the use of plant hormones during the propagation process, which can cause production losses and consequently will affect the attributes of plants production via in vitro technology [2].

Currently, there are numerous methods ~~available which~~utilized for ~~detecting~~ detection of the genetic fidelity of micropropagated plants [11]. Molecular markers like SSR and ISSR are ~~a~~ powerful techniques for identification of genetic variation [12][13][14][15]. DNA based molecular markers are anchored and ubiquitous to most of the living organisms and have become an important tool to check the genetic uniformity and true-to-type nature of the micropropagated plants [5]. Molecular markers suitable for generating DNA profiles have ~~proved~~ proven to be an effective tool in assessing the genetic stability of regenerated plants. The present investigation wasset-in-motion with the premise that, micropropagation of banana will be effective method for production of genetically stable (true to type) plants on a large scale. Furthermore, ISSR and SSR markers can be employed for testing of genetic fidelity of banana cv. Grand Naine.Therefore, ~~on this context~~ the present study investigation ~~was tackled with~~ed the following objectives.

a) Micropropagation of banana genotype Grand Naine (G-9)

b) Identification of molecular markers for deciphering genetic fidelity in micropropagated banana genotype Grand Naine (G-9)

~~The present investigation wasset-in-motion with the premise that, micropropagation of banana will be effective method for production of genetically stable (true to type) plants on a large scale. Furthermore ISSR and SSR markers can be employed for testing of genetic fidelity of banana cv. Grand Naine.~~

2. MATERIAL AND METHODS

Plant material

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~~In present investigation, d~~Different banana genotypes including Grand naine (G-9), Basrai and Shrimantee were utilized in this study. ~~pre owed collected from Vidarbh region, Maharashtra state of India.~~ Sword suckers of ~~banana~~ each genotype were used as explants. The sword suckers were collected from Vidarbh region, Maharashtra state of India.

Culture medium

~~In the present study f~~For establishment of explants, Murashige and Skoog (MS) media with full strength were pre owed in combination with plant hormone 5 mg/l BAP and 39 mg/l AdSO4. Furthermore, for rooting M.S. media fortified with growth harmones viz., 2 mg/l BAP, 1 mg/l IBA and 200 mg/l activated charcoal were used ~~under present investigation~~ (Demissie ~~A. G.~~, 2013). The pH of ~~the~~ both media was adjusted to 5.7– 5.8 with 0.1 N HCl or NaOH before adding 0.8% agar and autoclaved at 121°C for 15 min~~utes~~.

Molecular marker~~s~~ used for fidelity testing

Different ISSR and SSR primers were used ~~in present investigation viz.,~~for fidelity testing including UBC 866, UBC 825, ISSCR 8, ISSCR 4, UBC 816, P-16, P-17, UBC 840, IG-19, UBC 834, UBC 812, UBC 841 (ISSR), Mb1–5, Mb1-30, Mb1-50, Mb1-52, Mb1-69, Mb1-113, Mb1-139, Mb1-146, Mb1-148 and Mb1-49-2 (SSR).

Micropropagation of banana genotype Grand Naine

Sword suckers of banana genotypes were taken from the mother nursery block as shown in ~~fig~~ Fig 1 ~~(~~A~~)~~. These suckers were pretreated with different surface sterilizers such as 0.1% bavistin, 0.1% ~~streptocycline~~streptomycin, 0.1% $HgCl_2$, ~~as shown in f~~(Fig 1 ~~(~~B). The surface sterilized and aseptically excised explants (~~as shown in f~~Figs 1C & D) were transferred on the bottles containing medium prepared as per different treatments ~~as shown in f~~(Fig 1 E. Culture was incubated at 25 ± 2°C in an air-conditioned culture room with a light intensity of 2000-3000 lux by cool white florescent tubes. The light/dark cycles of photoperiod were maintained as 16/8 h~~rs~~ daily. Subculturing was done by trimming tip of emerging axillary bud and removal of dead tissue at base of explant. Proliferated buds were transferred to different rooting medium. Rooted plantlets were thoroughly washed to free from media. Explants were kept in 0.1% bavistin antifungal solution for 10-15 min~~utes~~. For primary hardening, plantlets were transferred to trays containing sterilized substrate ~~cocopit~~cocopeat and soil rite maintained in shade house under controlled condition. For secondary hardening, Plantlets were transferred to poly bags containing sand and soil (1:1) substrates. Plants were hardened by gradually increasing light intensity and reducing relative humidity ~~(~~from 40-30%~~)~~.

Comments:

Any phenotypic data collected?

Any experimental design used for the in-field establishment of the mother plants?

Please specify the experimental design used to appraise the genetic fidelity of different micropropagated banana genotypes comparing with mother plant during different stages of subculture.

~~**Statistical analysis**~~

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~~Statistical analysis was done by calculating the recorded data into 10 plantlets per three replications using completely randomized design (CRD).~~

DNA isolation

DNA isolation of mother plant (leaf) and tissue culture raised banana plantlets was done using CTAB extraction method [16] with ~~certain~~ slight modifications. About 0.2 g~~m~~ of tissue sample was weighed and macerated in mortar pestle. Further, 1 ml of pre-warmed CTAB extraction buffer was added into ~~it.~~each 1.5 ml microcentrifuge tubes ~~were~~, followed by ~~inverted~~ invertion of tubes for 2-3 ×~~times~~ and ~~incubated~~ incubation in water bath for 1 h~~our~~ at 65°C. The tubes were then centrifuged at 10000 rpm for 10 min at 4°C. Supernatant was taken and equal volume of chloroform isoamlyalcohol (24:1) was added in the supernatant followed by manual shaking. Centrifugation was carried out at 10000 rpm for 10 min at 4°C. The~~n~~ aqueous phase was removed and taken in another centrifuge tube without disturbing the interphase. Equal volume of prechilled isopropanol was added. Centrifugation was carried out at 10,000 rpm for 15 min at 4°C. The supernatant was removed and the pellet was washed with 70% ethanol. Pellet was air dried for 30 to 45 min and dissolved in TE buffer stored at 4°C for future use. ~~Afterword~~ To eliminate any trace of RNA, the DNA ~~confirmation if RNA trace was seen then~~samples, RNAse ~~treatment was given~~were added to the sample in a ratio of 10:1 ~~ratio~~ at 37°C for 1 h~~r~~.

DNA quantification

The 0.8 % agarose gel was used for DNA band separation. Quantity and quality of total DNA isolated from target tissue samples were assessed by spectrophotometer or nanodrop [Ref??]. The amount of DNA was calculated by using the formula given below,

$$DNA\left(\frac{ng}{\mu l}\right) = \frac{A260\ X50X\ dilutionfactor}{1000}$$

PCR amplification for ISSR and SSR markers

Synthesized primers were primarily dissolved in deionized water to get final stock concentration 100 pmol/ul. These stocks were diluted to pmol/ul in ratio 1:9. Different PCR components for preparation of PCR reaction mixture 10 ×~~X~~ PCR buffer 2.5 ml, ~~-~~2 mM dNTPs 2.0 ml, 1U Taq DNA polymerase 0.2 ml, 25 mM $MgCl_2$ 1.0 ml, Primer 0.8 ml, Genomic DNA/ Template 1.0 ml, Nuclease free water 12.5 ml with total 20 ml reaction mixture. The PCR was performed in Master cycler (Eppendorf, Germany) using a program for the ISSR and SSR primers. Following PCR program was perform initial denaturation 94°C for 4 min, denaturation 94°C for 1 min, annealing 45-65°C 1 min, extension 72°C for 2 min with 35 repeated cycles followed by final extension 72°C for 10 min. PCR product analysis was carried out on PAGE (8 % Polyacrylamide gel electrophoresis) followed by silver staining [17].

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Statistical analysis

Statistical analysis was done by calculating the recorded data into 10 plantlets per three replications using completely randomized design (CRD).

Data analysis

The gel image was analyzed using software SYNGENE (gel documentation unit). Clear, reproducible and scorable bands were considered for analysis [Ref??].

Comments
Specify data collection done: Phenotypic and molecular.
Cite accurate references for the analytical techniques used.

3. RESULTS AND DISCUSSION

~~The present investigation was performed to appraise the genetic fidelity of different micropropagated banana genotypes comparing with mother plant during different stages of subculture.~~

Micropropagation of banana genotype Grand Naine

Establishment of contamination free cultures is the first step in development of the regeneration protocol for any plant species. To avoid contamination during initiation, antibiotic viz., rifampicin (3 ml/l) and cefotaxime (2.5 ml/l) were added in initiation medium as shown in ~~fig~~ Fig 2-A. Inoculated bottles were kept for incubation in growth room under 16 h~~rs~~ photoperiods and temperature was maintained at 25 ± 2°C. The banana shoot-tip explants were creamy white in colour and turned brownish in color after 6 to 8 days ~~as shown in f~~(Fig 2-B). The leaf sheaths were turned green after 10 to 15 days of inoculation ~~as shown in f~~(Fig 2–C). Subculture was carried out when proliferation of shoots from the explants was done. Stimulation of axillary buds to develop in to shoot took place during initiation of multiplication stage. Initial sub culturing was done when the explants exhibited unwhorling of leaf sheaths after 21st day of inoculation ~~as shown in fig~~ (Fig 2–D). After 21st day of inoculation subculture was carried out by trimming basal dead tissues and brown portion of explants. Repeated ~~subculture~~ subculturing was carried out to control contamination, media depletion and browning. At this stage, ~~The~~ the surviving shoots ~~which survived~~ in initiation culture were subcultured ~~after 21th day~~. At second subculture which was carried after 50th day of inoculation, the multiple shoot clumps were separated and inoculated ~~on~~ in separate bottles. The observation~~-~~ recorded after 50th day~~s~~ of inoculation revealed that the central meristem produces clusters of proliferating buds and one to three axillary buds get regenerated from basal parts of explant around central apical meristem ~~as shown in f~~(Fig 2-F). ~~-~~At 50th day~~s~~ of inoculation, the second subculture was carried out to separate all multiple shoots ~~as shown in f~~(Fig 2–H). Proliferated multiples were separated and transferred into rooting medium containing different media ~~as shown in f~~(Fig 2–I). 90–% and 75% plants were successfully established during primary hardening (~~as shown in f~~Fig 2-J) in controlled as well as in natural field condition (~~as shown in f~~Fig 2-K) [5].

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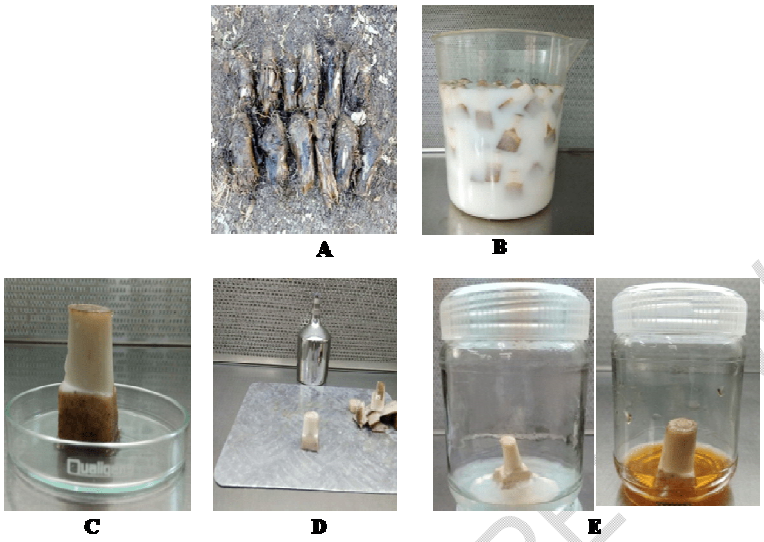


Fig. 1 Preparation of explant and inoculation of shoot tip from suckers of banana cv. Grand Naine A) Collection of explants B) Pretreatment C) Explant D) Final cut under LAF cabinet E) Inoculation of explant in solid & liquid medium

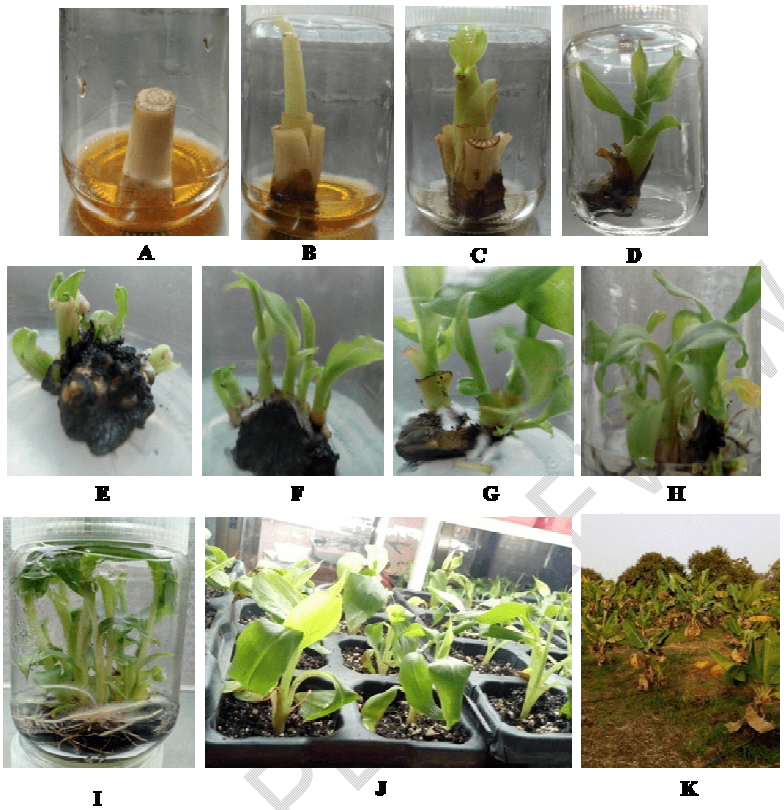


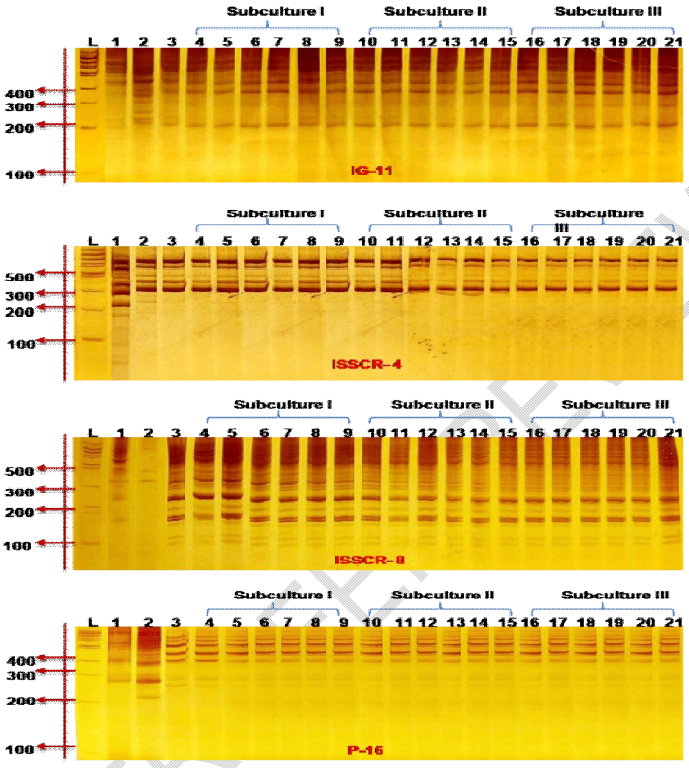
Fig. 2 Initiation, culture establishment & multiplication of banana cv. Grand Naine A) On 1st day B) After 8th days C) After 15th days D) After 21st days, Also different stages of multiplication after 8th days (E), 15th days (F), 21st days (G) & 30th days (H) days on multiplication medium & I) Rooting J) Primary hardening K) Secondary hardening

Genetic fidelity of banana genotypes Shrimantee, Basrai and Grand Naine

A ~~Total~~ total of 26 molecular markers comprising ~~(~~16 ISSR and 10 SSR~~)~~ [3][18] [19] ~~molecular markers~~ were screened for fidelity testing in Grand Naine and genetic identity of ~~different~~ genotypes ~~of banana~~ Shrimantee, Basrai and Grand Naine. ~~Total~~ Of the 26 markers,~~twenty~~ 20 primers (14 ISSR and 6 SSR) ~~primers were~~ produced scorable and clear bands with product size ranging from 100-2000 bp. The 20 primers ~~(ISSR and SSR) were~~ produced ~~totally~~ 146 clear, reproducible and scorable bands. The primers ISSCR-8 and IG-19 ~~were~~ amplified the highest ~~(~~of 12~~) number~~ of bands~~. W~~, whereas primers Mb1-05 and P-17 ~~were~~

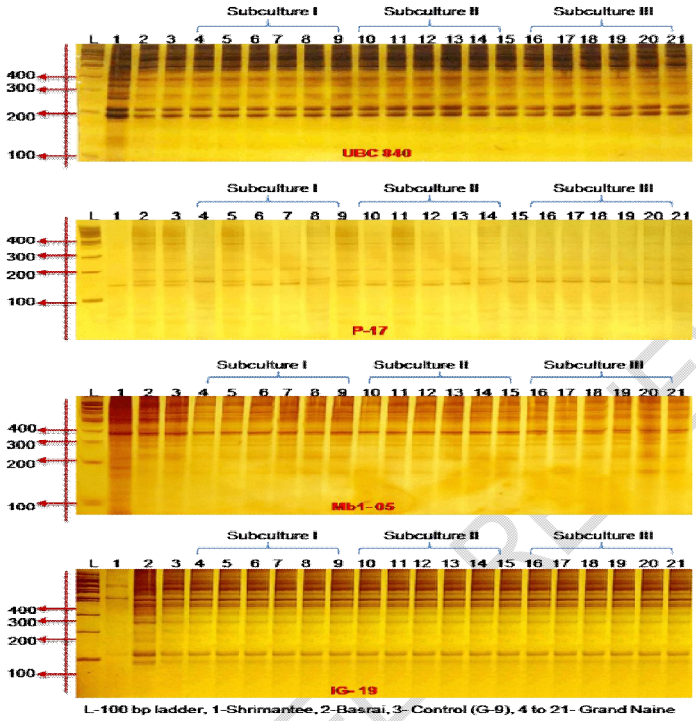
amplified the lowest ~~(~~of 3~~) number of~~ bands. The number of bands for each primer varied from 3 (Mb1-05 and P-17) to 12 (ISSCR-8 and IG-19) with an average of 7.3 bands per primer [12][13][20]. ~~Also for~~Genotypes Grand Naine, Shrimantee and Basrai exhibited

angenotype average of 4.45, 3.05 and 4.9 bands per primer, for Shrimantee genotype average 3.05 bands per primer and for Basrai genotype average 4.9 bands per primer were producedrespectively.



L-100 bp ladder, 1-Shrimantee, 2-Basrai, 3- Control (G-8), 4 to 21- Grand Naine

Fig. 3 Amplification pattern of banana genotypes using primer IG-11, ISSCR-4, ISSR-8 and P-16



L-100 bp ladder, 1-Shrimantee, 2-Basrai, 3- Control (G-9), 4 to 21- Grand Naine

Fig. 4 Amplification pattern of banana genotypes using primer UBC 840, P-17, MB1-05 & IG-19

What are the implications of findings on genetic fidelity in the studied materials?

4. CONCLUSION

During micropropagation, six randomly selected healthy tissue cultured plants were taken after three subculturing for genetic fidelity testing using ISSR and SSR markers. The banding pattern of these randomly selected plants was monomorphic with mother plant of genotype Grand Naine.

Screening of random selected samples of banana cultivars produces genetic stable, monomorphic and uniform clones. There were no changes found in the banding pattern of tissue culture plants so in vitro multiplication is the safest mode for multiplying of true to type plants. So all micropropagated banana cv. Grand Naine showed uniformity from that we can concluded that they are clones of each others. Molecular markers were utilized successfully in genetic fidelity of micropropagated plantlets of banana [2][14][21]. Therefore used molecular markers were found very effective in differentiating the Grand Naine from Shrimantee and Basrai and genetic fidelity testing of Grand Naine [22].

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Further study needed for assuring the somaclonal variation in tissue culture raised plants upto 5-6 subcultures.

Comments

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Comments
The authors need to include the genetic implications of their findings.

~~CONSENT (WHERE EVER APPLICABLE)~~

~~No, It is not applicable.~~

~~ETHICAL APPROVAL~~
~~It is not applicable~~

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