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

ABSTRACT

Micropropagation for extensive production of elite planting materials is an effective and superior alternative to suckers and conventional means of propagation of Musa spp. The present investigation was carried out to evaluate the genetic identity 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 Simple Sequence Repeat (SSR) and Inter Simple Sequence Repeat (ISSR). Moreover, for micropropagation explants (suckers) were inoculated on initiation medium containing MS (Murashige and Skoog) supplemented with 5 mg/l BAP (Benzyl amino purine) and 39 mg/l AdSO4 (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 90 % and 75 % plantlets were successfully established during primary hardening in controlled and natural field condition. Further, 26 primers were screened from that 20 primers (ISSR and SSR) showed 146 clear, reproducible and scorable bands ranging from 100-2000 base pairs 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 hormones, Genetic fidelity, Molecular markers

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1. INTRODUCTION

Banana is a monocotyledonous, perennial stooling herb that contains a wide range of triploid (2n=3x=33) cultivars that developed from interspecific and intraspecific 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 ranks first producing 27,575 thousand tons of bananas followed by China with 12,075 thousand tons. Tamil Nadu is the top banana producing state of India producing 8016.35 thousand tons, followed by Maharashtra 4,100 thousand tons [4]. Grand Naine an introduction from France is the most wide spreading commercial cultivar of India. It grows to 1.6 - 1.7 meters and solid green in color. It 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. The crop is one of the most important commercial cultivars 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. Phenotypic characters of Grand Naine resembles those of Dwarf Cavendish except for its robustness, finger of bigger size and heavy bunches. It bears bunches 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 [23]. 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 [24]. 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 utilized for detection of the genetic fidelity of micropropagated plants [11]. Molecular markers like SSR and ISSR are 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 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, the present study investigated following objectives.

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

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

2. MATERIAL AND METHODS

Plant material

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

Culture medium

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For establishment of explants, Murashige and Skoog (MS) media with full strength were prepared in combination with plant hormone 5 mg/l BAP and 39 mg/l AdSO4. Furthermore, for rooting M.S. media fortified with growth hormones viz., 2 mg/l BAP, 1mg/l IBA and 200mg/l activated charcoal were used. The pH of 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.

Molecular markers used for fidelity testing

Different ISSR and SSR primers were used 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 1 A. These suckers were pretreated with different surface sterilizers such as 0.1% bavistin, 0.1% estreptomycin, 0.1% HgCl2 Fig 1 B. The surface sterilized and aseptically excised explants Fig 1 C & D were transferred on the bottles containing medium prepared as per different treatments as shown in 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 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. For primary hardening, plantlets were transferred to trays containing sterilized substrate 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%.

DNA isolation

DNA isolation of mother plant (leaf) and tissue culture raised banana plantlets was done using CTAB extraction method[16]with slight modifications. About 0.2 g of tissue sample was weighed and macerated in mortar pestle. Further, 1 ml of pre-warmed CTAB extraction buffer was added into each 1.5 ml microcentrifuge tubes followed by invertion of tubesand incubation for 2-3 times in water bath for 1 h 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 isoamylalcohol (24:1) was added in the supernatant followed by manual shaking. Centrifugation was carried out at 10000 rpm for 10 min at 4°C.Then aqueous phase was removed and taken in another centrifuge tube without disturbing the interphase. An 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. To eliminate any trace of RNA, the DNA thensamples,RNAsewere added to the sample in a ratio of 10:1 at 37°C for 1 hr.

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 [25]. The amount of DNA was calculated by using the formula given below,

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

PCR amplification for ISSR and SSR markers

Synthesized primers were primarily dissolved in deionized water to get final stock concentrationof 100 pmol/ul. These stocks were diluted to pmol/ul in ratio 1:9. Different PCR components for preparation of PCR reaction mixture 10 PCR buffer 2.5ml, 2 mMdNTPs 2.0 ml, 1U Taq DNA polymerase 0.2 ml, 25mM MgCl2 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. PCR program was performed as the following: 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].

3. RESULTS AND DISCUSSION

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 2 A. Inoculated bottles were kept for incubation in growth room under 16 hrs photoperiods and temperature was maintained at 25 ± 2°C. The banana shoot-tip explants were creamy white in color and turned brownish in color after 6 to 8 days Fig 2 B. The leaf sheaths were turned green after 10 to 15 days of inoculation 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 Fig 2 D. After 21st day of inoculation subculture was carried out by trimming basal dead tissues and brown portion of explants. Repeated subculturing was carried out to control contamination, media depletion and browning. At this stage, the surviving shoots in initiation culture were sub-cultured. At second subculture which was carried after 50th day of inoculation, the multiple shoot clumps were separated and inoculated in separate bottles. The observation recorded after 50^{th} day of inoculation revealed that the central meristem produced clusters of proliferating buds and one to three axillary buds get regenerated from basal parts of explant around central apical meristem Fig 2 F. At 50^{th} day of inoculation, the second subculture was carried out to separate all multiple shoots Fig 2 H. Proliferated multiples were separated and transferred into rooting medium containing different media Fig 2 I. 90 % and 75% plants were successfully established during primary hardening Fig 2 J in controlled as well as in natural field condition Fig 2 K [5].

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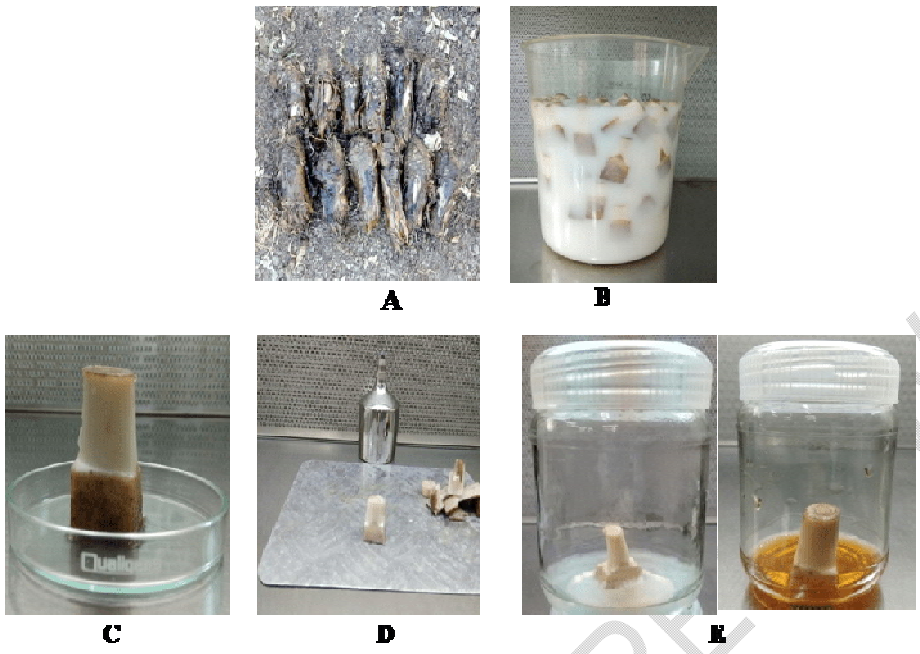

Fig. 1 Preparation of explant and inoculation of shoot tip from suckers of banana cv. Grand Naine A) Collection of explants B) Pretreatment C) Explant D) Final cut under LAF cabinet E) Inoculation of explant in solid & liquid medium

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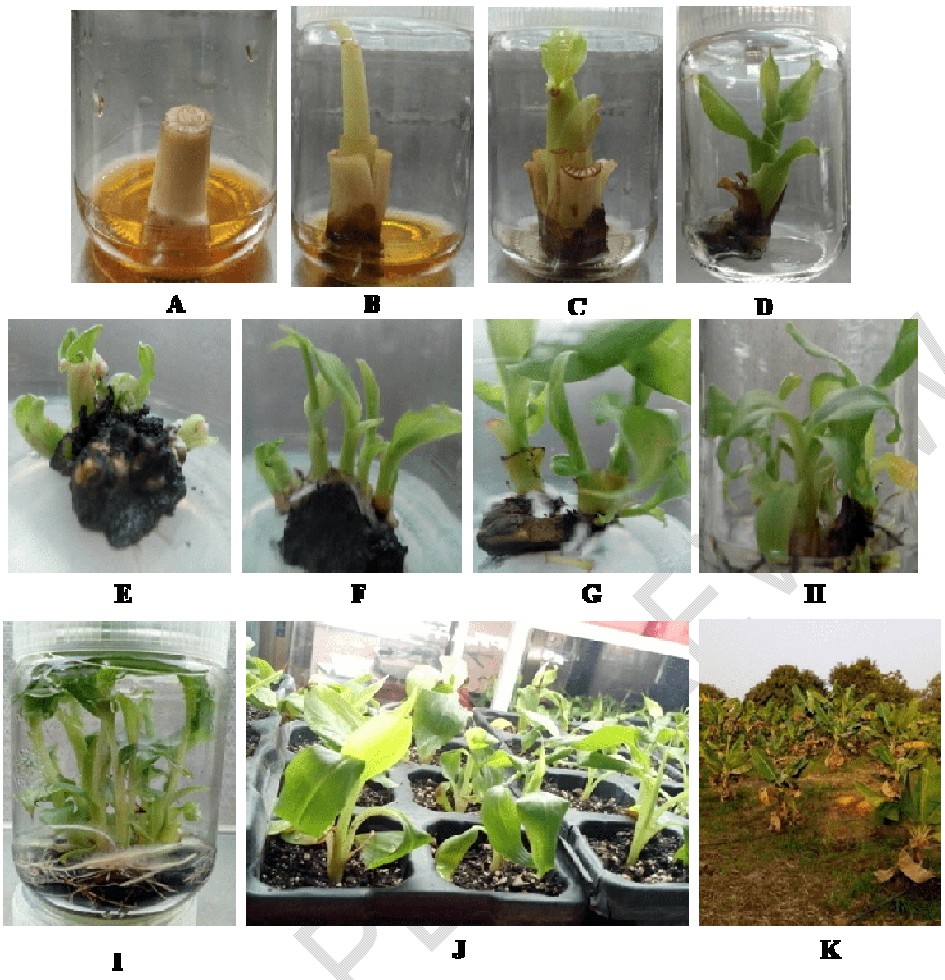

Fig. 2 Initiation, culture establishment & multiplication of banana cv. Grand Naine A) On 1st day B) After 8th days C) After 15th days D) After 21st days, Also different stages of multiplication after 8th days (E), 15th days (F), 21st days (G) & 30th days (H) days on multiplication medium & I) Rooting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

Genetic fidelity of banana genotypes Grand Naine

A total of 26molecular markers comprising16 ISSR and 10 SSR [3][18] [19] were screened for fidelity testing in Grand Naine and genetic identity of genotypes Shrimantee, Basrai and Grand Naine. The 20 primers (14 ISSR and 6 SSR) produced scorable and clear bands with product size ranging from 100-2000 bp. The 20 primers produced 146 clear, reproducible, and scorable bands. The primers ISSCR-8 and IG-19 amplified the highest of 12 of bands, whereas primers Mb1-05 and P-17amplified the lowest of 3 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].forGenotypes Grand Naine, Shrimantee and Basrai exhibited average of 4.45, 3.05 and 4.9 bands per primer, respectively.

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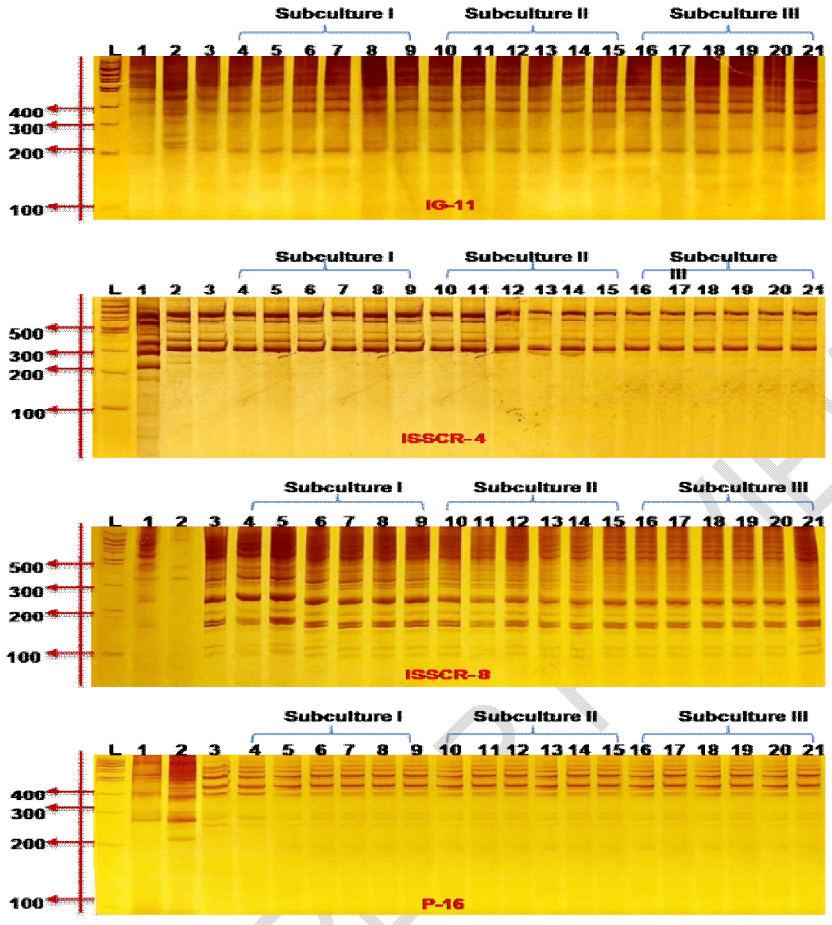

L-100 bp ladder, 1-Shrimantee, 2-Basrai, 3- Control (G-9), 4 to 21- Grand Naine

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

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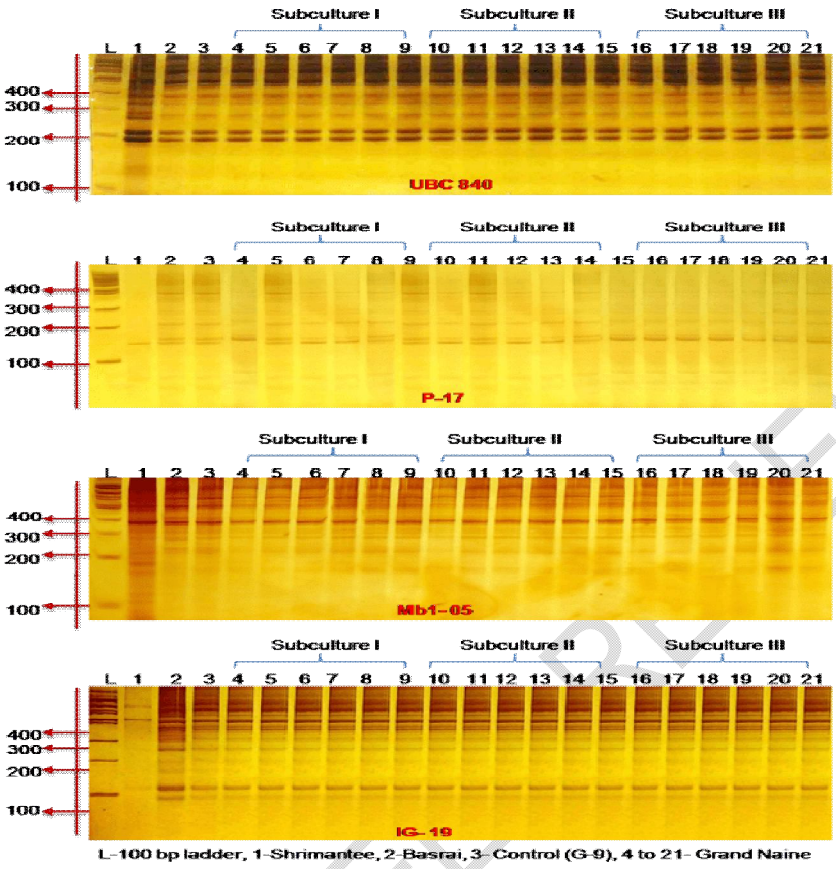

L-100 bp ladder, 1-Shrimantee, 2-Basrai, 3- Control (G-9), 4 to 21- Grand Naine

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

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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 produced 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. Findings revealed that 90 % and 75 % plantlets were successfully established during primary hardening in controlled and natural field condition. Further, 26 primers were screened from that 20 primers (ISSR and SSR) showed 146 clear, reproducible and scorable bands ranging from 100-2000 base pairs produced. The banding pattern of these randomly selected plants was monomorphic with mother plant of all selected genotype that confirmed the clonal uniformity.

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

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