

Standardization of DNA Isolation Protocol for Banana using Rapid Method and Fidelity Testing in Tissue Culture Developed Banana Plantlets of *Safed velchi*

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Abstract The present study was carried out to standardize a DNA isolation protocol for banana and to test fidelity of tissue cultured plantlets of banana cv *Safed velchi* using Inter Simple Sequence Repeat (ISSR) marker. Genomic DNA was extracted from 200 plantlets which were obtained from three different mother plants. A total of 100, 50 and 50 plantlets were selected from plant number 15, 16 and 21, respectively. DNA samples were bulked into ten lots of plant number 15, five lots of plant number 16 and five lots of plant number 21 and used for. A protocol using 0.095 g ml⁻¹ glucose, 0.025 g ml⁻¹ polyvinylpyrrolidone, 0.0045 g ml⁻¹ sodium bisulphite, 0.0055 g ml⁻¹ sodium dodecyl sulfate and 50 µl ml⁻¹ sarcosine produced good quality DNA. ISSR marker UBC-891 showed 0.12% average polymorphism in third lot of mother plant number 15. All other markers produced monomorphic banding pattern in remaining lots of mother plant number 15, 16 and 21. The results revealed that, the monomorphic banding patterns between the se-

lected plantlets and respective mother plant indicate the uniformity of tissue culture banana plantlets. It also indicates that, primer UBC-891 will be helpful in future to identify variation in tissue cultured plantlets of *Safed velchi*.

Keywords Banana, *Safed velchi*, Fidelity, Tissue culture, Genetic diversity.

Introduction

Banana (*Musa paradisiaca*) is the second most important fruit crop in India next to mango. Its year round availability, affordability, varietal range, taste, nutritive and medicinal value makes it the favorite fruit among all classes of people. It has also good export potential. Hi-tech cultivation of the crop is an economically viable enterprise leading to increase in productivity, improvement in produce quality and early crop maturity with the produce commanding premium price. Banana is the most important of the tropical fruits accounting for the world production of 40 million tonnes [1]. *Safed velchi* is preferably cultivated in Konkan region because of its test, high keeping quality and nutritive value. Due to maximum demand of *Safed velchi* in Konkan region, Plant Biotechnology Center Dr. B. S. K. K. V., Dapoli standardized micropropagation protocol and commercial production is started.

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Table 1. Details of 200 samples of 3 mother plants used in the study.

Sample number	Mother plants	No. of plants
1	Plant No.-15	100
2	Plant No-16	50
3	Plant No-21	50

At present, molecular techniques are powerful and valuable tools for the analysis of genetic fidelity of *in vitro* propagated plants. These are and influenced by environmental factors and generate reliable and reproducible results. Among the markers, inter simple sequence repeat (ISSR) has been mostly favored and have been successfully applied to detect genetic similarities or differences in tissue cultured materials of various plants [2]. To test the fidelity standard of micro propagated plantlets developed by tissue culture of banana cv *Safed velchi*, this research study was undertaken.

However, maintaining the genetic stability of tissue cultured plantlets is also very important and the information gained on genetic stability /variation will be valuable for quality control of the large scale propagation of banana. This is very useful for establishing a particular micro propagation system for the production of genetically identical and stable plants before it is released for commercial purposes and it is this point that we seek to address in this report.

Materials and Methods

Plant material

For the present experimental study, 200 tissue cultured banana plantlets of *Safed velchi* were obtained from Plant Biotechnology Center, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli. Dist. Ratnagiri (MS). Details of all 200 tissue cultured banana plantlets are presented in Table 1.

DNA extraction

DNA was isolated following the protocol with slight modifications. Three different solutions (T_1 , T_2 and T_3) were tested to extract tissue cultured banana leaf

Table 2. Chemical composition of the three extraction solutions T_1 - T_3 used to isolate genomic DNA from coconut leaves.

Component	T_1	T_2	T_3
Glucose	0.095 g ml ⁻¹	0.085 g ml ⁻¹	0.075g ml ⁻¹
Polyvinylpyrrolidone	0.025 g ml ⁻¹	0.035 g ml ⁻¹	0.045 g ml ⁻¹
Sodium bisulphite	0.0045 g ml ⁻¹	0.0040g ml ⁻¹	0.0035 g ml ⁻¹
Sodium dodecyl sulfate	0.0055 g ml ⁻¹	0.0050g ml ⁻¹	0.0045g ml ⁻¹
Sarcosine[(5% (w/v)]	50 µl ml ⁻¹	40µl ml ⁻¹	30µl ml ⁻¹

DNA (Table 2). Tender young leaf samples (0.1 g each) were collected from the tissue culture developed plantlets and used to extract genomic DNA. RNA was removed by treatment with 100 µg ml⁻¹ RNase (Merck Pvt. Ltd., Mumbai, India) and incubated at 37°C for 1 h. A total of 100, 50 and 50 plantlets were selected from plant no. 15, plant no. 16 and plant no. 21, respectively. DNA samples were bulked into ten lots of plant no. 15, five lots of plant no. 16 and five lots of plant no. 21 [3] and used for further analysis. The size range and concentration of genomic DNA in each bulked sample was determined after electrophoresis using a standard DNA ladder in 1% (w/v) agarose gels and by comparing the intensity of staining with 10 mg ml⁻¹ ethidium bromide.

PCR amplification

Fourteen inter-simple sequence repeat (ISSR) markers, composed of short, predefined tandem repeat sequences with an anchor sequence and representing different di- and tri-nucleotide microsatellites were used for PCR amplification. The ISSR markers were used for molecular characterisation and to measure genetic fidelity between and within samples of the three numbers of mother plants of *Safed velchi*.

A PCR protocol was standardised for all ISSR markers. Each 20 µl PCR contained 25 ng template DNA, 2.5 ml of 10×PCR buffer, 0.5 µl of 15 mM MgCl₂, 1 µl of 10 mM dNTPs (Bangalore Genei Pvt Ltd, Bangalore, India), 10 pmol of each ISSR or 5 pmol of each SSR primer (Bioresource Biotech Pvt Ltd, Pune, India) and 3.0 units of Taq polymerase (Bangalore Genei Pvt Ltd). Thermal profiles were standardized for each

Table 3. Annealing temperatures of the 14 ISSR primers used in this study. B = C, G, T; H = A, C, T; V = A, C, G; and D = A, G, T.

Primer name	Primer sequence (5' → 3')	Temperature (range (°C))	Standardised annealing temperature (°C)	GC content (%)
UBC-807	AGAGAGAGAGAGAGAGT	45-55	45.5	50.4
UBC-814	CTCTCTCTCTCTCTA	40-50	49.0	47.1
UBC-816	CACACACACACACAT	50-60	57.9	51.1
UBC-818	CACACACACACACAG	45.55	47.9	52.9
UBC-824	TCTCTCTCTCTCTCG	45-55	56.7	52.9
UBC-834	AGAGAGAGAGAGAGT	45-55	50.4	47.1
UBC-841	GAGAGAGAGAGAGAC	40-50	45.4	52.9
UBC-854	TCTCTCTCTCTCTCHG	45-55	54.8	55.5
UBC-857	ACACACACACACACVCG	45-55	51.7	55.5
UBC-879	CTTCACTTCACTCA	45-55	40.0	40.0
UBC-884	HBHAGAGAGAGAGAGAA	40-50	39.8	47.8
UBC-885	HBHAGAGAGAGAGAGAG	40-50	46.8	58.8
UBC-886	VDVCTCTCTCTCTCT	40-50	40.7	58.8
UBC-891	VHVGTGTGTGTGTGT	45-55	53.8	47.1

ISSR primer (i.e. marker) based on its melting temperature using a Master Cycler 2231 gradient-PCR machine (Eppendorf, Hamburg, Germany). The standard annealing temperatures of all ISSR primers are given in Table 3. The PCR-amplified products were separated by electrophoresis in 2% (w/v) agarose gels at 80 V. The gels were stained with 10 mg ml⁻¹ ethidium bromide and visualized under UV light using a Fire Reader gel documentation system (Uvitec Ltd, Cambridge, UK) and the data were stored for further analysis.

Data analysis

ISSR markers across the 200 samples of 3 mother plants were scored for their presence (1) or absence (0) of bands for each primer. By comparing the banding patterns of those samples for a specific primer specific bands were identified. The binary data so generated was used to estimate the levels of polymorphism by dividing the number of polymorphic bands by the total number of scored bands.

Results and Discussion

DNA isolation

The isolation of high-quality DNA is important for all molecular biological analyses, because contaminants such as proteins, polyphenols and polysaccharides

can interfere with key enzymes. Thus, it is important (i) to choose the most appropriate part of the plant to use as the source of DNA; and (ii) to establish an optimum extraction protocol to yield high-quality DNA.

DNA was isolated from young tender leaves of each tissue cultured banana plantlets using the rapid protocol with slight modifications in buffer composition and concentration. Various concentrations of glucose, polyvinylpyrrolidone (PVP) and sodium dodecyl sulfate (SDS), were tested (Table 2). A combination of 0.095 g ml⁻¹ glucose, 0.020 g ml⁻¹ PVP, 0.0040 g ml⁻¹ sodium bisulphite, 0.0050 g ml⁻¹ SDS, and 50 µl ml⁻¹ sarcosine produced the highest yields and best quantity DNA without any contamination by phenolic compounds. DNA samples were bulked into twenty lots of three mother plants.

PCR requires an appropriate, low quantity of DNA (10-30 ng). DNA concentrations above or below the optimum can result in incorrect amplification [4]. The optimum quantity of DNA was obtained by diluting each sample of DNA in double-distilled water.

Marker analysis

ISSR markers are very simple, fast, cost-effective, highly discriminative and reliable. Molecular techniques are powerful and valuable tools for the analy-

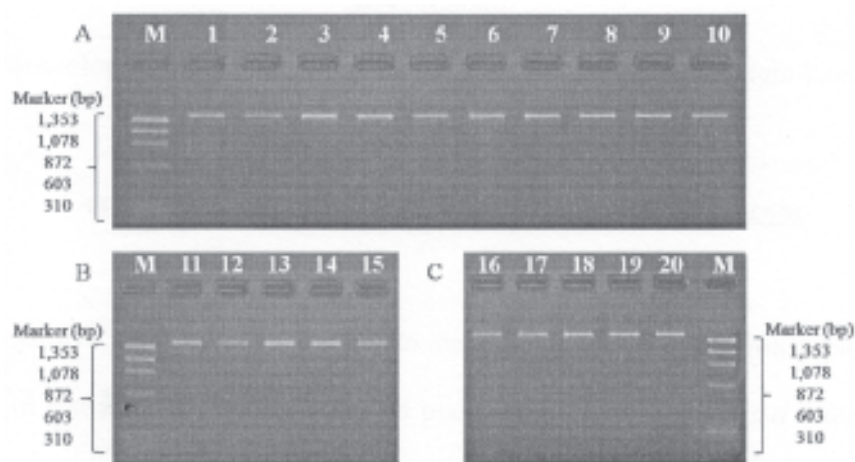


Fig. 1. ISSR assay of 20 lots of banana plantlets by using primer UBC-879. Lanes M, 1.3 kbp DNA ladder; lanes 1 - 20, amplicons of banana lots; A, lanes 1 - 10, mother plant 15; B, lanes 11 - 15, mother plant 16; C, lanes 16 - 20, mother plant 21.

sis of genetic fidelity of *in vitro* propagated plants [2]. PCR-based multiple loci techniques, which include random amplified polymorphic DNA (RAPD), ISSR and SSR markers, play important roles in crop improvement [5—7]. ISSR markers amplify different regions of the genome; their use allows better analysis of genetic stability/variation of the plantlets. The present investigation was aimed to test the genetic fidelity of *in vitro* regenerated plantlets of banana. ISSR marker analysis.

DNA samples were bulked into ten lots of plant no. 15, five lots plant no. 16 and plant no. 21 each. The primer-wise amplification detail of the bulked DNA of all tissue cultured banana plantlets and percent polymorphism across the 14 ISSR primers is presented in the Table 3. A total of 579 scorable DNA fragments were produced in 10 lots of mother plant number 15, 315 scorable DNA fragments in 5 lots of mother plant number 16 and 315 scorable DNA fragments in 5 lots of mother plant number 21.

Among them only 1 DNA fragment was found to be polymorphic in between third lot of mother plant number 15 by the primer UBC-891. Hence, a single polymorphic fragment produced by UBC-891 primer, while there was no any polymorphism shown by all the other primers. The average per cent polymorphism

across the 14 primers in between 10 lots of mother plant number 15 was 0.12% [3]. All other primers produced monomorphic banding pattern in remaining lots of mother plant number 15, 16 and 21.

The average size of product ranged from 200 bp (UBC-884, UBC-885, UBC-891) to 1800 bp (UBC-886) in mother plant No. 15, 300 bp (UBC-824, UBC-841, UBC-854, UBC-885, UBC-891) to 1800 bp (UBC-834) in both 16 and 21 no. mother plants are somewhat similar with the result of Ray et al. [5] (230 bp to 2700 bp).

The numbers of alleles produced by the markers were ranged from 1 to 7. While, the average number of alleles per marker were 4.38. Marker UBC-879 showed only a single allele and the maximum 7 alleles were produced by the markers UBC-818 and UBC-891. 14 ISSR primers detected uniformity due to the true to type behavior of tissue cultured plantlets. The results obtained from the present investigation fulfilling the basic objective of banana micropropagation that is production of genetically uniform plantlets. It is also observed that, ISSR-PCR analysis is an effective technique for testing fidelity / uniformity of tissue cultured plants. Overall, this study confirmed the test have been made mandatory to all the tissue culture laboratories so that genetically pure clones/plantlets are obtained.

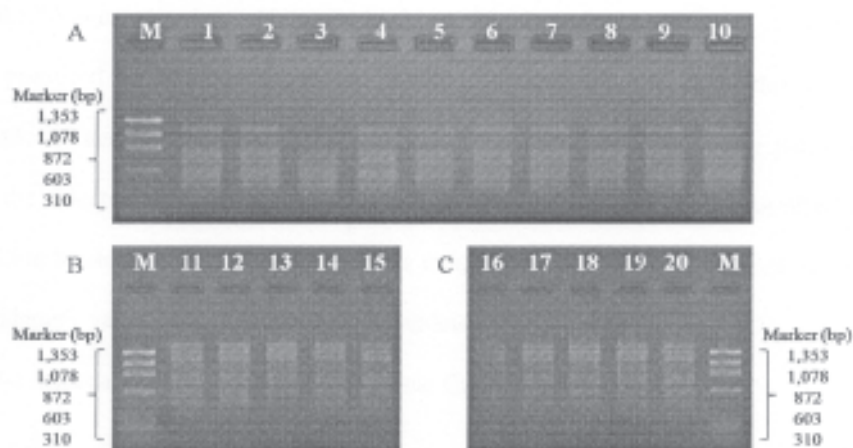


Fig. 2. ISSR assay of 20 lots of banana plantlets by using primer UBC-891. Lanes M, 1.3 kbp DNA ladder; lanes 1 - 20, amplicons of banana lots; A, lanes 1- 10, mother plant 15; B, lanes 11- 15, mother plant 16; C, lanes 16 - 20, mother plant 21.

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