Chapter 6

Micropropagation In Altered Growth Condition

Molecular Evaluation

6.1 Molecular evaluation

The methodology involving molecular markers, which proves efficacious in identifying polymorphic molecular loci among individuals or those genetically linked to pivotal genes, is progressively establishing itself as an indispensable asset in plant improvement initiatives (Emrey, 2022). Molecular markers serve a multifaceted role, not only aiding in the discernment of variability within germplasm and ensuring fidelity in micro-clones but also facilitating the identification of cultivars/genotypes and the selection of pivotal traits. Additionally, these markers prove instrumental in delving into the previously uncharted gene pool of wild or uncultivated plant species (Goswami *et al.,* 2022).

In the realm of plant biotechnology, *in vitro* propagation stands out as a highly viable technique for expeditiously generating a substantial quantity of genetically uniform plantlets. This efficiency surpasses that of conventional propagation methods, providing a rapid means to achieve a large-scale production of homogenous plant material (Kumar and Reddy, 2011). Regeneration *via* plant tissue culture frequently entails the occurrence of epigenetic or stable variations, attributed to various factors, including the micropropagation method employed, age of shoot cultures, genotype and media composition (Us-Camas *et al.,* 2014). Micropropagation through the stimulation of axillary buds is fevered due to its ability to assurance genetic stability. This is attributed to the conventional ontogenic pathway governing branch development through pre-existing lateral meristems. In contrast, alternative approaches to plant regeneration involve the cultivation of tissue sections devoid of a pre-existing meristematic region (adventitious origin) (Jiang *et al.,* 2023). Alternatively, originating from callus or cell cultures or derived therefrom (*de novo* origin) (Bhojwani and Dantu, 2013). Plant tissues exhibit heightened susceptibility to variations. Additionally, irregularities within the tissue culture environment and the resultant plants frequently escalate in frequency proportionally to the number of culture passages. Several publications have verified the appearance of genetic instability in plants subjected to micropropagation methodologies. (S. K. Sharma *et*

al., 2007; Olhoft and Phillips, 2018). Consequently, it is imperative to routinely subject plantlets derived from tissue culture to genetic fidelity assessments conducted randomly. This practice ensures the provision of genetically homogeneous planting material with a well-established source for subsequent deployment in commercial cultivation. In addition to morphological, cytological, and protein profiling, the application of advanced biochemical and DNA-based methodologies has facilitated a more intricate examination of the genetic stability of *in vitro* plant materials. In recent times, molecular markers derived from DNA have been employed to assess genetic fidelity in the course of micropropagation, with a particular emphasis on different plants species. The uniformity of regenerants achieved through the application of tissue culture biotechnology holds considerable importance in this context.

Various molecular markers, including but not limited to Restriction Fragment Length Polymorphisms (RFLPs), Randomly Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Inter-Simple Sequence Repeats (ISSRs), and Simple Sequence Repeats (SSRs), have been employed in the assessment of Soma clonal variations. However, the appropriateness and effectiveness of these markers are contingent upon their inherent properties. (Anca). Within the spectrum of molecular markers, PCR-based Random Amplified Polymorphic DNAs (RAPDs) and Inter-Simple Sequence Repeats (ISSRs) stand out as prevalent techniques employed for evaluating genetic fidelity in micro propagated plants. This preference arises from their inherent simplicity and cost-effectiveness.

The emergence of Polymerase Chain Reaction (PCR) technology, as pioneered by Mullis in 1990, has bestowed upon us a repertoire of straightforward methodologies for molecular assessment. Among these, the Random Amplified Polymorphic DNA (RAPD) technique has garnered significance in molecular analyses (Babu *et al.,* 2021). Identifies nucleotide sequence polymorphism within DNA amplification assays utilizing a singular primer with an arbitrary nucleotide sequence. In contrast to Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) analysis circumvents the need for restriction digestion, probe preparation, and hybridization steps. RAPDs have demonstrated efficacy in the identification and evaluation of genetic fidelity across a different plant species, demonstrated by instances such as *Lavandula officinalis* (Prasad *et al.,* 2015), *Picrorhiza kurroa* (Rawat *et al.,* 2013), *Cineraria maritima* (Srivastava *et al.,* 2009),

Rauwolifia tetraphylla (Rohela *et al.,* 2019), *Dioscorea deltoidea* (Nazir *et al.,* 2021) and *Pogostemon cablin* (Paul *et al.,* 2010). Random Amplified Polymorphic DNA (RAPD) markers have gained widespread popularity primarily due to their inherent simplicity. In contrast to alternative techniques, RAPD offers several advantageous features. Notably, RAPD assays do not necessitate prior knowledge of target DNA sequences, demand minimal quantities of DNA, exhibit cost-effectiveness per assay, and, owing to their PCR-based nature, are amenable to streamlined automation processes. Nevertheless, this methodology exhibits inherent constraints associated with its dominant characteristics and susceptibility to subtle variations in reaction conditions. These factors pose challenges to the consistent reproduction of banding patterns across distinct experiments, diverse PCR instrumentations, and various laboratory settings (S. Sharma *et al.,* 2019; Venkatesan *et al.,* 2022). Despite the acknowledged limitations, RAPD (Random Amplified Polymorphic DNA) remains the predominant method for evaluating the genetic fidelity of micro propagated plants. The inherent weaknesses can be moderated through particular adjustment of the reaction conditions, underscoring its continued utility in plant tissue culture (S. Sharma *et al.,* 2019; Venkatesan *et al.,* 2022). The Random Amplified Polymorphic DNA (RAPD) technique has been utilized for the assessment of genetic fidelity in an extensive array of micro propagated plants (Rawat *et al.,* 2013; Rohela *et al.,* 2019).

Martin *et al*. (2004) have reported the sustained genetic stability of *Prunus dulcis* plantlets originating from axillary branching. This stability was observed over extended periods of 4 and 6 years during *in vitro* culture, employing a comprehensive analysis involving 64 Random Amplified Polymorphic DNA (RAPD) primers. In the study conducted by Kawiak and Lojkowaska (2004), an assessment was undertaken on the genetic stability of plantlets regenerated from shoot tips and leaf explants of *Drosera binata* and *D. anglica*. The investigation involved the utilization of 20 arbitrary decamer primers to evaluate genetic markers. Notably, no discernible variation was observed among the *D. binata* plantlets. Conversely, polymorphic patterns were ascertained in the *D. anglica* plantlets subsequent to six culture passages. This elucidates the genomic dynamics and stability of the regenerated plantlets under the specified experimental conditions. Feyissa *et al*. (2007) Employed Random Amplified Polymorphic DNA (RAPD) markers for evaluating genetic

stability in micro propagated *Hagenia abyssinica* plants derived from both axillary and adventitious origins.

6.1.1 Materials and methods

To assess the impact of successive culture passages on genetic fidelity, micro-clones were select subsequent to the third culture passage in each experiment. For the extraction of genomic DNA, freshly harvested young leaves were procured from the field-cultivated mother plant of *Musa acuminata*. Additionally, samples were obtained from five arbitrarily chosen cultures, each representing distinct passages, within every accession under investigation. The selected leaves were enveloped in aluminum foil and preserved at -20°C. Subsequently, genomic DNA was extracted from the cryopreserved leaf material employing established protocols for DNA isolation (Dellaporta *et al.*, 1983; Saghai-Maroof *et al.*, 1984).

DNA Extraction

For the extraction of DNA, following the methodology outlined by Dellaporta et al. (1983), leaf tissue weighing 1 g was cryogenically ground into a fine whitish powder using liquid nitrogen. The resulting powder was promptly transferred, preventing any thawing, to a 15 ml DNA extraction buffer comprising 100 mM Tris–Cl (pH 8.0), 500 mM NaCl ,50 mM EDTA (pH 8.0), and 10 mM β–mercaptoethanol (added freshly). Subsequently, 500 µl of 20% SDS was introduced, and after thorough mixing, the solution underwent incubation at 65°C in a water bath. This step was succeeded by the addition of 2.5 ml of potassium acetate (5 M) and incubation on ice for 20 minutes. Following this, the reaction mixture underwent centrifugation at 14,000 rpm, and the resulting supernatant was filtered through muslin cloth. DNA precipitation was achieved using 0.6 volumes of chilled isopropanol, and the resultant pellet was collected by centrifugation at 10,000 rpm for 10 minutes at 4°C. The pellet was reconstituted in high salt TE buffer and centrifugation at 10,000 rpm to eliminate any remaining debris. DNA was once again precipitated from the supernatant, this time utilizing 3M sodium acetate (pH 5.2) and chilled isopropanol. The final pellet was suspended in 1 X TE buffer.

DNA Purification

In order to eliminate RNA impurities, present in the DNA, RNAase treatment was employed. Specifically, a solution of Pancreatic RNAase at a concentration of 5 mg/ml was introduced to the DNA suspension, achieving a final concentration of 100

µg RNAase/ml. Subsequently, the mixture underwent incubation at 37°C for a duration of one hour. To eliminate RNAase and other proteins as contaminants, the DNA solution underwent purification by the addition of an equivalent volume of chloroform: isoamyl alcohol (24:1). Subsequent to mixing through inversion, the resultant mixture was subjected to centrifugation at 10,000 rpm for 15 minutes at 4°C.

The supernatant aqueous phase was aseptically transferred into a separate microcentrifuge tube (MCT) utilizing wide-bore pipette tips. Subsequently, 1/10th volume of sodium acetate (3 M) and an equivalent volume of chilled isopropanol were added to the transferred aqueous phase. The resultant mixture was maintained at a temperature of -20°C for a duration of one hour. The genomic DNA was sedimented through centrifugation at 10,000 rpm for 10 minutes at 4°C, subjected to a washing step with 70% ethanol, air-dried, and subsequently reconstituted in 100 µl of 1X Tris-EDTA (TE) buffer.

DNA Quantification

DNA concentration was quantified employing a spectrophotometric approach with a UV-Vis Spectrophotometer (UV-1800, Shimadzu, Japan). The absorbance of the solution was assessed at wavelengths of 260 nm and 280 nm. The determination of DNA concentrations was performed utilizing the subsequent formula

$$\text{DNA concentration } (\mu g/ml) = \frac{50 \times \text{OD}_{260} \times \text{Dilution Factor}}{1000}$$

The optical density ratio at 260 nm to 280 nm (OD_{260}:OD_{280}) was determined, employing a consistent methodology for the quantification of all samples. Subsequently, DNA samples were appropriately diluted to attain a final concentration of 5 ng/µl, ensuring suitability for polymerase chain reaction (PCR) analysis. Only DNA samples exhibiting optimal quality, as discerned through agarose gel electrophoresis and displaying an OD_{260}/OD_{280} ratio approximating 1.8, were selected for subsequent analytical procedures.

Optimization of PCR Conditions for RAPD

Primers

The primers were acquired in a desiccated state as dry desalted powder, and a stock solution with a concentration of 100 pmol μl^{-1} was meticulously prepared. The dry powder was reconstituted by adding an appropriate volume of elution buffer to the vials, which were then allowed to stand at 4°C overnight. Subsequently, a thermal

shock treatment at 65°C was administered for 10 minutes to ensure the thorough dissolution of the primers. The resulting stock solution served as the basis for the formulation of working solutions with a concentration of 10 pmol μl^{-1}.

RAPD (Random Amplified Polymorphic DNA) primers were assessed for their suitability in conducting polymerase chain reaction (PCR) amplification of the entire genomic DNA extracted from *Musa acuminata*. For RAPD analysis OP1-OP9, (Table 6.1).

Optimized conditions for RAPD

RAPD profiles were generated via polymerase chain reaction (PCR) amplification following the methodology outlined by Williams et al. (1990), incorporating slight adjustments. The PCR reactions were conducted under optimized conditions in 0.2 ml polypropylene PCR tubes utilizing a Thermal Cycler. For each sample PCR reaction mention in table no.6.2.

6.1.2 Result

To assess the impact of distinct components on DNA amplification, a systematic approach was employed, wherein singular components were selectively altered while maintaining constancy in all other conditions, adhering to the optimized parameters delineated for Random Amplified Polymorphic DNA (RAPD) analysis.

Following the optimization of polymerase chain reaction (PCR) parameters, genomic DNA underwent PCR amplification employing Random Amplified Polymorphic DNA (RAPD) analysis. A total of 9 random decamer primers were use for the amplification of products. Each primer produced a unique set of amplification products. These selected RAPD primers yielded a total of 54 scorable bands with an average of 6 bands per primer ranging is size from 100 bp to 1200 bp (Table 6.3). Number of bands for each primer ranged from 6 (OP1) to 10 (OP 09) (Figure 6.1 a to c and Figure 6.2 a to f).

In all instances, the examined plantlets under diverse growth conditions exhibited compatible RAPD profiles in comparison to those of the mother plant. No discernible genetic variations were detected in any of the six conducted experiments. While subtle disparities in RAPD profiles were discerned concerning band intensity, the quantity and dimensions of the bands exhibited uniformity across all analysed samples. There was a lack of substantial variation discernible in any of the examined profiles. In the current investigation, variations in growth conditions within the culture

medium did not manifest discernible impacts on the genetic fidelity of micro propagated specimens of *Musa acuminata.*

Table 6.1 List of Random decamer Primers used for screening the PCR amplification of total genomic DNA in *Musa acuminata*

Name	Sequence	Tm	Molecular Weight
OP 01	TGCCGAGCTG	43.6	3044.01
OP 02	TTTGCCCGGA	39.5	3019.01
OP 03	ACCCCCGAAG	43.6	2981.95
OP 04	GGACCCTTAC	39.5	2987.98
OP 05	TTCGAGCCAG	39.5	3028.01
OP 06	GTGAGGCGTC	43.6	3084.08
OP 07	AAAGCTGCGG	39.5	3077.04
OP 08	CCGATATCCC	39.5	2947.95
OP 09	CTACGGAGGA	39.5	3077.04

Table 6.2 Concentration of PCR mixture for RAPD.

	Componet	Concentration
1.	Template DNA	25 ng
2.	PCR assay buffer	1X
3.	$MgCl_2$	2.5 mM
4.	dNTPs (dATP,dGTP, dCTP and dTTP)	200 μM
5.	Taq DNA Polymerase	1U
6.	Random Decamer Primer	20 pmol

Tabele 6. 3 RAPD analysis of genomic DNA extracted from micro-clones *Musa acuminata* using 09 random decamer primers.

Primers	Fragment size range (bp)
OP 01	110-550
OP 02	120-1000
OP 03	300-1500
OP 06	100-1200
OP 07	200-1200
OP 08	180-1200
OP 09	200-1200

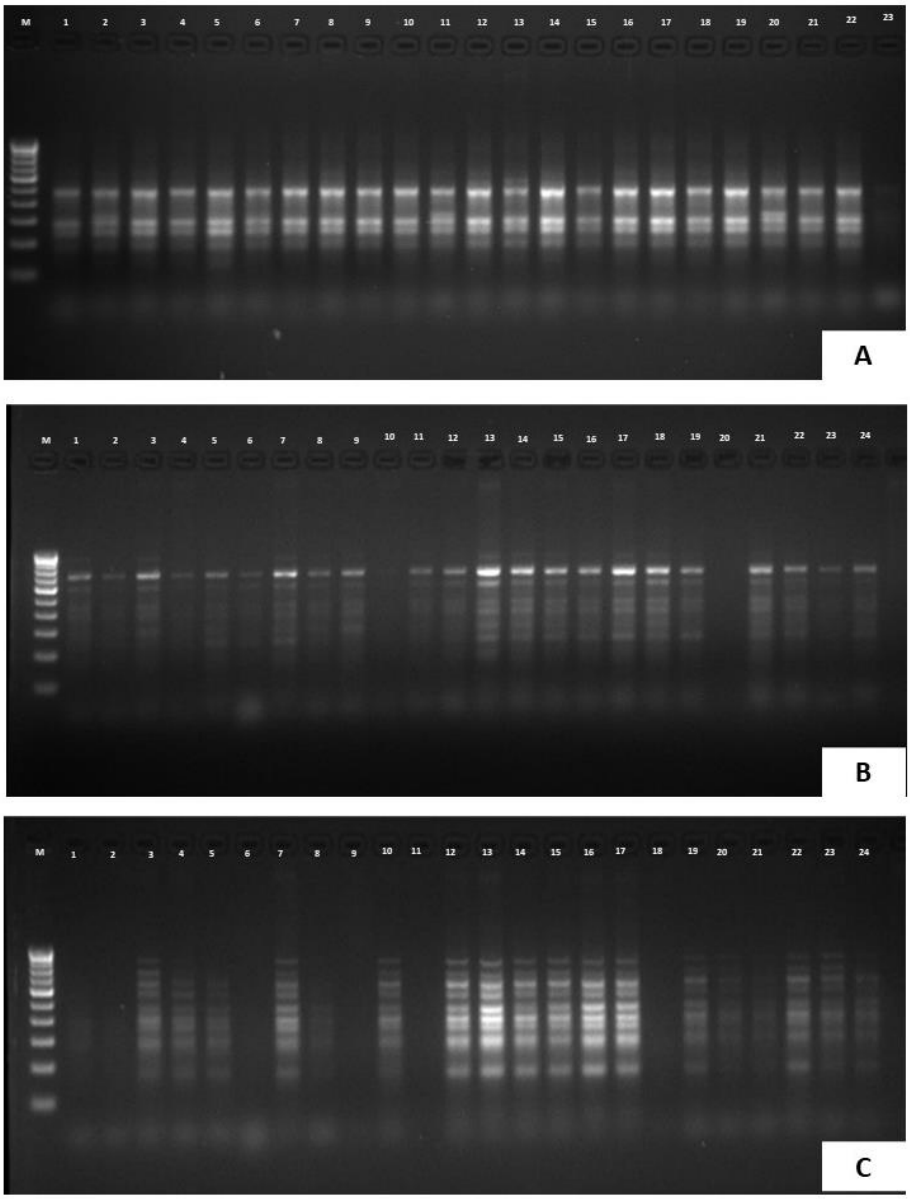

Figure: 6.1 Molecular evaluation of genetic fidelity in *in vitro* grown *Musa acuminata* during different growth condition. PCR amplification of genomic DNA using **(A)** RAPD primer OP1, **(B)** RAPD primer OP 2, **(C)** RAPD primer OP3. DNA amplified in - Lane M Mother plant, 1-4 micropropagules grown in liquid medium with different supporting material., Lane 5-10 micropropagules grown in different culture vessels. Lane 11-16 plant grown in different gelling agent; Lane 17-24 plant grown in LSE. Observations were recorded after three culture cycles *i.e.* 63 days

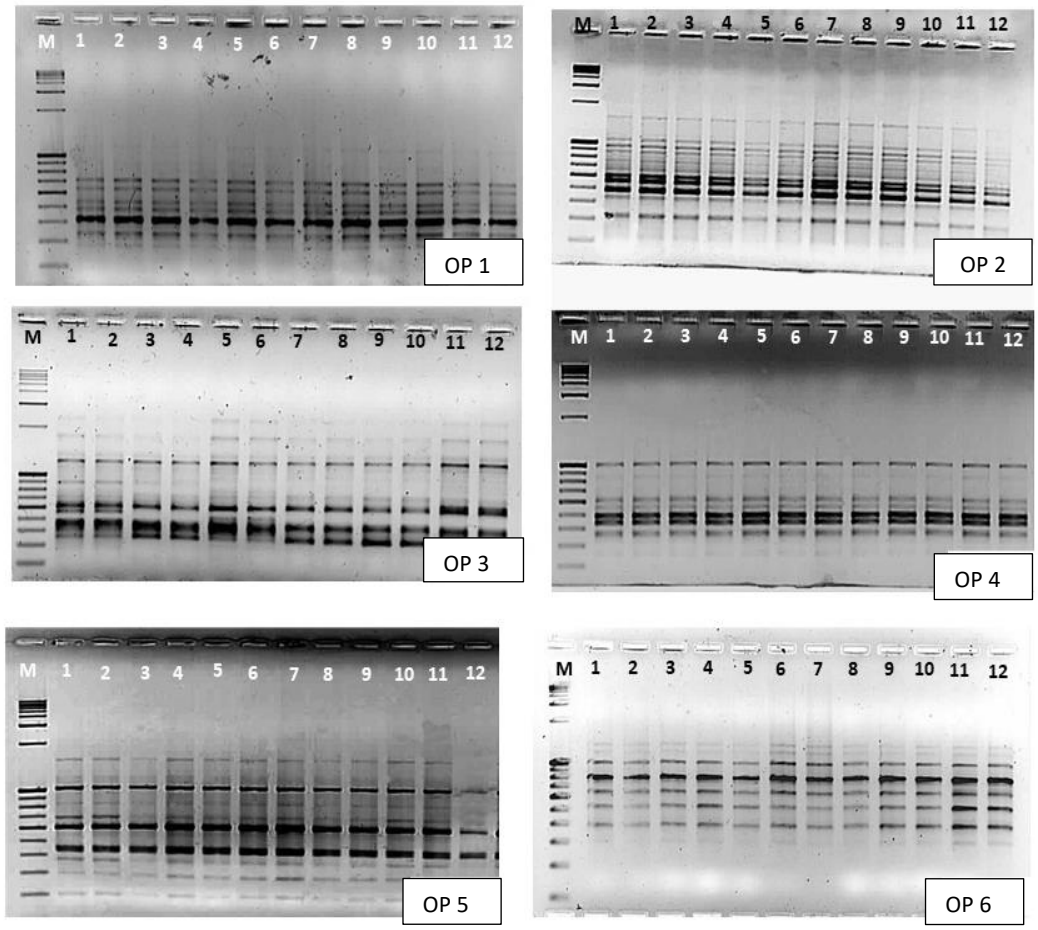

Figure: 6.2 Molecular evaluation of genetic fidelity in *Musa acuminata* grown under *in vitro* CO_2 Enrichment condition in liquid and solid medium. Lane M- mother plant, Lane 1-3 plant grown in 0% sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2, Lane 4-7 plant grown in 1% Sucrose with 0% CO_2, 0.03% CO_2; 0.5% CO_2; 2% CO_2, Lane 8-11 plant grown in 3% sucrose with 0% CO_2, 0.03% CO_2; 0.5% CO_2; 2% CO_2. and 12 Plant grown in 3% sucrose with ambient air. Observations were recorded after three culture cycles *i.e.* 63 days