Chapter 6

Micropropagation in Altered Growth Condition-Molecular Evaluation

6.1 Molecular evaluation of genetic fidelity

The methodology involving molecular markers, which is effective in identifying polymorphic molecular loci among individuals or those genetically linked to important genes, is increasingly recognized as an invaluable asset in plant improvement efforts (Emrey, 2022). Molecular markers play a versatile role, assisting in the detection of variability within germplasm, ensuring accuracy in micro-clones, and aiding in the identification of cultivars/genotypes and the selection of key traits. Furthermore, these markers are instrumental in exploring the previously unexplored gene pool of wild or uncultivated plant species (Goswami *et al*., 2022).

In the field of plant biotechnology, *in vitro* propagation stands out as an effective technique for rapidly producing a large quantity of genetically uniform plantlets. This method surpasses conventional propagation techniques, offering a swift means to achieve extensive production of homogeneous plant material (Kumar and Reddy, 2011). Regeneration through plant tissue culture often leads to the occurrence of epigenetic or stable variations, influenced by factors such as the micropropagation method used, age of shoot cultures, genotype, and media composition (Us-Camas *et al*., 2014). Micropropagation via the stimulation of axillary buds is preferred for its ability to ensure genetic stability, attributed to the conventional ontogenic pathway governing branch development through pre-existing lateral meristems. In contrast, alternative methods of plant regeneration include cultivating tissue sections lacking a pre-existing meristematic region, known as adventitious origin (Jiang *et al*., 2023). Another approach involves originating from callus or cell cultures, termed de novo origin (Bhojwani and Dantu, 2013). Plant tissues are prone to variations, and irregularities within the tissue culture environment can lead to increased frequency of abnormalities in resultant plants, particularly with multiple culture passages. Numerous studies have documented the occurrence of genetic instability in plants

subjected to micropropagation methods (Sharma *et al*., 2007; Olhoft and Phillips, 2018). As a result, it is essential to regularly subject plantlets obtained from tissue culture to random genetic fidelity assessments. This practice ensures the availability of genetically uniform planting material with a well-established source for subsequent use in commercial cultivation. Alongside morphological, cytological, and protein profiling, the utilization of advanced biochemical and DNA-based methods has facilitated a more detailed examination of the genetic stability of *in vitro* plant materials. In recent years, molecular markers derived from DNA have been utilized to evaluate genetic fidelity during micropropagation, with a specific focus on various plant species. The consistency of regenerants achieved through the application of tissue culture biotechnology holds significant importance in this regard.

Various molecular markers, such as 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 utilized in assessing somaclonal variations. However, the suitability and efficacy of these markers depend on their intrinsic characteristics (Anca). Among these molecular markers, PCR-based Randomly Amplified Polymorphic DNAs (RAPDs) and Inter-Simple Sequence Repeats (ISSRs) are commonly used techniques for evaluating genetic fidelity in micropropagated plants. This preference stems from their inherent simplicity and cost-effectiveness.

The advent of Polymerase Chain Reaction (PCR) technology, pioneered by Mullis in 1990, has provided us with a range of straightforward methodologies for molecular assessment. Among these, the Random Amplified Polymorphic DNA (RAPD) technique has gained significance in molecular analyses (Babu *et al*., 2021). RAPD identifies nucleotide sequence polymorphism within DNA amplification assays using a single primer with an arbitrary nucleotide sequence. Unlike Restriction Fragment Length Polymorphism (RFLP), RAPD analysis eliminates the need for restriction digestion, probe preparation, and hybridization steps. RAPDs have proven effective in identifying and evaluating genetic fidelity across various plant species, as demonstrated in studies on *Lavandula officinalis* (Prasad *et al*., 2015), *Picrorhiza kurroa* (Rawat *et al*., 2013), and *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 become widely popular primarily because of their inherent simplicity. Compared to alternative techniques, RAPD offers several advantageous features. Notably, RAPD assays do not require prior knowledge of target DNA sequences, need minimal quantities of DNA, are cost-effective per assay, and, being PCR-based, are adaptable to streamlined automation processes. However, this methodology has inherent limitations associated with its dominant characteristics and susceptibility to subtle variations in reaction conditions. These factors present challenges to consistently reproducing banding patterns across different experiments, diverse PCR instruments, and various laboratory settings (Sharma *et al*., 2019; Venkatesan *et al*., 2022). Despite the acknowledged limitations, RAPD (Random Amplified Polymorphic DNA) remains the primary method for evaluating the genetic fidelity of micropropagated plants. The inherent weaknesses can be mitigated through specific adjustments of reaction conditions, highlighting its continued usefulness in plant tissue culture (Sharma *et al*., 2019; Venkatesan *et al*., 2022). The RAPD technique has been employed for assessing genetic fidelity in a wide range of micropropagated plants (Rawat *et al*., 2013; Rohela *et al*., 2019).

Martin *et al*. (2004) documented the sustained genetic stability of *Prunus dulcis* plantlets derived from axillary branching. This stability was observed over extended periods of 4 and 6 years during *in vitro* culture, utilizing a comprehensive analysis involving 64 Random Amplified Polymorphic DNA (RAPD) primers. In a study conducted by Kawiak and Lojkowaska (2004), an assessment was carried out on the genetic stability of plantlets regenerated from shoot tips and leaf explants of *Drosera binata* and *D. anglica*. The investigation involved the use of 20 arbitrary decamer primers to evaluate genetic markers. Remarkably, no noticeable variation was detected among the D. binata plantlets. In contrast, polymorphic patterns were identified in the D. anglica plantlets after six culture passages. This sheds light on the genomic dynamics and stability of the regenerated plantlets under the specific experimental conditions. Feyissa *et al*. (2007) utilized Random Amplified Polymorphic DNA (RAPD) markers to assess genetic stability in micropropagated *Hagenia abyssinica* plants derived from both axillary and adventitious origins.

6.1.1 Materials and methods

To evaluate the effect of repeated culture passages on genetic fidelity, micro-clones were chosen after the third culture passage in each experiment. Genomic DNA was extracted from freshly harvested young leaves of the field-cultivated rose mother plant. Additionally, samples were collected from five randomly selected cultures, each representing different passages within every accession being studied. These chosen leaves were wrapped in aluminium foil and stored at -20°C. Later, genomic DNA was isolated from the cryopreserved leaf material using established protocols for DNA extraction (Dellaporta and Hicks, 1983).

DNA extraction

To extract DNA, we followed the procedure detailed by Dellaporta *et al*. (1983). Leaf tissue weighing 1 g was finely grind into a whitish powder using liquid nitrogen. This powder was immediately transferred, ensuring it remained frozen, into a 15 ml DNA extraction buffer containing 100 mM Tris–Cl (pH 8.0), 500 mM NaCl, 50 mM EDTA (pH 8.0), and 10 mM freshly added β–mercaptoethanol. Next, 500 µl of 20% SDS was added, and the solution was thoroughly mixed before incubating it at 65°C in a water bath. Subsequently, 2.5 ml of potassium acetate (5 M) was added, and the mixture was incubated on ice for 20 minutes. Afterward, the reaction mixture was centrifuged at 14,000 rpm, and the resulting supernatant was filtered through muslin cloth. DNA precipitation was conducted by adding 0.6 volumes of chilled isopropanol, followed by collecting the resulting pellet through centrifugation at 10,000 rpm for 10 minutes at 4°C. The pellet was then dissolved in high salt TE buffer and subjected to centrifugation at 10,000 rpm to remove any residual debris. Subsequently, DNA was precipitated once more from the supernatant, this time using 3M sodium acetate (pH 5.2) and chilled isopropanol. Finally, the resulting pellet was suspended in 1X TE buffer.

DNA purification

To remove RNA impurities from the DNA, RNAase treatment was utilized. Pancreatic RNAase solution at a concentration of 5 mg/ml was added to the DNA suspension, resulting in a final concentration of 100µg RNAase/ml. The mixture was then incubated at 37°C for one hour. To remove RNAase and other protein

contaminants, the DNA solution was purified by adding an equal volume of chloroform:isoamyl alcohol (24:1). After thorough mixing by inversion, the resulting mixture was centrifuged at 10,000 rpm for 15 minutes at 4°C.

The supernatant aqueous phase was carefully transferred into a separate microcentrifuge tube (MCT) using wide-bore pipette tips. Then, 1/10th volume of sodium acetate (3 M) and an equal volume of chilled isopropanol were added to the transferred aqueous phase. This mixture was kept at -20°C for one hour. Genomic DNA was then collected by centrifugation at 10,000 rpm for 10 minutes at 4°C, washed with 70% ethanol, air-dried, and finally reconstituted in 100 µl of 1X Tris-EDTA (TE) buffer.

DNA quantification

DNA concentration was measured using a spectrophotometric method with a UV-Vis Spectrophotometer (UV-1800, Shimadzu, Japan). The absorbance of the solution was evaluated at wavelengths of 260 nm and 280 nm. DNA concentrations were calculated using the following formula:

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

The ratio of optical density (OD) at 260 nm to 280 nm (OD (260):OD (280)) was calculated consistently for all samples to ensure accurate quantification. Following this, DNA samples were diluted as necessary to achieve a final concentration of 5 ng/µl, ensuring suitability for polymerase chain reaction (PCR) analysis. Only DNA samples demonstrating optimal quality, as determined by agarose gel electrophoresis and exhibiting an OD (260)/OD (280) ratio close to 1.8, were chosen for further analytical procedures.

Optimization of PCR conditions for RAPD

Primers

The primers were initially obtained in a desiccated state as dry desalted powder, and a stock solution was meticulously prepared with a concentration of 100 pmol μl^{-1}. The dry powder was reconstituted by adding an appropriate volume of

elution buffer to the vials, which were then left overnight at 4°C. Following this, a thermal shock treatment at 65°C was applied for 10 minutes to ensure complete dissolution of the primers. The resulting stock solution served as the foundation for formulating working solutions with a concentration of 10 pmol μl^{-1}.

RAPD (Random Amplified Polymorphic DNA) primers were evaluated for their appropriateness in amplifying the entire genomic DNA extracted from Rose via polymerase chain reaction (PCR). Specifically, for RAPD analysis, primers OPP7-OPP17 were considered (table 6.1).

Optimized conditions for RAPD

RAPD profiles were produced through polymerase chain reaction (PCR) amplification, following the procedure outlined by Williams *et al*. (1990), with minor modifications. The PCR reactions were carried out under optimized conditions in 0.2 ml polypropylene PCR tubes using a Thermal Cycler (table 6.2).

6.1.2 Results

To evaluate the influence of various components on DNA amplification, a methodical strategy was used. This involved individually modifying specific components while keeping all other conditions constant, following the optimized parameters established for Random Amplified Polymorphic DNA (RAPD) analysis. After optimizing the polymerase chain reaction (PCR) parameters, genomic DNA was amplified using Random Amplified Polymorphic DNA (RAPD) analysis. Nine random decamer primers were utilized for the amplification, with each primer generating a distinct set of amplification products.

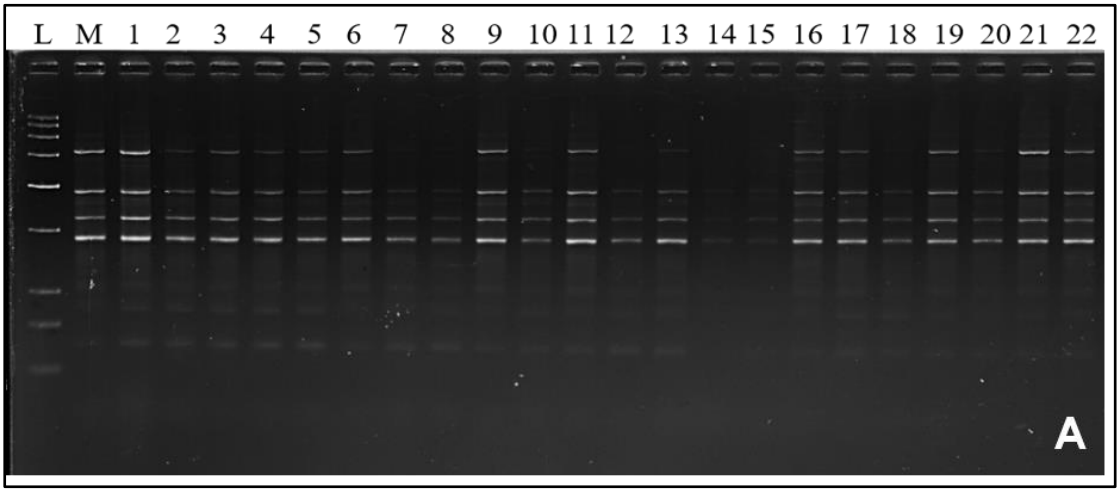
In every case, the plantlets grown under various conditions showed RAPD profiles that matched those of the mother plant. No genetic differences were found in any of the six experiments conducted. Although slight variations in band intensity were observed, the number and size of the bands remained consistent across all samples analysed. No significant differences were detected in any of the profiles examined. This study found that changes in growth conditions within the culture medium did not affect the genetic fidelity of micropropagated Rose specimens.

Table 6.1 List of random decamer primers used for screening the PCR amplification of total genomic DNA in Rose

Name	Sequence	Tm (°C)	Molecular Weight (daltons (g/M))
OPP 7	GTCCATGCCA	32	2987
OPP 9	GTGGTCCGCA	34	3041
OPP 11	AACGCGTCGG	34	3050
OPP 12	AAGGGCGAGT	32	3113
OPP 13	GGAGTGCCTC	34	3041
OPP 14	CCAGCCGAAC	34	2981
OPP 15	GGAAGCCAAC	32	3044
OPP 17	TGACCCGCCT	34	2963

Table 6.2 Concentration of PCR mixture for RAPD

Components	Concentration
Template DNA	25 ng
PCR assay buffer	1X
MgCl2	2.5 mM
dNTPs (dATP, dGTP, dCTP and dTTP)	200 µM
Taq DNA Polymerase	1U
Random Decamer Primer	20 pmol



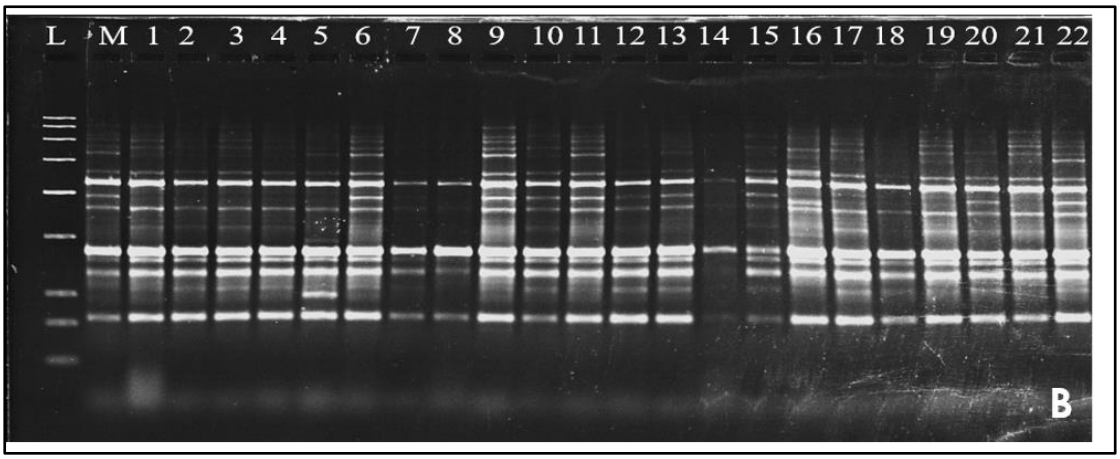


Figure 6.1(a) Molecular evaluation of genetic fidelity in *in vitro* grown Rose during different growth condition. PCR amplification of genomic DNA using (A) RAPD primer OPP-7, (B) RAPD primer OPP-9. Lane L – Marker, M-Mother plant, 1-7 micropropagules grown in different culture vessels in semi-solid medium, Lane 8-14 micropropagules grown in different culture vessels in liquid medium, Lane 15-18 plant grown in different support materials, Lane 19-22 plant grown in different gelling agents. Observations were recorded after two culture cycles *i.e.* 45 days

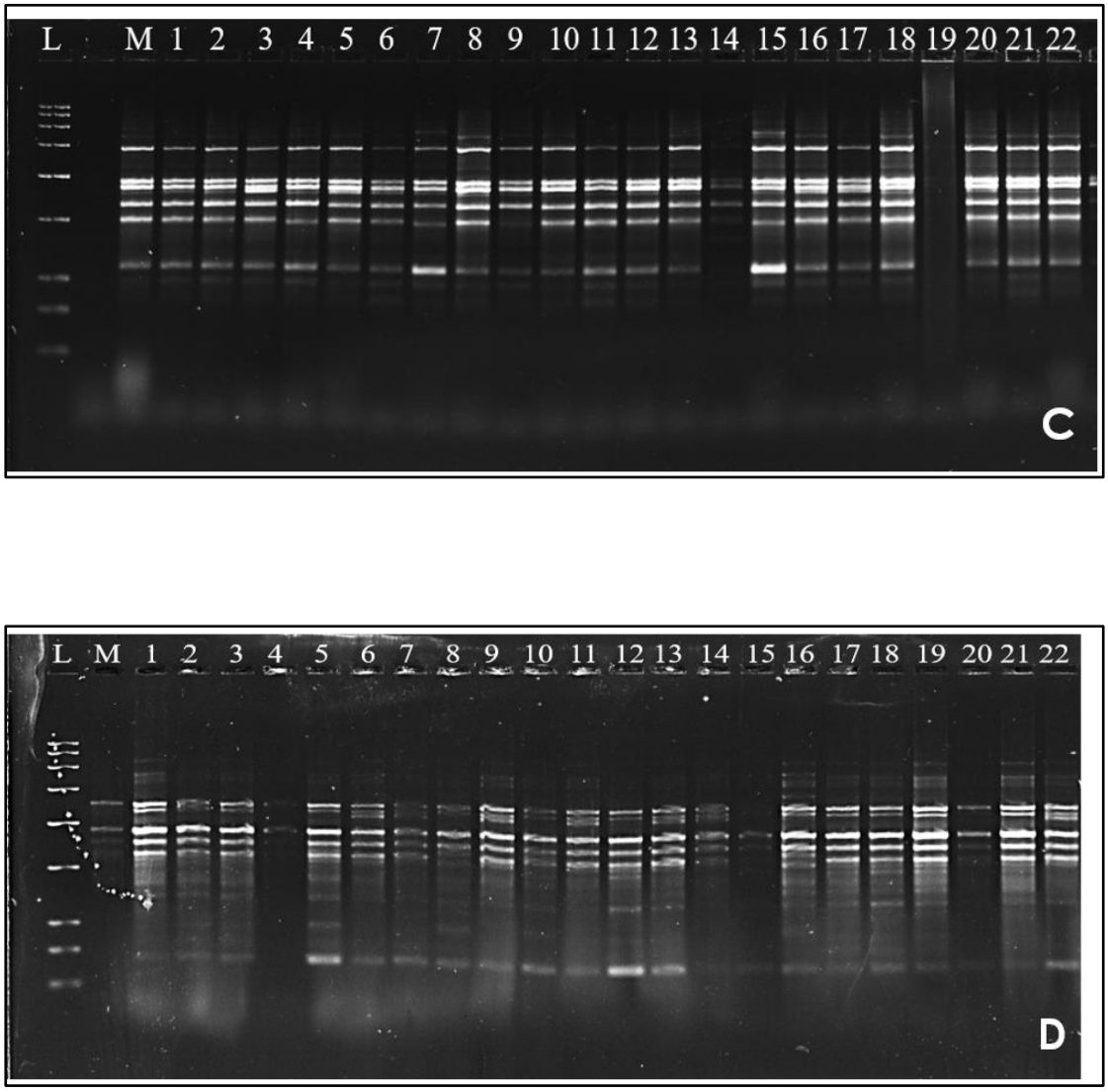


Figure 6.1(b) Molecular evaluation of genetic fidelity in *in vitro* grown Rose during different growth condition. PCR amplification of genomic DNA using (C) RAPD primer OPP-11 and (D) RAPD primer OPP-12. Lane L – Marker, M-Mother plant, 1-7 micropropagules grown in different culture vessels in semi-solid medium, Lane 8-14 micropropagules grown in different culture vessels in liquid medium, Lane 15-18 plant grown in different support materials, Lane 19-22 plant grown in different gelling agents. Observations were recorded after two culture cycles *i.e.* 45 days

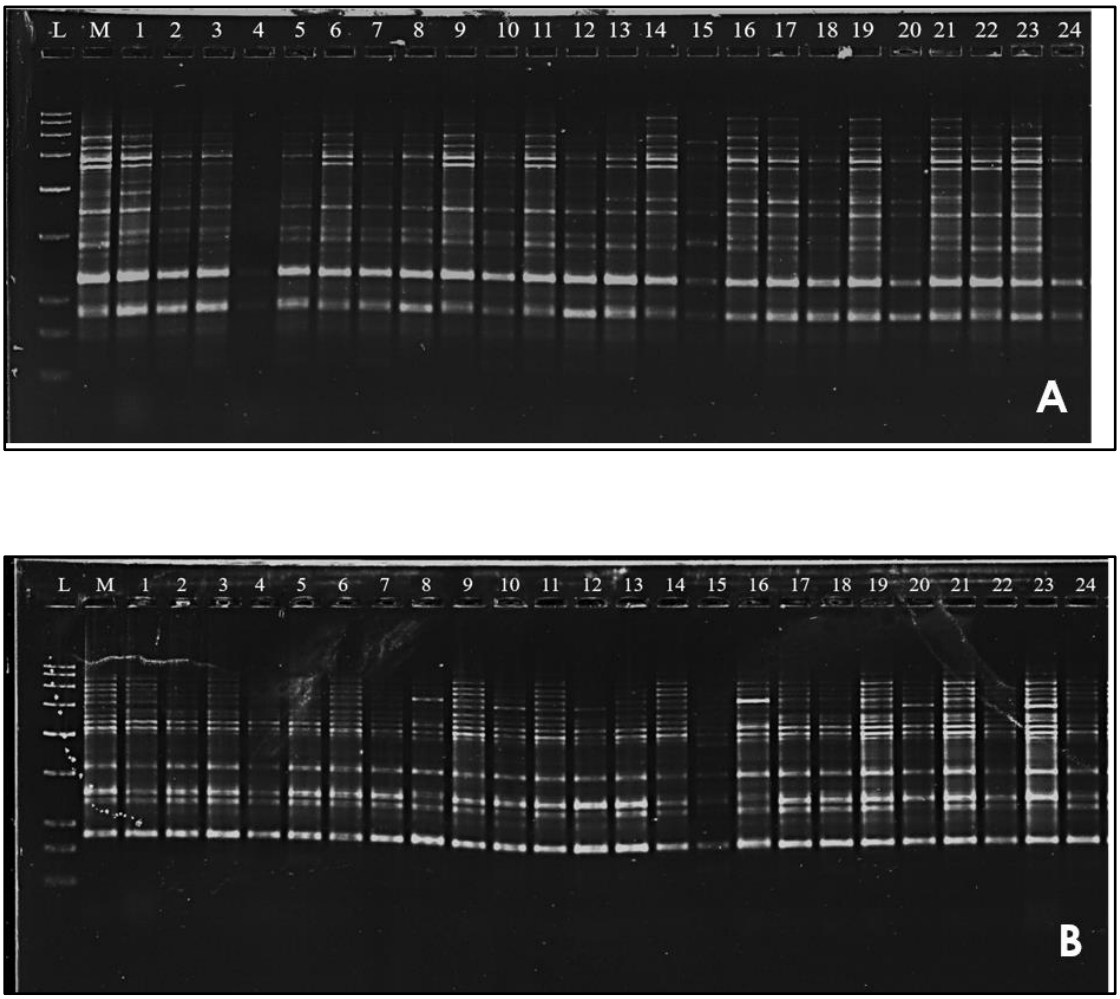


Figure 6.2(a) Molecular evaluation of genetic fidelity using RAPD primers in Rose grown under *in vitro* CO_2 enrichment condition with A: OPP-13, B: OPP-14. Lane L-Marker, M-mother plant, Lane 1-3 plant grown in 0% sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2, Lane 4-6 plant grown in 1% Sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2, Lane 7-9 plant grown in 3% sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2. and 10-12 plant grown in 1%;2%;3% sucrose with ambient air and liquid medium. Lane L – Marker, M- mother plant, Lane 13-15 plant grown in 0% sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2, Lane 16-18 plant grown in 1% Sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2, Lane 19-21 plant grown in 3% sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2. and 22-24

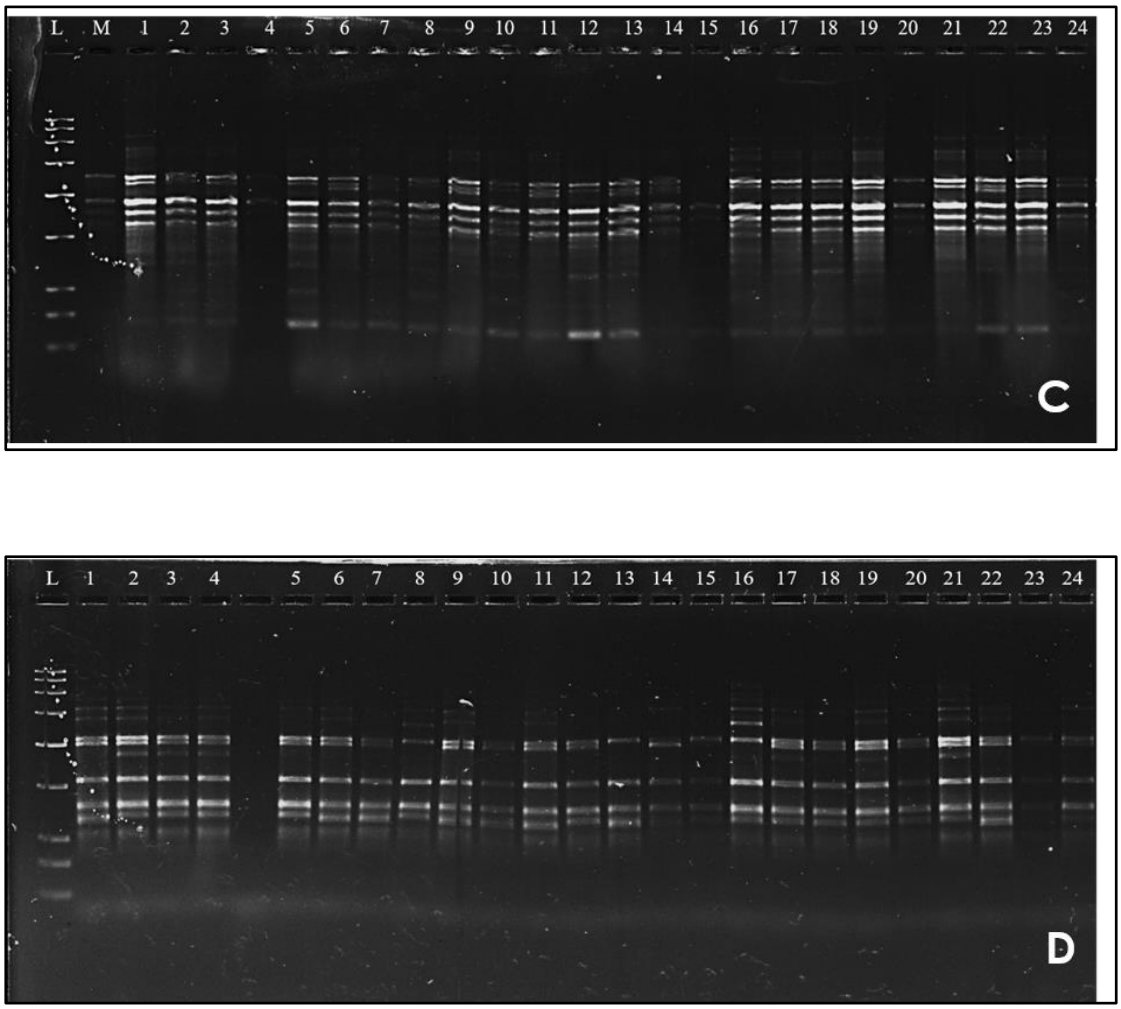


Figure 6.2(b) Molecular evaluation of genetic fidelity using RAPD primers in Rose grown under *in vitro* CO_2 enrichment condition with C: OPP-15 & D: OPP-17 primer in semi-solid medium. Lane L-Marker, M-mother plant, Lane 1-3 plant grown in 0% sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2, Lane 4-6 plant grown in 1% Sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2, Lane 7-9 plant grown in 3% sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2. and 10-12 plant grown in 1%;2%;3% sucrose with ambient air and liquid medium. Lane L – Marker, M- mother plant, Lane 13-15 plant grown in 0% sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2, Lane 16-18 plant grown in 1% Sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2, Lane 19-21 plant grown in 3% sucrose with 0.03% CO_2; 0.5% CO_2; 2% CO_2. and 22-24 Plant grown in 1%;2%;3% sucrose with ambient air Observations were recorded after two culture cycles *i.e.* 45 days