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Enhancing Gerbera Micropropagation Efficiency and Genetic Fidelity through Cytokinin and Auxin Combination Strategies

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The present investigation was carried out to assess the impact of 6-benzyladenine (BA) in conjunction with Indole-3-butyric acid (IBA) on gerbera explant establishment under micropropagation. Additionally, the effects of BA, either individually or in combination with Kinetin (KIN), on shoot proliferation in two Gerbera cultivars, namely Kormoran and Dolores was experimented too. Throughout the experiment, various morphological changes were documented occurring during these micropropagation phases and also monitored potential genetic alterations using SSR markers. The studies revealed that the combination of BA and IBA yielded exceptional results, achieving a 100% success rate in explant regeneration within the shortest time frame.

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Notably, when BA and IBA were applied at lower concentrations, the number of shoots generated was reduced. However, the most substantial proliferation of shoots was observed when the growth medium contained 4 mg of BA and 0.5 mg of IBA per litre. Furthermore, our investigation into genetic fidelity using SSR analysis revealed no detectable polymorphism between the mother plant and the micropropagated plantlets in both the Gerbera cultivars, affirming the reliability of the micropropagation method in preserving genetic consistency.

Keywords: Micropropagation; SSR markers; somaclonal variation.

ABBREVIATIONS

BA : 6-benzyladenine
IBA : Indole-3-butyric acid
SSR : Simple sequence repeats (marker)
PGR : Plant Growth Regulator
MS : Murashige and Skoog medium
CTAB: Cetyltrimethylammonium bromide

1. INTRODUCTION

Gerbera (*Gerbera jamesonii*), commonly known as African daisy or Transvaal daisy, belongs to the Asteraceae family and is a perennial plant characterized by its deeply lobed leaves. This plant boasts a unique appearance, having vibrant and cheerful daisy-like blooms, making it a beloved choice for landscaping. It is highly sought after flower which is majorly used as a decorative cut flower for bouquets and is also well-suited for planting in beds and pots. Gerbera flowers have a prolonged vase life, exhibit resilience during transportation, and offer a favorable market value [1]. The growing demand for Gerbera in the market has prompted researchers to explore ways to enhance both the quantity and quality of this crop. Among the various factors influencing its growth, the propagation method plays a pivotal role in determining quality and yield performance. However, the conventional method of propagation, such as rhizome divisions, falls short of meeting global demands, posing a challenge to the mass cultivation of Gerbera [2]. Hence, the large-scale production of quality Gerbera planting material within a short timeframe can be achieved through micropropagation techniques [3].

In micropropagation, one of the key determinants of morphogenesis is the application of plant growth regulators (PGRs). The balance between exogenous and endogenous hormone concentrations governs the induction of specific developmental pathways and the regeneration of particular organs [4,5]. Cytokinins, among the phytohormones, are recognized as major regulators of plant growth due to their cell division-inducing properties. Subsequently, their influence on various physiological, morphogenetic, and biochemical processes in plants, particularly in conjunction with other hormones, has been established [6,7]. Cytokinins promote the production of photosynthetically active pigments, inhibit chlorophyll breakdown, and enhance the concentration of total sugars in regenerated shoots by stimulating the photosynthetic system of plants [8,9]. Additionally, cytokinins play a vital role in protecting plants from oxidative stress by boosting the activity of antioxidative enzymes [10,11].

However, even under well-defined culture conditions, including the type and concentration of PGRs, variations in the regenerated material can occur, particularly at genetic and epigenetic levels [12-14]. Among the multiple variations, somaclonal variation which is characterized by genetic changes in somatic cells of vegetatively propagated plants is most commonly observed. Hence, it is crucial to characterize these modifications to establish genetic uniformity in micropropagated plants, ensuring their commercial quality [15]. In this context, DNA markers serve several purposes, including assessing genetic variability in breeding material, identifying cultivars, confirming hybrid origins, and verifying genetic fidelity in *in vitro* regenerated plants [16,17].

Among various DNA-based markers, Restriction Fragment Length Polymorphism (RFLP) are frequently used markers that can assess the genetic stability in tissue-cultured plants. However, this method involves expensive enzymes, a significant quantity of DNA, and radioactive materials, requiring extensive care and rendering it less suitable. In contrast, Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeats (ISSR), and Simple Sequence Repeats (SSR) markers offer advantages such as minimal DNA sample requirements, simplicity, speed, and non-

radioactivity, making them suitable for analyzing clonal fidelity [18].

Therefore, the present study is designed to investigate the impact of cytokinins on establishment, shoot proliferation, and genetic stability in *in vitro* culture. Additionally, the research aims to develop a specific micropropagation system for the production of genetically identical and stable Gerbera plants for commercial purposes.

2. MATERIALS AND METHODS

2.1 Plant Material

The present investigation was conducted on two cultivars of gerbera *viz*. **"Kormoran"** and "**Dolores"**, which were grown under polyhouse conditions at the Agri-Tourism Centre, Chaudhary Charan Singh Haryana Agricultural University, Hisar. Immature capitulum was used as an explant in the present study which were collected from plants grown under polyhouse conditions (Fig. 1).

2.2 Sterilization of Instruments and Glassware

All the glass wares *viz.* test tubes, beakers, flasks, jam bottles, measuring cylinders, etc. used for the experiment were washed with 0.1% teepol solution followed by washing with tap water. Sterilization of all the instruments and other accessories was carried out with the help of an autoclave at 121˚C for 20 min (15 lbs/inch^2). At the time of inoculation, all the instruments and the laminar surface were wiped with ethyl alcohol. A hot air oven was used to dry the culture vessels used during the experiment. In addition, the UV light of the laminar air-flow chamber was kept on for 30- 40 min before use. At the time of inoculation, all the forceps, scalpels, scissors, etc. were dipped in spirit inside the laminar chamber and were frequently flame sterilized during the operation.

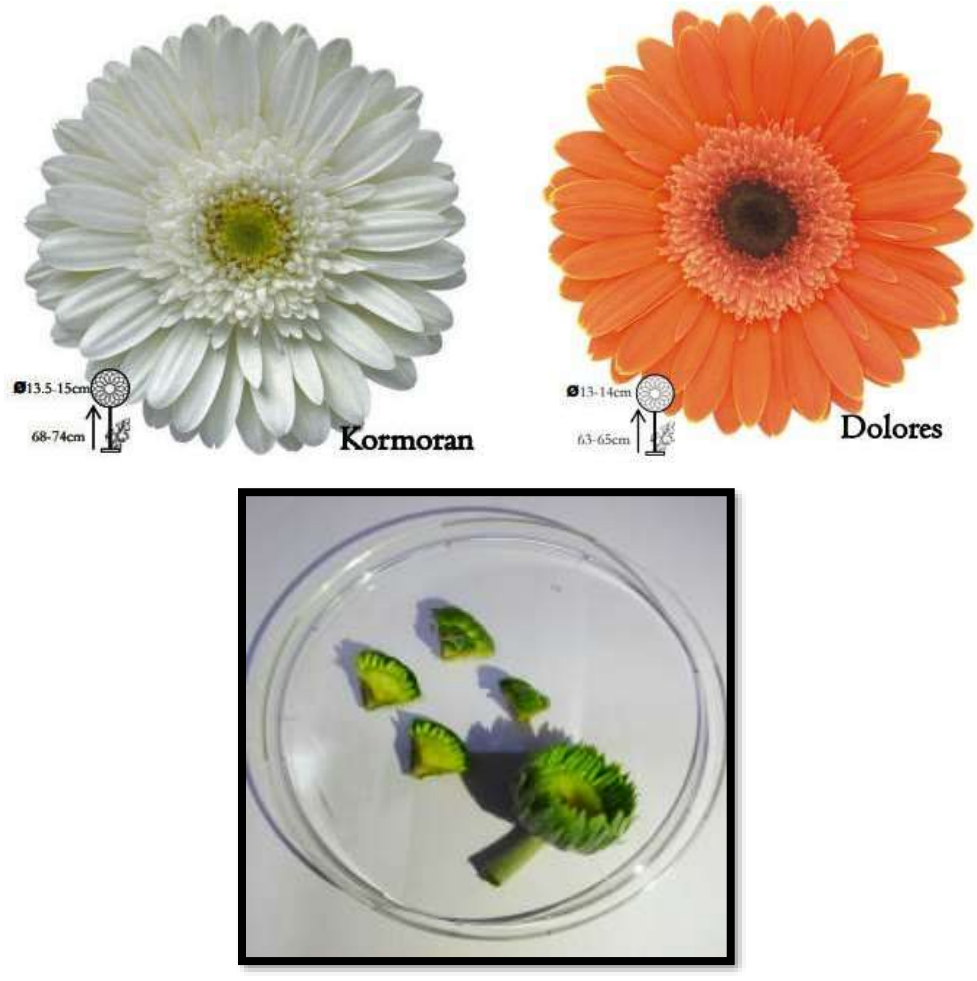

Fig. 1. a. Gerbera cv. Kormoran; b. Gerbera cv. Dolores; c. Capitulum used as explant

2.3 Preparation of Stock Solution

The stock solution of cytokinins (BA and KIN) was prepared by dissolving 50 mg of BA or KIN in a few drops of 1N HCl and then the final volume was made to 50 ml by adding double distilled water. Likewise, the stock solutions of IBA (Auxin) were prepared separately by dissolving 50mg of IBA in a few drops of 1N NaOH and the final volume was made to 50 ml by adding double distilled water. All the prepared solutions of MS basal medium and growth regulators were stored in a refrigerator at 4°C till further use.

2.4 Multiplication Phase Medium and Growth Conditions

Initially, well-prepared explants were treated with 0.1% teepol solution for 15 min and washed with tap water for 10-15 min to remove the residue of teepol. Then the explants were treated with Bavistin (0.1%) for 20 min followed by washing with running tap water. The pre-treated explants were sterilized under a laminar air flow cabinet with $HgCl_2$ (0.1%) for 5 min and subsequently washed 3-4 times with sterile distilled water to remove the residue of $HgCl_2$ before inoculation. Capitulum segments, established on the Murashige and Skoog medium (MS) [19] with 1mg to 5 mg/L BA with 0.5 mg/L IBA. For the multiplication phase the MS medium was enriched with 2 mg/L or 4 mg/L or 6 mg/L 6-benzyladenine (BA) alone or with combination of 1 mg/L, 2 mg/L, and, 3 mg/L Kinetin (KIN). The control treatment was the PGRs- free MS medium. Sucrose (30 g/L) served as the carbon source in the medium. The medium was solidified with Gelrite (3 g/L) and its pH was adjusted to 5.8 by 1N NaOH or 1N HCl before autoclaving. The 250 ml jars filled with 50 ml of the medium, closed with plastic covers and autoclaved for 20 min at 121 ˚C and 15 lbs per $inch^2$. During the establishment and multiplication phase, the jars with explants were kept in a growth chamber at 23±2 ˚C with 8h dark and 16h day light provided by fluorescent lamps. The percentage of regenerated plants, shoot lengths, and mean shoot numbers each containing 5 explants were evaluated after four weeks in triplicates.

2.5 Assessment of Genetic Stability of Microcuttings using SSR Markers

5 randomly selected six-week-old microshoots from each treatment of both cultivars were collected and 100 mg of the tissue were ground in liquid nitrogen and stored at -80 ˚C. Before the initiation of the cultures, material was also collected from the mother plants of both cultivars, additionally. 10 SSR primers were selected and tested (GEM1, GEM 2, GEM 17, GEM 52, GEM 70, GEM 98, GEM 106, GEM 140, GEM 179 and GEM 205) as depicted in Table 1. Further, Genomic DNA was extracted using modified CTAB method and the polymerase chain reaction was performed using 20 µl of the mixture consisting 1 µL of DNA, 0.3 µL Taq Polymerase, 0.8 µL of SSR primer, 0.5 µL of dNTPS, 14.4 µL of autoclaved water and 3 µL of buffer (x10 Taq buffer with 0.8 mM $MgCl^2$). The initial denaturation occurred at 4 min at 94 ˚C, followed by 40 cycles of 45 sec denaturation at 94 ˚C, 45 sec annealing at the temperature range from 45.92 ˚C to 59.52 ˚C, and 90 sec extensions at 72 ˚C, with a final extension at 72 ˚C for 5 min. Samples were stored at 4 ˚C till further analysis. SSR amplifications were performed for PCR products and amplification products for all samples were resolved on 2.5 % (w/v) agarose gels in 1x TBE buffer and stained with 0.5 μgL^{-1} ethidium bromide. A Gel Documentation system was used for the final visualization and photographs of the gel. DNA ladder (Himedia, India) of 1 kb was employed to determine the size of unknown DNA fragments on the agarose gel.

2.6 Statistical Analysis

All experiments under the current study were set up in a completely randomized design (Factorial). Data were subjected to one-way analysis of variance followed by the Turkey multiple range test ($\alpha \leq 0.05$) [20].

3. RESULTS

In our current investigation, noteworthy and positive effects were observed when the MS medium was enriched with various concentrations of 6-benzyladenine (BA) in combination with Indole-3-butyric acid (IBA), as opposed to the control group lacking any growth hormones. Notably, we observed a gradual decline in tissue necrosis in all treatment groups (Table 2). The percentage of successfully regenerated explants increased across all treatments for both cultivars. Remarkably, the inclusion of BA at 4 mg, coupled with IBA at 0.5 mg, resulted in a remarkable 100% explant regeneration rate in both cultivars. This combination of hormones also yielded the highest regeneration ratio, signifying the rapid

establishment of explants and the production of more than seven axillary shoots per explant in both cultivars. Additionally, the number of days required for explant establishment significantly decreased as the BA concentration increased, particularly up to 4 mg/L (Table 2).

Furthermore, all the treated explants exhibited a favorable response during the multiplication phase when cytokinin BA was applied either independently or in combination with Kinetin (KIN), in comparison to the control group (Table 3). In both cultivars, the most extended shoots bearing the highest number of shoots were observed when the MS medium was fortified with BA at 6 mg and KIN at 1 mg.

3.1 Assessment of Genetic Stability of Micro Cuttings using SSR Markers

As mentioned earlier, 10 SSR markers were used for assessment of genetic fidelity of micro propagated cuttings of gerbera. Among them, 4 primers showed amplification whereas 6 primers did not amplify (Table 4). These amplified primers had monomorphic band pattern (Figs. 2 & 3), which indicates true-to-type nature of *in vitro* raised plantlets of gerbera.

4. DISCUSSION

In current investigation, we found that all concentrations of 6-benzyladenine (BA) in combination with Indole-3-butyric acid (IBA) at 0.5 mg/L resulted in increased explant regeneration. These combinations were particularly effective in enhancing the propagation ratio, i.e., the number of new shoots per explant, while also reducing the time required for their establishment. When we combined these two growth regulators, BA and Kinetin (KIN), during the multiplication phase, we observed a significant and substantial increase in the number of new shoots compared to the control group without cytokinins (Table 3).

Table 1. SSR primers used for assessment of genetic stability

Sr. No.	Primer Name	Forward Sequence	Reverse Sequence
1	GEM 1	TTTCCTTTTCTTCCCAAATCAA	TTCTTCAACAGCCCACCTCT
2	GEM 2	GGTCAATTGCTCTGCTAGGG	GGAACACAATTACTGGTACAGTTGA
3	GEM 17	GGTTTGTATAAAGGGTTT	TCAATGCATTTAGATAATAA
4	GEM 52	AAGGCAGATGACCCTTCTCT	TTGCACCTTAAATCACACCA
5	GEM 70	GGCGGAGTGCTTGATAATTC	GCAACTACCTAAAAGTCGAATGC
6	GEM 98	CCTACAACCAAACCAAACCAT	GGTCCTCAGGAACGTTTTTC
7	GEM 106	GTGCCAGCATTTGAACTTCC	GCCCACATTGCAGTACCAGT
8	GEM 140	GCCTAAATGTAGTGTGCCATT	ACAACGAGCATTTTGAAGTTT
9	GEM 179	GGAAGAAGAAGGCGACATTG	CCCAGTCGTTGGTTATGCTT
10	GEM 205	AGCTGGACTAGATCATGTTG	TAGACCAAAATTCCATTCAT

Table 2. Effect of cytokinin (BA) in combination of auxin (IBA) on the establishment and axillary shoot formation gerbera cv. Kormoran and Dolores

MS medium with cytokinin [mg L^{-1}]	Kormoran			Dolores		
	Regenerated explants (%)	Number of shoots	Number of days taken for establishment	Regenerated explants (%)	Number of shoots	Number of days taken for establishment
Hormone-free MS	0.00^{f*}	0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}
1 BA + 0.5 IBA	71.11 ± 3.85^{e}	2.87 ±0.31^{e}	87.67 ± 0.61^{a}	68.89 ±3.85^{e}	2.93 ± 0.31^{e}	89.86 ± 0.50^{a}
2 BA + 0.5 IBA	82.22 ± 3.85^{d}	4.27 ±0.42^{d}	76.93 ± 0.31^{b}	80.00 ± 0.0^{d}	4.20 ± 0.20^{d}	77.66 ± 0.42^{b}
3 BA + 0.5 IBA	93.33 ± 6.67^{c}	6.13 ±0.12^{c}	72.33 ± 0.50^{c}	91.11 ±3.85^{c}	5.73 ± 0.31^{c}	72.73 ± 0.31^{c}
4 BA + 0.5 IBA	100.00 ± 0.0^{a}	8.00 ±0.20^{a}	61.13 ± 0.31^{e}	100 ± 0.0^{a}	7.73 ± 0.31^{a}	61.46 ± 0.50^{e}
5 BA + 0.5 IBA	97.77 ± 3.85^{b}	7.07 ±0.31^{b}	62.20 ± 0.53^{d}	95.55 ±3.85^{b}	7.20 ± 0.20^{b}	63.80 ± 0.53^{d}

** Means ± standard deviation within a column followed by the same letter are not significantlydifferent according to Tukey's multiple range test at α = 0.05*

Table 3. Effect of cytokinins (BA and KIN) on *in vitro* shoot multiplication in gerbera cv. Kormoran and Dolores

MS medium with cytokinins [mg L^{-1}]	Kormoran		Dolores	
	Shoot length (cm)	Number of shoots	Shoot length (cm)	Number of shoots
Hormone-free MS	1.37 ± 0.11^{k*}	1.00 ± 0.00^{j}	1.44 ± 0.13^{k}	1.00 ± 0.00^{g}
2 BA	1.68 ± 0.15^{j}	1.80 ± 0.20^{i}	1.64 ± 0.14^{j}	1.60 ± 0.20^{f}
2 BA + 1 KIN	1.74 ± 0.07^{j}	2.00 ± 0.20^{hi}	1.89 ± 0.07^{i}	2.27 ± 0.23^{e}
2 BA + 2 KIN	1.82 ± 0.11^{j}	2.20 ± 0.20^{h}	2.14 ± 0.07^{h}	2.40 ± 0.20^{e}
2 BA + 3 KIN	2.23 ± 0.06^{i}	2.67 ± 0.31^{g}	2.18 ± 0.09^{h}	2.93 ± 0.12^{d}
4 BA	2.41 ± 0.08^{h}	3.27 ± 0.12^{f}	3.19 ± 0.08^{g}	3.67 ± 0.12^{c}
4 BA + 1 KIN	2.90 ± 0.11^{g}	3.87 ± 0.12^{e}	3.31 ± 0.04^{g}	3.60 ± 0.20^{c}
4 BA + 2 KIN	3.25 ± 0.06^{f}	4.13 ± 0.23^{de}	3.62 ± 0.07^{f}	4.13 ± 0.12^{b}
4 BA + 3 KIN	3.67 ± 0.09^{e}	4.40 ± 0.20^{d}	4.05 ± 0.10^{e}	4.33 ± 0.31^{b}
6 BA	4.50 ± 0.04^{d}	5.20 ± 0.20^{b}	4.72 ± 0.04^{d}	5.27 ± 0.31^{a}
6 BA + 1 KIN	6.19 ± 0.03^{a}	5.53 ± 0.31^{a}	6.23 ± 0.06^{a}	5.43 ± 0.29^{a}
6 BA + 2 KIN	5.95 ± 0.12^{b}	5.40 ± 0.20^{ab}	6.02 ± 0.11^{b}	5.47 ± 0.31^{a}
6 BA + 3 KIN	5.34 ± 0.11^{c}	4.87 ± 0.12^{c}	5.71 ± 0.10^{c}	5.13 ± 0.31^{a}

** Means ± standard deviation within a column followed by the same letter is not significantlydifferent according to Tukey's multiple range test at α = 0.05*

Table 4. Summary table of SSR analysis for genetic fidelity testing of tissue cultured plantlets

Description	SSR primers
Total number of primers tested	10
Number of amplified primers	4
Number of primers showing monomorphic band pattern	4
Size of amplified fragment (GEM 2)	200 bp
GEM 52	200 bp
GEM 70	800 bp
GEM 106	190 bp

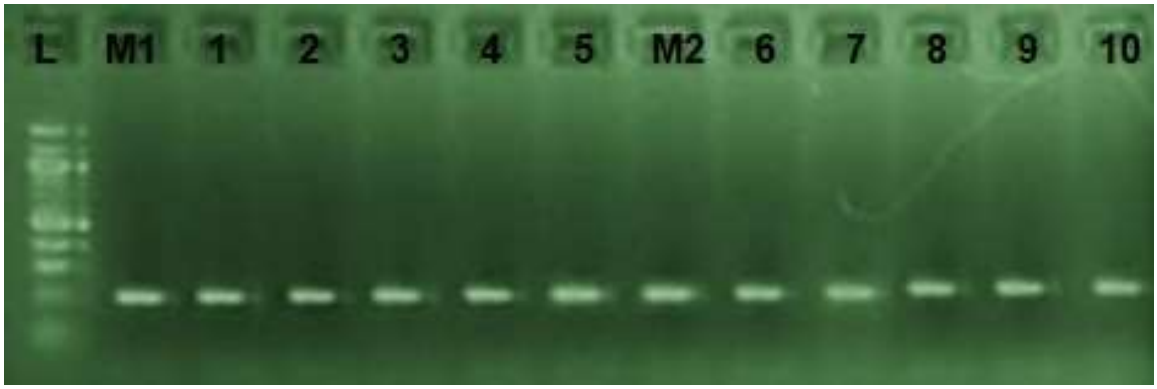

Fig. 2. Amplification of genomic DNA of mother plant and tissue culture raised plants atlocus GEM 2

L: Ladder; M1: Mother plant of cv. Kormoran; M2: Mother plant of cv.Dolores; 1-5: Tissue cultured plants of cv. Kormoran; 6-10: Tissue cultured plants of cv. Dolores

According to existing literature, BA and KIN are the most frequently recommended cytokinins for the micropropagation of Gerbera. Higher doses of BA have been known to promote greater shoot proliferation [21,22]. Additionally, they stimulate shoot growth, that is why the treatment involving BA at 6 mg and KIN at 1 mg resulted in the longest shoots. This stimulation of shoot elongation by BA and KIN in Gerbera, as well as by zeatin in Rhododendron indicum, has also been reported previously [23]. The quality of regenerated microcuttings is highly dependent on genetic stability, which ensures the repeatability of crucial features defining the original cultivar. In

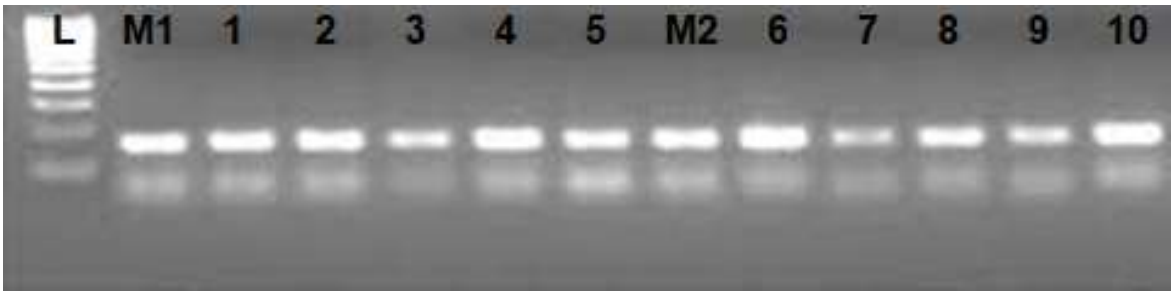

Fig. 3. Amplification of gemonic DNA of mother plant and tissue culture raised plants atlocus GEM 106

L: Ladder; M1: Mother plant of cv. Kormoran; M2: Mother plant of cv. Dolores; 1-5: Tissue cultured plants of cv. Kormoran; 6-10: Tissue cultured plants of cv. Dolores

clonal propagation, it is essential that the multiplied material is both phenotypically and genetically identical to the mother plant. However, as numerous authors have reported, this is not always the case [24-26,11].

To assess the impact of cytokinins on genetic stability during Gerbera micropropagation, an evaluation study was conducted after the proliferation phase. Cytokinin not only plays a crucial role in cell division and proliferation but also involved in the plant's response to abiotic stressors i.e., drought, extreme heat, or cold. Thus, cytokinin serves as a key regulator of both plant development and stress responses [27]. This dual role of cytokinins may contribute to the development of variability between micropropagated plants and the mother plant. This early assessment is significant as it enables the removal of regenerants that are genetically distinct from the stock plants during the early stages of propagation, saving time and resources by excluding material that does not exhibit the desired cultivar characteristics. In this context, SSR and ISSR markers offer valuable tools for evaluating genetic stability [13,11].

5. CONCLUSION

Based on the present study, it can be concluded that the presence of cytokinin (BA) in combination of low concentration of IBA (0.5 mg) in MS medium provided better and early establishment while in combination of KIN it also provides better proliferation than the control. Molecular studies revealed that SSR markers are the better option for the assessment of clonal fidelity of *in vitro* raised plants of gerbera. No polymorphism was detected among the mother plants and *in vitro* raised clones of each cultivar during SSR analysis. Out of ten SSR primers, four primers showed monomorphic banding patterns within *in vitro* raised clones comparable with the mother plant of each cultivar. Further, it can be concluded that there is no effect of cytokinins on genetic stability of *in vitro* raised plants of gerbera.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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