



An effective micropropagation protocol and determination of the clonal fidelity of in vitro developed microshoots of carnation (*Dianthus caryophyllus* L.) using SSR markers

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Abstract

Carnation is an important cut flower in the floriculture industry. A micropropagation method for carnation cv. 'Irene' has been standardized. Nodal plant explants were grown in Murashige and Skoog (MS) medium containing plant growth regulators (PGRs). Determination of clonal fidelity of regenerated plantlets was performed with the help of SSR markers. Early formation of micro-shoots with maximum regeneration was noted in the half MS medium in combination of benzyl amino purine (BAP) thidiazuron (TDZ) and silver nitrate (AgNO_3) in the concentrations 1.5, 0.25 and 0.5 mg L^{-1} , respectively; while BAP (2.5 mg L^{-1}), TDZ (1.0 mg L^{-1}) and AgNO_3 (1.5 mg L^{-1}) produced maximum length of the micro-shoots. Quick induction of roots and maximum number of roots per culture were achieved in half MS basal rooting medium supplemented with 1.5 mg L^{-1} of indole-3-butyric acid (IBA) and 0.5 mg L^{-1} of naphthalene acetic acid (NAA). Simple sequence repeat (SSR) markers for clonal fidelity analysis showed uniform genetic fidelity among the proliferated plantlets and the mother plant lines. The protocol can be suitable for commercial cultivation of carnation plants.

Keywords Carnation · Plant growth regulators · Clonal fidelity

Abbreviations

BAP Benzyl Amino Purine
TDZ Thidiazuron
 AgNO_3 Silver Nitrate

RAPD Randomly Amplified Polymorphic DNA
SSR Simple Sequence Repeats
ISSR Inter Simple Sequence Repeats
CTAB Cetyl Triethyl Ammonium Bromide

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Introduction

Floriculture has become an important industry worldwide since the development of plant cell culture technology. There are many constraints which restrict the progress of floriculture business. One of the major constraints in the flower industry is the non availability of quality planting materials. Increasing demand of the floriculture produce could be met only by the rapid production of qualitative and quantitative propagation of ornamental plants. Carnation being an important cut flower of the world as well as in Indian market is mainly propagated through micropropagation for commercial purpose. Many floriculturists in the present time follow the micropropagation technology as a potential tool for rapid multiplication and to produce disease free and true to type plantlets of ornamental plants. Therefore, development of good quality plant material of carnation through

micropropagation is an essential requirement to cater the need of cut flowers. Limited literature are available which mention optimum conditions for large scale propagation through in vitro culture of carnation using different explants viz. Nodal explants, meristem and leaf segments [12, 14, 28, 33]. In the recent past, plant growth regulators (PGRs), have been widely used by various workers for in vitro propagation in carnation [2]. There is a need to further standardize the micropropagation protocol of the present carnation cultivar because response for micropropagation and in vitro rooting vary due to physiological status of different genotype [5, 7].

The main aim of in vitro culture is to obtain true to type plants to maintain the uniformity in the microplants. But the occurrence of somaclonal variation is a potential drawback when propagation of an elite plant species is intended. Thus, the clonal fidelity i.e. genetic uniformity of the plantlets achieved from micropropagation must be screened to maintain the desired elite genotypes. Various molecular markers such as Polymerase Chain Reaction (PCR) based viz. Randomly Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Inter Simple Sequence Repeats (ISSR) and Amplified Fragment Length Polymorphisms (AFLPs) etc. are quite helpful for clonal character determination of micropropagated plantlets. These markers have several advantages over the others. They are very much useful to determine the genotyping and polymorphism detection in micropropagated plantlets. To establish the genetic fidelity among the micropropagated plantlets, SSR markers may be the most useful due to co-dominant in nature [22]. SSR markers are versatile and widely used to detect genetic stability among in vitro grown plantlets due to enviable characteristics such as their high reproducibility, high allelic diversity and very much abundance in the genome of different plants [17]. Thus, SSR markers are considered as one of the best choice to detect genetic similarity or dissimilarity including any mutations occurred during in vitro culture of micropropagated plants [3, 23, 27]. Keeping in view of the above facts the present study was to optimize the in vitro protocol for high regeneration capacity of carnation and to do the SSR analysis to detect genetic fidelity among the micropropagated microplants for their commercial exploitation.

Materials and methods

Source of germplasm

One most promising and standard cultivar of Carnation (*Dianthus caryophyllus* L) viz. 'Irene' having good commercial potential was selected for the first time for standardization of micropropagation protocol to develop large scale quality planting materials and clonal fidelity analysis.

Rooted cuttings of carnation were procured from Department of Floriculture and Landscaping at DYS Parmar Horticulture and Forestry University, Solan (HP).

Explants used

Stem cuttings were taken from healthy donor plants. Nodes were excised carefully from stem cuttings. All the nodal explants were treated in the Teepol (0.1%) solution for 10 min and washed with tap water and then treated with Bavistin (0.1%) for approximately 20 min. They were washed thrice under running tap water for 15–20 min. All the nodal explants were excised to a size of about 4–5 mm. Carnation nodes were surface sterilized with the help of 70 percent ethyl alcohol for approximately 1 min. Thereafter, explants were washed three to four times with molecular biology grade deionised water. Explants were then sterilized with the help of HgCl₂ (0.1%) solution for 1–2 min and rinsed 3–4 times in double distilled water properly before inoculation on the culture medium.

In vitro establishment and multiplication of cultures

For in vitro establishment of carnation cultures, surface sterilized nodal segments were put on a gelled half MS medium [31] supplemented with thirty six different concentration combinations of Benzyl Amino Purine (BAP), Thidiazuron (TDZ) and Silver Nitrate (AgNO₃) (0.25–2.5mgL⁻¹). For multiplications purpose, healthy shoots of approximately 3.5–4.0 cm were selected. Shoots were individually put on basal gelled Murashige and Skoog (1962) half medium containing different concentrations (0.25, 0.50, 1.0, 1.5, 2.0 and 2.5 mgL⁻¹) BAP in combination with TDZ (0.25, 0.50, 1.0, 1.5, 2.0 and 2.5 mgL⁻¹) and AgNO₃ (0.25, 0.50, 1.0, 1.5, 2.0 and 2.5 mgL⁻¹), in addition to control in different 36 concentration combinations. The media were named as MS1- MS36. All the cultures were kept at 25 ± 2⁰C for further growth under 16/8 h photo/dark period for 4 weeks. The observations were recorded for establishment of shoot buds of nodal segments of carnation. After 4 weeks of incubation, cultures were observed carefully and required data was recorded.

In vitro root formation and microplants acclimatization

In vitro multiplied micro-shoots were separated and transferred to half strength MS medium supplemented with different levels of IBA (0.5–2.0mgL⁻¹) and NAA (0.5–2.0mgL⁻¹) for induction of roots. Murashige and Skoog (1962) half medium containing different concentrations (0.50, 1.0, 1.5 and 2.0 and mgL⁻¹) IBA in combination with NAA (0.50 and 1.0 mgL⁻¹) in addition to control in different five

concentration combinations. The media were named as MS1–MS5. The concentration of Culture conditions were maintained at $25 \pm 2^\circ\text{C}$ under 16/8 h photo/dark period. Observations were recorded at 2 weeks old cultures of carnation for root formation.

Potting medium (sand: coco peat in ratio 1:1) was used to acclimatize the microplants. Soil mixture was properly autoclaved at 15 lbs pressure of per inch² at 121°C for 25 min to sterilize. In vitro grown micro-plants attained 4.0–5.0 cm along with 4–6 leaves were taken out from the culture tubes gently so that roots not damaged. Roots were thoroughly washed with tap water for around 6 min to remove the nutrient medium. Plants were kept in a beaker containing water for about 20 min, so that they do not wilt after transferring to soil. Thereafter, roots were treated with bavistin (0.2%) solution for 10 min to make them free from possible fungal infection. Well-developed microplants were transferred to small mouth plastic cups. Plantlets were placed in the soil mixture and carefully covered with polybags to maintain the proper relative humidity.

DNA isolation and selection of SSR primer sequence

Genomic DNA was isolated from young and fresh leaves through Cetyl Triethyl Ammonium Bromide (CTAB) method [10]. The agarose gel (0.8%) was used to test the quality of DNA. Bio-Rad's SmartSpec™ Plus spectrophotometer was used to check the purity of DNA. A PCR (polymerase chain reaction) was set as per specified program and amplicons were run under an electric field using electrophoresis unit at 80 V electric current. Twelve SSR primer pairs (Supplementary table1) were used in the present study, the sequence of these primers were taken as mentioned by Kimura et al. [20].

Clonal fidelity analysis with SSR markers

Selected SSR primers were used to estimate the clonal fidelity of in vitro grown mother as well as micropropagated plants. On the basis of prior amplification records, twelve SSR primer pairs were used and all primer pairs showed successful amplification of micropropagated microplants including mother microplants to check the clonal fidelity (Table 4).

A master mix (20- μl reaction mixture) was made to perform the PCR amplification reaction using in a reaction mixture. The amplification conditions were set to amplify DNA with a set reaction cycle (1) initial denaturation performed for 5 min at 94°C , (2) 35 cycles used at 94°C for 30 s, and 55°C temperature was set for 30 s for annealing the primer (although for each primer a separates annealing temperature was calculated and used), and (3) finally 72°C temperature used for 30 s, followed by a final extension step

at 72°C for 10 min. Agarose gel (3%) was used to separate the PCR products with the help of electrophoresis gel which was mixed with 0.5 mg mL^{-1} of Ethidium bromide and visualized under UV light trans-illuminator. For calculation purpose, only clearly visible and scorable bands at a particular position were used for calculation and scoring the clonal fidelity.

Experimental design and statistical analysis

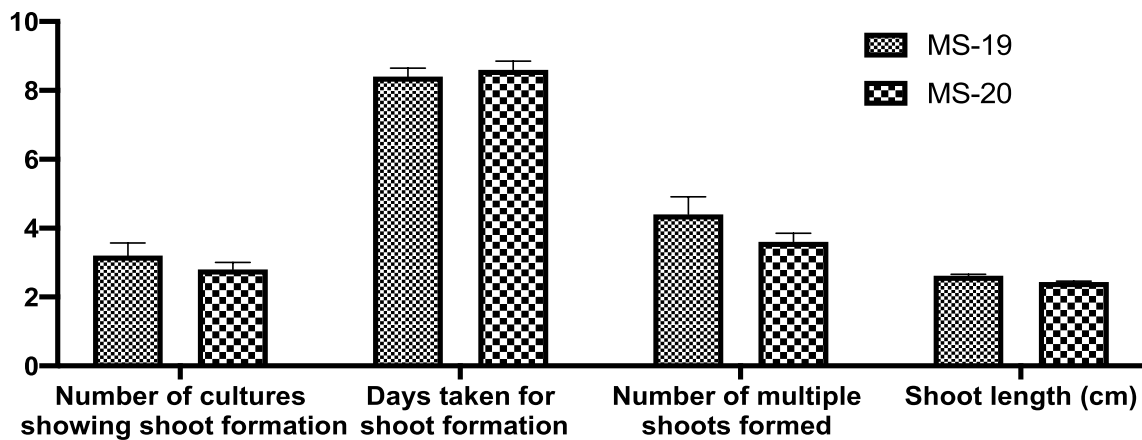
A completely randomized experimental design (CRD) was used in the present experiment with three replications. After that the data were statistically analyzed with the help of one way analysis of variance (ANOVA) followed by Duncan's multiple test range ($P \leq 0.05$) using SPSS (version 17.0) statistical programme. $P \leq 0.05$ was taken for consideration as significance values in compare to either control or among the PGRs or chemical treatments. SSR markers were analyzed by counting only reproducible and scorable bands of different sizes were scored manually on the basis of their presence (1) or absence (0) on the gel.

Results and discussion

Effect of BAP, TDZ and AgNO₃ microshoot initiation, days taken to microshoot formation, number of multiple microshoot formations and microshoot length

In the present investigation the effect of BAP, TDZ and AgNO₃ was studied in different combinations in carnation cv. Irene in vitro in half MS medium. The parameters included microshoot initiation, days taken to microshoot formation, number of multiple microshoot formations and microshoot length in 36 different culture media (MS1–MS36). Single nodal explants were inoculated in each culture tube for bud break and shoot proliferation. Results of the present study indicated that MS19 (1.5mgL^{-1} BAP; 0.25mgL^{-1} TDZ and 0.5mgL^{-1} AgNO₃) and MS20 (1.5mgL^{-1} BAP; 0.50mgL^{-1} TDZ and 0.5mgL^{-1} AgNO₃) were most suitable for the proliferation of the explants. The number of cultures showing micro shoot formation was 3.20 in MS19 with an average of 8.40 days for bud break and the number of multiple micro shoots formed were 440 with a maximum shoot length of 2.62 cm, while in MS 20 it took 8.60 days for bud break and multiple shoots formed with 2.44 cm as the maximum shoot length (Figs. 1 and 2).

Early work reported that BAP was very much effective to boost the elongation of shoots in carnation. TDZ, a substitute of phenyl urea (N phenyl-1,2,3-thidiazol-5-ylurea) derivative, showed a very strong activity as exhibited by cytokinins [30]. TDZ has been reported to be very much effective in



*The data represents mean value of five replications with a \pm standard error (SE)

Fig. 1 Effect of BAP, TDZ with AgNO_3 in micro shoot initiation in MS19 and MS20 cultures of Carnation cv. Irene

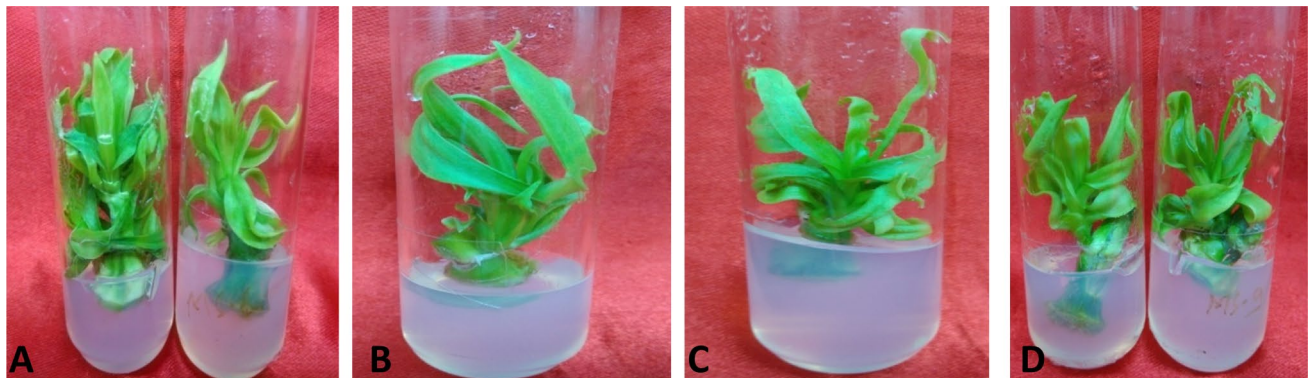


Fig. 2 In Vitro shoot induction in carnation variety “Irene” after 4-weeks of culture. **a** MS-19–1.5 mgL^{-1} BAP, 0.25 mgL^{-1} TDZ and 0.5 mgL^{-1} AgNO_3 ; **b** MS-20–1.5 mgL^{-1} BAP, 0.5 mgL^{-1} TDZ and

1.5 mgL^{-1} AgNO_3 ; **c** MS-22–1.5 mgL^{-1} BAP, 1.5 mgL^{-1} TDZ and 2.0 mgL^{-1} AgNO_3 ; **d** MS-21–1.5 mgL^{-1} BAP, 1.0 mgL^{-1} TDZ and 1.5 mgL^{-1} AgNO_3

shoot proliferation in a large number of different plant species [11]. Combination of BAP with TDZ in a gelled media was also effective to enhance the proliferation of microshoots of carnation and our results are in line with that of Hajong et al. [16]. In the present study, combined effect of AgNO_3 with cytokinin (BAP and TDZ) inhibited the ethylene action and promoted multiplication rate of microshoot formation. Poor regeneration frequency and shoot induction response of explants was observed in MS1 media (with minimum concentration of BAP, TDZ and AgNO_3). It may be attributed to the accumulation of ethylene that suppressed the growth of microshoots. Very low and high concentrations of BAP and TDZ showed poor results. Addition of AgNO_3 showed a stable microshoot proliferation, while, higher concentrations ($\geq 2.0 \text{ mgL}^{-1}$) had negative effects on the growth and development of explants. This was evidenced by the curly leaves, stunted growth and browning of tissue in the explants [34]. The explants cultured in MS-19 with BAP (1.5 mgL^{-1}); TDZ (0.25 mgL^{-1}) and AgNO_3 (0.5 mgL^{-1}) showed

maximum shoot length (2.62 cm). It decreased gradually both with increasing and decreasing combinations of BAP, TDZ and AgNO_3 and minimum shoot length (1.14 cm) was observed in MS-1. Cytokinin-like activity was exhibited by TDZ that causes shoot induction and proliferation in vitro in many plant species including ornamental plants [19].

Suppression of shoot organogenesis by the presence of ethylene under *in-vitro* environment is considered vital to many ornamental plants, including carnation. However, AgNO_3 is reported to be one of the effective inhibitors of ethylene in plant cell culture conditions. Ethylene activity is inhibited by the silver (Ag^+) ions thereby reducing its receptor binding capacity. Silver nitrate enhances microshoot proliferation when added with TDZ [24]. The beneficial and potent effects of AgNO_3 on organogenesis have been reported on many plant species [29]. Sarropoulou and Maloupa [36] reported that maximum shoot length could be achieved by BAP (2.2 μM) in combination with AgNO_3 (1.0 μM) and higher concentrations of both the chemicals decreased the microshoot length in *Sideritis Syriaca*.

In vitro rooting and acclimatization

Rooting of proliferated microshoots is strongly influenced by many factors and chemicals like the type of auxin used. Among the different combinations of IBA and NAA, the maximum number (81.20) of regeneration of rooting was recorded in MS-3, supplemented with IBA (1.0 mgL^{-1}) and NAA (0.5 mgL^{-1}), while, minimum (63.80) was observed in MS-1 (Table 1; Fig. 3a and b). Kharrazi et al. [18] reported excellent root regeneration capacity of NAA and IBA in carnation, but regeneration capacity was different in the two cultivars. This variation may be due to the differential activity of auxin and in the differences of the different organs as well on the genotypes. Differential rooting response among the genotypes may be also due to the variation in the concentration of auxin receptor proteins and the metabolic capabilities of the genotypes [13]. Our results are in confirmation with that of Kumar et al. [21]. They obtained best rooting response in half MS gelled media supplemented with 0.5 mgL^{-1} IBA and 0.5 mgL^{-1} NAA. Similarly, Murkute et al. [32] observed 7.8 roots/explants with 0.5 mgL^{-1} NAA and 1.0 mgL^{-1} IBA in *C. Karna*. In the present study, significant rooting was achieved using optimum concentrations of NAA and IBA in the medium. However, very low and high concentrations of both regulators showed adverse affect on rooting. The reason of this may be due to the synergistic effect of NAA and IBA as reported earlier [6]. Rooted plants after transferring to pots displayed normal growth and good survival during hardening (Fig. 2b). In our experiments the roots were not dense and we observed that the roots were not truly geotropic. The probable reason may be that roots were not completely exposed to dark during in vitro rooting process. Majority of the roots emerged near the surface of medium and a few were exposed to air within the culture tubes.

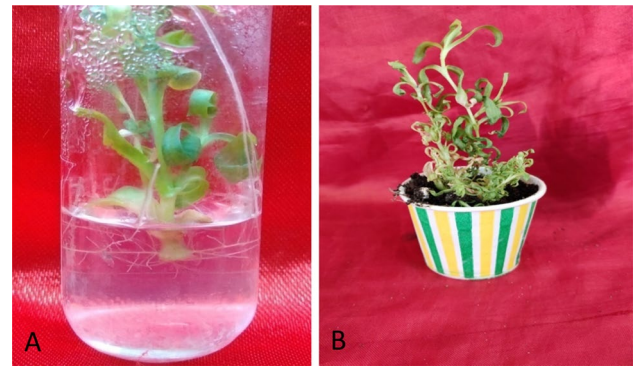


Fig. 3 In vitro root induction in carnation variety “Irene” after 2 weeks of culture: **a** MS3— 1.0 mgL^{-1} IBA and 0.5 mgL^{-1} NAA; **b** Acclimatized carnation plantlets

Assessment of genetic fidelity of in vitro grown microplants through SSR markers

To estimate the genetic fidelity of micropropagated carnation plants (Fig. 2a-d), twelve SSR markers (Supplementary table 1) were used in 11 randomly chosen plants as a different sub cultures (SC) from 1 to 11 including the mother plant

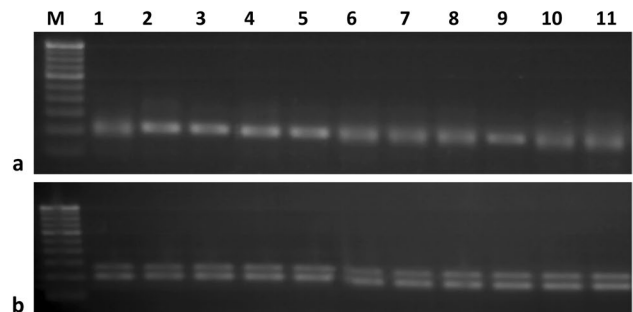


Fig. 4 DNA banding pattern generated through SSR primer **a** CB0016a and **b** CB0011a in mother plant and micropropagated plants of carnation; M: 100 bp Ladder; Lane1: Donor Mother Plant (DMC); Lane 2–11: SC1- SC10 (SC: Sub culture)

Table 1 Effect of NAA and IBA in different concentration combinations of IBA and NAA on in vitro rooting in carnation cv. Irene after 2 weeks

Media	Percent root generation	Days for root induction	Number of root/culture
MS-1: $\frac{1}{2}$ MS Basal Medium	63.80 ± 1.36^a	13.40 ± 0.25^o	4.40 ± 0.20^w
MS-2: $\frac{1}{2}$ MS Basal Medium + 0.5 mgL^{-1} IBA + 0.5 mgL^{-1} NAA	73.60 ± 0.75^c	12.60 ± 0.40^o	5.40 ± 0.25^x
MS-3: $\frac{1}{2}$ MS Basal Medium + 1.0 mgL^{-1} IBA + 0.5 mgL^{-1} NAA	81.20 ± 1.02^d	10.60 ± 0.25^n	7.60 ± 0.25^y
MS-4: $\frac{1}{2}$ MS Basal Medium + 1.5 mgL^{-1} IBA + 0.5 mgL^{-1} NAA	76.00 ± 0.63^c	8.60 ± 0.25^m	9.00 ± 0.32^z
MS-5: $\frac{1}{2}$ MS Basal Medium + 2.0 mgL^{-1} IBA + 1.0 mgL^{-1} NAA	69.60 ± 1.17^b	10.40 ± 0.40^n	5.80 ± 0.37^x

Values are the mean of five replicates with \pm standard error (SE). Means with different lower case letters within the columns represent significantly different according to Duncan's multiple range test ($p < 0.05$)

Table 2 Total number and size range of amplified fragments generated through SSR primers in micropropagated and mother plants of carnation cv. Irene

S. No.	Primer name	Number of amplified Band	Number of poly-morphic Band	Total number of amplified Band	Band size range
1	CF003a	1	0	11	100–200
2	CB003a	2	0	22	100–200
3	CB004a	1	0	11	100–200
4	CB011a	2	0	22	200–300
5	CB016a	2	0	22	100–390
6	CB018a	1	0	11	100–200
7	CB020a	2	0	22	100–200
8	CB026a	1	0	11	100–200
9	CB027a	1	0	11	100–200
10	CB041a	1	0	11	100–200
11	CB047a	2	0	22	100–200
12	CB057a	2	0	22	100–300
Total bands	–	18	0	198	–

(Supplementary table 2). Marker analysis clearly revealed that the regenerated microplants showed monomorphic amplicons, which were identical to the mother microplants (Fig. 4a and b).

The SSR primers varied from 1 to 2 bands with an average of 1.50 bands per primer pair (Table 2). Out of 12 SSR primers pairs, six primers showed single band, while rest of six primers showed double bands during amplification process. SSR markers are co-dominant in nature therefore at each of the locus two alleles were achieved. Some times in many conditions two alleles are visualized as a single band. In the present study a similar pattern was observed in six primer pairs, while rest of the primers did not show double banding pattern. A total of 198 bands (number of plants analyzed x number of scorable bands by all primers) were obtained from in vitro raised plantlets including mother plant and showed 18 bands by all the primers used in a single generation. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant. Results clearly indicated that the plantlets were true-to-type in nature i.e. similar to the mother plants. SSR markers showed more stable results as reported earlier by Bhatia et al. [4] who obtained 99.8% stability in gerbera by using RAPD and ISSR markers.

Our results proved the genetic equality among the various subcultured microplants. All the micropropagated plantlets were genetically similar to the respective mother plant thus providing substantially good evidence of absence of somaclonal variation during in vitro culture. It is well established that the genetic fidelity during in vitro propagation depends on the source of the explants and the regeneration methods [15]. Among the various in vitro propagation methods, nodal explants are widely used due

its simplicity and high multiplication efficiency [35]. Martins et al. [26] reported that plants established from nodal explants exhibited the lowest risk of genetic variation. However, Martin et al. [25] reported that plants derived from these tissues are not always genetically identical, as the plant growth hormones may be responsible for variation in micropropagated plants. Moreover, plants developed from a well organized meristem are not guaranteed to be genetically similar and true to the type as reported in several crop species [8, 9]. In the present study, we optimized the best chemical combinations of BAP, TDZ and AgNO₃ and achieved superior micropropagation better than those reported earlier [1]. The hormonal combinations used presently for in vitro multiplication were optimal for the maintenance of clonal fidelity in carnation.

In conclusion we may mention that the micropropagation protocol developed in this study is appropriate and suitable for mass clonal propagation of true-to-type carnation genotype ‘Irene’.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13237-021-00362-3>.

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Author’s contribution All the authors contributed the research equally.

Declarations

Conflicts of interest Authors solemnly declare no conflict of interests. Manuscript read and approved by all the contributors.

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